United Arab Emirates University
Scholarworks@UAEU

Theses | Electronic Theses and Dissertations

11-2021

ROLE OF AUTOPHAGY IN ACRIDOCARPUS ORIENTALIS-INDUCED ANTI-BREAST CANCER ACTIVITY

Suhib Hisham Ahmed Saeed Altabbal

Follow this and additional works at: https://scholarworks.uaeu.ac.ae/all_theses

 Part of the Biotechnology Commons

United Arab Emirates University

College of Science

Department of Biology

ROLE OF AUTOPHAGY IN ACRIDOCARPUS ORIENTALIS-INDUCED ANTI-BREAST CANCER ACTIVITY

Suhib Hisham Ahmed Saeed Altabbal

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

Under the Supervision of Dr. Yusra Al Dhaheri

November 2021

Declaration of Original Work

I, Suhib Hisham Ahmed Saeed Altabbal, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Role of Autophagy in Acridocarpus Orientalis-Induced Anti-Breast Cancer Activity*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Yusra Al-Dhaheri, in the College of Science at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

Student's Signature:  Date: 11/11/2021

Copyright © 2021 Suhib Hisham Ahmed Saeed Altabbal
All Rights Reserved

Advisory Committee

1) Advisor: Dr. Yusra Al Dhaheri

Title: Assistant Professor

Department of Biology

College of Science

2) Co-advisor: Prof. Rabah Iratni

Title: Professor

Department of Biology

College of Science

Approval of the Master Thesis

This Master Thesis is approved by the following Examining Committee Members:

1) Advisor (Committee Chair): Dr. Yusra Al Dhaheri
   Title: Assistant Professor
   Department of Biology
   College of Science

   Signature  Date 11-11-2021

2) Member: Dr. Mohammed Akli Ayoub
   Title: Associate Professor in Molecular Pharmacology and Physiology
   Department of Biology
   College of Science

   Signature  Date 11-11-2021

3) Member (External Examiner): Dr. Ali H. Eid
   Title: Associate Professor of Pharmacology
   Department of Basic Medical Sciences
   Institution: Qatar University/Qatar

   Signature  Date 11-11-2021

This Master Thesis is accepted by:

Dean of the College of Science: Professor Maamar Benkraouda

Signature maamar Benkraouda Date Dec. 16, 2021

Dean of the College of Graduate Studies: Professor Ali Al-Marzouqi

Signature Ali Hassan Date 16/12/2021

Copy ____ of ____

Abstract

Breast cancer is the most frequently diagnosed cancer in women worldwide. Triple Negative Breast Cancer (TNBC), which lacks the expression of the hormonal Estrogen Receptor (ER) and Progesterone Receptor (PR), and the amplification of Human Epidermal Growth Factor Receptor 2 (HER2), is not responsive to the hormonal therapy. Therefore, chemotherapy and radiotherapy, which cause severe side effects, are the current available choices to treat TNBC. Hence, there is an urgent need to find new therapeutic choices for TNBC. It is estimated that 50% of all drugs in clinical use during the 21st century are natural products and plants derived. *Acridocarpus orientalis* is a rare plant used in folk medicine to treat many health conditions. The aim of this study is to evaluate the anti-cancer activity of *Acriodocarpus orientalis* Ethanoic Extract (AOEE) against TNBC cell line MDA-MB-231, and to investigate the molecular mechanisms underlying its activity. The results revealed that AOEE inhibits cell proliferation in a concentration- and time-dependent manner. The anti-proliferative effect of AOEE was found to be accompanied with the induction of cell cycle arrest at the G1/S phase. These changes were accomplished with upregulation of p21^{WAF1} and p27 ^{Kip1}, downregulation of PCNA, Cyclin D1, phospho-Rb. Moreover, AOEE induces autophagy through upregulation of autophagy related proteins LC3-II, Beclin-1, p62. Cellular senescence was induced in AOEE treated MDA-MB-231 cells confirmed by p16 upregulation and senescence-associated β-galactosidase (SA-β-gal) expression in the treated cells. AOEE induced activation of ERK and p38 pathways, which might be involved in autophagy induction and senescence. In conclusion, AOEE inhibits the proliferation of MDA-MD-231 breast cancer cells through induction of autophagy, cellular senescence and DNA double stranded breaks, suggesting that *Acridocarpus orientalis* could be a potential source for novel chemotherapeutic agents against TNBC.

Keywords: *Acridocarpus orientalis*, triple-negative breast cancer, autophagy, cell cycle arrest, cellular senescence, DNA damage.

Title and Abstract (in Arabic)

دور الإلتهام الذاتي في التأثير المضاد لسرطان الثدي لنبات القفاص *Acridocarpus orientalis*

الملخص

يعتبر سرطان الثدي أكثر أنواع السرطانات تشخيصاً بين النساء في جميع أنحاء العالم. سرطان الثدي السلبي الثلاثي (TNBC)، الذي يفتقر إلى مستقبلات هرمون الاستروجين (ER) ومستقبل البروجسترون (PR)، وتضخيم عامل نمو البشرة البشري 2 (HER2)، لا يستجيب للعلاج الهرموني، والعلاجات الكيميائية التي تسبب آثارًا جانبية شديدة هي الخيارات الحالية الوحيدة المتاحة لعلاج سرطان الثدي السلبي الثلاثي. لذلك، هناك حاجة ملحة لإيجاد أهداف جديدة وخيارات علاجية جديدة لـ سرطان الثدي السلبي الثلاثي. تشير االدراسات إلى أن 50% من جميع الأدوية المستخدمة في القرن الحادي والعشرين هي منتجات طبيعية أو مشتقة من النباتات. (نبات القفاص) *Acridocarpus orientalis* هو نبات نادر يستخدم في الطب الشعبي لعلاج العديد من الحالات الصحية. الهدف من هذه الدراسة هو تقييم التأثير المضاد للسرطان لـ مستخلص الأوراق ل نبا القفاص ضد خلايا سرطان الثدي السلبي الثلاثي MDA-MB-231، والكشف عن الآليات الجزيئية الكامنة وراء التأثيرالمضاد للخلايا السرطانية. أظهرت نتائج هذه الدراسة أن مستخلص الأوراق ل نبات القفاص يمنع تكاثر الخلايا بطريقة تعتمد على التركيز والوقت. إضافة لذلك, أظهرت الدراسة أن التأثير المضاد لتكاثر خلايا السرطان الثدي السلبي الثلاثي مرتبطًا بإيقاف دورة الخلية في مرحلة G1/S. تم تحقيق هذا التأثير من خلال تحليل إنتاج البروتينات التي تنظم دورة انقسام الخلية مثل بروتين p21, p27 الذي زاد انتاجهم مع استخدام المستخلص، والذي أدى إلى تقليل تنظيم بروتينات PCNA و Cyclin D1 و phospho-Rb. بالإضافة إلى ذلك، أظهرت نتائج الدراسة أن مستخلص الأوراق ل نبات القفاص يحفز الالتهام الذاتي الذاتي والشيخوخة الخلوية في خلايا MDA-MB-231. على المستوى الجزيئي, أظهرت النتائج أنه تم زيادة إنتاج بروتينات LC3-II و Beclin-1 و p62 المرتبطة بالالتهام الذاتي، وزيادة إنتاج p16 و β-galactosidase (SA-β-gal) المرتبط بالشيخوخة في الخلايا المعالجة. كما أظهرت النتائج أن مستخلص نبات القفاص ينشط مسارات (ERK) و p38، التي قد تكون مرتبطة بتفعيل الالتهام الذاتي والشيخوخة الخلوية. في الختام، يمنع مستخلص الأوراق ل نبات القفاص تكاثر خلايا سرطان الثدي MDA-MD-231، ويرتبط التأثير بتحريض الالتهام الذاتي والشيخوخة

الخلوية وضرر للحمض النووي، مما يشير إلى أن نبات القفاص *Acridocarpus orientalis* يمكن أن يكون مصدرًا غنيا لمركبات نشطة جديدة لعلاج سرطان الثدي السلبي الثلاثي TNBC.

مفاهيم البحث الرئيسية: سرطان الثدي الثلاثي السلبي، الالتهام الذاتي، توقف الدورة الخلوية، الشيخوخة الخلوية، تلف الحمض النووي.

Acknowledgements

First, I would like to thank my family for all the support they provided me to complete the master program journey, without them, this journey would not be completed. My special thanks go to my advisor Dr. Yusra Al Dhaheri who guided me since day one. She provided me the support and assistance I needed, which helped me to accomplish this work, and for that, I am very grateful. I would also like to extend my gratitude and appreciation to my co-advisor Prof. Rabah Iratni for his continuous support and mentoring during my research thesis work. Special thanks to all lab mates for the all the guidance and support they provided, Sawsan, Abdul Rasheed, Halima, Aisha and Yaseen.

Dedication

To my beloved parents and family

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AOEE	*Acridocarpus orientalis* Ethanolic Extract
TNBC	Triple Negative Breast Cancer
ER	Estrogen Receptor
EGFR	Epidermal Growth Factor Receptor
PR	Progesterone Receptor
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
HER2	Human Epidermal Growth Factor Receptor-2

Chapter 1: Introduction

1.1 Overview of Cancer

Cancer is a large group of diseases with more than 277 cancer types is known up to date. According to 2020 global cancer statistics, prostate, breast, and colorectum cancers for both sexes are among the highest prevalence cancer types (Sung et al., 2021). Cancer is the second leading cause of death worldwide and expected be the leading cause of death by 2060. According to the American cancer society, growing of the global burden to cancer is expected by 2040 to reach 27.5 million new cases and 16.3 million deaths. The growing in cancer mortality and morbidity rates is explained by the growing and aging of the global inhabitance (American Cancer Society, 2018). Among all human diseases, cancer causes the highest rates of clinical, social, and economic burden (Mattiuzzi & Lippi, 2019). Negative impact on the quality of life in cancer patients by its symptoms, treatment and duration is definite (Lewandowska et al., 2020; Nayak et al., 2017). Cancer cells are transformed cells characterized by the unlimited growth potential. Other characteristics of cancer cells distinguish them from normal cells include their ability to migrate and invade the surrounding or distant tissues, resist cell death, sustain proliferative signaling, induce angiogenesis, and avoid growth suppressors or immune destruction (Markopoulos et al., 2017; Senga & Grose, 2021).

Cancer in solid organs forms tumor, which can be benign or malignant. Benign tumors are localized in the affected tissue without noticed spreading to other adjacent or distant tissues and they tend to grow slowly. On the other hand, malignant or cancerous tumors grow rapidly and can metastasize to the tissues and lymph nodes surrounding

them. Some types of benign tumors such as colon polyps can transform to malignant ones, making their diagnosis, early monitoring and treatment a priority (Patel, 2020). Metastasis is the spread of cancer cells from the primary site of tumor to the adjacent and surrounding tissues or to distant organs in the advance cases (Seyfried & Huysentruyt, 2013). The most common sites for the metastasis of most cancer types are liver, lungs, brain, and bones (Patel, 2020). During metastasis, cancer cells invade from their primary site to the surrounding tissue. Colonization in the distant organ after entering the circulation and survival from the immune cells must occur for cancer cells to metastasize (Elia et al., 2018). Mutations, which change the cell function and cause dysregulation in life cycle, are believed to have a major role in cancer pathogenesis and progression (Hassanpour & Dehghani, 2017). Cancer is multifactorial disease and several factors can interact to initiate and develop different types of cancer (Wu et al., 2016). Table 1 summarize the most common identified causes of cancer.

Table 1: Common causes of cancer (Blackadar, 2016; Hassanpour & Dehghani, 2017)

Viruses	• Human T-cell lymphotrophic virus • Human immunodeficiency virus • Hepatitis B virus • Hepatitis C virus • Human papillomavirus • Epstein-Barr virus • Human herpes virus
External factors	• Sunlight • Tobacco • Alcohol • Salted fish • Wood dust • Radiation rays
Food	• Beta carotene • Red meat • Processed meats • Low fiber diets

Table 1: Common causes of cancer (Blackadar, 2016; Hassanpour & Dehghani, 2017) (Continued)

Lifestyle	• Obesity • Increased adult height • Non-breast feeding • Sedentary lifestyles
Pharmaceuticals and carcinogenic chemicals	• Nitrogen mustards (β-chloroethyl sulphides and amines) • Melphalan • Busulfan • Cyclophosphamide • β-naphthylamine

1.2 Overview of Breast Cancer

Breast cancer is the most frequently diagnosed cancer in women worldwide. According to the Global Cancer Observatory, breast cancer accounts for 11.7% of new cancer cases in 2020 worldwide (WHO, 2020). More than 248,000 new cases were estimated in the United States during 2021, with more than 2,600 cases of them in men (Siegel et al., 2021). Although the incidence of breast cancer increases with advanced aging, breast cancer incidence rates among women aged 20 to 29 years, increased by 2% every year in the last 10 years, whereas the annual increasing in the incidence of breast cancer among women in their 30s for the same period is 0.2%. In adolescents, no increase in the incidence rates was noticed in the last 10 years (Miller et al., 2020; Society, 2018). While the incidence rate of breast cancer is higher in developed countries, the mortality rate of breast cancer is higher in less developed countries (Momenimovahed & Salehiniya, 2019). The development of more invasive forms of breast cancer is affected by race. Black women are at higher risk to develop invasive cancer from pre-malignant breast lesions (Dania et al., 2019).

1.2.1 Types and Clinical Staging of Breast Cancer

Breast cancer can be divided into several subtypes based on the expression of several molecular markers. Cellular expression status of Estrogen Receptor (ER) and/or Progesterone Receptor (PR) along with the amplification of Human Epidermal Growth Factor Receptor-2 (HER2) can divide the breast cancer into 3 types: HR+/HER2-, which express either estrogen or progesterone receptors; HER2+, which express HER2 receptor; and Triple-Negative Breast Cancer (TNBC), which lacks the expression of the two hormonal receptors and the amplification of HER2 (Waks & Winer, 2019).

Breast cancer clinical staging depends on many anatomical features including: the primary tumor extent and size, presence and extent of lymph node involvement, and presence or absence of metastases. TNM system created by the American Joint Committee on Cancer (AJCC), which depends on the anatomical spread of the disease, is the most used clinical staging system for breast cancer worldwide. In TNM system, (T) refers to tumor size and take value from 0-4, (N) refers to nodal status and can take value from 0-4 based on the lymph nodes affected by the cancer cells, and (M) refers to the presence of metastasis and can be 0 or 1. Other staging systems depend on both the TNM staging and the expression status of ER/PR and HER2 can be used to stage breast cancer, the American Joint Committee on Cancer (AJCC) staging system is one example of such staging systems (Boster et al., 2020; Giuliano et al., 2018).

Based on the TNM staging system, breast cancer can be simply divided into 4 stages as below (Boster et al., 2020).:

- Stage 0 represents cancer cells without involvement of basement membrane of breast tissue.
- Stage I represent small invasive tumor without lymph node involvement.

- Stage II represent small invasive tumor with involvement of regional lymph nodes.
- Stage III represent large invasive tumor with extensive nodal involvement is
- Stage IV or metastatic disease refers to breast cancer with metastases to distant organs.

1.2.2 Triple Negative Breast Cancer

Triple negative breast cancer is the most aggressive subtype of breast cancer. Despite the low incidence rates of TNBC among breast cancer subtypes, mortality rates for TNBC are remain the highest and the prognosis is the poorest among the other subtypes. Furthermore, more complex pathogeneses are believed to control the overgrowth of TNBC cells compared to the other breast cancer subtypes (Lee & Djamgoz, 2018; Waks & Winer, 2019). Since TNBC lack the expression of ER, PR and HER2 amplification, TNBC is not responsive to the hormonal therapy. The conventional chemotherapies, which cause severe side effect, are the only current available choices to treat TNBC. Therefore, new targets and therapeutic choices are needed to control TNBC (Waks & Winer, 2019). Additionally, based on the gene expression profile, TNBC can be subdivided into six subtypes, each subtype displays unique gene expression and ontologies. Surprisingly, each subtype has specific features, makes it susceptible to specific chemotherapies not the others. This can be explained by the specific signaling pathways controlling the overgrowth and excessive proliferation for each subtype (Diana et al., 2018; Lehmann et al., 2011). Table 1 summaries the main 6 subtypes of TNBC as divided based on the gene expression status.

Many germline mutations associated with TNBC were identified. *BRCA1/2* gene mutation, which is tumor suppressor gene related to the Homologous Recombination (HR) repair of double-strand DNA breaks, is highly prevalent and its frequency reaches up to 18.2% in TNBC. The current guidelines recommended *BRCA1/2* gene status screening for women with TNBC diagnosed at age below 60 years regardless the positive cancer family history. Other germline mutations such as *PALB2,* and *FANCM* genes mutations were identified among TNBC patients (Hahnen et al., 2017). Mutation in the tumor suppressor gene P53 in human cancer cells is also believed to be associated with the pathogenesis of TNBC. p53 has a key role in the regulation of cell cycle, senescence, and apoptosis in cancer cells (Zeng et al., 2019).

Table 2: Characteristics of different subtypes of TNBC based on the genes expression (Diana et al., 2018)

TNBC Subtype	**Characteristics**
Basal-like 1 (BL1)	- Heavily enriched in cell cycle-related genes and pathways involved in the repair of DNA damages - High ki-67
Basal-like 2 (BL2)	- Enriched in growth factor signaling, such as: epithelial growth factor (EGF), MET and insulin growth factor receptor (IGF1R) pathway - Enriched in signaling of glycolysis and gluconeogenesis
Immunomodulatory (IM)	- Enriched for gene involved in immune cell processes, such as: B, natural killer cell and T cell signaling; cytokine signaling; chemokine receptors and ligands; complement cascade and antigen presentation - High levels of infiltration of immune cells defined tumor-infiltrating lymphocytes (TILs)

Table 2: Characteristics of different subtypes of TNBC based on the genes expression (Diana et al., 2018) (Continued)

TNBC Subtype	Characteristics
Mesenchymal-like (M)	- High expression of vimentin - Decreased of expression of E-cadherin - Activation of: • c-MET • epithelial growth factor (EGF) • mammalian target of rapamycin (mTOR) • fibroblastic growth factor (FGF) • insulin growth factor (IGF) • transforming growth factor β (TGF-β) • Wnt/β catenin pathways
Mesenchymal stem-like (MSL)	- Similar to M with enrichment in genes involved in angiogenesis, including VEGFR2 and some components of immune signaling - High expression of stem cells genes - Low expression of proliferation genes and epithelial-related genes involved in the maintenance of cellular junction, such as claudin (claudin-low breast cancer)
Luminal androgen receptor (LAR)	- Androgen receptor (AR) positivity - Enrichment in hormonally regulated pathways, especially steroid synthesis and metabolism

1.2.3 Diagnosis of Breast Cancer

Imaging techniques including Mammography, Magnetic Resonance Imaging (MRI), Positron-Emission Tomography (PET), Computed Tomography (CT), and Single-Photon Emission Computed Tomography (SPECT) are the main techniques used to have definitive diagnosis for breast cancer (Jafari et al., 2018). Mammography is considered as the gold standard imaging modality for breast cancer diagnosis and proven to reduce mortality in breast cancer patients. MRI is considered more sensitive technique to detect smaller breast masses at earlier stage, duo to the high resolution of the results (Wellings et al., 2016). Biological markers including CA 27-29, CA 15-3,

CA27.29, carcinoembryonic antigen, tissue polypeptide specific antigen, p53, cathepsin D, cyclin E, nestin, Estrogen Receptor (ER), Progesterone Receptor (PR) and HER-2 are usually expressed in breast cancer patients and can be used as tool for better diagnosis and monitoring of the disease (Kabel, 2017). Depending on the risk of having breast cancer, women with *BRCA1* or *BRCA2* mutations are encouraged to screen for breast mass by clinical breast examination and mammography starting at age of 30 years and additional screening with MRI is recommended for higher risk patients (Jafari et al., 2018). New investigational biomarkers including Epidermal Growth Factor Receptor (EGFR) and 8-Hydroxy-2'-deoxyGuanosine (8-OHdG) might be used in the future to detect early stages of noninvasive breast cancer with high sensitivity (Bayo et al., 2018; Boster et al., 2020; Eldin et al., 2019).

1.2.4 Treatment of Breast Cancer

Treatment choices for breast cancer are highly variable. The most important factors determine the choice of treatment are the disease subtype and the disease stage. Additionally, the treatment goal differs among the disease stage at the time of diagnosis. Total cure from the disease is the treatment goal in stage I to III of the disease. Choices with curative intent include breast surgery, radiotherapy, and adjuvant/neoadjuvant systemic treatment (cytotoxic chemotherapy, endocrine treatment, and targeted agents). Furthermore, supportive care of cancer-related pain and chemotherapy related cytotoxicity and side effects are essential (Mutebi et al., 2020). In contrast, palliative care strategies including pain management, as well as psychosocial and spiritual support are the treatment choices used in the metastatic breast cancer. The treatment choices used in stage I to III can also be used in the metastatic breast cancer with specific goals. For example, surgery can be used to

remove breast masses with very few metastatic deposits; and hepatic surgery can be done if the disease presenting with liver metastases to prolong overall survival. In contrast, removal of primary tumor in metastatic disease is controversial and usually not recommended for cure goal. Radiation can be used if the primary breast mass is concurrent with few malignant cells. In case of bone metastasis, radiotherapy is used to palliate the symptoms associated with it. Interestingly, radiotherapy have curative role in case of Central Nervous System (CNS) metastasis. Hormonal therapy and chemotherapy may be used in metastatic breast cancer to prolong the overall survival and quality of life; depending on the subtype of the cancer cells (Sambi et al., 2019).

1.3 Phytomedicine

1.3.1 Overview of Phytomedicine

Phytomedicine is the traditional practice that involves the use of plants and herbs as medications. Before the scientific revolution, ancients' cultures relied on the error and trial to investigate the medicinal effects of the plants. In the ongoing era, several chemical, biological techniques and methods can be used to explore the active compounds responsible for the potential activities of the medicinal plants for the treatment of the diseases, making the plants rich sources for finding novel plant-based medications (Nigam, 2021). Many widely used medications such as Aspirin, Atropine, Codeine, Ephedrine and Digoxin are either plant derived or discovered after the study and analysis of the traditionally used plants (Saad et al., 2017). Its estimated that 50% of all drugs in clinical use during the 21st century are natural products and plants derived (Shakya, 2016).

1.3.2 Biological Activities of Medicinal Plants

Based on the active compounds synthesized by the plant, medicinal plants can have wide biological activities including antispasmodic, antimalarial, analgesic, diuretic, antiviral, anthelmintic, antibacterial, anticancer, antimalarial and anti-inflammatory properties (Shakya, 2016).

Acute diseases such as myocardial infarction and chronic diseases such as osteoarthritis, rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, chronic heart failure and cancers are associated with chronic inflammation and autoimmune responses. *Zingiber officinale Roscoe* (known as Ginger), *Curcuma longa Linn* and *Camellia sinensis* are few examples of medicinal plants containing potential anti-inflammatory active compounds such as Curcumin and epigallocatechin-3-gallate with varied molecular mechanisms (Tasneem et al., 2019). Some medicinal plants are characterized by their antipyretic and analgesic activities in addition to the anti-inflammatory properties they have. *Asparagus officinalis*, *Avena sativa*, *Brassica rapa*, *Capsicum annuum* and *Capsicum frutescens* are few examples of them (Al-Snafi, 2016).

More than 80 medicinal plants as reviewed by Jacob and Narendhirakannan (2019) found to have several effects on diabetes mellitus. Anti-diabetic, anti-hyperglycemic, hypoglycemic and insulin mimetic properties were screened from such plants. Additionally, such plants gained by the anti-lipidemic effects of them can reduce diabetes mellitus mediated hyperlipidemia. Medicinal plants contain active compounds effective for diabetes mellitus include *Coriandrum sativum L.* (Coriander), *Zingiber officinale Roscoe* (Ginger) and *Syzygium cumini (L.) Skeels* (Black plum) (Jacob & Narendhirakannan, 2019).

Other diseases causing high mortality rates such as atherosclerosis can be prevented by the active compounds in some medicinal plants. Phenols, flavonoids, and antioxidants are examples of plant-derived compounds proven to be effective in preventing atherosclerosis via reducing the levels of cholesterol and excessive free radicals' production, which are responsible for the development of vascular plaque, and by decrease the vascular resistance responsible for the development of the disease. Such plants include *Gynostemma pentaphyllum*, *Triticum aestivum* and *Panax* ginseng (Qadir et al., 2018; Sedighi et al., 2017).

1.3.3 Medicinal Plants for Cancer Prevention and Treatment

Numerous numbers of plants were studied and screened for potential compounds to prevent and treat cancer. Initial phase of carcinogenesis can be suppressed or reversed using natural plants-derived biological agents leading to prevention of tumorigenesis. Capsaicin derived from *Capsicum* (chili pepper), catechines derived from green tea and lycopene derived from tomatoes are examples for natural plant derived active compounds effective both for cancer prevention and treatment (Ranjan et al., 2019). Furthermore, many currently used chemotherapeutic agents used for many types of cancer are either natural products isolated from various plants or semisynthetic products based on the modification of the original compounds isolated from the plants. Vincristine and Vinblastine extracted from *Vinca rosea* are vinca alkaloids chemotherapeutic agents, both used for the treatment of various types of cancer such as acute lymphoblastic leukemia, lymphomas, breast cancer and others. Similarly, Paclitaxel is classified as taxane chemotherapeutic agent extracted from *Taxus brevifolia* and current guidelines include it within the treatment choices for several solid tumors such as lung, ovarian and breast cancers (Dragoi & Alexandru, 2020).

1.3.4 Molecular Mechanisms of Anti-Cancer Activity of Medicinal Plants

Due to the high diversity of the active compounds found in the medicinal plants, various molecular mechanisms and pathways are involved in their anti-cancer pharmacological effects. In addition to the induction of cell death pathways such as apoptosis and necrosis, other pathways involved in the inhibition of cancer cells proliferation, the active compounds in the medicinal plants can inhibit invasion and metastasis.

1.3.4.1 Apoptosis

Apoptosis is the programmed cellular death (type I cell death), which is activated by cellular stresses such as DNA damage or Endoplasmic Reticulum (ER) stress (Green & Llambi, 2015). Apoptosis induction can be stimulated by external (receptor mediated) or internal mechanisms. In the external pathway, apoptosis is activated by the interaction of the proapoptotic signaling molecules with specific receptors on the surface of the cells, whereas proapoptotic proteins are released from the internal organelles or expressed by specific genes in the internal pathway. Both internal and external mechanisms of apoptosis activate specific cytosolic caspase cascade to induce intracellular organelles degradation or to prepare the cell for phagocytosis (Savitskaya & Onishchenko, 2015). Figure 1 summarizes the major steps in the external and internal mechanisms of apoptosis (D'Arcy, 2019). *Garcinia quaesita* (Fruits Hexane) extract, a commonly used plant to flavor food in Sri Lanka., is one example of phytomedicinal plants shown to inhibit the growth of TNBC MDA-MB-231 cells via induction of apoptosis (Colamba Pathiranage et al., 2020).

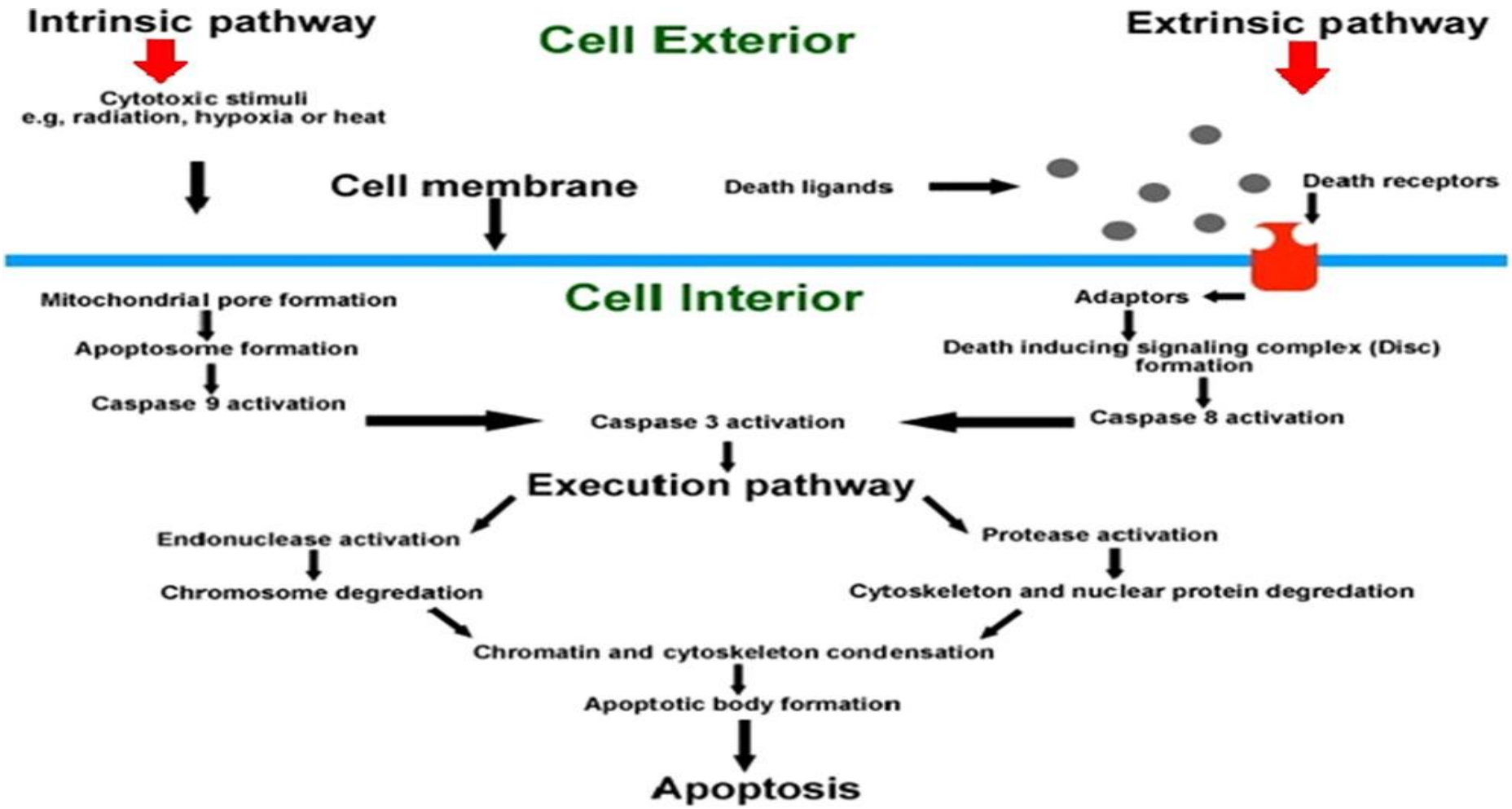

Figure 1: Major steps in the external and internal pathways of apoptosis (D'Arcy, 2019)

1.3.4.2 Autophagy

Autophagy is a process of recycling cellular components. The non-functional macroproteins or whole organelles are degraded inside lysosomes after sequestering them by the formation of the autophagosomes. Fusion of autophagosomes with the lysosomes to form the functional autolysosomes degrade the sequestered proteins. Despite the fact that autophagy is highly conserved catabolic process in eukaryotes, extensive autophagy can lead to cellular destruction in both cancer and aged cells (D'Arcy, 2019; Green & Llambi, 2015). Autophagic cell death (type II cell death) is another major cellular death pathway in mammalian cells targeted by the phytochemicals (Green & Llambi, 2015). Figure 2 summarizes the steps and signaling pathways involved in autophagy (Green & Llambi, 2015).

While autophagy is usually termed as macroautophagy, other forms of autophagy are also existed. Microautophagy is a more specific form of autophagy than macroautophagy. Microautophagy is triggered for recycling specific damaged organelles, for example, mitophagy is one type of microautophagy specific for mitochondria (D'Arcy, 2019).

Selective autophagy (chaperone-mediated autophagy) involves the degradation of misfolded proteins with the specific peptide motifs (such as KFERQ peptide motif) by selective interaction of specific receptor on the surface of phagophors and proteins motifs to guide them for degradation (Hosaka et al., 2020; Johansen & Lamark, 2020).

Chebulinic acid is a polyphenolic compound naturally found in many medicinal traditional plants such as *Phyllanthus emblica* and *Terminalia arborea*. Chebulinic acid was found to inhibit the growth and the metastatic potential of the TNBC cells MDA-MB-231 cells through Autophagy induction (Sharma et al., 2020). Interestingly,

some medicinal plants such as St. John's Wort can inhibit the growth of TNBC by targeting both autophagy and apoptosis (You et al., 2020).

1.3.4.3 Necrosis

Necrosis (type III cell death) is the non-programmed cell death in response to severe environmental or pathological changes inside the cells. Cell swelling, distension of various cellular organelles, clumping and random degradation of nuclear DNA, and extensive injury and/or rapture of the plasma membrane are the distinctive cellular changes accompanied with necrosis. Various regulators are responsible for specific subtypes of necrosis such as necroptosis and oncosis (D'Arcy, 2019).

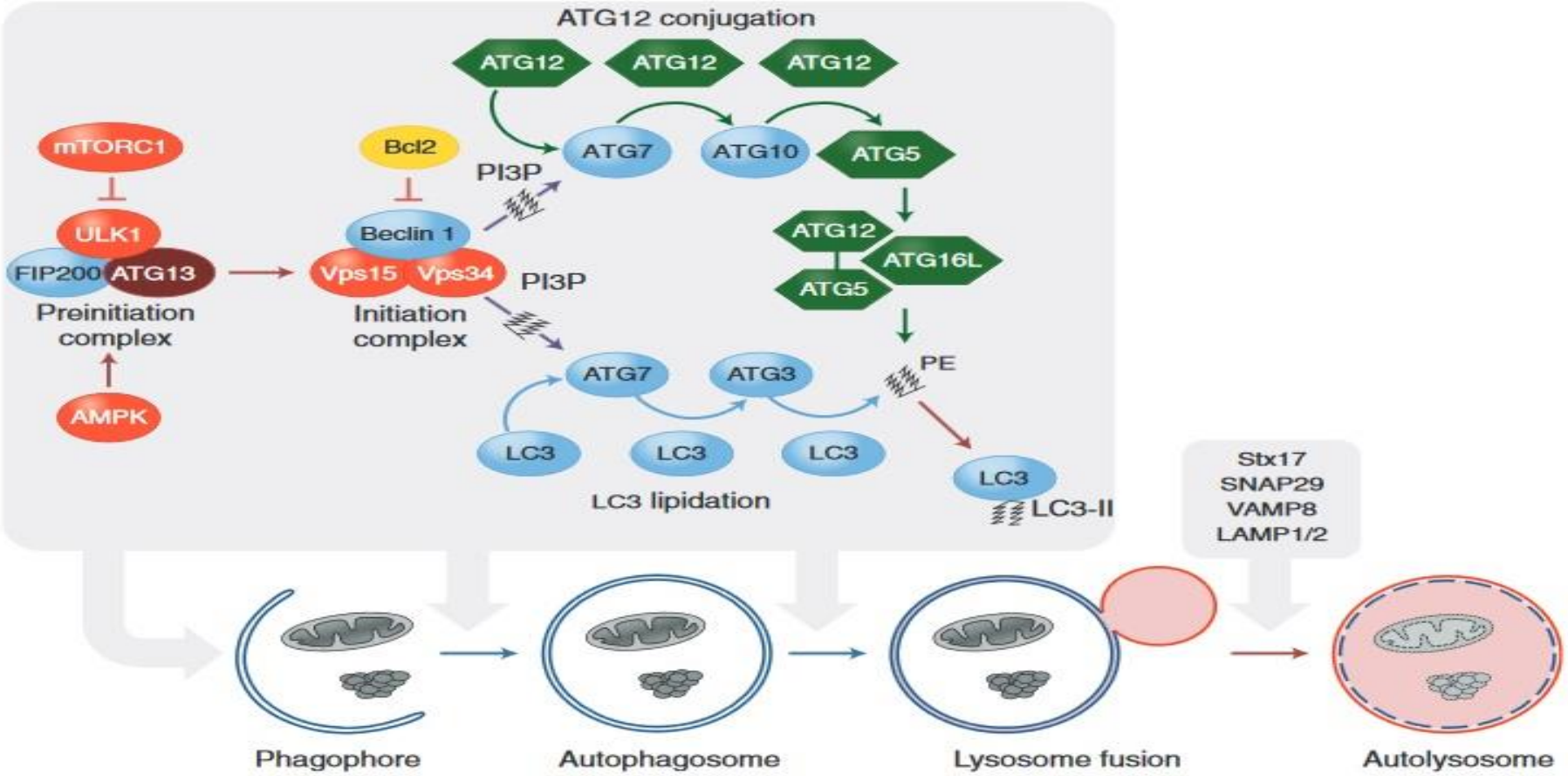

Figure 2: Steps involved in autophagy and it's signaling pathways (Green & Llambi, 2015)

1.3.4.4 DNA Damage

Normally, both healthy and cancer cells can be exposed to various endogenous and exogenous DNA damage stimuli such as Reactive Oxygen Species (ROS) and the exposure to the Ultraviolet (UV) radiation. If DNA damage occurs in normal cell, DNA repair is activated through specific network of proteins called the DNA Damage Response (DDR). DDR can also regulate the inhibition of the proliferation in DNA damaged cells. However, the DNA repair capacity in the cell is limited and excessive DNA damage can activate several growth inhibitory pathways such as apoptosis and senescence (Reuvers et al., 2020; Srinivas et al., 2019). DNA damage has emerged as effective target to inhibit the proliferation of cancer cells. Many anti-cancer treatment options such as radiotherapy and cytotoxic chemotherapy exert their effect by inducing several forms of DNA damage such as Single-Strand Breaks (SSBs) and Double-Strand Breaks (DSBs). For example, the antimetabolite 5-Fluorouacil (5-FU) can lead to chain termination, by inhibiting the synthesis of the nucleoside thymidine and by being incorporated in the DNA after metabolism, and therefore induce SSB and DSB (Reuvers et al., 2020). Kaempferol, a natural derived flavonoid, was reported to inhibit the proliferation of MDA-MB-231 TNBC cells by inducing DNA damage (Zhu & Xue, 2019).

1.3.4.5 Cell Cycle Arrest

For the normal cell to divide and duplicate, it must pass through sequence of events called “cell cycle” (van den Heuvel, 2005; Wenzel & Singh, 2018):

- G1 phase: during the G1 phase of the cell cycle, the cells grow in preparation for DNA replication. Depending on the environmental and developmental signals, cells in G1 may temporarily or permanently leave the cell cycle and

enter arrested phase known as G0 phase. For example, the removal of the growth factors signals during early G1 will enter the cells into G0 phase, while the removal of such signals in the late G1 phase will enter the cells into the S phase.

- S phase: during the S phase of the cell cycle, DNA replication occurs, and each chromosome duplicates to become two sister chromatids.
- G2 phase (separates the S and M phases): during the G2 phase of cell cycle, synthesis of the materials needed for mitosis, such as RNA and proteins, occurs.
- M phase (mitosis): during the M phase of the cell cycle, the duplicated sets of chromosomes separate to the two formed daughter cells. Figure 3 illustrates the cell cycle phases.

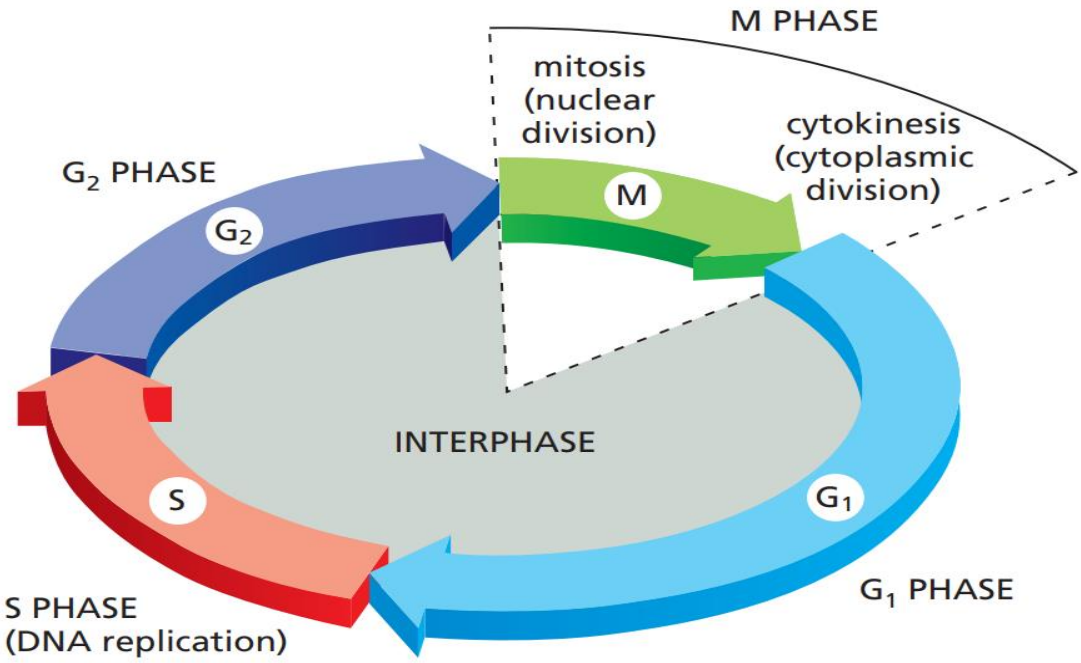

Figure 3: Cell cycle phases (Alberts et al., 2009)

Specific regulatory proteins such as Cyclins, Cyclin-Dependent Kinases (CDKs), oncogenes and tumor suppressor genes regulate the cell cycle progression through specific “checkpoints”, in which they either allow the cell cycle to proceed or not. Those checkpoints ensure that each phase is completed before the progression to the next phase. Each checkpoint in the cell cycle is regulated by various regulatory proteins and signals, which lead to either the progression of cell cycle or cell cycle arrest. For example, a regulatory sensory signal to error in the cell cycle phases (e.g., DNA damage) can induce cell cycle arrest at one or more checkpoint(s). During the G1 phase for example, specific Cyclins/CDKs complexes (Cdk4/cyclin D and Cdk2/cyclin E) must be formed to phosphorylate the Retinoblastoma protein (Rb), which activates the transcription of further proteins responsible for cell cycle transition through G1/S phases (Lim & Kaldis, 2013). Figure 4 shows the checkpoints involved in the cell cycle regulation.

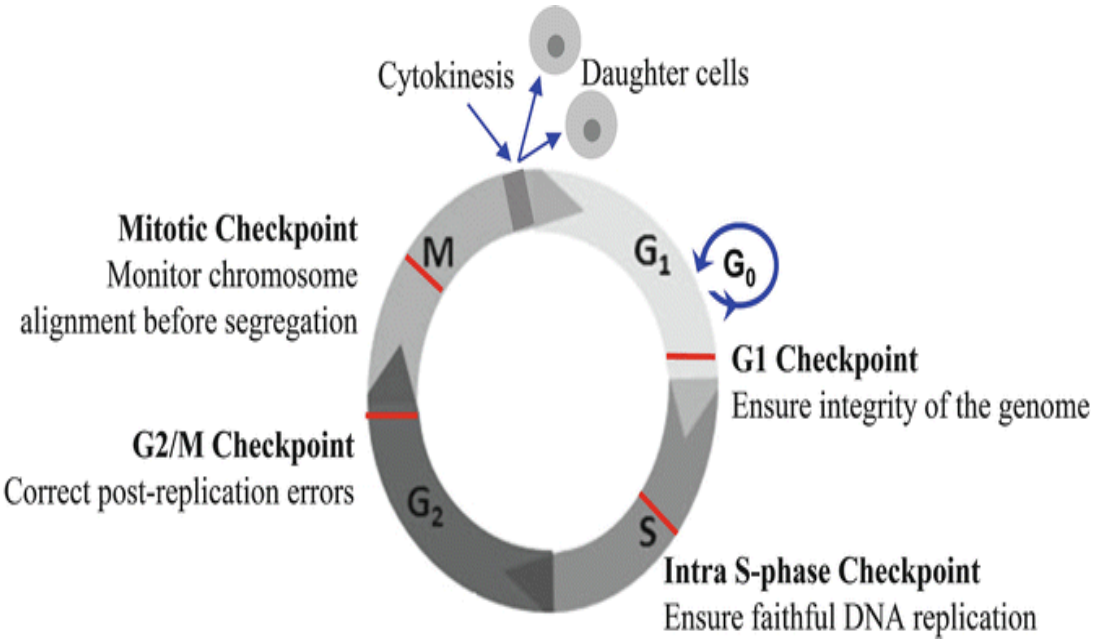

Figure 4: Cell cycle checkpoints (Ma, 2017)

Many medicinal plants with anti-cancer activities act by inducing cell cycle arrest at different checkpoints. For example, bacopaside II, derived from the medicinal plant *Bacopa monnieri*, was found to inhibit the growth and the proliferation of colon cancer cells by inducing G2/M arrest and apoptosis (Smith et al., 2018). Similarly, Ethyl Acetate Fractions of Ajwa Dates (EAFAD) induced S phase cell cycle arrest and decreased the percentage of cells in G2 phase in prostate cancer cell line PC3 (Mirza et al., 2018).

1.3.4.6 Cellular Senescence

Cellular senescence is irreversible cell cycle arrest in response to different intrinsic or external stresses (oxidative damage, ultraviolet and chemotherapeutic drugs) that cause persistent DNA damage. Interestingly, senescent cells, unlike quiescent cells, resist mitogenic or growth factor stimuli, which make them unable to enter the cell cycle again in response to such stimuli (Herranz & Gil, 2018). Figure 5 represents the molecular pathways controlling the growth arrest during senescence.

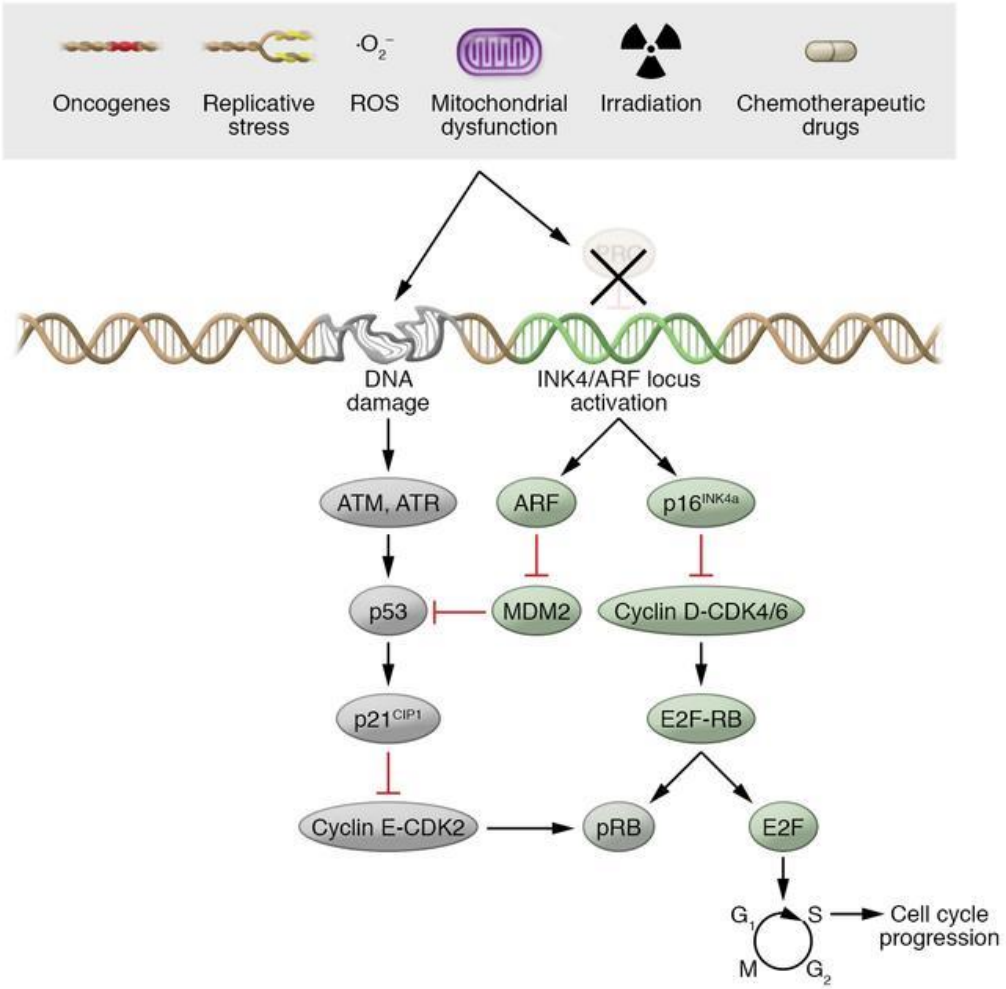

Figure 5: Molecular pathways controlling growth arrest during senescence (Herranz & Gil, 2018)

Senescent cells are characterized by the change in their morphological features such as cell flattening, and the enlargement caused by the rearrangement of the cytoskeleton. Other features of senescence include mitochondrial enlargement and dysfunction and the upregulation of the lysosomal enzyme Senescence-Associated β-galactosidase (SA-β-gal). Thus, SA-β-gal is considered as biological marker for senescent cells (Lee & Lee, 2019). Wogonin, a natural derived compound, was found to inhibit the proliferation of MDA-MB-231 cells by Reactive Oxygen Species (ROS) accumulation that lead to senescence induction (Yang et al., 2020).

1.4 *Acridocarpus orientalis*

1.4.1 Overview of *Acridocarpus orientalis*

Acridocarpus orientalis (Arabic name *Qafas*) is a rare plant that belongs to the family Malpighiaceae. It is a small perennial shrub with highly branched, hairy stems and yellow flowers (Rehman, Hussain, et al., 2019). Figure 6 shows *A. orientalis* growing in Oman. Even though *A. orientalis* is prone to extinction, 30 species of this genus are known to date and distributed in Africa, Asia, New Caledonia, and Arab countries including UAE and Oman (Kisksi et al., 2012; Rehman, Hussain, et al., 2019). In 2012, *A. orientalis* distribution in UAE was restricted to specific regions such as Jebel Hafit in Al-Ain, further information for the distribution and availability of *A. orientalis* are not available (Kisksi et al., 2012).

1.4.2 Chemical Constitutions of *Acridocarpus orientalis*

Phytochemical investigations of the chemical composition of *A. orientalis* collected from Oman revealed several active compounds (Table 3). Similarly, phytochemical screening *A. orientalis* fresh young leaves collected from Jabal Hafeet, Al-Ain revealed similar constituents including flavonoids, phenolics, and tannins (Lotfy, Al-Hammadi, et al., 2020). Phenolic content of *A. orientalis* stem and the leaves showed 128.5 ± 0.17 mg/100 g and 46.6 ± 0.14 mg/100 g, respectively (Ali et al., 2018). For *A. orientalis* extracted from Jabal Hafeet, quantification of total phenolic and flavonoid contents of the dry leaves extract revealed 154.2 mg/g total phenolic content and 79.9 mg/g total flavonoid content (Lotfy, Al-Hammadi, et al., 2020).

Figure 6: *Acridocarpus orientalis* growing in Al-Hamra, in AdDakhiliyah region of the Sultanate of Oman (Rehman, Mabood, et al., 2019)

Table 3: Chemical compositions of *A. orientalis* collected from Oman (Rehman, Hussain, et al., 2019)

Family	**Compounds**
Flavonoids	Quercetin Choerospondin Morin Morin-3-O -[alpha] -L-rhamnopyranoside Morin-3-O -[beta] -D-glucopyranoside
Triterpenoids	Botulin Betulinic acid
Benzoquinones	2,5-Dimethoxy-1,4-benzoquinone 2,6-Dimethoxy-1,4-benzoquinone
Long-chain alcohol	1-Docosanol
Steroidal glycoside	[beta] -Sitosterol-3-O -[beta] -D-glucopyranoside
Steroid	[beta] -Sitosterol

1.4.3 Biological Activities of *Acridocarpus orientalis*

Traditionally, *A. orientalis* has been used in folk medicine for many health conditions. In UAE and Oman, paste is made by crushing the seeds or seeds oil and applied on the forehead to relief headache (Divakar et al., 2016; Sakkir et al., 2012). Furthermore, *A. orientalis* leaves have been used to relieve swellings, muscle pains, and to treat arthritis (Rehman, Mabood, et al., 2019), dermatological and topical disorders, nerve disorders (hysteria and epilepsy) and eye and urinary disorders (Ghazanfar & Al-Al-Sabahi, 1993; Hinai et al., 2020). Additionally, villagers in Oman have used *A. orientalis* plant as a source of yellow dye (Divakar et al., 2016).

Despite the limited studies on *A. orientalis*, two flavonoids morin and morin-3-O-β-D-glucopyranoside isolated from the crude methanolic extract of *A. orientalis* collected from Oman showed various biological activities, including Antifungal, allelopathic and antioxidants effects (Hussain et al., 2014). Interestingly, ethanolic crude extract collected from Al Ain and Oman showed stronger antioxidant, anti-lipoxygenase and anti-inflammatory effects of *A. orientalis* compared to the activity of *A. orientalis* samples collected from Oman (Ksiksi & Hamza, 2012; Ksiksi et al., 2017). Furthermore, *A. orientalis* fresh young leaves ethanolic extract found to increase the levels of reduced Glutathione (GSH) in male albino mice model, indicating the hepatoprotective potential of this extract (Lotfy, Al-Hammadi, et al., 2020).

A study by Lotfy, Ksiksi et al. (2020) revealed significant antidiabetics effects of *A. orientalis* on sterptozocin induced diabetes mellitus rat model. Blood glucose levels and glucagon-immunoreactive cells were reduced after treatment with *A. orientalis* ethanolic extract. Additionally, the number of insulin-positive cells and the serum

level of superoxide dismutase were increased, indicating the promising effects of *A. orientalis* active compounds on diabetes (Lotfy, Ksiksi, et al., 2020). Moreover, α-glucosidase and urease enzymes inhibition by various *A. orientalis* fractions support the antidiabetic effects of the this plant (Rehman, Mabood, et al., 2019).

1.5 Statement of the Problem

Breast cancer cases among females are continuously increasing. Finding new novel therapeutic options for breast cancer with less side effects is urgently needed. Traditional medicinal plants are rich source of new compounds with novel mechanisms of action. *A. orientalis* is a rare medicinal plant in UAE with promising anticancer effects against various types of cancer cells. However, the anticancer effect of *A. orientalis* leaves extract against TNBC and its underlying molecular mechanism is not investigated.

1.6 Hypothesis

This study hypothesized that *A. orientalis* Ethanolic Extract (AOEE) can inhibit the proliferation of TNBC through inducing autophagy process and cell cycle arrest.

1.7 Research Objectives

The objectives of the current study are:

1. To determine cell growth inhibition activity of AOEE in three different subtypes of human breast cancer cell lines, MDA-MB-231, MCF-7 and Hs578t.
2. To assess whether AOEE induces autophagy in breast cancer using MDA-MB-231 cell line as *in vitro* model.

3. To evaluate expression levels of cell cycle control and autophagy related proteins markers, which are associated with the cell cycle and autophagy induction.
4. To investigate whether other oncogenic signaling pathways such as NF-kB, mTOR, and PI3K are implicated in AOEE mediated growth inhibition and autophagy.

Chapter 2: Materials and Methods

2.1 Preparation of *A. orientalis* Ethanolic Extract

An air-dried and ground aerial part of *A. orientalis* (10 g) was extracted with 70% (v/v) ethanol (200 ml). The mixture was macerated for 72 h at room temperature. The resulting extract was then filtered dried under reduced pressure in a rotary evaporator at 40°C and an aqueous ethanol crude extract was generated. This crude extract was weighed, dissolved in 50% ethanol (typically 50 mg/ml) and kept at −20°C for further analysis.

2.2 Cell Culture

The three human breast cancer cell lines used in this study (MDA-MB-231, MCF-7 and Hs578T) were maintained in Dulbecco Minimal Essential Medium (DMEM) at 37°C under a humidified atmosphere containing 5% CO_2. The DMEM (Gibco/Invitrogen, UK) was supplemented with 10% Fetal Bovine Serum (FBS) (Gibco/Invitrogen, UK) and antibiotics (100 U/ml penicillin/streptomycin) (Hyclone, Cramlington, UK). Daily monitoring of the cells was done using EVOS™ XL Core Imaging System (Invitrogen, UK), and media was changed when necessary. Cells were passed as required as follows: media was removed, Phosphate Buffer Saline (PBS) solution was used for washing the cells and then 0.25% Trypsin/EDTA (Gibco/Invitrogen) for 3-5 minutes at 37°C to allow cell to detach, then neutralized with 1:1 ratio of media and adjust the final volume with the growth medium. For each experiment, cells were counted and then seeded at desired density according to the cell/assay type.

2.3 Cellular Viability Measurement

$5 * 10^3$ MDA-MB-231 cells and $7 * 10^3$ Hs578t and MCF-7 cells were seeded in 100 μL of culture medium per well in triplicate in 96-well plates. The cells were left for 24 hours to attach after seeding, then the media was replaced with treatment-added media with increasing concentration of AOEE or an equivalent volume of vehicle (50% EtOH) for control cells and incubated for another 6, 24, 48, and 72 hrs. EVOS™ XL Core Imaging System (Invitrogen, UK) was used to observe the morphological changes upon treatment at several time points. CellTiter-Glo Luminescent cell viability assay (Promega Corporation, Madison, USA), which measure the amount of ATP signaling as indicative of the metabolic activity of the cells, was used to measure the viability at 6 (for MDA-MB-231 cells), 24, 48 and 72 hours post treatment. For each well, 1:1 ratio of new media and CellTiter-Glo reagent were added after removing the media. With gentle shaking, the plate was incubated after covering it with aluminum foil for 15 minutes at room tempreture. GloMax® Explorer Multimode Detection System (Promega Corporation, Madison, USA) was used to detect the luminescence of each well. Data was provided as proportional viability (%) by equating the data for treated cells to untreated cells, which is assumed to be 100%. All experiments were performed in triplicate. The results are representative of an average of at least three independent experiments. Cell viability was calculated as follow:

% of cell viability = (average luminescence values for treatment wells / average luminescence value for control) * 100.

The results were plotted against the range of AOEE concentrations in Excel.

2.4 Senescence Associated-β-galactosidase (SA-β-gal) staining

2.5 * 10^5 MDA-MB-231 cells were seeded and cultured in each well of 6-wells plate. The cells were left overnight to attach then treated with AOEE (200 and 400 μg/mL or 50% EtOH for 96 hours). Cells were washed with PBS, then fixed with 2% formaldehyde/0.2% glutaraldehyde for 5 min at room temperature (Al Dhaheri et al., 2013). The senescent cells were counted manually using microscope at 48, 72, and 96hours post-treatment.

2.5 Cell Lysate Preparation

1.8 * 10^6 of MDA-MB-231 cells were seeded in 10 cm tissue culture dish and treated with AOEE (200, 400 and 600 μg/ml) or vehicle (50% EtOH) for 48 hrs. After that, cells were washed twice with ice-cold PBS, scraped, pelleted, lysed with sonication in Radioimmunoprecipitation Assay (RIPA) lysis buffer (Pierce) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor (Roche). Cell lysates were centrifuged at 14,000 rpm at 4°C for 30 min after incubation for 30 min on ice. BCA protein assay kit (Thermo Scientific) was used to quantify protein concentrations of the supernatants, and the lysates were adjusted with lysis buffer. The supernatants were aliquoted and stored at - 80°C.

2.6 Western Blotting Analysis

Depending on the molecular weight of the protein of interest, 6 - 15% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels were prepared and loaded with equal amounts (15 μg) of cell lysates along with PageRuler plus prestained protein ladder (Thermo Scientific) for electrophoresis. The proteins separated in the gel were then transferred onto Polyvnylidene Fluoride (PVDF) membranes

(Millipore). The membranes were then blocked in 5% non-fat skim milk prepared in TBST (Tris-buffered saline with 0.05% Tween 20) for one hour at room temperature, then were washed 3 times with TBST to remove the excess blocking buffer. After that, the membranes were incubated with the specific diluted primary antibodies in blocking buffer overnight at 4°C. The membranes were then washed 3 times with TBST and anti- mouse, -rabbit or -goat Horseradish peroxidase-conjugated anti-IgG were used as secondary antibodies depending on the primary antibody used. Immunoreactive bands were detected by enhanced chemiluminescence ECL/ SuperSignal West Femto chemiluminescent substrate (Thermo Scientific) using C-DiGit® Blot Scanner (LI-COR). Restore western blot stripping buffer (Thermo Scientific) was used to strip the membranes when needed according to the manufacturer's instructions. Image Studio Digits Ver 5.2 software **(**LI-COR) was used to obtain the bands and measure their intensity.

Antibodies to Cyclin D1 (04-1151), $p21^{WAF1}$ (05-655), Phospho-Rb (Ser807/Ser811) (07-899) and PCNA were obtained from Millipore (Millipore, Hayward, CA, USA). Antibodies to β-actin-HRP (sc-47778), goat anti-mouse IgG- HRP (sc-2005), and goat anti-rabbit IgG-HRP (sc-23575), were obtained from Santa Cruz Biotechnology, Inc (USA). Antibodies to SQSTM1/p62 (ab91526) was obtained from Abcam (Abcam, Cambridge, UK). Antibodies to Cyclin E1 (#4129), Phospho-Histone H2A.X (Ser139) (#9718), LC3B (#2775), Beclin-1 (#3495), $p27^{Kip1}$ (#3686), Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (#9101) and Phospho-p38 MAPK (Thr180/Tyr182) (#9211) were obtained from Cell Signaling. Antibody to p16 (551154) was obtained from BD Biosciences.

2.7 Statistical Analysis

The data are expressed as the mean ± Standard Error (SEM), and are derived from at least three independent experiments, unless specified otherwise. The difference between experimental and control values was assessed by ANOVA followed by Least Significant Difference (LSD) post-hoc multiple comparison test. Student t-test was used to calculate the significance between the two independent groups. The statistically significant difference was set at p values of * 0.05, ** < 0.005, *** < 0.001 between control and treated groups.

Chapter 3: Results

3.1 AOEE Decreases Cell Viability of Human Breast Cancer Cell Lines

To investigate the anti-breast cancer activity of AOEE, three breast cancer cell lines (MDA-MB-231, MCF-7 and Hs578T) were treated with increasing concentration of AOEE and cell viability was measured at three time points (24, 48, and 72 hours) using CellTiter-Glo® assay. As shown in Figure 7 (a-c), AOEE decreases cell viability of the three human breast cancer cell lines in time- and concentration- dependent manner compared to control group (50% ethanol). Notably, the three human breast cancer cell lines showed relatively similar response to the AOEE treatment except for the highest concentration of AOEE of 600 μg/mL, where more cell viability reduction was detected in MDA-MB-231 and Hs578T cells, which implies a higher sensitivity of both cell lines to AOEE when used at high concentrations. The estimated IC_{50} (the concentration that leads to 50% inhibition) value for MDA-MB-231, Hs578T, and MCF-7 cells were approximately ~300, 350, and 325 μg/mL respectively at 72 hours with less than 20% cell viability of MDA-MB-231 observed at higher concentration (600 μg/mL) (Table 4). Since MCF-7 and MDA-MB-231 exhibited comparable sensitivity, this implies that estrogen receptor expression does not affect the AOEE-induced growth inhibition in human breast cancer cells. Collectively, these data indicate that AOEE exerts a negative action on the viability of the breast cancer cells independently of estrogen receptor and p53 status.

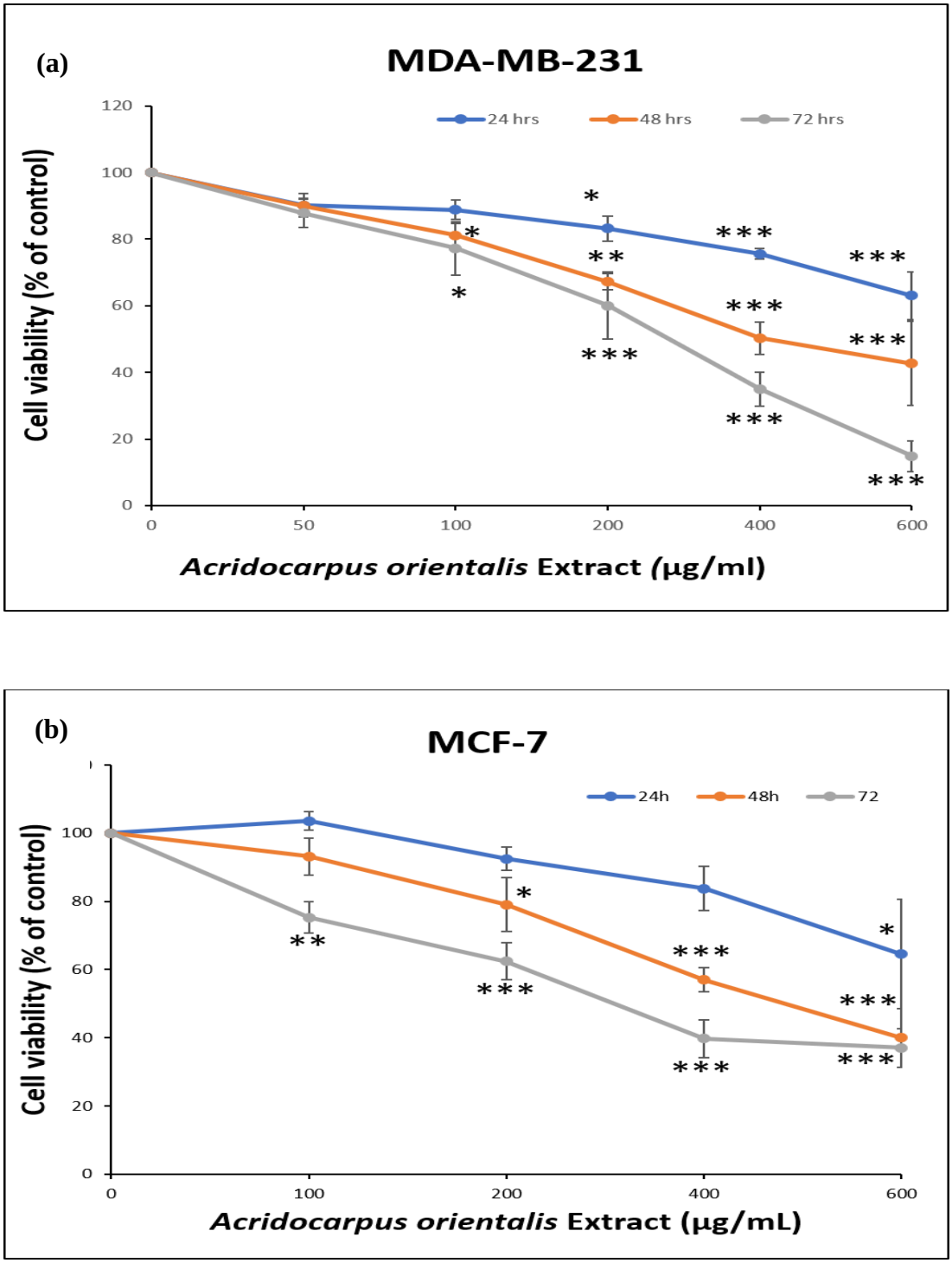

Figure 7: Inhibition of cell viability of human breast cancer cells by AOEE after 24, 48, and 72 hrs. (a) MDA-MB-231, (b) MCF-7, (c) Hs578T. Data represent the mean of three independent experiments carried out in triplicate. Statistical analysis for cell viability data was performed using one-way ANOVA followed by LSD Post-Hoc test (* $p< 0.05$, ** $p< 0.005$, *** $p< 0.001$).

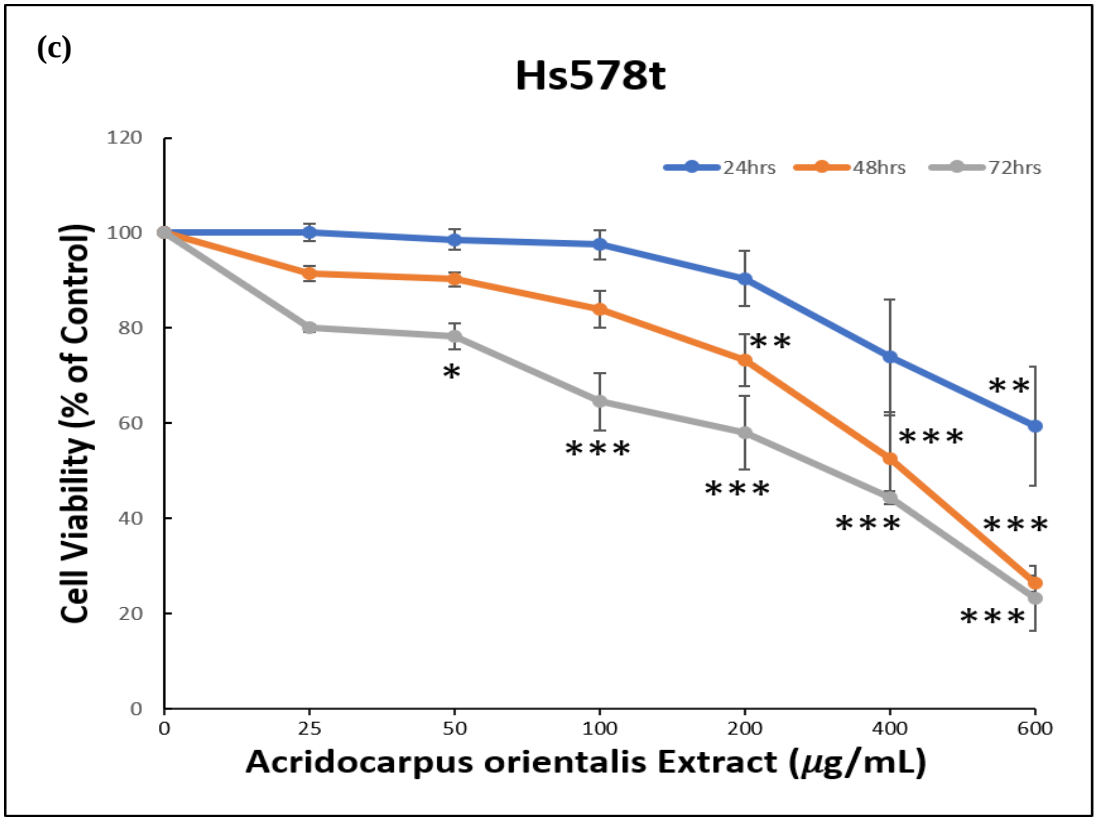

Figure 7: Inhibition of cell viability of breast cancer cells by AOEE after 24, 48, and 72 hrs. (a) MDA-MB-231, (b) MCF-7, (c) Hs578T. Data represent the mean of three independent experiments carried out in triplicate. Statistical analysis for cell viability data was performed using one-way ANOVA followed by LSD Post-Hoc test (* $p< 0.05$, ** $p< 0.005$, *** $p< 0.001$). (Continued)

Table 4: The estimated IC50 (µg/mL) ± SEM of AOEE in three breast cancer cell line, MDA-MB-231, MCF-7 and Hs578t at 72 hrs.

Cell line	**Estrogen Receptor Status**	**Estimated IC50 (µg/mL) at 72 hours**
MDA-MB-231	Negative	~311 ± 23
MCF-7	Positive	~390 ± 36
Hs578t	Negative	~325 ± 12

In this study, the mechanism(s) by which AOEE exerts its anti-cancer activity on the highly proliferative and invasive Estrogen Receptor (ER)-negative, mutant p53 breast cancer cell line MDA-MB-231, was investigated.

3.2 AOEE Induces Morphological Changes in MDA-MB-231 Cells

To investigate the molecular mechanism(s) responsible for the anti-cancer effect of AOEE in MDA-MB-231 cells, the morphological changes under the microscope at 48 hours post-treatment were monitored. As seen in Figure 8, the control group showed the morphological features of healthy and normally proliferated MDA-MB-231 cells. On the other hand, at low concentration of AOEE (200 μg/mL), the cells started to lose the cell-cell contact and they started to look flattened and more elongated, and cells showed a kind of membrane extensions as they lost cell to cell contact. Interestingly, the cytoplasmic vacuolation (black line arrow) was clear at 200 μg/mL of AOEE and the percentage of cells showed such vacuolation increased in concentration- dependent manner. The cytoplasmic vacuolation was reported to be induced by the induction of autophagy (Chen et al., 2005). At high concentrations of AOEE (400 and 600 μg/mL), the cells were enlarged with flattened shape (dashed black line arrow) compared to the untreated cells. The enlarged and flattened cell morphology is one of the characteristics of cells undergoing cellular senescence (Wang & Dreesen, 2018). The minimal appearance of morphological features associated with cell death, such as cellular shrinkage and floating cells, might indicates that the anticancer effect of AOEE is caused by non-apoptotic pathway(s). The molecular mechanisms for the anti-cancer effect of AOEE in MDA-MB-231 cells were further investigated in the next sections of the study.

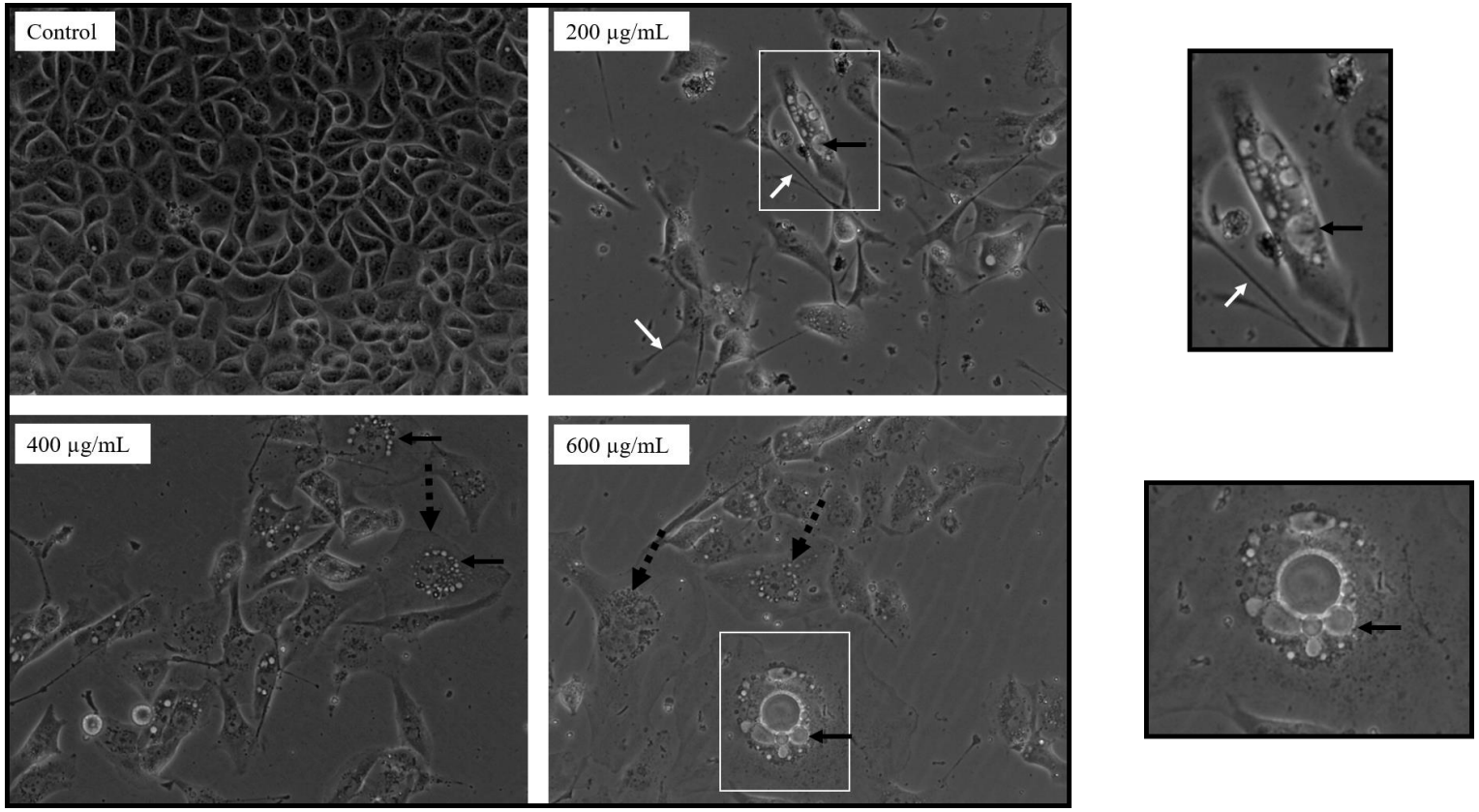

Figure 8: Representative micrographs of the cellular morphology of MDA-MB-231 treated with increasing concentrations of AOEE after 48 hrs. Cells were viewed using EVOS XL Core Cell Imaging System (Life Technologies). Magnification: 40X. The black arrows show cytoplasmic vacuolation, the black dashed-arrows show flattened enlarged cells. The white arrows show the cell extensions and elongation.

3.3 AOEE Inhibits the Proliferation of MDA-MB-231 Cells

The minimal appearance of cellular death morphological features under the microscope prompting us to count the number of viable cells in the control group and the treated group at different AOEE concentrations (100, 200, 400 and 600 μg/ml) and time points (24, 48 and 72 hours) after treatment. As shown in Figure 9 and Table 5, the number of cells after 24 hours of treatment did not change significantly, which indicates minimal effect of AOEE on the proliferation at this time point. Similar pattern was observed After 48 and 72 hours of treatment with 100 μg/ml indicating a minimal effect of AOEE on cell proliferation when treated with 100 μg/ml. However, number of cells treated with 200 and 400 μg/ml of AOEE decreased significantly compared to the control group, which indicates that cell growth inhibition started at 200 μg/mL after 48 hours. Interestingly, at highest concentration of AOEE (600 μg/ml), a significant reduction in the number of viable cells after 48 and 72 hours of treatment was observed. The results obtained confirm that AOEE inhibit the proliferation of MDA-MB-231.

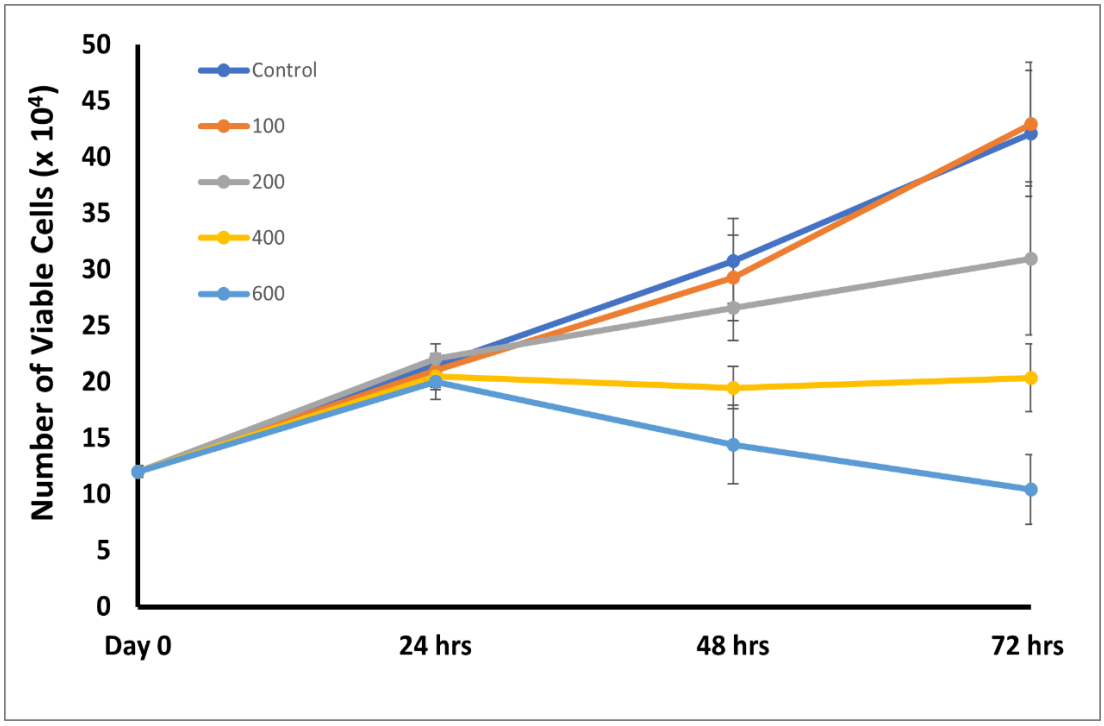

Figure 9: AOEE inhibits the proliferation of MDA-MB-231 breast cancer cells. MDA-MB-231 cells were treated with increasing concentration of AOEE for 24, 48, and 72 hrs, then, cell number was measured using Muse® Count & Viability Kit (Millipore). Data represent the mean of three independent experiments carried out in triplicate ± SEM.

Table 5: The number of viable cells in AOEE treated MDA-MB-231 cells. Data represent the mean of three independent experiments carried out in triplicate ± SEM.

	Total Viable Cells (x 10^4)			
	Day 0 (treatment)	Day 1	Day 2	Day 3
control	12.019 ± 0.51	21.51 ± 0.8	30.77 ± 3.8	42.13 ± 5.6
100		21.04 ± 1.5	29.28 ± 3.8	42.97 ± 5.5
200		22.09 ± 1.3	26.58 ± 2.9	30.97 ± 6.8
400		20.51 ±1.2	19.49 ± 1.9	20.38 ± 3.0
600		20.03 ± 1.6	14.43 ± 3.5	10.43 ± 3.1

Using Western blotting, the antiproliferative effect of AOEE on MDA-MB-231 cells was confirmed by analyzing the expression levels of Proliferating Cell Nuclear Antigen (PCNA) protein in the control and AOEE treated cells. PCNA plays a major

role in the proliferation of the cells through its role in nucleic acid metabolism during DNA replication and repair (Kelman, 1997). As shown in Figure 10, PCNA protein expression levels decreased in the treated MDA-MB-231 cells starting at 200 μg/mL of AOEE treatment.

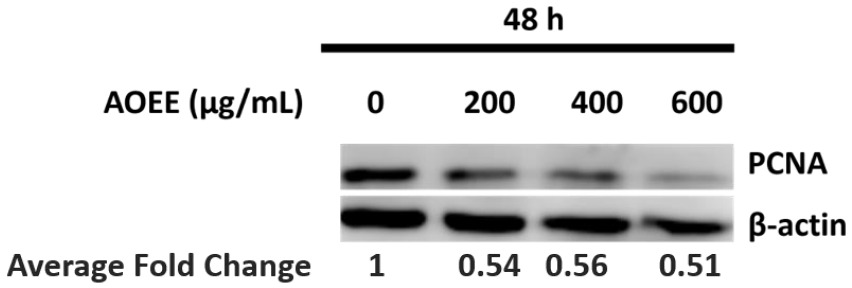

Figure 10: AOEE decreases the expression of PCNA in MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle or increasing concentrations of AOEE (200, 400, and 600 μg/mL) for 48 hrs, then the protein levels of PCNA were examined by Western blotting. β-actin was used as loading control. Three independent experiments were performed to calculate the average fold change.

3.4 AOEE Induces Autophagy in MDA-MB-231 Cells

Three major types of cell death are known to occur in cancer cells: caspase-dependent apoptosis, necroptosis, and autophagic cell death. For this, the type(s) of cell death induced by AOEE in breast cancer cells MDA-MB-231 was determined. To confirm that apoptosis is not involved in the AOEE-induced growth inhibitory effect on MDA-MB-231, cells were the pre-treated with Z-VAD-FMK - Pan-Caspase inhibitor, potent apoptosis inhibitor, and the viability of the cells were measured after 48 hours of AOEE treatment (400 and 600 μg/ml). As seen in Figure 11, Z-VAD-FMK pre-treatment showed no significant effect on cell viability after AOEE treatment

compared to the group treated without Z-VAD-FMK. Altogether, the results suggest that the inhibitory effect of AOEE on MDA-MB-231 cells is independent of apoptosis.

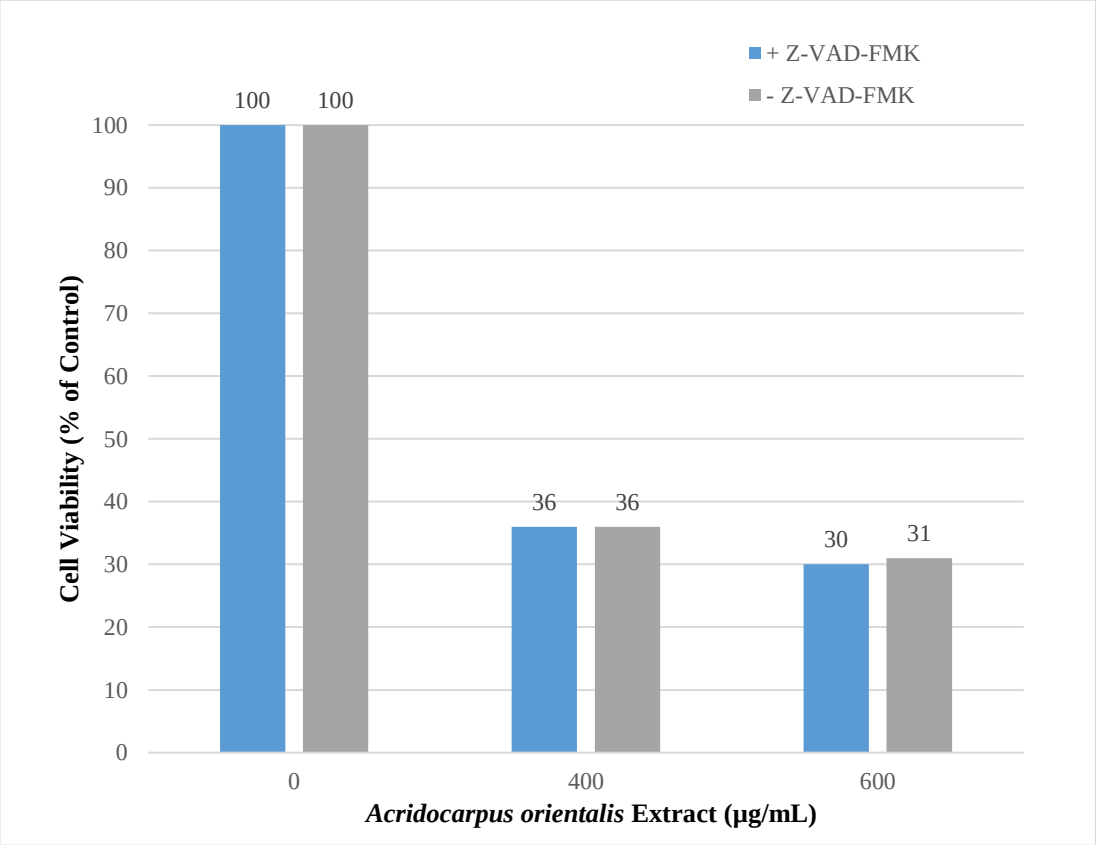

Figure 11: Cell viability of MDA-MB-231 cells treated with 400 and 600 µg/mL of AOEE with or without 50 µM of Z-VAD-FMK - Pan-Caspase inhibitor for 48 hrs. CellTiter-Glo Luminescent cell viability assay was used to measure the cell viability.

Necroptosis, a programmed form of necrosis, is shown to be specifically inhibited by necrostatin-1. Next, necroststin-1 was used to test if necroptosis contributes to AOEE-induced cell death. The results show that necrostatin-1 does not inhibit AOE-induced cell death (Figure 12) indicating that AOEE-induced cell death does not involve necroptosis.

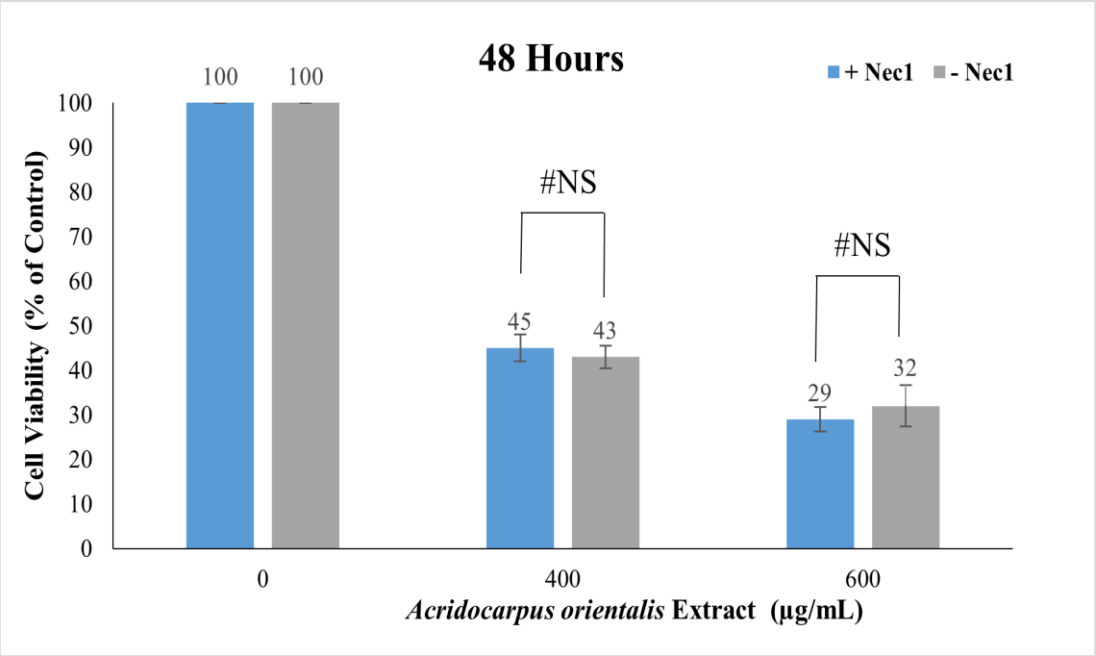

Figure 12: Cell viability of MDA-MB-231 cells treated with 400 and 600 µg/mL of AOEE with or without 50 µM of Necrostatin-1 (Nec-1) for 48 hrs. #NS: Non-significant. CellTiter-Glo Luminescent cell viability assay was used to measure the cell viability. Data represent the mean ± SEM of three independent experiments carried out in triplicate. Student t-test was used to evaluate the significance between the two groups.

To study whether autophagy is involved in AOEE-induced cell death, thr autophagy inhibitor 3-methyladenine (3-MA) was used confirm the role of autophagy as a key molecular mechanism in the anti-cancer effect of AOEE on MDA-MB-231 cells, the effect of 3-Methyladenine (3-MA), a widely used autophagy inhibitor acting by inhibiting class III Phosphatidylinositol 3-Kinases (PI3K) which block the formation of autophagosomes (Wu et al., 2010). The results showed that 3-MA decreases AOEE-induced proliferation inhibitory effect MDA-MB-231 cells (Figure 13). These findings suggest that autophagy contributes to anti-proliferative effect shown by AOEE treatment on MDA-MB-231.

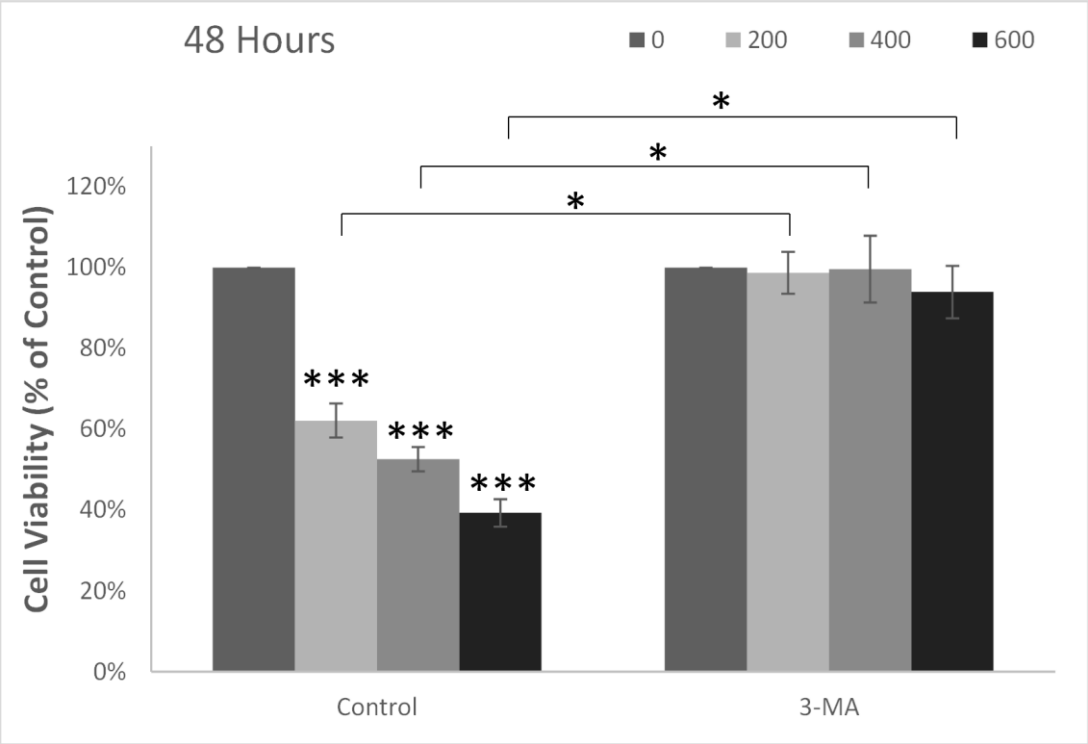

Figure 13: Cell viability of MDA-MB-231 cells treated with AOEE (200, 400 and 600 µg/mL) with or without 5 mM of 3-MA for 48 hrs. Data represent the mean ± SEM of three independent experiments carried out in triplicate. Student t-test was used to calculate the significance between the two groups (cells treated with AOEE without 3-MA and cells treated with AOEE and 3-MA) at each concentration (* $p < 0.05$). One-way ANOVA followed by LSD Post-Hoc test was used to calculate the significance between the treated and control groups (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$).

Next, to confirm autophagy induction in breast cancer cells MDA-MB-231, the alteration in the cellular levels of different autophagy related proteins and markers was analyzed using Western blotting. The formation of the autophagosomes at the early step of autophagy is followed by the fusion with the lysosomes to form autolysosomes. Autophagosomes formation requires the cleavage of microtubules-associated light chain-I (LC3-I) to form LC3-II, which is recruited and conjugated to phospholipids and incorporated into the autophagosomes (Tanida et al., 2008). Therefore, LC3-II is used as indicator for the induction of autophagy. As shown in Figure 14, the levels of

the conjugated form (LC3-II) clearly increased starting at cells treated with 200 μg/mL of AOEE, with average ~3-fold increase in cells treated with 600 μg/mL.

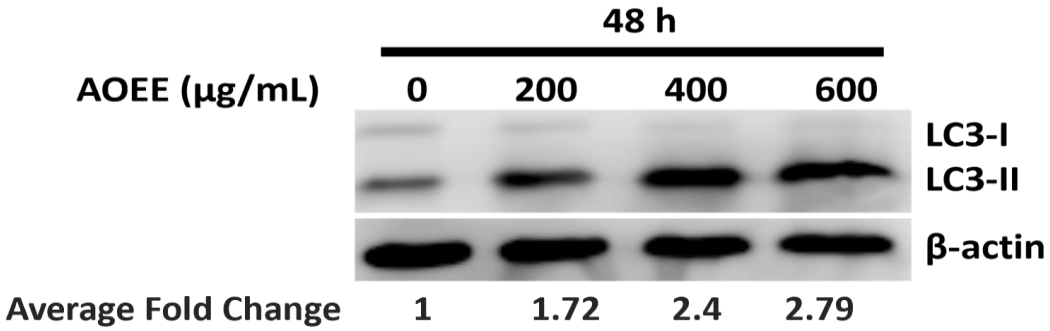

Figure 14: AOEE alters the levels of the conjugated form of LC3-I MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle or increasing concentrations of AOEE (200, 400, and 600 μg/mL) for 48 hrs, then the protein levels of LC3-I and LC3-II were examined by Western blotting. β-actin was used as loading control. Three independent experiments were performed to calculate the average fold change.

The alteration in the level of Beclin-1, a protein with autophagy-promoting activity, was also determined using Western blotting. As shown in Figure 15, the protein levels of Beclin-1 increased starting at 400 μg/mL of AOEE treatment. In addition to its role in autophagy induction, Beclin-1 is considered as mammalian tumor suppressor protein, plays a role in inhibiting the anti-apoptotic protein Bcl-2, therefore inhibit the proliferation of the cancer cells. Notably, several cancer cell lines are reported for Beclin-1 deficient (Liang et al., 1999).

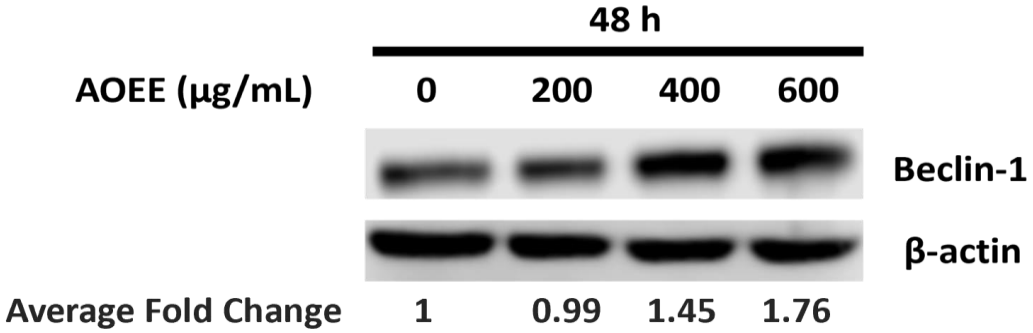

Figure 15: AOEE alters the levels of the Beclin-1 in MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle or increasing concentrations of AOEE (200, 400, and 600 µg/mL) for 48 hrs, then the proteins levels of Beclin-1were examined by Western blotting. β-actin was used as loading control. Three independent experiments were performed to calculate the average fold change.

In similar pattern to Beclin-1, the levels of p62, another important autophagy related protein, was increased starting at 400 µg/mL of AOEE treatment (Figure 16). The p62 or Sequestosome 1 (SQSTM1) protein helps in linking and localizing the ubiquitinated non-functional or misfolded proteins to be degraded inside the autolysosomes, by binding to ubiquitin (Bjørkøy et al., 2009). Even though p62 is degraded during autophagy and its accumulation may indicates lack of autophagy , p62 protein accumulation can indicate also an abortive (prolonged) autophagy (Benhalilou et al., 2019). Altogether, the results confirm that autophagy could be one mechanism by which AOEE exerts its anti-cancer activity against MDA-MB-231 cells.

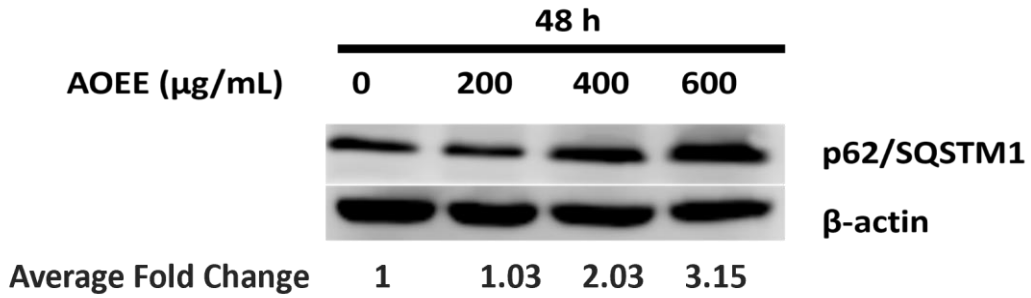

Figure 16: AOEE alters the levels of the p62/SQSTM1 in MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle or increasing concentrations of AOEE (200, 400, and 600 µg/mL) for 48 hrs, then the protein levels of p62/SQSTM1 were examined by Western blotting. β-actin was used as loading control. Three independent experiments were performed to calculate the average fold change

3.5 AOEE Induces Senescence in MDA-MB-231 Cells

To investigate the role of cellular senescence in the anti-cancer effect of the AOEE on MDA-MB-231 cells, the Senescence-Associated β-galactosidase (SA-βgal) activity, a widely used marker for senescent cells, was measured using SA-β-gal staining at AOEE concentrations of 200 and 400 µg/mL after 48, 72 and 96 hours of treatment. Figure 17 shows the expression of SA-β-gal (black arrows) seen under the microscope, where the blue stained cells represent cells positive for SA-β-gal, which indicates the induction of senescence in AOEE treated MDA-MB-231 cells in concentration- and time- dependent manner. As seen in Figure 18, the percentage of senescent cells (blue stained cells counted) significantly increased in both AOEE concentrations used starting from 48 hours. To further confirm the role of cellular senescence in the anti-cancer effect of the AOEE, the variation in the level of the tumor suppressor gene p16, an indicator for cellular senescence (Rayess et al., 2012), was assessed using Western blotting. The results shown in Figure 19 demonstrate an increase in the levels of p16 protein at AOEE concentrations of 400 and 600 µg/mL. Overall, these results confirm

that AOEE induces cellular senescence, suggesting its role in the AOEE-induced anti-cancer activity against MDA-MB-231 cells.

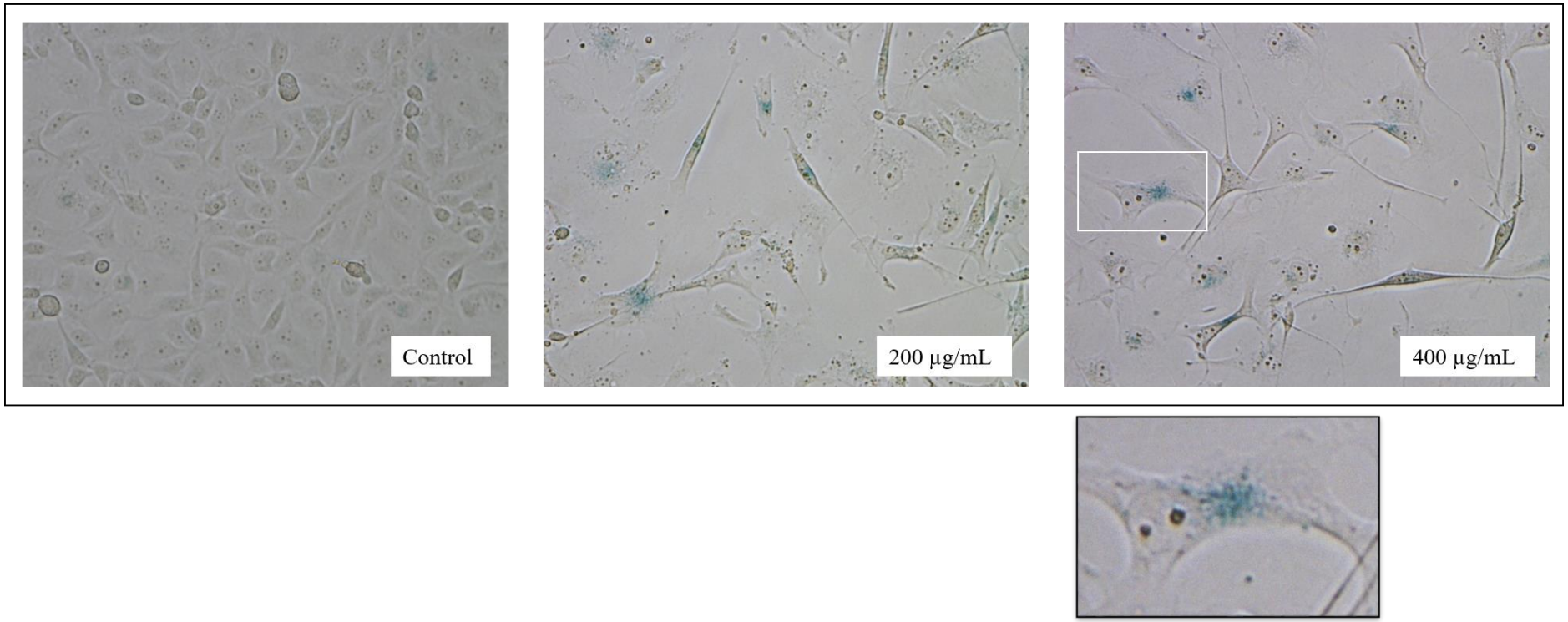

Figure 17: The expression of SA-β-gal in AOEE treated MDA-MB-231 cells after 96 hours of treatment. MDA-MB 231 cells were incubated with 400 µg/mL for 96 hrs and stained for SA-β-galactosidase activity to detect senescence after treatment.

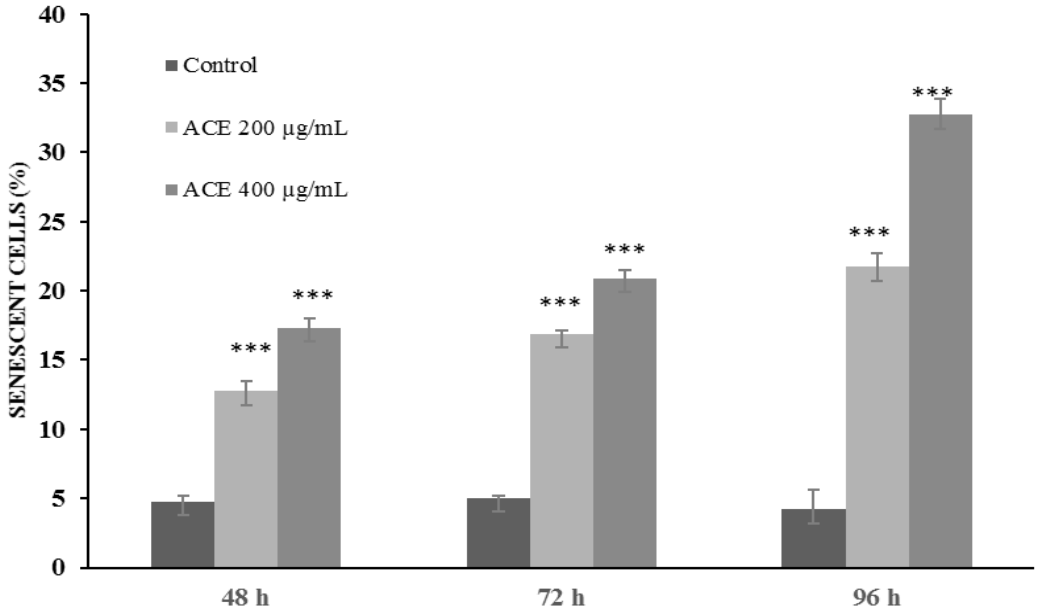

Figure 18: AOEE induces senescence in MDA-MB-231 breast cancer cells. MDA-MB 231 cells were treated with AOEE (200 and 400 µg/mL) for 48, 72 and 96 hrs and stained for SA-β-Galactosidase activity to detect senescence. Data are representative of two independent experiments. Statistical analysis to determine the significance was performed using student t-test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$).

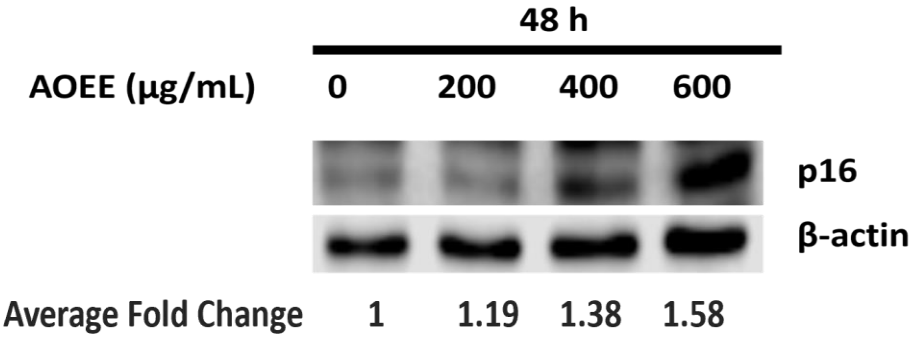

Figure 19: AOEE alters the levels of p16 in MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle or increasing concentrations of AOEE (200, 400, and 600 µg/mL) for 48 hrs, then the protein levels of p16 were examined by Western blotting. β-actin was used as loading control. Three independent experiments were performed to calculate the average fold change.

3.6 AOEE Induces G1/S Cell cycle arrest

To confirm further whether AOEE inhibited MDA-MB-231 cell proliferation by inducing cell cycle arrest, the alteration in the expression of the G1/S transition cell cycle regulatory proteins, Cyclin D1, Cyclin E1 and CDK2 were investigated using Western blotting. Cyclin D1 is one of the Cyclins proteins which accumulates in response to mitogenic growth factors to assemble with its cognate CDK4/6 subunit. The conjugation between Cyclin D1 and CDK4/6 is required for cells to progress through the restriction point at G1 phase. Interestingly, Cyclin D1 is overexpressed in many types of cancer due to several genetic alteration such as chromosomal translocation (Diehl, 2002). As seen in Figure 20, the level of Cyclin D1 decreased in the treated MDA-MB-231 cells in concentration- dependent manner, which further confirm the G1/S cell cycle arrest obtained by the cell cycle analysis.

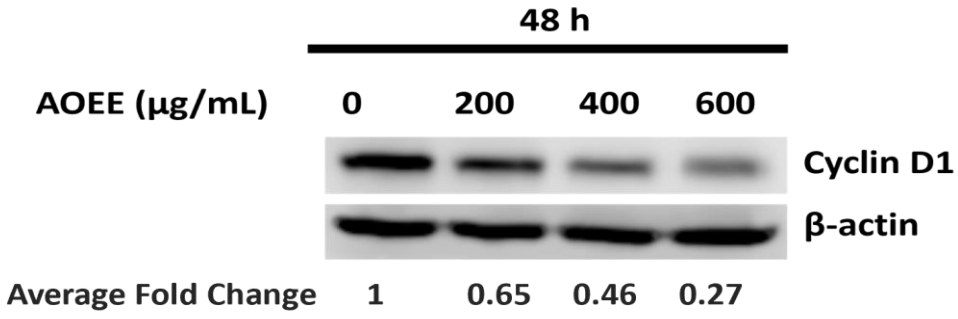

Figure 20: AOEE alters the levels of the Cyclin D1 in MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle or increasing concentrations of AOEE (200, 400, and 600 µg/mL) for 48 hrs, then the protein levels of Cyclin D1 were examined by Western blotting. β-actin was used as loading control. Three independent experiments were performed to calculate the average fold change.

Like Cyclin D1, Cyclin E1 is another cell cycle regulatory protein which conjugate with CDK2 for the cell cycle transition through G1/S phases (Milioli et al., 2020).

Surprisingly, the levels of Cyclin E1 and CKD2 proteins didn't change significantly upon treating MDA-MB-231 cells with AOEE, only slight increase in the protein level of Cyclin E1 when cells treated with 400 µg/mL of AOEE and slight decrease at 600 µg/mL concentration (Figure 21).

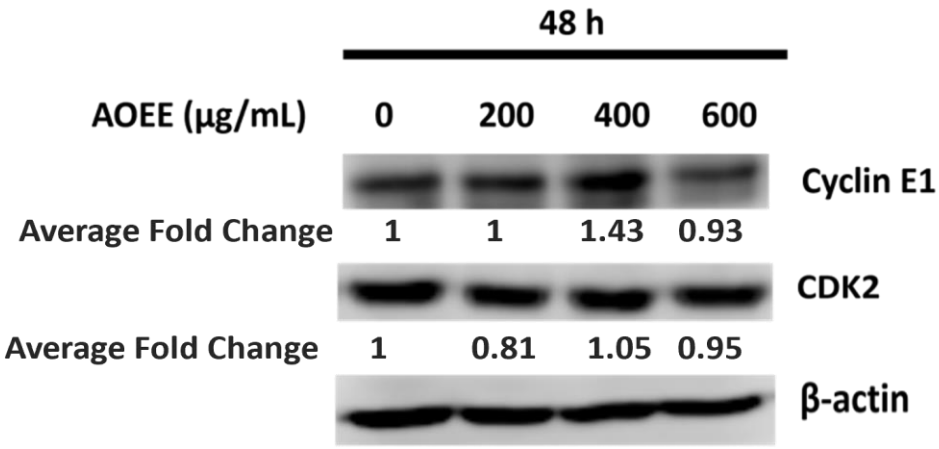

Figure 21: AOEE alters the levels of the Cyclin E1 and CDK2 in MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle or increasing concentrations of AOEE (200, 400, and 600 µg/mL) for 48 hrs, then the proteins levels of Cyclin E1 and CDK2 were examined by Western blotting. β-actin was used as loading control. Two independent experiments were performed to calculate the average fold change.

The alteration in the phosphorylated form of Retinoblastoma protein (p-Rb) was also investigated using Western blotting. For cells to enter the S phase of the cell cycle, both CDK4–Cyclin D and CDK2–Cyclin E must phosphorylate Rb protein. p-Rb next regulate the expression of many genes by controlling the activity of the transcription factor E2F (Rubin, 2013). As demonstrated in Figure 22, p-Rb levels decreased clearly in the treated MDA-MB-231 cells starting at 200 µg/mL of AOEE. In addition to its role in the metabolism of nucleic acid, PCNA has a role in the cell cycle progression in the S phase through the interaction with Cyclin A/CDK2 complex (Jurikova et al., 2016). As demonstrated here earlier in Figure 10, PCNA was downregulated in

response to AOEE treatment in MDA-MB-231 cells, which further confirm the cell cycle arrest at G1/S. Overall, the results confirm that AOEE induces cell cycle arrest at G1/S phase in MDA-MB-231 cells, which contributes to the inhibitory effect on the proliferation and growth of MDA-MB-231 cells induced by AOEE.

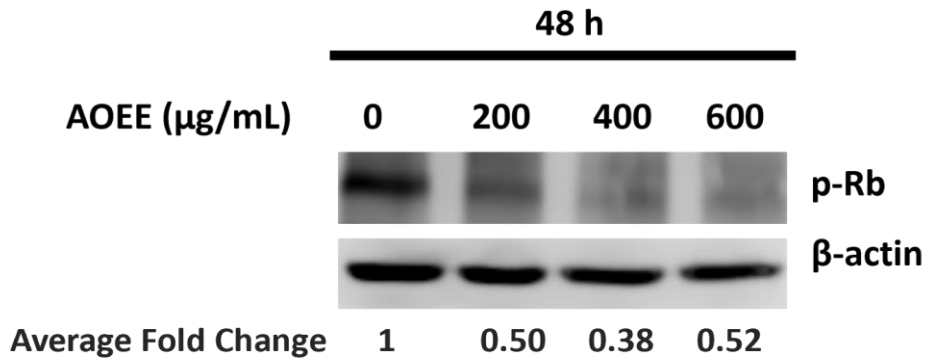

Figure 22: AOEE alters the levels of the p-Rb in MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle or increasing concentrations of AOEE (200, 400, and 600 µg/mL) for 48 hrs, then the protein levels of p-Rb were examined by Western blotting. β-actin was used as loading control. Three independent experiments were performed to calculate the average fold change.

3.7 AOEE Upregulates the Cyclin-Dependent Kinases Inhibitors, $p21^{WAF1}$ and $p27^{kip1}$, in MDA-MB-231 cells

Both $p21^{WAF1}$ and $p27^{Kip1}$ are cyclin-dependent kinases inhibitors and cell cycle regulators in which both proteins play a role in blocking the cell cycle at different stages in response to various stimuli (Zhang et al., 2021). To investigate the involvement of $p21^{WAF1}$ and $p27^{Kip1}$ in cell cycle arrest induced by AOEE in MDA-MB-231 cells, the alteration of their levels was investigated using Western blotting. As demonstrated in Figure 23 (a-b), both $p21^{WAF1}$ and $p27^{Kip1}$ were upregulated at AOEE concentrations of 200 and 400 µg/mL. Interestingly, the level of both proteins decreased at concentration of 600 µg/mL. In addition to its role in the induction of the cell cycle arrest, the upregulation of $p21^{WAF1}$ was reported to induce senescence

regardless to the p53 status (Fang et al., 1999). Furthermore, $p21^{WAF1}$ dependent senescence pathway was also reported, independently to pRB/p16 and p53 (Jia et al., 2011).

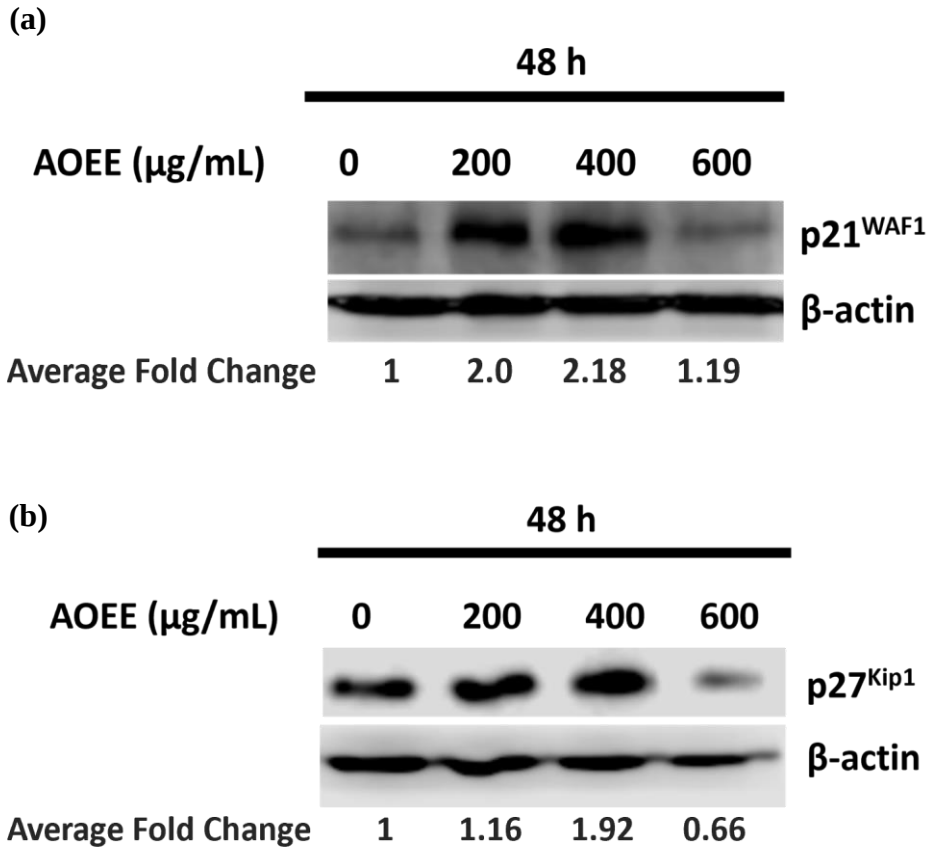

Figure 23: AOEE Upregulates the Cyclin-Dependent Kinases Inhibitors, $p21^{WAF1}$ and $p27^{kip1}$, in MDA-MB-231 cells. MDA-MB-231 cells were treated with vehicle or increasing concentrations of AOEE (200, 400, and 600 µg/mL) for 48 hrs, then the proteins levels of $p21^{WAF1}$ (a) and $p27^{Kip1}$ (b) were examined by Western blotting. β-actin was used as loading control. Three independent experiments were performed to calculate the average fold change.

3.8 AOEE Induces DNA Damage in MDA-MB-231 Cells

To investigate the possible inducers responsible for the cell cycle arrest/senescence induced by AOEE in MDA-MB-231 cells, the variation in the level of the

phosphorylated form of Histon-H2AX, which helps in DNA double-stranded breaks repair, was estimated using Western blotting. p-Histone-H2AX, also known as γ-H2AX, is considered as a novel biomarker for detecting DNA double-stranded breaks (Kuo & Yang, 2008). As seen in Figure 24, indeed p-Histone-H2AX level was dramatically increased starting at 400 µg/mL of AOEE compared to the untreated control cells. Therefore, the results suggest that DNA damage could be one key molecular initiator for the cell cycle arrest/senescence observed in the AOEE treated MDA-MB-231 cells.

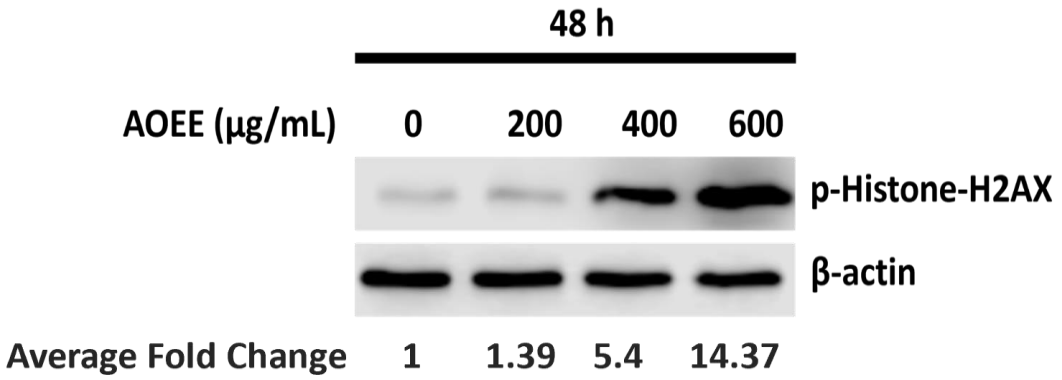

Figure 24: AOEE induces a dose-dependent activation of p-Histone-H2AX, a marker of DNA double-strands breaks. MDA-MB-231 cells were treated with vehicle or increasing concentrations of AOEE (200, 400, and 600 µg/mL) for 48 hrs, then the protein levels of p-Histone-H2AX were examined by Western blotting. β-actin was used as loading control. Three independent experiments were performed to calculate the average fold change.

3.9 AOEE Activates ERK and p38 Signaling Pathways in MDA-MB-231 Cells

The Extracellular Signal-Regulated Kinase (ERK1/2) and p38 pathways are both members of the Mammalian Family of Mitogen-Activated Protein Kinases (MAPKs) pathways, which regulate several cellular activities such as proliferation, differentiation, survival, and death in response to extracellular and intracellular stimuli

(Kim & Choi, 2010). Many studies suggested the role of ERK 1/2 and p38 pathways in regulating and induction of autophagy (Huang et al., 2015; Sun et al., 2018; Wang et al., 2017). To investigate the effect of AOEE on ERK1/2 and p38 pathways, the alteration in the phosphorylated form of both proteins was analyzed using Western blotting. As demonstrated in Figure 25 (a-b), p-ERK1/2 (a) and p-p38 (b) levels started to increase at AOEE concentration of 200 µg/mL. Altogether, the results suggest that ERK1/2 and p38 pathways might be involved in the anti-breast cancer activity of AOEE.

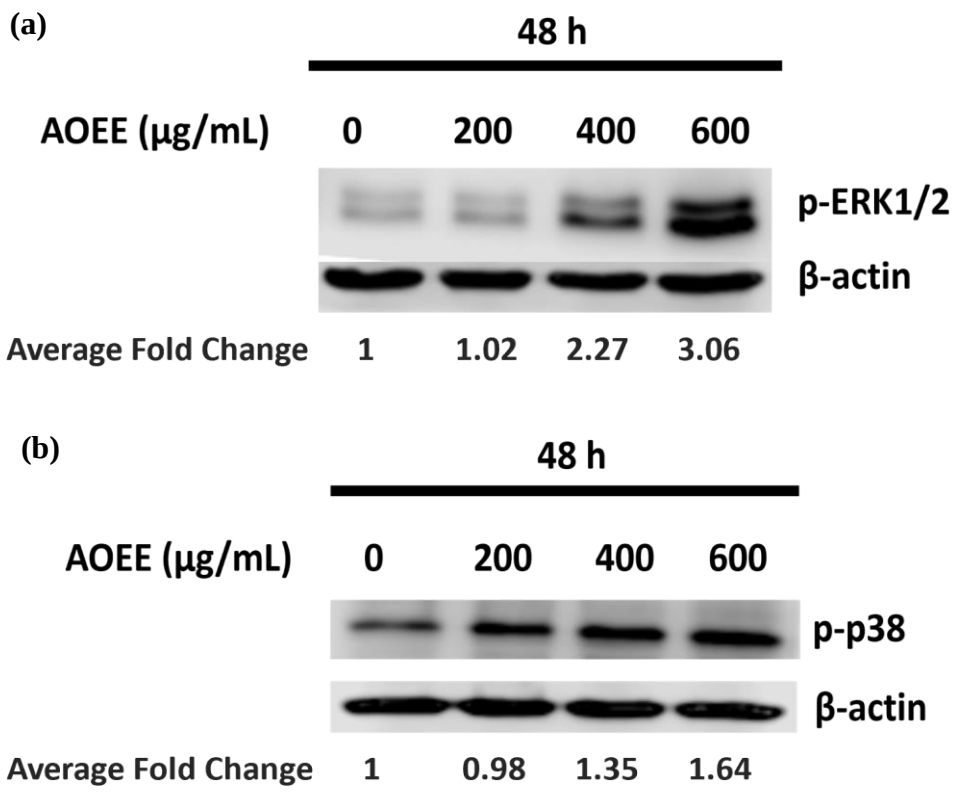

Figure 25: AOEE induces phosphorylation of p38 and ERK1/2 proteins. MDA-MB-231 cells were treated with vehicle or increasing concentrations of AOEE (200, 400, and 600 µg/mL) for 48 hrs, then the proteins levels of p-p38 and p-ERK1/2 were examined by Western blotting. β-actin was used as loading control. Three independent experiments were performed to calculate the average fold change.

Chapter 4: Discussion

Despite the extensive cancer research, important challenges are still going in cancer treatment. For example, conventional treatment choices of cancer such as chemotherapy causes severe side effects, which limit their use in high doses and the overall effect on metastatic cancers (Oun et al., 2018). Additionally, cancer is characterized by their plasticity, which leads to the development of chemotherapy resistance, and therefore treatment failure by various cellular mechanisms (Ramos & Bentires-Alj, 2015). TNBC is highly invasive in its nature and the absence of targeted therapy to defeat it makes the treatment challenging and highly subjected to relapse. Recently, several signaling pathways targeted by TNBC therapeutics were discovered, but they were hindered by either drug resistance or replace (Medina et al., 2020). Therefore, finding new treatment choices with novel targets to treat TNBC is necessary.

For decades, medicinal plants were used traditionally to treat many diseases. Despite the use of trial-and-error approach by the ancient people to discover the potential pharmaceutical effects of the medicinal plants, the current available biological techniques made it easier to screen for the biological activities of the plants and their derived compounds (Barkat et al., 2020). Interestingly, more than 50% of the currently used medications are plant derived. Most of the plants found to have anti-cancer potentials act by disrupting the abnormal cellular mechanisms which contributes to cancer cells hyperproliferation capacity. Large body of evidence revealed that medicinal plants often hit multiple molecular targets of multiple process associated with cancer development and progression such as proliferation, metastasis,

angiogenesis and cellular death (Karikas, 2010). Therefore, medicinal plants, indeed is a rich source for novel anti-cancer agents with novel molecular and cellular targets. *Acridocarpus orientalis* is a rare medicinal plant that is used in folk medicine to treat several illnesses. Furthermore, recent studies revealed anti-diabetic, antioxidant and hepatoprotective effects of *Acridocarpus orientalis* (Ksiksi & Hamza, 2012; Lotfy, Al-Hammadi, et al., 2020; Lotfy, Ksiksi, et al., 2020; Rehman, Mabood, et al., 2019). *Acridocarpus orientalis* possesses strong inhibiting effects against a variety of cancer cells, including colorectal adenocarcinoma (HT29) (Hussain et al., 2014; Rehman, Hussain, et al., 2019), colorectal adenocarcinoma (HCT116) (Hussain et al., 2014; Rehman, Mabood, et al., 2019), human hepatoma derived cell line (HepG2) (Hussain et al., 2014; Rehman, Mabood, et al., 2019), hormone responsive breast cancer (MCF-7) (Balhamar et al., 2019), triple hormone receptor-negative breast cancer (MDA-MB-231) (Balhamar et al., 2019), cervical cancer (HeLa) (Balhamar et al., 2019), and mouse mammary carcinoma cell line (4T1 cells) (Jamshidi-Adegani et al., 2020), however, the underlying anticancer target(s)/mechanism(s) remains unknown. Therefore, the aim of the current study is to investigate the effect of *A. orientalis* on TNBC and to elucidate the underlying anti-breast cancer molecular target(s)/mechanism(s).

Different molecular mechanisms and death pathways are responsible for the anticancer effects of *A. orientalis*. For example, induction of apoptosis and activation of autophagy are the key mechanisms of various fractions of *A. orientalis* extract against HeLa cell line (Balhamar et al., 2019). In 4T1 cells, apoptosis and cell cycle arrest in G0/G1-phase are the key mechanisms of proliferation inhibition upon *A. orientalis* leaves and stem extracts treatment (Jamshidi-Adegani et al., 2020). Novel molecular mechanisms to induce cellular death were also reported by Ksiksi and

Hamza (2012). The ethanolic extract of *A. orientalis* was reported by to inhibit the Histone Deacetylases (HDACs) enzymes, which therefore can induce cell death and inhibit the angiogenesis in cancer cells (Ksiksi & Hamza, 2012).

The findings of this study revealed significant proliferation and growth inhibitory effect of AOEE on TNBC cell lines (MDA-MB-231 and Hs578T) and ER positive breast cancer cell line (MCF-7). The growth inhibitory effect of AOEE on the three studied human breast cancer cell lines is independent of the ER-status and p53 status. However, each may act through different molecular mechanisms. The current study, focused on studying the molecular mechanism(s) and identifying molecular target(s) associated with the anti-breast cancer activity of AOEE on MDA-MB-231.

Autophagy is an evolutionarily conserved catabolic mechanism, by which cells recycle or degrade internal proteins or organelles. In autophagy, cytoplasmic materials are directed to the lysosomes for degradation. In addition to its role in recycling of misfolded and degraded proteins, the induction of autophagy was found to have antiproliferative effects, mainly by regulating the cell cycle progression or cell death induction (Kocaturk et al., 2019). Indeed, AOEE induces autophagy in MDA-MB-231 cells confirmed by the upregulation of LC3-II protein, a marker for the formation of the autophagosomes. Beclin-1, which has a role in the initial steps of the autophagosomes formation (Vega-Rubín-de-Celis, 2020), was also upregulated upon AOEE treatment, which suggests that the autophagy induced by AOEE is Beclin-1 dependent. Surprisingly, p62 responsible for the sequestration of the misfolded and non-functional proteins for degradation, along with sequestered proteins by the autolysosomes, was upregulated upon AOEE treatment. Of note, p62 accumulation indicates the induction of abortive autophagy. The use of the autophagy inhibitor 3-MA caused partial reversal of the viability of AOEE treated MDA-MB-231 cells,

which suggests that autophagy induced by AOEE is not protective mechanism and contributes to the growth inhibitory effect induced by AOEE. Ginkgolide B, a natural derived compound extracted from *Ginkgo biloba* leaves with anti-cancer potential, was reported to inhibit the proliferation of A549 and H1975 lung cancer cell lines by inducing Beclin-1 dependent autophagy accomplished by the upregulation of p62 protein (Wang et al., 2020).

In addition to the role of autophagy in the growth inhibitory effect of AOEE in MDA-MB-231 cells, the results of the present study demonstrated that AOEE induces cellular senescence in concentration- and time- dependent manner. Cellular senescence has emerged as a potent target for inhibiting the proliferation of cancer cells with low doses of radiotherapy or chemotherapy to minimize the side effects of both treatment choices, by inducing irreversible, or at least prolonged, cell cycle arrest (Zeng et al., 2018). Interestingly, AOEE has significantly increased the expression of SA-β-gal; a biomarker for the induction of senescence, in MDA-MB-231 cells. The results also demonstrated upregulation of p16 protein, which is highly expressed in senescent cells and known to inhibit the Cyclin D-CDK4/6 complex required for the cell cycle progression from G1 to S phase. G1/S cell cycle arrest and alteration in the cell cycle regulatory proteins were detected by AOEE. While the cell cycle regulatory protein, Cyclin D1, which plays a role in cell cycle progression from G1 to S phase, was downregulated in the treated cells, the CDKs inhibitors $p21^{WAF1}$ and $p27^{Kip1}$ were upregulated upon treating MDA-MB-231 cells with AOEE for 48 hours. Even though p53, which regulates the expression of $p21^{WAF1}$ and $p27^{Kip1}$ (Philipp-Staheli et al., 2004), is mutated in MDA-MB-231 cells, the upregulation of both proteins in the treated cells suggests p53-independent pathway for the induction of senescence and cell cycle arrest. PCNA, a marker for cell proliferation, plays major roles in the DNA

replication and replication-associated processes such as mismatch repair and chromatin assembly in the S phase of cell cycle (Boehm et al., 2016). Interestingly, $p21^{WAF1}$ found to regulate the function of PCNA, by inhibiting its binging with DNA polymerase δ (Wang et al., 2021). A clear reduction in the PCNA levels was detected at AOEE concentration of 200 μg/mL, which further confirm the antiproliferative effect of AOEE on MDA-MB-231 cells. Rb is an important downstream target for Cyclin D1 and Cyclin E1 that regulates the G1/S transition in the cell cycle. Phosphorylation of Rb; initiated by the effect of Cyclin D/CDKs complexes, followed by the hyperphosphorylation effect mediated by Cyclin E/CDK2 results in the release of E2F transcription factors required for the G1/S transition (Beasley et al., 2003). The results of this study demonstrated hypophosphorylation of Rb upon AOEE treatment, which indicates that the p16/Cyclin D1/Rb pathway plays a major role in the G1/S cell cycle arrest. The anti-cancer potential of a novel indenone derivative was also reported to induce senescence and G1 cell cycle arrest in MDA-MB-231 cells (Priyanga et al., 2020).

Double-strand breaks in DNA can result mainly from the exposure to exogenous agents such as radiation and certain chemicals or during DNA replication and repair (Cannan & Pederson, 2016). DNA double-strand breaks can highly affect the cell cycle progression, where a single double-strand break reported to be sufficient to induce cell cycle arrest in G1 or G2 (van den Berg et al., 2018). Additionally, DNA damage was reported to be linked to the induction of senescence via the activation of ATM/ATR/p53/p16 pathway (Von Zglinicki et al., 2005). The levels of the phosphorylated form of Hisotone-H2AX, which is used as biological marker for double-strand breaks in the cells, were increased starting at 200 μg/mL of AOEE. Thus, the results obtained from the present study suggest that DNA damage is the

initiator for the cell cycle arrest starting at 200 μg/mL. On the other hand, the absence of significant upregulation of p16 at 200 μg/mL of AOEE can be explained by the minimal concurrent DNA double-strand breaks at the same concentration.

The ERK1/2 and p38 pathways are members of the MAPK signaling pathway responsible for several cellular processes, including, cell proliferation, differentiation, migration, senescence and apoptosis, by delivering extracellular signals to the nucleus (Sun et al., 2015). The phosphorylation of ERK1/2 at threonine and tyrosine is required to acquire its biological functions. Importantly, ERK1/2 pathway was reported to have dual effect on cellular senescence; where it can promote or inhibit the induction of senescence via various mechanisms and under different dose- and duration-circumstances (Zou et al., 2019). Furthermore, ERK1/2 was previously shown to have a role in the induction of autophagy (Lee et al., 2013; Yuan et al., 2018). Similar to ERK1/2 pathway, p38 pathway was also reported to be involved in the regulation of the cell cycle and induction of cell cycle arrest in several phases. Surprisingly, p38 activation was reported to be reduced in many types of tumors, and the reduction of p38 activation is linked to proliferation in those tumors. Therefore, the activation of p38 pathway can activate senescence (Zarubin & Jiahuai, 2005). The complex relationship between p38 and autophagy is not clear yet and needs further investigation, but several reports found dual regulatory effect of p38 activation on autophagy, and autophagy induction effect on p38 activation (Sui et al., 2014; Webber, 2010). The upregulation in the phosphorylated p38 and ERK1/2, in AOEE treated MDA-MB-231, demonstrated in the present work suggests that both pathways might be related to the induction of senescence and autophagy.

In summary, AOEE inhibited the viability of three human breast cancer cell lines (MDA-MB-231, Hs578t and MCF-7) and the effect seems to be independent of the

ER expression status and p53 status. Different cellular mechanisms and pathways were associated with the growth inhibitory effect of AOEE in the high invasive triple-negative breast cancer cell line MDA-MB-231. The antiproliferative activity and cell cycle arrest induced by AOEE in MDA-MB-231 cells were accomplished by the downregulation of cyclin D1, PCNA, p-Rb and the upregulation of $p21^{WAF1}$ and $p27^{Kip1}$. Additionally, AOEE induced prolonged autophagy accomplished by the upregulation of LC3-II, Beclin-1 and p62. Moreover, AOEE induced DNA double stranded breaks and cellular senescence characterized by the upregulation of p-H-H2AX, SA-β-gal and p16 respectively in MDA-MB-231 cells. Finally, AOEE induced activation of ERK and p38 pathways, which might be related to the induction of autophagy and senescence. Further investigation on the exact role of the ERK and p38 pathways in the autophagy and senescence induced by AOEE is warranted. Figure 26 represents a hypothetical model summarizes the molecular events induced by AOEE in MDA-MB-231 cells.

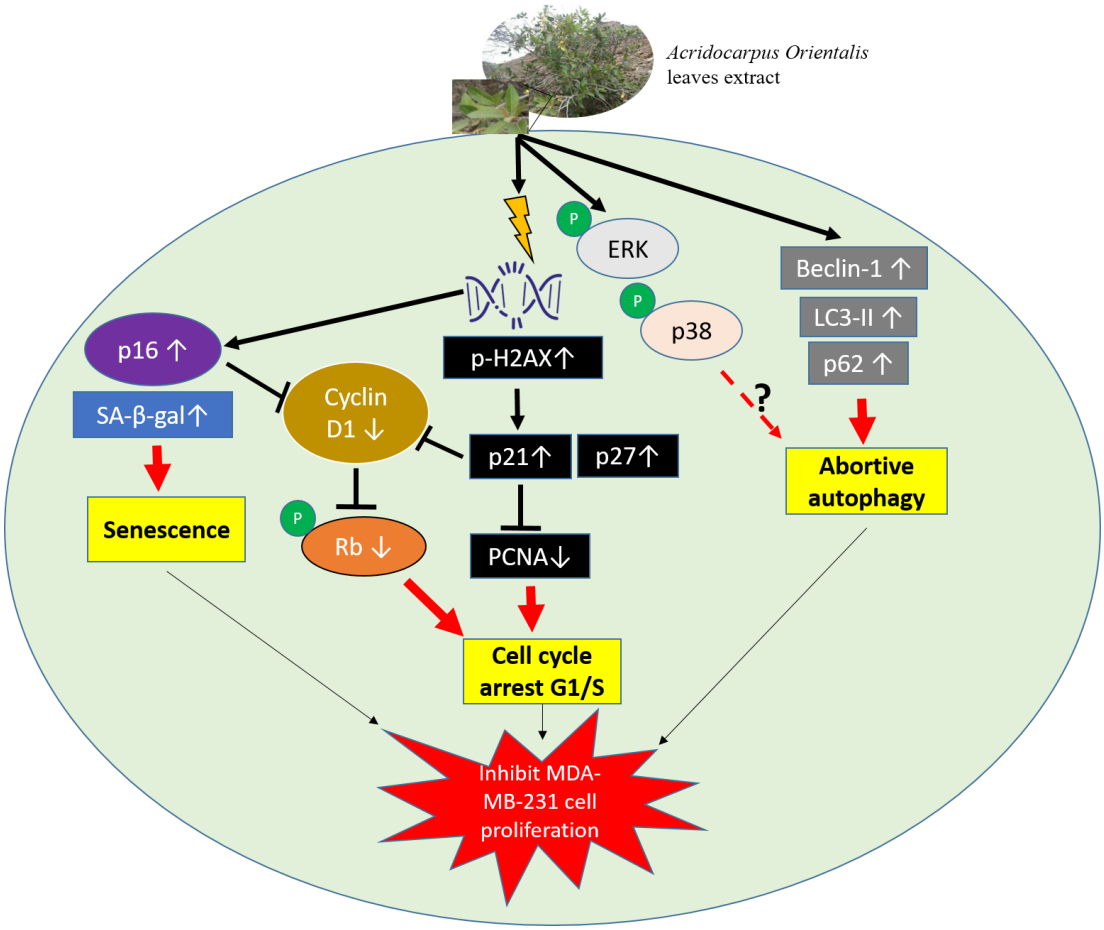

Figure 26: Proposed model demonstrating the underlying mechanism of action in AOEE-induced anti-breast cancer activity in MDA-MB-231 cells

Chapter 5: Conclusion

In conclusion, the present study reveals a negative action of AOEE on the viability of breast cancer cells *in vitro*. The study showed, for the first time, that AOEE inhibits MDA-MB-231, human breast cancer, cells proliferation and growth. The anti-proliferation effect of AOEE on MDA-MB-231 cells was associated with the cell cycle arrest, through modulation of cell cycle regulatory proteins, autophagy and cellular senescence induction, and DNA double stranded breaks. MAPK signaling pathway was also involved in the anti-breast cancer activity of AOEE. Therefore, AOEE could be a rich source for active compounds. Further identification and analysis of the active compounds responsible for the observed anti-cancer activity of AOEE is warranted. *In vivo* studies are also required to validate the *in vitro* AOEE anti-breast cancer effect.

References

Al-Snafi, A. E. (2016). Medicinal plants possessed anti-inflammatory antipyretic and analgesic activities (part 2)-plant based review. *Sch Acad J Pharm, 5*(5), 142-158.

Al Dhaheri, Y., Attoub, S., Arafat, K., AbuQamar, S., Eid, A., Al Faresi, N., & Iratni, R. (2013). Salinomycin induces apoptosis and senescence in breast cancer: upregulation of p21, downregulation of survivin and histone H3 and H4 hyperacetylation. *Biochimica et Biophysica Acta (BBA)-General Subjects, 1830*(4), 3121-3135.

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2009). *Essential Cell Biology 3rd edn (New York: Garland Science)*. New York: Garland Science.

Ali, L., Mabood, F., Rizvi, T. S., Rehman, N. U., Arman, M., Al-Shidani, S., Al-Abri, Z., Hussain, J., & Al-Harrasi, A. (2018). Total polyphenols quantification in Acridocarpus orientalis and Moringa peregrina by using NIR spectroscopy coupled with PLS regression. *Chemical Data Collections, 13*, 104-112.

American Cancer Society. (2018). *Global cancer facts & figures 4th edition*. [online]. (Accessed 18/9/2021). Retrieved from: https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/global-cancer-facts-and-figures/global-cancer-facts-and-figures-4th-edition.pdf

Balhamar, S. O. M. S., Panicker, N. G., Akhlaq, S., Qureshi, M. M., Ahmad, W., Rehman, N. U., Ali, L., Al-Harrasi, A., Hussain, J., & Mustafa, F. (2019). Differential cytotoxic potential of Acridocarpus orientalis leaf and stem extracts with the ability to induce multiple cell death pathways. *Molecules, 24*(21), 3976. doi.org/10.3390/molecules24213976

Barkat, M. A., Goyal, A., Barkat, H. A., Salauddin, M., Pottoo, F. H., & Anwer, E. T. (2020). Herbal Medicine: Clinical Perspective & Regulatory Status. *Combinatorial Chemistry & High Throughput Screening*. 24(10), 1573-1582.

Bayo, J., Castano, M., Rivera, F., & Navarro, F. (2018). Analysis of blood markers for early breast cancer diagnosis. *Clinical and Translational Oncology, 20*(4), 467-475.

Beasley, M. B., Lantuejoul, S., Abbondanzo, S., Chu, W.-S., Hasleton, P. S., Travis, W. D., & Brambilla, E. (2003). The P16/cyclin D1/Rb pathway in neuroendocrine tumors of the lung. *Human pathology, 34*(2), 136-142.

Benhalilou, N., Alsamri, H., Alneyadi, A., Athamneh, K., Alrashedi, A., Altamimi, N., Al Dhaheri, Y., Eid, A. H., & Iratni, R. (2019). Origanum majorana ethanolic extract promotes colorectal cancer cell death by triggering abortive autophagy and activation of the extrinsic apoptotic pathway. *Frontiers in oncology, 9*, 795. doi: 10.3389/fonc.2019.00795

Bjørkøy, G., Lamark, T., Pankiv, S., Øvervatn, A., Brech, A., & Johansen, T. (2009). Monitoring autophagic degradation of p62/SQSTM1. *Methods in enzymology, 452*, 181-197.

Blackadar, C. B. (2016). Historical review of the causes of cancer. *World journal of clinical oncology, 7*(1), 54. doi: 10.5306/wjco.v7.i1.54

Boster, B. L., Patel, N. K., & Michaud, L. B. (2020). Breast Cancer. In J. T. DiPiro, G. C. Yee, L. M. Posey, S. T. Haines, T. D. Nolin, & V. Ellingrod (Eds.), *Pharmacotherapy: A Pathophysiologic Approach, 11e*. McGraw-Hill Education.

Cannan, W. J., & Pederson, D. S. (2016). Mechanisms and consequences of double-strand DNA break formation in chromatin. *Journal of cellular physiology, 231*(1), 3-14.

Chen, Y., Yang, L., Feng, C., & Wen, L.-P. (2005). Nano neodymium oxide induces massive vacuolization and autophagic cell death in non-small cell lung cancer NCI-H460 cells. *Biochemical and biophysical research communications, 337*(1), 52-60.

Colamba Pathiranage, V., Lowe, J. N., Rajagopalan, U., Ediriweera, M. K., Senathilake, K., Piyathilaka, P., Tennekoon, K. H., & Samarakoon, S. R. (2020). Hexane Extract of Garcinia quaesita Fruits Induces Apoptosis in Breast Cancer Stem Cells Isolated from Triple Negative Breast Cancer Cell Line MDA-MB-231. *Nutrition and Cancer, 73*(5), 845-855.

D'Arcy, M. S. (2019). Cell death: a review of the major forms of apoptosis, necrosis and autophagy. *Cell biology international, 43*(6), 582-592.

Dania, V., Liu, Y., Ademuyiwa, F., Weber, J. D., & Colditz, G. A. (2019). Associations of race and ethnicity with risk of developing invasive breast cancer after lobular carcinoma in situ. *Breast Cancer Research, 21*(1), 1-7.

Diana, A., Franzese, E., Centonze, S., Carlino, F., Della Corte, C. M., Ventriglia, J., Petrillo, A., De Vita, F., Alfano, R., & Ciardiello, F. (2018). Triple-negative breast cancers: systematic review of the literature on molecular and clinical features with a focus on treatment with innovative drugs. *Current oncology reports, 20*(10), 1-11.

Diehl, J. A. (2002). Cycling to cancer with cyclin D1. *Cancer biology & therapy, 1*(3), 226-231.

Divakar, M. C., Amani Al-Siyabi, S., Varghese, S., & Al Rubaie, M. (2016). The practice of ethnomedicine in the Northern and Southern provinces of Oman. *Oman medical journal, 31*(4), 245. doi: 10.5001/omj.2016.49

Dragoi, A., & Alexandru, O. (2020). PLANT-DERIVED CHEMOTHERAPEUTICS DRUGS FOR CANCER CHEMOTHERAPY. *Medico Oncology, 1*(1), 28-37.

Eldin, E. E. M. N., El-Readi, M. Z., Eldein, M. M. N., Alfalki, A. A., Althubiti, M. A., Kamel, H. F. M., Eid, S. Y., Al-Amodi, H. S., & Mirza, A. A. (2019). 8-hydroxy-2'-deoxyguanosine as a discriminatory biomarker for early detection of breast cancer. *Clinical breast cancer, 19*(2), 385-393.

Elia, I., Doglioni, G., & Fendt, S.-M. (2018). Metabolic hallmarks of metastasis formation. *Trends in cell biology, 28*(8), 673-684.

Fang, L., Igarashi, M., Leung, J., Sugrue, M. M., Lee, S. W., & Aaronson, S. A. (1999). p21 Waf1/Cip1/Sdi1 induces permanent growth arrest with markers of replicative senescence in human tumor cells lacking functional p53. *Oncogene, 18*(18), 2789-2797.

Ghazanfar, S. A., & Al-Al-Sabahi, A. M. (1993). Medicinal plants of northern and central Oman (Arabia). *Economic Botany, 47*(1), 89-98.

Giuliano, A. E., Edge, S. B., & Hortobagyi, G. N. (2018). of the AJCC cancer staging manual: breast cancer. *Annals of surgical oncology, 25*(7), 1783-1785.

Green, D. R., & Llambi, F. (2015). Cell death signaling. *Cold Spring Harbor perspectives in biology, 7*(12), a006080. doi: 10.1101/cshperspect.a006080

Hahnen, E., Hauke, J., Engel, C., Neidhardt, G., Rhiem, K., & Schmutzler, R. K. (2017). Germline mutations in triple-negative breast cancer. *Breast Care, 12*(1), 15-19.

Hassanpour, S. H., & Dehghani, M. (2017). Review of cancer from perspective of molecular. *Journal of Cancer Research and Practice, 4*(4), 127-129.

Herranz, N., & Gil, J. (2018). Mechanisms and functions of cellular senescence. *The Journal of clinical investigation, 128*(4), 1238-1246.

Hinai, A. A., Lupton, D. A., & Al Issai, G. (2020). Indigenous knowledge and folk use of medicinal plants in the Eastern Hajar Mountains, Oman. *Journal of Medicinal Plants, 8*(4), 104-110.

Hosaka, Y., Araya, J., Fujita, Y., Kadota, T., Tsubouchi, K., Yoshida, M., Minagawa, S., Hara, H., Kawamoto, H., & Watanabe, N. (2020). Chaperone-Mediated Autophagy Suppresses Apoptosis via Regulation of the Unfolded Protein Response during Chronic Obstructive Pulmonary Disease Pathogenesis. *The Journal of Immunology, 205*(5), 1256-1267.

Huang, Y.-C., Yu, H.-S., & Chai, C.-Y. (2015). Roles of oxidative stress and the ERK1/2, PTEN and p70S6K signaling pathways in arsenite-induced autophagy. *Toxicology letters, 239*(3), 172-181.

Hussain, J., Ali, L., Khan, A. L., Rehman, N. U., Jabeen, F., Kim, J.-S., & Al-Harrasi, A. (2014). Isolation and bioactivities of the flavonoids morin and morin-3-O-β-D-glucopyranoside from Acridocarpus orientalis—a wild Arabian medicinal plant. *Molecules, 19*(11), 17763-17772.

Jacob, B., & Narendhirakannan, R. (2019). Role of medicinal plants in the management of diabetes mellitus: a review. *3 Biotech, 9*(1), 4. doi: 10.1007/s13205-018-1528-0

Jafari, S. H., Saadatpour, Z., Salmaninejad, A., Momeni, F., Mokhtari, M., Nahand, J. S., Rahmati, M., Mirzaei, H., & Kianmehr, M. (2018). Breast cancer diagnosis: Imaging techniques and biochemical markers. *Journal of cellular physiology, 233*(7), 5200-5213.

Jamshidi-Adegani, F., Vakilian, S., Rehman, N. U., Al-Broumi, M., Al-Kindi, J., Alam, K., Mozafarinahavandi, P., Hasan, A., Al-Riyami, H., & Hussain, J. (2020). Secondary metabolites from acridocarpus orientalis inhibits 4T1 cells and promotes mesenchymal stem cells (MSCs) proliferation. *Molecular Biology Reports, 47*(7), 5421-5430.

Jia, L., Li, H., & Sun, Y. (2011). Induction of p21-dependent senescence by an NAE inhibitor, MLN4924, as a mechanism of growth suppression. *Neoplasia, 13*(6), 561-569.

Johansen, T., & Lamark, T. (2020). Selective autophagy: ATG8 family proteins, LIR motifs and cargo receptors. *Journal of molecular biology, 432*(1), 80-103.

Jurikova, M., Danihel, Ľ., Polák, Š., & Varga, I. (2016). Ki67, PCNA, and MCM proteins: Markers of proliferation in the diagnosis of breast cancer. *Acta histochemica, 118*(5), 544-552.

Kabel, A. M. (2017). Tumor markers of breast cancer: New prospectives. *Journal of Oncological Sciences, 3*(1), 5-11.

Karikas, G. A. (2010). Anticancer and chemopreventing natural products: some biochemical and therapeutic aspects. *J BUON, 15*(4), 627-638.

Kelman, Z. (1997). PCNA: structure, functions and interactions. *Oncogene, 14*(6), 629-640.

Kim, E. K., & Choi, E.-J. (2010). Pathological roles of MAPK signaling pathways in human diseases. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1802*(4), 396-405.

Kisksi, T., Guenaoui, C., & Fawzi, N. (2012). Early growth stages of the rare Acridocarpus orientalis in the UAE-A First step towards conservation. *Nat. Resour, 3*, 1-5.

Kocaturk, N. M., Akkoc, Y., Kig, C., Bayraktar, O., Gozuacik, D., & Kutlu, O. (2019). Autophagy as a molecular target for cancer treatment. *European Journal of Pharmaceutical Sciences, 134*, 116-137.

Ksiksi, T., & Hamza, A. A. (2012). Antioxidant, lipoxygenase and histone Deacetylase inhibitory activities of Acridocarpus orientalis from Al Ain and Oman. *Molecules, 17*(11), 12521-12532.

Ksiksi, T., Rasheed Palakkott, A., BT Ppoyil, S., & Alhammadi, R. (2017). Immature leaves of Acridocarpus orientalis A. Juss. exhibit high antioxidant and anti-LOX properties. *Current Bioactive Compounds, 13*(2), 144-151.

Kuo, L. J., & Yang, L.-X. (2008). γ-H2AX-a novel biomarker for DNA double-strand breaks. *In Vivo, 22*(3), 305-309.

Lee, A., & Djamgoz, M. B. (2018). Triple negative breast cancer: emerging therapeutic modalities and novel combination therapies. *Cancer treatment reviews, 62*, 110-122.

Lee, J.-W., Kim, K.-S., An, H.-K., Kim, C.-H., Moon, H.-I., & Lee, Y.-C. (2013). Dendropanoxide induces autophagy through ERK1/2 activation in MG-63 human osteosarcoma cells and autophagy inhibition enhances dendropanoxide-induced apoptosis. *PloS one, 8*(12), e83611. doi: 10.1371/journal.pone.0083611

Lee, S., & Lee, J.-S. (2019). Cellular senescence: A promising strategy for cancer therapy. *BMB reports, 52*(1), 35. doi: 10.5483/BMBRep.2019.52.1.294

Lehmann, B. D., Bauer, J. A., Chen, X., Sanders, M. E., Chakravarthy, A. B., Shyr, Y., & Pietenpol, J. A. (2011). Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *The Journal of clinical investigation, 121*(7), 2750-2767.

Lewandowska, A., Rudzki, G., Lewandowski, T., Próchnicki, M., Rudzki, S., Laskowska, B., & Brudniak, J. (2020). Quality of Life of Cancer Patients Treated with Chemotherapy. *International Journal of Environmental Research and Public Health, 17*(19), 6938. doi: 10.3390/ijerph17196938

Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., & Levine, B. (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature, 402*(6762), 672-676.

Lim, S., & Kaldis, P. (2013). Cdks, cyclins and CKIs: roles beyond cell cycle regulation. *Development, 140*(15), 3079-3093.

Lotfy, M., Al-Hammadi, R., Palakkott, A. R., Yasin, J., Al-Hammadi, S., & Ksiksi, T. (2020). Hepatoprotective potentials of Acridocarpus orientalis in mice. *Clinical Phytoscience, 6*(1), 1-9.

Lotfy, M., Ksiksi, T. S., Palakkot, A. R., D'Souza, C. M., Mohsin, S., & Adeghate, E. A. (2020). Anti-diabetic Effect of Acridocarpus Orientalis. *The Open Medicinal Chemistry Journal, 14*(1). doi: 10.2174/1874104502014010132

Ma, W. (2017). Cell Cycle Checkpoint. In M. Schwab (Ed.), *Encyclopedia of Cancer* (pp. 897-901). Springer Berlin Heidelberg. doi: 10.1007/978-3-662-46875-3_996

Markopoulos, G. S., Roupakia, E., Tokamani, M., Chavdoula, E., Hatziapostolou, M., Polytarchou, C., Marcu, K. B., Papavassiliou, A. G., Sandaltzopoulos, R., & Kolettas, E. (2017). A step-by-step microRNA guide to cancer development and metastasis. *Cellular Oncology, 40*(4), 303-339.

Mattiuzzi, C., & Lippi, G. (2019). Current cancer epidemiology. *Journal of epidemiology and global health, 9*(4), 217-222.

Medina, M. A., Oza, G., Sharma, A., Arriaga, L., Hernández Hernández, J. M., Rotello, V. M., & Ramirez, J. T. (2020). Triple-negative breast cancer: a review of conventional and advanced therapeutic strategies. *International journal of environmental research and public health, 17*(6), 2078. doi: 10.3390/ijerph17062078

Milioli, H., Alexandrou, S., Lim, E., & Caldon, C. E. (2020). Cyclin E1 and cyclin E2 in ER+ breast cancer: prospects as biomarkers and therapeutic targets. *Endocrine-related cancer, 27*(5), 93-112.

Miller, K. D., Fidler-Benaoudia, M., Keegan, T. H., Hipp, H. S., Jemal, A., & Siegel, R. L. (2020). Cancer statistics for adolescents and young adults, 2020. *CA: a cancer journal for clinicians, 70*(6), 443-459.

Mirza, M. B., Elkady, A. I., Al-Attar, A. M., Syed, F. Q., Mohammed, F. A., & Hakeem, K. R. (2018). Induction of apoptosis and cell cycle arrest by ethyl acetate fraction of Phoenix dactylifera L.(Ajwa dates) in prostate cancer cells. *Journal of ethnopharmacology, 218*, 35-44.

Momenimovahed, Z., & Salehiniya, H. (2019). Epidemiological characteristics of and risk factors for breast cancer in the world. *Breast Cancer: Targets and Therapy, 11*, 151. doi: 10.2147/BCTT.S176070

Mutebi, M., Anderson, B. O., Duggan, C., Adebamowo, C., Agarwal, G., Ali, Z., Bird, P., Bourque, J. M., DeBoer, R., & Gebrim, L. H. (2020). Breast cancer treatment: A phased approach to implementation. *Cancer, 126*, 2365-2378.

Nayak, M. G., George, A., Vidyasagar, M., Mathew, S., Nayak, S., Nayak, B. S., Shashidhara, Y., & Kamath, A. (2017). Quality of life among cancer patients. *Indian journal of palliative care, 23*(4), 445. doi: 10.4103/IJPC.IJPC_82_17

Nigam, M. (2021). Phytomedicine: Scope and current highlights. In *Preparation of Phytopharmaceuticals for the Management of Disorders* (pp. 39-54). Elsevier.

Oun, R., Moussa, Y. E., & Wheate, N. J. (2018). The side effects of platinum-based chemotherapy drugs: a review for chemists. *Dalton transactions, 47*(19), 6645-6653.

Patel, A. (2020). Benign vs Malignant Tumors. *JAMA oncology, 6*(9), 1488-1488.

Philipp-Staheli, J., Kim, K.-H., Liggitt, D., Gurley, K. E., Longton, G., & Kemp, C. J. (2004). Distinct roles for p53, p27 kip1, and p21 Cip1 during tumor development. *Oncogene, 23*(4), 905-913.

Priyanga, J., Kumar, B. S., Mahalakshmi, R., Nirekshana, K., Vinoth, P., Sridharan, V., Bhakta-Guha, D., & Guha, G. (2020). A novel indenone derivative selectively induces senescence in MDA-MB-231 (breast adenocarcinoma) cells. *Chemico-Biological Interactions, 331*, 109250. doi: 10.1016/j.cbi.2020.109250

Qadir, M. I., Manzoor, A., & Akash, M. S. H. (2018). Potential role of medicinal plants for anti-atherosclerosis activity. *Bangladesh Journal of Pharmacology, 13*(1), 59-66.

Ramos, P., & Bentires-Alj, M. (2015). Mechanism-based cancer therapy: resistance to therapy, therapy for resistance. *Oncogene, 34*(28), 3617-3626.

Ranjan, A., Ramachandran, S., Gupta, N., Kaushik, I., Wright, S., Srivastava, S., Das, H., Srivastava, S., Prasad, S., & Srivastava, S. K. (2019). Role of phytochemicals in cancer prevention. *International journal of molecular sciences, 20*(20), 4981. doi: 10.3390/ijms20204981

Rayess, H., Wang, M. B., & Srivatsan, E. S. (2012). Cellular senescence and tumor suppressor gene p16. *International journal of cancer, 130*(8), 1715-1725.

Rehman, N. U., Hussain, H., Ali, L., Khan, A., Mabood, F., Shinwari, Z. K., Hussain, J., & Al-Harrasi, A. (2019). Chemical Constituents of Acridocarpus orientalis and Their Chemotaxonomic Significance. *Chemistry of Natural Compounds, 55*(3), 586-588.

Rehman, N. U., Mabood, F., Khan, A. L., Ali, L., Gillani, S. A., Abbas, G., Khan, A., Al-Harrasi, A., & Hussain, J. (2019). Evaluation of biological potential and physicochemical properties of Acridocarpus orientalis (Malpighiaceae). *Pak. J. Bot, 51*(3), 1099-1106.

Reuvers, T. G., Kanaar, R., & Nonnekens, J. (2020). DNA damage-inducing anticancer therapies: from global to precision damage. *Cancers, 12*(8), 2098. doi: 10.3390/cancers12082098

Rubin, S. M. (2013). Deciphering the retinoblastoma protein phosphorylation code. *Trends in biochemical sciences, 38*(1), 12-19.

Saad, B., Zaid, H., Shanak, S., & Kadan, S. (2017). Introduction to medicinal plant safety and efficacy. In *Anti-diabetes and anti-obesity medicinal plants and phytochemicals* (pp. 21-55). Springer.

Sakkir, S., Kabshawi, M., & Mehairbi, M. (2012). Medicinal plants diversity and their conservation status in the United Arab Emirates (UAE). *Journal of Medicinal Plants Research, 6*(7), 1304-1322.

Sambi, M., Qorri, B., Harless, W., & Szewczuk, M. R. (2019). Therapeutic options for metastatic breast cancer. In *Breast Cancer Metastasis and Drug Resistance* (pp. 131-172). Springer.

Savitskaya, M., & Onishchenko, G. (2015). Mechanisms of apoptosis. *Biochemistry (Moscow), 80*(11), 1393-1405.

Sedighi, M., Bahmani, M., Asgary, S., Beyranvand, F., & Rafieian-Kopaei, M. (2017). A review of plant-based compounds and medicinal plants effective on atherosclerosis. *Journal of research in medical sciences: the official journal of Isfahan University of Medical Sciences, 22*. doi: 10.4103/1735-1995.202151

Senga, S. S., & Grose, R. P. (2021). Hallmarks of cancer—the new testament. *Open Biology, 11*(1), 200358. doi: 10.1098/rsob.200358

Seyfried, T. N., & Huysentruyt, L. C. (2013). On the origin of cancer metastasis. *Critical reviews in oncogenesis, 18*(1-2), 43.

Shakya, A. K. (2016). Medicinal plants: future source of new drugs. *International Journal of Herbal Medicine, 4*(4), 59-64.

Sharma, A., Mishra, T., Thacker, G., Mishra, M., Narender, T., & Trivedi, A. K. (2020). Chebulinic acid inhibits MDA-MB-231 breast cancer metastasis and promotes cell death through down regulation of SOD1 and induction of autophagy. *Cell biology international, 44*(12), 2553-2569.

Siegel, R. L., Miller, K. D., Fuchs, H. E., & Jemal, A. (2021). Cancer statistics, 2021. *CA: a cancer journal for clinicians, 71*(1), 7-33.

Smith, E., Palethorpe, H. M., Tomita, Y., Pei, J. V., Townsend, A. R., Price, T. J., Young, J. P., Yool, A. J., & Hardingham, J. E. (2018). The purified extract from the medicinal plant Bacopa monnieri, bacopaside II, inhibits growth of colon cancer cells in vitro by inducing cell cycle arrest and apoptosis. *Cells, 7*(7), 81.

Srinivas, U. S., Tan, B. W., Vellayappan, B. A., & Jeyasekharan, A. D. (2019). ROS and the DNA damage response in cancer. *Redox biology, 25*, 101084. doi: 10.1016/j.redox.2018.101084

Sui, X., Kong, N., Ye, L., Han, W., Zhou, J., Zhang, Q., He, C., & Pan, H. (2014). p38 and JNK MAPK pathways control the balance of apoptosis and autophagy in response to chemotherapeutic agents. *Cancer letters, 344*(2), 174-179.

Sun, C., Li, C., Li, X., Zhu, Y., Su, Z., Wang, X., He, Q., Zheng, G., & Feng, B. (2018). Scutellarin induces apoptosis and autophagy in NSCLC cells through ERK1/2 and AKT Signaling Pathways in vitro and in vivo. *Journal of Cancer, 9*(18), 3247. doi:10.7150/jca.25921

Sun, Y., Liu, W.-Z., Liu, T., Feng, X., Yang, N., & Zhou, H.-F. (2015). Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. *Journal of Receptors and Signal Transduction, 35*(6), 600-604.

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians, 71*(3), 209-249.

Tanida, I., Ueno, T., & Kominami, E. (2008). LC3 and Autophagy. In *Autophagosome and phagosome* (pp. 77-88). Springer.

Tasneem, S., Liu, B., Li, B., Choudhary, M. I., & Wang, W. (2019). Molecular pharmacology of inflammation: Medicinal plants as anti-inflammatory agents. *Pharmacological research, 139*, 126-140.

van den Berg, J., G. Manjón, A., Kielbassa, K., Feringa, F. M., Freire, R., & Medema, R. H. (2018). A limited number of double-strand DNA breaks is sufficient to delay cell cycle progression. *Nucleic acids research, 46*(19), 10132-10144.

van den Heuvel, S. (2005). Cell-cycle regulation. *WormBook: The Online Review of C. elegans Biology* [online] . (Accessed 15/9/2021). Retrieved from: https://www.ncbi.nlm.nih.gov/books/NBK19719/

Vega-Rubín-de-Celis, S. (2020). The role of Beclin 1-dependent autophagy in cancer. *Biology, 9*(1), 4. doi: 10.3390/biology9010004

Von Zglinicki, T., Saretzki, G., Ladhoff, J., di Fagagna, F. d. A., & Jackson, S. (2005). Human cell senescence as a DNA damage response. *Mechanisms of ageing and development, 126*(1), 111-117.

Waks, A. G., & Winer, E. P. (2019). Breast cancer treatment: a review. *Jama*, *321*(3), 288-300.

Wang, A. S., & Dreesen, O. (2018). Biomarkers of cellular senescence and skin aging. *Frontiers in Genetics*, *9*, 247. doi: 10.3389/fgene.2018.00247

Wang, X.-Y., Zhang, X.-H., Peng, L., Liu, Z., Yang, Y.-X., He, Z.-X., Dang, H.-W., & Zhou, S.-F. (2017). Bardoxolone methyl (CDDO-Me or RTA402) induces cell cycle arrest, apoptosis and autophagy via PI3K/Akt/mTOR and p38 MAPK/Erk1/2 signaling pathways in K562 cells. *American journal of translational research*, *9*(10), 4652-4672.

Wang, X., Shao, Q.-H., Zhou, H., Wu, J.-L., Quan, W.-Q., Ji, P., Yao, Y.-W., Li, D., & Sun, Z.-J. (2020). Ginkgolide B inhibits lung cancer cells promotion via beclin-1-dependent autophagy. *BMC Complementary Medicine and Therapies*, *20*(1), 1-11.

Webber, J. L. (2010). Regulation of autophagy by p38α MAPK. *Autophagy*, *6*(2), 292-293.

Wellings, E., Vassiliades, L., & Abdalla, R. (2016). Breast cancer screening for high-risk patients of different ages and risk-which modality is most effective? *Cureus*, *8*(12). doi: 10.7759/cureus.945

Wenzel, E. S., & Singh, A. T. (2018). Cell-cycle checkpoints and aneuploidy on the path to cancer. *In Vivo*, *32*(1), 1-5.

World Health Organization (WHO). (2020). *Cancer today - Breast fact sheet*. [online]. (Accessed 15/9/2021). Retrieved from: https://gco.iarc.fr/today/data/factsheets/cancers/20-Breast-fact-sheet.pdf.

Wu, S., Powers, S., Zhu, W., & Hannun, Y. A. (2016). Substantial contribution of extrinsic risk factors to cancer development. *Nature*, *529*(7584), 43-47.

Wu, Y.-T., Tan, H.-L., Shui, G., Bauvy, C., Huang, Q., Wenk, M. R., Ong, C.-N., Codogno, P., & Shen, H.-M. (2010). Dual role of 3-methyladenine in modulation of autophagy via different temporal patterns of inhibition on class I and III phosphoinositide 3-kinase. *Journal of Biological Chemistry*, *285*(14), 10850-10861.

Yang, D., Guo, Q., Liang, Y., Zhao, Y., Tian, X., Ye, Y., Tian, J., Wu, T., & Lu, N. (2020). Wogonin induces cellular senescence in breast cancer via suppressing TXNRD2 expression. *Archives of Toxicology*, *94*(10), 3433-3447.

You, M., Lee, Y.-H., Kim, H.-J., Kook, J. H., & Kim, H.-A. (2020). St. John's Wort Suppresses Growth in Triple-Negative Breast Cancer Cell Line MDA-MB-231 by Inducing Prodeath Autophagy and Apoptosis. *Nutrients, 12*(10), 3175. doi: 10.3390/nu12103175

Yuan, Y., Ding, D., Zhang, N., Xia, Z., Wang, J., Yang, H., Guo, F., & Li, B. (2018). TNF-α induces autophagy through ERK1/2 pathway to regulate apoptosis in neonatal necrotizing enterocolitis model cells IEC-6. *Cell Cycle, 17*(11), 1390-1402.

Zarubin, T., & Jiahuai, H. (2005). Activation and signaling of the p38 MAP kinase pathway. *Cell research, 15*(1), 11-18.

Zeng, S., Shen, W. H., & Liu, L. (2018). Senescence and cancer. *Cancer translational medicine, 4*(3), 70. doi: 10.4103/ctm.ctm_22_18

Zeng, Y., Wang, C.-L., Xian, J., Ye, Q., Qin, X., Tan, Y.-W., & Cao, Y.-D. (2019). Positive correlation between programmed death ligand-1 and p53 in triple-negative breast cancer. *OncoTargets and therapy, 12*, 7193. doi: 10.2147/OTT.S209484

Zhang, M., Zhang, L., Hei, R., Li, X., Cai, H., Wu, X., Zheng, Q., & Cai, C. (2021). CDK inhibitors in cancer therapy, an overview of recent development. *American journal of cancer research, 11*(5), 1913-1935.

Zhu, L., & Xue, L. (2019). Kaempferol suppresses proliferation and induces cell cycle arrest, apoptosis, and DNA damage in breast cancer cells. *Oncology research, 27*(6), 629-634.

Zou, J., Lei, T., Guo, P., Yu, J., Xu, Q., Luo, Y., Ke, R., & Huang, D. (2019). Mechanisms shaping the role of ERK1/2 in cellular senescence. *Molecular medicine reports, 19*(2), 759-770.

UAEU عمادة المكتبات Libraries Deanship
جامعة الإمارات العربية المتحدة United Arab Emirates University
Digital Library Services Section - قسم الخدمات المكتبية الرقمية