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The Antioxidant and Pro-apoptotic effects of *Sternbergia clusiana* Bulb Ethanolic Extract on Breast Cancer Cells *in vitro*

By

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The Antioxidant and Pro-apoptotic effects of *Sternbergia Clusiana* Bulb Ethanolic Extract on Breast Cancer Cells in vitro

Mona Barrak El Samarji

Abstract

Extensive research has revealed that *Amaryllidaceae* species exhibit numerous therapeutic properties through several mechanisms. Among the various *Amaryllidaceae* species, *Sternbergia clusiana* has been recently given a great amount of attention in the medicinal field due to its richness in small chemical molecules, namely Amaryllidaceae alkaloids; these have been reported to possess an antioxidant and anti-inflammatory role, in addition to a prominent anti-cancer effect on different types of cancer cell lines. The current study investigates the selective anti-proliferative effect of *Sternbergia clusiana* bulb ethanolic extract (SbBEE) on breast cancer cells cultured in vitro (MDA-MB-231 and MCF-7) and reveals the underlying mechanism responsible for the induction of cell death. MDA-MB-231 cells treated with SbBEE exhibited morphological and biochemical hallmarks of apoptosis that were detected through DNA fragmentation, the hyper-expression of phosphatidylserine on the outer leaflet of cells membrane and the upregulation of proteins able to mediate cell death through apoptosis (c-PARP, Bax/ Bcl2). The prominent decrease in ROS levels upon treatment confirmed the antioxidant property of the extract being investigated, highlighting the promising effect of SbBEE in breast cancer treatment.

Keywords: *Sternbergia clusiana*; Breast cancer; MDA-MB-231; MCF-7; Apoptosis

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List of Abbreviations

ABTS	2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
ANOVA	Analysis of variance
Bax	Bcl-2 associated X protein
BSA	Bovine Serum Albumin
DMEM	Dulbecco's modified Eagle medium
DCFDA	20,70-dichlorodihydrofluorescein diacetate)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2-diphenyl-1-picrylhydrazyl
ECL	Enhanced Chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal Bovine Serum
IC50	Half maximal inhibitory concentration
MSC	Mesenchymal Stem Cells
NAC	N-acetyl cysteine
PBS	Phosphate buffered saline
PI	Propidium iodide
PARP	Poly (ADP-ribose) polymerase
ROS	Reactive oxygen species
SbBEE	*Sternbergia clusiana* Bulb Ethanolic Extract
TBHP	Tert-butylhydrogen peroxide

CHAPTER ONE

INTRODUCTION

1.1.Breast Cancer

Based on epidemiological studies conducted by the International Agency for Research on Cancer (IARC) in 2018, breast cancer was shown to be the most common cancer diagnosed in women worldwide and the first cause of women death from cancer to become a prominent contributor to more than 50% of the global incidence and mortality burden in 2018 (Ferlay et al., 2019).

Table 1: Estimated new breast cancer cases and deaths among women worldwide (2018) along with uncertainty intervals (95% UI, all ages, thousands), age standardized rates (ASRs, per 100,000) and cumulative risks (CR) to age 75 (%), adapted from (Ferlay et al., 2019).

Breast Cancer	**Females**			
	Numbers	95% UI	ASRs	CR (%)
Estimated new cases	2,088.8	(2,003.7-2,177.6)	46.3	5.03
Estimated Deaths	626.7	(606.1-648.0)	13.0	1.41

1.2.Risks and Causative factors

Knowing that the same hormones regulating mammary glands development -since the early postnatal stages- control breast tumorigenesis; any aberrant expression of growth and ovarian hormones can alternatively lead to carcinogenesis (Brisken & O'Malley, 2010). Endocrine activity in women is tightly associated with reproductive stages mainly between menarche and menopause where the secreted steroid hormones control breast development and the risk of carcinogenesis is at its most threatening peak (Hamajima et al., 2012). Other exogenic factors such as oral contraceptives, ovulation stimulating drugs or post-menopausal therapy affect hormonal exposure which leads to an aberrant cellular response that is manifested through malignancies (Reeves et al., 2012).

The main non-hormonal dependent breast cancer risk factors have a genetic or epigenetic origin, primarily due to the accumulation of irreversible mutations translated into a truncated protein. The non-sporadic process is initially encoded within a set of well-defined genes (BRAC1 and 2, PTEN, STK11, CDH1…) showing lethal modification either on the DNA level or at the level of their expression (Mavaddat et al., 2010). Nevertheless, family history remains one of the strongest epidemiological evidences determining risk factors where 1:4 of all breast cancers cases showed a familial predisposition to high-risk genes mutation (Antoniou & Easton, 2006).

Over the past decade, several studies have been conducted and have highlighted a blurred correlation between anthropometric parameters and the risk of developing breast cancer. In the first place comes obesity which has always been associated with cardiovascular diseases and diabetes mainly in developed countries. Overweight was established as a major factor inducing breast cancer among women (Picon-Ruiz et al., 2017), and this is

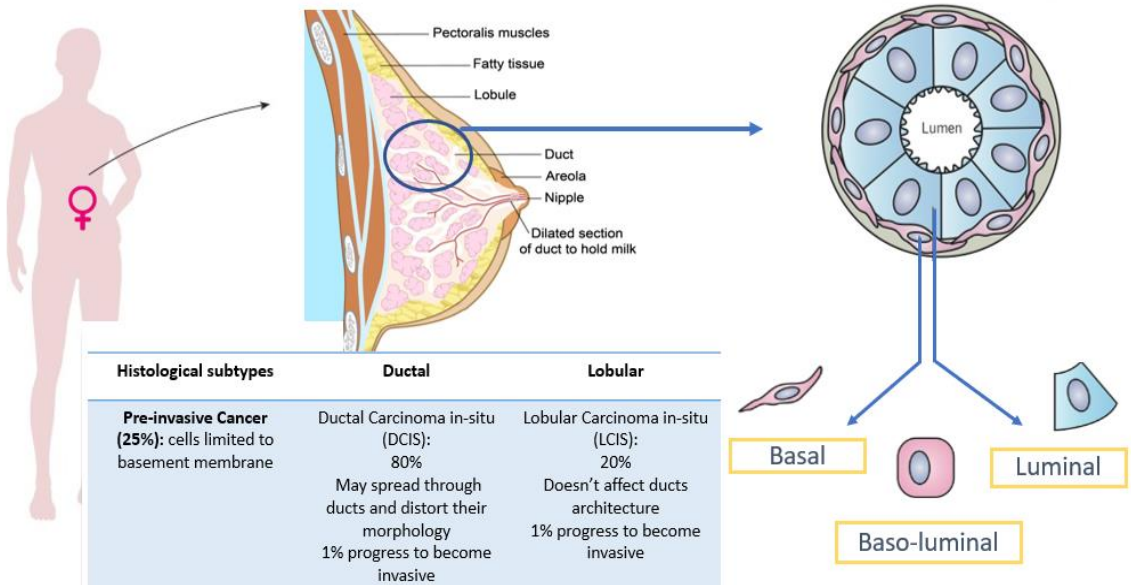
mainly due to the reduced secretion of anti-oncogenic adipokines namely adiponectin in addition to the hypersecretion of cancer-causing adipokines such as leptin that promotes postmenopausal breast cancer and tumor growth. Hence, the association between increased adiposity and the elevated risk of developing breast cancer in women is now well established (Schmidt et al., 2015).

1.3.Subtypes and heterogeneity

Diversity and plasticity are key reinforcements of homeostasis through which the organism responds to intrinsic and extrinsic stimuli and escapes cellular stresses. However, one notable feature of tissues undergoing tumorigenesis is their unconditional ability to increase cellular plasticity, self-renewal and differentiation as a response to many epigenetic alterations (Brooks et al., 2015). Cancer cells exhibit an enormous level of heterogeneity that manifests by mutable biological and clinical features making its classification into -more or less-homogenic types a high necessity in order to understand this complex pathology.

1.3.1. Histological Subtypes and invasiveness

Histological classification is based on the fact that breast carcinogenesis is a multistep process passing through several stages incorporating defined tissue kinds and growth patterns. It starts with hyper-proliferating mammary epithelial cells that accumulate in either the lobular or ductal lumen, forming in-situ lobular carcinoma LCIS or in-situ ductal carcinoma DCIS respectively, causing modifications in ducts' morphology **(Figure 1)**. Following further events of multiplication, propagation and infiltration, cancer cells penetrate into the duct walls and delocalize to become invasive and spread to other tissue types (Malhotra et al., 2010).



Histological subtypes	Ductal	Lobular
Pre-invasive Cancer (25%): cells limited to basement membrane	Ductal Carcinoma in-situ (DCIS): 80% May spread through ducts and distort their morphology 1% progress to become invasive	Lobular Carcinoma in-situ (LCIS): 20% Doesn't affect ducts architecture 1% progress to become invasive
Invasive Cancer (75%): cells extend beyond the basement membrane	Invasive Ductal Carcinoma (IDC): 79% Usually from DCIS precursor, cause fibrous response and a palpable mass on examination. Metastatic through lymph and blood.	Invasive Lobular Carcinoma (ILC) 10% Usually from LCIS precursor, minimal fibrous response, rarely form palpable mass. Metastatic through abdominal viscera to GI, ovaries, uterus.

Figure 1: Histological characterization and pathogenesis of Breast Cancer adapted from (*Breast cancer | McMaster Pathophysiology Review*, n.d.).

One of the most important uses of the histopathological classification is its role in defining breast cancer stages and prognosis, whereby Nottingham histologic score (or histologic grade) and the definition of four stages of breast carcinoma were developed **(Figure 2)**. Three parameters are considered when determining the pathological score: auxiliary lymph node status and degree of its involvement, nuclear pleomorphism and mitotic count predicted by the tumor size (Oluogun et al., 2019). Accordingly, four stages were described ranging from a non-invasive tumor (Stage 0), where both normal and malignant cells coexist, (eg: DISC) to the most advanced type (Stage IV) having the highest metastatic rate. Furthermore stages I, II and III are divided into subcategories depending on the number of lymph nodes involved (Akram et al., 2017).

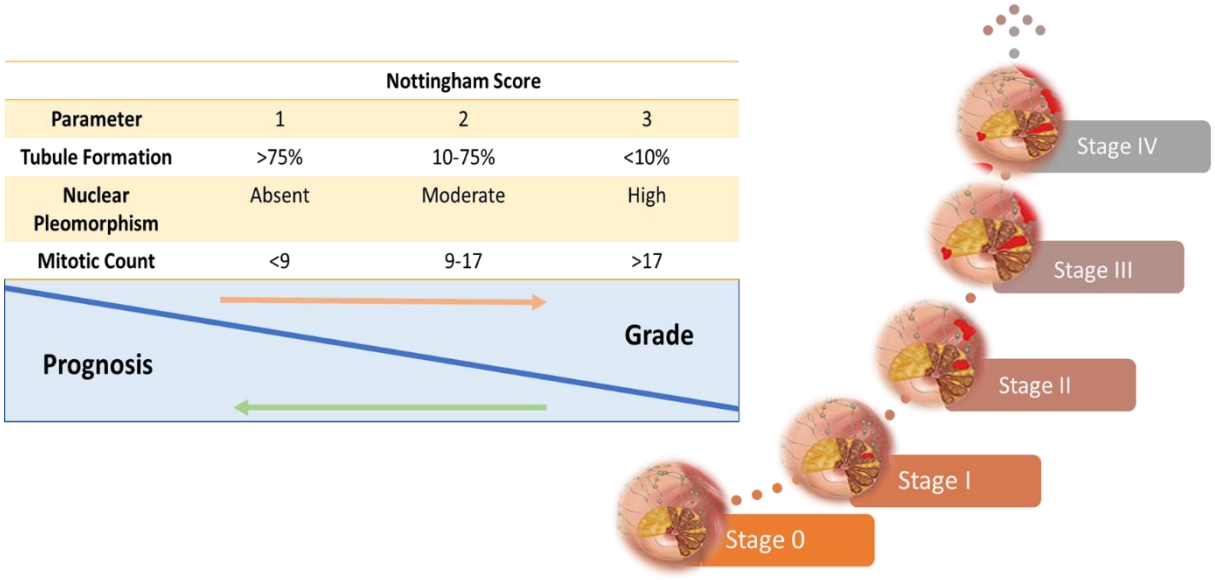


Figure 2: Nottingham scoring gradient assumed in determining breast cancer stages adapted from (*Staging & Grade - Breast Pathology | Johns Hopkins Pathology*, n.d.).

1.3.2. Molecular Subtypes

In humans, three different hormone receptors are used as prognostic biomarkers in order to molecularly classify breast cancer **(Figure 3)**: estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2). Breast cancer cells having none of the three receptors are designed as triple negative breast cancers (TNBC) which are considered to be one of the most aggressive types of breast cancer (Subramani et al., 2017). Further classification based on gene expression profile and molecular analyses showed four other subtypes including luminal A, luminal B, HER2 enriched, and basal-like triple negative cells. Luminal cancer subtype is mainly characterized by the double expression of ER and PR receptors with a high to intermediate prognosis, slower proliferation rate when compared to other subtypes and lower recurrence rate (Fragomeni et al., 2018). The third type, characterized by highly distinguishable proliferation rate, HER2 enriched tumors result from the overexpression c-ERBB2 proto-oncogene affecting cell-cycle control and checkpoints (Tan & Yu, 2013).

Basal-like and TNBC are two terms that were used interchangeably to describe breast tumors lacking the three major receptors but harboring unique oncogenic mutations such as the *BRCA1* gene (17q21) involved in DNA repair events and p53 gene leading to the exceptional pathological expression of "basal-like" cytokeratins (CK 5,6) (Anders & Carey, 2009).

Molecular Subtypes	Receptor Expression based on immunohistochemical characterization		
	ER	PR	HER2
LUM A	+	+	-
LUM B	+	+	+
HER2/neu	-	-	+
TNBC	-	-	-

ER= Estrogen Receptor (+)= Present (-)= Absent
PR=Progesterone Receptor HER2= Human Epidermal growth factor Receptor 2
LUM A & B= Luminal A & B TNBC= Triple Negative Breast Cancer

Figure 3: Molecular subtypes of Breast Cancer based on immunohistochemical characterization adapted from (Kumar et al., 2015).

1.4.Breast Cancer cells and ex-vivo research

Despite the questions and doubts that have been raised over how representative immortalized cell lines are of human breast cancer heterogeneity and complexity in their original in-vivo atmosphere, traditionally established cancer cell lines are still excessively used to test main biological features and mechanisms.

The first breast cancer cell line to be established in 1958 gave rise to the BT-20 cell-line followed by MDAs (MD Anderson series) in 1973 and MCF-7 in 1978 (Holliday & Speirs, 2011).

1.4.1. MDA-MB-231 and MCF-7

Table 2: Phenotypic heterogeneity between two types of breast cancer cells MDA-MB-231 and MCF-7 cells (Theodossiou et al., 2019).

MDA-MB-231	**MCF-7**
Ductal invasive / Breast Carcinoma	Ductal invasive / Breast Carcinoma
Hormone Independent:	Hormone dependent:
ER-, PR-, HER-	ER+, PR+, HER+
Express mesenchymal phenotype	Express epithelial phenotype

MCF-7 **(Table 2)** were the first established ER responsive mammary cells whose proliferation and invasiveness are directly stimulated by the levels of estrogen hormone. Later studies showed that MCF-7 are triple positive breast cancer expressing the three main hormone receptors (ER, PR and HER), making them a good target for anti-cancerous hormonal therapies. Relying on oxidative phosphorylation, MCF-7 are more described as Pasteur type of cells metabolically dependent on ATP under normal conditions while shifting to a highly stimulated glycolytic activity under hypoxic circumstances (Sakamoto et al., 2011).

On the other hand, MDA-MB 231 cells **(Table 2)** were identified as TNBC lacking the three hormones' receptors leading to a high resistance against a wide range of anti-estrogen agents such as tamoxifen which is a selective modulator of estrogen receptor.

Metabolically, MDA-MB 231 cells are more Warburg type totally dependent on glycolysis providing ATP in the presence or absence of oxygen (Theodossiou et al., 2019).

1.4.2. Cell Cycle Regulation

The tight regulation of the mammalian cell cycle is directly attributed to multiple signaling proteins, namely cyclins and cyclin-dependent kinases, which ensure the integrity of the process and establish molecular checkpoints for the transition between the four distinct cell-cycle phases (G0/G1, S, G2 and M). Therefore, any mutation or genetic lesion affecting these proteins dysregulates the signaling pathway leading to the genesis of malignancies and other detrimental biological issues (Otto & Sicinski, 2017). Cell cycle analysis and interpretation in immortalized mammary epithelial cell lines have shown chromosomal abnormalities (chromatid cohesion), aberrant cell cycle progression kinetics, and defective checkpoints at G_2 or M phases (Bower et al., 2017) .

1.4.3. Programmed Cell Death (PCD)

Cell death is a natural process occurring eventually at the end of a long journey of non-controllable mitotic events. However, the programmed cell death is triggered upon exposure to particular extrinsic or intrinsic stresses leading to unrepairable errors and modifications affecting cellular mechanisms. Depending on the molecular triggers, the morphological features and the process by which the cell switches on its self-destruction, different types of programmed cell death were identified noting mainly apoptosis, autophagy and necroptosis (Savitskaya & Onishchenko, 2015).

When alteration occurs on the level of DNA, either through chromatin condensation-Pyknosis- or DNA fragmentation, apoptosis is the most common event to be turned on. Apoptotic cells can be dynamically and morphologically distinguished since most of these dying cells show a dynamic membrane blebbing accompanied with cell shrinkage, decreased connections between the cells and their extracellular matrix in addition to a hyper-exposure of phosphatidyl serine towards the exterior is stimulated (Nikoletopoulou et al., 2013). Apoptosis is a highly sophisticated process involving extensive protein cross-linking. Therefore, scientists were interested in differentiating the mechanisms leading to apoptosis based on the type of proteins involved within each pathway.

Two main mechanisms of apoptosis were identified: an intrinsic- mitochondrial pro-enzymes dependent- pathway and an extrinsic- death receptor dependent- pathway (Fulda, 2010). Each pathway triggers its own initiator caspases (8 in extrinsic and 9-10 in intrinsic) which in turn will stimulate the effectors or executioners (caspase-3, 6, or 7) and inflammatory caspases (caspase-1,4,5) (Prokhorova et al., 2018). However, another type of apoptosis was shown to occur without the activation of the caspase cascade, hence the name of caspase-independent apoptotic pathway, which is similar in that criteria to programmed necrosis. The most notable difference between apoptosis and necroptosis is the release of cellular content leading to local inflammation in necroptosis and revealing the activation of inflammatory cell invasion (Liu et al., 2018).

Another type of PCD is autophagy that acts as a catabolic process preserving cell homeostasis, while initiating a set of reactions leading to cell starvation, organelles and DNA damage as well as proteins degradation. Driven by a wide set of well conserved proteins, autophagy activates a multi-step procedure through which unwanted molecules are sequestered within an isolation membrane called the autophagosome. Lysozymes

eventually fuse with the isolation sac forming the autolysosome where the reactions of degradation take place (Shimizu et al., 2014).

1.5.Treatment

Given the complexity and the heterogeneity of malignant cells regarding their phenotype, morphology and inter-cellular relationship, cancer patient treatment should ideally consider the entire tumor ecosystem in addition to specific biomarkers. Subsequently, monotherapies tend to fail in inhibiting cancer cells proliferation and invasion while causing detrimental effects on normal ones.

1.5.1. Targeted therapies and resistance

The discovery of many crucial genomic and molecular bio-markers had led to a better understanding of tumorigenesis and the emerging of specific targeted therapies aiming to suppress tumor development without affecting normally developing cells. Diverging from broad chemotherapy that has been always used to treat all types of breast cancer, researchers are now more interested in therapeutic options targeting the expression type specific receptors such as ER, PR and HER2+. One of these options is endocrine therapy (ET) that works by reducing the expression these markers using receptor-modulators and down-regulators, aromatase inhibitors or basic oophorectomy.

In order to decrease resistance against hormonal therapies, many attempts were done to combine ET with other therapies targeting intracellular pathways. Two key targets were determined: the use of specific inhibitors that interfere with the rapamycin/PIK3CA (mTOR/PIK3CA) pathway and targeting cyclin-dependent kinase 4/6 (CDK4/6) in order to restrict cell cycle progression (El Hachem et al., 2019). TNBC cells lacking the three validated molecular markers (ER,PR and HER) were shown to express to express either

epidermal growth factor receptor (EGFR) or cytokeratin 5/6 (Cheang et al., 2008) rendering them susceptible to another family of specific inhibitors such as tyrosine kinase inhibitors (TKIs) or cetuximab (anti-EGFR monoclonal antibody) (Chavez et al., 2010).

1.5.2. Plant based therapy

Aiming to reduce the side effects brought by chemotherapeutic drugs in addition to their unbearable cost, oncologists were interested in finding alternative and complementary treatments for cancer with promising results. In the 21st century, one of the oldest and most native medical science, Ayurveda, was reconsidered where natural drugs extracted from plants are used to suppress tumorigenesis (Jain et al., 2010). Nowadays, more than 35000 medicinal plant species screened by the National Cancer Institute (NCI) were shown to have anticancer properties (Pawar et al., 2018).

During the past the two years, our lab was interested in investigating the anti-cancerous effect of different Mediterranean plants on the proliferation and the survival of malignant cells. In what follows is a brief overview of the promising plants that we have evaluated in our lab.

1.5.2.1.Annona cherimola

Annona cherimola, a green edible fruit with large black seeds, was shown to have pro-apoptotic effects on malignant cells. An ethanolic extract prepared from *Annona*'s seeds (ASEE) was able to cause death of AML cells via the activation of both intrinsic and extrinsic pathways of apoptosis, detected by increased expression of pro-apoptotic proteins such as p53 and c-PARP, and inhibition of ROS levels (Haykal et al., 2019). Later, another ethanolic extract prepared from *Annona*'s leaves (AELE) appeared to have a pro-apoptotic effect through the upregulation of Bax/Bcl2 and c-PARP expression in

treated AML cells (Ammoury et al., 2019). A similar mechanism was detected upon treating breast cancer cells, both MDA-MB-231 and MCF-7, by (AELE) which selectively stimulated an intrinsic pathway of apoptosis achieved by the upregulation of Bax/Bcl2 ratio, cytochrome-c and the tumor suppressor p-21 (Younes et al., 2020) .

1.5.2.2.Malva pseudolavatera

A methanolic extract prepared from *Malva pseudolavatera* leaves (MMLE) was tested on AML cells and showed a selective, time and dose dependent anti-cancerous effect (El Khoury et al., 2020). *Malva*, a plant already known for its antioxidant and anti-inflammatory properties, stimulated ROS levels and induced an intrinsic Bax/Bcl2 dependent apoptotic pathway in Monomac-1 leukemic cells.

1.5.2.3.Flax Seeds

In the study conducted by Tannous et al. (Tannous et al., 2020), three lignans, namely Enterodiol (END), Enterolactone (ENL) and Secoisolariciresinol diglucoside (SDG), extracted from flaxseeds appeared to have a promising anti-cancerous effect on AML cells. The derivative with the most promising effect, ENL, executed an anti-proliferative effect on both KG-1 and Monomac-1 cells in a dose and time-dependent manner through the induction of apoptosis. The experiments carried out confirmed the externalization of phosphatidylserine, the increase in ROS levels, the hyper-expression of pro-apoptotic proteins (Bax, c-casp3 and c-PARP) and the release of mitochondrial cytochrome-c into the cytosol.

1.6.On the Genus *Sternbergia* (Amaryllidaceae) and its subtypes

Figure 4: *Sternbergia clusiana* flower and bulbs brought in November 27-2018.

Named after the Hungarian botanist Count Caspar von Sternberg (1761-1838**),** the genus *Sternbergia,* that was first described by Waldstein & Kitaibel (1803-1805), embraces mostly autumn flowering species characterized by a yellow wine glass shape and a well-developed perigonium (Youssef et al., 2017). It belongs to the family of plants known as *Amaryllis,* and specifically to the subfamily *Amaryllidaceae*. This genus comprises four well-defined species including: *S.fisheriana, S.lutea, S.candida* in addition to the largest flowering member *S.clusiana* **(Figure 4)**.

Since 1877, many studies were conducted to determine the active components contained within the bulbs and leaves of several *Amaryllidaceae* species that appeared to be highly rich in isoquinoline alkaloids (Kornienko & Evidente, 2008). The majority of the alkaloids occurring in the Amaryllidaceae are not known to be found in any other plants family and they are mainly classified into 9 distinct types : norbelladine, lycorine, homolycorine, crinine, haemanthamine, narciclasine, tazettine, montanine and galanthamine (Bastida et al., 2011).

Further investigations performed in the last few years showed the cytotoxic and chemotherapeutic properties of these alkaloids, in addition to their interference with the

biological and synthetic pathways of many crucial proteins involved in multiple diseases (Hulcová et al., 2019; Martinez-Peinado et al., 2020).

Table 3: Total phenol and total flavonoid contents of the methanolic extracts of Sternbergia species adapted from (Orhan et al., 2011).

Sternbergia Extracts	Total Phenol Content (mg/1g of methanolic extract)	Total Flavonoid Content (mg/1g of methanolic extract)
S. candida	35.36±1.23	0.78±0.08
S. clusiana	**37.15±1.55**	**0.95±0.01**
S. fischeriana	28.93±1.06	1.37±0.96
S. lutea subsp. lutea	82.14±1.26	2.03±0.38
S. lutea subsp. sicula	86.07±1.07	1.17±0.05

1.7. Medicinal properties of *Sternbergia* species

1.7.1. Antioxidant and anti-inflammatory activity of *Sternbergia* extracts

Homeostasis in living organisms is mainly based on three main processes: the synthesis, the degradation and the accumulation of material. Any minor impairment in the biological balance is capable to provoke deleterious effects. Oxidative stress is a major problem affecting oxygen-metabolism, caused by the imbalance of reactive oxygen species (ROS) production versus its accumulation in living cells (Pizzino et al., 2017). The excessive cellular stress is highly related to harmful pathological phenomena such as atherosclerosis and rheumatoid arthritis, but most importantly its contribution to inflammatory diseases due to the inhibition of anti-inflammatory cytokines (Lobo et al., 2010). The ultimate mechanism to neutralize oxidative stress is through the destruction of free radicals, a process maintained by endogenous antioxidants that can be supported by exogenous antioxidants. Vitamin A, Vitamin C, lycopene, flavonoids and many other naturally occurring reducing agents were shown to exert an antioxidative defense mechanism as "radical scavengers" when introduced through dietary intake (Bouayed & Bohn, 2010). Experimentally, ethanolic extracts from bulbs, fruits and stems of Amaryllidaceae plants, *P. maritimum*, showed promising antioxidative capacity in a concentration dependent manner (Leporini et al., 2018). Other experiments using bulbs ethanolic extracts from *Sternbergia* species, *S.lutea*, confirmed an ultimate free radical scavenging activity (Aydin et al., 2015).

1.7.2. Anti-microbial and anti-fungal activity of *Sternbergia* extracts

Due to their phytochemicals-rich nature, *Sternbergia* species were tested for their protective effects against invasive fungi and microbes. A recent study (Can Ağca et al., 2020) demonstrated the ability of *S. lutea* aqueous and ethanolic bulbs extracts to stabilize the membrane of hemolyzed erythrocytes in a percentage of stabilization of 80.4% and 71.3% respectively, thus revealing their significant anti-inflammatory properties. *S.lutea* was also shown to be effective against the Gram-positive *S. aureus* and Gram-negative bacteria such as *E. coli* and *S. typhimurium.* On the other hand, *S.clusiana* was shown to be relatively ineffective against most of Gram-positive and Gram-negative bacteria, while exerting a highly significant anti-candida activity. Another Sternbergia member with anti-fungal properties, *S.vernalis*, was able to inhibit the germination phase of *C.albicans* by reducing hyphae tail length as an important mechanism to stop candida virulence (Kaskatepe et al., 2019).

1.7.3. Its role in Alzheimer's disease

Most Alzheimer patients suffer from a severely altered cholinergic function that can be enhanced either through the stimulation of cholinergic receptors or inhibition of the enzyme-acetylcholinesterase, degrading the neurotransmitter acetylcholine at cholinergic synapses. Many studies were carried to determine natural agents that are able to hinder the breakdown of acetylcholine by its degrading enzyme. Eventually, plants of the family Amaryllidaceae were shown to contain alkaloids, mainly galanthamine and lycorine, that exhibit cholinesterase inhibiting activity, and among all the species of this family, *Sternbergia* species including *S. clusiana* gained attention as a source of metabolites of these alkaloid contents (Haznedaroglu & Gokce, 2014). Besides their involvement in

treating dementia in Alzheimer's disease, several alkaloids extracted from different Amaryllidaceae species exhibit a neuroprotective effect against oxidative stresses (Cortes et al., 2018).

1.7.4. Anti-cancer properties

Alkaloids derivates from natural Amaryllidaceae extracts were suspected to have a potent anti-cancer activity. Several in vitro studies targeting cervical adenocarcinoma, African monkey kidney epithelium and human T-cell leukemia showed the effectiveness of Lycorine and Narciclasine as antiproliferative and pro-apoptotic agents (Evidente & Kornienko, 2009).

The anti-cancer effect of Lycorine was thoroughly studied in order to understand its mode of action. Lycorine was shown to be a highly selective in inhibiting the growth of a wide range of cancers and severe melanoma while exerting a mild effect on normal healthy tissues. Showing a high potency at very low concentrations, lycorine was identified as a strong inducer of programmed cell death, mainly apoptosis, and a potent inhibitor of invasion and metastasis. It was also able to affect the cytostatic state of apoptosis-resistant malignant cells, hence inhibiting their migration and proliferation (Roy et al., 2018).

Being highly rich in lycorine (Çitoğlu et al., 1996) , *Sternbergia clusiana* were also suspected to have an anti-cancerous effect that needs to be investigated.

CHAPTER TWO

MATERIAL AND METHODS

2.1. Plant Material

Sternbergia Clusiana (Ker Gawl.) plants were collected from Falougha, Lebanon (33.825008◦ N, 35.751962◦ E, 149 m above sea level) during November 2018 and identified by Dr. Nisrine Machaka-Houri, a botanist and expert on Lebanese flora, according to the indications and characteristics described by Post.

2.2. Sternbergia Bulb Ethanolic Extract Preparation (SbBEE)

Bulbs were taken from *Sternbergia clusiana,* washed with distilled water, dried and grinded. A mass of 36.47g of grounded bulbs was mixed in 350ml of 70% ethanol and thermo-shaken at 200 rpm for one week at room temperature (25°C). Ethanol was evaporated from a volume of 50ml of the mixture by applying mechanical rotation under vacuum (Roto-evaporation). The extract was re-dissolved in 2.5ml DMSO under sonication and 25ml of cell-culture media (RPMI, 10% DMSO). The solution was filtered with a sterile cheese cloth (Volume brought to 90ml) and centrifuged at 24,446 x g then filtered with a syringe filter (0.45µm) to a volume of 87 ml. The pure extract (232.7µg/ml) was labeled as SbBEE and placed in aliquots at -80°C for later uses. Each aliquot was diluted 10 times in DMEM before applying the extract to the cells in order to maintain a final concentration of DMSO less than 1%.

2.3. Cell Lines

MDA-MB-231 and MCF-7 breast cancer cells were obtained from ATCC for culture and upcoming experiments. Both MDA-MB-231 and MCF-7 were established from a pleural effusion of two distinct Caucasian females of 51-year-old and 69-years old respectively suffering from metastatic mammary adenocarcinoma.

2.4. Cell Culture

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, GibcoTM, Dublin, Ireland) and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin from Pen-Strep, Lonza, Basel, Switzerland and 0.1mg/ml Kanamycin from Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator at 37 ◦C (5% CO2). Cell viability and morphology were checked routinely and prior to each experiment using the ZOE Fluorescent Cell Imager (Abcam, Cambridge, UK), along with Trypan Blue exclusion method as previously described by Hodroj et al. (Hodroj et al., 2018).

2.5. Culture of Mesenchymal Stem Cells (MSCs) isolated from Rat Bone Marrow

MSCs isolation was approved by the university's Animal Care and Use Committee (ACUC) and complied with the Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2010). The cells were isolated from a 12-week-old rat that was provided by the animal facility at the Lebanese American University and sacrificed under CO_2 asphyxiation. Briefly, femoral and tibial bones were aseptically isolated and washed with 70% ethanol then placed in sterile phosphate buffered saline (PBS, Lonza) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza). After removing the bone epiphyses

with sterilized scissors, the bone marrows were flushed out using a needle filled with Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco^{TM}) , 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza). The cells collected were then incubated in vented flasks at 37◦C with 5% CO_2. After 5 days of daily medium change, MSCs were identified by their spindle-shaped morphology as observed using the ZOE fluorescent cell imager (Bio-Rad, Irvine, CA, USA) (Haykal et al., 2019; Najar et al., 2019; Soleimani & Nadri, 2009)

2.6. Cytotoxicity Assay

Cell viability was assessed using an assay based on the cleavage of the stable tetrazolium salt MTS into to a soluble formazan in metabolically active cells. The cells were seeded in a 96 well-plate at a confluency of 0.5×10^5 cells/ml before applying SbBEE at increasing concentrations of (0.15-0.5-2.5% v/v). Interference wells were considered where the extract increasing concentrations were added to DMEM only (as background), in addition to negative and positive controls. The cells were incubated with various SbBEE concentrations for two different time frames (24h and 48h). After the completion of the proper incubation time, the supernatant was removed and a volume of 100ul of MTS-PMS (Acros, Fisher, Germany) (20:1) mixture was added in each well and left for 1h30min. The amount of formazan produced was detected by measuring the absorbance at a wavelength of 492 nm using a Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific, Bremen, Germany). Each experiment was performed in triplicates in order to determine the average percentage proliferation of cancerous cells at different concentrations of SbBEE used.

2.7. Cell Cycle Arrest detection by PI Staining and Flow Cytometry

MDA-MB-231 cells (2× 10^5 cells/ml) were seeded in 6-well plates and treated with increasing concentrations of SbBEE and 0.4 μM topotecan that was used as positive control. The cells were washed with PBS, detached with trypsin-EDTA and then quenched using media before centrifugation at 414.05 x g. The pellet was resuspended in 600 μl of PBS and left overnight after fixation with ice-cold absolute ethanol. The next day, the samples were centrifuged at 736.09 x g for 10min and the pellet was suspended in 500 μl of PBS. The cells in each sample were counted using trypan blue method, and the adequate volume of Propidium iodide (PI, Abcam, Cambridge, UK) staining buffer was added according to the manufacturer's instructions. The Guava easyCyte™ flow cytometer was used to assess the DNA content in each vial and that was classified as follows: sub-G0/G1 phase cells (Pre-G or dead cells) have <2n, G0/G1 phase cells have 2n, S phase cells have between 2n and 4n, and G2/M phase cells have 4n.

2.8. Cell Death ELISA

DNA fragmentation was assessed using the Cell Death ELISA kit (Roche), following the manufacturer's instructions. Cells were seeded at a confluency of 2× 10^5 cells/ml overnight before applying the treatment at increasing concentrations and a positive control Topotecan (20 μM). After 24 h, the incubation media was removed into labelled conicals, the cells were washed with PBS (1%) and detached using Trypsin (1X). Conicals containing the mixture of media, PBS and detached cells were centrifuged at 414.05 x g for 5min. The supernatant was discarded and the pellet was resuspended in media and centrifuged again.at 736.09 x g for 5min. Cells in the pellet were then lysed using the incubation buffer before centrifugation at 24,446 x g for 10min. The DNA-rich

supernatant was plated into a histone coated microplate (prepared one day before and left overnight at 4◦C) and incubated for 120min at room temperature. The microplate was washed thoroughly three times before the application of the peroxidase linked anti-DNA antibody solution. The plate was covered and left for incubation at room temperature for another 120min. Following incubation, the microplate was washed again and ABTS buffer was then added 15min prior to absorbance reading at 405nm using the Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific, Bremen, Germany). Quantification was done by calculating the enrichment factor of fragmented DNA using the following formula:

$$\text{Enrichment Factor} = \frac{\text{(Absorbance of treated cells)}}{\text{(Absorbance of non-treated cells)}}$$

2.9. Imaging of Annexin V/PI Staining under fluorescent microscopy

MDA-MB-231 cells were seeded overnight in a 96 well-plate before applying SbBEE in increasing concentrations. The cells were incubated for 24h then washed twice with PBS solution. Annexin V-FITC/PI kit (Abcam, Cambridge, UK) was used to stain the treated cells compared to control and visualized with the ZOE fluorescent microscope under bright-field conditions, green and red filters for Annexin-V and PI detection respectively.

2.10. Apoptosis detection by dual Annexin V/PI Staining and Flow Cytometry

After the incubation of MDA-MB-231 (2×10^5 cells/ml) with the treatment for 24h, the cells were washed with PBS, detached using trypsin (1%) then quenched with media before centrifugation at 736.09 x g. The pellet was resuspended in 500µl of PBS on ice and an adequate volume (2×10^5 - 5×10^5 cells/ml) was taken to be mixed with Annexin-V binding buffer in order to obtain a total volume of 300 µl/sample. Cells in suspension

were then stained with Annexin V-FITC (Annexin-V–fluorescein isothiocyanate [FITC] Apoptosis Detection Kit, Abcam) according to the manufacturer's instructions (5 µl/sample). Guava easyCyte™ flow cytometer was used to analyze the samples.

2.11. Western Blot

MDA-MB-231 cells were plated in petri-dishes at a density of 2×10^5 cells/ml before treatment with increasing concentrations of SbBEE for 24 h. The concentrations used were: 3.49 µg/ml <11.63 µg/ml ~ IC50< 58.18 µg/ml. Cells were incubated with the lysis buffer from the Qproteome mammalian protein prep kit (Qiagen, Hilden, Germany) for 5min on ice, and a scraper was used to detach the cells and allow better lysis. Lysed cells mixtures were then centrifuged at 24,446 x g for 10min. The protein fraction obtained in the supernatant was quantified using the DC (Detergent Compatible) protein assay (Bio-Rad). Extracted proteins were then separated by SDS-PAGE (10%) for 120min under two phases: samples were allowed to traverse the stacking gel for 20min at 90V, once in the resolving phase the voltage was switched to 120V. After run completion, proteins were transferred to PVDF membranes that were blocked with 5% skimmed milk for 1h at room temperature while shaking, then incubated with primary antibodies (Table 4) diluted in BSA solution (5%) overnight. Membranes were washed 4 times in a 40min time interval with PBS-0.1%TWEEN to remove unspecific bound antibodies and incubated for another hour with the specific secondary antibody (Bio-Rad, Irvine, CA, USA) followed by exposure for image development using Clarity™ Western ECL substrate (Abcam) on a ChemiDoc machine (Bio-Rad). Quantification using the ImageJ program allowed us to calculate the relative expression of proteins, as compared to the loading control β-actin.

Table 4: Primary Antibodies used in Western-Blot.

Primary Antibodies	Company
Anti-β-actin	Santa Cruz Biotechnology, Dallas, Tx, USA
Anti-cleaved poly (ADP-ribose) polymerase (PARP)	Abcam,Cambridge, UK
Anti-Bax	Elabscience, Houston, TX, USA
Anti-Bcl2	Elabscience, Houston, TX, USA
Anti-Caspase-3	Abcam,Cambridge, UK
Anti-Caspase-8	Elabscience, Houston, TX, USA
Anti-Caspase-9	Elabscience, Houston, TX, USA
Anti-Beclin-1	Cell Signaling
Anti-Cytochrome c	Abcam,Cambridge, UK

2.12. Cytochrome-c release analysis through Cytosolic and Mitochondrial fractionation

MDA-MB-231 cells were seeded in petri-dishes at a density of 2×10^5 cells/ml before treatment with increasing concentrations of SbBEE: 3.49µg/ml,11.63µg/ml, and 58.18µg/ml. After 24h of incubation, proteins were extracted using a protocol adapted from Abcam subcellular fractionation protocol. A lysis buffer (4.77×10^{-3} g/ml Hepes, 0.75×10^{-3} g/ml KCl, 0.19×10^{-3} g/ml MgCl2, 0.29×10^{-3} g/ml EDTA, 0.2µl/ml DTT and 1µl/ml Protease's inhibitor) was prepared and used to incubate the cells for 20 min on ice and allow lysis. A series of centrifugation steps were done in order to separate the subcellular fractions: after a centrifugation of 5min at 1656.2 x g, the supernatant containing both cytosolic and mitochondrial contents was recentrifuged at 7441.5 x g for another 5min to obtain the cytosolic fraction in the supernatant and the mitochondrial one in the pellet. The pellet was resuspended in 500 µl TBS/0.1SDS and homogenized well

on ice. The proteins extracted were then quantified using the DC (Detergent Compatible) protein assay (Bio-Rad). Western immunoblots were run, as described in the previous section, in order to detect the release of cytochrome c (Abcam, Cambridge, UK) from the mitochondria to the cytosol of the cells.

2.13. Reactive Oxygen Species Detection

The DCFDA Cellular ROS Detection Assay kit (Abcam, Cambridge, UK) was used to detect the levels of Reactive Oxygen Species (ROS) in MDA-MB-231 cells upon treatment with SbBEE. Cells plated at a density of 0.5×10^5 cells/ml a 96-well plate, were incubated with the reduced agent 20,70-dichlorodihydrofluorescein diacetate (H2DCFDA) for 45min before adding the increasing concentrations of the extract. TBHP, a potent ROS inducer, was used as a positive control (30µM) in addition to a ROS inhibitor NAC (1mM). Two hours later, the amount of H2DCFDA oxidized into DCFDA, as a result of ROS reduction, was measured using the Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific, Bremen, Germany).

2.14. Statistical Analysis

Several trials were performed before establishing the results shown below with a minimum of (n=3) depending on each experiment. Statistical analyses were performed using GraphPad Prism 8 to represent graphical data along with appropriate statistical analyses where means of replicates were coupled to standard deviation (STDEV) and significance represented by p-values was calculated by t-tests or two-way ANOVA. The following table represents p-values along with their related significance.

p-values	Representation	Significance
0.01 < p < 0.05	*	Significant
0.001 < p < 0.01	**	Very Significant
0.0001 < p < 0.001	***	Highly Significant
p < 0.0001	****	Extremely Significant

CHAPTER THREE

RESULTS

3.1. SbBEE exerts a selective anti-proliferative effect on MDA-MB-231 and MCF-7 cells as compared to its effect on MSCs

A cell viability reagent, MTS, was used to detect the cytotoxic effect exerted by SbBEE on breast cancer cells both MDA-MB-231 and MCF-7 via spectrophotometry. A significant decrease in viable cells was detected in a dose and time-dependent manner with an IC50 of 23.97 µg/ml and 5.956 µg/ml for MDA-MB-231 at 24h and 48h respectively **(Figure 5A)**. The effect was similarly significant on MCF-7 that showed an IC50 of 29.12 µg/ml at 24h and 3.406 µg/ml at 48h **(Figure 5B)**. MSCs were used to show the selectivity of the extract in inhibiting the proliferation of malignant cells. SbBEE showed no cytotoxic effect on normal MSCs cells when treated with highly saturated doses of the extract **(Figure 5C)**.

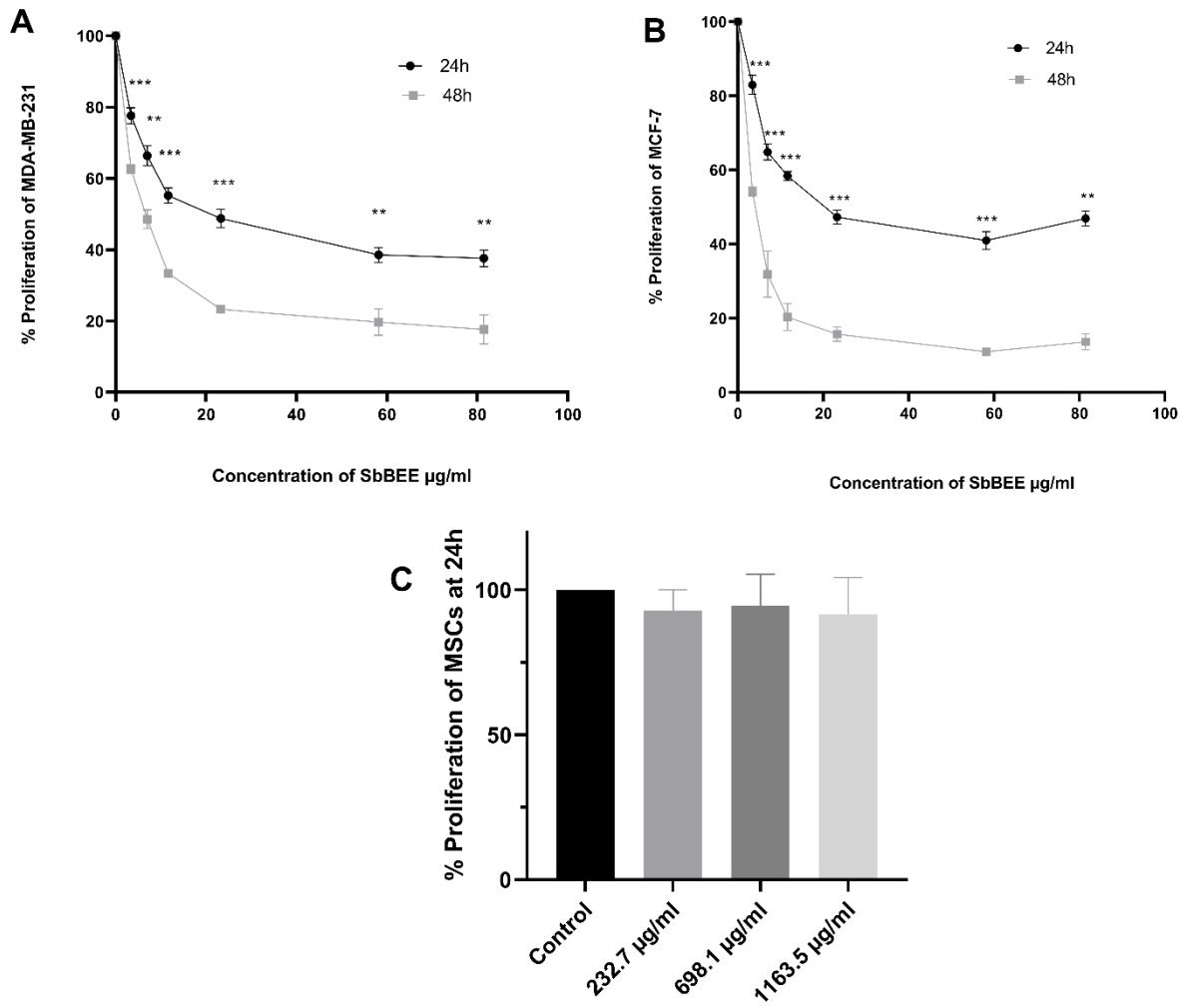


Figure 5: Cytotoxicity Assay. Proliferation (%) of MDA-MB-231 after 24 and 48 hours (A), of MCF-7 after 24 and 48h (B), and mouse Mesenchymal stem cells (MSC) after 24h (C) of incubation with increasing concentrations of SbBEE. Values were reported as mean ± SD. Significant differences were reported with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$ and *** indicating a p-value: $0.0001 < p < 0.001$.

3.2. SbBEE induces cellular fragmentation without significantly affecting the cell cycle progression

In order to determine whether the *Sternbergia* extract affects MDA-MB-231 cell cycle progression, propidium iodide staining followed by flow cytometry was used. Stained cells were classified according to the amount of DNA they present following 24h of treatment with increasing concentrations of SbBEE. **Figure 6(A and B)** illustrates the distribution of cells in each phase of the cell division cycle (pre-G0, G0-G1, S, G2-M) and that was quantified as it's graphically represented in **Figure 6C**. The results obtained showed an increase in the percentage of cells at the Pre-G0 phase (DNA <2n) that doesn't appear to be significant until a treatment concentration of 58.18µg/ml was applied. This increase in the pre-G0 phase was coupled to a lightly significant decrease in the percentage of cells in the G0-G1 phase that shifts from 45.2% in control samples to 35.5% in samples treated with 58.18µg/ml of SbBEE. These results suggest that SbBEE promotes cellular fragmentation.

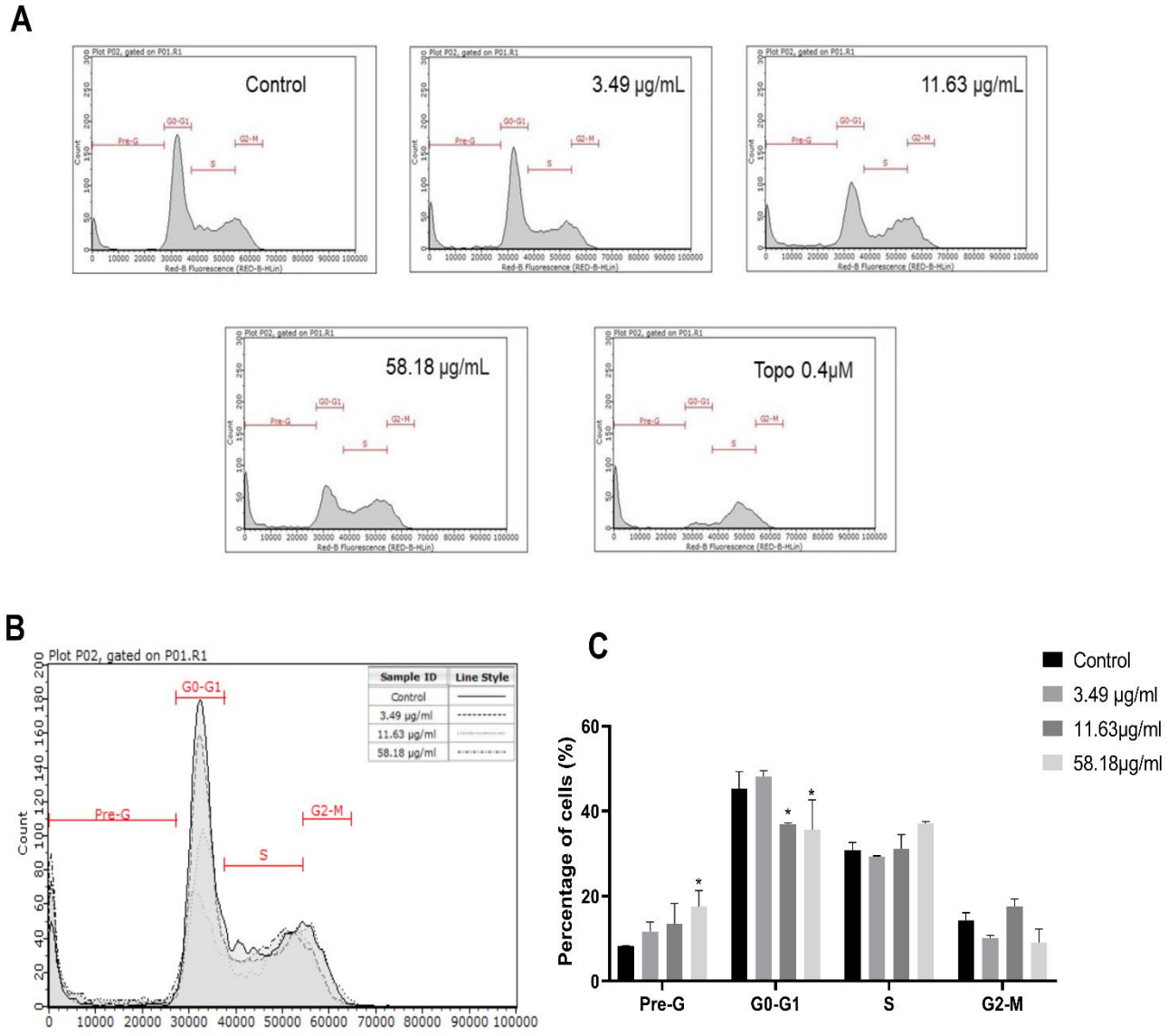


Figure 6: Cell cycle analysis of PI-stained MDA-MB-231 cells treated with increasing concentrations of SbBEE at 24h under flow cytometry (A.B). Distribution of MDA cells percentages within each phase of the cell cycle (C). Values were reported as mean ± SD. Significant differences were reported with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$ and *** indicating a p-value: $0.0001 < p < 0.001$.

3.3. SbBEE promotes DNA fragmentation in breast cancer cells

In order to determine the mechanism by which SbBEE exerts its cytotoxic effect on malignant cells, cell death assay (ELISA) was done to reveal the fragmentation of cellular DNA as a hallmark of apoptosis. The amount of fragmented DNA was quantified by calculating the enrichment factor representing the ratio of the absorbance of treated MDA-MB-231 to that of non-treated ones. The increase in DNA fragmentation was shown through an approximately 2-folds increase in the enrichment factor at IC50 **(Figure 7)**, a value that continues to increase upon increasing SbBEE concentration to reach 2.7-folds. These values were compared to the enrichment factor (4.29-fold) reached upon treating cells with 20µM of the positive control Topotecan.

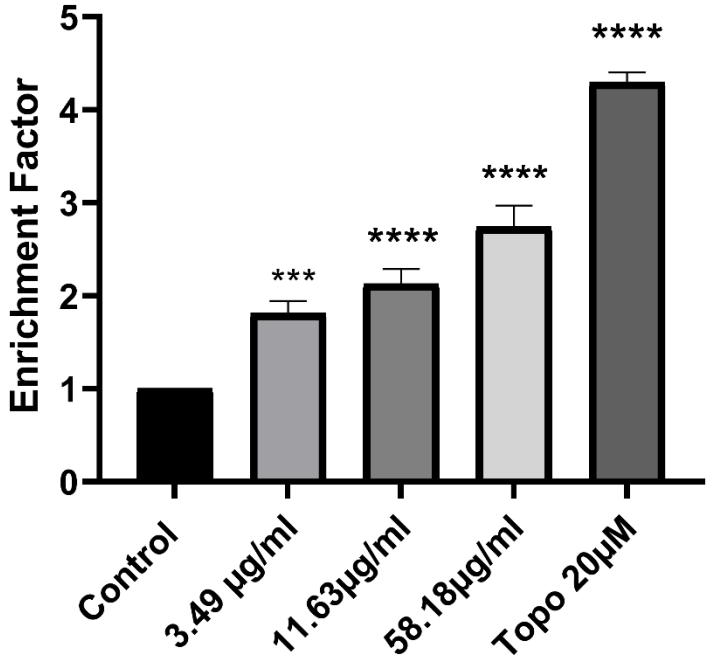


Figure 7: Cell Death ELISA. DNA fragmentation enrichment factor in MDA cells after 24h of treatment with SbBEE. Values were reported as mean ± SD. Significant differences were reported with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$, *** indicating a p-value: $0.0001 < p < 0.001$ and ****indicating a p-value: $p < 0.0001$.

3.4. SbBEE significantly induces apoptosis in treated MDA-MB-231

In order to confirm the apoptotic programmed cell death induced by SbBEE, a qualitative dual staining assay was done under fluorescent microscopy. Visualization of stained MDA-MB-231 cells revealed an important increase in AnnexinV binding to the outer cellular membrane in addition to propidium iodide penetration into the treated cells **(Figure 8A)**. Quantification showed that the fluorescence intensity of AnnexinV staining upon increasing SbBEE concentrations increases significantly to reach a value of 132.43 $\times 10^6$ at 58.18µg/ml treatment concentration, a value that is far more significant than the one reached by PI (71.54×10^6) at the same SbBEE concentration **(Figure 8B)**.

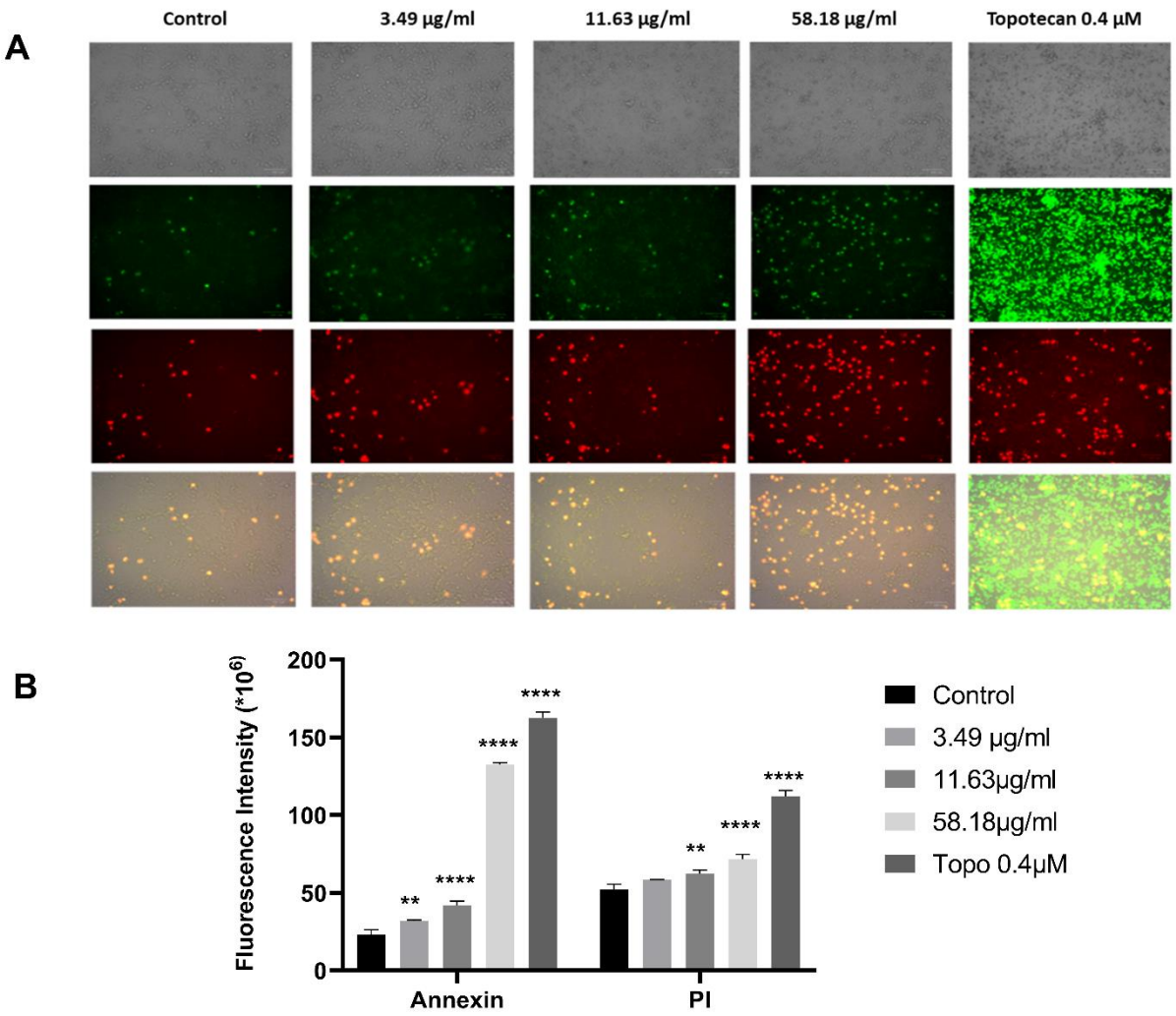


Figure 8: Annexin (Green)/PI (Red) staining under fluorescent microscopy (A). Quantification of fluorescence intensity in MDA cells after 24h of treatment with SbBEE (B). Values were reported as mean ± SD. Significant differences were reported with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$, *** indicating a p-value: $0.0001 < p < 0.001$ and ****indicating a p-value: $p < 0.0001$.

Another assay was performed in order to quantitatively assess the induction of the apoptotic mechanism through flow cytometry. After treatment, MDA-MB-231 cells were stained with Annexin V and PI, followed by classification into 4 different quadrants **(Figure 9A)** as follows: dual negative staining (living cells), annexin+/PI- staining (early apoptotic cells), annexin+/PI+ staining (late apoptotic cells) and annexin+/PI+ staining (necrotic cells) (starting with the quadrant at the bottom left and moving in a counterclockwise direction). In order to compare the distribution of cells according to their staining, a bar graph was plotted and it showed a potent decrease in living cells percentage accompanied with a significant increase in total apoptotic cells (both early and late) upon treatment with SbBEE: the percentage of living cells shifted from 92% (Control) to 56% (with 58.18µg/ml of SbBEE) while the percentage of apoptotic cells increased from 6.68 % to reach a value of 40.5% **(Figure 9B)**.

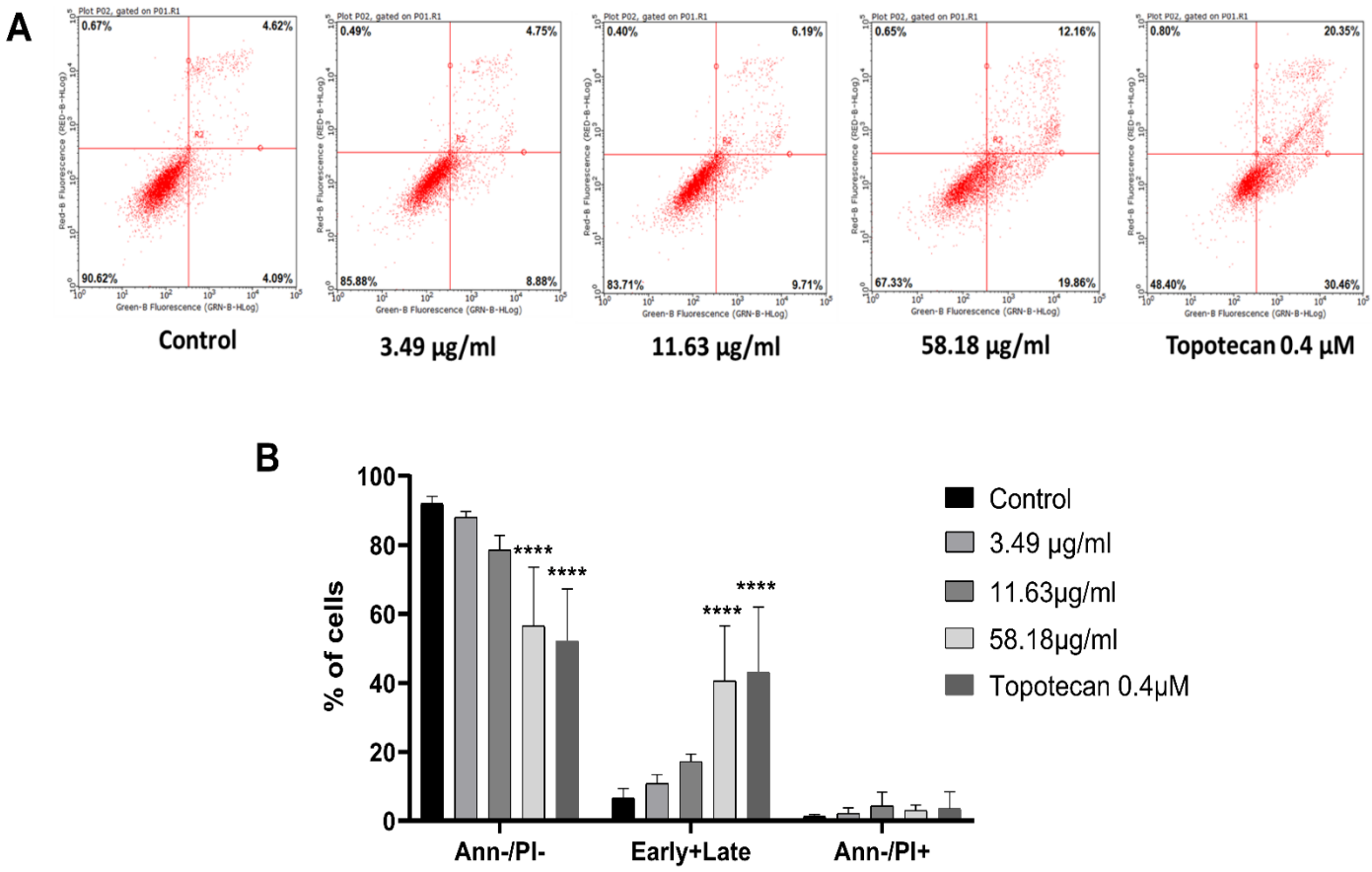


Figure 9: AnnexinV/PI staining under flow cytometry (A). Quantification of MDA cells percentage within each quadrant representing different stages of apoptosis achieved upon treatment with increasing SbBEE concentrations at 24h (B). Values were reported as mean ± SD. Significant differences were reported with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$, *** indicating a p-value: $0.0001 < p < 0.001$ and ****indicating a p-value: $p < 0.0001$.

3.5. SbBEE upregulates the expression of apoptosis inducing proteins

In order to determine the cellular proteins involved in the apoptotic signaling pathway induced by SbBEE on MDA-MB-231 cell line, a Western Blot analysis was performed following 24h of treatment with increasing concentrations of the extract. As shown in **Figure 10 (A and B)**, SbBEE induced an upregulation of cleaved PARP (c-PARP) while exerting an unsignificant effect on caspases (3-8-9) and the autophagy regulator Beclin-1 **(Figure 10 A, D)**. SbBEE upregulated also the expression of the pro-apoptotic protein Bax while not affecting the anti-apoptotic protein Bcl-2 expression which led to a significant increase in the Bax/Bcl-2 ratio **(Figure 10 A, C)**.

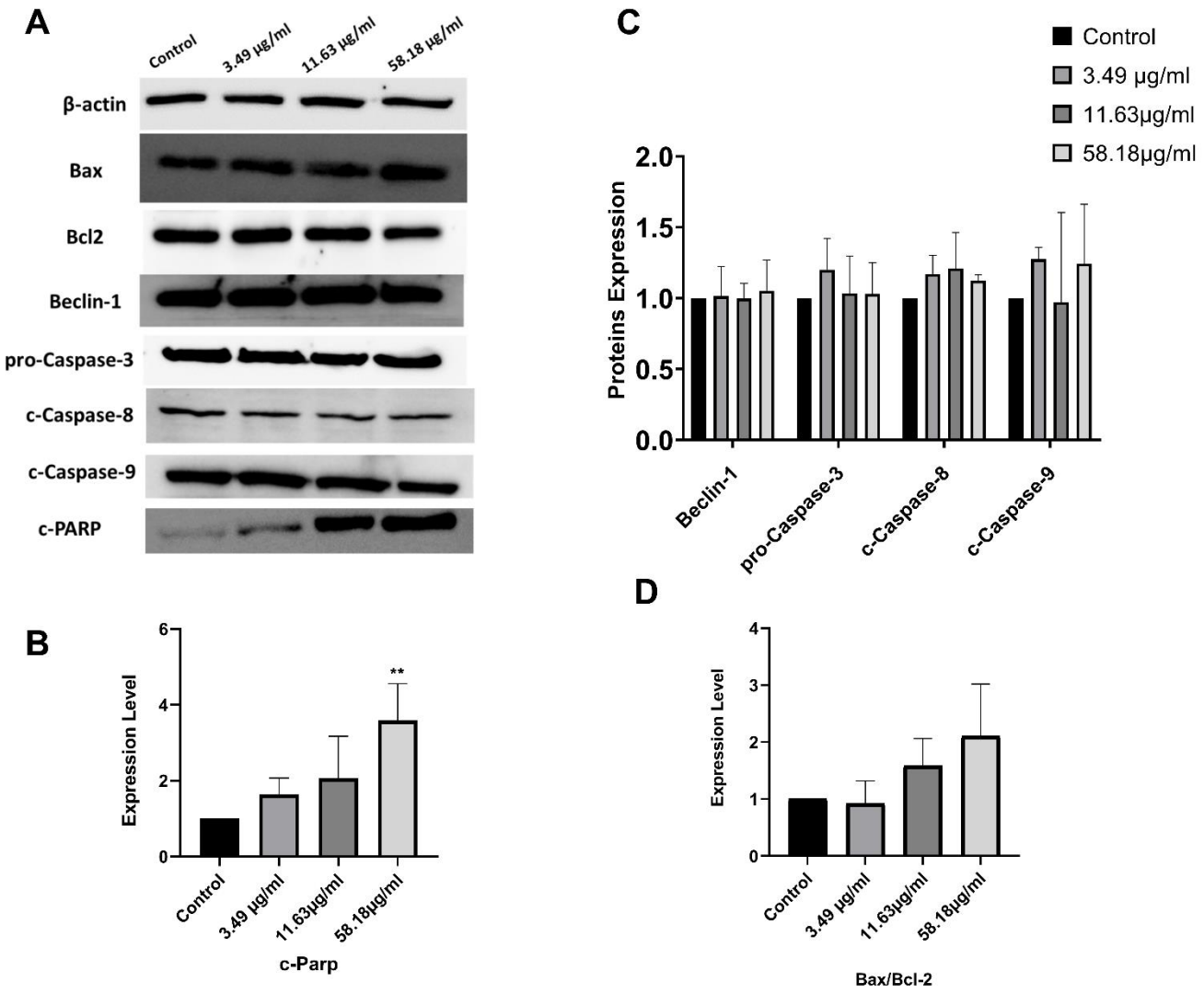


Figure 10: Western blot analysis and quantification of expression levels of apoptosis-regulating proteins in MDA-MB-231 cells treated with SbBEE for 24 h. Values were reported as mean ± SD. Significant differences were reported with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$ and *** indicating a p-value: $0.0001 < p < 0.001$.

3.6. SbBEE induces a cytochrome independent apoptotic pathway

While caspase expression was shown to be unaffected by the treatment, a subcellular fractionation assay was done to reveal the nature of the apoptotic pathway triggered by SbBEE and its dependency on cytochrome-c. Mitochondrial and cytosolic fractions were isolated and analyzed for the levels of cytochrome-c through Western-blots. Cyto-c levels remained almost constant in the mitochondria upon treatment with SbBEE and its expression was decreasing significantly in the cytosol. In contrast to SbBEE **(Figure 11)**, Cisplatin induced a significant release of cytochrome-c from the mitochondria into the cytosol.

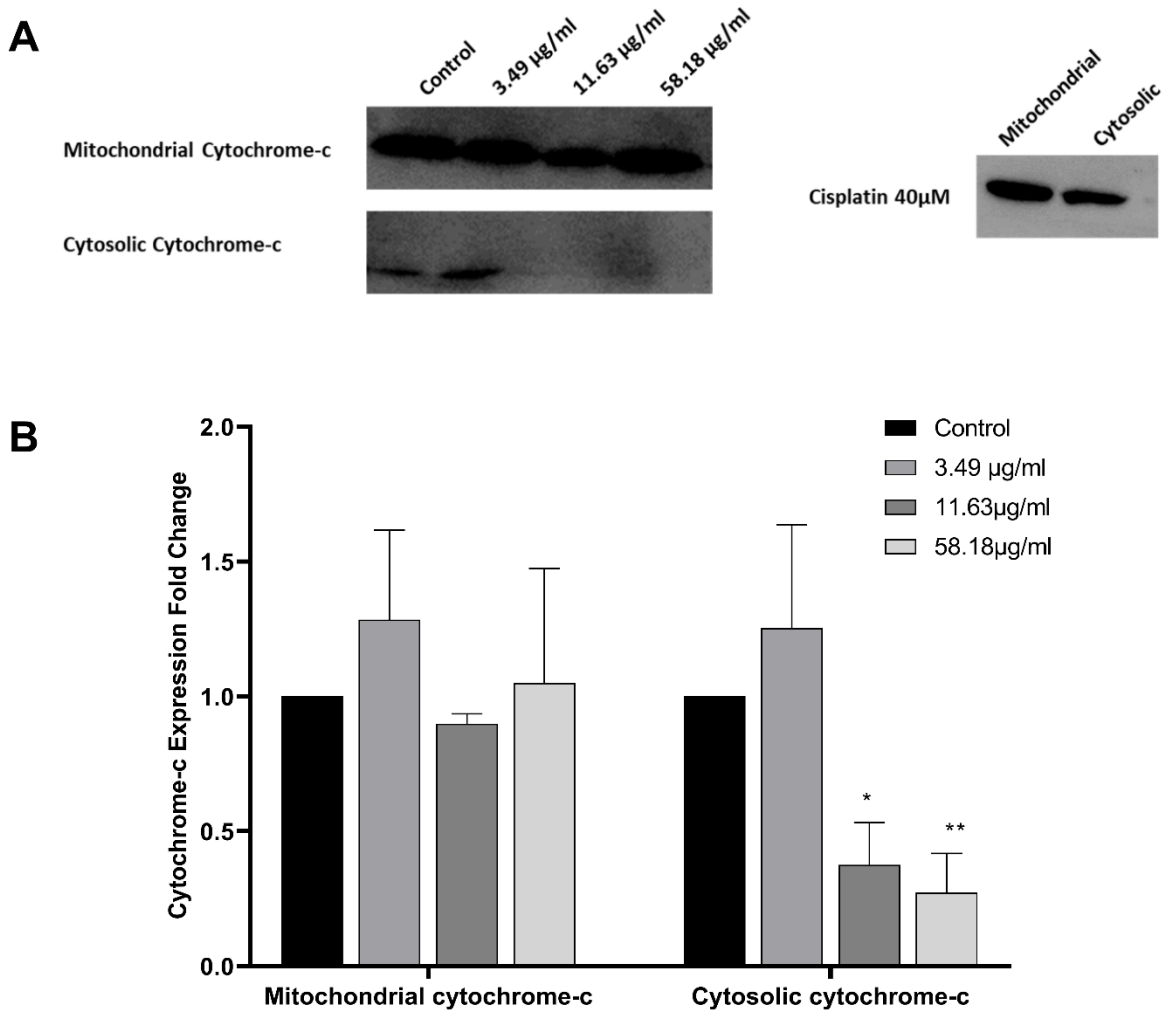


Figure 11: Western Immunoblotting of cytochrome-c in the mitochondrial versus cytosolic fraction of MDA-MB-231 cells treated with SbBEE. Values were reported as mean ± SD. Significant differences were reported with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$ and *** indicating a p-value: $0.0001 < p < 0.001$.

3.7. SbBEE inhibits ROS levels in MDA-MB-231 cells

In order to elucidate the effect of SbBEE on oxidative stress, the levels of reactive oxygen species were measured after 24h of treating MDA cells with increasing concentrations of *Sternbergia* extract when compared to ROS levels upon treatment with a potent inducer (TBHP) and an effective inhibitor (NAC). Results showed 0.9-fold decrease to 0.67 and 0.49 respectively when treated with increasing concentrations of the treatment (3.49 µg/ml <11.63 µg/ml ~ IC50< 58.18 µg/ml). These values were compared to the levels of ROS (0.19-fold and 1.5-fold) reached upon treating MDAs with 1Mm of the negative control (NAC) 30µM of the positive control (TBHP) respectively **(Figure 12)**. This down-regulation of ROS reveals the antioxidant potential of SbBEE.

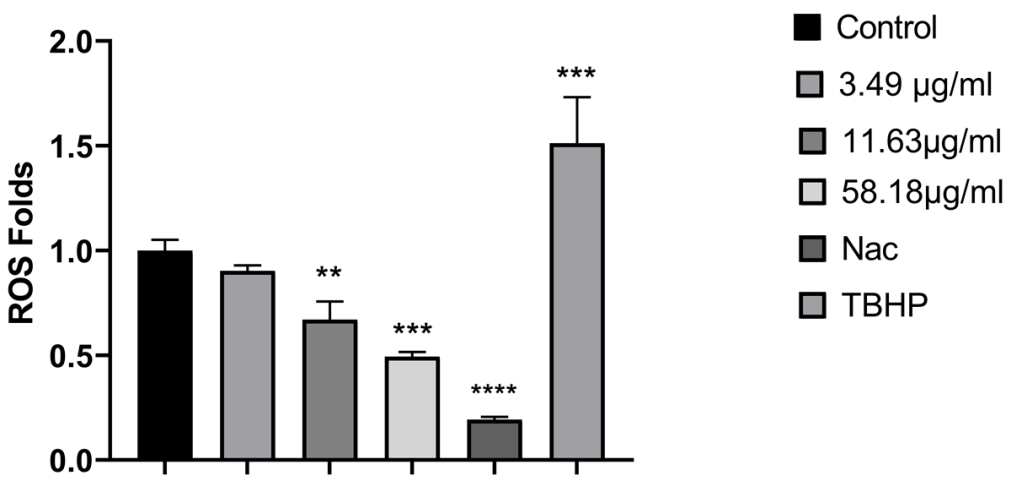


Figure 12: ROS levels detecting assay. Levels of Reactive oxygen species in MDA cells after 24h of treatment with SbBEE. Values were reported as mean ± SD. Significant differences were reported with * indicating a p-value: $0.01 < p < 0.05$, ** indicating a p-value: $0.001 < p < 0.01$, *** indicating a p-value: $0.0001 < p < 0.001$ and ****indicating a p-value: $p < 0.0001$.

CHAPTER FOUR

DISCUSSION

Phytochemical studies of plants are becoming more intensive due to their selective anticancer property and richness in bioactive natural compounds with minimal side effects (Kooti et al., 2017). According to recent experimental studies in addition to many traditional recommendations, several medicinal plants have been identified to have a preventive, antiproliferative, pro-apoptotic, or anti-metastatic effects on different tumoral cases (Hosseini & Ghorbani, 2015). Having a long and prominent place in the history of traditional and herbal medicine, Amaryllidaceae species gained a high medicinal value which is tightly associated with the presence of structurally unique complexes named Amaryllidaceae alkaloids (AA) (Cahlíková et al., 2020). Research has developed into inspecting the potential characteristics of Amaryllidaceae plants extracts including *Sternbergia clusiana* in order to prepare essential naturally derived drugs for several diseases including cancer. The aim of this study was to investigate the effective medicinal role of *Sternbergia clusiana* bulb Ethanolic Extract (SbBEE) in the treatment of breast cancer and study its mechanism of action in vitro.

This antiproliferative effect of Amaryllidaceae species was previously displayed through a significant growth inhibitory and cytotoxic action against a wide range of cancer cell lines including breast carcinoma cells MCF-7 and T47-D (Cedrón et al., 2015). Lycorine, a prominent Amaryllidaceae alkaloid, was also reported to exhibit promising anti-proliferative effects on breast cancer through the blockage of the Src/FAK (focal adhesion kinase)-involved pathway in MDA-MB-231 cells while preserving its selective potency

in vivo (Ying et al., 2017). These findings were supported in our study where SbBEE exerted a significant dose and time-dependent anti-proliferative effect on both in MDA-MB-231 and MCF-7 malignant cells. The extract selectivity was tested on MSCs that showed resistance against its cytotoxic effect even at very high doses strengthening the advantage of naturally based drugs in targeting cancer cells over basic non-selective chemotherapeutic drugs.

Noting the similar cytotoxic effect exerted on both MDA-MB-231 and MCF-7, the lethality of TNBC subtype and the chemoresistance it presents against many successful cancer treatments (Nedeljković & Damjanović, 2019), the rest of the experiments were carried out using the MDA-MB-231 cells in order to detect the mechanism of SbBEE leading to the death of TNBC cells. Remaining analyses were performed by treating the cells for 24 hours to be able to elucidate the underlying molecular changes triggered by SbBEE leading to the prominent cell death reported at 48 hours.

Several previously reported findings have revealed that the apoptotic pathway may be a key factor in programmed cancer cell death initiated by Amaryllidaceae alkaloids (J. Nair et al., 2016). Several experiments were performed in our study to decipher the underlying molecular mechanism by which the antiproliferative effect was exerted; the data obtained revealed a pro-apoptotic mode of action. We first investigated cell cycle progression using flow cytometry; SbBEE was shown to induce a significant increase in the pre-G0 phase indicating cellular fragmentation which could results from apoptotic cell death. Chromosomal DNA digestion into oligo-nucleosomal size fragments is an integral hallmark of apoptosis (Zhang & Xu, 2000) noticed through CDE assay which showed the efficacy of SbBEE in initiating genomic fragmentation. Dose-dependent apoptosis was further verified by fluorescence microscopy and flow cytometry through dual Annexin

V/PI staining, which confirmed the translocation of phosphatidylserine to the outer leaflet of treated cells membranes. Increasing SbBEE concentrations ((3.49 –58.18 μM) caused increasing apoptosis rates (13.63 % - 32.02%), indicative of a proportional relationship between SbBEE concentration and tumor cell apoptosis.

Cytotoxic cancer therapy involves numerous underlying mechanisms for the complex initiation of apoptosis which depends on the activation of pro-apoptotic cellular agents and the inhibition of anti-apoptotic moieties. In order to unravel the molecular pathway involved in apoptotic cell death upon SbBEE treatment, the expression of several proteins playing a role in the apoptotic pathway was examined. The results of Western Blots obtained upon SbBEE treatment revealed a caspase independent programmed death pathway in which c-PARP and Bax were upregulated. Moreover, no cytochrome-c release from the mitochondria was observed.

PARP proteins have a particular role in DNA repair mechanism allowing survival of tumoral cells (Morales et al., 2014) . This mechanism of defense is inactivated during apoptosis by cleavage of PARP producing an 89 kDa and a 24 kDa fragments comprising the catalytic domain and the DNA binding domain respectively (Soldani & Scovassi, 2002), a process that was clearly detected in our study. The cleavage of PARP was previously shown sufficient to induce an intrinsic apoptotic pathway that is unaffected by caspase inhibitors but involving Bax that was revealed upregulated in SbBEE treated cells (Xiang et al., 1996) (Yang et al., 2004) (Qi & Liu, 2006) . It has been widely proposed that the relative expression of death promoting proteins such as Bax governs the susceptibility of the cell to overcome the restriction or resistance to apoptosis brought by the overexpression of anti-apoptotic Bcl-family proteins (Khanzadeh et al., 2018). Previous studies explained the role of Bcl2 in regulating mitochondrial membrane

permeability and preventing proteolytic activation of caspase-3 within caspase-independent apoptotic pathways (Wang et al., 2016). These findings explain the constant expression of the inactivated pro-caspase-3 and accumulation of cytochrome-c in the mitochondria while Bcl2 expression remained insignificantly affected by SbBEE.

In addition to caspase-3, other caspases are usually recruited within the apoptotic cell death promoting the activation of an extrinsic pathway. Caspases 8 and 9 that are stimulated upon ligation of death receptors, are cleaved in order to trigger the execution phase of apoptosis. Treatment of MDA-MB-231 cells with SbBEE did not alter the expression of the active caspases 8 and 9 cleaved forms, indicating the exclusive activation of the intrinsic pathway leading to cell death.

Recent literature has reported the role of autophagy in programmed cell death since it is tightly associated with apoptosis, and its suppression is directly correlated to malignant transformation (Q. Chen et al., 2018). Hence, autophagy was also suspected to be involved in SbBEE mediated cancer cell death. The hyperexpression of the autophagy inductor Beclin-1 reduced the malignant phenotype in MCF-7 cells and inhibited their proliferation, a process that is naturally occurring in normal breast epithelia (Qi & Liu, 2006). The constant expression of Beclin-1 in SbBEE treated MDA-MB-231 indicates that autophagy was not involved in the programed cell death in our study.

Finally, reactive oxygen species (ROS) levels were quantified since they can act as important mediators of apoptosis (Brodská & Holoubek, 2011). The results indicated antioxidant characteristic of SbBEE which goes in line with a previous study aiming to determine the antioxidant capability of Sternbergia Clusiana bulbs extracts prepared in different alcoholic solvents and which showed that the ethanolic extract has the highest potential to stabilize the free radical DPPH (R.Mammadov, Y.Kara, 2011). Investigations

on many alkaloids isolated from the bulbs of different Amaryllidaceae species reported an interesting correlation between the antioxidant effect of these agents and their cytotoxic activity against a wide range of tumors (M. X. Chen et al., 2018).

CHAPTER FIVE

CONCLUSION

The anticancer properties of phytochemicals within Amaryllidaceae species have been recognized for centuries. Our study showed that *Sternbergia Clusiana* presents a considerable potential for the treatment of breast cancer through a caspase independent apoptotic mechanism. Further characterization experiments are warranted in order to identify and isolate the active compounds of the bulb extract used in the present study. In addition, much remains to be learned about pharmacokinetics in vivo, optimal dosages for living organisms and the long-term repercussions of the compounds proposed for tumoral treatment. It is suggested that the amalgamation of phytochemicals may have more potent effects than the same phytochemical taken separately. Based on this belief, combinations of the bioactive phytochemicals within SbBEE with other anticancer compounds may have more effect and yield more potent therapeutic complexes for cancer.

BIBLIOGRAPHY

Akram, M., Iqbal, M., Daniyal, M., & Khan, A. U. (2017). Awareness and current knowledge of breast cancer. In *Biological Research* (Vol. 50, Issue 1, p. 33). BioMed Central Ltd. https://doi.org/10.1186/s40659-017-0140-9

Ammoury, C., Younes, M., El Khoury, M., Hodroj, M. H., Haykal, T., Nasr, P., Sily, M., Taleb, R. I., Sarkis, R., Khalife, R., & Rizk, S. (2019). The pro-apoptotic effect of a Terpene-rich Annona cherimola leaf extract on leukemic cell lines. *BMC Complementary and Alternative Medicine*, *19*(1), 365. https://doi.org/10.1186/s12906-019-2768-1

Anders, C. K., & Carey, L. A. (2009). Biology, metastatic patterns, and treatment of patients with triple-negative breast cancer. *Clinical Breast Cancer*, *9*(SUPPL.2), S73. https://doi.org/10.3816/CBC.2009.s.008

Antoniou, A. C., & Easton, D. F. (2006). Models of genetic susceptibility to breast cancer. In *Oncogene* (Vol. 25, Issue 43, pp. 5898–5905). Oncogene. https://doi.org/10.1038/sj.onc.1209879

Aydin, Ç., Ermiş, A., & Mammadov, R. (2015). Phenolic Contents and Antioxidant Properties of Sternbergia lutea (L.) Ker-Gawl. Ex Sprengel Ethanol Extract. In *International Journal of Secondary Metabolite* (Vol. 2).

Bastida, J., Berkov, S., Torras, L., Belén, N., Jean, P., De Andrade, P., Martínez, V., Codina, C., & Viladomat, F. (n.d.). *3. Chemical and biological aspects of Amaryllidaceae alkaloids*.

Bouayed, J., & Bohn, T. (2010). Exogenous antioxidants - Double-edged swords in cellular redox state: Health beneficial effects at physiologic doses versus deleterious effects at high doses. In *Oxidative Medicine and Cellular Longevity* (Vol. 3, Issue 4, pp. 228–237). Hindawi Limited. https://doi.org/10.4161/oxim.3.4.12858

Bower, J. J., Vance, L. D., Psioda, M., Smith-Roe, S. L., Simpson, D. A., Ibrahim, J. G., Hoadley, K. A., Perou, C. M., & Kaufmann, W. K. (2017). Patterns of cell cycle checkpoint deregulation associated with intrinsic molecular subtypes of human breast cancer cells. *Npj Breast Cancer*, *3*(1), 1–12. https://doi.org/10.1038/s41523-017-0009-7

Breast cancer | McMaster Pathophysiology Review. (n.d.). Retrieved June 27, 2020, from http://www.pathophys.org/breast-cancer/

Brisken, C., & O'Malley, B. (2010). Hormone action in the mammary gland. In *Cold Spring Harbor perspectives in biology* (Vol. 2, Issue 12). Cold Spring Harbor Laboratory Press. https://doi.org/10.1101/cshperspect.a003178

Brodská, B., & Holoubek, A. (2011). Generation of Reactive Oxygen Species during

Apoptosis Induced by DNA-Damaging Agents and/or Histone Deacetylase Inhibitors. *Oxidative Medicine and Cellular Longevity*. https://doi.org/10.1155/2011/253529

Brooks, M. D., Burness, M. L., & Wicha, M. S. (2015). Therapeutic Implications of Cellular Heterogeneity and Plasticity in Breast Cancer. In *Cell Stem Cell* (Vol. 17, Issue 3, pp. 260–271). Cell Press. https://doi.org/10.1016/j.stem.2015.08.014

Cahlíková, L., Kawano, I., Řezáčová, M., Blunden, G., Hulcová, D., & Havelek, R. (2020). The Amaryllidaceae alkaloids haemanthamine, haemanthidine and their semisynthetic derivatives as potential drugs. In *Phytochemistry Reviews* (pp. 1–21). Springer. https://doi.org/10.1007/s11101-020-09675-8

Can Ağca, A., Yazgan Ekici, A. N., Yılmaz Sarıaltın, S., Çoban, T., Saltan İşcan, G., & Sever Yılmaz, B. (2020). Antioxidant, anti-inflammatory and antidiabetic activity of two Sternbergia taxons from Turkey. *South African Journal of Botany*. https://doi.org/10.1016/j.sajb.2020.04.002

Cedrón, J., Ravelo, Á., León, L., Padrón, J., & Estévez-Braun, A. (2015). Antiproliferative and Structure Activity Relationships of Amaryllidaceae Alkaloids. *Molecules*, *20*(8), 13854–13863. https://doi.org/10.3390/molecules200813854

Chavez, K. J., Garimella, S. V., & Lipkowitz, S. (2010). Triple negative breast cancer cell lines: One tool in the search for better treatment of triple negative breast cancer. *Breast Disease*, *32*(1–2), 35–48. https://doi.org/10.3233/BD-2010-0307

Cheang, M. C. U., Voduc, D., Bajdik, C., Leung, S., McKinney, S., Chia, S. K., Perou, C. M., & Nielsen, T. O. (2008). Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. *Clinical Cancer Research*, *14*(5), 1368–1376. https://doi.org/10.1158/1078-0432.CCR-07-1658

Chen, M. X., Huo, J. M., Hu, J., Xu, Z. P., & Zhang, X. (2018). Amaryllidaceae alkaloids from Crinum latifolium with cytotoxic, antimicrobial, antioxidant, and anti-inflammatory activities. *Fitoterapia*, *130*, 48–53. https://doi.org/10.1016/j.fitote.2018.08.003

Chen, Q., Kang, J., & Fu, C. (2018). The independence of and associations among apoptosis, autophagy, and necrosis. In *Signal Transduction and Targeted Therapy* (Vol. 3, Issue 1, pp. 1–11). Springer Nature. https://doi.org/10.1038/s41392-018-0018-5

Çitoğlu, G. S., Sener, B., Tankerl, M., Çitoğlul, G., Gümüşel2, B., & Şener3, B. (1996). Alkaloids of Sternbergia clusiana and their Analgesic Effects O Swets &Zeitlinger Aı,x,tı,oIDS oF SrnnxnnRGIA CLUSIANA AND THEIR ANİICUSIC EFFECTS. *Article in Pharmaceutical Biology*. https://doi.org/10.1076/phbi.34.3.194.13207

Cortes, N., Castañeda, C., Osorio, E. H., Cardona-Gomez, G. P., & Osorio, E. (2018). Amaryllidaceae alkaloids as agents with protective effects against oxidative neural

cell injury. *Life Sciences*, *203*, 54–65. https://doi.org/10.1016/j.lfs.2018.04.026

El Hachem, G., Gombos, A., & Awada, A. (2019). Recent advances in understanding breast cancer and emerging therapies with a focus on luminal and triple-negative breast cancer. *F1000Research*, *8*. https://doi.org/10.12688/f1000research.17542.1

El Khoury, M., Haykal, T., Hodroj, M. H., Najem, S. A., Sarkis, R., Taleb, R. I., & Rizk, S. (2020). Malva pseudolavatera Leaf Extract Promotes ROS Induction Leading to Apoptosis in Acute Myeloid Leukemia Cells In Vitro. *Cancers*, *12*(2), 435. https://doi.org/10.3390/cancers12020435

Evidente, A., & Kornienko, A. (2009). Anticancer evaluation of structurally diverse Amaryllidaceae alkaloids and their synthetic derivatives. *Phytochemistry Reviews*, *8*(2), 449–459. https://doi.org/10.1007/s11101-008-9119-z

Ferlay, J., Colombet, M., Soerjomataram, I., Mathers, C., Parkin, D. M., Piñeros, M., Znaor, A., & Bray, F. (2019). Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. In *International Journal of Cancer* (Vol. 144, Issue 8, pp. 1941–1953). https://doi.org/10.1002/ijc.31937

Fragomeni, S. M., Sciallis, A., & Jeruss, J. S. (2018). Molecular Subtypes and Local-Regional Control of Breast Cancer. In *Surgical Oncology Clinics of North America* (Vol. 27, Issue 1, pp. 95–120). W.B. Saunders. https://doi.org/10.1016/j.soc.2017.08.005

Fulda, S. (2010). Evasion of apoptosis as a cellular stress response in cancer. In *International Journal of Cell Biology*. https://doi.org/10.1155/2010/370835

Hamajima, N., Hirose, K., Tajima, K., Rohan, T., Friedenreich, C. M., Calle, E. E., Gapstur, S. M., Patel, A. V., Coates, R. J., Liff, J. M., Talamini, R., Chantarakul, N., Koetsawang, S., Rachawat, D., Marcou, Y., Kakouri, E., Duffy, S. W., Morabia, A., Schuman, L., … Fukao, A. (2012). Menarche, menopause, and breast cancer risk: Individual participant meta-analysis, including 118 964 women with breast cancer from 117 epidemiological studies. *The Lancet Oncology*, *13*(11), 1141–1151. https://doi.org/10.1016/S1470-2045(12)70425-4

Haykal, T., Nasr, P., Hodroj, M. H., Taleb, R. I., Sarkis, R., Moujabber, M. N. E., & Rizk, S. (2019). Annona cherimola Seed Extract Activates Extrinsic and Intrinsic Apoptotic Pathways in Leukemic Cells. *Toxins*, *11*(9), 506. https://doi.org/10.3390/toxins11090506

Haznedaroglu, M. Z., & Gokce, G. (2014). Comparison of anti-acetylcholinesterase activity of bulb and leaf extracts of Sternbergia candida Mathew & T. Baytop. *Acta Biologica Hungarica*, *65*(4), 396–404. https://doi.org/10.1556/ABiol.65.2014.4.4

Hodroj, M. H., Jardaly, A., Raad, S. A., Zouein, A., & Rizk, S. (2018). Andrographolide potentiates the antitumor effect of topotecan in acute myeloid leukemia cells through an intrinsic apoptotic pathway. *Cancer Management and Research*, *10*, 1079–1088.

https://doi.org/10.2147/CMAR.S160924

Holliday, D. L., & Speirs, V. (2011). Choosing the right cell line for breast cancer research. In *Breast Cancer Research* (Vol. 13, Issue 4, p. 215). BioMed Central. https://doi.org/10.1186/bcr2889

Hosseini, A., & Ghorbani, A. (2015). Cancer therapy with phytochemicals: evidence from clinical studies. *Avicenna Journal of Phytomedicine*, *5*(2), 84–97. https://doi.org/10.22038/ajp.2015.3872

Hulcová, D., Maříková, J., Korábečný, J., Hošťálková, A., Jun, D., Kuneš, J., Chlebek, J., Opletal, L., De Simone, A., Nováková, L., Andrisano, V., Růžička, A., & Cahlíková, L. (2019). Amaryllidaceae alkaloids from Narcissus pseudonarcissus L. cv. Dutch Master as potential drugs in treatment of Alzheimer's disease. *Phytochemistry*, *165*, 112055. https://doi.org/10.1016/j.phytochem.2019.112055

J. Nair, J., van Staden, J., & Bastida, J. (2016). Apoptosis-Inducing Effects of Amaryllidaceae Alkaloids. *Current Medicinal Chemistry*, *23*(2), 161–185. https://doi.org/10.2174/0929867323666151118121124

Jain, R., Kosta, S., & Tiwari, A. (2010). Ayurveda and cancer. In *Pharmacognosy Research* (Vol. 2, Issue 6, pp. 393–394). Wolters Kluwer -- Medknow Publications. https://doi.org/10.4103/0974-8490.75463

Kaskatepe, B., Yilmaz, B. S., Acikara, O. B., Iscan, G. S., Vlainic, J., & Kosalec, I. (2019). Antifungal activity of some Sternbergia taxa: Effects on germ tube and biofilm formation. *Brazilian Journal of Pharmaceutical Sciences*, *55*, 17200. https://doi.org/10.1590/s2175-97902019000117200

Khanzadeh, T., Hagh, M. F., Talebi, M., Yousefi, B., Azimi, A., Feizi, A. A. H. P., & Baradaran, B. (2018). Investigation of BAX and BCL2 expression and apoptosis in a resveratrol- and prednisolone-treated human T-ALL cell line, CCRF-CEM. *Blood Research*, *53*(1), 53–60. https://doi.org/10.5045/br.2018.53.1.53

Kooti, W., Servatyari, K., Behzadifar, M., Asadi-Samani, M., Sadeghi, F., Nouri, B., & Zare Marzouni, H. (2017). Effective Medicinal Plant in Cancer Treatment, Part 2: Review Study. In *Journal of Evidence-Based Complementary and Alternative Medicine* (Vol. 22, Issue 4, pp. 982–995). SAGE Publications Ltd. https://doi.org/10.1177/2156587217696927

Kornienko, A., & Evidente, A. (2008). Chemistry, biology, and medicinal potential of narciclasine and its congeners. *Chemical Reviews*, *108*(6), 1982–2014. https://doi.org/10.1021/cr078198u

Kumar, N., Patni, P., Agarwal, A., Khan, M. A., & Parashar, N. (2015). Prevalence of molecular subtypes of invasive breast cancer: A retrospective study. *Medical Journal Armed Forces India*, *71*(3), 254–258. https://doi.org/10.1016/j.mjafi.2015.04.006

Leporini, M., Catinella, G., Bruno, M., Falco, T., Tundis, R., & Loizzo, M. R. (2018). Investigating the Antiproliferative and Antioxidant Properties of Pancratium maritimum L. (Amaryllidaceae) Stems, Flowers, Bulbs, and Fruits Extracts. *Evidence-Based Complementary and Alternative Medicine*, *2018*. https://doi.org/10.1155/2018/9301247

Liu, C., Zhang, K., Shen, H., Yao, X., Sun, Q., & Chen, G. (2018). Necroptosis: A novel manner of cell death, associated with stroke (Review). In *International Journal of Molecular Medicine* (Vol. 41, Issue 2, pp. 624–630). Spandidos Publications. https://doi.org/10.3892/ijmm.2017.3279

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. In *Pharmacognosy Reviews* (Vol. 4, Issue 8, pp. 118–126). https://doi.org/10.4103/0973-7847.70902

Malhotra, G. K., Zhao, X., Band, H., & Band, V. (2010). Histological, molecular and functional subtypes of breast cancers. In *Cancer Biology and Therapy* (Vol. 10, Issue 10, pp. 955–960). Taylor & Francis. https://doi.org/10.4161/cbt.10.10.13879

Martinez-Peinado, N., Cortes-Serra, N., Torras-Claveria, L., Pinazo, M. J., Gascon, J., Bastida, J., & Alonso-Padilla, J. (2020). Amaryllidaceae alkaloids with anti-Trypanosoma cruzi activity. *Parasites and Vectors*, *13*(1), 299. https://doi.org/10.1186/s13071-020-04171-6

Mavaddat, N., Antoniou, A. C., Easton, D. F., & Garcia-Closas, M. (2010). Genetic susceptibility to breast cancer. In *Molecular Oncology* (Vol. 4, Issue 3, pp. 174–191). John Wiley and Sons Ltd. https://doi.org/10.1016/j.molonc.2010.04.011

Morales, J. C., Li, L., Fattah, F. J., Dong, Y., Bey, E. A., Patel, M., Gao, J., & Boothman, D. A. (2014). Review of poly (ADP-ribose) polymerase (PARP) mechanisms of action and rationale for targeting in cancer and other diseases. *Critical Reviews in Eukaryotic Gene Expression*, *24*(1), 15–28. https://doi.org/10.1615/CritRevEukaryotGeneExpr.2013006875

Najar, M., Fayyad-Kazan, H., Faour, W. H., Merimi, M., Sokal, E. M., Lombard, C. A., & Fahmi, H. (2019). Immunological modulation following bone marrow-derived mesenchymal stromal cells and Th17 lymphocyte co-cultures. *Inflammation Research*, *68*(3), 203–213. https://doi.org/10.1007/s00011-018-1205-0

Nedeljković, M., & Damjanović, A. (2019). Mechanisms of Chemotherapy Resistance in Triple-Negative Breast Cancer-How We Can Rise to the Challenge. In *Cells* (Vol. 8, Issue 9). NLM (Medline). https://doi.org/10.3390/cells8090957

Nikoletopoulou, V., Markaki, M., Palikaras, K., & Tavernarakis, N. (2013). Crosstalk between apoptosis, necrosis and autophagy. In *Biochimica et Biophysica Acta - Molecular Cell Research* (Vol. 1833, Issue 12, pp. 3448–3459). https://doi.org/10.1016/j.bbamcr.2013.06.001

Oluogun, W. A., Adedokun, K. A., Oyenike, M. A., & Adeyeba, O. A. (2019). Histological classification, grading, staging, and prognostic indexing of female breast cancer in an African population: A 10-year retrospective study. *International Journal of Health Sciences*, *13*(4), 3–9. http://www.ncbi.nlm.nih.gov/pubmed/31341449

Orhan, I. E., Yilmaz, B. S., Altun, M. L., Saltan, G., & Şener, B. (2011). Anti-acetylcholinesterase and antioxidant appraisal of the bulb extracts of five Sternbergia species. *Records of Natural Products*, *5*(3), 193–201.

Otto, T., & Sicinski, P. (2017). Cell cycle proteins as promising targets in cancer therapy. In *Nature Reviews Cancer* (Vol. 17, Issue 2, pp. 93–115). Nature Publishing Group. https://doi.org/10.1038/nrc.2016.138

Pawar, S. R., Jangam, S. S., & Waghmare, S. A. (2018). Anti-Cancer Herble Drugs: an Overview. *Journal of Drug Delivery and Therapeutics*, *8*(4), 48–58. https://doi.org/10.22270/jddt.v8i4.1749

Picon-Ruiz, M., Morata-Tarifa, C., Valle-Goffin, J. J., Friedman, E. R., & Slingerland, J. M. (2017). Obesity and adverse breast cancer risk and outcome: Mechanistic insights and strategies for intervention. *CA: A Cancer Journal for Clinicians*, *67*(5), 378–397. https://doi.org/10.3322/caac.21405

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D., & Bitto, A. (2017). Oxidative Stress: Harms and Benefits for Human Health. In *Oxidative Medicine and Cellular Longevity* (Vol. 2017). Hindawi Limited. https://doi.org/10.1155/2017/8416763

Prokhorova, E. A., Kopeina, G. S., Lavrik, I. N., & Zhivotovsky, B. (2018). Apoptosis regulation by subcellular relocation of caspases. *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-018-30652-x

Qi, R., & Liu, X. Y. (2006). New advance in caspase-independent programmed cell death and its potential in cancer therapy. *Int J Biomed Sci*, *2*(3), 211–216. http://www.ncbi.nlm.nih.gov/pubmed/23674984%5Cnhttp://www.ncbi.nlm.nih.gov/pmc/articles/PMC3614612/pdf/IJBS-2-211.pdf

R.Mammadov, Y.Kara, H. E. V. (2011). *(20) Study on the Phenolic Content, Antioxidant and Antimicrobial Effects of Sternbergia clusiana | Request PDF*. 12 August 2011. https://www.researchgate.net/publication/288582936_Study_on_the_Phenolic_Content_Antioxidant_and_Antimicrobial_Effects_of_Sternbergia_clusiana

Reeves, G. K., Pirie, K., Green, J., Bull, D., & Beral, V. (2012). Comparison of the effects of genetic and environmental risk factors on in situ and invasive ductal breast cancer. *International Journal of Cancer*, *131*(4), 930–937. https://doi.org/10.1002/ijc.26460

Roy, M., Liang, L., Xiao, X., Feng, P., Ye, M., & Liu, J. (2018). Lycorine: A prospective natural lead for anticancer drug discovery. In *Biomedicine and Pharmacotherapy*

(Vol. 107, pp. 615–624). Elsevier Masson SAS. https://doi.org/10.1016/j.biopha.2018.07.147

Sakamoto, T., Niiya, D., & Seiki, M. (2011). Targeting the Warburg effect that arises in tumor cells expressing membrane type-1 matrix metalloproteinase. *Journal of Biological Chemistry*, *286*(16), 14691–14704. https://doi.org/10.1074/jbc.M110.188714

Savitskaya, M. A., & Onishchenko, G. E. (2015). Mechanisms of apoptosis. In *Biochemistry (Moscow)* (Vol. 80, Issue 11, pp. 1393–1405). Maik Nauka Publishing / Springer SBM. https://doi.org/10.1134/S0006297915110012

Schmidt, S., Monk, J. M., Robinson, L. E., & Mourtzakis, M. (2015). The integrative role of leptin, oestrogen and the insulin family in obesity-associated breast cancer: Potential effects of exercise. *Obesity Reviews*, *16*(6), 473–487. https://doi.org/10.1111/obr.12281

Shimizu, S., Yoshida, T., Tsujioka, M., & Arakawa, S. (2014). Autophagic cell death and cancer. In *International Journal of Molecular Sciences* (Vol. 15, Issue 2, pp. 3145–3153). Molecular Diversity Preservation International. https://doi.org/10.3390/ijms15023145

Soldani, C., & Scovassi, A. I. (2002). Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: An update. In *Apoptosis* (Vol. 7). Kluwer Academic Publishers.

Soleimani, M., & Nadri, S. (2009). A protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow. *Nature Protocols*, *4*(1), 102–106. https://doi.org/10.1038/nprot.2008.221

Staging & Grade - Breast Pathology | Johns Hopkins Pathology. (n.d.). Retrieved July 5, 2020, from https://pathology.jhu.edu/breast/staging-grade/

Subramani, R., Nandy, S. B., Pedroza, D. A., & Lakshmanaswamy, R. (2017). Role of growth hormone in breast cancer. In *Endocrinology* (Vol. 158, Issue 6, pp. 1543–1555). Endocrine Society. https://doi.org/10.1210/en.2016-1928

Tan, M., & Yu, D. (2013). *Molecular Mechanisms of ErbB2-Mediated Breast Cancer Chemoresistance*. https://www.ncbi.nlm.nih.gov/books/NBK6194/

Tannous, S., Haykal, T., Dhaini, J., Hodroj, M. H., & Rizk, S. (2020). The anti-cancer effect of flaxseed lignan derivatives on different acute myeloid leukemia cancer cells. *Biomedicine and Pharmacotherapy*, *132*, 110884. https://doi.org/10.1016/j.biopha.2020.110884

Theodossiou, T. A., Ali, M., Grigalavicius, M., Grallert, B., Dillard, P., Schink, K. O., Olsen, C. E., Wälchli, S., Inderberg, E. M., Kubin, A., Peng, Q., & Berg, K. (2019). Simultaneous defeat of MCF7 and MDA-MB-231 resistances by a hypericin PDT–tamoxifen hybrid therapy. *Npj Breast Cancer*, *5*(1), 1–10.

https://doi.org/10.1038/s41523-019-0108-8

Wang, Q., Zhang, L., Yuan, X., Ou, Y., Zhu, X., Cheng, Z., Zhang, P., Wu, X., Meng, Y., & Zhang, L. (2016). The relationship between the Bcl-2/Bax proteins and the mitochondria-mediated apoptosis pathway in the differentiation of adipose-derived stromal cells into neurons. *PLoS ONE*, *11*(10). https://doi.org/10.1371/journal.pone.0163327

Xiang, J., Chao, D. T., & Korsmeyer, S. J. (1996). BAX-induced cell death may not require interleukin 1-converting enzyme-like proteases (apoptosiscysteine proteaseFASmitochondriareactive oxygen species). In *Cell Biology* (Vol. 93).

Yang, Y., Zhao, S., & Song, J. (2004). Caspase-dependent apoptosis and -independent poly(ADP-ribose) polymerase cleavage induced by transforming growth factor β1. *International Journal of Biochemistry and Cell Biology*, *36*(2), 223–234. https://doi.org/10.1016/S1357-2725(03)00215-2

Ying, X., Huang, A., Xing, Y., Lan, L., Yi, Z., & He, P. (2017). Lycorine inhibits breast cancer growth and metastasis via inducing apoptosis and blocking Src/FAK-involved pathway. *Science China Life Sciences*, *60*(4), 417–428. https://doi.org/10.1007/s11427-016-0368-y

Younes, M., Ammoury, C., Haykal, T., Nasr, L., Sarkis, R., & Rizk, S. (2020). The selective anti-proliferative and pro-apoptotic effect of A. cherimola on MDA-MB-231 breast cancer cell line. *BMC Complementary Medicine and Therapies*, *20*(1), 343. https://doi.org/10.1186/s12906-020-03120-1

Youssef, S., Mahmood, A., & Vela, E. (2017). On the genus Sternbergia (Amaryllidaceae) in Iraq. *Anales Del Jardin Botanico de Madrid*, *74*(1), 1–6. https://doi.org/10.3989/ajbm.2451

Zhang, J. H., & Xu, M. (2000). DNA fragmentation in apoptosis. In *Cell Research* (Vol. 10, Issue 3, pp. 205–211). Science Press. https://doi.org/10.1038/sj.cr.7290049