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MOLECULAR MECHANISIMS UNDERLYING THE ANTI-CANCER ACTIVITY OF GUM ARABIC FROM ACACIA SP. IN TRIPLE-NEGATIVE BREAST CANCER CELLS

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

Under the Supervision of Dr. Yusra Al Dhaheri

June 2021

Declaration of Original Work

I, Sawsan Yaslam Hussein AlYafii , the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Molecular Mechanisms Underlying the Anti-Cancer Activity of Gum Arabic from Acacia sp. in Triple-Negative Breast Cancer Cells*", 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 been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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   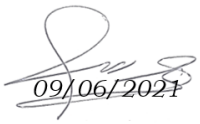

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Abstract

Metastatic breast cancer is the leading cause of cancer-related deaths among women worldwide. Triple-negative breast cancer (TNBC) is the most aggressive, accounting for 15-20% of all breast cancer cases. As TNBC cells lack the expression of hormone receptors estrogen receptors (ER) and progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2), TNBCs are unresponsive to hormonal therapy and often become highly resistant when exposed to standard chemotherapy, which has been identifies as a major obstacle in TNBC treatment. Gum Arabic, a natural exudate produced from plants, is widely used traditionally for religious, cosmetics as well as medical purposes since ages. Although it is well reported for its biological activities and medical value, no studies have been carried to assess its anticancer potential. In search of new and novel compounds to target TNBCs effectively and less toxicity, this study aims to evaluate the anticancer activity of Gum Arabic extract (GAE) and against breast cancer MDA-MB-231 cell line, a triple-negative human breast cancer cell line, and to elucidate the molecular targets underlying its mechanism of action. The results revealed that GAE inhibits cell proliferation in a concentration- and time-dependent manner. The anti-proliferative effect of GAE was found to be linked to cell cycle arrest at the G1/S phase along with induction of apoptosis confirmed in the cells as suggested by caspase 3/7 activation and cleaved caspase 3 and cleaved PARP detection. Cell cycle inhibitory protein $p21^{WAF1}$ was increased in GAE treated cells compared to untreated cells while cyclin D1 and c-myc were downregulated. Furthermore, the study found that Wnt/β-catenin signaling was markedly inhibited and could induce loss of expression of the canonical Wnt-directed targets genes cyclin D1, c-myc, and survivin in MDA-MB-231 cells treated with GAE. In conclusion, GAE inhibits the proliferation of MDA-MD-231 breast cancer cells that is associated with the suppression of Wnt/β-catenin signaling suggesting that Gum Arabic could be a potential new chemotherapeutic agent against highly chemoresistant triple negative breast.

Keywords: Gum Arabic, triple-negative breast cancer, apoptosis, cell cycle, cell cycle arrest, anti-breast cancer agent.

Title and Abstract (in Arabic)

دراسة الآليات على المستوى الجزيئي التي يقوم عليها النشاط المضاد للسرطان للصمغ العربي على خلايا سرطان الثدي الثلاثي السلبية

الملخص

سرطان الثدي سريع الانتشار هو السبب الرئيسي للوفيات المرتبطة بالسرطان بين النساء في جميع أنحاء العالم. سرطان الثدي الثلاثي السلبي (TNBC) هو الأكثر خطورة، حيث يمثل 15- 20% من جميع حالات سرطان الثدي. نظرًا لأن خلايا TNBC تفتقر إلى التعبير عن مستقبلات هرمون الاستروجين (ER) ومستقبلات البروجسترون (PR) ومستقبلات عامل نمو البشرة البشري 2 (HER2)، فإن TNBCs لا تستجيب للعلاج الهرموني وغالبًا ما تصبح شديدة المقاومة عند تعرضها للعلاج الكيميائي المستخدم حالياً، والذي يمثل عقبة رئيسية في علاج TNBC. يستخدم الصمغ العربي، وهو إفراز طبيعي ينتج من النباتات، على نطاق واسع للأغراض الدينية ومستحضرات التجميل وكذلك للأغراض الطبية منذ العصور. على الرغم من أنه تم دراسة أنشطته البيولوجية وقيمته الطبية، إلا أنه لم يتم إجراء دراسات لتقييم إمكاناته المضادة للسرطان. بحثًا عن مركبات جديدة ومبتكرة لاستهداف TNBCs بشكل فعال وأقل سمية، تهدف هذه الدراسة إلى توضيح النشاط المضاد للسرطان لمستخلص الصمغ العربي (GAE) ضد سرطان الثدي MDA-MB-231، وهي خلية سرطان الثدي البشري الثلاثية السلبية. لتوضيح الأهداف الجزيئية الكامنة وراء آلية عملها. أظهرت النتائج أن GAE يثبط تكاثر الخلايا بطريقة تعتمد على التركيز والوقت. أظهرت الدراسة ان التأثير المضاد للتكاثر لـ GAE مرتبطًا بتوقف دورة الخلية في مرحلة G1 / S جنبًا إلى جنب مع تحريض موت الخلايا المبرمج المؤكد في الخلايا كما هو مقترح بواسطة تنشيط caspase 3/7 وcaspase PARP 3 المشقوق. تمت زيادة البروتين المثبط لدورة الخلية $p21^{WAFA}$ في الخلايا المعالجة بـ GAE مقارنة بالخلايا غير المعالجة بينما تم تقليل تنظيم cyclin D1 و c-myc. علاوة على ذلك، أثبتت الدراسة أن إشارات Wnt / β-catenin الخلوية قد تم تثبيطها بشكل ملحوظ ويمكن أن تؤدي إلى فقدان التعبير عن جينات الأهداف الموجهة لـ Wnt cyclin D1 و c-myc و survivin في خلايا MDA-MB-231 المعالجة بـ GAE. في الختام، يمنع GAE تكاثر خلايا سرطان الثدي MDA-MD-231 المرتبط بقمع إشارات Wnt / β-catenin مما يشير إلى أن الصمغ العربي يمكن أن يكون عامل علاج كيميائي جديد محتمل ضد الثدي الثلاثي السلبي المقاوم للأدوية الكيميائية.

مفاهيم البحث الرئيسية: الصمغ العربي، سرطان الثدي ثلاثي السلبية، موت الخلايا المبرمج، دورة حياة الخلية، إيقاف دورة حياة الخلية، عامل مضاد لسرطان الثدي.

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Dedication

To my beloved parents Yaslam and Rasha, for their continues support, prayers, and for believing in me, Sister Manal, and my family, for care, help, and support all the time

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List of Abbreviations

BAX	Bcl-2 Associated X Proteins
BC	Breast Cancer
Bcl-2	B-Cell Lymphoma 2 Protein
BLBCs	Basal-Like Breast Cancers
CDKs	Cyclin-Dependent Kinases
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EpCAM	Epithelial Cell Adhesion Molecule
ER	Estrogen Receptor
HER2	Human Epidermal Growth Factor Receptor 2
HRT	Hormone Replacement Therapy
IGF	Insulin-Like Growth Factor
MAPK	Mitogen-Activated Protein Kinase
NCDs	Noncommunicable Diseases
NFκB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
PARP	Poly- ADP Ribose Polymerase
PDGF	Platelet-Derived Growth Factor
PI3K	Phosphoinositide 3-Kinase
PKB	Protein Kinase B

PKC	Protein Kinase C
PR	Progesterone Receptor
pRb	Phospo- Retinoblastoma Protein
RAGE	Receptor for Advanced Glycation End Products
TNBC	Triple-Negative Breast Cancer
TP53	Tumor Suppressor TP53
UAE	United Arab Emirates
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

Chapter 1: Introduction

1.1 Cancer

Cancer is a disease which causes a group of abnormal cells to divide uncontrollably and it is characterized by disregarding the normal rules of cell division [1]. Cancer is a term that specifically refers to a new cellular growth which has the ability to spread throughout the body to new areas (metastasize) where they form new colonies, and infiltrate surrounding tissues [2]. Cancer cells which originate within tissues, as they grow and divide at an abnormal accelerated rate, are “poorly differentiated” which have abnormal morphology and lacks the normal tissue structures, and have irregular membranes, cytoskeletal proteins, and morphology. This abnormality in cells can be progressive with a slow transition from normal cells to benign tumors and then malignant tumors [3], [4].

Any tumor can be either benign or malignant. A benign tumor remains restricted to its original location. It does not invade nearby normal tissue nor spread to another body sites. In contrast, a malignant tumor has the capability of both invading nearby normal tissues and spreading throughout the body through the lymphatic or circulatory systems. Unlike benign tumors, only malignant tumors are referred to as cancers, due to their ability to invade surrounding tissues and metastasize, which is the primary cause of cancer morbidity and mortality (Figure 1) [5], [6].

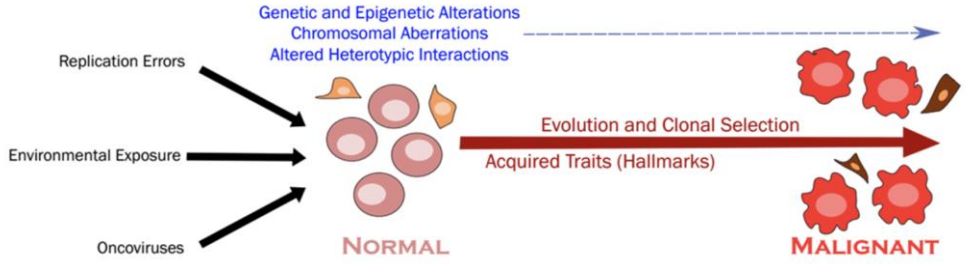

Figure 1: The transformation process. Different insults continuously act on cells leading to transformative alterations in (epi) genetics, chromosomal numbers and arrangements, and heterotypic interactions which, along the path towards malignancy, undergo cycles of evolutionary clonal selection leading to the acquisition of cancer-competent traits, the hallmarks of cancer [7].

1.2 Hallmarks of cancer

The hallmarks of cancer consist of six biological capabilities obtained during the development of tumors (Figure 2). The hallmarks establish an organizing principle for explaining the complexity of neoplastic diseases [7].

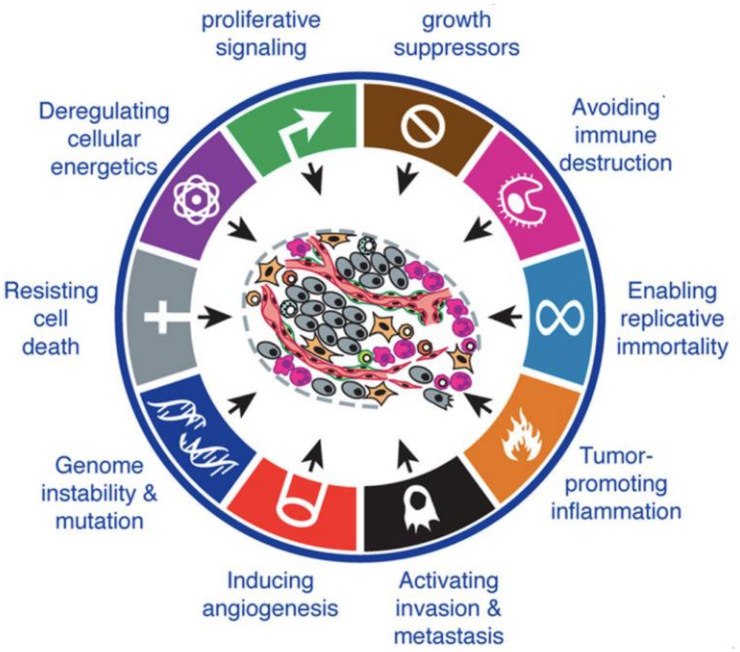

Figure 2: Hallmarks of cancer [7].

1.2.1 Self-sufficiency in growth signals

Normal cells depend on growth signaling of a regulated cell cycle in order to control proliferation and maintain tissue homeostasis, but in case of cancer, such balance is disrupted. Currently in cancer cells, the growth and signaling pathways that control cell proliferation suppress one or more driving alterations within their compartments giving them a survival edge. Those compartments include growth ligands, their receptors or the cytosolic signaling molecules [8].

Several types of extracellular matrix receptors (integrins) that are expressed by cancer cells is switched, so it can favor the receptors that transmit pro-growth signals [7]. Even if growth signals are present, the cell cycle regulation through regulatory proteins maintains the division cycles in and keep it in check. Checkpoint disruption and deregulation of the cell cycle are important for cancer cells to grow [8].

1.2.2 Insensitivity to anti-growth signals

Proliferation is blocked by antigrowth signals, by two mechanisms. Cells are arrested during the active proliferative cycle and forced out into the quiescent (G0) state from which they may reemerge on some future occasion when extracellular signals permit [7]. By disrupting the pRb pathway, E2F transcription factor is released and hence this gives cell proliferation the capability of rendering cells which can normally operate along this pathway to become insensitive to antigrowth factors and block advance through the G1 phase of the cell cycle [7].

1.2.3 Evasion of apoptosis

Apoptosis is a form of programmed cell death that occur in organisms to eliminate unnecessary or infected cells from the body over the course of development

or as a result of undergoing cellular stress. Cancer cells are always under constant stress, such as genomic instability, oncogenic stress, and cellular hypoxia [9]. Although the intrinsic pathway of apoptosis would be normally activated as a response to these internal apoptotic stimuli, cancer cells can sometimes avoid this cellular response by disabling the apoptotic pathways [9].

1.2.4 Limitless replicative potential

The maintenance of telomeric DNA underlies the ability of tumors to possess unlimited replicative potential, one of the hallmarks of cancer. Telomere length and structure are maintained by the reverse transcriptase telomerase and a multiprotein telomere complex termed shelterin. Telomerase activity has been elevated in the majority of tumors. Unlike normal tissues, telomeres are critically shortened in tumors, hence providing a compelling principle to target the telomerase/telomere pathway for broad-spectrum cancer therapy [10]. At some point during the course of multistep tumor progression, evolving premalignant cell populations exhaust their endowment of allowed doublings and can only complete their tumorigenic agenda by breaching the mortality barrier and acquiring unlimited replicative potential [7].

1.2.5 Sustained angiogenesis

Malignant tumors often outgrow their blood supply and in order to grow it should actively recruit vasculature. Angiogenesis is promoted by tumor cells by up regulating the pathways that promote blood vessel formation such as increased expression of vascular endothelial and fibroblast growth factors [11].

1.2.6 Tissue invasion and metastasis

Malignant cells possess key hallmarks, which is uncontrolled growth potentials and the ability to invade surrounding tissues and metastasize. The degree and timing of invasion and metastasis may vary due to the genetic and epigenetic heterogeneity within the tumor. Moreover, it can be associated with further signals from extrinsic factors, such as those within that particular tumor microenvironment (Figure 3) [5].

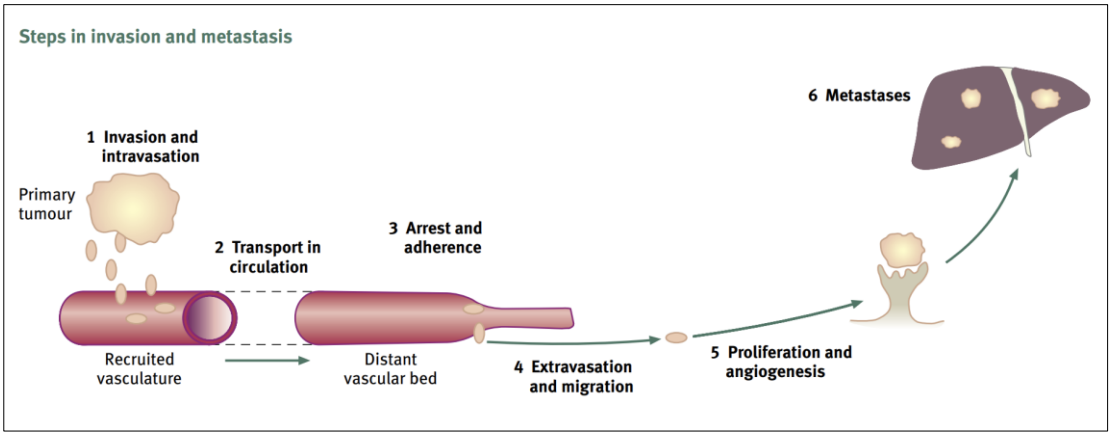

Figure 3: Cancer invasion and metastasis. Abnormal cells start proliferated in a non-controlled manner, forming a primary tumor. Cancer cells invade near tissues; after that, cancer cells interred the bloodstream and translocated into distant sites, and develop new colonies [12].

1.3 Types of cancer

There are more than 100 different types of cancer. Cancer is named for the tissue or organ where it originates from. Cancers may also be described by the type of cell that formed them, such as a squamous or an epithelial cell [2], [13]. From a histological viewpoint, there are hundreds of several cancers, which are categorized into five major groups [14]:

- Carcinoma is cancer that begins in the skin or in tissues that cover internal organs [15]. It is identified as a stage 0 cancer, because it is located only in

the layer of skin where it started. Carcinoma can originate in and spread to any area throughout the body. Most common types of carcinoma are skin cancer, and breast cancer [16].

- Sarcoma refers to cancer that originates in connective tissues such as bones, cartilage, and muscle. The most common sarcoma that occurs in young adults often develops as a painful mass on the bone [14]. Sarcomas are divided into two main types: bone cancers as well as soft tissue sarcomas. These are also classified into more than 70 sarcoma subtypes which are named based on the nearby tissue, the type of cells creating the tumor, or the affected area of the bone [17].
- Leukemia are cancers that begin in the blood forming tissue of the bone marrow, which is caused by the buildup of huge numbers of abnormal white blood cells in the bone marrow and blood. As a result, normal blood cells are crowded out [13]. After, the leukemia cells interrupt the development of normal cells [18]. There are four common types of leukemia, which are categorized based on the time of how the disease gets worse, and on the type of blood cell the cancer starts in [13].
- Lymphoma is a wide term used to describe cancer that begins in cells of the lymphatic system. The two major types are Hodgkin lymphoma and Non-Hodgkin lymphoma [19]. Non-Hodgkin lymphomas starts when T cell or B cell (type of white blood cell) becomes abnormal [20].
- Melanoma is cancer that begins in cells that become melanocytes, which are specialized cells that make melanin (the pigment that gives skin its color) [13]. These abnormal cancerous growths develop when unrepaired DNA damage to skin cells which is often caused by ultraviolet radiation,

triggers mutations that direct the skin cells to multiply rapidly to form malignant tumors [21].

1.4 Cancer epidemiology

Cancer is a non-communicable disease (NCDs). The occurrence and mortality of cancer significantly vary between countries and across cancer types. Cancer considers as the first leading cause of premature death (i.e., at ages 30–69 years) in 55 countries and the second in 79 countries worldwide [22], [23]. Cancer causes around 1 in every 6 death worldwide, higher than AIDS, tuberculosis, and malaria combined [24]. According to the International Agency for Research on Cancer (IARC), 18.0 million new cancer cases and 9.5 million cancer-related death (about 26,000 cancer deaths a day) were recorded worldwide in 2018 [22], [24], [25]. By 2040, it is estimated that the global burden of cancer will grow to around 30 million new cancer cases and 16.4 million cancer deaths due to the population's growth, aging and the increased risks of modifiable factors [24], [25].

In the United Arab Emirates (UAE), cancer is the third leading cause of death after cardiovascular diseases and injury. In 2017, it was estimated that cancer caused 16% of total NCDs mortality in the UAE [26]. In 2020, according to World Health Organization (WHO), 4807 new cancer cases and 1896 cancer-related death were reported [27]. WHO estimated that by 2040, the increase would be almost 300% to indices 19,067 new cases, whereas mortality would be increased by 380% to occupy 10,014 death cases [25].

1.5 Cancer risk factors

Cancer is a multifactorial disease, as the main leading cause of cancer is not entirely understood. Many factors can play a role in increasing the risk for cancer development [22]. These factors are divided into adjustable and nonadjustable factors. Adjustable risk factors include tobacco consumption and smoking, excess body weight, unhealthy diet, physical inactivity, exposure to environmental pollutants, and ultraviolet (UV) radiation [24]. Nonadjustable factors such as inherited genetic mutations, hormones, and immune conditions. Both adjustable and nonadjustable factors can interact together or in sequence to initiate or promote cancer growth [27].

1.6 Cancer treatment

Current available treatments for cancer include radiotherapy, surgery, and chemotherapy [28]. Radiotherapy was first used for cancer treatment over 100 years ago by using crude radium as the radiation source [29]. Radiotherapy must be performed as a mandatory step in treatment after conventional surgery for an invasive tumor, regardless of the characteristics of the disease, because it decreases the rate of local recurrence and by this way, specific mortality [30]. The therapeutic potential of chemotherapy against cancer is seriously not satisfying enough due to the nonspecific drug distribution, heterogeneity of cancer, and multidrug resistance (MDR) [28]. In fact, American Cancer Society's main objective is to detect for example breast cancer in its onset stages, and this is an opportunity to cure the disease with relatively simple surgical procedures [31]. Meanwhile, according to modern cancer therapy, not every malignant tumor has an available cure. Therefore, treatment needs to be cautiously chosen to maximize the chance for a cure while providing a maximum quality of life [32].

1.7 Breast cancer

Breast cancer is a malignant tumor that develops from breast cells. Usually, breast tumors start from ductal hyperproliferation. It develops into benign tumors or metastatic carcinomas after constant stimulation of various carcinogenic factors (Figure 3). These factors result in a series of genetic events that lead to alterations in proto-oncogenes and tumor suppressor genes [33], [34].

1.7.1 Breast cancer epidemiology

About one in eight women are diagnosed with breast cancer during their lifetime [35]. According to the American Cancer Society, more than 8 million women were diagnosed with cancer in 2018. Breast cancer showed to be the most frequent malignancy diagnosed in women worldwide. It accounts for about 25% of all women cancer cases, 15% of all women cancer-related deaths, making breast cancer the first leading cause of cancer-related deaths worldwide [24], [36]. These statistics make breast cancer a significant public health concern [37]. Despite the great improvement in early diagnosis and effective treatment in recent years, approximately a third of all women with breast cancer develop metastatic cancer, which eventually leads to death. [38], [39]. Previous years showed an increase in survival rate, but sadly survivors have a higher risk of cancer re-occurrence than those who don't have prior cancer history [40]. The 5-year survival depends on the extent of the disease, whether it is "localized" or "regional. Countries with more-developed health services showed a higher 5-year survival rate (around 30% higher) compared with developing countries, in addition to a decrease in the mortality rate. It is estimated that by 2030, the number of breast cancer incidence will increase between 2.4 million per year to 3.2 million per year [41].

1.7.2 Breast cancer in the UAE

In the UAE, breast cancer is the most prevalent cancer among females, with 38.86% of all reported female cancers and the second cause of death among the UAE women [27], [42]. Breast cancer incidence and mortality in UAE have a lower age-standardized rate than the European countries and the US with almost one decade [43], [44]. It was reported that 55.8% of all breast cancer cases were in women under 50, and 21.5% were in women between 30 and 40 [44]. By 2030, It is expected that the number will double for breast cancer cases in the UAE [25].

1.7.3 Breast cancer risk factors

Several risk factors can increase the risk of developing breast cancer [35]. These risk factors can be divide into intrinsic (unmodifiable) and extrinsic (modifiable). The unmodifiable are factors that a person cannot control or change, while the modifiable are factors that a person can handle [45].

1.7.3.1 Non-genetic intrinsic risk factors

Several factors are unmodifiable, including age, gender, reproductive factors, and endogenous hormone exposure, personal or family history of breast disease. Age and gender are the most potent risk factor for breast cancer [46]. Being a woman increases the risk of developing cancer about 100 times more than in men. Age increases the risk gradually, from rare before 20 to 100 times more by the age of 45-55 [47], [48]. Breast cancer cases are primarily diagnosed in women age 55 and older, and by the age of 90, one-fifth of women are affected [48]. Moreover, personal history raises the risk of developing breast cancer. Having cancer in one breast increases the risk of developing new cancer in the second breast or another part of the same breast.

Besides, breast cancer survivors have a significant risk of developing breast cancer again [35], [46]. It is well document that having a positive first-degree relative (mother, sister, or daughter) or two first-degree relatives diagnosed with breast cancer will increase the risk of developing breast cancer by almost double or 3-fold respectively [46]. Along with the risks mentioned above, benign proliferative lesions of the mammary gland and chronic inflammation are also correlated with metastatic breast cancer and lower survival [45]. The expression of estrogen throughout a woman's lifetime has implications in the development of or the protection against breast cancer [35]. Long exposure duration to higher concentrations of endogenous estrogen increases the risk of developing breast cancer [47], which usually happens in women with early menarche (≤ 11 years) or late menopause (≥55) [34], [47]. Increased risk of developing breast cancer is also linked to higher testosterone levels in women at premenopausal and postmenopausal [35], [47].Women who have first birth at a young age have a lower risk for developing breast cancer. However, getting first birth at older ages increases the risk of developing breast cancer more than the nulliparous women [35], [46]. Other unmodifiable factors are race and ethnicity. Breast cancer prevalence varies with the ethnic difference. Breast cancer is more common among whites than African- Americans. However, African-American women are more commonly to develop breast cancer under the age of 45 and have a higher prevalence of triple-negative breast cancers. Therefore, it is more likely for African-American women to die from breast cancer at any age. Other races have a lower risk of developing and dying from breast cancer, such as Asian, Hispanic, and Native American women [46], [47]. The variance among ethnic groups is estrogen and progesterone receptor subtypes and status in each group [47].

1.7.3.2 Extrinsic risk factors

The main extrinsic risk factors are behavioral and lifestyle habits. Modern lifestyle includes excessive use of alcohol, low physical activity, and consuming a lot of dietary fat leading to obesity (being overweight). As a result, fat tissues can cause an increase in estrogen levels in women, which can increase the risk of developing breast cancer [33]. Exposure to ionizing radiation such as medical radiation treatment or nuclear explosion increases cancer risk [35], [49]. Moreover, after menopause, hormone replacement therapy (HRT) increases the risk of breast cancer, chances of dying from breast cancer, and the probability of getting diagnosed at a late stage [46]. Long-term use of HRT increases the risk of developing breast cancer, 15% in 5 years and 34% in the case of 10 years [50]. However, the risk is eliminated within five years after stopping HRT usage. Likewise, oral contraceptive increases the risk slightly, but it abolished after stopping oral contraceptive usage [46]. Moreover, having no children or having them at a late age slightly increases the risk [33], whereas becoming pregnant at an early age or having many pregnancies reduces breast cancer risk [46]. Also, exposure to chemicals such as organochlorines, dioxins, and organo-chlorine can increase breast cancer risk as they can lead to oncogenes mutations [47], [49].

1.7.4 Classification of breast cancers

Breast cancer is a heterogeneous disease with specific histological and biological features. These properties can vary according to the genetic, epigenetic, and transcriptome changes, which ultimately cause differences in a patient's clinical behavior, diagnosis, prognosis, and treatment response [51-54]. Today, breast cancer is classified according to histological type and grade, stage biomarker receptor status, and gene expression profiling [54], [55], based on histological features into triple-

negative breast cancer (TNBC), hormone-receptor-positive, and human epidermal growth factor receptor-2 overexpressing (HER2+) [56]. There are two factors that determine the appropriate survival outcome in breast cancer: (1) detection of disease at an early stage with screening mammography, and (2) advances in adjuvant systemic treatment including hormone therapy, chemotherapy, and HER2-targeted therapy that eliminates micrometastases after definitive surgery for breast cancer (Table 1) [57].

1.7.4.1 Molecular classification of breast cancers

The molecular classification of cancer helps in identifying the alteration in gene expression in cancer cells. The molecular classification made considerable differences in prognosis, medication responses, and clinical outcomes for each cancer subtype (Table 2) [58]. Besides, identifying the expression alteration plays a role in selecting the most effective treatment, reducing relapse, or help in designing a specific selective therapy for each patient [53], [58]. According to various gene expression analysis, breast cancer is divide into five subtypes; i) luminal A, ii) luminal B, where estrogen (ER), progesterone receptors (PR) are frequently positive, iii) human epidermal growth factor receptor 2 overexpression (HER-2 positive) with ER- /PR-negative, iv) basal-like and v) normal breast tissue-like [51], [54]. Clinically, HER2 and basal-like tumors showed the worst aggressive behavior among the five subtypes. In contrast, basal-like tumors contain one of the most aggressive cancer types, triple-negative breast cancer [55].

Table 1: Description of therapies available to treat breast cancer, the corresponding drugs, and mode of action on the tumor cells [59]

Therapies available	Drugs used	
Chemotherapy	• Alkylating agents (e.g., Cisplatin and Carboplatin)	• Keep the cell from reproducing by damaging its DNA.
	• Antimetabolites (e.g., 5-fluorouracil (5-FU), Capecitabine and Gemcitabine)	• Interfere with DNA and RNA by acting as a substitute for the normal building blocks of DNA and RNA.
	• Anti-tumor antibiotics: Anthracyclines such as Doxorubicin and Epirubicin Not anthracyclines	• Not like antibiotics. They work by changing the DNA inside cancer, keeping it from growing and multiplying.
	• Topoisomerase inhibitors (e.g., Mitoxantrone)	
	• Mitotic inhibitors: 1) Taxanes such as Docetaxel and Paclitaxel	• Interfere with topoisomerases.
	2) Vinca alkaloids such as Viblastine	• They stop cells from dividing to form new cells but can damage cells in all phases by Keeping enzymes from making proteins.
Immunotherapy	• PD-L1 inhibitors (e.g., Atezolizumab)	• Immune checkpoint inhibitors. Help to restore the immune response against breast cancer cells.
Endocrine therapy	• Tamoxifen Fulvestrant	• Drugs that block estrogen receptors, stopping estrogen from fueling breast cancer cells.
	• Aromatase inhibitors	• Lower estrogen levels by stopping estrogen production.
Target therapy	• Trastuzumab (Herceptin) Pertuzumab Ado- trastuzumab emtansine Lapatininb	• Monoclonal antibody that acts directly against HER2 protein.
	• CDK4/6 inhibitors (e.g. Palbociclib and Ribociclib)	• Blocks the cyclin-dependent kinases (CDKs), particularly CDK4 and CDK6, on hormone receptor-positive breast cancer cells, stopping cells from dividing.
	• Everolimus	• For women who went through menopause and had advanced receptor-positive, HER2 (56) breast cancer. The drug block mTOR.
	• Olaparib or Talazoparib	• For women with BRCA gene mutations. These drugs are PARP inhibitors • For cancers with PIK3CA gene mutation.
	• Alpelisib	• Is a PIK3CA inhibitor.

1.7.4.1.1 Triple-negative-tumors

Approximately 60% to 90% of basal-like tumors are triple-negative cases [60]. Triple-negative breast cancer (TNBC), which is the cancer that lack the expression of progesterone receptor and estrogen receptor (ER) and absence of ERBB2 (HER2) overexpression and/or gene amplification. TNBC accounts for 15–20% of breast cancer cases and affects more frequently younger patients around the age of 40 [61], [62]. TNBC contributes to a large proportion of breast cancer deaths despite its small proportion among all breast cancers [63].

Table 2: Molecular/intrinsic subtypes of breast cancers [46]

Subtypes	**Molecular Signatures**	**Characteristics**	**Treatment Options[a]**
Luminal A	ER+, PR±, HER2-, Low Ki67	≈70%, Most common Best prognosis	Hormonal Therapy Targeted Therapy
Luminal B	ER+, PR±, HER2±, High Ki67	10%– 20%	Hormonal Therapy Targeted Therapy
HER2	ER-, PR-, HER2±	Lower survival than Luminal A 5%–15%	Targeted Therapy
Triple Negative	ER-, PR-, HER2-	15%–20% More common in black women Diagnosed at younger age Worst prognosis Rare	Limited Targeted Therapy
Normal-like	ER+, PR±, HER2-, Low Ki67	Low proliferation gene cluster expression	Hormonal Therapy Targeted Therapy

[a]Besides conventional surgical and non-surgical treatment

TNBCs are extremely proliferative and characterized by its aggressive nature (mostly grade III), often exhibit metastasis to secondary sites such as the brain and lung, early recurrences, and poor survival prognosis, unique molecular profile, and lack of targeted therapies and develop consistently resistance against chemotherapeutic agents [62], [64]. Common treatments like hormone therapy and drugs that target progesterone, estrogen, and HER-2 are ineffective, because the tumor cells lack the necessary receptors [65], making it one of the most challenging groups of breast cancers to treat. As a result, chemotherapy using cytotoxic drug that inhibit cancer cells is mainly used in the treatment of TNBCs [60], [62], [66], [67].

Currently, some targeted therapies were approved for TNBC clinical use, including poly- ADP ribose polymerase (PARP) inhibitors and bevacizumab – a monoclonal antibody– (vascular endothelial growth factor (VEGF inhibitor). At the same time, several other potential targets are under investigation and evaluation [74], including the receptor for advanced glycation end products (RAGE), which involve in apoptotic activity in TNBC [68] and Epithelial cell adhesion molecule (EpCAM), a cell surface molecule, its over-expression associated with proliferation and malignancy in epithelial cancers [59].

1.7.5 Anticancer drugs from plants

Natural products represent a useful source of active molecules and play a key role in the drug discovery process [69], [70]. Plants were the first medicinal agents to treat and prevent an uncountable number of diseases and health conditions [71], [72] Natural compounds as bioactive ingredients isolated from natural sources (fungi, plants, and marine life forms) have become one of the primary cancer researchers' interests [73], [74] which have transformed and improved the field of anticancer

therapeutics [75]. Nowadays, about 60% of all recent discovered anticancer drugs derive from natural compounds such as plants or plants ‘derived compounds [76]. However, only 10% of the plants have been studied for different disease treatments [77].

1.7.5.1 Molecular mechanisms of anti-cancer effects of natural products

Naturally occurring substances demonstrated anti-cancer effects by inhibiting several stages of carcinogenesis, including initiation, promotion, and progression by affecting cellular and molecular events [78].

1.7.5.1.1 Apoptosis (programmed cell death)

Apoptosis is a tightly organized multi-step pathway where cells commit to death. This programmed cell death mechanism is exceptionally fateful through development. However, it is also essential in adult multicellular organisms’ homeostasis as it eliminates unnecessary, unwanted, and damaged cells [79]. It occurs in a controlled manner to minimize damage and disruption to neighboring cells. Critical characteristics of apoptosis include changes in the plasma membrane, cytoplasmic and nuclear shrinkage, condensation of the nucleus, followed by DNA fragmentation [80]. During apoptosis, cells become rounded and detach from surrounding cells due to plasma membrane blebbing. The translocation of phosphatidylserine (PS) from the inner to the outer side of the plasma membrane is a dominant apoptosis signal that allows the recognition by phagocytic cells to engulf and consume apoptotic cells. This process is orchestrated primarily, however not exclusively, by the cysteine proteases family members who are known as caspases [81].

1.7.5.1.2 Cell cycle regulation

Cell proliferation involves the reproduction of a cell to form two daughter cells. The proliferation process is a tightly regulated by a series of events that ensure a healthy DNA replication and cell division. The cell cycle occurs through several sequential phases that are highly regulated by several specific cyclin-dependent kinases (CDKs) and cyclin proteins. S and M phases consider as the major phases in the cell cycle. In the S phase, DNA replication and chromosome duplication take place. While in the M phase, the nuclear division (mitosis) and actual cell division occurs. In most cells, gap phases occur between each phase and another, like G1 before the S phase and G2 before the M phase. The purpose of these gaps is to provide additional time for cells to grow and regulate transitions that control the progression to the next stage of the cell cycle. G1 phase consider as the initial phase in the cell cycle, as it determines if the cell will continued division or exit from the cell cycle. Cells go into prolonged G1 or withdrawal into a non-dividing state called G0 in case of nonstable growth conditions [82], [83].

In some cases, cells may pause the cell cycle progression to respond to intracellular or external stimuli. The pause is referred to as a “checkpoint”, which allows the cell to fix any damaged DNA or acquire a sufficient growth factor level before transitioning to the next phase. If the cells are not able to repair the DNA damages, the cell will activate apoptotic-signaling cascades to prevent generating daughter cells with damaged DNA. Checkpoint is highly crucial in the cell cycle to maintain genomic stability, preventing carcinogenesis. In most tumors, these checkpoints are deregulated [83].

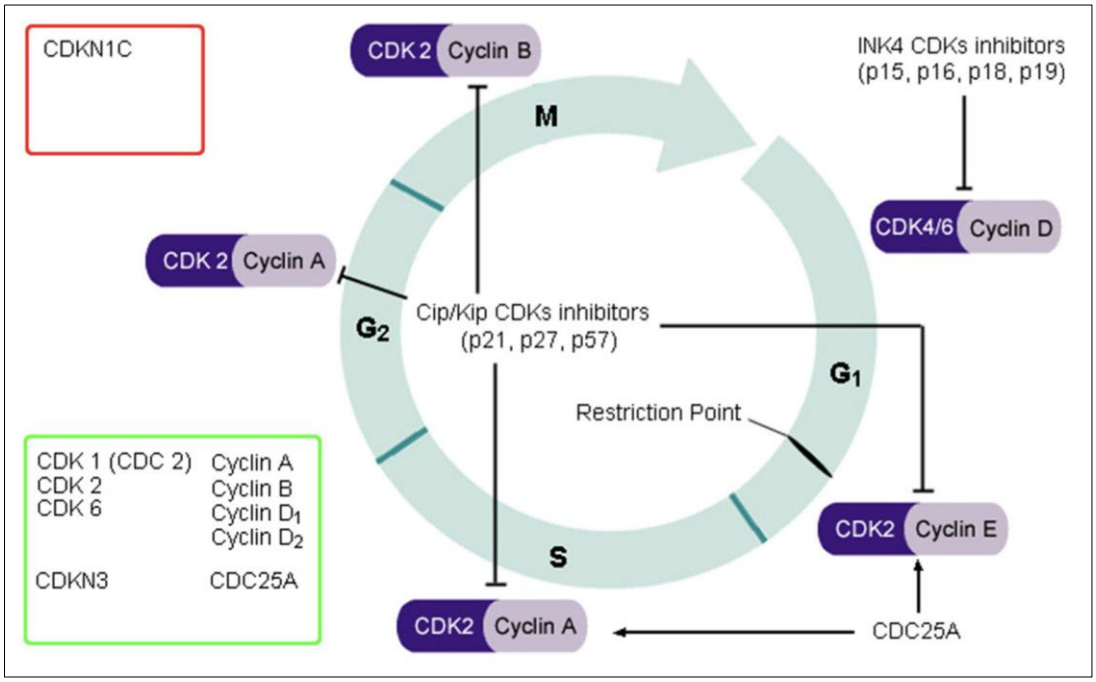

Figure 4: Cell cycle regulation by several specific cyclin-dependent kinases (CDKs) and cyclins proteins [84]

1.7.5.1.3 DNA damage

Different stresses can alter DNA which results in DNA damages. Stressors included chemicals, radiations such as UV light, and reactive oxygen species (ROS), among others. Under normal conditions, cells can repair these damages and maintain genomic stability [85]. When cells are under stress, they activate several mechanisms to repair damaged DNA and protect the genetic information, checkpoint proteins pusses the cell cycle progression to allow the DNA repair. If cells are unable to fix the damaged DNA, the cell activates apoptosis pathway to eliminate potentially dangerous mutations [86]. Cancer can result due to the accumulation of non-repaired damaged DNA in these stressed cells. Changes in typical DNA sequence can alter several genes, including proto-oncogenes and tumor suppressor genes.

Most cancer tumors have mutations in DNA damage repair and signaling pathways that contribute to their aggressive phenotype [85],[87]. Moreover, particular DNA repair pathways enable cancer cells to survive DNA damage induced by chemotherapeutic treatments, making them resistant to such drugs [88].

1.7.5.2 Review on anti-cancer activity of natural compounds derived from plants

The anticancer potential of plant-derived natural compounds (such as luteolin and curcumin) has been associated to their capability to target multiple impairment regulatory mechanisms of signaling pathways (such as mitogen-activated protein kinase (MAPK) and Hedgehog) causing suppression of proliferation, inflammation, cell growth, migration, angiogenesis, epithelial-mesenchymal transition (EMT) and metastasis, and activation of apoptosis in TNBCs [69]. Plant-derived compounds, including the epipodophyllotoxin analogues and the vinca bisindole alkaloids have been among the most extensively used cancer chemotherapeutics available, and interest in these compounds and other natural products to defeat cancer is increasing [89].

Gallic acid isolated from *Phaleria macrocarpa* showed anti-cancer effect in several human cancer cell lines including, lung cancer, leukemia, and colon adenocarcinoma cell lines and prevents malignancy transformation cancer development [90]. Moreover, in breast cancer, *P. macrocarpa* showed anti-proliferative, anti-angiogenic, and pro-apoptotic effects. Apoptosis induced by *P. macrocarpa* was through intrinsic apoptotic signaling pathway by caspase-9 activation, downregulation of B-cell lymphoma 2 protein (Bcl-2), and upregulation of Bcl-2 associated X proteins (BAX), followed by further activation of caspases 3, 6, 7,

and finally activating DNAase to cause DNA fragmentation, thus inducing apoptosis [91].

Curcumin, the principal curcuminoid of turmeric (*Curcuma longa*), emonstrated anti-cancer activity in several studies. Curcumin exerts its anticancer action by targeting multiple pathways (such as MAPK and NF-κB pathways) and modifies various target proteins and genes including growth factors and their receptors (PDGF, EGF, VEGF), cell surface adhesion molecules (E-cadherin, β-catenin), and protein kinases (CDKs, EGFR, PKC, p38 MAPK) [92]. Curcumin suppresses cell growth, migration, and proliferation. It also promotes apoptosis and inhibits angiogenesis, invasion, and metastasis in cancers through the modulation of some molecular targets. Curcumin treatment sufficiently reduced cell proliferation and promoted apoptosis *in vitro* via the modification of the EGFR-MAPK signaling, as indicated by curcumin treatment-associated reduce in the levels of phosphorylated EGFR and ERK1/2 [93]. In breast cancer cell line (MCF-7), curcumin showed cytotoxicity and anti-proliferation activity through increased caspase 3/9 activity and induction of apoptosis, downregulation of p21 and upregulating the PTEN/Akt signaling pathway [94].

In a study done by Solowey et al. [95] *Urtica membranacea* (Urticaceae), *Artemesia monosperma* (Asteraceae), and *Origanum dayi post* (Labiatae) showed anticancer activity against several human tumor cells and primary tumor cultures [95]. *U. membranacea* and *A. monosperma* showed to induce apoptosis, while *O. dayi* showed to activate the caspase-3-independent mechanism [95].

Catharanthus roseus contain vinca alkaloids, vinblastine, and vincristine, the first agents to advance into clinical use. It showed anticancer activity in leukemias,

lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma.

Paclitaxel is a chemotherapy drug produced initially from the bark of the Pacific yew, *Taxus brevifolia* Nutt. Paclitaxel is significantly active against ovarian cancer, advanced breast cancer, small and non-small cell lung cancer [96].

Leaves of *Azadirachta indica* (Neem) showed anticancer activity on prostate cancer cells (PC- 3). It induces cell death by apoptosis, resulting in increased DNA fragmentation and a decrease in cell viability [97]. The flowers of Neem also showed some chemopreventive agents effective in inhibiting liver and mammary gland carcinogenesis in rats [97].

Hedyotis diffusa, contains Methyl anthraquinonesare, the active component responsible for inducing apoptosis in many cancer types. In breast cancer cell line MCF-7, cells were arrested at the S phase, and apoptosis was activated via caspase-4/Ca^{2+}/calpain pathway. *H. diffusa* also showed an anti-cancer effect in cervical, colorectal cancer, and Hela cells [90].

Lycopene, which is found widely in fruits and vegetables, exhibits anti-carcinogenic, and chemoprevention properties in *vitro* and *in vivo* models. The mechanism of action occurred by changes in antioxidant and phase II detoxifying enzymes by regulating the nuclear factor E2-related factor 2-antioxidant response element (Nrf2-ARE) system. This induces cell cycle arrest at the G0/G1 phase and downregulation of growth factor (PDGF, VEGF, and IGF)- by regulating cyclin D1 and the PI3K/AKT/PKB and Ras/RAF/MAPK signaling pathways [92], [98]. Lycopene showed promising anticancer activity in prostate, breast, gastric, colorectal, liver, renal, bladder, and lung cancer [98]. In the US, Lycopene has entered Phase II clinical trials to prevent and treat prostate cancer [92].

Protopanaxadiol and protopanaxatriol were obtained from *Asian ginseng*. Protopanaxadiol induces cancer cell apoptosis and inhibits proliferation by targeting the Wnt/β-catenin signaling pathway, down-regulating of Akt activity, and inhibiting P-glycoprotein (P-gp) [92]. A mixture of protopanaxadiol and protopanaxatriol (PandimexTM) has been approved conditionally in China for the treatment of advanced cancers of the breast, colon- rectum, lung, and pancreas. It is ongoing in Phase I clinical trial in the US for advanced lung, gastric, breast, and pancreatic cancers combined with paclitaxel or alone [92].

1.7.5.3 Gum Arabic

Acacia gum (Figure 5A), known as Gum Arabic (GA), is an edible air-dried gummy exudate secreted by the hardened sap (Figure 5B) of various acacia tree species [99]. *Acacia senegal* and *Acacia seyal* (family Leguminosae) are the only two species of *Acacia* gums authorized by Food and Agriculture Organization (FAO) in 1999 and recognized as safe by the Food and Drug Administration (FDA) [100]. The gum is produced by the *Acacia* tree's trunk or branches due to stress conditions as heat, drought, insects and mold infections, injury, or amputation [101]. *Acacia* trees are natives to the barren areas of the sub-saharian zone, from Senegal to East Africa, and beyond to India and Pakistan [102]. It also found in Arabian Peninsula, such as Oman [103].

Sudan is considered the largest producer, followed by Chad and Nigeria. Together they produce nearly 95% of all GA exported to the worldwide market [101], [102]. It is shipped as a primary product to industrial countries, mainly Western Europe and the US [104].

Acacia seyal gum is a natural, nontoxic hydrocolloid complex [102], with low stickiness and viscosity, utterly soluble in want without any taste or odor (Figure 5C) [105]. Chemically, *Acacia seyal* gum (Asey) is found in nature as heteropolysaccharide and glucuronic acid residues [106]. It is composed of 37–44% D-galactose, L- 33–48% arabinose, 2–3% L-rhamnose, 7–13% D-glucuronic acid, and 6%4-O-methyl-D-glucuronic acid, with a small fraction of proteins 1%, and 4% of minerals consisting of a complex mixture of potassium, calcium and magnesium salts [106], [107]. The fractionation of *A. seyal* gum by hydrophobic interaction chromatography (HIC) and size exclusion chromatography (SEC) separated it into three fractions, which were specific as arabinogalactan (AG), arabinogalactan-protein (AGP), and glycoprotein (GP) [106], [108].

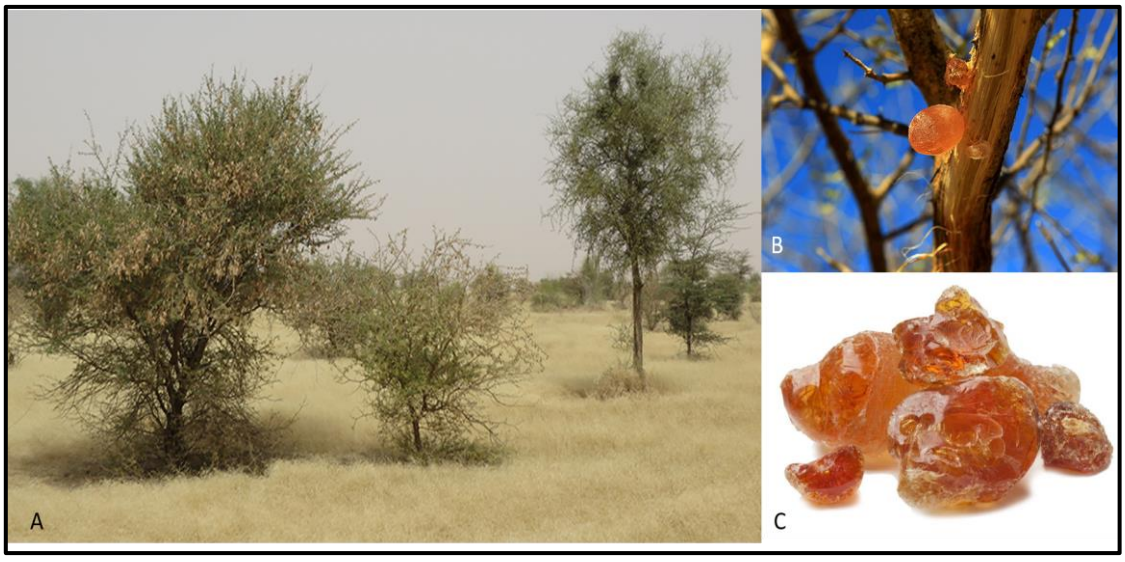

Figure 5: *Acacia* Gum Arabic. (A) *Acacia* trees in a savannah of Africa [109]. (B) Gum Arabic hardened sap [110]. (C) Gum Arabic exudates [111].

The variance in gum's physical, biochemical composition, and molecular characteristics depend on a number of factors such as *Acacia* species trees age, location, weather conditions, and the way of tapping. Moreover, the post-harvesting proceeding includes storage situation, maturation period, filtration, high-pressure homogenization, spray drying, irradiation, or heat treating affect the gums characteristics [112]. Due to these exceptional physical and chemical functional properties, the gum was employed in a variety of applications such as food, pharmaceutical, lithography, textile, cosmetics, and other industrial applications [102], [107], [105]. It is an excellent emulsifier, film former, thickener, flocculant, and surface-coating agent, food stabilizer with E-code E414. It is also used to produce drinks, candy, flavor encapsulations, bakery products, and brewing [102].

In the medical field, GA was demonstrated to effectively treat diarrhea, dysentery, and wounds [113]. Besides, recent studies highlighted the antioxidant, cytoprotective, nephroprotective, hepatoprotective, antiulcer, immunomodulatory, antibacterial, antimalarial [113-115], and has a role in the metabolism of lipids reducing body mass index (BMI) [116], [117]. It shows a positive effect in the treatment of several degenerative diseases such as chronic kidney disease [104], [116], type 2 Diabetes mellitus (DM) [118], [119], cardiovascular and gastrointestinal disorder [120]. Moreover, GA shows good use as a nanoparticle carrier for drug delivery [99], [120]. However, very few studies focused on the anti-cancer activity of GA and its mechanism, and only one study was done on breast cancer cells with the use of stem barks of *Acacia seyal* GA [121], and no study, to the best of our knowledge, test the anti-cancer effect of the dried gummy exudate.

1.7.5.4 Safety of Gum Arabic

FDA recognized Gum Arabica as one of the safest nutritional fibers [122], and they confirmed that it is safe as a food supplement. In addition, GA was approved as the safest feed additives from the FAO/WHO. Codex Alimentarius Committee (INS414) and the European Union (E414) approved the use of Gum Arabic in food [123].

1.8 Hypothesis

Gum Arabic from *Acacia sp.* exerts anti-cancer activity against triple-negative breast cancer cells MDA-MB-231 through modulating the cell cycle and inducing the apoptosis pathway.

1.9 Objectives of the study

Overall objective of this study is to investigate the potential molecular mechanisms underlying the anti-cancer activity of Gum Arabic from *Acacia sp.* in triple-negative breast cancer.

A. To evaluate the anti-cancer effect of Gum Arabic extract against selected human cancer cell lines

The study will test the anti-cancer effect of Gum Arabic extract on selected human cancer cell lines (breast cancer cells MDA-MB-231, MCF-7, Hs578T). The effect of Gum Arabic extract will be tested on cell viability using different cell-based assays. Increasing concentrations of Gum Arabic extract will be tested on each cell line for different period of time points.

B. To investigate the possible mechanism(s) of action of growth-inhibitory effect induced by Gum Arabic extract

In this objective, the aim is to study the mechanism(s) of actions by which the *Acacia sp*. gum extract (commonly known as Gum Arabic) exerts its growth inhibitory effect in triple-negative breast cancer cells. To approach this, apoptosis, colony formation assay, and cell cycle will be evaluated *in vitro* upon exposure to increasing concentrations of Gum Arabic extract at different time points.

C. To identify the biochemical events and downstream biomarkers associated with the cell's response to the anti-cancer effect of Gum Arabic extract

Because most anticancer agents are known to alter gene expression, the study will examine the effects of the *Acacia sp.* Gum extract on the expression of pathways (survival, proliferation) - specific genes in triple-negative breast cancer cell line using Western blot.

Chapter 2: Materials and Methods

2.1 Preparation of Gum Arabic extract

5.154 g of GA was finely ground and kept in 200 mL of bench ethanol for three days. The mixture was filtered, and the filtrate was evaporated under a vacuum to yield 1.580 g of fluffy beige solid. Gum Arabic stock solution (mg/mL) was prepared by dissolving GA in DMSO. Aliquots were stored at - 20°C until later use.

2.2 Cells culture reagents, and antibodies

Human breast cancer cells MDA-MB-231(basal-like) and MCF-7 (luminal A) were maintained in Dulbecco Minimal Essential Medium (DMEM) (Hyclone, Cramlington, UK). The medium was supplemented with 10% fetal bovine serum (FBS) (Hyclone, Cramlington, UK) and antibiotics (100 U/ml penicillin/streptomycin) (Hyclone, Cramlington, UK). Cells were maintained at 37°C under a humidified atmosphere containing 5% CO_2. Cells were monitored daily under EVOS XL Core Cell Imaging System (Life Technologies), and media was changed when necessary. Cells were passaged using phosphate buffer saline solution (PBS) 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 assay type.

For Western blot analysis, antibody to cyclin B1 (05-373), Cyclin D1 (04-1151) cyclin A1 (2477862), p21 (05-655), and c-myc (AB3252) were obtained from Millipore (Millipore, Hayward, CA, USA). Antibodies to β-catenin (sc-7963), 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 cleaved PARP (Ab4830) were obtained from Abcam (Abcam, Cambridge, UK). Antibodies to survivin (2803), Anti-rabbit (7074p2) were obtained from Cell Signaling.

2.3 Measurement of cellular viability

To measure cell viability *in vitro*, all cells were seeded in triplicate in 96-well plates at a density of 5 x 10^3cells per well for MDA-MB-231 and Hs578t cells and 7 x 10^3 cells per well for MCF-7 in 100 μL of culture medium. Twenty-four hours after the seeding, cells were treated with increasing AGE concentrations (20, 30, 50, 70,100 μg/mL) or an equivalent volume of vehicle (DMSO) for control cells and incubated for another 24, 48, and 72 hrs. Cell morphological changes were observed at the indicated time points and imaged by EVOS XL Core Cell Imaging System (Life Technologies). After treatment, cell viability was determined using a CellTiter-Glo Luminescent cell viability assay (Promega Corporation, Madison, USA). In this assay, CellTiter-Glo measures the amount of ATP signaling, which indicates the metabolically active cell. Briefly, at each time point, the media was removed by aspiration from each well; a 1:1 ratio of new media and CellTiter-Glo reagent were added to each well. The plate was then incubated on gentle shaking at room temperature for 2 minutes. After that, the plate was incubated for additional 10 minutes at room temperature. Luminescence was measured using the GloMax® Explorer Multimode Detection System. 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 = (luminescence value for treatment / luminescence value for control) * 100. The results were plotted against the range of GAE concentrations in Excel.

2.4 Measurement of caspase 3/7 activity

Caspase 3/7 activity was measured using a luminescent caspase-Glo 3/7 assay kit (Promega Corporation, Madison, USA) following the manufacturer's instructions. Briefly, MDA-MB-231 cells (5 x 10^3 in each 96 well plate) were treated with indicated concentrations of GAE (50, 70, 100 μg/mL) or an equal volume of vehicle (DMSO) as a control for 24 and 48. To measure caspase 3/7 activity, we added a 1:1 ratio volume of Caspase-Glo® 3/7 reagents to each well of a 96-well plate. Then covered and mixed in an orbital shaker for 10 minutes at room temperature. Luminescent signal was measured as described above, and the relative caspase 3/7 activity was normalized to the number of viable cells per well. Caspase 3/7 activity was calculated and expressed as fold of induction compared to the control.

2.5 Colony formation assay

MDA-MB-231 cells were seeded in 6-well plates at a density of 200 cells/well. Cells were allowed to grow and form colonies for ten days. On day 10, cells were refreshed with frsh media containing the indicated concentrations of AGE or an equal volume of vehicle (DMSO) then allowed to grow for additional 7 days. At the end of the experiment, plates were washed two times with PBS, cells were fixed for 15 min with 4% formalin, and then stained with 0.01% crystal violet for 30 min.

In another setting, MDA-MB-231 cells were seeded in 6-well plates at a density of 2,000 cells/well. After 24 hrs incubation, cells were treated with indicated concentrations of GAE (30, 50, 70, 100 μg/ml) or an equal vehicle volume (DMSO).

Seven days later, plates were washed twice with PBS, fixed for 15 min with 4% formalin, and stained with 0.01% crystal violet for 30 min.

2.6 Cell cycle analysis

The cell cycle distribution analysis in control and GAE-treated MDA-MB-231 cells was performed with the Muse™ Cell Analyzer (Millipore, Hayward, CA, USA) using the Muse™ Cell Cycle Kit (Millipore, Hayward, CA, USA) according to the manufacturer's instructions. Briefly, cells ($3x10^5$) grown onto 6-well plates were treated with GAE indicated concentrations of GAE for 48 hrs. Cells were then collected by trypsinization washed in 1X PBS and fixed in ice-cold 70% ethanol (1:1) in - 20°C, at least 3 hrs before staining. To prepare cells for staining, cells were washed off from the ethanol by several wash/centrifugation steps. After that, the Muse cell cycle test reagent was added to each test tube conating the cell pellet. Cells were incubated for 30 min at room temperature in the dark. After staining, the cells were processed for cell cycle analysis. Percentage of cells in G0/G1, S and G2/M phases were determined using the FlowJo software.

2.7 Cell lysate preparation

MDA-MB-231 Cells (1.8×10^6) were treated with GAE (50, 70, 100 μg/ml) or vehicle (DMSO) for 48 hrs. Then, 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). After incubation for 30 min on ice, cell lysates were centrifuged at 14,000 rpm at 4°C for 20 min. The supernatants were quantified for protein

concentration using the BCA protein assay kit (Thermo Scientific), and the lysates were adjusted with lysis buffer. The supernatants were aliquoted and stored at - 80°C.

2.8 Western blotting analysis

Equal amounts of cell lysates (15 μg) were electrophoresed through 6-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels along with PageRuler Plus Prestained Protein Ladder (Thermo Scientific). Proteins were transferred onto polyvinylidene fluoride PVDF membranes (Millipore) and 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. Incubation with specific diluted primary antibodies was performed in blocking buffer overnight at 4°C. Horseradish peroxidase-conjugated anti-IgG was used as a secondary antibody. Immunoreactive bands were detected by enhanced chemiluminescence ECL/ SuperSignal West Femto chemiluminescent substrate (Thermo Scientific). When needed, membranes were stripped in Restore western blot stripping buffer (Thermo Scientific) according to the manufacturer's instructions.

2.9 Statistical analysis

The data are expressed as the mean ± standard error of the mean ± standard error of the mean (SEM), and are derived from at least three independent experiments, unless specified otherwise. Student's *t*-test was used for comparison of two 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 GAE decreases cell viability of estrogen positive and negative breast cancer cells

The study started by investigated the anti-breast cancer potential of GAE on different breast cancer cell lines (MDA-MB-231, Hs578T and MCF-7) using the CellTiter-Glo® assay. Cells were seeded in triplicates into 96-wells plates and treated with increasing GAE concentrations for 24, 48 and 72 hrs, and then cell viability was measured. As shown in Figure 6, GAE treatment led to significant decrease in cellular proliferation in a concentration and time-dependent manner in all three breast cancer cell lines in relative to vehicle control (DMSO). The response of the three cell lines to the GAE treatment showed clear sensitivity to AGE. Notably, the inhibitory effect of GAE in MDA-MB-231 and Hs567T cells was comparable to or greater than that in MCF-7, indicating that GAE may be an effective cytotoxic agent against TNBC. More cell viability reduction was detected in MDA-MB-231 and Hs578T cells at higher concentrations (70 and 100 μg/mL), which implies a higher sensitivity of both cell lines to GAE when used at high concentrations. The estimated IC_{50} (the concentration that leads to 50% inhibition) value for MDA-MB-231 cells and Hs578T were approximately 75 μg/mL at 48 hrs with less than 20% cell viability observed at higher concentration used (100 μg/mL) (Table 3). In contrast, the estimated IC_{50} of MCF-7 cells was ~100 μg/mL at 48 hrs. Since MCF-7 and MDA-MB-231 exhibited comparable sensitivity, the study also conclude that estrogen receptor expression does not affect the GAE-induced growth inhibition in human breast cancer cells. Collectively, these data indicate that GAE exerts an anti-breast cancer effect.

Table 3: Molecular characteristics of the breast cancer cell lines and the estimated IC_{50} values of GAE after 48 hrs

Cell lines	p53 status	ER-α	Estimated IC50 (µg/mL)
MDA-MB-231	mutant	negative	~75
MCF-7	wild-type	positive	~100
Hs578T	mutant	negative	~75

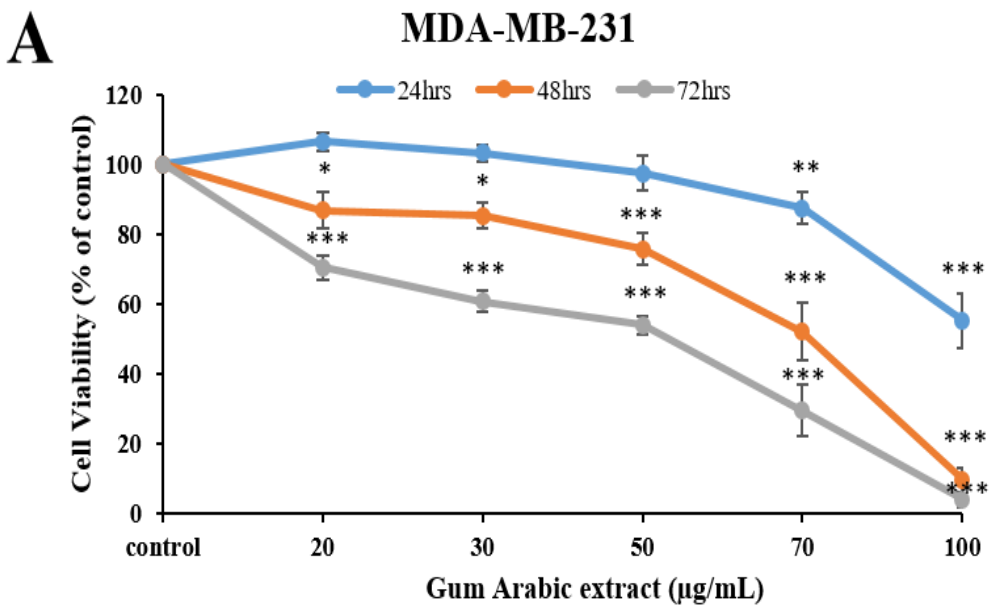

Figure 6: Growth inhibitory effect of GAE. (A) MDA-MB-231, (B) MCF-7, and (C) Hs578T cell growth determined by cell viability assay. Exponentially growing cells of the three breast cancer cell lines were cultured in 96 well plated and treated with and without the indicated concentrations of GAE for 24, 48 and 72 hrs. The statistically significant difference was set at *p* values of * 0.05, ** < 0.005, *** < 0.001 between control and treated groups.

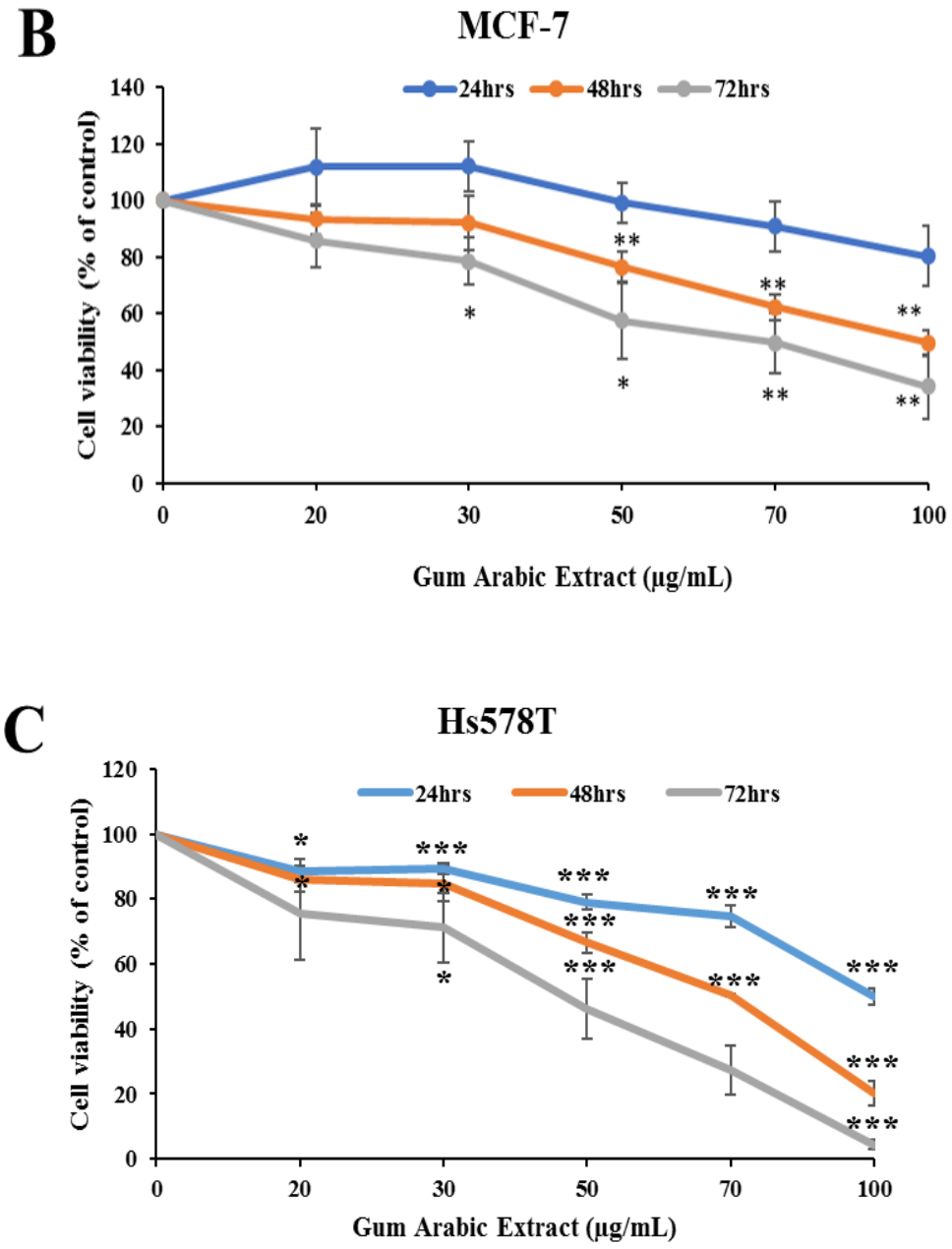

Figure 6: Growth inhibitory effect of GAE. (A) MDA-MB-231, (B) MCF-7, and (C) Hs578T cell growth determined by cell viability assay. Exponentially growing cells of the three breast cancer cell lines were cultured in 96 well plated and treated with and without the indicated concentrations of GAE for 24, 48 and 72 hrs. The statistically significant difference was set at *p* values of * 0.05, ** < 0.005, *** < 0.001 between control and treated groups. (continued)

The study further investigated the mechanisms by which GAE exerts its anti-cancer activity on the highly proliferative and invasive Estrogen Receptor (ER)-negative, mutant p53 breast cancer cell line MDA-MB-231.

3.2 GAE induces morphological changes in breast cancer cells MDA-MB-231

The examined treatment revealed any morphological changes during the exposure times of the MDA-MB-231 to GAE. For that, the morphology of the treated cells was monitored overtimes. As shown in Figure 7, untreated MDA-MB-231 cells (control) remained healthy and maintained their original morphology, appeared elongated-spindle shape, adherent, and maintain cell-to cell contact throughout the treatment periods, indicating a regular rate of proliferation (Figure 7). In contrast, treated MDA-MB-231 cells underwent morphological alterations in concentration and time-dependent manner. Cells lost their original shape starting from 24 hrs at high concentrations (70, 100 μg/mL) of GAE treatment. At low concentrations treatment after 48 hrs, cells lost their elongated-spindle morphology with long and fine membrane extensions, cells looked enlarged and flattened compared to untreated cells and starts to loose cell-to cell contact started from 50 μg/mL. At higher concentrations (50, 70,100 μg/mL) showed an apparent reduction of cell size (cell shrinkage) and number, and membrane blabbing, with floating cells (dead), which were more observed when the incubation time extended to 72 hrs. All these observed morphological changes were typical signs associated with cell death; possibly through apoptosis.

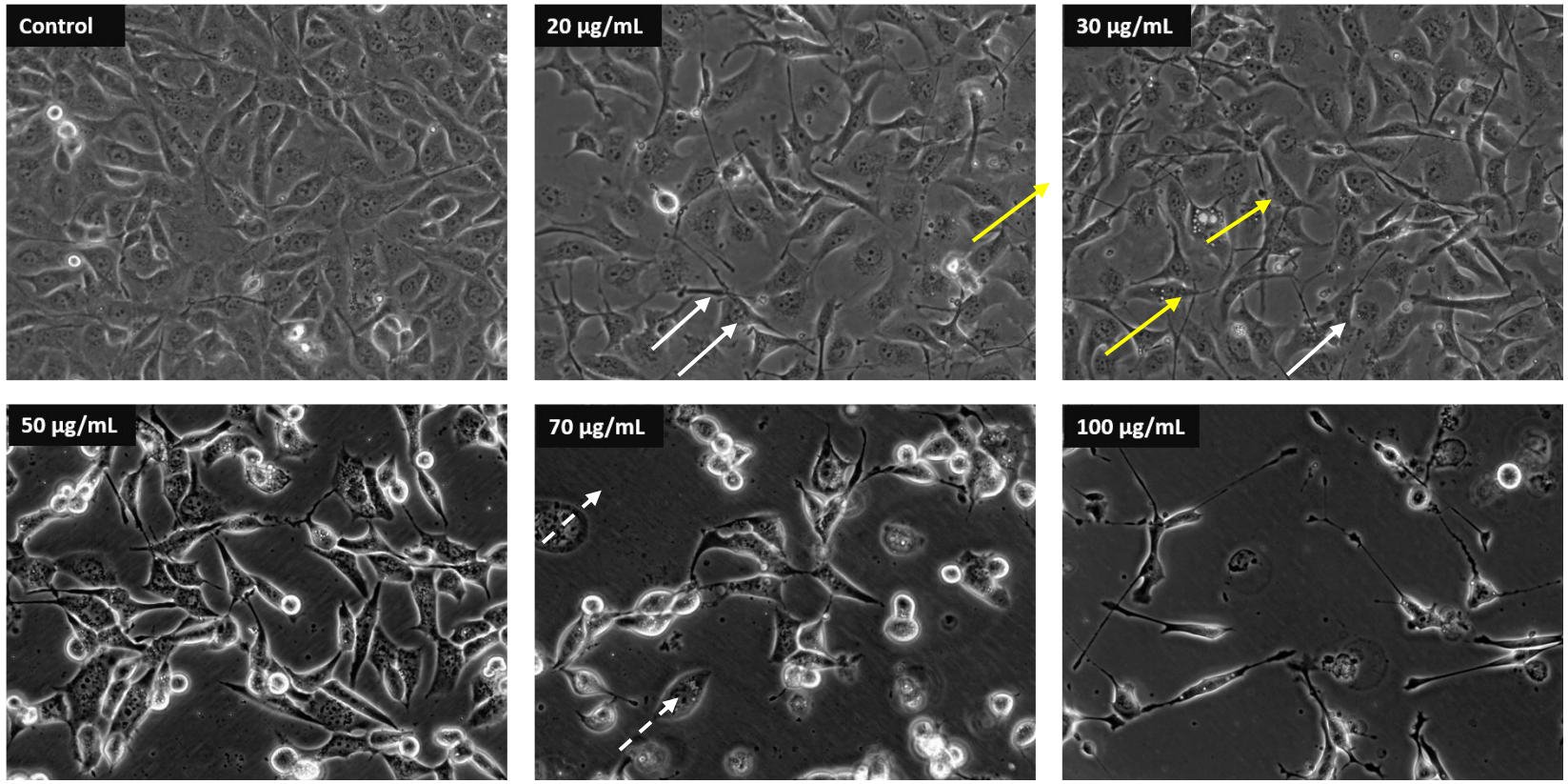

Figure 7: Representative micrographs of the cellular morphology of MDA-MB-231 treated with increasing concentrations of GAE after 48 hrs. Cells were viewed using EVOS XL Core Cell Imaging System (Life Technologies). Magnification: 40X. The white dashed-arrows show dying cells, the yellow arrows show elongated-spindle shape cells, and the white arrows show flattened enlarged cells.

3.3 GAE reduces and inhibits MDA-MB-231 colony formation

To further confirm the anti-cancer effect of GAE on human breast cancer cells MDA-MB-231 cells, long-term inhibitory effects of GAE, a 14-days colony formation assay was carried out. Results showed that GAE could reduce the clonogenic ability of MDA-MB-231, from concentration as low as 30 µg/ml. In addition, no colonies were formed at concentrations higher than 50 µg/ml (Figure 8A).

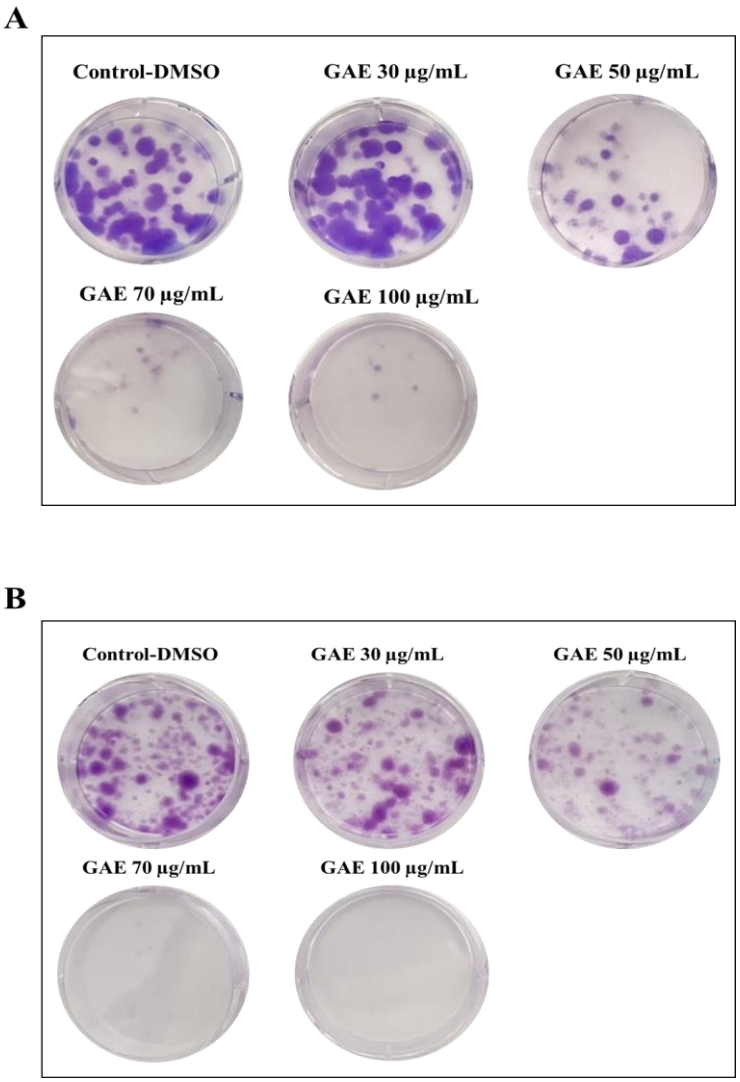

Figure 8: Colony formation assay. The figures show a clear reduction in colony formation in MDA-MB-231 cell treated with different concentration of GAE (A) a 14-days colony formation assay on MDA-MB-231 treated on day 10 with the indicated concentrations of GAE (B) Effect of increasing concentrations of GAE on MDA-MB-231 cells ability to form colonies.

To test the direct effect of GAE on colongenic ability of MDA-MB-231 cells, cells were treated with increasing concentrations of GAE. Results revealed that GAE treatment GAE reduces colony formation of MDA-Mb-231 cells in a concentration- and –time-dependent manner (Figure 8B). At concentration > 50 µg/mL, a clear decline trend was observed in the number of colonies compared with the untreated groups (control), with greater effect seen at 70 and 100 µg/mL. Taken together, these results confirm the growth inhibitory effect of GAE on MDA-MB-231 cells.

3.4 GAE induces G1/S arrest in breast cancer cells

To investigate the mechanism(s) underlying the GAE growth inhibitory activity on breast cancer cells, the effect of GAE on cell cycle progression was examined. To this end, MDA-MB-231 cells were treated with the indicated concentrations of GAE for 48 hrs and then followed by performing cell cycle analysis. The results showed that treating the cells with AGE caused significant inhibition of cell cycle progression in MDA-MB-231 cells at 48 hrs leading to an increase in the G1 population. The G1 cells population rose from 44% ± 1.9 in control cells to 50% ± 1.7, 50% ± 2.2 and 48% ± 0.95 in cells treated for 48 hrs with 20, 30 and 50 µg/mL GAE (Figure 9 A, B), respectively.

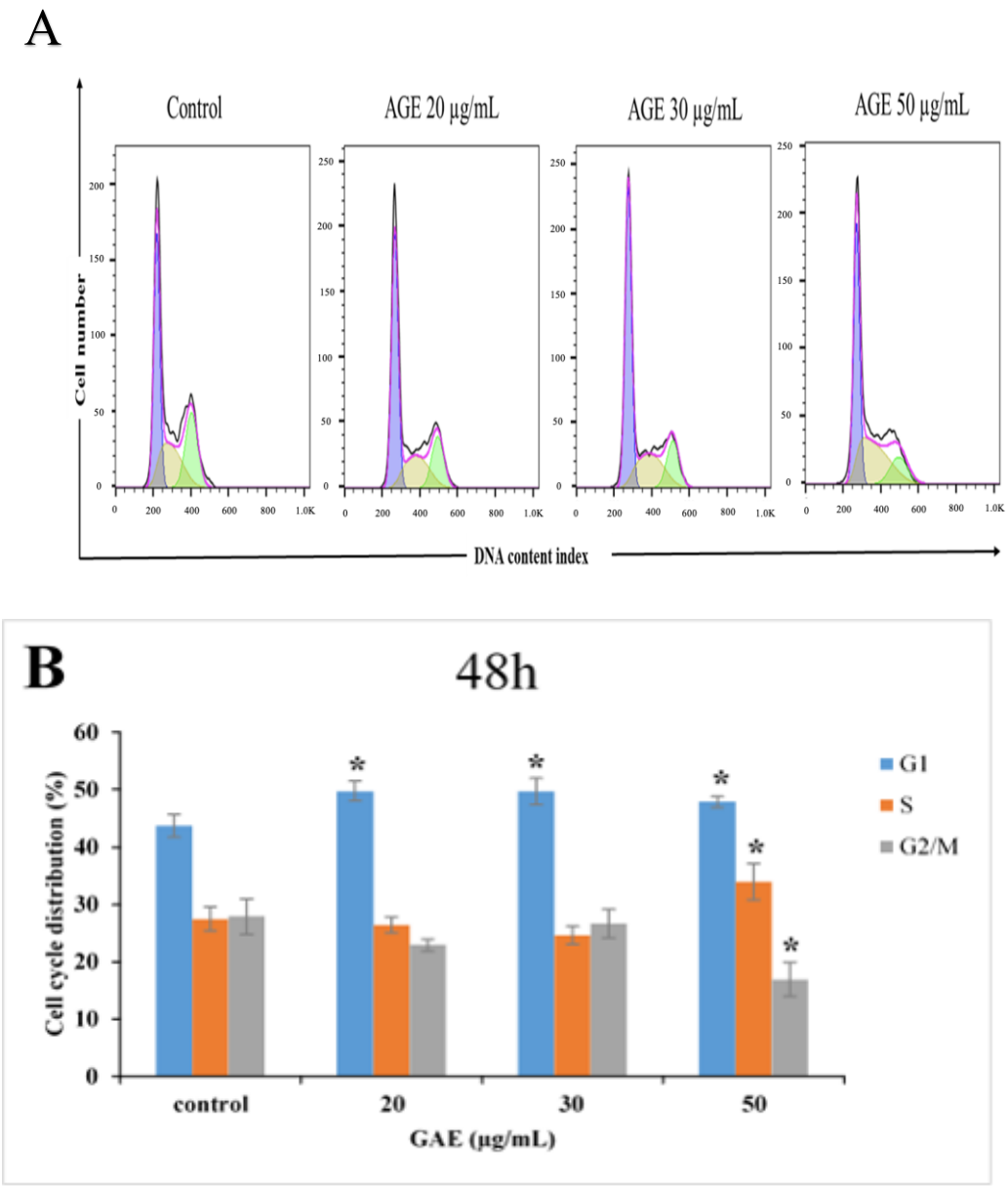

Figure 9: GAE treatment induces G1/S arrest in MDA-MB-231 cells. MDA-MB 231 cells were treated with GAE at the indicated concentrations for 48 hrs, and then analyzed with Muse TM Cell Analyzer as described in Materials and Methods. (A) Cell cycle distribution analysis of GAE-induced G1/S cell-cycle arrest. (B) Percentages of cell populations in each phase of cell cycle. The statistically significant difference was set at *p* values of *< 0.05 between control and treated groups.

3.5 GAE alters the expression of cell cycle regulatory proteins

Several cell cycle regulatory proteins known to regulate cellular proliferation. Next, the study investigated, by western blotting, the effect of GAE on selected proteins know to regulate cellular proliferation. Cyclin D1 is known to be essential for cell cycle progression [124]. Cyclin D1 overexpression occurs in many types of human cancer. Cyclin D1 was reported to shorten the G1 phase of cell cycle, and therefore, inhibition of this protein results in blocking G1-S transition [125]. As shown in Figure 10, cyclin D1 protein level decreased upon the treatment with GAE in a dose-dependent manner after 48 hrs. Further, other proteins involved in cell cycle regulation were examined, cyclin A1 and cyclin B1. Cyclin A1 is essential in S and G2/M phases. Dysregulation in cyclin A1is implicated in carcinogenesis, progression, and metastasis of many solid tumors types [126]. Cyclin B1 plays a role in cell transition from the G2 phase to mitosis (M phase), and it is usually overexpressed in primary breast cancer cells and cancer cell lines [127]. As shown in Figure 10, both cyclins A1 and B1 proteins were down-regulated after 48 hrs in MDA-MB-231 cells in response to GAE treatment in a dose-dependent manner. Notably, all cyclins D1, A1 and cyclin B1 protein levels were almost unmeasurable with high concentrations of GAE (70 and 100 μg/mL). These results confirm that GAE induces cell cycle arrest, suggesting that it could be one of the mechanisms by which GAE exerts its anti- cancer activity against MDA-MB-231 cells.

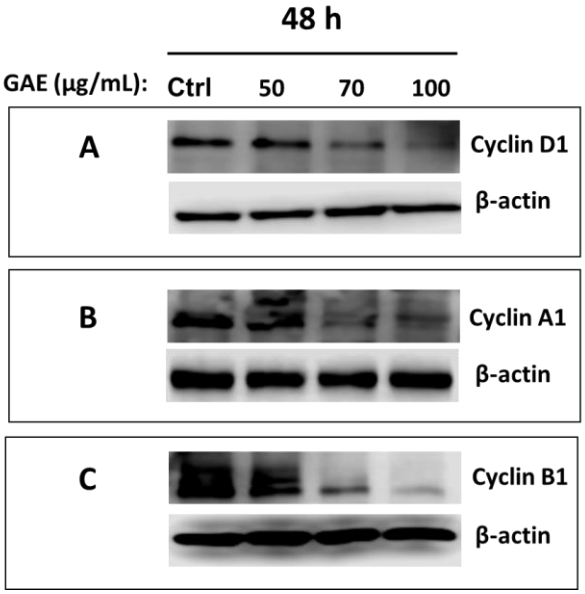

Figure 10: GAE alters the expression of several proteins involved in cell cycle progression. MDA-MB-231 cells were treated with vehicle or increasing concentrations of GAE (50, 70, and 100 µg/mL) for 48 hrs, then the proteins levels of cyclins (A) D1, (B) A1, and (C) B1 were examined by Western blotting. β-actin was used as loading control.

3.6 GAE inhibits cell proliferation, downregulates c-myc and up-regulates $p21^{WAF1}$ in breast cancer cells MDA-MB-231

c-myc overexpression stimulates cell progression, whereas the down-regulation or inactivation of this protein decreases cell proliferation or lead to growth arrest [128]. c-myc protein was reported to controls G1-S transition through activating downstream targets and inhibition of cyclin-dependent kinase (Cdk) inhibitor $p21^{WAF1}$ [129]. $p21^{WAF1}$ CDK inhibitor acts as tumor suppressor protein by inhibiting cell cycle progression during G1 and S phases transition [130]. The expression of $p21^{WAF1}$ was checked the to determine whether the growth inhibition was mediated through induction of $p21^{WAF1}$. The results shown in Figure 11 demonstrate a significant increase of $p21^{WAF1}$ protein at concentrations of 50 and 70 µg/mL. However, the $p21^{WAF1}$ protein level was abolished in cells treated with 100 µg/mL of GAE. Therefore, GAE inhibits breast cancer cells by inhibiting c-myc protein expression and

inducting of $p21^{WAF1}$ protein expression independent of p53 activity as tumor suppressor protein in these cells.

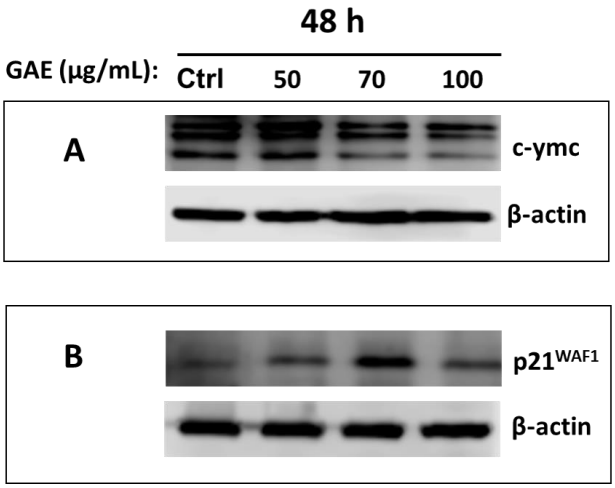

Figure 11: GAE alters the expression of regulatory proteins involve in cell proliferation. MDA-MB-231 cells were treated with vehicle or increasing concentrations of GAE (50, 70, and 100 µg/mL) for 48 hrs, then the proteins levels of c-myc (A) and $p21^{WAF1}$ (B) were examined by Western blotting. β-actin was used as loading control.

3.7 GAE triggers survivin down-regulation in breast cancer cells MDA-MB-231

Survivin a cancer anti-apoptotic marker belongs to the family of inhibitor of apoptosis proteins (IAP). Survivin plays a crucial role in regulating cell division, inhibiting apoptosis, and promote invasion and metastasis [131]. Survivin overexpression was reported in most human cancers, including breast cancer [132], which is implicated in cell cycle arrest at G2/M and apoptosis inhibition. In contrast, decreases in survivin expression results in apoptosis. It was reported that survivin suppresses apoptosis by preventing the activation of caspase-3 and caspase-7 or interacting with cyclin-dependent kinase (CDK)-4 and CDK-2 to avoid the activation of apoptotic signaling pathways [131]. Therefore, the possible role of survivin in GAE-induced cell cycle arrest and apoptosis was examined. Toward this, the expression of

survivin in response to increasing concentrations of GAE after 48 hrs of treatment was analyzed, by Western blotting. Interestingly, survivin showed a differential effect by GAE on MDA-MB-231 cells (Figure 12). A low concentration of GAE led to an increase in the level of survivin, which might conferre resistance to cell death to this population of cells. However, higher concentrations of GAE result in a vast decrease in surviving protein expression that might suggest its role in inducing cell death. Therefore, GAE targets one of the important breast cancer oncoproteins survivin in basal-like breast cancer cells.

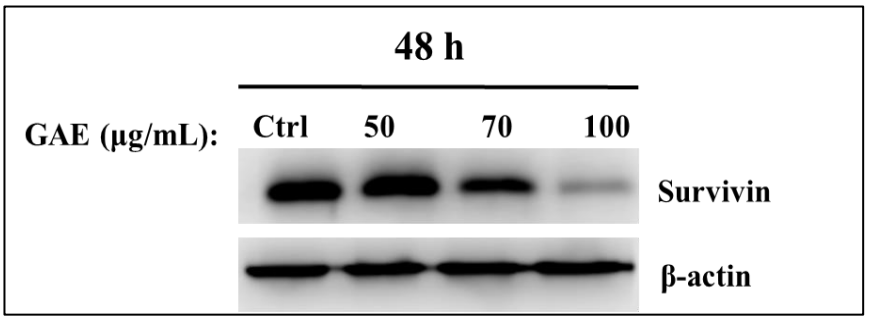

Figure 12: Differential regulation of survivin expression by GAE in MDA-MB-231 cells. Western blot analysis shows a differential effect on survivin expression by different concentrations of GAE in MDA-MB-231 cells. Whole-cell proteins were extracted from GAE or vehicle (DMSO)-treated cells and subjected to Western blot analysis. β-actin was used as loading control.

3.8 GAE down-regulates β-catenin protein level in breast cancer cells MDA-MB-231

Wnt/ β-catenin pathway has a well-established role in tumorigenesis. Abnormal expression of Wnt/β-catenin signaling was proven to contribute to the initiation, progression, and metastasis in various human cancers, including breast cancer [133], [134]. Indeed, targeting Wnt/ β-catenin signaling pathway is of great significance in the treatment of cancer. Toward this, Western blotting was used to measure the protein level of β-catenin in response to increasing GAE concentrations

after 48 hrs. The results showed that GAE caused a massive decrease in β-catenin protein expression in human breast cancer MDA-MB-231 cells compared to DMSO-treated (control) cells (Figure 13). Notably, the expression of β-catenin totally vanished at 100 µg/mL. These data clearly indicate that GAE treatment resulted in the inhibition of β-catenin signaling in human breast cancer cells. To confirm the GAE-down regulation effect on β -catenin signaling, the study sought whether GAE treatment can affect β-catenin target genes. β-catenin is responsible for regulating downstream genes such as c-myc, cyclin D1 and survivin [133]. The results showed down-regulation of c-myc, cyclin D1, and survivin protein expression. These results indicate that GAE treatment downregulates β-catenin and its target genes in human breast cancer cells.

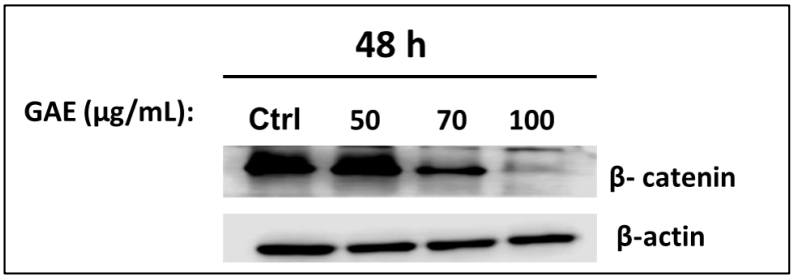

Figure 13: GAE inhibits the β-catenin signaling pathway**.** Concentration-dependent decrease of β- Catenin protein in GAE-treated MDA-MB-231 cells. Cells were treated with vehicle (DMSO) or indicated concentrations of GAE for 48 hrs, then whole-cell proteins were extracted from GAE or vehicle (DMSO)-treated cells and subjected to Western blot analysis for β- catenin. β-actin was used as loading control.

3.9 GAE activates caspase 3/7 and minimal PARP cleavage in breast cancer MDA-MB 231 cells

Caspase-3/7 is essential in apoptotic cell death pathway. Therefore, in this study, caspase 3/7 activity was evaluated in MDA-MB-231 cells incubated with 50, 70, and 100 µg/mL of GAE for 24 and 48 hrs to determine whether apoptosis was associated with caspase 3/7 activation. The results show that after 24 hrs, caspase 3/7

was activated significantly at 100 µg/mL of GAE. Treatment after 48 hrs increases caspase 3/7 activity considerably at 50, 70, and 100 µg/mL compared to the control by 1.3, 1.5, and 3.6 folds, respectively (Figure 14). Moreover, Western blot analysis showed a cleaved caspase 3 indicating the activation of caspases upon GAE treatment during the 48 hrs (Figure 15A). Further, PARP expression was checked the by Western blot, a well-known apoptosis marker, to confirm apoptosis induction in treated MDA-MB-231 cells (Figure 15B). The results revealed a slight induction of PARP cleavage at 100 µg/mL of GAE. Altogether, these findings suggest that GAE activates apoptosis through a caspase-dependent pathway.

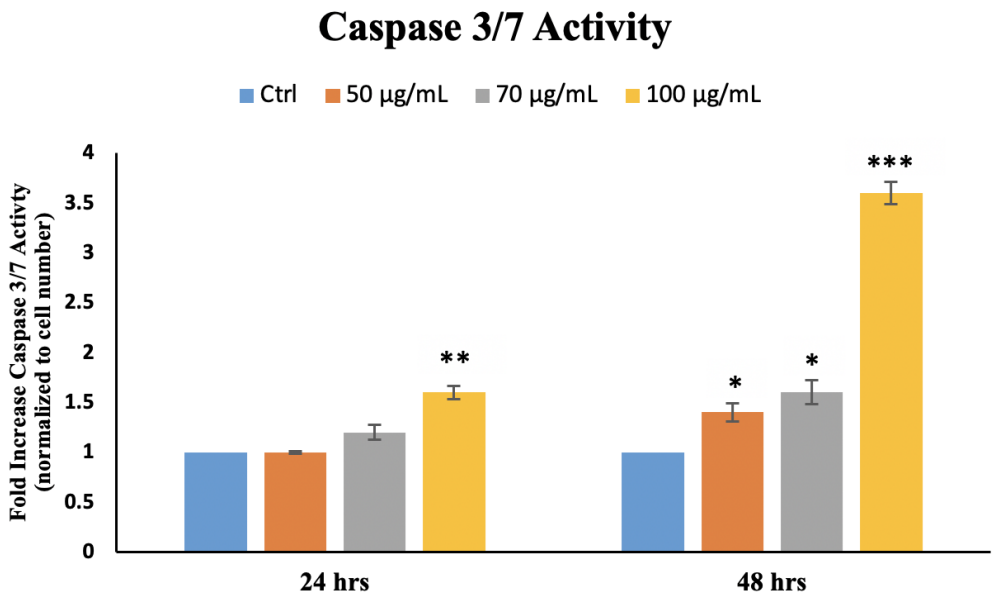

Figure 14: GAE activated caspase 3/7 in MDA-MB-231 cells. Caspase 3/7 activity in MDA-MB-231 cells after exposure to GAE (50 to 70 µg/mL) for 24 and 48 hrs, relative to a similar amount of viable DMSO-treated cells. The relative caspase 3/7 activity was normalized to the number of viable cells per well and is express as fold of induction compared to the control. The statistically significant difference was set at *p* values of * 0.05, ** < 0.005, *** < 0.001 between control and treated groups.

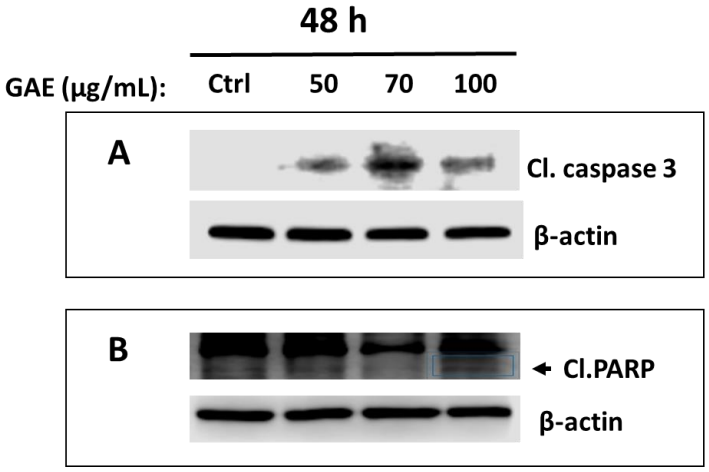

Figure 15: Western blot analysis of cleaved caspase 3 and PARP after 48 hrs of GAE treatment in MDA-MB-231 cells. (A) cleaved caspase 3, (B) PARP, MDA-MB-231 cells were treated for 48 hrs with different concentrations of the GAE and proteins were extracted from GAE or vehicle (DMSO)-treated cells and subjected to Western blot analysis using cleaved caspase 3 (A) and PARP (B) antibodies. β-actin was used as loading control.

Chapter 4: Discussion

Currently, the treatment of cancer mainly includes radiotherapy, surgery and chemotherapy. However, the curative effects of the existing chemotherapeutic drugs in treating such multifactorial disease with agents targeting a single target might provide partial treatment. Unfortunately, cure rates are disappointing in many cases as patients usually develop drug resistance along with the numerous side effects of treatment. TNBC is the most aggressive type of breast tumor with a high metastatic rate, early recurrences, and poor survival prognosis and contributes to a large proportion of breast cancer deaths despite its small proportion among all breast cancers [62], [66], [67]. TNBC lack any standard targeted systemic therapy and is usually treated with chemotherapy or radiotherapy, which causes severe side effects, and showed limited long-term success [64], [135]. Therefore, searching for highly efficient anti-cancer drugs remains one of the biggest challenges in medicine and deserves a high priority of research to combat the enormous human suffering and related health care. Among the potential alternatives that have emerged for treating cancer are anti-cancer agents from natural sources such as plants and dietary phytochemicals, marine organisms and microorganisms. Through history, nature has been an attractive and rich source of new therapeutic compounds that have found many applications in cancer therapy as a tremendous scaffold diversity is found in millions of species of plants, animals, marine organisms and microorganisms and are becoming an important area for drug discovery [136].

Increasing knowledge and understanding on the hallmark characteristics of cancer has led to the use of natural products such as phytochemicals and plants in cancer therapy, which modulate several molecular targets and exert anticancer

activities. Natural products have shown to exert therapeutic potential via modulation of core hallmark capabilities of cancer. The activity comes from their ability to suppress proliferative signaling, induce cell death, up-regulate growth suppressors, deactivate invasion and metastasis, interact replicative immortality and attenuate angiogenesis [136], [137]. Recent studies have significantly improved our understanding of anti-cancer activities and underlying molecular mechanisms of many anti-cancer agents derived from natural sources [138]. Targeted therapies against signaling pathways involved in cell proliferation, metastasis, apoptosis, and cell cycle regulation may provide a new insight into cancer therapeutics. If the study can precisely activate or inhibit molecules that mediate the diversity of cell death outcomes, perhaps it can succeed in more effective and less toxic chemotherapeutic regimens.

Natural exudates, such as gums and resins produced from plants, have been traditionally used for religious, cosmetics as well as medical purposes since ages. Different types of gums and resins from plants have been used in traditional medicine to treat variety of conditions including inflammatory diseases like arthritis, asthma, chronic pain, bowel conditions and many other diseases. Although the use of plants exudates and resins in traditional medicine has been commonly observed, few pharmacological and chemical studies have been conducted on plant exudates and resins concerning their scientific merits especially in the field of cancer therapy. Gum Arabic is an edible, dried, gummy biopolymer exudates obtained from trees and branches of *Acacia senegal* and *Acacia seyal*. In previous studies, Gum Arabic has been found to possess several biological activities [139-141]. However, the anti-cancer activity of GA has not been evaluated yet especially on TNBC.

This study initial results, showed for the first time, that GAE exhibits significant growth inhibitory effects on MDA-MB-231 and Hs578T (triple negative) and MCF-7 (ER positive) breast cancer cell lines. As the growth inhibitory effect of GAE in MDA-MB-231 was greater than that in MCF-7, the study focused on the effect of GAE in TNBC MDA-MB-231 cell line. The two cell lines have different molecular and histological characteristics. While, MDA-MB-231 cell line originated from adenocarcinoma, estrogen, progesterone, and HER-2 receptors with a mutant p53 gene (gain of function mutant p53), MCF-7 cells originated from adenocarcinoma, estrogen receptor positive, and progesterone receptor negative harboring wild type p53 gene.

In MDA-MB-231 cells, GAE induces cell cycle arrest in G1/S phase accompanied with alterations in several cell cycle regulatory proteins. A body of evidence supported G1/S transition: (i) cyclin D1 downregulation in which its inhibition was reported to play a role in G1/S transition. (ii) c-myc downregulation, that is reported to be correlated with cellular proliferation, and it controls the G1/S transition, and the (iii) upregulation of the CDK inhibitor $p21^{WAF1}$, whose upregulation can lead to apoptosis and disrupt CDKs/cyclin complexes necessary for cell cycle progression during G1 and S phases transition. Cyclin D1 was downregulated upon GAE treatment, which could be the leading cause for the G1 block as it was required for G1 to S transition. The downregulation of cyclin B1 and cyclin A1, both are involved in the G2/M phase transition, indicate that additional mechanisms might be involved with GAE exposure. Interestingly, Kaempferol and Curcumin were also reported to induce both G1/S arrest G2/M phase arrest in HT-29 cells and HOS cells, respectively [142], [143]. The cell cycle arrest data are in agreement with the growth inhibition data shown by cell viability.

In MDA-MB-231, GAE could induce apoptotic cell death via activation of apoptotic pathway. GAE significantly activates caspase 3/7 activity. Executioner caspases-3 and -7 is activated in both the intrinsic and extrinsic apoptosis pathway by caspase 9 or caspase 8. The activated caspase3/7 quickly start to break down proteins resulting in eliminating abnormal cells [79]. A minimal cleavage of PARP was detected by Western blot analysis. poly [ADP-ribose] polymerase (PARP) is involve in repairing damaged DNA. It gets activated in response to DNA damage, and its also a key marker in apoptosis activation [144]. The activation of PARP in important for cell survival, as cells is very sensitive to its inhibition. PARP overexpression is usually correlated with TNBC [145]. PARP cleavage lead to the inactivation of enzyme by destroying its ability to respond to DNA strand breaks [146]. Further investigation of which apoptosis pathways was triggered by GAE is ongoing in the lab. Moreover, possible roles of ROS and DNA damage in GAE-induced anti-proliferative and cell death are warranted.

Currently, majority of drugs used to treat cancer work at the molecular level to induce cell death and inhibit the cell cycle progression, two fundamental strategies for preventing malignant cell growth in the first place. Interestingly, numerous herbal extracts from edible plants and their compounds have shown anti-cancer activities by targeting these two events [59], [72].

Survivin is a member of the inhibitor of apoptosis (IAPs) proteins family that represent anti-apoptotic proteins. Survivin has a unique structure making it a critical factor in the modulation of cell division, cell cycle progression, and protection against apoptosis by blocking caspase activation [147]. It has been assumed that survivin also plays a role as a mitotic inducer [148]. In most breast cancer cases, survivin was overexpressed, and its expression was correlated with tumor progression,

chemotherapeutic resistance, and poor clinical outcomes [149]. Thus, targeting survivin protein can serve as a promising treatment strategy for breast cancer patients. The results demonstrated an initial increase in the survivin level at a lower concentration of GAE (50 µg/mL), which might be explained by the cells' attempt to survive. However, survivin was dramatically decreased at higher concentrations of GAE treatment (70 and 100 µg/mL). *Origanum majorana* plant extract and Salinomycin, a natural compound, were reported to induce survivin downregulation in breast cancer cell line MDA-MB-231 [39], [150]. Therefore, the study suggest that GAE exerts its anti-cancer effect at least partially through survivin's downregulation.

Wnt/ *β*-catenin pathway is highly regulated by β-catenin protein, which is responsible for regulating mammary development and tumorigenesis. Dysregulation of Wnt/ *β*-catenin was associated with breast cancer progression and metastasis [133], [134]. In the present study, it was revealed that GAE decreased the b-catenin expression in breast cancer cells MDA-MB-231. Notably, the inhibitory effect of GAE on *β*-catenin signaling occurred at concentrations comparable to those required for inhibiting cell viability and inducing apoptosis in these cells. These results indicate that the anti-breast cancer activity of GAE was associated, at least partially, with its inhibitory effects on Wnt/ *β*-catenin signaling pathway. Cyclin D1, c-myc and survivin are established targets of Wnt signaling and their elevated expression are characterstics of advanced breast cancer. The results support the idea that GAE may suppress c-myc**,** cyclin D1, and survivin expression by blocking Wnt/β-catenin signaling. Increased activity of the Wnt/ β- catenin signaling pathway is a common prporty of drug resistant cancers, thus it is conceivable to use GAE as adjuvant with the chemotherapy regimen that could improve the treatment of advanced breast cancer. A study has also reported

that Sulforaphane downregulated the Wnt/ β- catenin self-renewal pathway in breast cancer stem cells [151].

In summary, the data are consistent with a model shown in Figure 16. For the first time, it was demonstrated that GAE inhibited the viability of the highly proliferative and invasive human breast cancer cell line MDA-MB-231. The cell cycle arrest at G1/S induced by GAE was accompanied by downregulation of cyclin D1, c-myc, and upregulation of $p21^{WAF1}$. Moreover, a differential expression of survivin was observed in cells treated with different GAE concentrations where survivin was induced at low concentration and then abolished at higher concentrations. Finally, GAE caused a massive decrease of β-catenin protein expression.

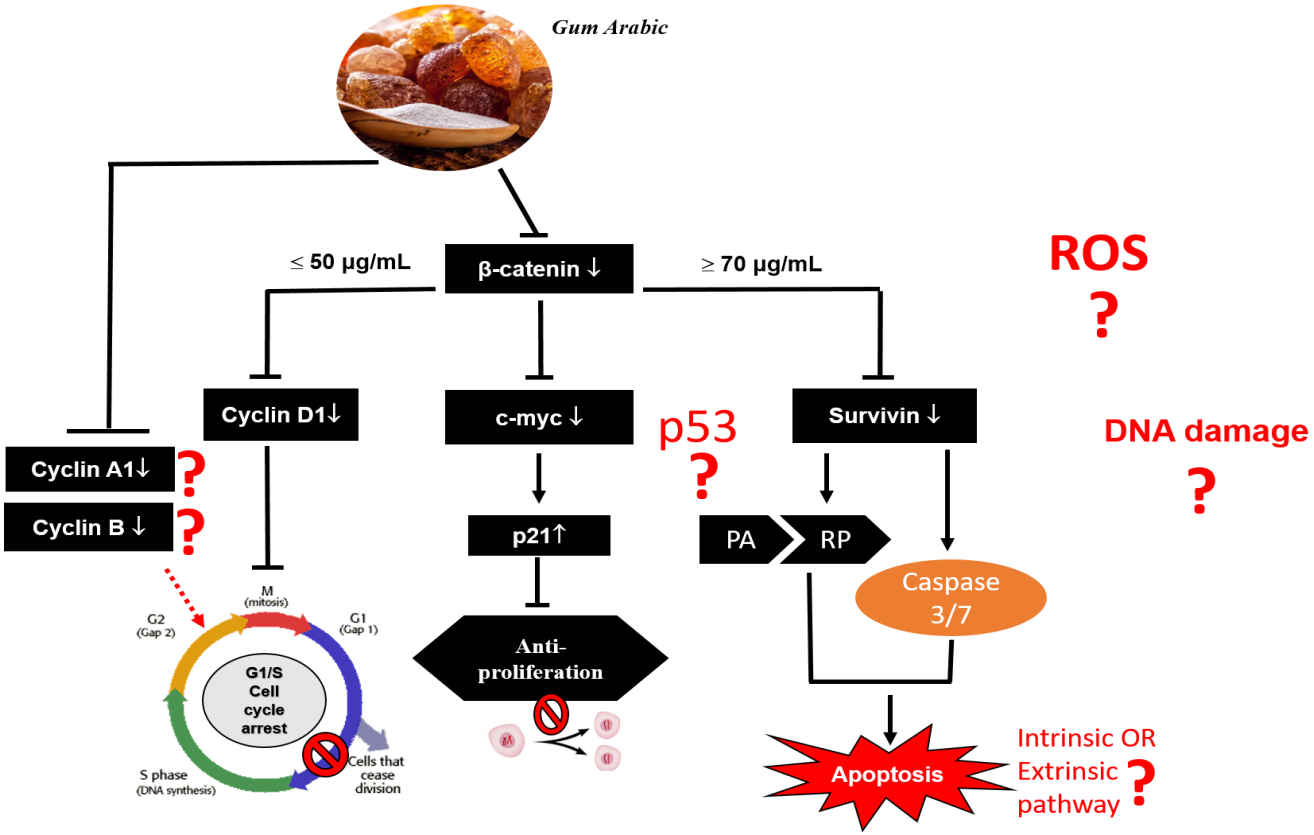

Figure 16: Proposed model demonstrating the underlying mechanism of action in GAE-induced anti-breast cancer activity in MDA-MB-231 cells

Chapter 5: Conclusion

In conclusion, the present study demonstrated, for the first time, the potential role of Gum Arabic as an anti-breast cancer agent in *vitro*. The study showed that treatment of MDA-MB-231 TNBC cells with GAE inhibits cell proliferation, induces cell cycle arrest and apoptosis through the down-regulation of cell cycle regulatory proteins, downregulation of survivin and blocking β-catenin signaling pathway. Further experiments are warranted to study the detailed molecular mechanisms of action and downstream key players. Investigating Gum Arabic anti-cancer effect *in vivo* is required to validate the *in vitro* findings.

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