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DECIPHERING THE MOLECULAR MECHANISM THROUGH WHICH RHUS CORIARIA EXERTS ITS ANTI-CANCER ACTIVITY

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Khawlah Najib Saeed Athamneh

This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Under the Supervision of Professor Rabah Iratni

April 2019

Declaration of Original Work

I, Khawlah Najib Saeed Athamneh, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*Deciphering the Molecular Mechanism through which Rhus coriaria Exerts its Anti-cancer Activity* ", hereby, solemnly declare that this dissertation is my own original research work that has been done and prepared by me under the supervision of Professor 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 Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions.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 dissertation.

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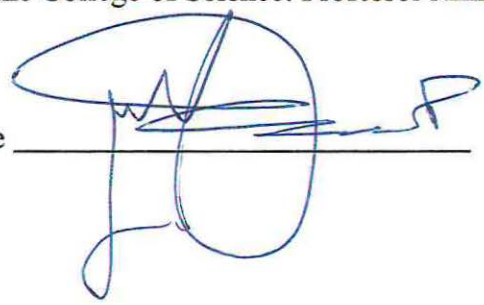
Signature  Date 22/5/2019

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Abstract

Cancer remains a major health problem around the globe. Among various types of treatments, plants have been shown to have great capacity in cancer treatment, one of which, is *Rhus coriaria.* Commonly known as sumac, *Rhus coriaria* is a culinary herb that is known to possess different therapeutic values including anti-oxidant and anti-microbial activities.

In this PhD project, we tested the effect of *Rhus coriaria* extract (RCE) on the migration, invasion and metastasis of MDA-MB-231 human breast cancer cells. We showed that non-cytotoxic concentrations of RCE inhibited migration and invasion, blocked adhesion to fibronectin and downregulated MMP-9. Additionally, we found that RCE reduced VEGF production and downregulated the inflammatory cytokines TNF-α and IL-6. The suggested mechanism for the effect of RCE appears to be through inhibiting NFκB and STAT3 pathways. Moreover, we extended our study and investigated the anti-cancer effect of RCE on HT-29 and Caco-2 human colorectal cancer cells. We found that RCE inhibited the viability and colony growth of colon cancer cells. RCE also induced Beclin-1-independent autophagy and caspase-7-dependent apoptosis. The suggested mechanism through which RCE exerts its effect is by inactivating AKT/mTOR pathway and downregulating Beclin-1, p53 and pro-caspase-3 through targeting them to proteasome-dependent degradation. Proteasome inhibition restored these proteins to level comparable to control cells and reduced RCE-induced cell death and blocked the activation of autophagy and apoptosis. Proteasomal degradation of mTOR was concomitant with an overall increase in proteins ubiquitination which target the proteins for degradation by the proteasome.

In conclusion, these preliminary results make *Rhus coriaria* a promising therapeutic candidate against both breast and colorectal cancer.

Keywords: *Rhus coriaria*, breast cancer, colorectal cancer, apoptosis, autophagy, proteasome.

Title and Abstract (in Arabic)

دراسة الآلية الجزيئية التي يقوم من خلالها نبات السماق بنشاطه المضاد لمرض السرطان

الملخص

يعد مرض السرطان خطراً صحياً في جميع أنحاء العالم، ومازالت عملية البحث عن علاجات لهذا المرض مستمرة، ومن ضمن هذه العلاجات استخدام النباتات ومشتاقاتها لصناعة أدوية مضادة للسرطان. أحد هذه النباتات والذي لاقى اهتماماً هو نبات السماق أو كما يعرف باسمه العلمي (*Rhus coriaria*) حيث أشارت الدراسات السابقة على فاعليته كعلاج مضاد للأكسدة ومضاد حيوي.

ومن هذا المنطلق قمنا في هذه الأطروحة بدراسة أثر مستخلص نبات السماق على نمو وانتشار خلايا الثدي السرطانية MDA-MB-231. وأشارت نتائج هذه الدراسة إلى أن استخدام تركيزات منخفضة لا تقتل الخلايا من مستخلص السماق حالت دون غزو وانتشار الخلايا السرطانية ومنعت ارتباط هذه الخلايا ببروتين ال fibronectin كما أنها أدت إلى خفض مستويات بروتين ال MMP-9. بالإضافة إلى ذلك فقد وجدنا أن السماق يقلل من إفراز كل من VEGF وTNF-α وIL-6. وعلى ما يبدو فإن الآلية المقترحة لعمل مستخلص السماق تتمثل في تثبيط مسارات NFκB وSTAT3. بالإضافة إلى ذلك قمنا بدراسة أثر مستخلص السماق على خلايا سرطان القولون HT-29 وCaco-2، ووجدنا أن مستخلص السماق يمنع نمو خلايا سرطان القولون المفردة منها والتي كونت مستعمرات، كما وجدنا أن المستخلص أدى إلى عملية الالتهام الذاتي التي لا تعتمد على بروتين Beclin-1 وأدى إلى موت الخلايا المبرمج المعتمد على caspase-7. واستناداً على هذه النتائج فإن الآلية المقترحة التي من خلالها يمارس المستخلص تأثيره هو تعطيل مسار AKT / mTOR وتقليل مستويات البروتينات التالية Beclin-1 وp53 وpro-caspase-3 عن طريق تحليل هذه البروتينات في البروتيوزوم. وللتحقق من ذلك قمنا بتثبيط البروتيوزوم وعالجنا خلايا سرطان القولون بمستخلص السماق، ووجدنا أن مستويات البروتينات عادت إلى مستويات تشبه المجموعة الضابطة من الخلايا والتي بها نقارن الخلايا المعالجة. كما أن استخدام مثبط البروتيوزوم أدى إلى التقليل من موت الخلايا كما منع حدوث

عمليتي الالتهام الذاتي وموت الخلايا المبرمج. ووجدنا أيضاً أن انخفاض مستوى بروتين mTOR، كان مصاحبًا للزيادة الكلية في مستوى بروتينات ubiquitin والتي تعمل على توجيه البروتينات للتحليل بواسطة البروتيوزوم.

في الختام، فإن هذه النتائج الأولية من البحث تلقي الضوء على نبات السماق وتجعله مرشحًا واعداً للعلاج والوقاية ضد سرطان الثدي وسرطان القولون.

مفاهيم البحث الرئيسية: نبات السماق، سرطان الثدي، سرطان القولون، الموت المبرمج للخلايا، الالتهام الذاتي للخلايا، البروتيزوم.

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Last but not least my parents and siblings, who never seized to help me along the way I am deeply grateful. In addition, special thanks are extended to all my friends for supporting and encouraging me and among all of them is Safeya Alhamoudi for being there through good times and bad supporting me and being by my side always.

Dedication

To my beloved parents and family

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List of Abbreviations

ATG	Autophagy associated protein
ECM	Extracellular Matrix
EMT	Epithelial mesenchymal transition
ER	Estrogen Receptors
HER2	Human Epidermal Growth Factor-2
MMP	Matrix Metalloprotease
PARP	Poly (ADP-ribose) Polymerase
PR	Progesterone Receptor
RCE	*Rhus coriaria* Extract
TEM	Transendothelial migration
TNBC	Triple Negative Breast Cancer
TNF-a	Tumor Necrosis Factor - a
Ub	Ubiquitin
UPS	Ubiquitin Proteasome System
UV	Ultra Violet
VEGF	Vascular Endothelial Growth Factor

Chapter 1: Introduction

1.1 Cancer

1.1.1 A brief history of cancer

Cancer has been known to humanity since ancient times. Cancer develops when cells in a specific part of the body turn to grow out of control. One of the primary proofs of the existence of cancer was revealed in fossilized bone tumors in human mummies in ancient Egypt. As it was documented in ancient manuscripts, bony skull damage was seen in the head and neck. The disease was firstly entitled cancer by the Greek physician Hippocrates, father of medicine, who used Greek words “carcinoma” and “Karakinos” to describe a tumor; these terms were used to describe crab movement [1-3].

1.1.2 Epidemiology

Cancer continues to be a health burden globally; it is the second leading cause of death accounting to an estimated 9.6 million deaths in 2018 [4]. Cancer is a multifactorial disease that is characterized by uncontrolled cellular division, invasion and spreading of those cells from their primary site to other sites in the body to establish new colonies of cancer cells [5].

1.1.3 Hallmarks of cancer

Cancer cells are distinguished from normal cells by gaining specific hallmarks. These hallmarks include acquiring autonomous growth by secreting their own signals and growth factors to maintain their proliferation state. Moreover, cancer cells can

escape inhibitory signals that might otherwise stop their growth. In addition, evading apoptosis by escaping the regulation of the tumor suppressor genes and enabling unlimited replicative potential through upregulation of oncogenes. Additionally, inducing angiogenesis which is the process of formating new blood vessels to get nutrient supply that will promote their tumorigenesis. Cancer also invade local tissues and migrates to distant organs in a process called metastasis [6]. Emerging hallmarks of cancer were also described; these hallmarks include genome instability and mutation, promoting inflammation, avoiding immune destruction and reprogramming energy metabolism [7].

1.1.4 Classification of cancer

Cancers are very diverse; more than one hundred different types of cancers have been identified. According to the type of tissue in which they originate, cancers were classified into five main groups, which are carcinoma, sarcoma, myeloma, leukemia and lymphoma. Carcinoma starts in epithelial tissues, while sarcoma is found in mesoderm derived cells such as bone and muscle. Myeloma originates in the plasma cells of bone marrow. Moreover, leukemia is a cancer that is found in the bone marrow and lymphoma develops in the glands or nodes of the lymphatic system [8, 9].

1.1.5 Causes of cancer

While the real cause of cancer is still unknown, many factors have been associated with cancer. These factors include genetic mutations in tumor suppressor genes and oncogenes. Tumor suppressor genes are present in the cells to promote cell death and suppress cell division such as tumor protein 53 (P53) and retinoblastoma protein (RB), while oncogenes are important in promoting cell division and

proliferation such as c-myc and Ras. Life style can also be a risk factor such as smoking, UV radiation and obesity. Chemical agents that can cause cancer are called carcinogens and these includes cadmium, arsenic, nitrosamines and aflatoxins. Viruses can also lead to cancer development such as human papillomavirus which can cause cervical cancer, Helicobacter pylori virus which can cause gastric cancer and hepatitis virus B which can cause hepatocellular carcinoma [8].

1.2 Breast cancer

Breast cancer remains one of the most common cancers as well as one of the leading causes of worldwide cancer-related mortality. It is the second most common cancer worldwide accounting for 2.09 million cases in 2018 and it is the fifth most common cause of cancer-related deaths accounting for 627,000 deaths in 2018 [10]. There are a number of factors correlated with an increased risk of breast cancer such as age, family history, exposure to radiation [5] and lifestyle [6] .

Breast cancers are heterogeneous and diverse group of diseases that come with 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 [11, 12]. Breast tissues are made up mainly of lobules, ducts and stroma. From these different types of breast cells breast cancer originates [13, 14]. Breast cancers can be classified according to their histopathology and protein profile and gene expression [15].

1.2.1 Histopathological classification of breast cancer

Breast cancers can be classified according to their histopathological characteristics into lobular neoplasia, ductal carcinoma *in situ* and invasive ductal carcinoma. Lobular neoplasia is composed of noninvasive lesions, such as atypical lobular hyperplasia, lobular carcinoma *in situ* and invasive lobular carcinoma. Ductal carcinoma *in situ* is characterized by the proliferation of malignant cells within the ducts without invasion of the surrounding stromal tissue. Finally, the invasive ductal carcinoma which is the most common invasive carcinoma of the breast [16-18].

1.2.2 Molecular classification of breast cancer

The molecular classification of breast cancer is based on examining the alterations of gene expression that drive cancer. This is important as it has prognostic significances beyond the traditional prognostic indexes and can aid in the determination of the most suitable treatment for the individual. Using hierarchical cluster analysis; breast cancers can be classified into five molecular subtypes: luminal A and luminal B, basal-like, human epidermal growth factor receptor 2 (HER2) overexpression, and normal breast-like [11, 19, 20].

1.2.3 Triple negative breast cancers

Triple negative breast cancer (TNBC) represents a heterogeneous subtype of breast cancers that belongs mainly to the basal-like breast cancers and is associated with an aggressive clinical conditions, where targeted therapies are currently limited [21]. TNBC is a diagnosis of exclusion since those cells are known to lack the expression of estrogen receptor (ER), progesterone receptor (PR) and human

epidermal growth factor receptor (HER2) [22]. TNBC is considered to be highly aggressive and have high proliferative index compared to other breast cancers [23]. Moreover, TNBC is characterized by distinctive patterns of metastasis which usually include brain, lung and bone metastasis [24]. Additionally, they have poor prognosis and relapse very quickly compared to other breast cancers [25]. Some of the TNBCs are known to have BRCA1 mutations. Mutations in BRCA1, a gene that is essential for DNA repair mechanism, accumulates DNA errors and causes genetic instability which could lead to tumor growth [26].

1.3 Colorectal cancer

Colorectal cancer is the third most common cancer globally where 1.80 million cases were recorded in 2018 [10]. It is the second leading cause of cancer related deaths where 862,000 deaths were documented in 2018 [10]. Colorectal cancer affects both sexes equally with poor survival rate once it metastasizes [27].

Colorectal cancer is a complex disease; it starts growing in the lining of the colon and the rectum in a form of a polyp, which is a non-cancerous mass bulging in the lumen. It is worth mentioning that not all polyps will develop into cancer [28]. During the development of colorectal adenocarcinoma, gastrointestinal epithelial cells acquire consecutive genetic and epigenetic mutations in oncogenes and tumor suppressor genes; these mutations in some cases might give the cells proliferative and self-renewal abilities. Therefore, the transitioning epithelium cells become hyper-proliferative which develops into a benign adenoma that might evolve into malignant carcinoma that can spread and metastasize forming new tumor colonies in neighboring organs [29].

1.3.1 Risk factors

Genetic and environmental factors are crucial in the etiology of colorectal cancer. A subgroup of colorectal cancer patients is affected by a hereditary colorectal cancer syndrome. Lynch syndrome is the most common syndrome which is caused by a mutation in one of the genes that are crucial in DNA mismatch-repair such as: *MLH1*, *MSH2*, *MSH6*, *PMS2* or *EPCAM*. Errors in mismatch repair mechanism during replication gives rise to the accumulation of DNA mutations [27].

Colorectal cancer has seen an increase in incidents that now it became one of the predominant cancers. A range of environmental lifestyle factors influence the risk of developing colorectal cancer such as aging, poor diet and lifestyle, smoking, low rate of physical activities and obesity [30].

1.3.2 Mechanisms and pathophysiology of colorectal cancer

Colorectal cancer develops when the previous mentioned risk factors promote the acquisition of cancer hallmarks in colon epithelial cells. One possible way is through the sequential and progressive accumulation of genetic mutations and epigenetic alterations that aid in the activation of oncogenes and inactivation of tumor suppressor genes [7, 31]. However, the majority of colorectal cancer follows what is known as the classic model of formation, in which they arise from polyps that if left unchecked might develop into an early adenoma which is less than 1 cm in size, with tubular histology. Then the adenoma might obtain enough hallmarks to progress to an advanced adenoma which is roughly the same size as the early adenoma, but, with villous histology, before they are finally and fully becoming colorectal cancer. The process from polyp formation to the development of colorectal cancer might take

between 10 to 15 years [32]. Other types of colorectal cancer have been shown to evolve from a subset of polyps called sessile serrated polyps [33] and are classified into three categories: hyperplastic polyps, sessile serrated adenomas and traditional serrated adenomas [34]. Sessile serrated polyps have the ability to transform into colorectal cancer through the following sequence: hyperplastic polyp to sessile serrated polyp to adenocarcinoma [35].

1.3.3 Molecular classification of colorectal cancer

Colorectal cancer can be classified into four different subgroups according to their molecular features; these groups are hypermutable microsatellite stable, hypermutable microsatellite unstable, microsatellite stable or chromosome unstable, and CpG island methylator phenotype cancers. Mutations between the molecular subclasses can differ dramatically which suggest that each subclass has its own set of cooperating drivers [36]. Mutations as the ones that present in APC and SMAD4, are common among all the molecular subgroups, while there are other mutations that are restricted to one subgroup [37]. Not only genetic mutations occur in colorectal cancer, but also epigenetic mutations which occur in polyps and colorectal cancer and seem to cooperate in driving the polyp to develop into a cancer prototype. An example of an epigenetic modification is the DNA methylation of CpG islands that can result in transcriptional silencing. Aberrant gene methylation seems to increase most significantly during the progression of early adenoma to advanced carcinoma [38].

1.4 Molecular targets in cancer treatment

1.4.1 Apoptosis

Apoptosis is a tightly orchestrated multi-step pathway where cells commit to self-suicide. This mechanism of programmed cell death is extremely crucial during development; however, it is also important in adult multicellular organisms' homeostasis. Key characteristics of apoptosis are cellular shrinkage, condensation of the nucleus and DNA fragmentation [39, 40]. Cells that undergo apoptosis initially become rounded and retracted from neighboring cells which is accompanied by plasma membrane blebbing [41, 42]. A dominant signal of apoptosis is the translocation of phosphatidylserine from the inner to the outer side of the plasma membrane. This 'eat-me' signal functions as a recognition signal for phagocytic cells to engulf apoptotic cells [43]. Apoptosis occur in a controlled manner to minimize damage and disruption to neighboring cells [39]. Apoptosis is orchestrated primarily by members of cysteine proteases family known as caspases [44]. Apoptosis can be initiated through one of two pathways; the extrinsic and the intrinsic pathways.

1.4.1.1 Apoptotic signaling pathways

1.4.1.1.1 Extrinsic pathway

The extrinsic pathway requires external stimulation; this will cause the extracellular death ligands such as tumor necrosis factor-α (TNF-α) or FasL to bind to transmembrane death receptors. Binding of death receptors with their corresponding ligands provokes the recruitment of adaptor proteins, such as the Fas-associated death domain protein (FADD), which in turn recruits caspase-8 and promotes its activation.

Active caspase-8 proteolytically activates caspase-3 and -7, which cause further caspase activation events that results in substrate proteolysis and cell death [42].

1.4.1.1.2 Intrinsic pathway

The intrinsic pathway is engaged by a wide array of stimuli that are sensed intracellularly, including cytokine deprivation, DNA damage and endoplasmic reticulum stress. These stimuli activate BH3-only family members, which inhibit the pro-survival BCL-2-like proteins, thereby enabling the activation of the pro-apoptotic effectors BAX and BAK, which then disrupt the mitochondrial outer membrane and cause the release of cytochrome c. Cytochrome c promotes caspase-9 activation on the scaffold protein apoptotic protease activating factor 1 (APAF1) which propagates a proteolytic cascade of further caspase activation events [45].

1.4.1.2 Apoptosis in cancer

Apoptosis is widely considered as a positive process that both prevents and treats cancer. Undoubtedly, having a beneficial role, apoptosis can also cause unwanted effects that may even promote cancer. For instance, apoptotic cells release different ‘eat-me’ and ‘find-me’ molecules to signal their removal by phagocytes. These signals can have various tumorigenic effects, including turning tumor-associated macrophages towards a pro-oncogenic state. Additionally, death receptors can function as promoting growth, invasion and survival of cells. Beyond defining the dark side of apoptosis, some of these oncogenic effects also offer promising potential to fight cancer through understanding how cancer cells tolerate failed apoptosis and survive, which could provide new strategies to sabotage this process to kill cancer cells [46].

1.4.2 Autophagy

Lysosomes were first discovered and named by Christian de Duve and his group in 1955 [47] and for that he earned a Nobel Prize in physiology or medicine in 1974 [48]. De Duve also called the delivery of intracellular material to the lysosome "autophagy" as early as 1963, and he studied the regulation of autophagy by nutrient availability [49]. Nevertheless, the mechanism of lysosomal delivery remained unknown and research on autophagy did not receive much attention for more than 30 years. After that Yoshinori Ohsumi's group conducted a genetic screening to dissect autophagy process in yeast, and they identified 15 autophagy- related proteins (ATGs) essential for autophagy process [50]. In 2016, Ohsumi was awarded a Nobel Prize in physiology or medicine for his discovery of mechanisms of autophagy [51].

Autophagy is a highly regulated cellular process that can either result in the degradation of proteins or it can specifically target distinct organelles i.e. mitochondria in mitophagy and the endoplasmic reticulum in reticulophagy [52]. Autophagy starts with the engulfment of damaged or unnecessary cellular content into a double-membrane vesicle named autophagosome. Autophagosome is transported and fused with the lysosome to form single-membrane autolysosome, in which the content would be degraded and recycled [53]. Fundamentally, autophagy is a cellular survival mechanism, where it mediates the turnover of protein aggregates that otherwise might lead to cellular dysfunction. Nevertheless, if the cell acquired too many faults that exceed its ability to salvage, then the cell could die due to autophagy destroying large proportions of the cytoplasm that would result in an irreversible cellular atrophy; consequently, collapse of crucial cellular functions and cell death [54, 55]. Autophagy occurs in several steps that are depicted in Figure 1.

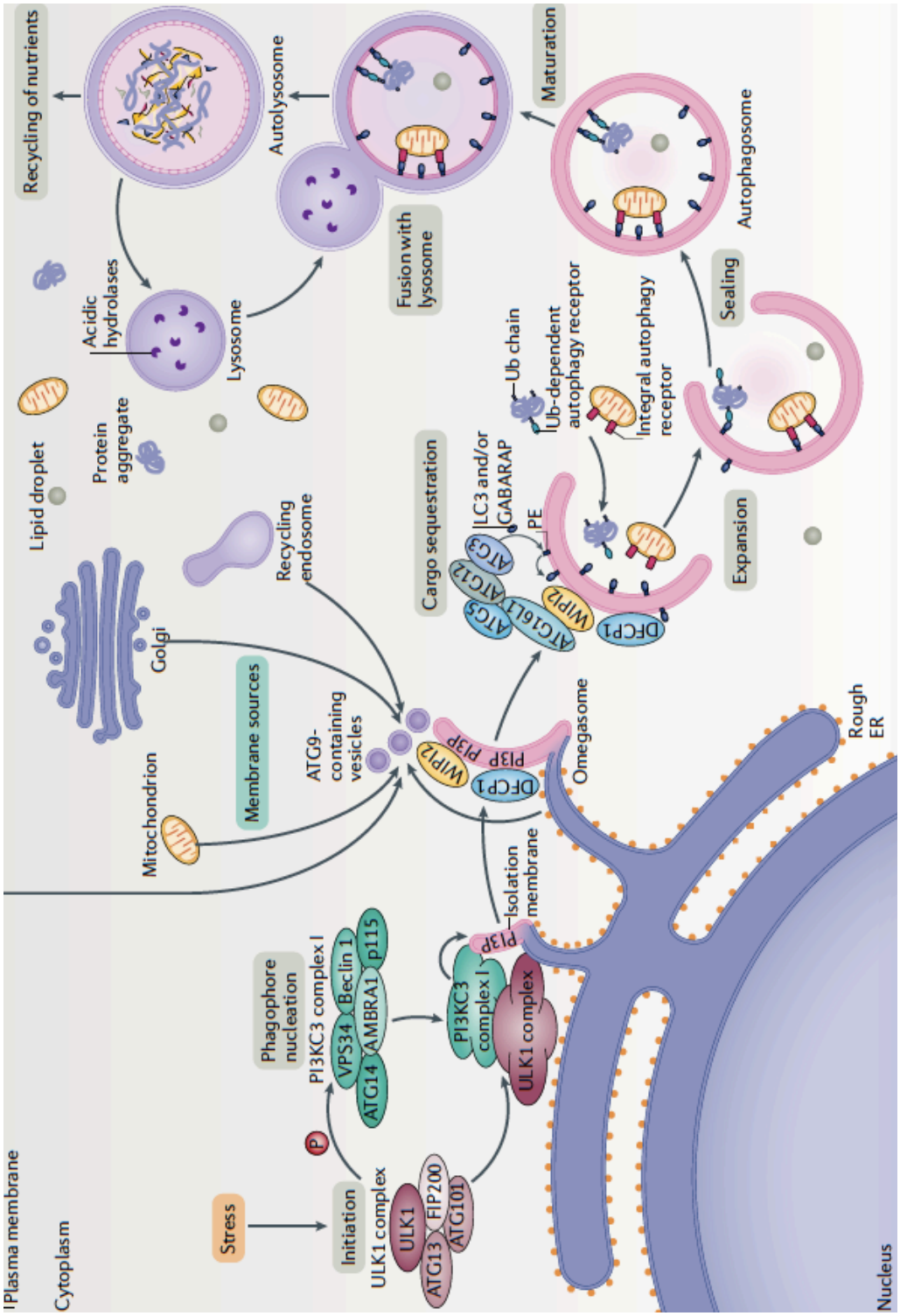


Figure 1: Overview of autophagy process [56]

1.4.2.1 Autophagic pathway

1.4.2.1.1 Initiation

Signals that activate autophagy originate from different conditions of stress, such as starvation, oxidative stress, hypoxia and protein aggregation [56]. The initiation of autophagic process requires the ULK complex [57]. Under nourished conditions, the ULK complex is bound to the mammalian target of rapamycin complex 1 (mTORC1) and is, thus, inactive. However, upon amino acid starvation, the mTORC1 becomes inactive and dissociates from the ULK complex, which results in increased ULK1 and ULK2 kinase activity. The carboxy-terminal domain of ULK1 and ULK2 binds to the cellular membranes which is thought to mediate the recruitment of the complex to the site of autophagosome initiation [58].

1.4.2.1.2 Nucleation

Once activated and targeted to the site of autophagosome initiation, phagophore nucleation starts by the activation of Beclin-1 complex through its phosphorylation. Beclin-1 complex consist of class III PI3K, vacuolar protein sorting 34 (VPS34), Beclin-1, ATG14, activating molecule in Beclin-1-regulated autophagy protein 1 (AMBRA1) and general vesicular transport factor (p115) [59, 60]. ULK1 was shown to phosphorylate VPS34 which enhances the activity of the PI3K complex. This will lead to the activation of local phosphatidylinositol-3-phosphate (PI3P) production at a characteristic endoplasmic reticulum structure called the omegasome. These previous events drive the nucleation of the phagophore membrane and the recruitment of additional ATG proteins and autophagy-specific PI3P effectors, such as

double FYVE-containing protein 1 (DFCP1) and WD-repeat domain phosphoinositide-interacting proteins (WIPI) [56].

1.4.2.1.3 Expansion

Following nucleation, WIPI2 binds to ATG16L1 directly; thus, recruiting the ATG12~ATG5–ATG16L1 complex that enhances the ATG3-mediated conjugation of ATG8 family proteins. These proteins include microtubule- associated protein light chain 3 (LC3) proteins and γ- aminobutyric acid receptor- associated proteins (GABARAPs) to membrane- resident phosphatidylethanolamine (PE). This enables them to associate with the autophagosomal membrane in lipidized forms, therefore, the cytosolic LC3-I will be converted into the lipidized LC3-II which is a characteristic signature of autophagic membranes. The association of these cytosolic proteins and protein complexes with the membrane occurs while the isolation membrane is expanding [61]. Several cellular membranes, including the plasma membrane, mitochondria, recycling endosomes and the golgi complex, contribute to the elongation of the autophagosomal membrane by donating membrane material [56].

1.4.2.1.4 Cargo sequestering

During phagophore expansion, LC3-II binds to the adaptor protein p62/sequestosome1 (P62/SQSTM1), which is involved in trafficking proteins to the proteasome and serves to facilitate the autophagic degradation of ubiquitinated protein aggregates [62]. The p62/SQSTM1 is normally degraded during autophagy and accumulates when autophagy is impaired, as has been shown in autophagy-deficient mice [63]. Aside from their contribution to phagophore expansion, ATG8 also facilitate cargo recruitment in selective autophagy and LC3-II is critically involved in

the sequestration of specifically labelled cargo into autophagosomes via LC3-II interacting region that contains cargo receptors which themselves recognize the cargo through ‘eat- me’ signals such as ubiquitin (Ub) or galectins [56].

1.4.2.1.5 Sealing

Before the closure of the phagophore, the ATG proteins that are bound to the membrane dissociate, but LC3-II and its family members remain attached to the membrane. LC3-II remains bound after the closure of the phagophore to the inner membrane of the autophagosome [64]. LC3-II family members are thought to help in the expansion and closure of the phagophore and their retention inside the closed autophagosome provides an important and widely used marker for identifying autophagosomes in cells [65].

1.4.2.1.6 Maturation and fusion

Autophagosomes undergo maturation by fusion with lysosomes to form autolysosomes. After expansion and sealing of the phagophore, the machinery responsible for lysosomal delivery which consists of microtubule- based kinesin motors is recruited. Moreover, the machineries that mediate fusion with the lysosome are recruited as well and those are: SNAREs (syntaxin 17 (STX17) and synaptosomal-associated protein 29 (SNAP29)) on the autophagosome, vesicle associated membrane protein 8 (VAMP8) on the lysosome, and the homotypic fusion and protein sorting (HOPS) complex which mediates membrane tethering to support SNARE- mediated fusion. ATG8 drives maturation by linking the autophagosome to kinesins through autophagy- specific kinesin adaptors [66, 67]. Post- translational modifications of ATG8 further regulate autophagosome maturation, as the phosphorylation of LC3 on

residue Thr50 by the Ste20 Hippo kinase orthologues serine/threonine protein kinase 3 (STK3) and STK4 was recently found to be essential for autophagosome–lysosome fusion and for clearance of intracellular bacteria by autophagy. However, these processes are still under investigation and need further characterization [68].

In the autolysosomes, the inner membrane and the luminal content of the autophagic cargo are degraded by acidic hydrolases and recycled nutrients are released back to the cytoplasm to be used again by the cell [69].

1.4.2.2 Autophagy role in cancer

The role of autophagy in cancer is complex and may differ depending on the tumor type and conditions. It is suggested that upregulation of autophagy can either be a cell protective mechanism or an alternative cell death mechanism. As a cytoprotective function; it is believed that autophagy has tumor- suppressive potential before the onset of tumorigenesis, and loss of autophagy has been associated with increased risk of cancer as autophagy protects cells from the genotoxic stress that can lead to oncogenic transformation [70, 71]. However, some investigators have hypothesized that once the tumor has formed, it will use autophagy process as a survival mechanism to overcome the stresses imposed during cancer progression, as well as those caused by radiation or chemotherapy [72]. Other researchers have suggested that autophagy might suppress tumorigenesis by inducing cell death [73].

Therefore, depending on the type of tumor and its developmental stage, activation or inactivation of autophagy can contribute differently to tumorigenesis. A better understanding of the autophagy in tumor models is crucial in identifying new and effective therapeutic strategies for cancer treatment.

1.4.3 Ubiquitin proteasome system

The ubiquitin proteasome system (UPS) is a highly regulated and extremely selective cellular mechanism of protein degradation. The degradation of most nuclear and cytosolic proteins is the responsibility of the UPS, including misfolded proteins, short-lived as well as long-lived proteins. Protein degradation by the UPS is highly precise, involving the concerted functions of series of enzymes. Proteins are marked for degradation by the attachment of a Ub chain to them [74, 75].

The UPS degrades a massive variety of proteins that contain specific degradation signals, or degrons. A degron is defined as a minimal element within a protein that is sufficient for recognition and degradation by the proteasome. To allow substrate unfolding and translocation into the proteasome, degrons require specific E3-binding determinants, an appropriate Ub modification site and a proteasomal degradation initiation site. The most common acceptor site for polyubiquitin chain addition is on the lysine. Degron activity is regulated in many ways. One way is through post-translational modifications which activate many degrons such as protein phosphorylation, hydroxylation and proteolytic cleavage [76].

Ubiquitination is recognized by receptors on the proteasome. Proteins are unfolded and passed into the catalytic chamber for digestion. Proteolysis is irreversible, making it a powerful mechanism for imposing directionality on a system, as shown by its use in controlling the cell cycle [77].

1.4.3.1 The mechanism of UPS

The initiation of proteins degradation starts by attaching those proteins to polymers of the highly conserved Ub protein which are firstly activated by Ub-

activating enzyme E1. This covalent modification targets the conjugated protein to the 26S proteasome, a protease complex. The attachment of Ub commonly occur on Lysine side chain of the protein. The activated Ub is then transferred to the active site Cystine residue in the second protein, an E2 Ub-conjugating enzyme. With the aid of Ub-protein ligase E3, E2 catalyzes the ligation of the polyubiquitin chain onto the protein that is destined for degradation. The polyubiquitinated substrate can bind directly to the Ub receptors in the 26S proteasome or to adaptor proteins that contain polyubiquitin-binding domain and proteasome-binding domain. After binding, protein unfolding occurs and the polyubiquitin chain is removed by proteasome-associated de-ubiquitylating enzymes (DUBs). DUBs is important in maintaining a sufficient pool of free Ub molecules inside the cell and they translocate the unfolded protein to the central proteolytic chamber, where the targeted protein will be cleaved into shorter peptides [76, 78, 79].

1.4.3.2 Spatial control of proteolysis

Some substrates are only recognized at particular locations in the cell by the UPS. This is because the UPS components localize to specific places in the cell, or it is because the substrates can only be modified in a specific compartment. Localized proteolysis is difficult to be measured directly, therefore, in most cases spatial control is inferred from the localizations of substrate and UPS constituents. E3 are most often localized to distinct subcellular structures or compartments; E1, Ub, most E2s and the proteasomes are more uniformly distributed. Evidence also indicates that the E3s carry out the rate-limiting step in Ub-mediated proteolysis and may bind and recruit proteasomes to the substrate. Therefore, analyzing E3 behavior should provide insights into how the proteolysis of substrates is controlled [80].

1.4.4 Metastasis

At early stages of tumor progression, cancer cells multiply near the site of origin; this results in a primary tumor where cancer cells proliferate and expand within the organ of origin. These primary tumors correspond approximately to 10% of cancer deaths, while the remaining 90% of deaths were found in patients that have metastasized tumors. Metastasis is the process by which tumor cells from a primary site invade and migrate to other parts of the body [5, 81].

Cell migration depends on the type of malignant tumor and the neighboring tissue and is defined by distinct patterns in the activity of extracellular proteases, matrix-cell adhesion mediated by integrins, cell-cell adhesion mediated by cadherins, cellular polarity and cytoskeletal arrangements [82].

Tumor cells undergo major steps during metastasis which are invasion, intravasation, transport through the blood stream, extravasation and metastatic colonization [8]. Therefore, better understanding of the molecular mechanisms of metastasis is important to develop new treatments and prevention strategies against cancer.

1.4.4.1 Invasion

Invasive ability of cancer cells is considered as key features of metastatic cascade. In order for cells of primary tumor to invade surrounding tissue, they must escape from the normal molecular restrictions that link nearby cells to each other. Thus, these tumors cells need to remodel their cell-matrix and cell-cell adhesion interactions to gain invasive capabilities [5]. This occur through Epithelial–mesenchymal transition (EMT) which is a reversible cellular process that converts epithelial cells

into mesenchymal cell transiently [83]. During EMT, epithelial cells progressively lose their brick epithelial shape in monolayer cultures and convert into a spindle-shaped, mesenchymal morphology cells. Remodeling of cell–cell and cell–extracellular matrix (ECM) interactions occur during EMT, which leads to the detachment of epithelial cells from each other and the underlying basement membrane and their migration by activating a new transcriptional program [84, 85]. E-cadherins are cell adhesion molecules that are crucial to hold epithelial cells together through tight junctions. When EMT is activated, the expression of E- cadherin is repressed, which leads to the loss of the typical epithelial morphology. The cells acquire a spindle- shaped mesenchymal morphology and express markers such as N- cadherin, vimentin and fibronectin which are associated with the mesenchymal cell state [84].

Invasion of cancer cells into adjacent tissues requires the action of several hydrolytic enzymes or proteases, which are released either by cells around the tumor or by the tumor cells themselves [86]. Matrix metalloproteinases (MMPs) and serine proteases are two families of proteolytic enzymes implicated in metastasis [87]. MMPs production is induced via a protein named ECM metalloprotease inducer (EMMPRIN). These MMPs act on degrading the structural components of ECM and basement membrane and cleave other proteins found outside the cells, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) [88].

1.4.4.2 Intravasation

To intravasate, tumor cells need to invade the ECM towards blood vessels. Tumors induce local angiogenesis, and these new blood vessels have weak cell–cell junctions through which cancer cells can enter the vascular system [89]. Factors such as transforming growth factor-β (TGFβ) or VEGF, reduce endothelial barrier function;

thus, increasing the number of cancer cells entering into blood vessels and increase metastasis [90].

1.4.4.3 Transport

Circulation and transport of tumor cells through the bloodstream and lymphatic vessels flows in one direction [8]. Upon entering the bloodstream, tumor cells are exposed to shearing forces and interactions that might cause their destruction. However, cancer cells are able of resisting this destruction by attaching to the endothelial cells of blood vessels and thereby protecting themselves from the immune system [91].

1.4.4.4 Extravasation

The attachment of tumor cells to endothelial cells is the first step of the extravasation process and is followed by transendothelial migration (TEM). Indeed, tumor cells do not damage or induce vascular leak at the site of extravasation; however, they induce local vessel remodeling [92]. Adhesion of tumor cells to the endothelium requires the expression of ligands and their receptors on tumor cells and endothelial cells. Ligands and receptors contributing to this process include selectins, integrins, cadherins, CD44 and immunoglobulin superfamily receptors [93]. Chemokines, as well, have important roles in regulating extravasation [94].

1.4.4.5 Colonization

During cancer progression, tumor colonization in a secondary organ marks the difference between a possibly curable tumor and a generally incurable disease [95]. Primary tumor secretes growth factors to direct progenitor hematopoietic cells from

bone marrow toward the bloodstream to the site of metastasis. By this, a preparation of a premetastatic microenvironment occurs even before the arrival of the tumor cells [96, 97]. Steps of metastasis process are depicted in Figure 2.

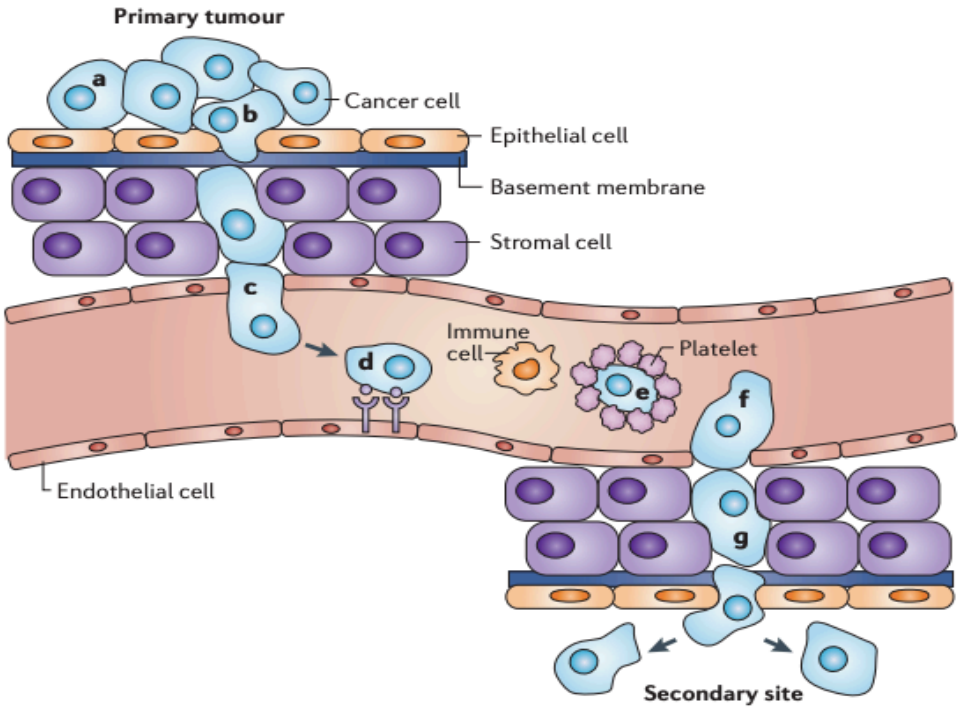


Figure 2: Schematic diagram of cancer metastasis [98]

1.4.5 Angiogenesis

Like normal organs, tumors also need to have their blood supply to satisfy their need for nutrients and oxygen and other metabolic functions [99]. This is achieved through angiogenesis which is the process of developing new blood vessels from a pre-existing vascular network [100]. The regulation of angiogenesis depends on a net balance between anti-angiogenic factors and pro-angiogenic factors. However, in cancer, the balance is tilted toward angiogenic factors to drive vascular growth [101].

Pro-angiogenic factors involve VEGF, TNF-α and other factors that are secreted from tumor cells or mobilized from the ECM [8].

1.5 Therapy

Many types of cancer treatment are used -independently or combined- depending on the type of cancer and how advanced it is. Surgery is the most common type of therapy for colorectal cancer and breast cancer where the tumor alongside with some of the healthy tissues are removed [8]. Radiation therapy exerts its effect by damaging DNA of all cells in the body, while healthy cells can repair the damage, cancer cells will accumulate DNA damage that would eventually be lethal to them [8]. Chemotherapy targets fast dividing cells aiming at inhibiting mitosis and cellular growth and by that stopping the progression of cancer [8]. Although great advancements in cancer treatment and control have been achieved, the undesired side effects that are accompanied by such treatments have serious effects on the health of the person. Therefore, alternative therapies that include less toxic and more potent anticancer drug are needed to be developed [102, 103].

1.5.1 Targeted therapy

Targeted therapy refers to major modalities of medical treatment that block the growth and metastasis of cancer by targeting specific molecules that are critical for cancer growth and survival. Different from traditional chemotherapy, targeted therapy focuses on molecular abnormalities specific to cancer which make it more effective than chemotherapy and radiotherapy and less harmful to normal cells [104]. Targeted cancer therapy was first recognized in 1998, when trastuzumab, a monoclonal antibody against HER2, was approved by the FDA for treating patients with HER2- positive

metastatic breast cancer [105]. In 2001, imatinib, which was the first designed small-molecule inhibitor that targets constitutively activated Bcr-Abl, was approved for the treatment of chronic myeloid leukemia [106]. Since then, over 30 targeted drugs were approved for clinical use, either alone or in combination with chemotherapy for treating different human cancers which made a revolution in anti-cancer drug development [104]. Nonetheless, targeted therapy remains challenged because of the extremely small proportion of patients that can benefit from it and the high failure.

1.5.2 Plant therapy

For thousands of years, mankind looked to plants for utilizing their medicine. Plants were used starting from the leaves to the roots, and they were extracted as crude extracts such as tinctures, teas, powders and other forms of formulations. The use of plants for medicinal purposes kept evolving throughout history, in the early 19th century active compounds were isolated and purified, beginning with the purification of morphine from opium. Medicinal plants are a rich source of a wide variety of active compounds referred to phytochemicals which can offer a lot of possibilities in the development of drugs to treat several diseases including cancer [103, 107].

An ideal phytochemical is one that possesses anti-tumor properties with minimal or no toxicity and has a defined mechanism of action. Hence, identification and development of new chemotherapeutic agents from plants have gained significant recognition in the field of cancer therapy and become a major area of experimental cancer research especially in developed countries where they have considerably improved quality of the herbal medicines used in the treatment of cancer. Recently, scientists all over the world are concentrating on the herbal medicines to fight against cancer [108].

Moreover, plant derived drugs have been used in cancer treatment such as Taxol that is isolated from the bark of *Taxus brevifolia* Nutt [109], Camptothecin isolated from the Chinese *Camptotheca acuminata* Decne [110], Combretastatin isolated from *Combretum caffrum* [111] and Vinblastine and Vincristine that are isolated from *Catharanthus roseus* [112]. Several other types of promising bioactive compounds of plant origin are currently in clinical trials or preclinical trials or undergoing further investigation [113].

By understanding the complex synergistic interaction of various constituents of anti-cancer herbs, new novel herbal anti-cancer agents can be discovered and designed to attack the cancerous cells without affecting normal cells of the body.

1.5.2.1 *Rhus coriaria*

Rhus coriaria, which is commonly known as sumac, is a Mediterranean shrub that belongs to the Anacardiaceae family and traditionally has been used as a spices and flavoring agents [114]. Sumac is a shrub with height range of 3-4 meters, pinnate leaves are in pairs of 6 or 8 small leaflet, and with cluster of white flowers in terminal. The fruits are spherical and become reddish drupe when ripe [115].

1.5.2.1.1 Chemical composition of *Rhus coriaria*

Rhus coriaria plant is rich in phytochemical compounds. This was identified through many studies that were conducted in order to identify the chemical compounds present in *Rhus coriaria* plant in a process to link them to different biological activities. One of the earliest researches conducted on this matter was in 1896 when Perken et al., identified myricetin as the coloring agent which was detected in the extract leaves of the Italian sumac and they identified gallic acid to

be present in the extract as well [116]. Since then, researchers continued on this road by investigating the other chemical compositions of different parts of the sumac organs. This was up to 2014 when Abu-Reidah et al., carried out a comprehensive study to investigate the phytochemical components of the sumac fruit extract. They identified 211 phenolic and other phyto-constituents, most of which have been described for the first time in *Rhus coriaria* fruits [117].

1.5.2.1.2 Biological activities of *Rhus coriaria*

Rhus coriaria plant has therapeutic values and also has been used as a traditional medicine for hundreds of years which attract more attention to it recently. The biological activities of *Rhus coriaria* are often attributed to their vital compounds, these exhibited activities can be a result of the contribution of different compounds at once or the reaction of one compound only. It has been established that *Rhus coriaria* has many biological properties such as anti-bacterial activities [118, 119], anti-diabetic activities [120], anti-fungal activities[121], anti-oxidant activities [122, 123], cardioprotective activities [124-126], anti-nociceptive activities [127], neuroprotective activities [128-131] and dental protection activities [132, 133].

Chapter 2: Objectives

We have recently shown that ethanolic extract of *Rhus coriaria* extract (RCE) induces cell cycle arrest along with concomitant autophagic cell death of TNBC cells. However, whether it can modulate the metastatic phenotype of these cells remained largely obscure. Here, we sought to determine the effect of RCE on the malignant behavior of MDA-MB-231 breast cancer cells and determine the underlying mechanisms. Moreover, we extended our study and investigated the anti-cancer effect of RCE on HT-29 and Caco-2 human colorectal cancer cells.

The hypotheses of this work are stated as the following:

Hypothesis 1: RCE might inhibits breast cancer cell metastasis.

Hypothesis 2: RCE might exerts anti-cancer activities against colorectal cancer.

Chapter 3: Materials and Methods

3.1 Cell culture and reagents

Human breast cancer cells MDA-MB-231 and Human colorectal cancer cells HT-29 and Caco-2 were maintained in DMEM (Hyclone, Cramlington, UK). Media were supplemented with 10% fetal bovine serum (FBS) (Hyclone, Cramlington, UK), 100 U/ml penicillin/streptomycin (Hyclone, Cramlington, UK). MG-132 was obtained from Cell Signaling, 3-Methyl adenine (3-MA) from Millipore (Hayward, CA, USA) and Chloroquine (CQ) from Sigma-Aldrich (Saint-Quentin Fallavier, France).

Antibodies to NF-κ B, phospho-p65, Flottilin-2, p62/SQSTMI, cleaved PARP and TNF-α were obtained from Abcam (Cambridge, UK). Antibodies to P300, STAT3, pSTAT3, β-actin, goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP were obtained from Santa Cruz Biotechnology, Inc (California, USA). Antibodies against LC3, ATG5, ATG7, P27, AKT, Beclin-1, Rab9, Ubiquitin, mTOR, phospho-mTOR (Ser 2448), caspase-3, caspase-7, p53, Beclin-1 and control siRNA were obtained from Cell Signaling (Massachusetts, USA). Those against cleaved caspase 3, Cyclin D1, HIF-1α, Acetyl H3, Acetyl H4, and phospho-AKT (Ser 473) were obtained from Millipore (Hayward, CA, USA). Antibody against PARP (full-length and cleaved) was purchased from BD Pharmingen (New jersey, USA).

3.2 Preparation of the RCE

Fruits of *Rhus coriaria* were collected from a private farm located in Ma'rakeh, Tyre, Lebanon after the approval of its owner. The plant is neither

endangered nor protected by any laws and it is readily and commercially available in the market. Ten grams of the dried fruit of *Rhu coriaria* were ground to a fine powder. The powder was then suspended in 100 mL of 70% ethanol and the mixture was kept in the dark for 72 h at 4 °C. Then, the mixture was then filtered, and the filtrate was evaporated to dryness at room temperature. The red residue was kept under vacuum for 2–3 h and its mass was recorded. The residue was stored at −20 °C until further use.

3.3 Matrigel invasion assay

To test invasion in MDA-MB-231 cells, BD matrigel Invasion Chamber (8-μ m pore size; BD Biosciences, Bedfrord, MA, USA) was used according to manufacturer's instructions. In brief, MDA-MB-231 cells (1×10^5 /well) were placed in 0.5 mL of media that contains 0.2% ethanol as control or RCE (200μg/mL) and then were seeded into the upper chambers of the system; while the bottom wells were filled with media supplemented with 10% FBS as a chemo-attractant and then incubated at 37 °C for 24 h. Non-penetrating cells were removed from the upper surface of the filter. Cells that have migrated though the matrigel were fixed with 4% formaldehyde and stained with DAPI nuclear stain. DAPI fluorescence was detected by a filter with excitation wavelength of 330–380 nm and barrier filter of 400 nm. Stained nuclei were counted in 10 random fields per well using inverted fluorescence microscope (Nikon Ti-U, Nikon) at X200 magnification. For quantification, the assay was done in duplicates and repeated three times.

3.4 Wound healing migration assay

MDA-MB-231 cells were grown in six-well plates until they reached confluency. A scratch was done through the confluent monolayer with a sterile plastic pipette tip. For each sample, three wounds were made. Then, the plates were washed with 1XPBS and incubated at 37 °C in fresh DMEM supplemented with 10% FBS in the presence of ethanol or different concentrations of RCE. Three arbitrary places were marked where the width of the wound was photographed with an inverted microscope (Nikon Ti-U, Nikon) at X40 magnification. The closure of the wound was determined by measuring the distance (μm) between the edges of the wound at time 0, 6 and 10 h, using the NIS-Elements BR 3.0 software (Nikon). Quantification of the distance migrated by the cells was done as follow: D = (Size of the wound at t = 0 h –size of the wound at t = 6 or 10 h).

3.5 Adhesion to fibronectin assay

96-well plates were coated with fibronectin that was dissolved in 1X PBS and incubated at 37 °C overnight. After that, plates were blocked with 3% BSA for 3 h at room temperature. MDA-MB-231 cells at density of 5×10^3/well were then seeded in growth medium and incubated for 60 minutes in the humidified incubator. Cells were then washed 1X PBS to remove non-adherent cells. Attached cells were stained with 1% crystal violet and observed under the microscope. At least five random fields were counted, and the experiment was repeated three times.

3.6 Measurement MMP-9 by ELISA

Cells were seeded in 6-well plates in the presence of 0.2% ethanol or different concentrations of RCE for 24 h. The conditioned medium was collected

and the levels of secreted MMP-9 were determined using immunoassay kits (Abcam, Cambridge, UK) according to the manufacturer's protocol. The optical density at 450 nm of each sample was measured using an AMP Platos R 496 microplate reader (AMP Diagnostics, Poland). The proteins present in the conditioned media were concentrated using the Amicon Ultra-15 protein purification and concentration column (Millipore, USA) and protein concentration was assayed using the BCA protein assay kit (Thermo Scientific, USA). Levels of MMP-9 were normalized to the total protein level in each sample. The assays were performed in triplicates and three independent experiments were performed. Data are presented as mean values ± SEM.

3.7 Transient transfection assay

MDA-MB-231 cells (1.5×10^5/well) were seeded in 12-well plates the day before transfection. Cells were then transfected with the pGL4.32[luc2P/NFκB-RE/Hygro] expression plasmid using Fugene HD according to the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, 18 h post-transfection, cells were incubated for another 24 h in fresh complete media containing increasing concentrations of RCE. Luciferase activity was measured using Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Renilla luciferase reporter was used as an internal control, to which firefly luciferase values were normalized. Experiments were repeated three times and the average of three means is represented ± SEM.

3.8 Quantitative immunoassay for VEGF

Cells (1.5×10^5/well) were seeded in 24-well plates overnight in serum-containing culture media and then, the media was replaced by serum-free media. Cells were treated with vehicle (ethanol) or indicated concentrations of RCE, and the conditioned media was collected at 24 h. The level of VEGF therein was measured using a VEGF enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The optical density at 570 nm of each sample was measured using an AMP Platos R 496 microplate reader (AMP Diagnostics, Poland). The proteins present in the conditioned media were concentrated using the Amicon Ultra-0.5 protein purification and concentration column (Millipore, USA) and protein concentration was assayed using the BCA protein assay kit (Thermo Scientific, USA). Levels of VEGF were normalized to the total protein level in each sample. The assays were performed in triplicates and three independent experiments were performed. Data are presented as mean values ± SEM.

3.9 ELISA quantification of IL-6

Cells (2×10^5/well) were cultured in a 6-well plate overnight and then serum-starved for 24 h in the presence of vehicle (ethanol) or indicated concentrations of RCE. Levels of IL-6 in the collected media were measured using ELISA quantification kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The optical density of each sample was measured using an AMP Platos R 496 microplate reader (AMP Diagnostics, Poland). The proteins present in the conditioned media were concentrated using the Amicon Ultra-0.5 protein purification and concentration column (Millipore, USA) and protein

concentration was assayed using the BCA protein assay kit (Thermo Scientific, USA). Levels of the IL-6 were normalized to the total protein level in each sample. Assays were performed in triplicates and three independent experiments were performed. Data are presented as mean values ± SEM.

3.10 Measurement of cellular viability

Cells were seeded in triplicate in 96-well plates at a density of 7,000 cells/well. 24 h later, cells were treated with or without various concentrations of RCE for different durations. Cell viability was measured with the Cell cytotoxicity assay kit (Abcam, UK) according to the manufacturer's instructions. The results are representative of an average of at least 4 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 with 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). 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.

3.11 Colony formation assay

HT-29 cells were seeded in 6-well plate at a density of 2000 cells/well and allowed to grow for 7 days to form colonies before RCE is added. The growth media was replenished every 3 days. After 1 week, various concentrations of RCE were

added in freshly added medium and the colonies were allowed to grow for 5 additional days. Colonies were photomicrographed at day 0 (colonies at day 7), three days (colonies at day 10) and 5 days (colonies at day 12) using Evos light microscope. Then, colonies were washed 3 times with PBS, fixed for 15 min with 4% formalin and stained with 0.01% crystal violet for 30 min. Colonies in each well were counted and their surface area was determined using the imageJ software. The experiment was carried in triplicate and repeated three times.

3.12 Colony formation assay in soft agar

Assays were performed in six-well plates. The lower (base) layer consisted of 1 ml 2.4% Noble agar. The base layer was overlaid with a second layer consisted of 2.9 ml growth medium, 0.3% Noble agar, and 3×10^4 HT-29 cells. Growth medium was then added, and plates incubated at 37^o C. Cells were allowed to grow to form colonies for 10 days before RCE was added. At day 13, colonies were treated with or without RCE (300 and 450 µg/mL) for 5 days. Following treatment, plates were washed twice with PBS and then colonies were fixed with 10% ice-cold methanol for 10 min and washed once with PBS. Colonies were allowed to stain for 1 h in solution containing 2% Giemsa. Colonies in each well were counted using the imageJ software. The experiment was carried in duplicate.

3.13 Detection of autophagic vacuoles

HT-29 cells ($2 \text{ X } 10^4$) were grown in 8 chambers slides (Millipore) followed by treatment with or without RCE for 24 h. Following treatment cells were washed and stained for autophagic vacuoles using the autophagy detection kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Fluorescent autophagic

vacuoles were examined under Olympus fluorescence microscope CKX 53 (Olympus).

3.14 Quantification of apoptosis by annexin V labeling

Apoptosis was examined using the Annexin V & Dead Cell kit (Millipore, Hayward, CA, USA) according to the manufacturer's instructions. Briefly, HT-29 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).

3.15 Quantification of caspase 3/7 activity

HT-29 cells were seeded at a density of 5,000 cells/well into 96-well plate in triplicate and treated with or without RCE for 48 h. Caspase 3/7 activity was measured using a luminescent caspase-Glo 3/7 assay kit (Promega Corporation, Madison, USA) following the manufacturer's instructions. Briefly, caspase reagents were added to triplicate 96 wells. The plate was mixed on an orbital shaker and incubated for 2.5 h at room temperature in the dark. Luminescent signal was measured using the GloMax Multi-detection System (Promega).

3.16 Immunofluorescence staining

HT-29 cells (2×10^4) were grown on 8 well labteck chamber slide (Becton Dickinson) for 24 h, then treated with or without RCE for 24 h. Cells were then fixed in 10% formalin solution (4% paraformaldehyde) (Sigma-Aldrich; Saint-Quentin Fallavier, France) for 5 min at room temperature followed by permeabilization in

PBS containing 0.1% Triton X-100 for 5 min at room temperature (RT). Cells were then washed three times with PBS, blocked with 5% non-fat dry milk in PBS for 30 min at RT and then incubated with the primary antibody diluted in 1% non-fat dry milk/PBS for 2 h at 37 °C. Following incubation, cells were washed three times with PBS and incubated for 45 min at RT in the presence of fluorescein-conjugated secondary antibody diluted at 1:200 in 1% nonfat dry milk/PBS. After washing with PBS, cells were mounted in Fluoroschield with DAPI (Sigma-Aldrich; Saint-Quentin Fallavier, France) and examined under Olympus fluorescence microscope CKX53 (Olympus).

3.17 Knockdown of Beclin-1

HT-29 cells (250,000) were seeded in 6-well cell culture plate in serum-containing growth media and allowed to grow to 50% confluency. Then, cells were transfected with siRNA I (100 nM) using lipofectamine 2000 transfection reagent (Invitrogen, Life technologies) as described by the manufacturer for 48 h at 37 °C in 5% CO2 before treatment for 48 h with and without 300 and 450 µg/mL RCE in fresh complete media.

3.18 RNA extraction and qRT-PCR

Total RNA from vehicle- or RCE-treated HT-29 cells were prepared using Trizol reagent (Life Technologies, Inc.) as described by the manufacturer. The expression of specific genes was determined by qRT-PCR using the GoTaq 1-Step RT-qPCR system (Promega Corporation, Madison, USA) as per the manufacturer's instructions. Amplification was carried out on the Stratagene Mx3000 P (Agilent technology). Briefly, the amplification reaction consisted of 100ng of total RNA

and 0.2 μM primers in a final volume of 25 μl reaction. GAPDH was used as an endogenous reference for normalization. Expression levels were calculated by the comparative cycle threshold method, and normalization to the control was performed. A minimum of three technical replicates was used for each sample. Primer sequences are as follow: GAPDH (Forward): 5′-cacccactcctccacctttg-3′; GAPDH (Reverse): 5′-ccaccaccctgttgctgtag-3′; mTOR (Forward): 5′-ctgggactcaaatgtgtgcagttc-3′; mTOR (Reverse): 5′-gaacaatagggtgaatgatccggg-3′; Beclin-1 (Forward): 5′-acagtggacagtttggcaca-3′; Beclin-1 (Reverse): 5′-cggcagctccttagatttgt-3; p53 (Forward): 5′-gttccgagagctgaatgagg-3′; p53 (Reverse): 5′-ttatggcgggaggtagactg-3′.

3.19 Whole cell extract and Western blotting analysis

Cells (2×10^6) were seeded in 10 cm culture dishes and cultured for 24 h before treatment. After incubation with RCE for the indicated time, cells were washed twice with ice-cold PBS, scraped, pelleted and lysed in RIPA (Pierce) buffer supplemented with protease inhibitor cocktail 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). Aliquots of 25 μg of total cell lysate were resolved onto 6–15% SDS-PAGE along with PageRuler Plus Prestained Protein Ladder (Thermo Scientific). Proteins were transferred to nitrocellulose membranes (Thermo Scientific) and blocked for 1 hour at room temperature with 5% non-fat dry milk in TBST (TBS and 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) and chemiluminescence was detected using the LiCOR C-DiGit blot scanner. Where needed, membranes were stripped in Restore Western blot stripping buffer per the manufacturer's instructions. Protein quantification was carried out using the ImageJ software.

3.20 Statistical analysis

Statistical analysis was done using SPSS version 21. Data were reported as group mean ± SEM. The data were analyzed via one-way ANOVA followed by LSD's Post-Hoc multiple comparison test. (*** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ indicate a significant difference).

Chapter 4: Results

4.1 The effect of RCE on breast cancer

4.1.1 RCE induces autophagy that is independent of ATG5 and ATG7

Previously RCE was investigated for the first time for its activity against triple negative MDA-MB-231 breast cancer cell line. It was reported that RCE inhibited the viability of MDA-MB-231 cells and induced senescence in those cells. Moreover, it induced autophagy which was the main cellular death mechanism. To investigate more into the mechanism of autophagy, the expression of ATG5 and ATG7 proteins -which are known autophagy marker- were assessed. We found that RCE induced autophagy independently of ATG5 and ATG7 proteins, as Figure 3 shows a decrease in the expression level of ATG5 and ATG7 proteins.

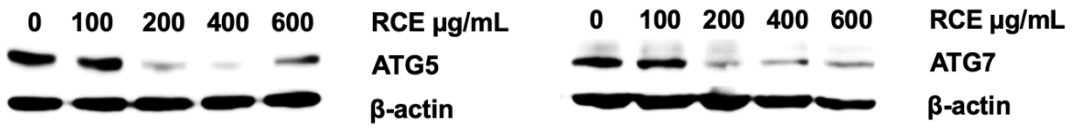


Figure 3: Western blot analysis of ATG5 and ATG7 in MDA-MB-231. Cells were treated with increasing concentrations of RCE (100, 200, 400 and 600 µg/mL) for 48 h.

Next, we checked the level of Rab9 which has been connected with the formation of autophagosomes. Our results showed that Rab9 expression increased upon RCE treatment.

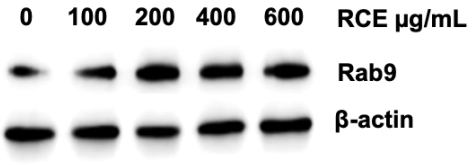


Figure 4: Western blot analysis of Rab9 in MDA-MB-231. Cells were treated with increasing concentrations of RCE (100, 200, 400 and 600 µg/mL) for 48 h.

4.1.2 RCE inhibits the acetylation of Histone 3 and Histone 4 variants

In an attempt to understand the mechanism by which RCE is affecting gene expression, the status of acetylated H3 and H4 histone variants was assessed, since impairment in acetylation status has been linked to cancer development and inhibition of acetylation was a cancer treatment target. Fortunately, we found that RCE caused a decrease in the acetylation profile of both H3 and H4 proteins (Figure 5).

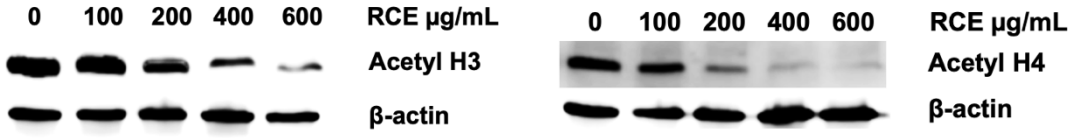


Figure 5: Western blot analysis of Acetyl H3 and Acetyl H4 in MDA-MB-231. Cells were treated with increasing concentrations of RCE (100, 200, 400 and 600 µg/mL) for 48 h.

P300 is a Histone Acetyl Transferase (HAT) enzyme that is known to acetylate H3 and H4 histone variants. Scoring for P300 showed a decrease in its expression level upon RCE treatment (Figure 6).

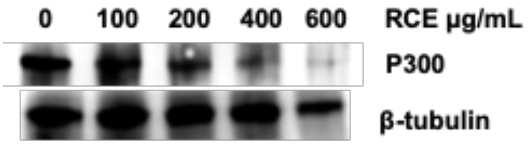


Figure 6: Western blot analysis of P300 in MDA-MB-231. Cells were treated with increasing concentrations of RCE (100, 200, 400 and 600 µg/mL) for 48 h.

4.1.3 RCE inhibits the migration ability of MDA-MB-231 cells

Cell migration plays an important role in metastasis; therefore, we tested the effect of RCE on migration of MDA-MB-231 cells using wound-healing migration assay. Non-cytotoxic concentrations (100 µg/mL and 200 µg/mL) of RCE were used to rule out the possibility that migration inhibition is due to cell death (Figure 7).

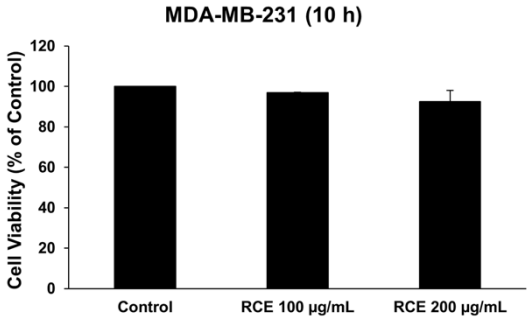


Figure 7: Cell viability for MDA-MB-231 cells after treatment with RCE.

After reaching their confluency, a wound was created in MDA-MB-231 cells by scratching using a pipette tip and the cells were incubated in DMEM supplemented with 10% FBS with control and indicated concentrations of RCE.

Figure 8 A and B shows that RCE treatment reduced migration upon increasing RCE concentrations.

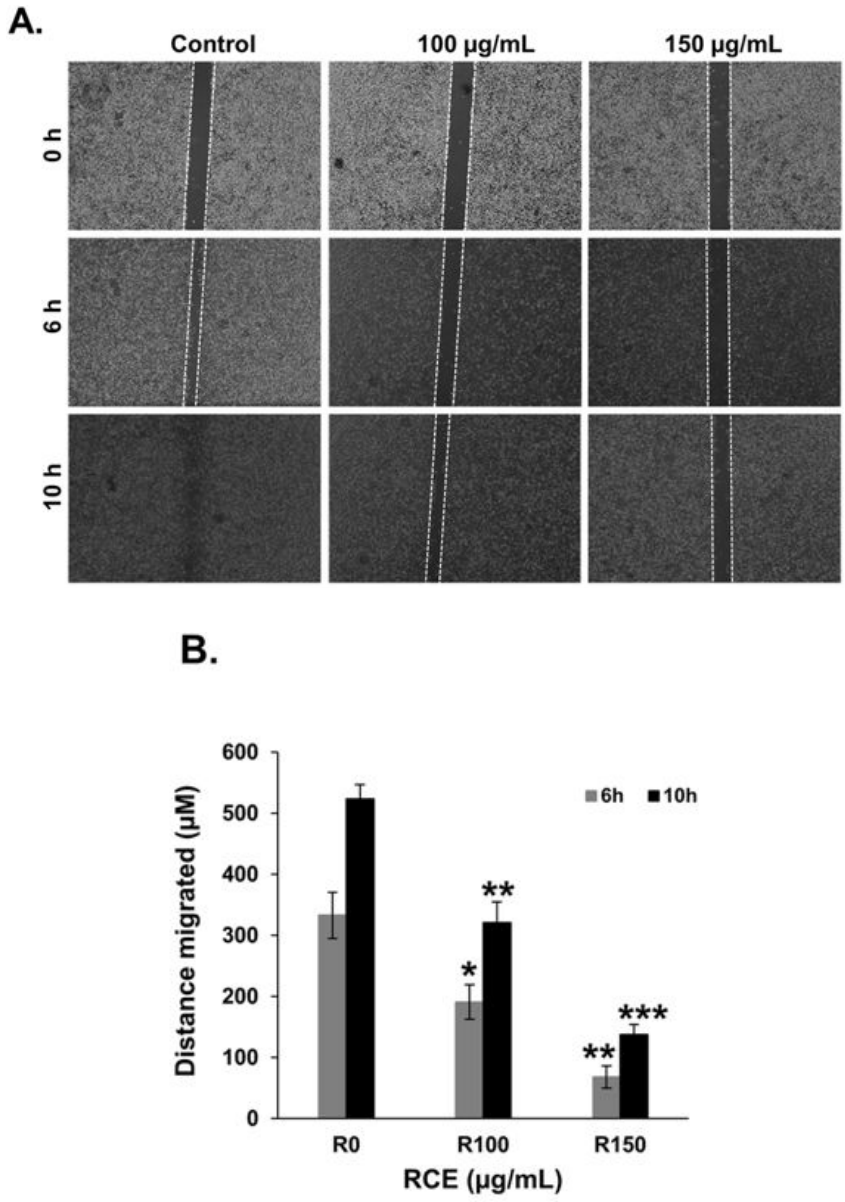


Figure 8: RCE inhibited the migration of MDA-MB-231 cells using wound healing assay. (A) The wound was measured with an inverted microscope at X40 magnification. (B) Values represent the mean ± SEM distance (µm) that cells have migrated in 6 and 10 h. Data are representative of three independent experiments. (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$).

4.1.4 RCE reduces invasion, downregulates MMP-9 and decreases the adhesion to fibronectin

Then, we tested the effect of RCE on MDA-MB-231 cells invasion using matrigel-coated Boyden chamber in the presence of ethanol and different concentrations of RCE. The number of cells that passed the matrigel coated membrane was reduced in RCE treated cells, which shows that RCE inhibits the invasive ability of MDA-MB-231 cells efficiently.

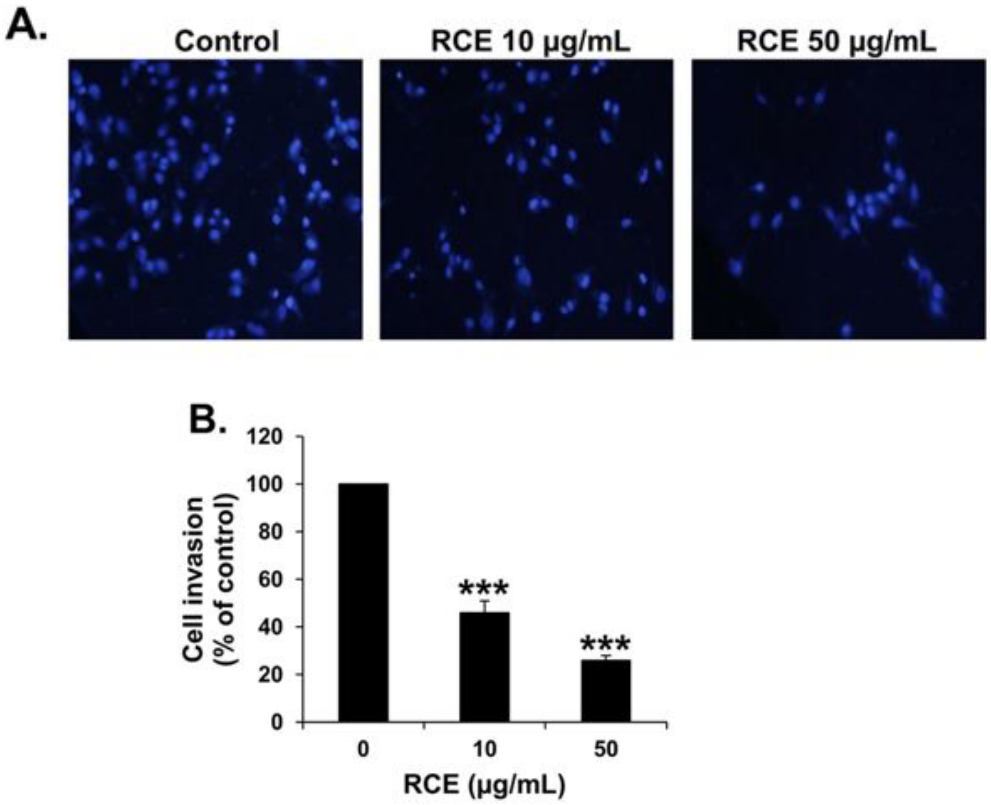


Figure 9: RCE inhibited the invasion potential of MDA MB-231 cells. (A) MDA-MB-231 cells were incubated for 24 h with or without RCE. Cells that invaded into the matrigel were scored. Invaded cells were stained with DAPI and were photographed at X100 magnification under an inverted microscope. (B) Quantification of invaded MDA-MB-231 into the matrigel. Values represented in percent were calculated from three independent experiments and are represented as mean ± SEM. (***$p < 0.001$).

Matrix metalloproteinase-9 (MMP-9), plays an important role in breast cancer cell invasion and metastasis. To test the effect of RCE on breast cancer invasion through the expression of MMP-9, we examined the expression level of MMP-9 in conditioned media on MDA-MB-231 cells treated with RCE. We found that secreted MMP-9 was reduced in response RCE treatment (Figure 10).

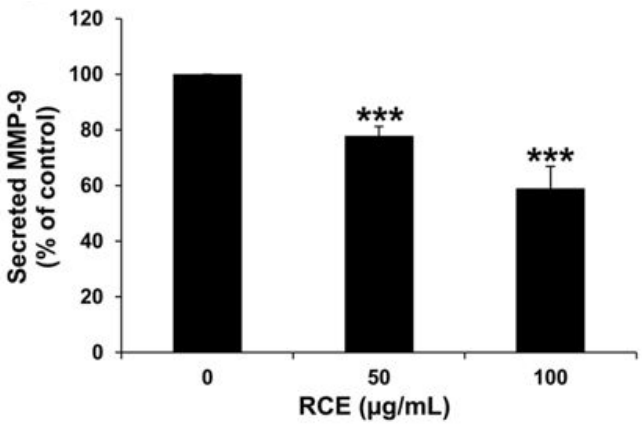


Figure 10: Effect of RCE on the secretion of MMP-9 in RCE-treated MDA-MB-231 cells. The levels of secreted MMP-9 was determined using immunoassay kits. Experiments were repeated three times in triplicate and the average of three means is represented ± SEM. (***$p < 0.001$).

Cell transport and adhesion to components of ECM, such as fibronectin, and to the basement membrane represent an important event in tumor invasion and metastasis. Thus, we examined the ability of MDA-MB-231 to adhere to fibronectin in RCE treated cells. We found that RCE inhibited the adhesion of MDA-MB-231 cells to fibronectin (Figure 11 A, B). This effect was very fast as it appeared within the first 60 min of contact, at time at which no cell death occurred.

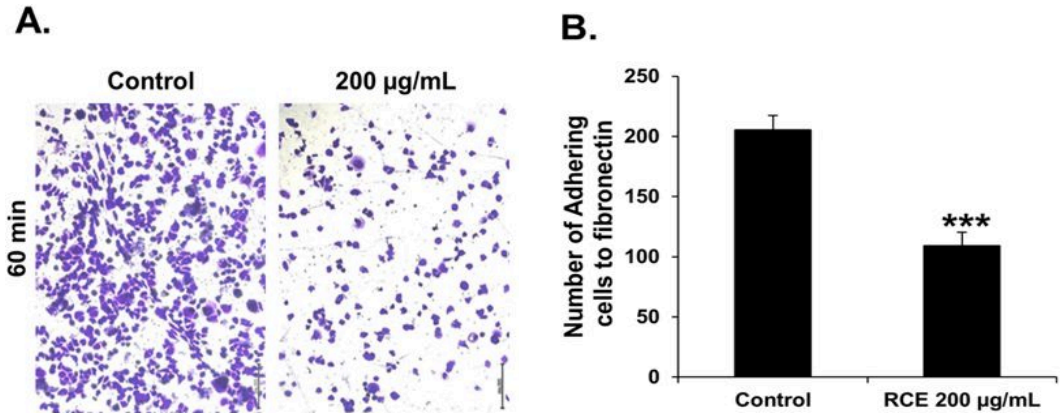


Figure 11: RCE inhibits adhesion of MDA-MB-231 cells to fibronectin. A) Effects of RCE on MDA-MB-231 cells adhesion to wells coated with fibronectin. MDA-MB-231 cells were seeded on the fibronectin-coated wells in the presence or absence of RCE. Attached cells were stained with crystal violet and photographed with an inverted microscope at X100 magnification. (B) Quantification of attached MDA-MB-231 cells to fibronectin. The number of adherent MDA-MB-231 cells to fibronectin was determined by counting at least 5 random fields per well at X200 magnification with an inverted microscope. Data represent a mean of cells counted and are representative of three independent experiments. (***$p < 0.001$).

4.1.5 RCE suppresses VEGF production in MDA-MB-231 cells

Cancer growth and metastasis depend on angiogenesis; thus, inhibiting angiogenesis would inhibit tumor expansion. VEGF which is a pro-angiogenic growth factor has a crucial role in angiogenesis. Therefore, we checked the effect of RCE on VEGF production by MDA-MB-231 cells. Figure 12 shows that treatment with RCE reduced VEGF secretion by MDA-MB-231 cells.

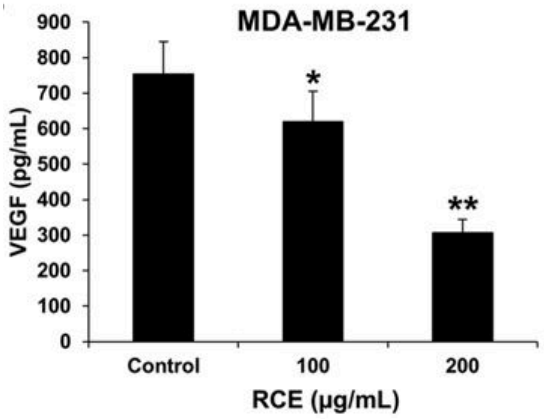


Figure 12: Reduced VEGF secretion in RCE-treated MDA-MB-231 cells. Quantification of basal level of VEGF secretion. MDA-MB-231 cells were treated with vehicle or the indicated concentrations of RCE for 24 h and then secreted VEGF, in the conditioned medium, was analyzed by ELISA. Data represents means ± SEM of three independent experiments. (*$p < 0.05$, **$p < 0.005$).

4.1.6 RCE downregulates the expression of TNF-α and reduces IL-6 production in MDA-MB-231 cells

Several studies had reported that the cytokine TNF-α is involved in cancer cell migration and invasion in different cancer types including breast cancer. Therefore, TNF-α might be considered as a therapeutic target for breast cancer treatment. Since we found that RCE reduced cell MDA-MB-231 cells migration and invasion, we examined the effect of RCE on TNF-α protein expression. Figure 13 shows that RCE induced a reduction in TNF-α protein expression in MDA-MB-231 cells.

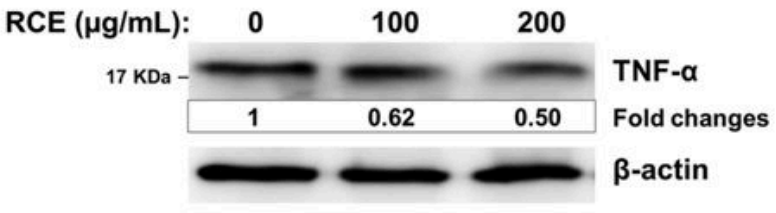


Figure 13: Western blot quantification showing a decrease in TNF-α protein in RCE-treated MDA-MB-231 cells.

After that, we tested the level of IL-6 which is another cytokine that is produced by breast cancer cells and has been shown to increase proliferation and metastasis in breast cancer cells. We found that RCE reduced the level of IL-6 in MDA-MB-231 cells (Figure 14).

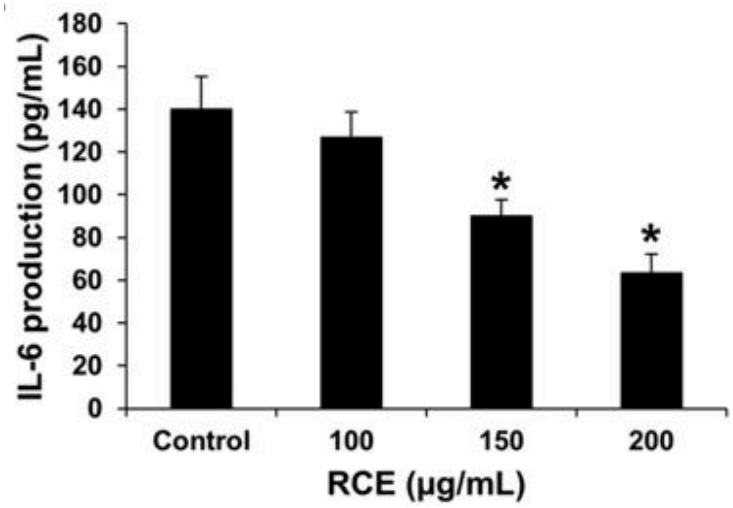


Figure 14: Reduction of IL-6 production in MDA-MB-231 cells. IL-6 production was quantified by ELISA. Data represents means ± SEM of three independent experiments. (*$p < 0.05$).

4.1.7 RCE attenuates STAT3 activation and inhibits NFκB pathway in MDA-MB-231 cells

STAT3 is a transcription factor that is activated by phosphorylation and is considered to be a mediator of tumorigenesis since it is involved in promoting cellular proliferation, resistance to apoptosis, invasion and migration of cancer cells [134]. Therefore, STAT3 is recognized as a potential target for cancer treatment. For that reason, we analyzed the level of pSTAT3 in MDA-MB-231 cells treated with RCE. We found that the phosphorylation of STAT3 was reduced upon RCE treatment (Figure 15).

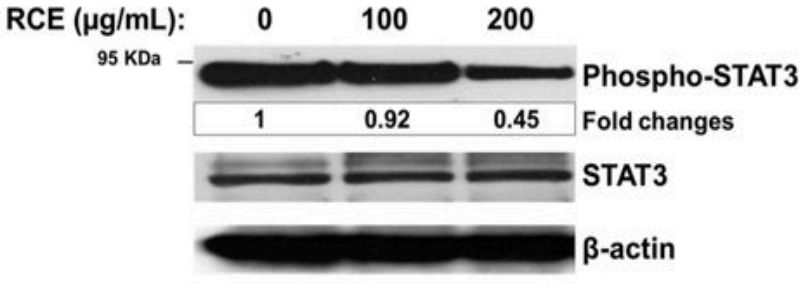


Figure 15: Concentration-dependent decrease of phospho-STAT3 in RCE-treated MDA-MB-231 cells.

NFκB signaling pathway is known to regulate the expression of different genes involved in cancer cells invasion. Therefore, we first examined the status of phospho-p65 in RCE-treated MDA-MB-231 cells. We found that RCE inhibited the p65 phosphorylation (Figure 16 A). After that, we measured the ability of RCE to inhibit the transcriptional activity of NFκB. Therefore, MDA-MB-231 cells were transfected transiently with an NFκB reporter expression vector. Figure 16 B showed that RCE repressed NFκB-dependent transcription of the luciferase reporter.

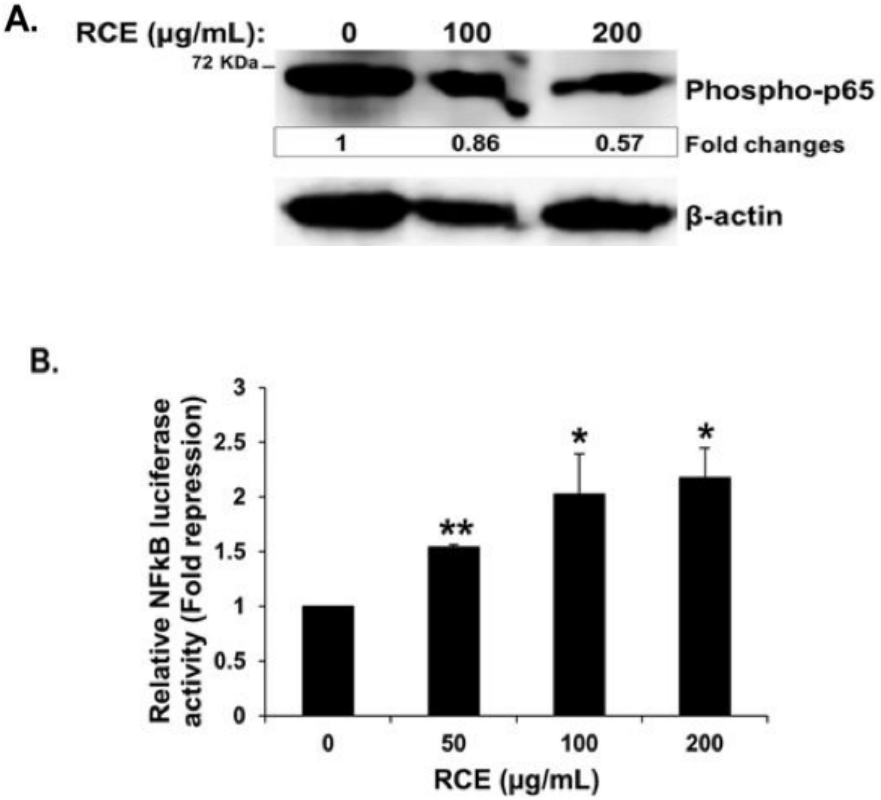


Figure 16: Inhibition of the NFκB signaling pathway by RCE. A) Western blot analysis showed a decrease of phospho-p65 (NFκB) in MDA-MB-231 cells in response to RCE treatment. (B) Inhibition, by RCE, of NFκB transcriptional activity in MDA-MB-231 cells. MDA-MB-231 cells were transfected with the pGL4.32[luc2P/NFκB-RE/Hygro] expression plasmid and luciferase activity were measured 18 h post-transfection. Columns represents mean; bars represent SEM of three independent experiments. (*$p < 0.05$, **$p < 0.005$).

4.1.8 RCE downregulates Flotillin-2 and HIF-1α

Flotillin-2 is a major scaffold protein on lipid rafts which was initially identified as a protein that was upregulated during axon regeneration after optic nerve lesion. Studies shown that dysregulation in Flotillin-2 protein contributed to the formation of cancer-specific cellular characteristics and was closely associated with tumor development, invasion, and metastasis [135]. For that reason, the expression of Flotillin-2 was assessed after treatment with RCE. Results indicated that RCE caused

a decrease in Flotillin-2 level (Figure 17) which can be used as a treatment target since studies had shown that Flotillin-2 was upregulated in breast cancer patients.

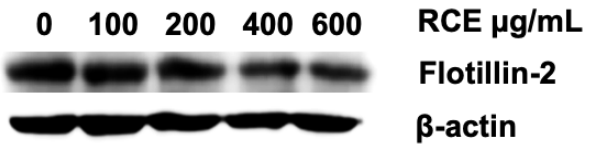


Figure 17: Western blot analysis showing a concentration-dependent decrease of Flotillin-2 in MDA-MB-231 cells in response to RCE treatment.

Hypoxia-inducible factor-1(HIF-1) has been recognized as an important cancer drug target. It has been shown that elevated levels of HIF-1 was associated with tumor metastasis, angiogenesis, poor patient prognosis as well as tumor resistance therapy. Hypoxia is a common characteristic in many types of solid tumors. As an adaptive response to hypoxic stress, hypoxic tumor cells activate several survival pathways to carry out their essential biological processes one of which HIF-1α pathway which is considered as a crucial survival pathway for novel strategies of cancer therapy to be developed [136]. Therefore, the level of HIF-1α protein was examined upon RCE treatment and it was found that HIF-1α decreased in a concentration- dependent manner.

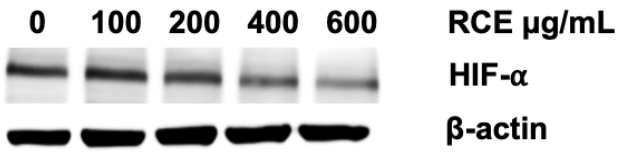


Figure 18: Western blot analysis showing a concentration-dependent decrease of HIF-1α in MDA-MB-231 cells in response to RCE treatment.

4.2 The effect of RCE on colon cancer

4.2.1 RCE inhibited the cellular viability of HT-29 and Caco-2 colon cancer cells

RCE at first was tested for its anti-colon cancer effect on the viability of HT-29 and Caco-2 (Figure 19 A and B) using an assay that monitor the cell metabolic activity. Exposure of HT-29 or Caco-2 cells to RCE decreased cellular viability in a time and concentration-dependent manner. The calculated IC_{50} values for the HT-29 cells were 518 µg/mL at 24, 346 µg/mL at 48 and 271 µg/mL at 72 h. While Caco-2 cells has IC_{50} of 384 µg/mL at 24 and 316 µg/mL at 48 h, respectively.

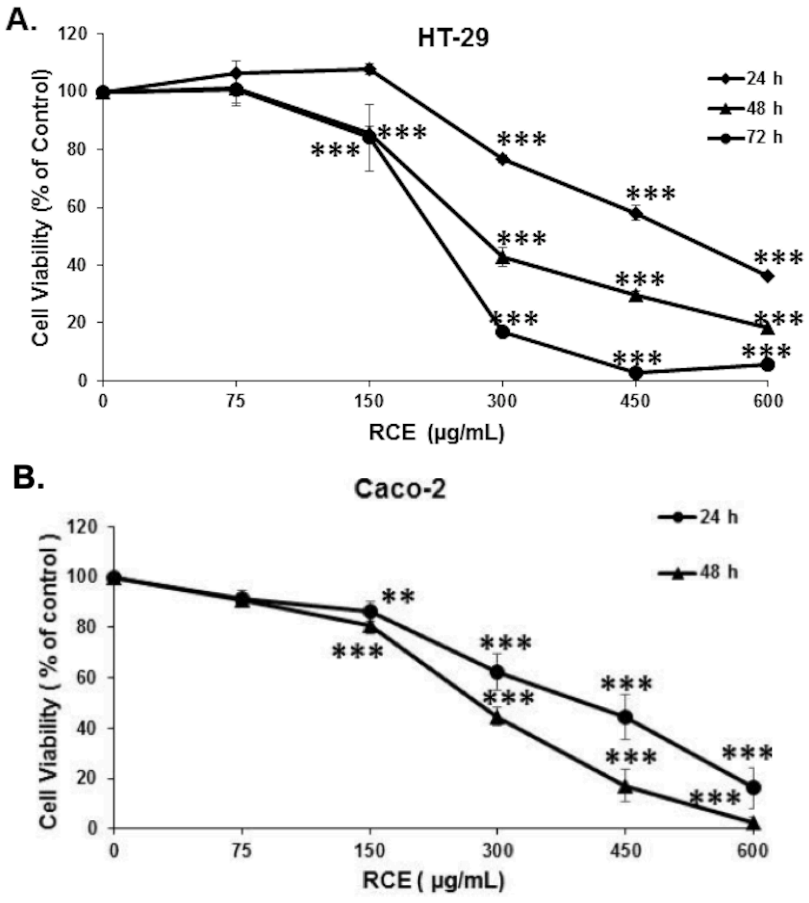
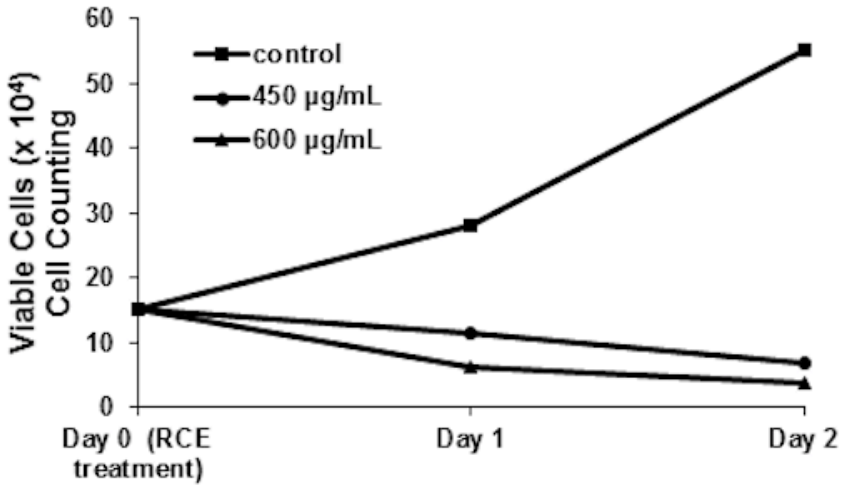


Figure 19: Inhibition of cellular viability by RCE. (A) Exponentially growing HT-29 and (B) Caco-2 colon cancer cells were treated with and without the indicated concentrations of RCE. Data represent the mean of six independent experiments carried out in triplicate. (**p < 0.005, ***p < 0.001).

Cell viability was tested using an assay that differentially stains viable and non-viable cells based on their permeability to two DNA binding dyes. We found that there was a decline in the number of viable HT-29 cells upon RCE treatment which indicate that cell death occurred, when comparing them to the number of cells counted at the day 0 which is the day of treatment (Figure 20).



Total Viable Cells (x 10^4)			
	Day 0 (treatment)	Day 1	Day 2
Control	15.125 ± 1.14	28.175 ± 2.93	55.2 ± 1.52
450		11.5 ± 0.54	7.02 ± 0.16
600		6.285 ± 0.52	3.905 ± 0.31

Figure 20: Determination of cellular viability through cell counting. HT-29 cells were exposed to RCE for 24 h and 48 and cell viability was monitored using the Muse cell analyzer. Data represent the mean ± SEM of three independent experiments.

Observation of HT-29 (Figure 21 A) and Caco-2 (Figure 21 B) cells upon RCE treatment using light microscopy showed morphological changes in both cell lines compared to control cells. Actually, a subpopulation of HT-29 and Caco-2 cells treated with RCE showed cytoplasmic vacuolation (dashed arrows). Higher concentrations of RCE (600 µg/mL), showed a subpopulation of cells that appeared smaller and rounded, which is a characteristic of dying cells (arrowheads).

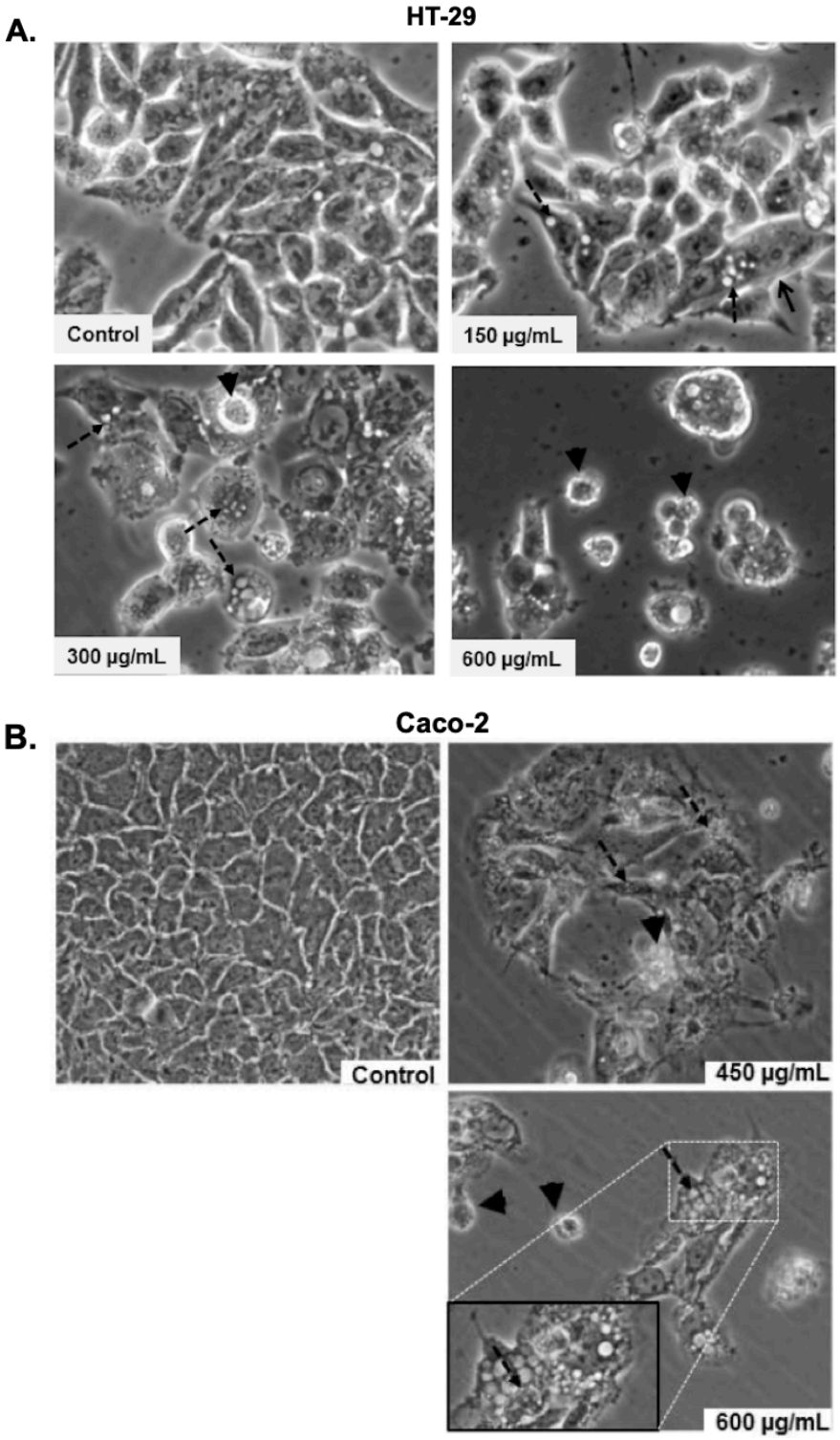
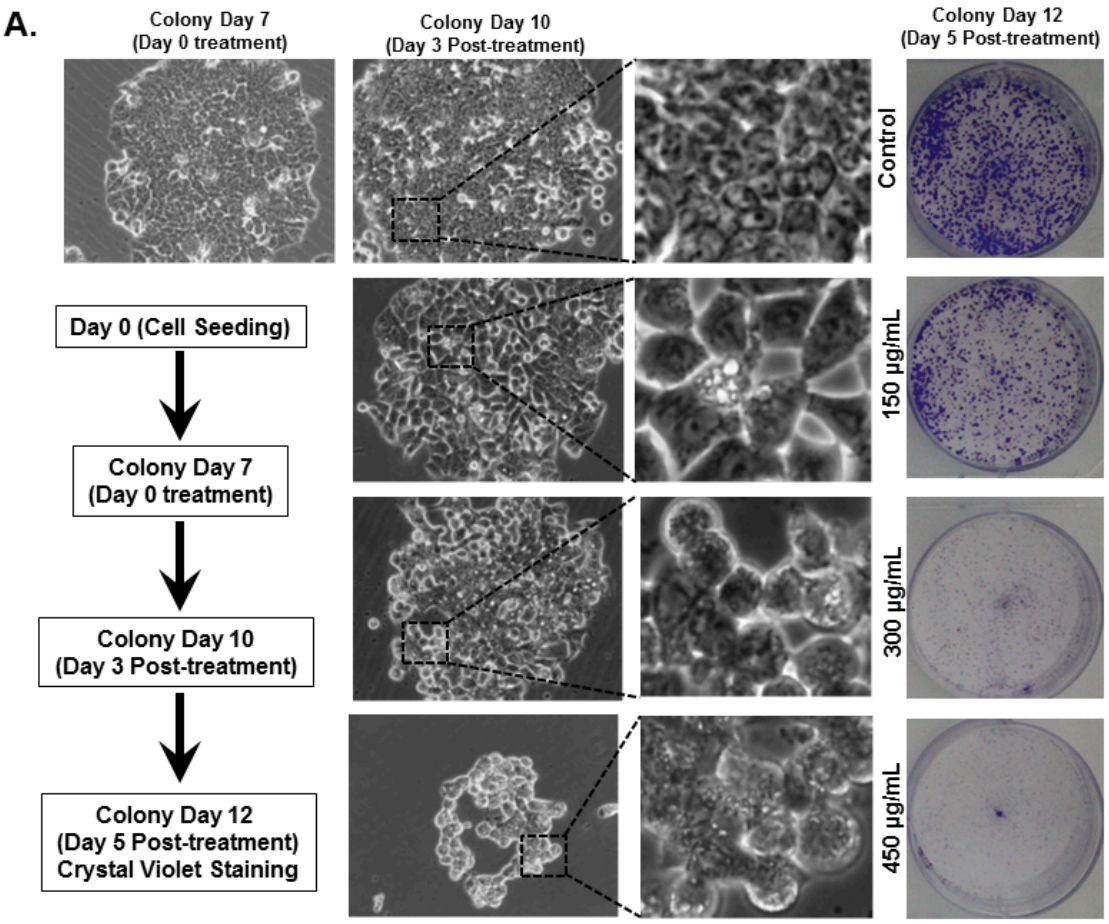


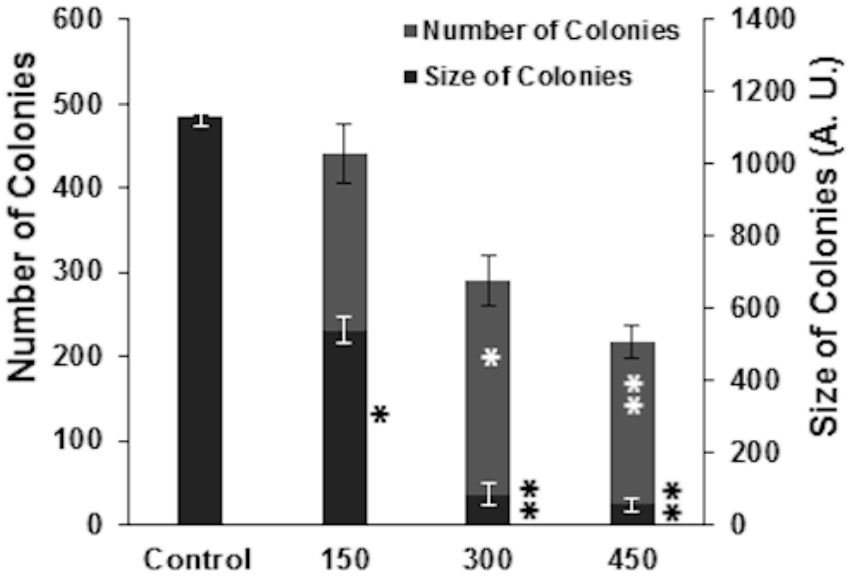
Figure 21: RCE induced morphological changes on human (A) HT-29 and (B) Caco-2 colon cancer cells using EVOS XL Core Cell Imaging System at X40.

4.2.2 RCE inhibits HT-29 colony growth

To further confirm the anti-cancer potential of RCE, we wanted to test the effect of RCE on the proliferative capacity of HT-29 colonies formed in culture. For that, HT-29 cells were grown for seven days to form colonies and then treated for five days with different concentrations of RCE. Figure 22 A and B showed that RCE treatment caused a significant decrease in the number and size of colonies in a concentration dependent manner. This significant reduction in number and size of colonies is clearly indicative of massive cell death. Additionally, microscopic observation of the treated colonies showed cellular vacuolation which suggest autophagy induction.



B.



	Number of Colonies	Colony size (Arbitrary Unit)
Control	462 ± 23.6	1130 ± 144.8
150 µg/mL	441 ± 36.2	538 ± 67.9
300 µg/mL	290 ± 30.3	82 ± 5.4
450 µg/mL	217 ± 19.4	56 ± 4.4

Figure 22: RCE inhibits HT-29 colony growth. (A) HT-29 colonies were first allowed to form in normal media for seven days. Formed colonies were then treated with or without different concentrations of RCE and allowed to grow for five more days before crystal violet staining. Size and morphology of the growing colonies were followed over time under the microscope at X40 magnification. (B) Inhibition of colony growth was assessed by measuring the number and size (surface area) of the colonies obtained in control and RCE-treated plate. Data represent the mean of three independent experiments carried out in triplicate. (*$p < 0.05$, **$p < 0.005$).

Colony formation by HT-29 was also done using soft agar colony formation assay. Similarly, RCE markedly reduced the number of colonies when grown on soft agar (Figure 23 A and B).

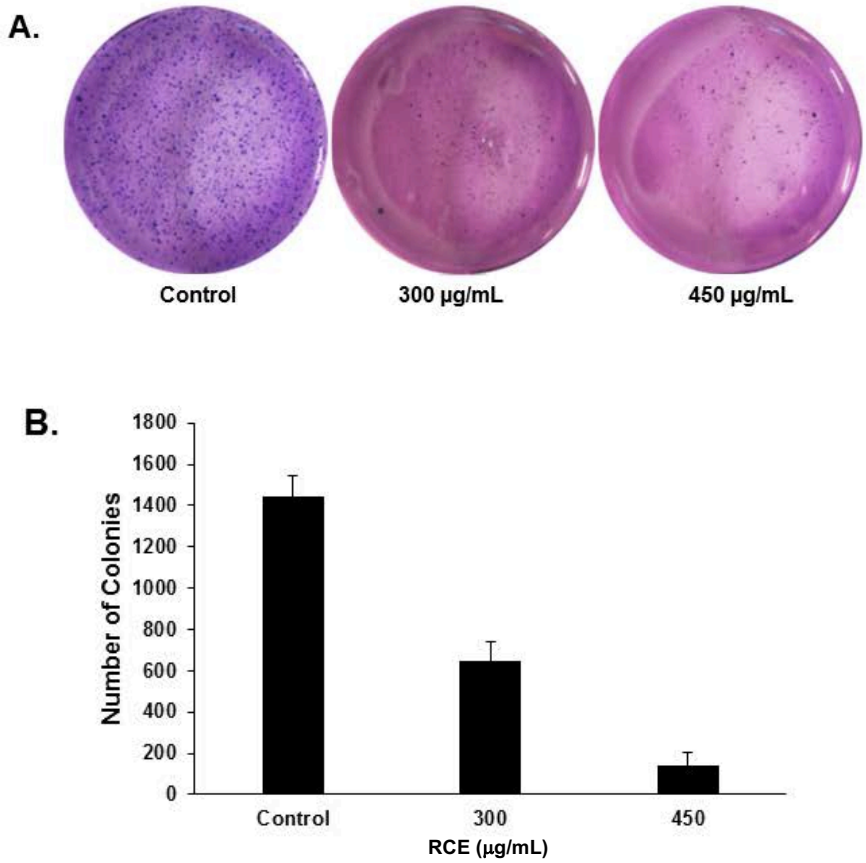


Figure 23: RCE inhibits HT-29 colony growth in soft agar. HT-29 colonies were first allowed to form in normal media for 13 days. Formed colonies (A) were then treated with or without RCE at the indicated concentrations and allowed to grow for 5 more days before staining. Inhibition of growth was assessed by measuring the size of the colonies in control and RCE-treated plate.

4.2.3 RCE induces Beclin-1 independent autophagy

We have shown in the previous results that RCE induced morphological changes in HT-29 and Caco-2 colon cancer cells (Figure 21) indicated by the massive cytoplasmic vacuolation which suggest the induction of autophagy (dashed

arrows). To confirm the autophagic origin of those vacuoles, a fluorescence marker of autophagy vacuoles was used. Figure 24 showed that exposure of HT-29 cells to RCE for 24 h led to an accumulation of autophagic vacuoles, thus confirming the induction of autophagy by RCE in colon cancer cells.

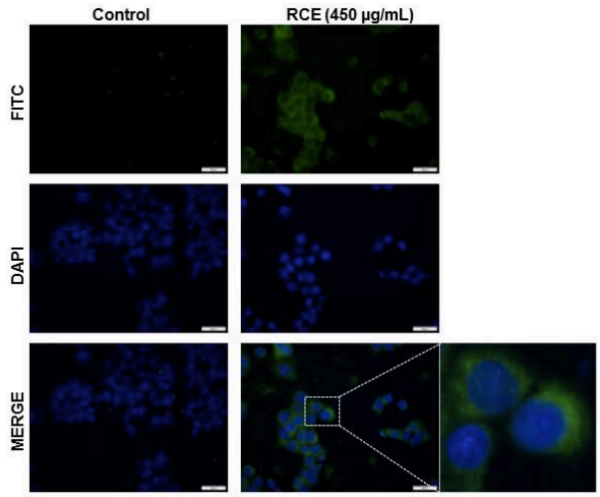


Figure 24: RCE induced the formation of autophagic vacuoles in HT-29. HT-29 cells were seeded in 8 chambers slide followed by treatment with or without 450 µg/mL RCE. Following treatment cells were washed and stained for autophagic vacuoles. Fluorescent autophagic vacuoles were examined under Olympus CKX54 fluorescence microscope.

After that the protein expression of specific markers for autophagy were examined. As described earlier, the conversion of LC3-I into LC3-II is a key characteristic in autophagy, therefore, we analyzed the accumulation of LC3-II by Western blotting. Figure 25 A shows that RCE induced an accumulation of LC3-II starting at 300 µg/mL of RCE in HT-29 Cells. Similarly, RCE also induced an accumulation of the LC3-II in Caco-2 cells (Figure 25 A). Moreover, immunofluorescence staining for endogenous LC3B revealed clear LC3-positive puncta in RCE treatment in HT-29 cells. Endogenous LC3B was hardly detectable in control cells (Figure 25 B).

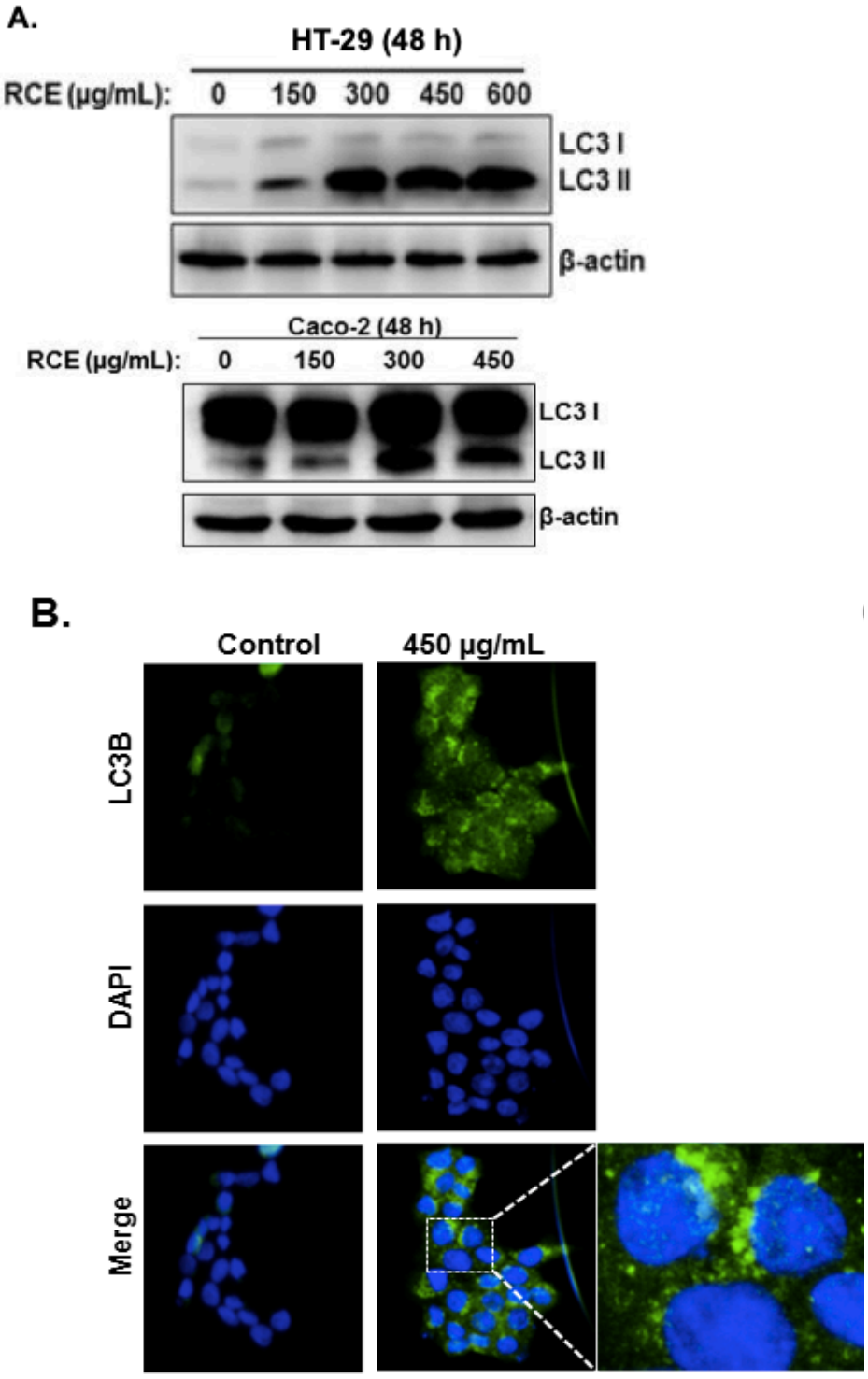


Figure 25: Detection of LC3-II. (A) Western blotting analysis of LC3-II expression in RCE-treated HT-29 and Caco-2 cells. (B) Immunofluorescence staining of LC3B in RCE-treated HT-29 cells. HT-29 cells were treated with RCE (450 μg/mL) for 24 h and then cells were stained with antibody specific for LC3B and DAPI.

Moreover, the expression p62(SQSTM1) was also evaluated. Figure 26 shows a decrease in p62(SQSTM1) level at 300 µg/mL, suggesting that autophagy is induced by a concentration ≥300 µg/mL of RCE.

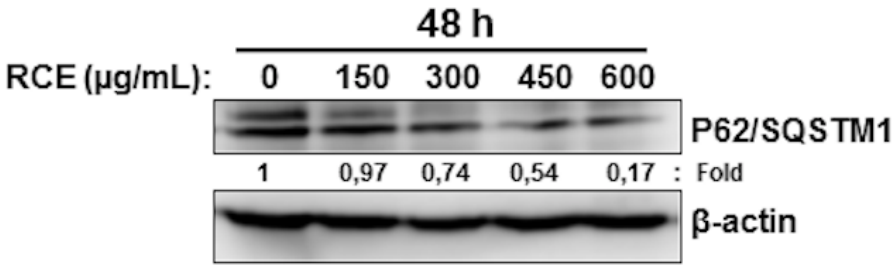


Figure 26: Western blotting analysis of P62 expression in RCE-treated HT-29 cells.

After that, we assessed the expression of Beclin-1, the autophagy effector that plays a key role in autophagosome formation as described earlier. Surprisingly, we found that Beclin-1 levels in HT-29 cells decreased after treatment with RCE at concentration of 300 µg/mL RCE (Figure 27 A). Similarly, Beclin-1 decrease was also observed in RCE-treated Caco-2 cells starting at 300 µg/mL RCE (Figure 27 A). Beclin-1 protein downregulation was also confirmed by immunofluorescence staining of Beclin-1 in HT-29 cells treated with 450 µg/mL RCE (Figure 27 B).

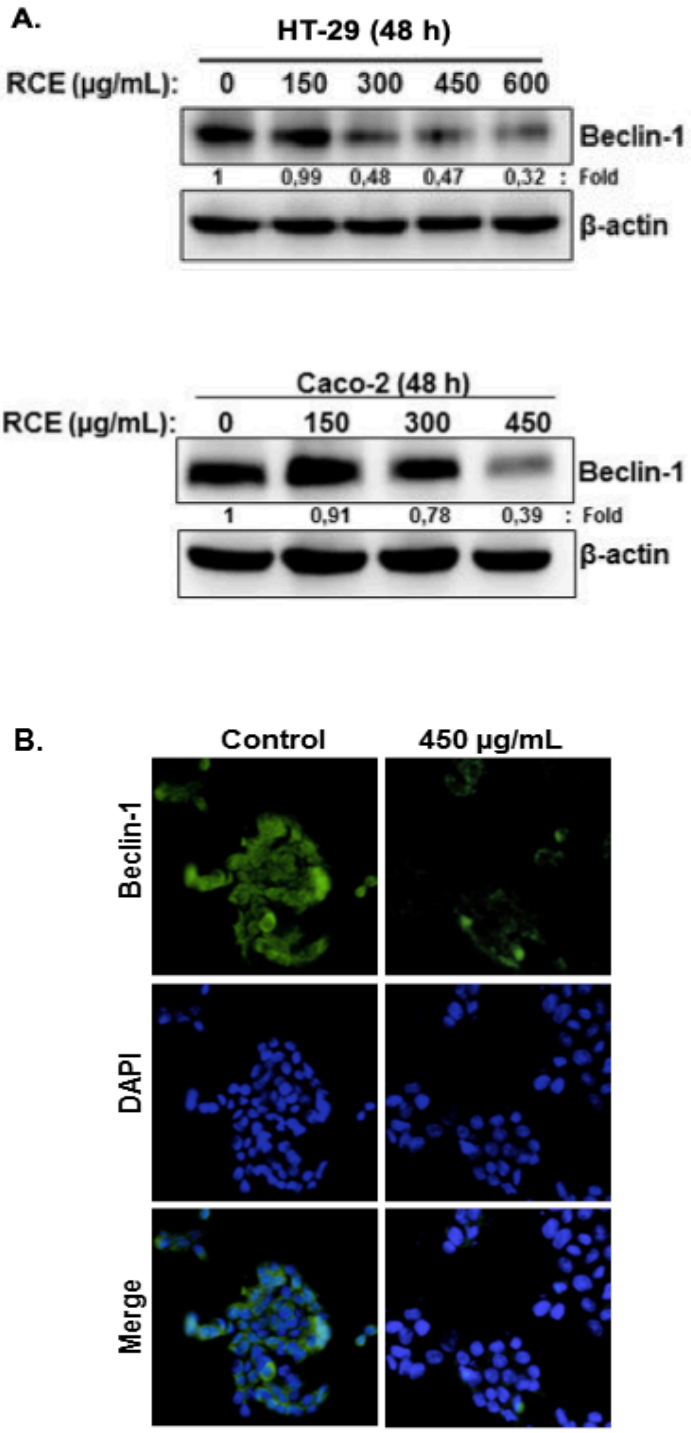


Figure 27: RCE downregulates Beclin-1. (A) Western blotting analysis of Beclin-1 expression in RCE-treated HT-29 and Caco-2 cells. (B) Immunofluorescence staining of Beclin-1 in RCE-treated HT-29 cells. HT-29 cells were treated with RCE (450 µg/mL) for 24 h and then cells were stained with antibody specific for Beclin-1 and DAPI.

To confirm that Beclin-1 is not required for the autophagy that is induced by RCE, beclin-1 protein was knocked down in HT-29 cells using Beclin-1-specific siRNA. As it is shown in Figure 28, knockdown of Beclin-1 did not inhibit LC3-II accumulation and hence RCE-induced autophagy.

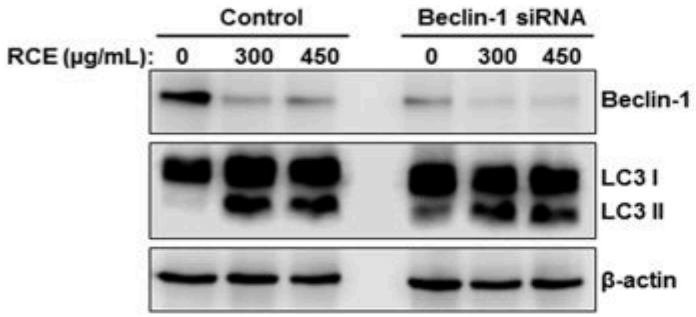


Figure 28: Western blotting analysis of LC3-II expression after knockdown of Beclin-1 using specific siRNA in RCE-treated HT-29 cells.

To investigate the mechanism of Beclin-1 downregulation after RCE treatment, the level of Beclin-1 transcript was firstly examined in HT-29 cells using qRT-PCR. As shown in Figure 29 A, the level of Beclin-1 mRNA stayed the same after RCE treatment which indicate that Beclin-1 downregulation is a posttranscriptional event. Then, we checked if the downregulation of Beclin-1 is a result of autophagolysosomal degradation. That was assessed by inhibiting the autophagolysosome formation using chloroquine (CQ) autophagy inhibitor and then measuring the level of Beclin-1. As it is shown in Figure 29 B, blocking autophagy by CQ failed to restore Beclin-1 protein levels after RCE treatment. Moreover, autophagosome formation inhibition by 3-methyl adenine (3-MA), another autophagy inhibitor, also failed to restore Beclin-1 protein level (Figure 29 C), which suggest that downregulation of Beclin-1 is autophagy-independent. Then we examined the possibility of targeting Beclin-1 for proteasome degradation after

RCE treatment. For that, cells were first pre-treated with MG-132 (15 µM), proteasome inhibitor, and then treated with RCE. We found that proteasome inhibition abrogated the RCE-induced decrease of Beclin-1, whose level remained comparable to control cells (Figure 29 D). This result clearly indicates that RCE targets Beclin-1 to proteasome degradation.

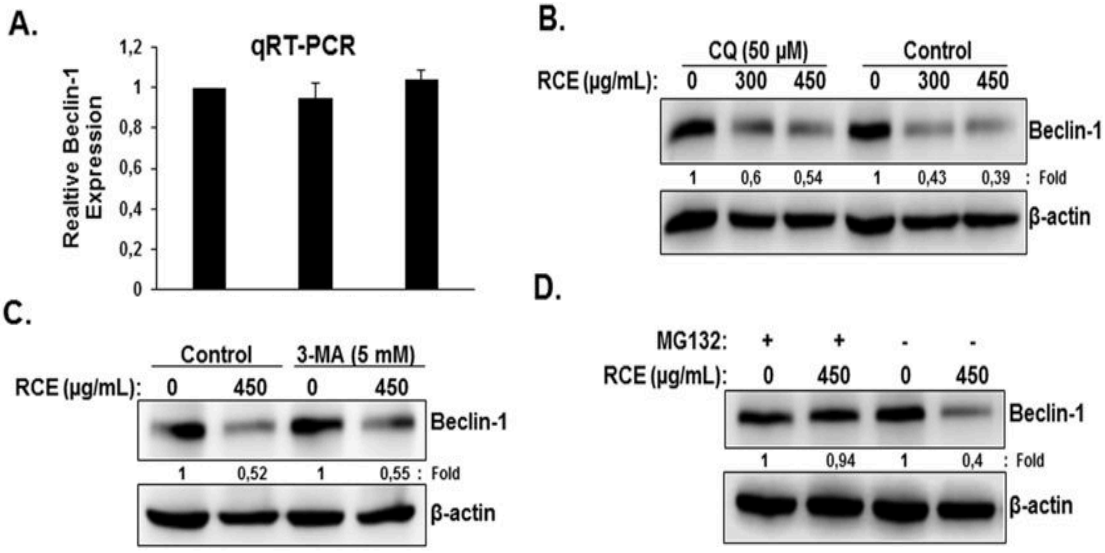


Figure 29: RCE targeted Beclin-1 to proteasome degradation in HT-29 cells. (A) qRT-PCR showed no effect on the levels of Beclin-1 transcripts. GAPDH was used as internal normalization control. (B, C) Downregulation of Beclin-1 is autophagy-independent. Cells were pretreated with or without CQ or 3-MA for 1 h then RCE was added at the indicated concentrations for 48 h. Proteins were extracted and Beclin-1 protein level was determined by Western blot. (D) RCE targets Beclin-1 to proteasome degradation and inhibitors of the proteasome (MG-132) restore Beclin-1 protein levels.

4.2.4 RCE induces caspase-7-dependent apoptosis in HT-29 cells

Next, we investigated the reason behind the inhibition of cell viability after RCE treatment and whether it was associated with apoptosis induction. An increase in the apoptotic populations of HT-29 cells was observed after RCE treatment starting at 300 µg/mL using Annexin V staining which indicates that these cells committed apoptosis (Figure 30).

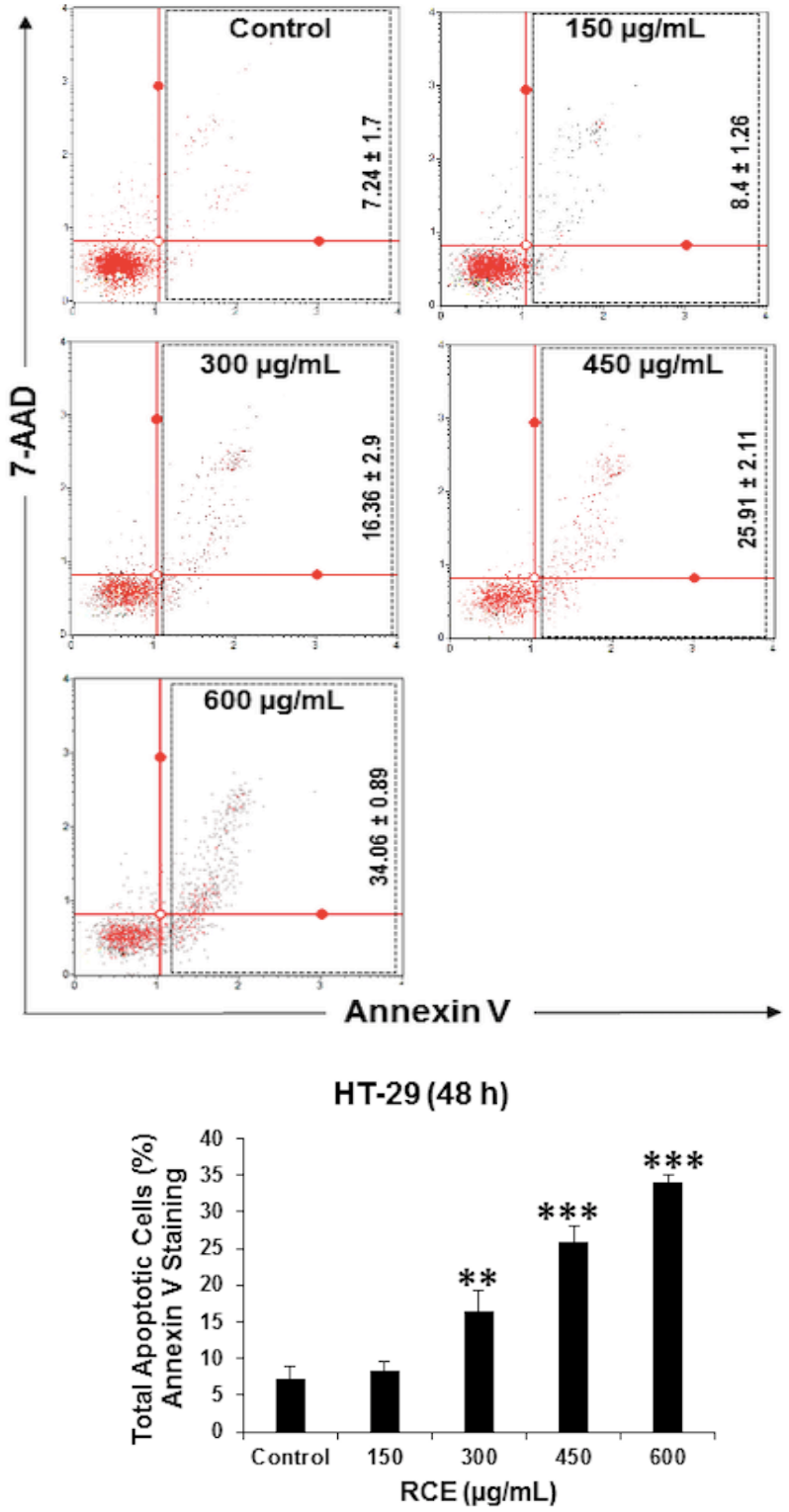


Figure 30: Induction of apoptosis by RCE in HT-29 cells. Annexin V binding was carried out using Annexin V & Dead Cell kit. Cells were treated with or without increasing concentrations of RCE for 48 h. Detached and adherent cells were collected and stained and then the events for total apoptotic cells were counted with the Muse™ Cell Analyzer. (**$p < 0.005$, ***$p < 0.001$).

To further confirm apoptosis induction upon RCE treatment in HT-29 cells, cleaved PARP, a marker of apoptosis, was checked. Figure 38 B showed that RCE induced a dose-dependent increase in cleaved PARP expression. After that we assessed the activation of caspase 3/7 using a caspase 3/7 activity assay (Figure 31 A), a significant increase in caspase 3/7 activity was observed after RCE treatment at concentrations of 300 and 450 µg/mL RCE by 3 and 5 folds, respectively. Moreover, Western blot analysis showed that RCE caused a decrease in caspase-3 levels. Interestingly, the decrease in the pro-form was not associated with increase in the processed active form. Based on this result, it appears that apoptosis that is induced by RCE is independent of caspase-3 activation. This inspired us to assess the activation of caspase-7. As shown in Figure 31 B, the expression level of cleaved caspase-7 increased obviously in RCE-treated HT-29 cells, suggesting that RCE induced a caspase-7- dependent apoptosis in colon cancer.

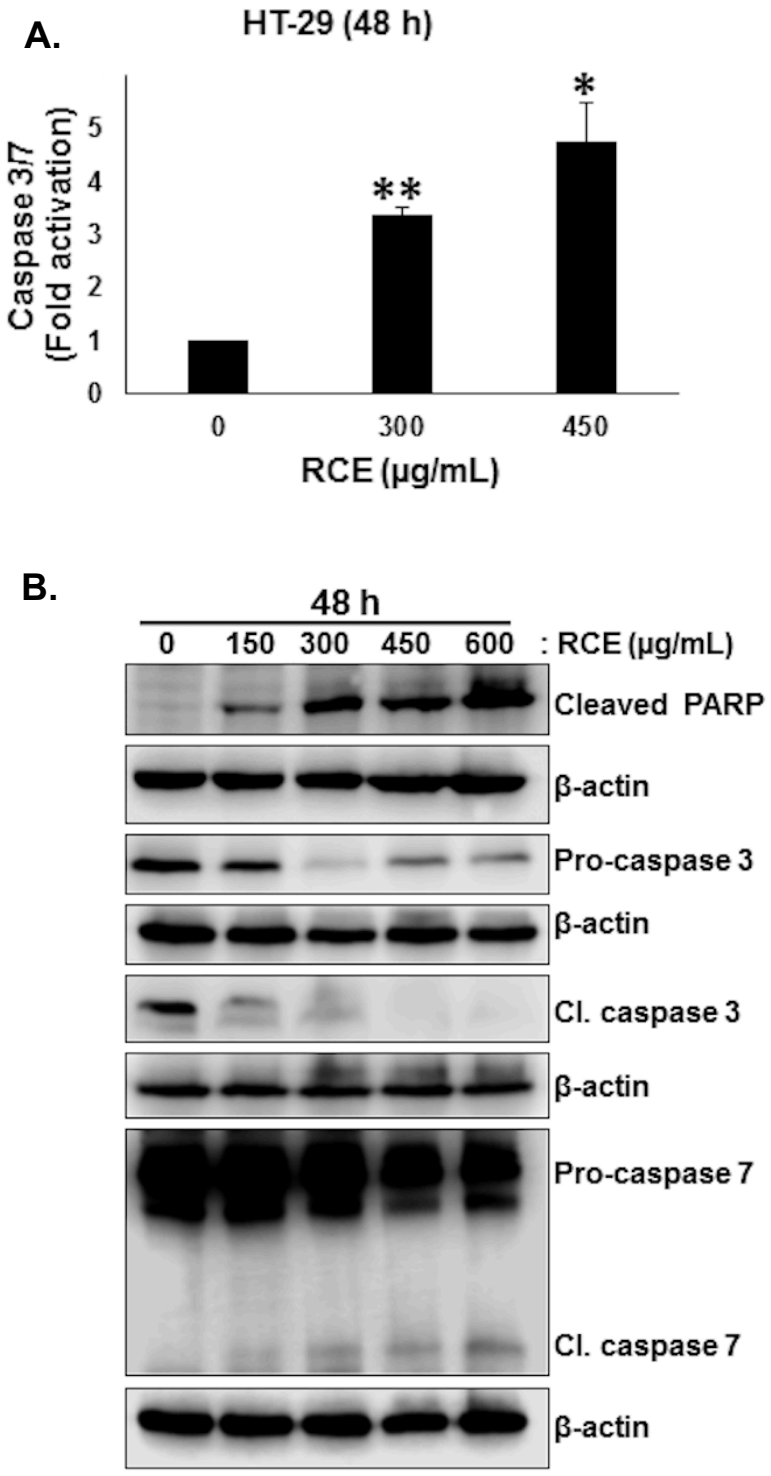


Figure 31: Induction of caspase-7-mediated apoptosis by RCE in HT-29 cells. (A) Stimulation of caspase 3/7 activity in HT-29 cells after exposure to RCE for 48 h. The relative caspase 3/7 activity was normalized to the number of viable cells and was expressed as fold of activation compared to the control cells. (*$p < 0.05$, **$p < 0.005$) (B) Western blot analysis of caspase-3, -7 activation and PARP cleavage in RCE-treated HT-29 cells after 48 h treatment.

4.2.5 Inhibition of autophagy rescues RCE- induced cell death in HT-29 cells

Since RCE induced apoptosis and autophagy, and because those two events are known to induce cell death, we aimed to investigate the contribution of apoptosis and autophagy to the viability inhibition activity of RCE. Therefore, we aimed to determine the timing at which autophagy and apoptosis occurred. For that, time-course analysis was performed for both events and the induction of autophagy and apoptosis was monitored over time. HT-29 cells were treated with 450 µg/mL of RCE and autophagy was detected through the conversion of LC3-I into LC3-II while apoptosis was examined through PARP cleavage. Autophagy was evident after 12 h after-treatment (Figure 32, lower panel), on the other hand apoptosis (Figure 32, upper panel), occurred 48 h after-treatment. These data indicate that autophagy is an early event that precedes apoptosis induction in response to RCE.

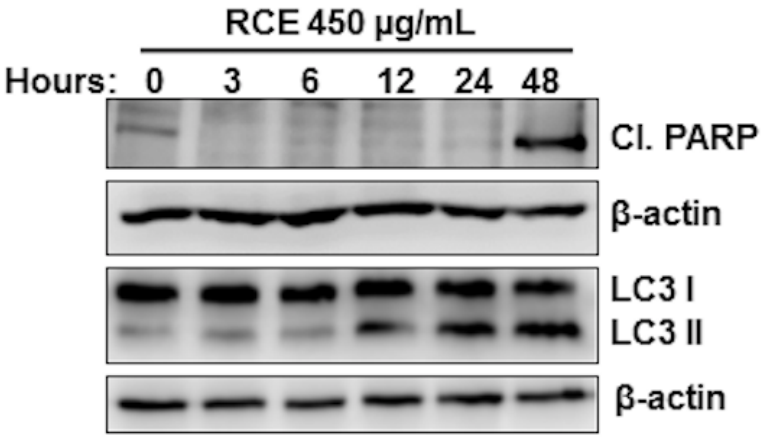


Figure 32: Time-course analysis of PARP cleavage and LC3-II accumulation in RCE-treated HT-29 cells. Cells were treated with 450 µg/mL RCE and proteins were extracted at the indicated time-points (3, 6, 12, 24 and 48 h).

Treatment by RCE at concentration of 300 and 450 µg/mL, induced a significant cell death (~60 and 70% inhibition of cell viability) after 48 h of RCE treatment (Figure 19 A) while apoptosis accounted for only ~16 and 25% as determined by Annexin V staining (Figure 30). This observation led us to test if these two cell death mechanisms are activated independently or if they are linked together. To answer this question, we tested the effect, of CQ, 3-MA and Z-VAD-FMK (pan-caspase inhibitor) on cell viability. Blocking autophagy was further assessed by evident decrease in the conversion of LC3-I to LC3-II by CQ and 3-MA whereas blocking apoptosis was assessed by the absence of cleaved PARP (Figure 33).

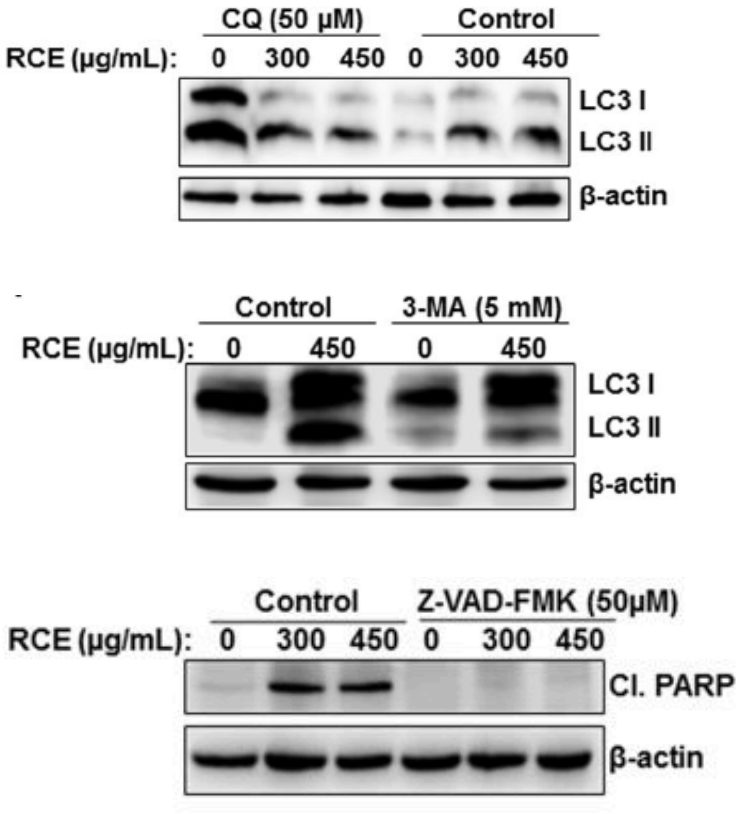


Figure 33: Analysis of LC3-II accumulation in HT-29 cells pre-treated with autophagy inhibitors (CQ or 3-MA) and pancaspase inhibitor. Proteins were extracted and LC3-II accumulation was determined by Western blot. Western blot quantification of cleaved PARP in cells pretreated with and without pan-caspase inhibitors to confirm apoptosis.

Cell viability improved significantly after autophagy inhibition. On the other hand, inhibition of apoptosis had almost no effect on cell death when compared to control cells treated with RCE only in HT-29 cells (Figure 34). This result is unexpected because at RCE concentrations of 300 and 450 µg/mL, apoptosis accounted for ~ 16 and 25% of cell death, respectively (Figure 30) in HT-29 cells. Similar result was also obtained with Caco-2 cells (Figure 34).

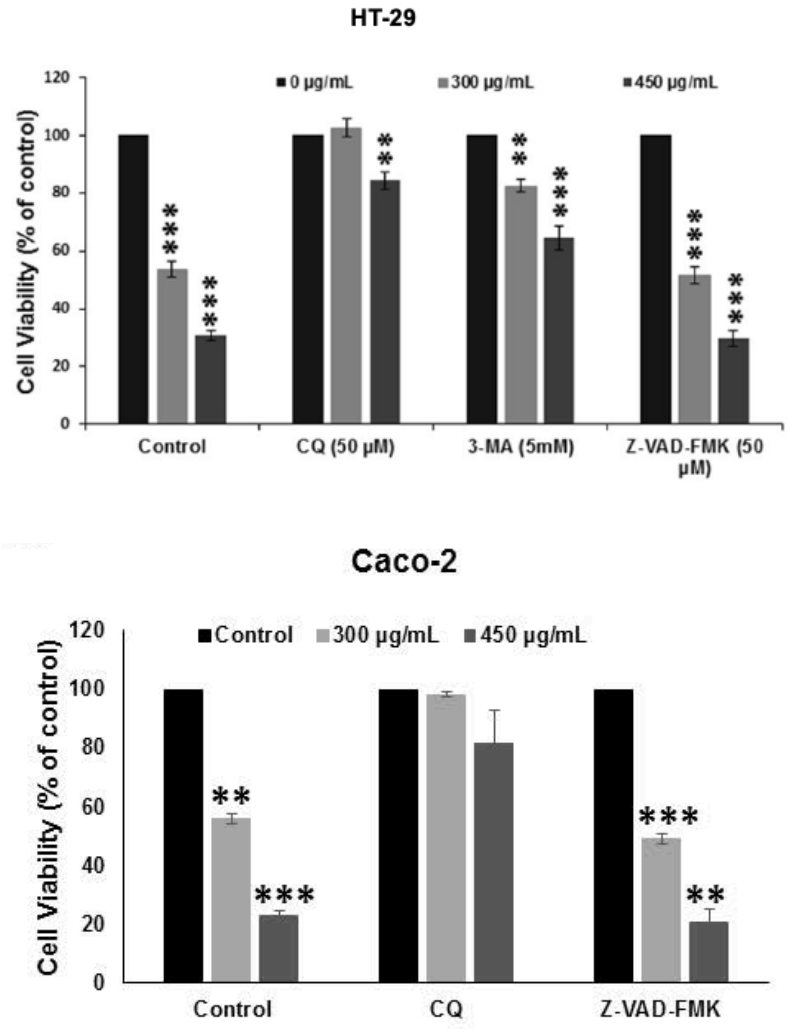


Figure 34: Inhibition of autophagy but not apoptosis reduces cell death induced by RCE. HT-29 cells were pretreated with CQ, 3-MA or the pan-caspase inhibitor (Z-VAD-FMK) and Caco-2 was pretreated with CQ or the pan-caspase inhibitor (Z-VAD-FMK) and then treated for 48 h with 300 or 450 µg/mL RCE. (**$p < 0.005$, ***$p < 0.001$).

It is also noteworthy to mention that, even though CQ induced PARP cleavage in control cells, RCE treatment did not lead to a further increase of the level of cleaved PARP (Figure 35), suggesting that the inhibition of autophagy led to the inhibition of RCE-induced apoptosis and therefore suggesting that apoptosis induction is autophagy-dependent.

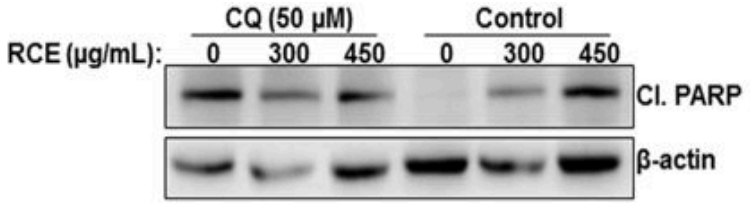


Figure 35: Western blot of cleaved PARP in cells pretreated with and without autophagy inhibitor.

4.2.6 RCE induces proteasome-dependent degradation of mTOR, Akt, p53 and caspase-3 in HT-29 cells

Next, we examined the mechanism through which RCE might exert its effects on autophagy and apoptosis in particular. mTOR kinase is a downstream target of the PI3K/AKT signaling pathway and has a role as a negative regulator of autophagy, was reported to regulate colorectal cancer tumorigenesis [137]. Therefore, we decided to test the effect of RCE on PI3K/AKT/mTOR pathway in HT-29 cells. For that, we examined the phosphorylation level of mTORC1 at Ser2448. Treatment with RCE led to a decrease in the level of mTORC1 phosphorylation in HT-29 and Caco-2 cells dramatically, which suggest an inhibition of mTOR activity upon RCE treatment. Unexpectedly, we observed a significant decrease in the total mTOR protein level in HT-29 and Caco-2 cells after RCE treatment. Likewise, a decrease in AKT, upstream regulator of mTOR

pathway, phosphorylation as well as total AKT protein was observed in HT-29 and Caco-2 cells treated with RCE (Figure 36).

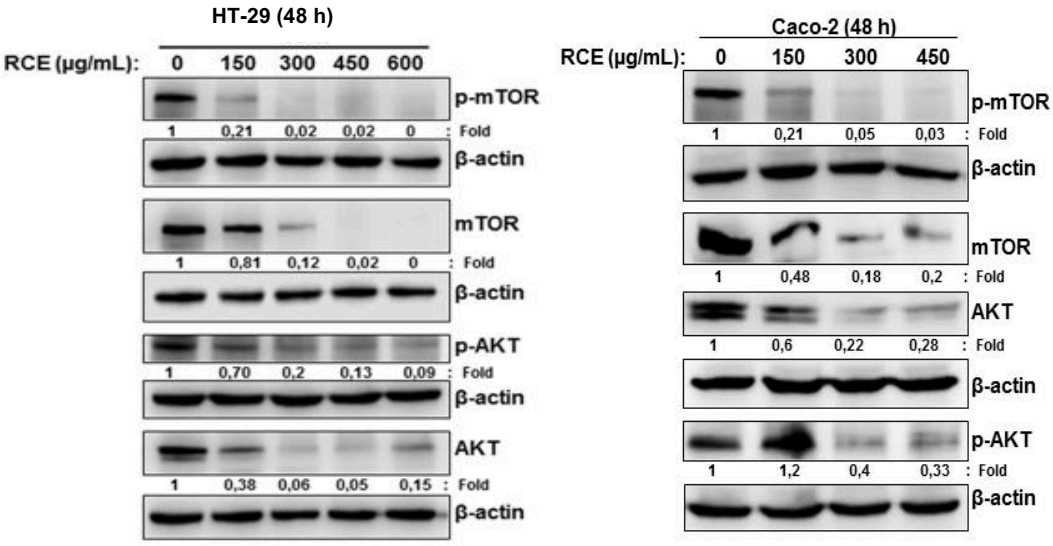


Figure 36: Concentration-dependent decrease of phospho-mTOR, total mTOR, phospho-AKT and total AKT protein in RCE-treated HT-29 and Caco-2 cells.

Then, we tested if the inhibition of mTOR was at the transcription level. Toward this qRT-PCR analysis of mTOR was carried on. Figure 37 showed that mRNA transcript level in HT-29 cells treated with RCE had no significant difference in mRNA levels compared to the control.

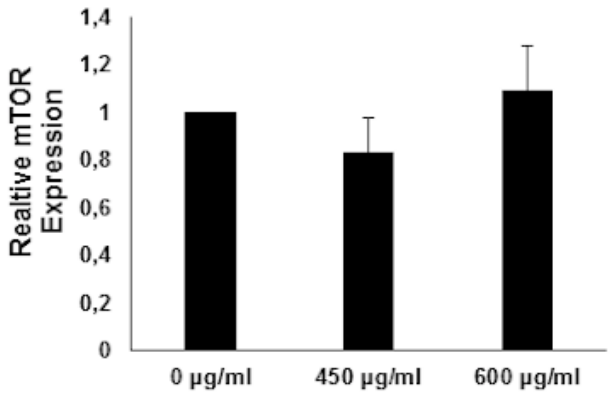


Figure 37: Downregulation of mTOR is transcription-independent. Total RNA from RCE-treated and untreated cells were amplified by qRT-PCR the mTOR transcripts using mTOR specific primers. GAPDH was used as internal normalization control.

Also, blocking late stage autophagy by CQ and early stage autophagy by 3-MA (Figure 38) failed to restore mTOR and AKT protein expression levels, which shows that the decrease of these two protein levels after RCE treatment is not a result of autophagolysosomal degradation.

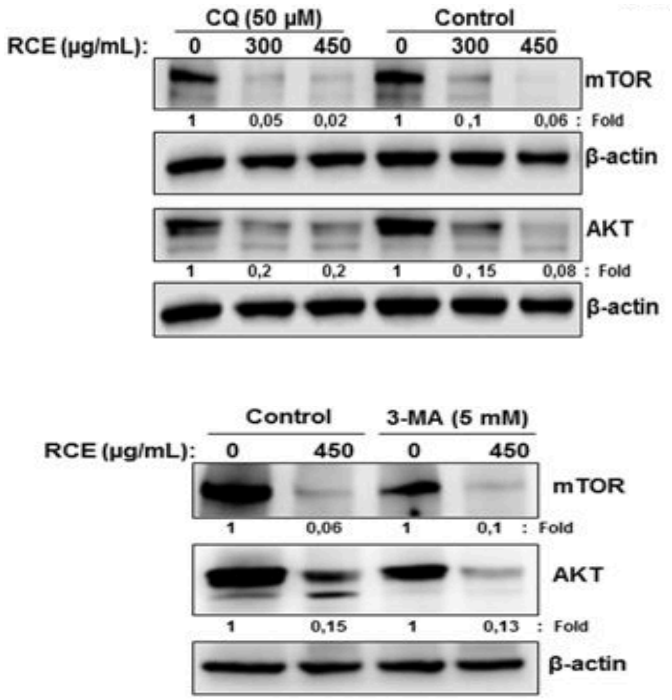


Figure 38: RCE-mediated decrease in the protein level of mTOR and AKT is autophagy-independent. Cells were pretreated with or without CQ or 3-MA for 1 h and then RCE was added at the indicated concentration for 48 h. Proteins were extracted and mTOR and AKT protein level was determined by Western blot.

Then, we sought to test if the of the proteasome is involved in the decrease of mTOR and AKT proteins, therefore, HT-29 cells were first pre-treated with MG-132, proteasome inhibitor, and then treated with or without RCE. Results shown in Figure 39 demonstrates that treatment with MG-132 was able to restore these two proteins to a level comparable to the control.

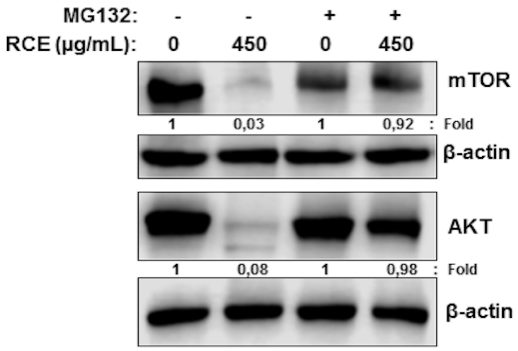


Figure 39: RCE targets mTOR and AKT to proteasome degradation. HT-29 cells were pre-treated for 1 h with MG-132 prior to treatment with RCE.

Next, we examined protein expression level of mutant p53 in RCE-treated HT-29 cells. Results in Figure 40 A showed a decrease in the expression of mutant p53 protein. The observed decrease was not a result of decreased gene expression, since qRT-PCR analysis showed no change in mRNA level of p53 transcripts between treated cells and control (Figure 40 B).

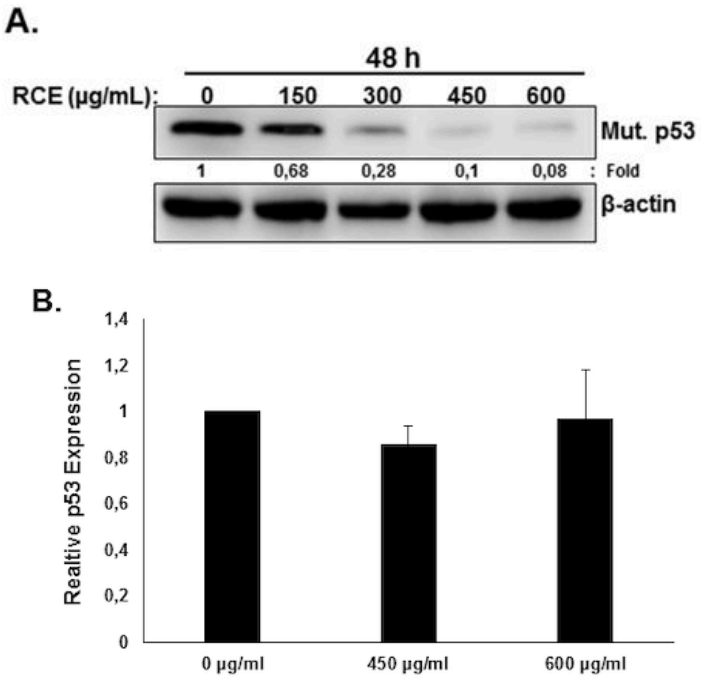


Figure 40: RCE reduced the level of p53. (A) Downregulation of p53 in RCE-treated HT-29 cells. (B) qRT-PCR analysis of p53 transcript in RCE-treated HT-29 cells.

Interestingly, proteasomal inhibition by MG-132 rescued mutant p53 from degradation (Figure 41), which suggests that mutant p53 is targeted to proteasomal degradation by RCE as well.

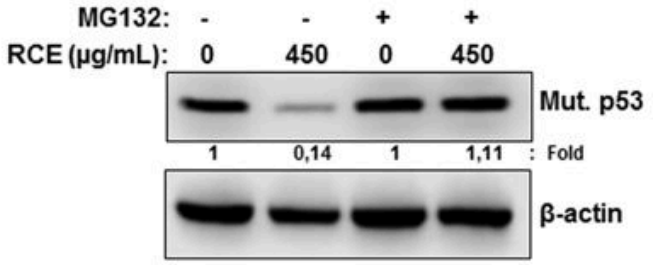


Figure 41: RCE targets mutant p53 to proteasome degradation. HT-29 cells were pre-treated for 1 h with or without MG-132 prior to treatment with RCE.

We showed previously that a downregulation of pro-caspase-3 in RCE treated cells without increase in the active form (Figure 31). This drove us to investigate if pro-caspase-3 is targeted to proteasomal degradation. Indeed, we found proteasomal inhibition by MG-132 restored pro-caspase-3 protein to a level comparable to control cells (Figure 42), indicating that pro-caspase-3 is also targeted for proteasomal degradation by RCE.

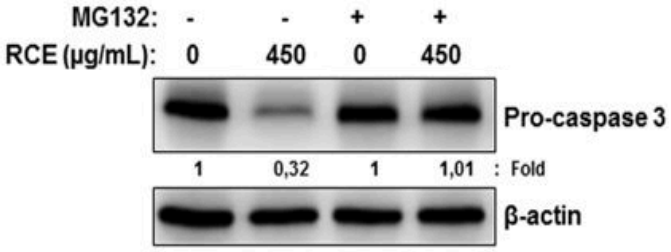


Figure 42: RCE targets Pro-caspase-3 to proteasome degradation. HT-29 cells were pre-treated for 1 h with or without MG-132 prior to treatment with RCE.

4.2.7 The activation of proteasome-mediated proteolysis of mTOR by RCE leads to the activation of autophagy and subsequent apoptosis in HT-29 cells

To determine the order of events at which autophagy and proteasomal degradation occurs in HT-29 cells, a time course experiment for protein expression was conducted. It was found that mTOR and its phosphorylated form were the first to be downregulated. Actually, the degradation of total mTOR occurred as early as 3 h upon RCE-treatment followed by a decline in its active form which was detected after 6 h post-treatment (Figure 43). Conversely, AKT levels and its active form, pro-caspase-3 and p53 proteins expression started to decrease after 12 h post-RCE treatment (Figure 43). We also found that Beclin-1 downregulation, which occurred as early as 6 h post-RCE treatment (Figure 43, lower panel), started before autophagy. Autophagy was triggered after 12 h post-treatment of RCE and that was determined by LC3-II accumulation (Figure 32). Together these results suggest that the inactivation of mTOR might serve as a trigger for downstream event (proteasomal degradation, autophagy and apoptosis) induced by RCE.

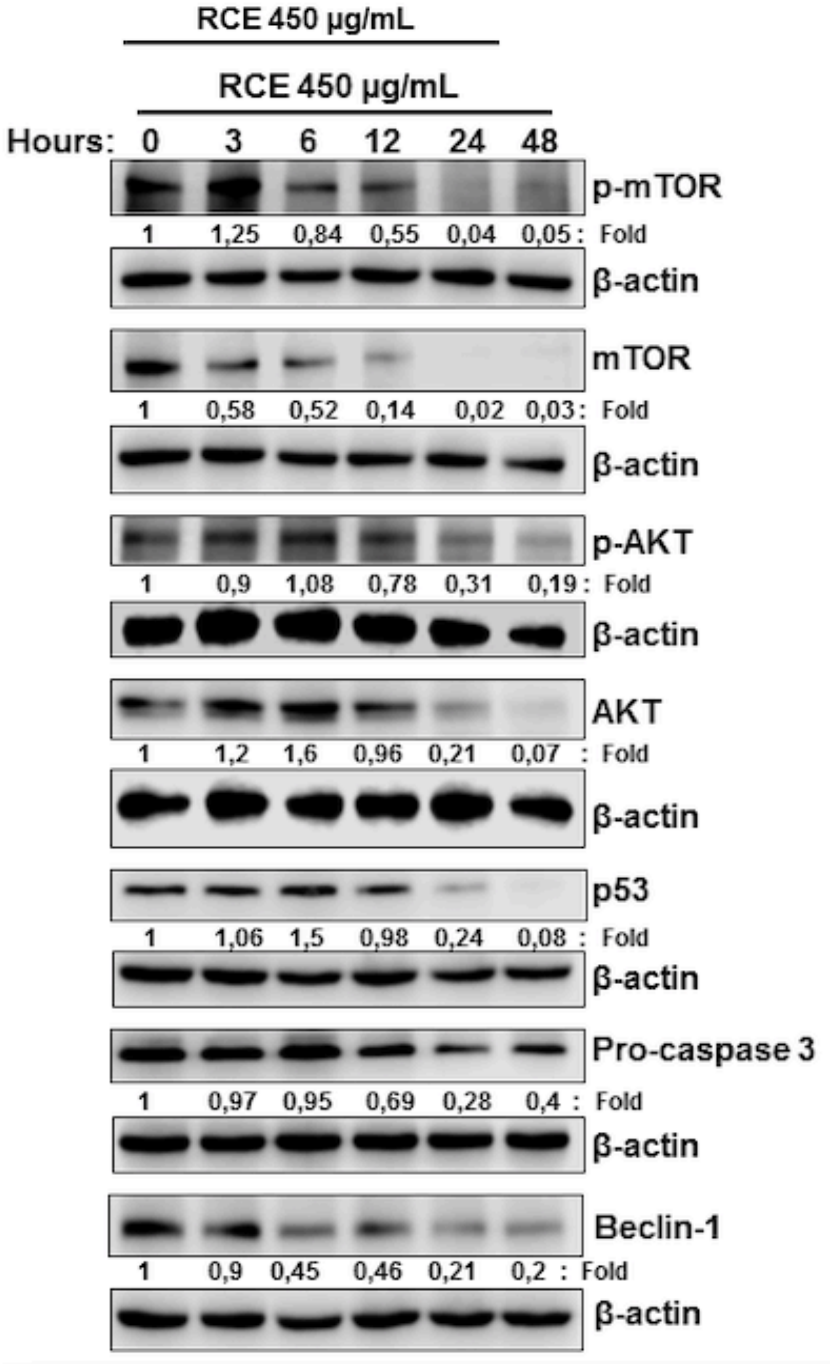


Figure 43: Inactivation of mTOR through proteasome degradation precedes autophagy. Time-course analysis of phospho-mTOR, total mTOR, phospho-AKT, total AKT, mutant p53, Beclin-1 and pro-caspase-3 in RCE-treated HT-29 cells. Cells were treated with 450 µg/mL RCE and proteins were extracted at the indicated time-points (3, 6, 12, 24 and 48 h).

To test the above hypothesis, we examined the possibility that the rescued mTOR protein from proteasomal degradation, by MG-132, could be phosphorylated. As shown in Figure 44 (upper panel), the inhibition of the proteasomal machinery restored phosphorylated mTOR to a level comparable to the control. Then we checked if the restoration of the active mTOR has an impact on autophagy activation and consequently on the induction of apoptosis. Restoration of phospho-mTOR was associated with a significant decrease in the conversion of LC3-I to LC3-II (Figure 44, middle panel), and with reduced level of active caspase-7 as well (Figure 44, lower panel). To further confirm that autophagy and apoptosis were blocked, cell viability was measured in cells treated first with MG-132 and then with RCE.

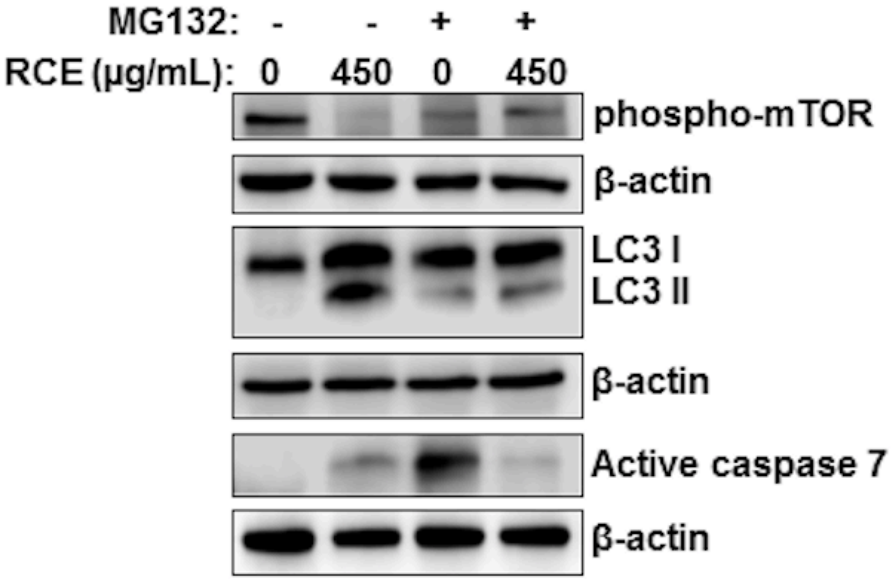


Figure 44: Inhibition of the proteasome rescue phospho-mTOR and block autophagy and apoptosis induced by RCE. HT-29 cells were pre-treated for 1 h with or without MG-132 prior to treatment with RCE.

Interestingly, cell viability was found to be significantly improved after proteasomal inhibition (Figure 45).

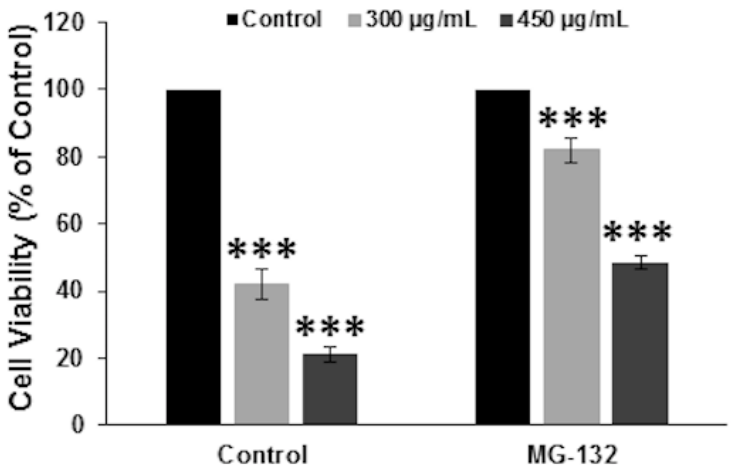


Figure 45: Inhibition of proteasome reduces cell death induced by RCE. HT-29 cells were pretreated for 1 h with or without MG-132 prior to treatment with RCE for 48 h. (***$p < 0.001$).

Recent work showed that mTOR decreased activity increases the overall protein ubiquitination and degradation by the UPS [138]. This drove us to test if mTOR inactivation by RCE enhances overall protein ubiquitination. HT-29 cells were treated with 300 and 450 µg/mL RCE and overall protein ubiquitination profile was determined. We found that RCE treatment resulted in marked increase in the total content of ubiquitinated protein (Figure 46). Increase in ubiquitinated protein profile was also observed in Caco-2 cells (Figure 46).

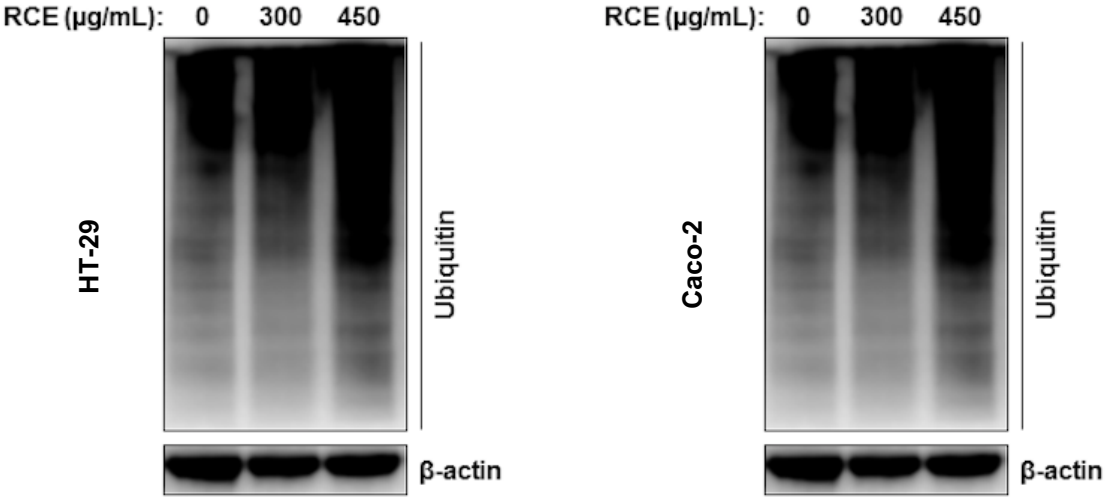


Figure 46: RCE treatment increases the cellular level of ubiquitinated proteins in HT-29 and Caco-2 cells.

Additionally, a time course experiment showed that increased protein ubiquitination profile might be detected as early as 3 h which also coincides with a time at which a decrease of mTOR protein was observed (Figure 47).

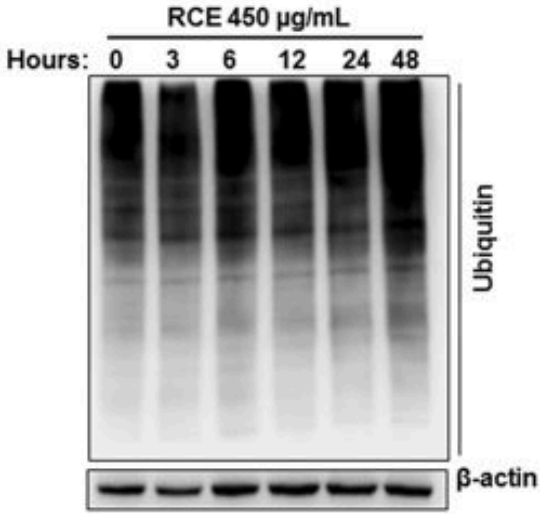


Figure 47: Time-course analysis of protein ubiquitination in RCE-treated HT-29 cells. Cells were treated with 450 µg/mL RCE and proteins were extracted at the indicated time-points (3, 6, 12, 24 and 48 h).

4.2.8 RCE downregulates cyclin D1 and p27

The earliest known and understood about the function of cyclin D is promoting cell proliferation as a regulatory partner for CDK4 or CDK6. Moreover, cyclin D1 can bind to p27 (a tumor suppressor protein that regulates G0 to S phase transitions) independently of CDK4 or CDK6, which promote cell migration [139]. Interestingly, we found that cyclin D1 was downregulated as the concentration of RCE increased in HT-29 cells. Moreover, the level of p27 was also downregulated after RCE treatment in the same cells (Figure 48 A and B).

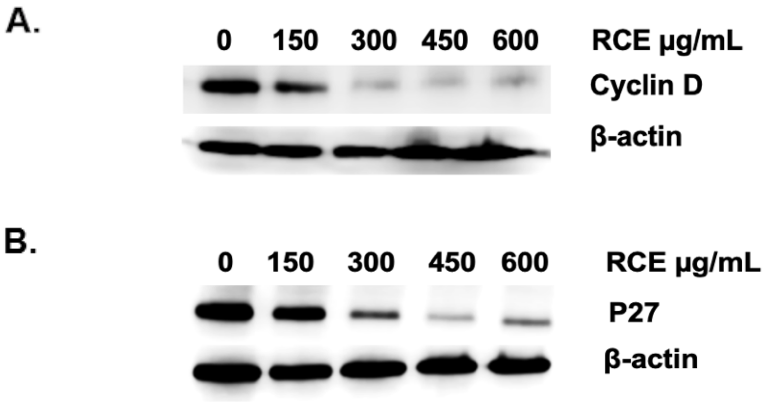


Figure 48: Downregulation of (A) cyclin D1 and (B) p27 upon RCE treatment. Cells were treated with increasing concentrations of RCE (100, 200, 400 and 600 µg/mL) for 48 h.

Chapter 5: Discussion

5.1 Breast cancer

In this study, we showed that at non-cytotoxic concentrations of RCE, migration and invasion were inhibited in MDA-MB-231 cells and their adhesion to fibronectin was abrogated. Furthermore, we found that RCE reduced VEGF production in MDA-MB-231 and downregulated MMP-9, TNF-α and IL-6. Interestingly, our investigation revealed that NFκ B and STAT3 pathways were inhibited in MDA-MB-231 in response to RCE.

An ever-increasing amount of evidence supports the claim that plants are, indeed, an essential player in the search for better cancer treatment or even a cure. Many of these plants or plant-derived drugs are acting through modulating programmed cell death. Autophagy or as referred to as programmed cell death II is the process in which subcellular membranes undergo dynamic morphological changes that lead to the degradation of cellular proteins and cytoplasmic organelles. Thus, plants present themselves as candidates for cancer thereby, with great potential and investigating their pharmacological capacity is of impending importance [140]. Autophagy was the main cellular death mechanism induced in MDA-MB-231 TNBC cell line upon RCE treatment. Autophagy is characterized by the induction of autophagosomes, which will fuse with the lysosomes to form autolysosomes, and to degrade the content of the autophagosome [141]. Induction of autophagy results in recruitment of ATGs to the phagophore assembly site (PAS) to help in the nucleation of an isolation membrane that forms a cup- shaped structure termed the phagophore.

ATG5 and ATG7 which are key proteins that are needed in phagophore expansion during autophagy process [56].

In this study we found that autophagy was induced in MDA-MB-231 cell line independently of ATG5 and ATG7. Indeed, RCE caused a decreased in ATG5 and ATG7 accompanied with an increase in Rab9 level. These results are in agree with Nishida et. al., group who showed that mouse cells lacking ATG5 or ATG7 can still form autophagosomes/ autolysosomes and perform autophagy-mediated protein degradation when subjected to certain stressors. Moreover, they showed that autophagosomes seemed to be generated in a Rab9-dependent manner by the fusion of isolation membranes with vesicles derived from the trans-Golgi and late endosomes [142].

Although at the genetic level cancer is caused by diverse mutations, epigenetic modifications are characteristic of all cancers, from apparently normal precursor tissue to advanced metastatic disease, and these epigenetic modifications drive tumor cell heterogeneity. The recent discovery of several mutated epigenetic modifiers in human cancer provides a potential mechanism by which DNA mutation might lead to epigenetic alterations. Environmental factors, such as carcinogens, diet, ageing, injury and inflammation, cause epigenetic reprogramming. The machinery for maintaining epigenetic integrity can be stably disrupted in either of two ways: by mutation or by epigenetic change itself with positive feedback [143].

Histone modification is important to show the status of chromatin structure. Compared to methylation and phosphorylation, histone acetylation is probably the best understood. Histone acetylation is usually associated with active transcription, which is mediated by histone acetyltransferases (HATs) [144]. HATs can act as an oncogene;

abnormal acetylation of histones at proto-oncogenes or acetylation of other tumorigenic players can lead to hyperactivity of these genes or proteins and consequently will enhance carcinogenesis. Abnormal recruitment to the wrong loci or excess of the HATs due to pathological overexpression are typical mechanisms [145]. P300 is one of the well-known HATs and has been an area of study. Studies have shown that P300 was targeted in prostate cancer treatment [146]. Additionally, another study had shown that high expression of p300 in breast cancer may be important in the acquisition of a recurrence phenotype and suggested that the high expression of p300 is an independent biomarker for poor prognosis of breast cancer patients [147]. Here, we showed that both acetyl H3 and acetyl H4 were downregulated after cancer treatment with RCE. Moreover, P300 expression level has declined as well, suggesting that targeting acetylation in breast cancer might be of an importance in cancer treatment. Histone modifications contribute to cancer metastasis by controlling different metastatic phenotypes as those modifications were shown to have a role in EMT and cancer metastasis [148]. This area needs further investigation to understand the molecular mechanism by which histone modifications promotes cancer progression and metastasis.

As described earlier, metastasis requires several crucial events such as cancer cell adhesion, proteolytic degradation of ECM and angiogenesis. Current cancer treatment drugs target cancer progression by blocking cell cycle, inducing cell death and inhibiting tumor invasion and angiogenesis. Several natural therapeutic compounds have been reported to target these events. Cancer's ability to adhere to components of the ECM is a required for cancer's migration and thus represents one of the central steps in metastasis.

It has also been shown that the interaction of fibronectin which is a component of the ECM with specific cell surface receptors such as integrins enhance the metastatic potential of breast cancer cells and blocking this interaction through integrin antibodies can inhibit the adhesion and migration of breast cancer cells [149]. In agreement with our data, it was shown that at non-cytotoxic concentrations of RCE adhesion of MDA-MB-231 to fibronectin as well as their migration were inhibited. This inhibition may partly account for the anti-metastatic potential of RCE on breast cancer cells.

Invasion involves the degradation of the ECM through many proteases, of which MMP-9 appears to play a key role [150]. It has been shown that increased expression of MMPs promote cell growth, aggressiveness and metastatic potential of breast cancer cells [151]. Thus, inhibiting these proteases is an essential approach in fighting breast cancer. Here, we demonstrate that RCE decreased MMP-9 levels and consequently reducing ECM degradation.

Angiogenesis is a process by which new blood vessels are formed and it is essential for tumor growth and metastasis. Prevention of this process would ultimately inhibit both tumor growth and metastasis [152]. One way through which angiogenesis can be blocked is by targeting pro-angiogenic factors secreted by tumor cells such as VEGF. Actually, VEGF is considered to be a major pro-angiogenic factor expressed in 60% of breast cancer patients [153]. Importantly, we found that RCE markedly reduced VEGF production in MDA-MB-231 cells and therefore suggesting that one possible mechanism through which RCE inhibits TNBC tumor growth is to block angiogenesis process. Hypoxia-inducible factor-1 (HIF-1) has been known as an important cancer drug target. Studies have shown a strong correlation between

elevated levels of HIF-1 and tumor metastasis, angiogenesis and poor patient prognosis. Moreover, HIF-1 has been taken as a key factor in regulation of VEGF and VEGFR and other angiogenic factors. Recent advances in cancer biology highlighted the HIF-1α pathway as an important survival pathway for which treatment strategies to inhibit HIF-1α could be developed for cancer treatment [136, 154]. Interestingly, we found that the expression of HIF-1α was also reduced after RCE treatment. Suggesting that inhibition of VEGF and HIF-1 α could participate at least partly in the inhibition of angiogenesis.

Accumulating evidences suggest a strong association between cancer progression and inflammation [155]. Increased production of inflammatory cytokines, such as IL-6 and TNF-α, are known to promote migration, invasion and metastasis of different types of cancer including breast cancer [156]. Additionally, many studies have identified IL-6 and TNF-α as key factors of poor prognosis in TNBC, given their role in promoting invasion and metastasis [157-159]. Therefore, inhibition of these signaling pathways offers a promising strategy for TNBC treatment. Additionally, Hartman et al. reported that TNBC progression relies on coordinate autocrine expression of IL-6 and inhibition of this cytokine lead to inhibition in colony formation *in vitro* and tumor growth *in vivo* of TNBC cells [160]. Likewise, inhibition of breast tumor growth by ulinastatin and docetaxel was associated with decrease in the expression level of IL-6 and TNF- α [160]. Moreover, TNF- α induces the production of IL-6 through ERK1 in breast cancer [161]. Interestingly, here we showed that RCE inhibited both TNF-α and IL-6 production in MDA-MB-231 cells. Therefore, we postulate that RCE inhibition of IL-6 occurs by its ability to suppress the TNF signaling route. Altogether, our data suggests that a possible mechanism through

which RCE inhibits invasion and tumor growth is by inhibiting IL-6 and TNF-α pathways.

NFκB which is a transcription factor that play an important role transmitting signals of inflammatory cytokines to the nucleus. NFκB signaling pathway can be activated by TNF-α and is responsible for the activation of several genes involved in metastasis and its inactivation have been associated with the suppression of metastasis in breast cancer cells [162]. Additionally, the inactivation of NFκB in breast cancer cells has been linked with the inhibition of the expression of many targeted genes involved in metastasis and tumor growth such as MMP-9, VEGF and IL-6 [163]. In this study, we showed that RCE inhibited the NFκ B signaling through downregulation of phospho-p65 as well as MMP-9, VEGF and IL-6 which are the downstream targets of NFκB. It is noteworthy to mention that RCE inhibited TNF-α in MDA-MB-231 cells as well. It seems that NFκB inhibition could account partly for the anti-metastatic effects of RCE. We can hypothesize that one possible mechanism by which RCE exerts its anti-metastatic and anti-tumor growth of TNBC involves the downregulation of TNF-α.

Signal transducer and activator of transcription 3 (STAT3) is responsible for mediating the transcription downstream of several cytokine, growth factor, and oncogenic stimuli. Constitutive activation of STAT3 was described in different cancers including breast cancer. The critical role of STAT3 in cancer cell survival, proliferation, invasion, metastasis and angiogenesis is well-established. Due to this central role, STAT3 is widely considered a good target for anti-cancer therapy [164]. Signaling through the IL-6/JAK/STAT3 pathway have been implicated in breast cancer development and this pathway is thought to be activated by the binding of IL-

6 to their receptors. Those receptors directly or indirectly induce STAT3 activation in different breast cancers. Abnormal STAT3 signaling promotes breast cancer progression through deregulation of downstream target genes which control proliferation such as Survivin, Cyclin D1 and c-Myc, angiogenesis such as HIF-1α and VEGF and EMT such as MMP-9 [165]. Interestingly, we found that RCE inhibited the phosphorylation of STAT3 in MDA-MB-231 cells, thus suggesting that STAT3 inactivation might contribute to the anti-cancer effect of RCE. Most importantly, IL-6 production was also reduced in response to RCE. Thus, our data suggest that IL-6 production reduction might contribute partly to STAT3 inhibition in TNBC.

Flotillin-2, a major protein on lipid rafts, that have role in a number of cellular mechanisms that are dysregulated in tumor cells, such as altered protein signaling and trafficking. It is possible that abnormalities of Flotillin-2 protein contribute to the formation of cancer-specific cellular characteristics [166]. Studies have shown that Flotillin-2 may serve as a potential predictor of prognosis in early-stage breast cancer [167]. Moreover, Wang et al. have shown that breast cancer cells show higher expression of flotillin-2 compared to normal cells, and overexpression of flotillin-2 has been related to clinical stage, classification, tissue differentiation and the expression of human EGFR [166]. As a regulator of lung metastasis, it was found that the decrease in flotillin-2 protein expression reduces the metastatic ability of breast cancer *in vivo* [168]. It was also reported that depletion of flotillin-2 lead to an impaired cell migration of breast cancer [169]. Taken the previous studies together, Flotillin-2 appears to be a hot target in cancer treatment. In this study, RCE led to a decrease in the expression level of Flotillin-2 in MDA-MB-231 cells, suggesting that Flotillin-2 might participate at least partly to the inhibition of metastatic potential of MDA-MB-231 cells.

5.2 Colon cancer

In the present study, we examined the potential anti-cancer activity of RCE on colon cancer. Our findings demonstrated that RCE inhibited the viability and colony growth of colon cancer cells through inactivation of proteasome-dependent degradation of mTOR. Actually, we found that RCE treatment, stimulated protein ubiquitination and proteasome degradation of proteins including caspase-3, AKT and p53 at the beginning. We suggest that this early event serves as a trigger for promoting non-canonical Beclin-1-independent autophagy and subsequent autophagy-dependent caspase-7-dependent apoptosis which ultimately leads to cell death in colon cancer cells.

Herbal extracts and their compounds are well known for their efficiency in inhibiting cell growth and promoting cancer cell death through different mechanisms including autophagy and apoptosis. In this study, RCE was tested against breast cancer and colon cancer cells. It is worth to mention that *Rhus coriaria* was shown to be safe to consume by both humans and animals. In fact, rats fed with doses up to 1 g/kg of lyophilized extract showed no signs of toxicity or mortality [170].

One crucial factor in the coordination of overall protein turnover is Ser/Thr protein kinase mammalian target of rapamycin (mTOR), which promotes growth and regulates amino acid, glucose, nucleotide, fatty acid and lipid metabolism [171]. In order to be activated, mTORC1 translocate from the cytoplasm to the lysosomal surface, where it is activated by growth factors via PI3K– AKT signaling [172, 173]. Activated mTOR coordinates the overall protein turnover and thus promoting cell growth and proliferation [171]. On the other hand, inhibition of mTOR can induce autophagy in eukaryotic [174]. The PI3K/AKT/mTOR pathway is hyperactivated in

many cancers, including colorectal cancer, and is crucial for cancer progression and cancer cell survival [174]. Therefore, mTOR has emerged as a potential target for drug development. Several mTOR inhibitors have already gone through clinical trials for treating various cancers including colorectal cancer [175]. Temsirolimus and Everolimus, are two commercially available mTOR inhibitors approved by the European Medicines Agency in the European Union, and Food and Drug Administration in the United States [176]. Rapamycin, the first discovered natural inhibitor of mTOR, was shown to suppress advanced stage colorectal cancer [177]. Other mTOR inhibitors have been used in colorectal cancer treatment and the role of mTOR inhibitors continues to evolve, as new compounds are synthetized. In this study we found that AKT and mTOR were targeted to proteasome-dependent degradation, along with other proteins upon RCE treatment. Interestingly, our data reveled that mTOR degradation occurred as early as 3 h after-treatment alongside an increase in the level of protein ubiquitination. AKT depletion occurred only 12 h after treatment with RCE, a time at which autophagy was already induced. This might suggest that mTOR suppression occurs through an AKT-independent mechanism. Which suggest that the effect of RCE in colon cancer may be initiated, at least partly, through the degradation and consequent inactivation of mTOR. Our data are in agreement with this claim, since proteasomal inhibition by MG-132 restored total mTOR level, restored the phosphorylated mTORC1, blocked autophagy and reduced cell death in HT-29 cells induced by RCE.

Increasing number of anticancer therapies has been shown to stimulate autophagy pathways that mediate autophagic cell death [178]. In this study we showed that RCE induced autophagy in colon cancer cells. Based on the findings that include intracellular cytoplasmic vacuolation, modulation of autophagy-specific markers such

as conversion of LC3-I to LC3-II and induction of p62 (SQSTM1) accumulation. This agrees with the finding on breast cancer cells in which RCE also induced autophagy. In contrast to breast cancer in which RCE induced beclin-1 dependent autophagy [179], here we show that RCE induced beclin-1 independent autophagy in colon cancer cells. Indeed, we found that RCE promoted beclin-1 degradation by the proteasome and inhibition of the proteasome using MG-132 restored beclin-1 to a level comparable to non-treated control cells. Similar findings were found by other studies on resveratrol, a natural compound, which was shown to induce canonical autophagy in human colorectal cancer cells [180] and non-canonical beclin-1 independent autophagy in breast cancer cells [181]. It appears that the type of autophagy induced in response to anti-cancer drugs depends mainly on the cell type.

Increasing number of studies showed that autophagosome induction can still occur in the absence of key autophagy actors such as beclin-1 [182]. Non-canonical beclin-1-independent autophagy has also been reported in cell treated with compounds that possess anti-cancer activities such as carnosol [182] and cobalt chloride [183]. It is worthy to mention that non-canonical beclin-1-independent autophagy was induced in beclin-1-depleted HeLa cells in response to cobalt chloride [184]. In agreement with the previous mentioned studies, we found that RCE targeted beclin-1 to degradation by the proteasome and also induced beclin-1 independent autophagy in colon cancer cells. Based on these results, we can suggest that non-canonical autophagy can be induced when the function of canonical autophagy proteins is compromised.

Apoptosis and autophagy are two different mechanisms with different key players and cross-talk between them exists, however, the interplay between these two mechanisms remains a big challenge for cancer therapy. Autophagy has a dual role in

the cancer cell, it either contributes to cytoprotective events that promote cancer cell survival and avoiding apoptosis or it can stimulate a pro-death signal pathway in cancer cells which ultimately lead to cell death. Additionally, under some circumstances, autophagy and apoptosis can exert synergetic effects, while in other cases autophagy can be triggered only when apoptosis is suppressed [185, 186]. Therefore, the relation between autophagy and apoptosis may depend upon the cell type, nature and duration of stimulus [187]. In this study we found apoptosis is induced upon RCE treatment, however it is not the main mechanism of cell death. As we found that the main mechanism of cell death might occur as a result of excessive autophagy. This claim is based on several results. First, we illustrated that autophagy occur before apoptosis. Time course experiments provided the evidence that autophagy activation occurred after 12 h of RCE treatment and the inhibition of cell viability was observed already after 24 h after RCE treatment. On the other hand, apoptosis was detected after 48 h, shown by the activation of apoptotic markers such as caspase-7 activation and PARP cleavage. In addition to that, autophagy inhibition by CQ rescued cancer cell death induced by RCE while pre-treating cells with pan-caspase inhibitor had almost no effect on cell death. Our results also showed that even though caspase-3 is depleted due to its proteasome-dependent degradation in HT-29 cells treated with RCE, the mechanism for apoptosis is still functional. Here we showed that HT-29 cells were able to induce apoptosis through caspase-7 dependent pathway. In agreement with our result, a study showed that induction of caspase-7-dependent apoptosis was observed in caspase-3 deficient breast cancer cells when treated with Styrylpyrone Derivative (SPD), a plant-derived active compound [188]. These results suggest that apoptotic cell death comes as secondary response to the increased intracellular stresses and therefore accumulation of cellular damage due to longer exposure of the cells to RCE.

The balance between the regulation of protein turnover and nutrient availability determines the overall status of cell growth. When nutrients are abundant, protein synthesis rates elevate, while protein degradation are kept to minimal. Whereas in energy-stressed cells, synthesis drops with rise in overall degradation [189]. One crucial factor in the coordination of overall protein turnover is Ser/Thr protein kinase mammalian target of rapamycin (mTOR), which promotes growth and suppresses autophagy [171].

mTOR inhibition, due to cell starvation or direct experimental inhibition, is known to induce autophagy and stimulates protein breakdown [190]. Recent studies have established that proteolysis through the UPS is also regulated by mTORC1. Conversely, it is yet to be concluded whether it stimulates or suppresses the UPS activities, due to the contradictory nature of these studies, where the first study reported that inhibition of mTORC1 reduced proteolysis through suppressing the expression of proteasome [53], whilst the second reported the opposite, sighting that the inhibition of mTOR stimulates and enhances both autophagy and proteolysis by UPS [138]. Our results are in agree with the second study. We found that the earliest effect that was observed at 3 h after RCE treatment was an increase in the overall level of protein ubiquitination and the degradation of total mTOR protein. The mechanism by which inhibition of mTOR stimulates proteasome degradation deserves more investigations.

Until recently, autophagy and UPS degradation pathways were referd to as independent events. Conversely, recent studies showed that ubiquitination can target several proteins for degradation through both mechanisms [191]. In our case, we speculate that autophagy may be induced as secondary event that serves as back-up

mechanism to help removing aggregated or misfolded proteins when the function of the proteasome system is overwhelmed due to excessive accumulation of damages. Indeed, we showed that autophagy was blocked when proteasomal function was inhibited by MG-132.

The tumor suppressor protein p53 which is mutated in about 50% of human cancers [192] had been found to be chemo-resistant to many used anti-cancer drugs and was known to enhance cell division and invasion [193]. Depletion of mutant p53 was shown to reduce cell proliferation, inhibit tumorigenicity and increase the susceptibility of colon cancer cells to anticancer drugs [194]. Therefore, mutant p53 is considered to be a potential target for cancer treatment [195]. Studies have shown that mutant p53 evade proteasomal-dependent degradation, which allow it to accumulate in response to stresses and this accumulation seems to have a role in cancer development and progression [196]. Moreover, studies showed that oxidative stress and DNA damage promote stabilization of mutant p53 that is required for its oncogenic function [197]. A current therapeutic strategy to inhibit mutant p53 function is to target it to degradation through autophagy and proteasomal processes [139]. In this study we showed that RCE promoted proteasomal degradation of mutant p53 in HT-29 cells, since inhibition of the proteasomal activity rescued p53 from degradation and reduced cell death in RCE treated cells. However, inhibition of autophagy had no effect on p53 protein level. The molecular mechanism through which RCE targets mutant p53 to proteasomal degradation is still not know and deserves further exploration.

Cyclin D1 is often deregulated in cancer and considered to be a biomarker of cancer progression. The capacity of cyclins to activate the cyclin-dependent kinases (CDKs) is a well-documented mechanism for their oncogenic activities and provides

an attractive therapeutic target. It has been illustrated that some genes such as thrombospondin and the Rho effector ROCK2 respond to cyclin D1 promote migration and invasion [139]. It was also shown that migration of epithelial cells and macrophages was reduced in the absence of cyclin D1 [198]. Interestingly, cyclin D1 was not able to promote migration after p27 knockdown [199] suggesting that cyclin D1 and p27 are related and both might be required for migration process. p27 which is a cyclin-dependent kinase (Cdk) inhibitor have been shown to regulate cell proliferation, cell motility and apoptosis [200]. In this study, we found that both cyclin D1 and p27 were downregulated in HT-29 cells upon RCE treatment; which suggest that the inhibition of migration might occur partly by inhibiting those proteins. However, the mechanism of action and how they are involved needs further investigation.

Chapter 6: Conclusion

Chemoprevention by edible phytochemicals is now believed to be an accessible approach for cancer control. Various phytochemicals derived from edible plants have been shown to interfere with different stages of tumorigenesis. Many cellular processes and mechanisms have been shown to account for the anti-carcinogenic actions of dietary components, but attention has recently been focused on intracellular signaling cascades as common molecular targets for various chemo-preventive phytochemicals.

Here, we demonstrate that RCE exerts a potent anti-angiogenic, anti-metastatic and anti-tumor growth effects on TNBC by targeting multiple key pathways employed by TNBC to acquire a rather aggressive phenotype. Moreover, our data also shows the effect of RCE on mutant p53 colon cancer cells. RCE, stimulates overall intracellular protein ubiquitination associated with proteasome degradation of component of negative regulator of autophagy pathway.

Our findings provide the first instance of a potential role for *Rhus coriaria* as an anti-cancer agent against breast and colon cancer and certainly deserves more attention for further explorations to identify novel effective therapeutic compound(s) against TNBC and colon cancer.

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List of Publications

Hussein, M., Awwad, F., Jithin, D., El Hasasna, H., Athamneh, K., and Iratni, R.."Breast cancer cells exhibits specific dielectric signature in vitro using the open-ended coaxial probe technique from 200 MHz to 13.6 GHz. Scientific Reports 9:4681 (2019).

Athamneh, K., El Hasasna, H., Al Samri, H., Attoub, A., Arafat, K., Benhalilou, N., Al Rashedi, A., Al Dhaheri, Y., Abuqamar, S., Eid, A., and Iratni, R.. "Rhus coriaria increases protein ubiquitination, proteasomal degradation and triggers non-canonical Beclin-1-independent autophagy and apoptotic cell death in colon cancer cells." Scientific Reports 7:11633 (2017).

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