Lebanese American University

*Sternbergia Clusiana* Bulb Ethanolic Extracts Induce Apoptosis in MCF-7 Breast Cancer Cells: Molecular Mechanisms and Chemical Characterization

By

Nazira Issam Elias

A thesis
Submitted in partial fulfillment of the requirements for the degree of Master of Science in Molecular Biology

School of Arts and Sciences
May 2022
Student Name: Nazira Issam Elias

I.D. #: 201608562

Thesis Title: Sancha klugii bulb ethanolic extracts induce apoptosis in MCF7 breast cancer cells: molecular mechanisms and chemical characterization

Program: MS Biological Sciences

Department: Natural Sciences

School: Arts and Sciences

The undersigned certify that they have examined the final electronic copy of this thesis and approved it in Partial Fulfillment of the requirements for the degree of:

MS

in the major of Biological Sciences

Thesis Advisor's Name: Sandra Rizk-Jamati

Signature: [Redacted]

Date: 5 / 5 / 2022

Committee Member's Name: Ahmad Houri

Signature: [Redacted]

Date: 5 / 5 / 2022

Committee Member's Name: Christian Khallil

Signature: [Redacted]

Date: 5 / 5 / 2022
By signing and submitting this license, you (the author(s) or copyright owner) grants the Lebanese American University (LAU) the non-exclusive right to reproduce, translate (as defined below), and/or distribute your submission (including the abstract) worldwide in print and electronic formats and in any medium, including but not limited to audio or video. You agree that LAU may, without changing the content, translate the submission to any medium or format for the purpose of preservation. You also agree that LAU may keep more than one copy of this submission for purposes of security, backup and preservation. You represent that the submission is your original work, and that you have the right to grant the rights contained in this license. You also represent that your submission does not, to the best of your knowledge, infringe upon anyone’s copyright. If the submission contains material for which you do not hold copyright, you represent that you have obtained the unrestricted permission of the copyright owner to grant LAU the rights required by this license, and that such third-party owned material is clearly identified and acknowledged within the text or content of the submission. IF THE SUBMISSION IS BASED UPON WORK THAT HAS BEEN SPONSORED OR SUPPORTED BY AN AGENCY OR ORGANIZATION OTHER THAN LAU, YOU REPRESENT THAT YOU HAVE FULLFILLED ANY RIGHT OF REVIEW OR OTHER OBLIGATIONS REQUIRED BY SUCH CONTRACT OR AGREEMENT. LAU will clearly identify your name(s) as the author(s) or owner(s) of the submission, and will not make any alteration, other than as allowed by this license, to your submission.

Name: Nazira Issam Elias
Signature:
Date: 08/05/2022
PLAGIARISM POLICY COMPLIANCE STATEMENT

I certify that:

1. I have read and understood LAU’s Plagiarism Policy.
2. I understand that failure to comply with this Policy can lead to academic and disciplinary actions against me.
3. This work is substantially my own, and to the extent that any part of this work is not my own I have indicated that by acknowledging its sources.

Name: Nazira Issam Elias
Signature: 
Date: 

Dedication Page

To my Dad in Heaven and my Mom
ACKNOWLEDGMENT

First and foremost, I want to thank God Almighty for giving me the strength to complete my research, especially after losing one of the dearest people to my heart, my Dad.

I would also like to express my sincere gratitude to a number of people without whom this project would not have been possible.

Special thanks to my advisor, Prof. Sandra Rizk-Jamati, for lending me a helping hand and offering me constant support and guidance all throughout the journey of writing my project. Her advice and insight into this subject matter increased my knowledge and helped me grow as a person and a young professional.

Besides my advisor, I would like to thank my committee members, Dr. Ahmad Houri and Dr. Christian Khalil, for taking part in this process and reading my thesis.

Thanks to my colleague, Maria Younes, from the Byblos lab team, who tolerated the ton of questions and extra work I presented to her.

And finally, thanks to my Mom, my grandparents, my sister, Larissa and my friend, Elsie, who were always there for me and offered me unconditional love and support during this period.
Sternbergia Clusiana Bulb Ethanolic Extracts Induce Apoptosis in MCF-7 Breast Cancer Cells: Molecular Mechanisms and Chemical Characterization

Nazira Issam Elias

ABSTRACT

*Sternbergia* is a genus of plants belonging to the Amaryllidaceae family which is a popular ornamental plant. Plant leaf and bulb extracts of various *Sternbergia* species have been proven to possess diverse significant biological effects and have been historically employed in traditional medicine. However, the anti-tumor effects of *Sternbergia clusiana* bulbs in particular has not been investigated to date. The present study explores the anti-cancer activity of the ethanolic extract of *Sternbergia clusiana* bulbs (SbBEE) on breast cancer MCF-7 cell line *in vitro* and deciphers the underlying molecular mechanism associated with it. Treatment of MCF-7 cells with SbBEE showed a dose- and time-dependent anti-proliferative effect along with the occurrence of numerous apoptotic hallmarks. A dose-dependent increase in apoptosis via induced DNA fragmentation was revealed via Cell Death ELISA as well as an increase in cellular fragmentation using flow cytometry. On a molecular level, western blot analysis showed an upregulation of pro-apoptotic proteins such as poly(ADP-ribose) polymerase (PARP), Bax/Bcl-2 ratio and caspase-9, with no alterations in caspase-8 levels, revealing the activation of the intrinsic apoptotic pathway. Moreover, SbBEE exposure caused a significant reduction in ROS production proposing a potent anti-oxidant potential. A number of chemical compounds that contributed to SbBEE’s pro-apoptotic activity on MCF-7 cells were detected by liquid chromatography.
coupled with mass spectrometry, including lycorine, 9S,13R-12-Oxophytodienoic acid (OPDA), fumaritine N-oxide, rhamnetin and ferulic acid. The data presented in this study reveal promising anti-cancerous effects of SbBEE on MCF-7 breast cancer cells in vitro that should be further evaluated in vivo.

Keywords: Sternbergia Clusiana, ROS, MCF-7 Cells, Medicinal Plants, Breast Cancer, Apoptosis
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I- Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Background on Cancer</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Types of Cancer</td>
<td>2</td>
</tr>
<tr>
<td>1.3 Breast Cancer</td>
<td>3</td>
</tr>
<tr>
<td>1.3.1 What is Breast Cancer?</td>
<td>3</td>
</tr>
<tr>
<td>1.3.2 Breast Cancer Types and Classifications</td>
<td>3</td>
</tr>
<tr>
<td>1.3.3 What Causes Breast Cancer?</td>
<td>7</td>
</tr>
<tr>
<td>1.4 Cell Cycle</td>
<td>8</td>
</tr>
<tr>
<td>1.4.1 Checkpoints and Cyclin-Dependent Kinases (CDKs)</td>
<td>9</td>
</tr>
<tr>
<td>1.4.2 Dysregulation of CDKs in Breast Cancer</td>
<td>10</td>
</tr>
<tr>
<td>1.5 Apoptosis</td>
<td>11</td>
</tr>
<tr>
<td>1.6 Therapy for Breast Cancer</td>
<td>13</td>
</tr>
<tr>
<td>1.7 Plant-Based Therapy</td>
<td>15</td>
</tr>
<tr>
<td>1.7.1 Brief Historical Review</td>
<td>16</td>
</tr>
<tr>
<td>1.7.2 Phytochemicals and Anticancer Agents in Plants</td>
<td>16</td>
</tr>
<tr>
<td>1.8 Plant Under Investigation: Sternbergia Clusiana</td>
<td>19</td>
</tr>
<tr>
<td>1.8.1 Components, Properties and Uses</td>
<td>20</td>
</tr>
<tr>
<td>1.8.2 Anticancer Properties</td>
<td>21</td>
</tr>
<tr>
<td>1.8.3 Additional Anticancer Potential of the Amaryllidaceae Family</td>
<td>23</td>
</tr>
<tr>
<td>1.9 Purpose of the Study</td>
<td>24</td>
</tr>
<tr>
<td>II-Materials and Methods</td>
<td>25</td>
</tr>
<tr>
<td>2.1 Plant Material</td>
<td>25</td>
</tr>
<tr>
<td>2.2 Sternbergia Bulb Ethanolic Extract Preparation (SbBEE)</td>
<td>25</td>
</tr>
<tr>
<td>2.3 Breast Cancer Cell Culture</td>
<td>26</td>
</tr>
<tr>
<td>2.4 MTS Assay</td>
<td>26</td>
</tr>
<tr>
<td>2.5 Cell Death ELISA</td>
<td>27</td>
</tr>
<tr>
<td>2.6 Cell Cycle Analysis</td>
<td>27</td>
</tr>
<tr>
<td>2.7 Western Blot</td>
<td>28</td>
</tr>
<tr>
<td>2.8 Reactive Oxygen Species (ROS) Detection</td>
<td>29</td>
</tr>
<tr>
<td>2.9 Statistical Analysis</td>
<td>30</td>
</tr>
</tbody>
</table>
2.10 Liquid Chromatography Mass Spectrometry Analysis ........................................ 30

III-Results.......................................................................................................................... 32

3.1 The anti-proliferative effect of *Sternbergia* Bulb Ethanolic Extract on MCF-7 cells. 32

3.2 *Sternbergia* Bulb Ethanolic Extract induces a dose-dependent cellular fragmentation ................................................................................................................ 33

3.3 *Sternbergia* Bulb Ethanolic Extract induces DNA fragmentation in MCF-7 cells 34

3.4 *Sternbergia* Bulb Ethanolic Extract promotes the activation of the mitochondrial apoptotic pathway in MCF-7 cells................................................................. 35

3.5 *Sternbergia* Bulb Ethanolic Extract has an antioxidant effect on MCF-7 cells 36

3.6 Chemical Elucidation of SbBEE using Liquid Chromatography Mass Spectrometry Analysis ................................................................................................................ 37

IV-Discussion..................................................................................................................... 41

V-Conclusion ...................................................................................................................... 48

References.......................................................................................................................... 49
List of Figures

Figure 1: Cell cycle progression through checkpoints ................................................................. 9
Figure 2: Bar graph showing the anti-proliferative effect of SbBEE on MCF-7 cells after 24 h and 48 h ........................................................................................................................................ 32
Figure 3: Results of flow cytometry on MCF-7 cells upon treatment with SbBEE .......................... 33
Figure 4: Cell cycle analysis of MCF-7 treated with SbBEE for 24 h ........................................... 34
Figure 5: Cell Death ELISA on MCF-7 treated with increasing concentrations of SbBEE and a positive control treated with Cisplatin for 24 h ............................................................... 35
Figure 6: Western blot analysis and quantification of expression levels of apoptosis-regulating proteins in MCF-7 cells treated with SbBEE for 24 h ....................................................... 36
Figure 7: Fold change of ROS MCF-7 treated with increasing concentrations of SbBEE and two positive control treated with TBHP and NAC for 24 h ................................. 37
List of Tables

Table 1: Anticancer properties of cancer drugs derived from plants (Yin et al., 2013) ................................................................. 17
Table 2: Chemical constituents of SbBEE as identified via liquid chromatography . 39
Table 3: Some compounds identified by LCMS and their corresponding structures 40
Chapter 1

Introduction

1.1 Background on Cancer

The ancient Egyptian Edwin Smith Papyrus, written in approximately 3000 BC, stands today as the oldest known description of diseases and the earliest record of human cancer: breast cancer (Hajdu, 2011). While paleopathologic findings prove cancer to have existed in animals (e.g. dinosaurs) for far longer than mentioned, the Papyrus disclaimed the presence of a cure at that time (Myres, 1933).

In fact, the name cancer was not yet used then. According to the American Cancer Society (2014), it is credited to the “Father of Medicine” and Greek physician, Hippocrates (460-370 BC), who utilized the Greek term carcinos (i.e. crab) to represent certain tumors. The latter was then interpreted in Latin by Celsus (28-50 BC), a Roman physician, and cancer became the common terminology (Sallam, 2010).

Multiple theories and discoveries on the origin of cancer arose over the years; this has contributed greatly to the field’s evolution. From being identified as a humoral disease at first, cancer’s progression included becoming a parasitic disease, a disease of cells, a disease of stem cells and, finally, a systemic disease (Mitrus et al., 2012).

Cancer is currently defined as a group of diseases that result from the uncontrollable proliferation of cancer cells and their transformation into malignant ones. These cells eventually grow into a tumor capable of undergoing metastasis (Lobo et al., 2007). However, despite this being a known factor, the initiation and development of cancer results from unknown aspects to this day. Thus, malignant tumors are known to be
cancerous and capable of spreading throughout the body, whereas tumors which are the opposite, non-cancerous and incapable of spreading, are called benign.

1.2 Types of Cancer

While the previously mentioned Edwin Smith Papyrus included only one form of cancerous tumors associated with the human breast, the 1500 BC Ebers Papyrus took a step further to highlight the possibility of skin, stomach, uterus and rectum cancer (Dawson, 1938). Today, though, over a hundred type of cancer exists.

Cancers are mainly classified into two different categories: hematologic cancers and solid tumor cancers. The first type is cancer associated with blood cells, such as leukemia. The second type is cancer that occurs in other body tissues or organs (e.g. breast and lung cancers). Although the two have similarities, each is unique in their growth, speed, the way they spread and their treatment methods.

According to the National Cancer Institute (n.d.), common cancer examples are Bladder Cancer, Breast Cancer, Colon and Rectal Cancer, Endometrial Cancer, Kidney Cancer, Leukemia, Liver Cancer, Lung Cancer, Melanoma, Non-Hodgkin Lymphoma, Pancreatic Cancer, Prostate Cancer and Thyroid Cancer. As shown, this type of tumors is usually classified and named after organs and tissues where they form (e.g. in the brain, brain cancer might be initiated.). However, with a total of 284,200 estimated new cases in the U.S. in 2021 alone, the most prevailing cancer is known to be breast cancer in men and women.
1.3 Breast Cancer

1.3.1 What is Breast Cancer?

Breast cancer is defined as a malignant tumor that develops from abnormal cell growth; it forms inside the breast tissue and is capable, through metastasis, of transferring to and growing in its vicinity (Simpson et al., 2005). This malignant tumor can occur in either one or both breasts and affects both men and women despite being the most common in women. An estimate of 43,600 deaths in American women was predicted for year 2021 (National Cancer Institute, n.d). However, breast lumps are mostly non-cancerous (i.e. benign) but can certainly lead to an elevated risk of getting breast cancer.

Numerous types of breast cancer have been determined over the years, making it a rather complex disease with many heterogeneities (Simpson et al., 2005); (Holliday & Speirs, 2011). To understand this diversity, breast cancer has long been modelled in laboratories using gene expression profiling via established cell lines. Although there exists a decent number of breast cancer cell lines, the first of which being BT-20 in 1958 (LASFARGUES & OZZELLO, 1958), the most popular cell line across the globe remains MCF-7. The latter was established at the Michigan Cancer Foundation in 1973 (Soule et al., 1973).

1.3.2 Breast Cancer Types and Classifications

Each type of breast cancer is first identified by the cells in which the cancer is formed. For instance, it is noteworthy to mention that the majority of breast cancers are carcinomas, meaning they begin in cells that align body organs and tissues. This specific type of carcinomas forms in the cells of milk ducts or lobules within the breast,
and they are named *adenocarcinoma*. Moreover, breast cancer is also classified based on whether the cancer has spread (i.e. invasive or infiltrating) into its surroundings or not (i.e. in situ).

The following are the various kinds of breast cancer (“Types of Breast Cancer”, 2021):

   a. Ductal Carcinoma in Situ (DCIS):

   DCIS is often signified as intraductal carcinoma as well (Burstein et al., 2004). As its name implies, DCIS is a non-invasive cancer that forms female breasts, specifically in the lumens of the ducts, without spreading to the outside. In spite not being life-threatening, DCIS is known to promote the development of a lethal invasive breast cancer, which makes it pre-invasive. However, the likelihood of this transformation and of it recurring is highly dependent on DCIS’s multiple features. Thus, it is, by itself, heterogeneous too.

   b. Invasive Ductal Carcinoma (IDC):

   Similarly, invasive ductal carcinoma occurs in the milk ducts of the breasts. However, IDC moves forward to invade the rest of the breast tissues, which makes it invasive. This type of breast cancer is also capable of spreading to lymph nodes and even other body areas. As reported by the American Cancer Society, a ratio of about 80:100 (i.e. 80%) of breast cancers are known to be of type IDC although 25.9% of IDC is diagnosed primarily, before surgery, as DCIS (Brennan et al., 2011). To this date, blood tests have not been successful in identifying risk factors associated with IDC (Takada et al., 2020).

   Invasive ductal carcinoma can be classified into five subtypes: Tubular, Medullary, Mucinous, Papillary and Cribriform Carcinoma of the Breast.
c. Invasive Lobular Carcinoma (ILC):

Invasive lobular carcinoma comes second after IDC in terms of the number of cases, which are about a percentage of 10 to 15 of every case of breast cancer. The two are very similar, but a difference mainly lies in ILC being in the lobules.

d. Inflammatory Breast Cancer (IBC):

IBC is known to be a rare but hostile kind of breast cancer in which the breast looks inflamed (Hester et al., 2021). It accounts for only 1% of all cases of breast cancer, according to the American Cancer Society, but can grow and move to surrounding tissues within relatively short periods of time such as days.

e. Triple-Negative Breast Cancer (TNBC):

With a high metastatic ability and poor prognosis features, triple-negative breast cancer is another aggressive breast cancer type which is rare. Its name implies that it tests negative for estrogen and progesterone receptors (i.e. ER and PR, respectively) as well as for HER2 protein (Yin et al., 2020).

Molecular subtypes of breast cancer are:

a. Luminal A:

Breast cancer of the type Luminal A tests positive for ER and PR hormones but negative for HER2 with very scarce Ki67 protein.

b. Luminal B:

Luminal B breast cancer, like Luminal A, tests positive for ER and PR, either positive or negative for HER2, but with immense Ki67 levels. Their growth is relatively faster, but they have poor prognosis compared to Luminal A (J. J. Gao & Swain, 2018).

c. Triple-negative/basal-like:

It is the same as the previously mentioned triple-negative breast cancer.
d. HER2-enriched (HER2+):
HER2-enriched breast cancer tests positive for HER2 protein but negative for the receptors of the other hormones, namely ER and PR.

e. Normal-like:
It is almost like Luminal A but with worse prognosis.

Other types of breast cancer:

a. Lobular Carcinoma in Situ (LCIS):
LCIS forms in the breast lobules also, but it is in fact not a malignant type cancer since it is still in situ. However, it does increase the risk of having a future invasive breast cancer.

b. Recurrent Breast Cancer:
This type occurs as a recurrence of a previously diagnosed breast cancer, either in the same breast, in the opposite breast or in the chest wall.

c. Metastatic Breast Cancer:
Metastatic breast cancer, or stage IV breast cancer, characterizes a cancer which has extended its reach to other body parts than the breast; common examples are the lungs, bones or brain.

d. Male Breast Cancer:
Although it is infrequent, breast cancer in men is not impossible. It occurs in 1% of men (Giordano et al., 2002).

Additional types of breast cancer which are also considered rare are: Paget's Disease of the Nipple, Phyllodes Tumors of the Breast and Angiosarcoma
Note that the most common subtype of breast cancer is the ER$^+$ type (Hanker et al., 2020), and the cell line MCF-7’s huge popularity is highly dependent on its hormone sensitivity for ER (Levenson & Jordan, 1997). Moreover, although the classification of breast cancer into different types is of significance to determining the next steps of therapy, the grade and stage of cancer remain better means of determining the treatment strategy to be followed and predicting the outcome (Roylance et al., 1999); (Buerger et al., 2001); (Reis-Filho & Lakhani, 2003).

1.3.3 What Causes Breast Cancer?
Several risk factors can play a role in forming cancer in human breasts; some are controllable, while others are not. For instance, being born a female alone is one of the major uncontrollable reasons behind an increased risk of breast cancer due to the high prevalence of breast cancers in females compared to males. Another uncontrollable aspect would be the breast density, due to the fact that in breasts of higher density, cancer detection through mammograms is much harder (Momenimovahed & Salehiniya, 2019). Moreover, certain reproductive factors can influence breast cancer: females who begin menstruating at a very young age, women who start their menopause late, women who have full-time pregnancies later in their lifetime and women who have never had children are more prone to getting breast cancer than others (Kelsey et al., 1993). In addition to that, aging is linked to a higher possibility of breast cancer with a low survival rate; the majority of casualties due to breast cancer can be found in women above the age of 65 (Karuturi et al., 2016). Being exposed to radiation in the chest area or to diethylstilbestrol (DES) in the past, having a past with breast cancer in the family or as an individual and genetic mutations can be added altogether to the long list of uncontrollable breast cancer risk factors.
As for those risks that can be controlled, these are also an extensive list that include mostly: consuming alcohol daily, being obese, using certain oral contraceptives, having breast implants, undergoing hormonal replacement therapy during menopause and abstaining from breastfeeding (Key et al., 2001).

### 1.4 Cell Cycle

A total of eight hallmarks have been established throughout the years to describe the contributing factors and biological characteristics of cancer development and progression. These are namely adequate growth signals, resistance to antigrowth factors, avoiding apoptosis, uninterrupted angiogenesis, unlimited proliferation, tissue invasion and metastasis, escaping from the immune system and metabolism rewiring (Hanahan & Weinberg, 2000); (Hanahan & Weinberg, 2011). Thus, genomic instability during the regular cell cycle is an important process to consider when studying cancer, regardless of its type.

During a cell cycle, a cell normally undergoes two main phases in order to divide; these phases are the interphase, in which the cell spends 95% of its time, and mitosis (M). The interphase consists of gap 1 (G1), synthesis (S) and gap 2 (G2) and prepares the cell for division through induced cell growth and DNA replication. Mitosis, on the other hand, is made up of four sub-phases: prophase (Pro), metaphase (Meta), anaphase (Ana) and telophase (Tel), and it is where an accurate separation of sister chromatids into two genetically identical daughter cells actually occurs. However, cells not ready for further division after G1 may enter and reside in a quiescence phase (G0), or restriction point, with the potential to re-enter active cell proliferation (Tomura et al., 2013).
1.4.1 Checkpoints and Cyclin-Dependent Kinases (CDKs)

Figure 1 below further elaborates on the cell cycle and shows that different phases are typically separated by multiple checkpoints (Matellán & Monje-Casas, 2020) such as G1/S, G2/M and metaphase/anaphase (WENZEL & SINGH, 2018); these checkpoints are responsible for insuring a safe and successful cell cycle by repairing damages to the DNA and promoting apoptosis when needed (Visconti et al., 2016) and, hence, promote genomic stability.

The progression of a cell cycle and its regulation is made possible with the presence of these checkpoints and enzymes like cyclin-dependent kinases (CDKs). CDKs are energized upon binding with one of the many cyclins present, and distinct levels of cyclins and CDKs and specific cyclin/CDK heterodimers characterize each cell cycle phase. The active complexes induce and regulate the transition between phases through the phosphorylation of targets such as retinoblastoma (RB or RB1), the tumor suppressor protein.
For instance, of the 20 CDKs and 29 cyclins found in human cells (L. Cao et al., 2014), from the G0 or G1 phase, CDK4 and CDK6 stimulate the cell-cycle progression into the S-phase along with D-cyclins (cyclin D1, cyclin D2, and cyclin D3) and regulation by INK4 family inhibitors (CKIs) (i.e. p16^{INK4A}, p15^{INKB}, p18^{INK4C} and p19^{INK4D}) (Malumbres & Barbacid, 2001). This leads to the formation of complexes of cyclin D/CDK4/6 and the phosphorylation of RB1 proteins and others (e.g. p107 and p130) which activate transcription factors like E2Fs that inhibit this transition and induce apoptosis when needed (Ahlander & Bosco, 2009).

1.4.2 Dysregulation of CDKs in Breast Cancer

Dysregulation in cyclin/CDK heterodimers disturbs the cell cycle and results in various breast cancer phenotypes; tumorigenesis and breast cancer maintenance occur upon the CDK4/6–cyclinD/INK4/pRB/E2F pathway, or its controllers, being deregulated (Santo et al., 2015). The latter could be through a depression in proteins of INK4 and CIP/KIP families or through CDK4/6 expansion (Asghar et al., 2015); (Cancer Genome Atlas Network, 2012). Studies reveal that CDK4/6–RB axis genetic deregulation is unique in each type of breast cancer in which the expression of an alteration in a single gene and/or a combination of multiple gene mutations is/are involved (Dukelow et al., 2015). In the case of ER⁺ breast cancer, for example, the rate of the progression from G1 to S phase is increased in the presence of estrogen. Estrogen binds to ER-alpha and leads to cyclin D1 transcription and a stimulated expression of multiple genes driven by receptors and involved in the process of cell proliferation and continuity. An amplification of cyclin D1 (Ding et al., 2020), an estrogen receptor 1 (ESR1) protein overexpression and a high phosphatidylinositol-4,5-bisphosphate 3-
kinase catalytic subunit alpha (PIK3CA) expression (Cancer Genome Atlas Network, 2012) are both detected in ER⁺ breast cancers.

However, the breast cancer subtype ER⁺ has relatively better genomic stability than others like HER2+ and TNBC in which the CDK4/6–RB axis also mediates cell growth. In HER2+ breast cancer, amplification of CDK4 and erb-b2 receptor tyrosine kinase 2 occurs. Mutations of cyclin D1, PIK3CA, p53 and phosphatase and tensin homolog (PTEN) are also detected (Ding et al., 2020). On the other hand, TNBC involves several of such mechanisms, especially the frequent mutation or deletion of RB1 (Robinson et al., 2013), a high proliferation rate and a defective BRCA1 (i.e. tumor suppressor breast cancer 1 is among the DNA damage response genes) pathway (Fedele et al., 2019). As a result, the compromise in the integrity of the cell cycle is inevitable, resulting in aggressive types of cancers.

1.5 Apoptosis

Apoptosis is of great significance to a successful cell cycle and a healthy organism/human being. It is the process in which unwanted, defective cells commit suicide or die as a means of maintaining a balance between cell divisions and cell deaths and, hence, preventing the occurrence of cancer and other diseases (Hassan et al., 2014). Therefore, inducing apoptosis in cancer cells is among the primary goals of the treatment of cancer (Rozeboom et al., 2019).

Two main pathways are included in the mechanism of apoptosis, which are the intrinsic pathway and the extrinsic pathway (Jan & Chaudhry, 2019):

The intrinsic pathway is dependent on the mitochondria and is mostly due to intercellular stresses including DNA damage and endoplasmic reticulum stress. It is
mediated by proteins released by the mitochondria such as those belonging to the protein family of B-cell lymphoma 2 (i.e. Bcl-2) (Maniam & Maniam, 2021). The latter involves pro-apoptotic proteins like Bcl-2, Bax, Bak, and Bcl-xL that control the release of cytochrome c release through regulating the mitochondrial outer membrane permeabilization (MOMP). A complex called apoptosome is relied upon to carry out the apoptotic downstream pathway; it is formed by cytochrome c, procaspase-9 and Apaf-1 (i.e. apoptotic protease-activating factor) (Su et al., 2015).

As for the extrinsic pathway, it is triggered by extracellular receptors of death, mostly those belonging to the TNF receptor superfamily. The latter bind to death ligands and create a death-inducing signaling complex (DISC) consisting of procaspase-8/10 and Fas-associated death domain (FADD) proteins. Subsequently, either downstream effector caspases are activated to by DISC to induce apoptosis or the mitochondria-mediated pathway (i.e. intrinsic pathway) is activated upon DISC cleaving the Bcl-2 family protein Bid into tBid (Su et al., 2015).

Additionally, a third pathway exists which is the execution pathway, which is the final point where the two pathways, intrinsic and extrinsic, meet. It begins with the activation of executor caspases, most importantly caspase-3, via initiator caspases. Caspase-3’s significance lies in its activation of endonuclease CAD (i.e. Caspase-activated DNase), resulting in the degradation of nuclear material and proteins in addition to the condensation of chromatin and, hence, apoptosis (Jan & Chaudhry, 2019).

However, a dysregulation of these pathways can eventually result in neoplastic cells multiplying by resisting to apoptosis. This often leads to tumorigenesis and obstructs
active cancer treatment. Therefore, among the primary goals of cancer treatment is the induction of cancer cells’ apoptosis while controlling apoptosis in normal cells.

1.6 Therapy for Breast Cancer

Multiple procedures and drugs have been implemented to treat cancer over the years, and that began long before modern treatment methods came into existence. For instance, before the 19th century, the Egyptians used a variety of salts, cautery and knives (Hajdu, 2004). The Greeks also used cautery, lotions and knives. The latter was used in case of deep tumors although not all types of cancers were thought to be treatable then. Breast cancer, in particular, was regarded as life-threatening by Hippocrates.

However, with the advancement in knowledge about cancer in general and breast cancer in specific, scientists have developed advanced treatment methods to those used before, and some therapeutic strategies are still under study till this day. It has come to an understanding that various types of cancers require different types of therapy and that some are best treated with surgery, while others need drugs or even both, in addition to radiotherapy.

Breast cancer alone can be treated using different procedures for its diverse types and the unique biological properties each type of breast cancer holds (Sørlie et al., 2006). Normally, treatment is decided upon based on the type of breast cancer the patient has, the stage of the breast cancer (i.e. if it has undergone metastasis), the grade of the cancer, if the cancer cells have receptors for certain hormones or for drug therapies that target them. Other considerations are related to the patient’s general health and if their body can handle a particular treatment and their menopausal status.
Generally, therapy is divided into two categories: local and systemic (Waks & Winer, 2019). Local treatments treat a specific area of the body or location. Examples of such treatments are surgery and radiation therapy. As for systemic treatments, they are those that can treat cancer cells throughout the whole body. Chemotherapy, immunotherapy, hormone therapy and targeted therapy are all examples of breast cancer systemic treatments.

In the case of breast cancer of type ER+, the most commonly used therapy is hormone therapy, which aims at reducing the levels of estrogen in the human body or blocking estrogen from inciting breast cancer cells’ growth. Tamoxifen (TAM) is among the highly used anti-estrogenic compounds in breast cancer treatment; it proved to be very effective in inducing apoptosis, suppressing proliferation and inhibiting metastasis in MCF-7 cells by competing with estrogen to bind to ER (Li et al., 2017).

However, resistance towards TAM could be developed among several ER+ patients. Moreover, TAM showed negative results on ERα36 breast cancer, for instance, as it contributed to the disease’s progression through the upregulation of the transcription of ALDH1A1 (Wang et al., 2018). These facts add to the need for an alternative or a second-line therapy at times, such as radiation or chemotherapy which mainly operate by damaging DNA (Bosco et al., 2007). On the other hand, an additional and very promising therapy when combined with the TAM is the use of CDK4/6 inhibitors (e.g. palbociclib). In breast cancer cell lines resistant to TAM, palbociclib supports the inhibition of the cell cycle and the dephosphorylation of RB. The latter is not the same in tumors without functional RB (Cadoo et al., 2014); (Dukelow et al., 2015).

As for the case of TNBC, while the traditional modes for treatment included surgery combined with radiation therapy and chemotherapy, new methods involving targeted
therapy and immunotherapy have proven to be the most effective options to tackle its heterogeneities. For example, the use of PARP inhibitors (e.g. olaparib) for germline BRCA1/2 mutations in metastatic Her2-negative breast cancer has received approval by the United States Food and Drug Administration (FDA). Studies have shown that inhibiting PARP results in double-stand breaks (DSBs) accumulating in replicating cells and a severe, selective toxicity (Turner et al., 2008). In other words, PARP inhibition induces apoptosis by blocking the DNA repair function (Yin et al., 2020). Moreover, less toxicity in olaparib has been found overall than in chemotherapy, thus, making it not only more selective but also more suitable. Research on olaparib as a monotherapy as well as with neoadjuvant therapy like chemotherapy is still ongoing (Robson et al., 2019).

On the other hand, atezolizumab has been approved by FDA as a part of immunotherapy to treat PD-L1+ tumors associated with a higher grade, large size, HER2-positive status and ER-negative status (Sabatier et al., 2015). Atezolizumab promotes T cell activity by blocking the interaction between PD-L1 and PD-1, thereby creating a positive immune response capable of killing the tumor. Again, attempts to find suitable combinations between atezolizumab and chemotherapy remain (Won & Spruck, 2020); (Manjunath & Choudhary, 2021).

1.7 Plant-Based Therapy

The use of the previously mentioned cancer therapies is usually accompanied by a variety of side effects. Common ones are hair loss, headache, nausea, fatigue, and several other systemic negative manifestations (Chan & Ismail, 2014). For that,
attention has been shifting towards alternative treatment options that cause less damage, which is where medicine extended from plants in nature comes in handy.

1.7.1 Brief Historical Review

In fact, plant-based therapy has been used for ages by the Egyptians, Chinese, Indians, Greeks and Central Asians to treat various diseases, including cancer (Ang-Lee et al., 2001); (Jamshidi-Kia et al., 2018). The Indian city, Nagpur, is home to the Sumerian clay slab on which the 5000-year-old written evidence of drug preparation using medicinal plants was found (Qiu, 2007). Moreover, thousands of years ago, the ancient Egyptians and Chinese used plant-derived therapeutics, some of which are still in use today. The Ebers Papyrus, for example, includes these remedies which were based on aloe vera, garlic, basil, onions, etc. The Greeks also put plants into use in their medicine years ago, starting with Hippocrates and his pupil, Aristotle, to Theophrastus, who founded the School of Medicinal Plants, and Pedanius Dioscorides, who wrote the famous De Materia Medica, describing a list of medicinal plants and their corresponding uses for therapy (Ríos & Recio, 2005); (Jamshidi-Kia et al., 2018).

1.7.2 Phytochemicals and Anticancer Agents in Plants

Plants are known to be rich in phytochemicals, which are bioactive natural chemicals present in foods and plants. These phytochemicals are an essential part of nutrition used by human-kind for the vast health benefits, antioxidant potential and anticancer compounds they entail (Choudhari et al., 2020).

Phytochemicals have proven to encompass great anti-proliferative and pro-apoptotic abilities and a higher selectivity compared to present therapy strategies such as
chemotherapy (S. Singh et al., 2016). Moreover, they are relatively inexpensive and widely available. However, major concerns remain regarding the possible toxicity of phytochemicals and their poor aqueous solubility, which is the focus of present studies in the scientific community (Choudhari et al., 2020).

Despite that, phytochemicals have shown noteworthy results in cancer therapy, especially breast cancer therapy. Examples of phytochemicals used as therapeutics for breast cancer can be found in table 1 below:

<table>
<thead>
<tr>
<th>Plant-Derived Components</th>
<th>Anticancer Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Isoflavone</em></td>
<td>Decreases the likelihood of breast cancer</td>
</tr>
<tr>
<td><em>Alkaloids and Coumarins</em></td>
<td>Prevent cancer cell growth</td>
</tr>
<tr>
<td><em>Flavonoids, Artemisunate and Polyphenols</em></td>
<td>Reduce the proliferation of breast cancer cells</td>
</tr>
<tr>
<td><em>Terpenoids</em></td>
<td>Induce MCF-7 cell apoptosis</td>
</tr>
</tbody>
</table>

*Table 1: Anticancer properties of cancer drugs derived from plants (Yin et al., 2013)*

Today, vinca alkaloids, taxane diterpenoids, epipodophyllotoxin and camptothecin derivatives are the major classes of anticancer compounds or phytochemicals derived
from plants which are used as standard-of-care regimens for treating cancer (Choudhari et al., 2020). Other classes of phytochemicals like ombretastatins, homoharringtonine, and ingenol mebutate are also often employed in cancer therapy.

For instance, vinflunine, a generation of vinca alkaloids, showed promising anticancer activity with minimal side effects for treating breast cancer when mixed with capecitabine (Campone et al., 2012). Like other vinca alkaloids, vinflunine induces apoptosis by binding to tubulin specifically and blocking microtubule polymerization. The latter results in a cell cycle arrest at G2/M leading to cell death (Kruczynski et al., 1998). Vinflunine also revealed less neurotoxicity compared to other vinca alkaloids as it binds relatively weakly to tubulin (Kruczynski & Hill, 2001). These properties helped in characterizing vinflunine according to its antiangiogenic and antimetastatic activities (Gourmelon et al., 2016).

In addition to that, the treatment of breast cancer in France has used elliptinium, from the Fijian Bleekeria vitensis plant (Cragg & Newman, 2005). The alkaloid ellipticine derivative is known to induce DNA breakages and inhibit its replication as well as the synthesis of RNA and protein (PubChem, n.d.-a).

Another example includes polyphenols found in green tea, in which a reduction of breast cancer risk occurred in Chinese women who consumed tea and have a minimum of one low-activity catechol-o-methyl transferase (COMT) allele (Wu et al., 2003). Polyphenols are found in a wide range of fruits and vegetables and have striking anticarcinogenic properties (Zabaleta et al., 2020). They interfere with the redox balance, cell cycle arrest, pro-apoptotic, ER expression modifications, with HER2 signaling and others (Losada-Echeberría et al., 2017). Moreover, considerably low or no toxicity is presented by polyphenols, which makes them great candidates for therapy (I. Arora et
Among the most important polyphenols is flavonoid genistein, present in soybeans. This substance was found to decrease glucose uptake in two breast cancer cell lines: ER\(^+\) MCF-7 and ER\(^-\) MDA-MB-231, hence, inhibiting their proliferation (Lim et al., 2006); (Keating & Martel, 2018).

Also, nab-paclitaxel, a taxane, is among the most efficacious medicine proven to treat all types of breast cancer, including metastatic breast cancer (Gradishar et al., 2012). It has shown unique anti-tumor properties, pro-apoptotic behavior, anti-metastatic properties and low toxicity over the course of 16 years, both as a mono-chemotherapy and as a combination with other drugs (Lu et al., 2021). It is mainly characterized by an induced delivery of albumin to tumors via transcytosis (i.e. transportation mediated by receptors) which is absent in paclitaxel (Abu Samaan et al., 2019).

1.8 Plant Under Investigation: \textit{Sternbergia Clusiana}

\textit{Sternbergia} is a genus of plants belonging to the Amaryllidaceae family. It constitutes eight different species recognized globally (Oran & Fattash, 2005), including \textit{Sternbergia clusiana}, which are scattered across the Mediterranean basin and central and southwestern Asia. These species exhibit a yellow color, except for \textit{Sternbergia candida}, which is white. They also commonly bloom in the fall, excluding a couple that only bloom in the spring.

\textit{Sternbergia clusiana}, or \textit{S. clusiana} (Ker Gawl.) Ker Gawl. ex Spreng, is a yellow bulbous plant which flowers in late autumn and among the largest in the genus \textit{Sternbergia}. It is native to the rocky slopes and open fields of Lebanon, Syria, Palestine, Turkey, Iran and the Aegean Islands (Karuturi et al., 2016).
This species has also been spotted in Jordan and Iraq. However, it is quite rare and is classified as potentially endangered, which struck interest for studies on how to conserve it or regenerate it (Oran & Fattash, 2005); (S. Youssef et al., 2017). The latter is primarily due to its history in medicine as well as its wide range of biological properties.

1.8.1. Components, Properties and Uses

*Sternbergia clusiana* is among the most popular and beautiful ornamental plants. However, like other species of *Sternbergia*, *Sternbergia clusiana* has a rich phytochemistry with a significant therapeutic activity. This enabled its usage as a medicinal plant for many years.

The species of *Sternbergia*, in general, possess various yet significant biological effects and have been historically employed in traditional medicine. In fact, the family to which these plants belong to, the Amaryllidaceae family, is known to have abundant amounts of alkaloids (Orhan et al., 2011). These alkaloids are prevalent for their apoptotic, anti-inflammatory, cytotoxic and anti-acetylcholinesterase or AChE inhibition activities (McNulty, 2007).

Studies on extracts of *Sternbergia clusiana*, in specific, have revealed diverse but positive antimicrobial, antioxidant and free-radical scavenging effects, due to its high phenol content ranging between 250.1 and 1126.9 gallic acid equivalents (GAE) in milligrams per a 100-gram extract sample (Mammadov et al., 2011). *Sternbergia clusiana* was reported to be affluent in several of these significant alkaloid compounds, including haemanthidine and lycorine, known as Amaryllidaceae alkaloids (G. Çitoğlu et al., 1998). These alkaloids as well as extracts of these species have revealed excellent
analgesic (i.e. pain-relieving) characteristics also. Haemanthidine and lycorine even proved higher analgesic activities than aspirin (Tanker et al., 1996). Moreover, galanthamine is another alkaloid found in *Sternbergia clusiana*. It is known as an anti-AChE agent, used as a drug to treat Alzheimer’s disease. Thus, *Sternbergia clusiana* extracts revealed promising AChE inhibition activity (Orhan et al., 2011). Adding to that, numerous other alkaloids like 11-hydroxyvittatine, crinine, isotazettine, haemanthamine and narciclasisine have also been detected in *Sternbergia clusiana* extracts (Tanker et al., 1996).

1.8.2. Anticancer Properties

Little to no studies on the genus *Sternbergia* and *Sternbergia clusiana* in particular have been published regarding their anticancer properties. However, studies on alkaloids present in this species have revealed promising anticancer activity.

For instance, the anti-tumor effect of the alkaloid lycorine, which is found in *Sternbergia clusiana*, was first revealed in 1976 (Jimenez et al., 1976), and was later proven to be effective against various cancer cells. The alkaloid is praised for its selectivity towards cancer cells; it is capable, for example, of reducing cancer growth with a trivial toxicity against normal breast cells (Ying et al., 2017). Lycorine also presents less toxicity and side effects and is useful even in low concentrations. An additional criterion of lycorine is its selectivity against resistance; it can fight cancer cells with apoptosis-resistance (Lamoral-Theys et al., 2009) and many others. Moreover, lycorine was proven to be efficient when tested on various types of cancers (Roy et al., 2018). For breast cancer cell lines including MCF-7, it showed abilities inhibiting proliferation and invasion by inducing cell death and obstructing the
Src/FAK-involved pathway (Ying et al., 2017). Another *in vitro* study showed lycorine’s abilities in cell death induction by apoptosis of leukemia cells via the activation of various caspases, especially caspase-3. Using a 5 µM concentration of lycorine on the promyelocytic leukemia HL-60 cell line resulted in a cell cycle arrest at the G2/M phase (Ren et al., 2014). Research also showed lycorine’s ability to reduce cyclin D1 and CDK4 protein levels and enhance the expression of p21, a CDK inhibitor, in multiple myeloma cell line ARH-77, hence, blocking further proliferation (Luo et al., 2015). Not only that, but lycorine’s hydrochloride salt also suppressed ovarian cancer Hey1B cell proliferation by inhibiting the expression of cyclin D3 and inducing that of p21 (Z. Cao et al., 2013).

Haemanthamine, another alkaloid found in *Sternbergia clusiana*, holds anticancer properties of its own. It is considered an anticancer agent for its ability to reduce resistance in cancer cells to apoptosis (Pellegrino et al., 2018). For instance, the exposure of acute T-cell leukemia Jurkat cell line to it activated caspases, reduced the potential of the mitochondrial membrane, blocked the G1 and G2/M cell cycle phase and enhanced apoptosis. Haemanthamine also revealed cytotoxic activity against other types of cancer cell lines, including MCF-7 of breast cancer (Havelek et al., 2014).

Similarly, the alkaloid haemanthidine, also found in *Sternbegia clusiana*, has displayed analogous biological characteristics to haemanthamine’s in leukemic Jurkat cell lines (Havelek et al., 2014). That is besides showing cytotoxic properties and inhibition of growth on lymphoma cell lines (Van Goietsenoven et al., 2010).

Furthermore, *Sternbergia clusiana* contains the alkaloid narciclasine, which is considered an inducer of apoptosis with lower cytotoxicity on normal cells. It has been proven to encourage cell death in MDA-MB-231 and MCF-7 breast cancer cell lines.
and in PC-3 human prostate cancer cells, mainly through activating the death receptors and mitochondrial or intrinsic pathways (Dumont et al., 2007).

Last but not least, crinine alkaloids have proven promising antiproliferative activity. A study revealed crinine-type alkaloids’ capabilities in treating cancers resistant to apoptosis, such as glioblastoma. Bulbispermine, a crinine alkaloid, acts by inhibiting glioblastoma cells’ proliferation via cytostatic effects. The rigidification of the actin cytoskeleton may be behind the occurrence of such effects (Luchetti et al., 2012).

1.8.3. Additional Anticancer Potential of the Amaryllidaceae Family

While the anticancer properties of *Sternbergia clusiana* have been identified through a number of its constituents, extended research on the Amaryllidaceae family, in general, shows further potential not yet revealed in the aforementioned *Sternbergia clusiana*. In addition to the alkaloids found in *Sternbergia clusiana*, the Amaryllidaceae family includes distichamine, pretazettine, pancratistatin, narciprimine, bulbispermine and several others (Habartová et al., 2016).

To begin with, although distichamine is considered rare in the Amaryllidaceae family, in vitro studies have proven its anticancer activity against many cell lines, including MCF-7 and HeLa cells. Moreover, in CEM human leukemia cells, it exhibited the ability to disturb the cell cycle and activate caspases 3 and 7 to induce apoptosis (He et al., 2015),(Nair et al., 2012). Next, pretazettine showed an inhibition of HeLa cells’ growth and therapeutic abilities against various types of leukemia and lung carcinoma (Bastida et al., 2011). Furthermore, according to previous studies, pancratistatin holds the ability to mediate apoptosis by activating caspases, promoting the permeabilization of the mitochondrial membrane and
producing reactive oxygen species in leukemic cells (Griffin et al., 2010), neuroblastoma SHSY-5Y cells (McLachlan et al., 2005), human prostate cells (Griffin et al., 2011) and breast cancer MCF-7 cells. Pancrastatin’s effect against MCF-7 cell lines was especially displayed upon combining with Tamoxifen (Siedlakowski et al., 2008). Additionally, antiproliferative abilities upon using MTT assay against MCF-7, HeLa and A341 cells were disclosed by narciprimine of the Amaryllidaceae family alkaloids (Bozkurt et al., 2012). Also, bulbispermine was proven to have cytotoxicity against HL-60 human leukemia cells and to induce cell death in glioblastoma cells like T98G and U373, which are resistant to apoptosis. The latter is generated by cytostatic effects resulting from increased polymerized actin amounts and the cytoskeleton organization rigidification (Luchetti et al., 2012).

1.9 Purpose of the Study

The conducted studies on alkaloids belonging to the Amaryllidaceae family have shown great potential in cancer therapy. However, prospects for Sternbergia clusiana’s capabilities in treating MCF-7 breast cancer cells, in particular, require further research. Therefore, the purpose of this study is to fulfill the gaps found in literature on the cytotoxic effect of Sternbergia clusiana against MCF-7 cell lines and decipher the underlying molecular mechanisms involved. The experimental analyses done include cytotoxicity assays, Cell cycle analysis, Cell death ELISA, Western Immunoblotting, and ROS induction in MCF-7 cell lines.
Chapter Two

Materials and Methods

2.1 Plant Material

*Sternbergia clusiana* (Ker Gawl.) plants were identified in Lebanon by Dr. Nisrine Machaka-Houri, who is a well-known botanist and professional in the country’s flora. These plants were retrieved in November, 2018, from the Lebanese region Falougha, located 33.825008° N, 35.751962 E, 149 m above sea level.

2.2 *Sternber gia* Bulb Ethanolic Extract Preparation (SbBEE)

*Sternbergia clusiana* bulbs were extracted, washed with distilled water and left to dry before grinding. A total of 36.47g of these grounded bulbs was mixed in 350ml of 70% ethanol. It was also thermo-shaken at room temperature at 200 rpm for one whole week. Moreover, mechanical rotation under vacuum, or roto-evaporation, was applied for the evaporation of ethanol from 50ml of the plant mixture. 2.5ml DMSO under sonication and 25ml of cell-culture media (RPMI, 10% DMSO) were used to redissolve the plant extract.

The volume of the solution was filtered down to 90ml using a sterile cheese cloth, which was further brought to a volume of 87ml upon centrifuging it at 24.446 g and filtering it with a 0.45um syringe filter.

In addition to that, the 232.7ug/ml pure extract was placed in aliquots at -80°C for future uses and labelled as SbBEE. However, each of these aliquots underwent a dilution of 10 times in DMEM before applying the extract to the cells. The latter was conducted mainly to maintain a final concentration of DMSO less than 1%.
2.3 Breast Cancer Cell Culture

The hormone dependent MCF-7 cells were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were cultured (37 °C, 5% CO2) in Dulbecco’s Modified Eagle Medium (DMEM, Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (FBS, Gibco™) in addition to 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza, Allendale, NJ) (Idriss et al., 2020). Cells growing in monolayer were regularly checked under the microscope and were split every 3 days at a confluency of 70–80%. Cells were washed and detached using Phosphate Buffered Saline (PBS, Lonza, Belgium) and trypsin-EDTA, respectively. Before proceeding in any experiment, cell viability was checked using the Trypan Blue exclusion method by mixing 10 µL of the cells with 10 µL of trypan blue and counting viable cells using a hemocytometer counting chamber.

2.4 MTS Assay

MCF-7 cells were seeded at a concentration of $1 \times 10^5$ cells/ml in a 96-well plates in triplicates and incubated overnight at 37°C with 5% CO2. Subsequently, the cells were treated the next day with SbBEE prepared in a fresh medium at increasing concentrations (0.15, 0.3, 0.5, 1 and 2.5 %v/v) for 24 h. MTS assay was implemented in order to assess the MCF-7 cell viability by measuring the conversion of the soluble tetrazolium salt into formazan by metabolically active cells. MTS (Promega) was prepared according to instructions set by the manufacturer by mixing a solution of PMS, MTS and DMEM at a ratio of 1:20:100. This mixture was incubated for 1 h and then the Varioskan™ LUX multimode microplate reader was allocated to measure the absorbance at 492 nm (Khalil, 2015).
The percentage of cell viability was calculated as follows:

\[
\frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100
\]

2.5 Cell Death ELISA

6-well plates were used to seed MCF-7 cells at a density of 1.5x10^5 cells/ml, in which they were also incubated overnight. Three various concentrations of SbBEE closest to IC50 (0.5, 1.5 and 2.5 %v/v) were then added to the plated cells, and a concentration of 30 µM of Cisplatin was employed as the positive control. Next, via the Cell Death ELISA kit and prior to the isolation of cytosolic histone-associated-DNA content, cells were gathered and lysed in incubation buffer within 24 h. Wells coated with biotin-associated anti-histone antibodies were then used to incubate the extracted DNA in the lysate. Following that, incubation was performed with anti-DNA antibodies linked to peroxidase enzyme, and the excess unbound DNA was washed with washing buffer prior to introducing the peroxidase substrate. Measuring the absorbance at 405 nm was made by spectrophotometry via Multiskan™ FC microplate photometer. As for the calculation of the DNA fragmentation enrichment factor, it was performed as the ratio of absorbance present in the treated samples to that found in the untreated controls (Hodroj et al., 2020).

2.6 Cell Cycle Analysis

6-well plates were used to seed MCF-7 cells at a density of 1.5x10^5 cells/ml, in which they were also incubated overnight. The cells were treated for 24 h with 0.5, 1.5 and 2.5 %v/v of SbBEE, and 30 µM of Cisplatin was used as a positive control. The cells were washed with 600 µL PBS and fixed using 1400 µL of ice cold ethanol
(97%) (Lonza, Basel, Switzerland). They were left overnight to allow their fixation at a temperature of -80°C. The cells were then treated with 100 µg/mL of ribonuclease I, RNase, (Roche, Penzberg, Germany) followed by staining with 500 µL of propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO, USA) (Idriss et al., 2020).

To detect the distribution of cells in each phase of the cell cycle, DNA content was evaluated through the use of an Accuri C6 flow cytometer. The latter indicated the following: cells before the G0/G1 phase had < 2n, cells within the G0/G1 phase had 2n, cells in the S phase had between 2n and 4n, whereas cells in the G2/M phase had 4n (Haykal et al., 2019).

2.7 Western Blot

A 6-well plate was used to seed MCF-7 cells at a density of 3 x 10^5 cells/mL. These cells were incubated overnight, followed by treatment with SbBEE at three increasing concentrations (0.5, 1.5 and 2.5 %v/v) for 24 h; these concentrations were chosen since they are the closest to IC50. DMEM and media were added to the control cells.

Total proteins were extracted by utilizing the Q-proteome Mammalian Protein kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions; extracted proteins were quantified using the DC (Detergent Compatible) protein assay (Bio-Rad). After that, the separation of the proteins was executed using 10% SDS-PAGE at a running voltage of 120V, and the membranes were transferred to PVDF (Polyvinylidene fluoride), in which they were blocked with 5% Bovine serum albumin (BSA) for 1 h at room temperature. The membranes were then incubated with primary antibodies anti-β-actin (Santa Cruz Biotechnology), anti-Bax (rabbit-Abcam), anti-Bcl2 (mouse- Elabscience), and antiPARP (rabbit- abcam), anti-
Casp9 (mouse-Elabscience), anti-cCasp8 (mouse -Elabscience) overnight in the fridge at 4°C (Ghanem et al., 2019).

The following day and for a duration of 1 h at room temperature, the membranes were washed 4 times with a washing solution (1 × PBS and 0.5% Tween-20) and later incubated with a secondary antibody (Bio-Rad, Hercules) at the recommended concentration. An additional wash was performed preceding the imaging using Clarity™ Western ECL Substrate (Abcam, Cambridge, UK) on ChemiDoc machine (BioRad, Hercules, CA, USA). The quantification of the blot bands was performed using the ImageJ computer program, enabling the calculation of the relative expression of proteins (Younes et al., 2020).

2.8 Reactive Oxygen Species (ROS) Detection

To determine the level of reactive oxygen species in the MCF-7 cells, an employment of the DCFDA cellular ROS detection assay Kit took place. The cells were treated with 0.5, 1.5 and 2.5 %v/v of SbBEE, and two different controls were used, namely TBHP and NAC. The first positive control was 20 μM of Tert-butyl hydrogen peroxide (TBHP), a known ROS inducer and the second one NAC (10mM) is a known ROS inhibitor. Cells were washed with buffer and then incubated with DCFDA for 45 mins.

The conversion of H$_2$DCFDA (2’,7’-dichlorodihydrofluorescein diacetate), a non-fluorescent molecule, into a highly fluorescent 2’,7’-dichlorofluorescein (DCF) molecule in the presence of ROS was quantified via spectrofluorometry using the Varioskan LUX multimode microplate reader (Thermo Fisher Scientific, Bremen, Germany) (El Khoury et al., 2020).
2.9 Statistical Analysis

The experiments were performed three distinct times (n=3). GraphPad Prism 8 (San Diego, CA, USA) was allocated for statistical analysis. The error bars were indicated as the mean ±SEM, and, based on the experiment, t-tests or two-way ANOVA was used to calculate the p-values.

Significant variations were reported, with *, **, *** and **** indicating the p-values: 0.01 < p < 0.05, 0.001 < p < 0.01, 0.0001 < p < 0.001 and p < 0.0001, respectively.

2.10 Liquid Chromatography Mass Spectrometry Analysis

Nucleodur C18 Gravity-SB (300 Å, 1.8 μm, 2x100 mm, Macherey-Nagel, Düren, Germany) was injected with a 2.5 μg sample via a Dionex Ultimate 3000 analytical RSLC system (Dionex, Germering, Germany) alongside a HESI (i.e. heated electrospray ionization) source (Thermo Fisher Scientific, Bremen, Germany). A gradient ranging between 3% and 50% of solvent B was applied within 35 min to implement the separation at a flow rate of 300 μl/min. Following that, steps consisted of washing the column and re-equilibration. Two solvents were utilized: solvent A and solvent B. Solvent A consisted of both water and 0.1% formic acid. As for solvent B, it contained acetonitrile and 0.1% formic acid. The QExactive HF-HT-Orbitrap-FT-MS (Thermo Fisher Scientific, Bremen, Germany), a benchtop instrument, was adopted for the analysis of eluting compounds. The first scan (MS1) was executed at a resolution of 60,000, a 3e6 AGC (i.e. automatic gain control) and 200 ms as the maximum injection time. The second scan (MS2) was completed in mode “Top10” with an isolation window of 2 m/z. The latter is in addition to the usage of 15,000 resolution, a 5e5 AGC, 50 ms maximum time of injection and

30
averaging 2 μscans. For fragmentation, the method employed was the higher-energy collisional dissociation (HCD) with a 28% normalized collision energy. On the other hand, mzCloud as well as ChemSpider database for chemicals were put into use to perform compound analysis (El Khoury et al., 2020).
Chapter Three

Results

3.1 The anti-proliferative effect of *Sternbergia* Bulb Ethanolic Extract on MCF-7 cells.

The effect of SbBEE on MCF-7 cell proliferation was assessed using the MTS cell viability reagent. A dose-and-time dependent significant decrease in the proliferation of MCF-7 cells was noticed upon treatment with various extract concentrations. The lowest concentration of treatment (0.15%v/v) exhibited a decrease in percent proliferation reaching 77.46% and 55.3% after 24h and 48h, respectively. Whereas, at the highest dose of SbBEE treatment (2.5 %v/v) the percent proliferation decreased till 63.49% and 11.81% after 24 h and 48 h, respectively. This extract was shown to induce a dose-and time-dependent anti-proliferative effect on MCF-7 cells after 24 h and 48 h.

The following experiments were performed using 0.15, 0.5 and 2.5 %v/v, the closest concentrations to the IC50, for 24 h.

![Figure 2: Bar graph showing the anti-proliferative effect of SbBEE on MCF-7 cells after 24 h and 48 h](image-url)
3.2 *Sternbergia* Bulb Ethanolic Extract induces a dose-dependent cellular fragmentation

To further investigate the effect of SbBEE extract on MCF-7 cells, cell cycle progression and DNA content were analyzed using flow cytometry to check if the cells are arrested at any stage of the cell cycle or fragmented. A significant decrease in the G0/G1 phase from 35.4% reaching 24.2% was shown when the cells were treated with the highest concentration of SbBEE (2.5%v/v) compared to the control. However, a significant increase in the pre-G phase from 5.5% to 19.1% was noticed upon treatment with the highest dose of the extract. This shows that SbBEE induces cellular fragmentation in a dose-dependent manner on MCF-7 cells.

![Flow cytometry results](image)

*Figure 3: Results of flow cytometry on MCF-7 cells upon treatment with SbBEE*
Figure 4: Cell cycle analysis of MCF-7 treated with SbBEE for 24 h

3.3 *Sternbergia* Bulb Ethanolic Extract induces DNA fragmentation in MCF-7 cells

DNA fragmentation is a major hallmark of apoptosis and Cell Death ELISA was performed to further quantify and confirm the increase in the level of DNA fragmentation. The enrichment factor is the ratio of absorbance measured for each concentration to the untreated control which reveal the abundance of cytosolic nucleosomes in the cells upon exposure to SbBEE. A significant dose dependent increase was shown compared to the control after treating MCF-7 cells with SbBEE for 24 h. An increase in DNA fragmentation was observed from 0.75-fold to 3.3-folds upon treatment with 0.5 and 2.5 %v/v, respectively for 24 h. While the ratio increased significantly, reaching 4.8-folds when the cells were treated with Cisplatin (30µM).
3.4 *Sternbergia* Bulb Ethanolic Extract promotes the activation of the mitochondrial apoptotic pathway in MCF-7 cells

In order to decipher the molecular mechanism of the apoptotic pathway initiated by SbBEE, Western blot analysis was performed. The results showed a 2-fold increase in the ratio of Bax to Bcl2; proteins involved in the regulation of the mitochondrial membrane permeability. Similarly, an upregulation of the cleaved caspase 9 expression was shown reaching a 3-fold increase at the highest concentration of the extract. Additionally, a 2-fold increase in the expression of cleaved-parp was observed upon treatment with 2.5% v/v SbBEE.

To further investigate the activation of the extrinsic apoptotic pathway, cleaved caspase 8 expression was assessed and found to be constant while increasing the concentration of treatment.

These results confirm the activation of the mitochondrial pathway upon treatment with increasing doses of SbBEE for 24 h.
3.5 *Sternbergia* Bulb Ethanolic Extract has an antioxidant effect on MCF-7 cells

In addition to pro-apoptotic activity of the extract on MCF-7 cells, the antioxidant effect was further investigated. The DCFDA Cellular ROS Detection Assay kit was used to quantify the Reactive oxygen species (ROS) production in MCF-7 cells treated with increasing concentrations of SbBEE. Results revealed a significant decrease of ROS level reaching 0.67 and 0.37- folds when treated with 0.15 and 2.5 \%v/v for 24 h. This shows that the extract has a dose-dependent antioxidant effect on MCF-7 cells.
Figure 7: Fold change of ROS MCF-7 treated with increasing concentrations of SbBEE and two positive control treated with TBHP and NAC for 24 h

3.6 Chemical Elucidation of SbBEE using Liquid Chromatography Mass Spectrometry Analysis

The chemical composition of SbBEE was assessed using liquid chromatography coupled to mass spectrometry. Results show the major and minor components of the extract, which include lycorine, 9S,13R-12-Oxophytodienoic acid (OPDA), fumaritine N-oxide, 4-Indolecarbaldehyde, rhamnetin, ferulic acid, adenosine and several others shown in the table below.

<table>
<thead>
<tr>
<th>Name</th>
<th>Formula</th>
<th>RT [min]</th>
<th>Area (Max.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bis(4-ethylbenzylidene)sorbitol</td>
<td>C24 H30 O6</td>
<td>37.878</td>
<td>3174434618</td>
</tr>
<tr>
<td>4-oxo-4,5,6,7-tetrahydrobenzo[b]furan-3-carboxylic acid</td>
<td>C9 H8 O4</td>
<td>11.438</td>
<td>2230875723</td>
</tr>
<tr>
<td>Lycorine</td>
<td>C16 H17 N O4</td>
<td>2.837</td>
<td>2150797725</td>
</tr>
<tr>
<td>3-oxoindane-1-carboxylic acid</td>
<td>C10 H8 O3</td>
<td>31.823</td>
<td>1490616529</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>C9 H11 N O2</td>
<td>2.793</td>
<td>1081816069</td>
</tr>
<tr>
<td>13(S)-HOTrE</td>
<td>C18 H30 O3</td>
<td>38.526</td>
<td>926272408.7</td>
</tr>
<tr>
<td>(+/-)9-HpODE</td>
<td>C18 H32 O4</td>
<td>38.535</td>
<td>890320782.9</td>
</tr>
<tr>
<td>2-Amino-1,3,4-octadecanetriol</td>
<td>C18 H39 N O3</td>
<td>31.808</td>
<td>610188836.2</td>
</tr>
<tr>
<td>13,14-dihydro Prostaglandin F1α</td>
<td>C20 H38 O5</td>
<td>35.606</td>
<td>511502894.1</td>
</tr>
<tr>
<td>9S,13R-12-Oxophytodienoic acid</td>
<td>C18 H28 O3</td>
<td>39.803</td>
<td>461820295.2</td>
</tr>
<tr>
<td>Name</td>
<td>C</td>
<td>H</td>
<td>N</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>------------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>Cetrimonium</td>
<td>C19 H41 N</td>
<td>39.798</td>
<td></td>
</tr>
<tr>
<td>3-hydroxy-4-(3-hydroxyphenyl)-1,2-dihydroquinolin-2-one</td>
<td>C15 H11 N</td>
<td>9.26</td>
<td></td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>C7 H6 O3</td>
<td>36.532</td>
<td></td>
</tr>
<tr>
<td>D-(+)-Tryptophan</td>
<td>C11 H12 N</td>
<td>5.543</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-(-)-Quinic acid</td>
<td>C7 H12 O6</td>
<td>3.174</td>
<td></td>
</tr>
<tr>
<td>4-(tert-butyl)phenyl 3,5-dimethylisoxazole-4-carboxylate</td>
<td>C16 H19 N</td>
<td>3.226</td>
<td></td>
</tr>
<tr>
<td>Sedanolide</td>
<td>O3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C12 H18 O2</td>
<td>38.527</td>
<td></td>
</tr>
<tr>
<td>Corchorifatty acid F</td>
<td>C18 H32 O5</td>
<td>31.371</td>
<td></td>
</tr>
<tr>
<td>2,2,6,6-Tetramethyl-1-piperidinol (TEMPO)</td>
<td>C9 H19 N O</td>
<td>29.349</td>
<td></td>
</tr>
<tr>
<td>α-Hydroxymidazolam</td>
<td>C18 H13 C</td>
<td>10.109</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F N3 O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>C10 H10 O4</td>
<td>11.449</td>
<td></td>
</tr>
<tr>
<td>Rhamnetin</td>
<td>C16 H12 O7</td>
<td>18.912</td>
<td></td>
</tr>
<tr>
<td>4-Indolecarbaldehyde</td>
<td>C9 H7 N O</td>
<td>16.502</td>
<td></td>
</tr>
<tr>
<td>3-Methoxy-5,7,3',4'-tetrahydroxy-flavone</td>
<td>C16 H12 O7</td>
<td>27.133</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>C6 H13 N O2</td>
<td>2.327</td>
<td></td>
</tr>
<tr>
<td>L-Pyroglutamic acid</td>
<td>C5 H7 N O3</td>
<td>2.204</td>
<td></td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>C9 H11 N O2</td>
<td>2.135</td>
<td></td>
</tr>
<tr>
<td>Corchorifatty acid F</td>
<td>C18 H32 O5</td>
<td>26.367</td>
<td></td>
</tr>
<tr>
<td>Ethyl 9H-beta-carboline-3-carboxylate</td>
<td>C14 H12 N</td>
<td>19.841</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,8,9-tri hydroxy-10-propyl-3,4,5,8,9,10-hexahydro-2H-oxecin-2-one</td>
<td>C12 H20 O5</td>
<td>19.341</td>
<td></td>
</tr>
<tr>
<td>Cinchophen</td>
<td>C16 H11 N</td>
<td>10.536</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9S,13R-12-Oxophytodienoic acid</td>
<td>C18 H28 O3</td>
<td>31.311</td>
<td></td>
</tr>
<tr>
<td>4-Acetyl-3-hydroxy-5-methylphenyl β-D-glucopyranoside</td>
<td>C15 H20 O8</td>
<td>8.52</td>
<td></td>
</tr>
<tr>
<td>trans-Cinnamaldehyde</td>
<td>C9 H8 O</td>
<td>11.449</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C15 H10 O7</td>
<td>16.992</td>
<td></td>
</tr>
<tr>
<td>3,4-Dihydroxybenzaldehyde</td>
<td>C7 H6 O3</td>
<td>8.401</td>
<td></td>
</tr>
<tr>
<td>Jasmonic acid</td>
<td>C12 H18 O3</td>
<td>24.837</td>
<td></td>
</tr>
<tr>
<td>Corchorifatty acid F</td>
<td>C18 H32 O5</td>
<td>36.331</td>
<td></td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>C8 H9 N O</td>
<td>15.696</td>
<td></td>
</tr>
<tr>
<td>4-Indolecarbaldehyde</td>
<td>C9 H7 N O</td>
<td>5.467</td>
<td></td>
</tr>
<tr>
<td>2-benzyl-6-hydroxy-2-azabicyclo[2.2.2]octan-3-one</td>
<td>C14 H17 N</td>
<td>2.876</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sedanolide</td>
<td>C12 H18 O2</td>
<td>16.589</td>
<td></td>
</tr>
<tr>
<td>Azelaic acid</td>
<td>C9 H16 O4</td>
<td>18.216</td>
<td></td>
</tr>
<tr>
<td>L-Norleucine</td>
<td>C6 H13 N O2</td>
<td>2.884</td>
<td></td>
</tr>
<tr>
<td>Sedanolide</td>
<td>C12 H18 O2</td>
<td>27.958</td>
<td></td>
</tr>
<tr>
<td>trans-Anethole</td>
<td>C10 H12 O</td>
<td>34.624</td>
<td></td>
</tr>
<tr>
<td>Tetradecanedioic acid</td>
<td>C14 H26 O4</td>
<td>29.245</td>
<td></td>
</tr>
<tr>
<td>Compound Name</td>
<td>Structure (PubChem, n.d.-d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lycorine</td>
<td><img src="image" alt="Lycorine Structure" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9S,13R-12-Oxophytodienoic acid (OPDA)</td>
<td><img src="image" alt="OPDA Structure" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fumaritine N-oxide</td>
<td><img src="image" alt="Fumaritine Structure" /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Chemical constituents of SbBEE as identified via liquid chromatography
<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Indolecarbaldehyde</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Rhamnetin</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Ferulic acid</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Adenosine</td>
<td><img src="image" alt="Structure" /></td>
</tr>
</tbody>
</table>

*Table 3: Some compounds identified by LCMS and their corresponding structures*
Chapter Four

Discussion

Natural products and derivatives have become widely popular in medicine for their safety and efficacy, and they have been recognized as a significant source of new cancer therapy and drug leads (Can Ağca et al., 2021); (Habartová et al., 2016). Studies investigating molecular constituents in plants of the Amaryllidaceae family have revealed therapeutic potential due to their alkaloid content, including *Sternbergia* species (G. S. Çitoğlu et al., 2012); (Can Ağca et al., 2021). In this study, the ethanolic extract of *Sternbergia clusiana* bulbs, SbBEE, was examined for its potential anti-proliferative and pro-apoptotic properties on MCF-7 breast cancer cell lines since leaves from different *Sternbergia* species and plants of the Amaryllidaceae family have shown cytotoxic effects on other cancer cell lines (Can Ağca et al., 2021); (G. S. Çitoğlu et al., 2012); (Nair et al., 2012).

MCF-7 cells have proven to be an adequate model for investigations on breast cancer (Shirazi et al., 2011). Previous studies have adopted this well-known human breast cancer cell line for being characteristic of differentiated mammary epithelium tissues. The latter includes tissues involved in the expression of estradiol and estrogenic receptors (Brandes & Hermonat, 1983).

In this study, SbBEE was shown to exhibit dose- and time-dependent anti-proliferative effects on the employed MCF-7 cancer cell lines using the MTS cell viability reagent. In fact, the anti-proliferative impact of various species belonging to the Amaryllidaceae family was shown to be promising by a number of studies wherein the percentage of proliferation in cancer cells decreased with respect to time and concentration of plant extract used (Tayoub et al., 2018); (D. T. A. Youssef et al.,...
It is important to mention that the most prominent effect was detected with the highest dose after 48 h, revealing that therapeutic levels of proliferation inhibition are best reached after an incubation of 48 h. Nonetheless, the remaining experiments of this study were performed by incubating SbBEE for 24 h in aims of elucidating its mechanism of action and in order to detect earlier the induced apoptosis at a molecular level.

Among the most substantial targets of anticancer therapy is the activation of programmed cell death or apoptosis (Pfeffer & Singh, 2018). The latter was the main mechanism revealed to be involved in SbBEE’s therapeutic effect on MCF-7 cells. This was shown through an evident cell cycle arrest, the activation of the mitochondrial pathways, in addition to an antioxidant effect.

To begin with, a dose-dependent cellular fragmentation was proven upon treating MCF-7 cells with SbBEE, displaying a significant decrease in the G0/G1 phase and a significant increase in the pre-G0 phase via cell cycle analysis using flow cytometry. Also, another hallmark of apoptosis, DNA fragmentation, was noticed using Cell Death ELISA in which an increase in the level of DNA fragmentation was observed. Collectively, these findings show that both cellular fragmentation and DNA fragmentation might have contributed to the induction of apoptosis in MCF-7 cells in this study. The latter could be primarily explained by previous research which associates a G0/G1 phase arrest and DNA fragmentation with a decrease in cell viability and the occurrence of damaged or fragmented cells, thus, leading to apoptosis activation (Younes et al., 2020); (Ahamad et al., 2014); (S. Arora & Tandon, 2015); (Pucci et al., 2000).
Various proteins that activate apoptotic pathways are known to lead to such apoptotic trademarks. For instance, the cleavage of poly (ADP-ribose) polymerase (PARP) is one of the characteristics of programmed cell death (Skidmore et al., 1979). Prior research has revealed the contribution of PARP to DNA repair through salvaging cells with DNA damage (Min & Im, 2020). Apoptosis is induced upon the obstruction of this DNA repair when cleaved PARP (c-PARP) in Monomac-1 binds to DNA double strand breaks. Hence, the increase in the expression of cleaved-PARP shown in the results upon treatment with SbBEE implies that DNA repair has ceased, paving the way for apoptosis in the cells (Smulson et al., 1998).

Furthermore, Bcl-2 and Bax are identified as apoptotic markers in which the Bcl-2/Bax ratio regulates apoptosis mediated by the mitochondrial pathway (Wang et al., 2016). Since Bax increases mitochondrial membrane permeability, whereas Bcl-2 decreases it, an upregulation in Bax/Bcl-2 has been reported to favor apoptosis (Prokop et al., 2000); (Matsumoto et al., 2004); (Khor et al., 2007); (Katkoori et al., 2010); (L. Singh et al., 2015). A similar alteration has been detected throughout this study upon SbBEE treatment.

Moreover, the cysteine-aspartic protease, caspase-9, is activated in the apoptosome and is identified as an initiator of the intrinsic pathway of apoptosis (Su et al., 2015); (Avrutsky & Troy, 2021). Thus, the increase in the cleaved caspase 9 expression displayed in the results indicates that the apoptotic pathway induced is rather intrinsic. On the other hand, caspase-8 is another initiator caspase that is a major key inducer of extrinsic apoptosis (Muzio et al., 1996); (Boldin et al., 1996). This implies that an increase in cleaved caspase-8 expression promotes apoptosis in the extrinsic pathway. However, the results from the western blot in this study showed a constant
expression of cleaved caspase-8, supporting the claim that the apoptotic pathway triggered by SbBEE is intrinsic.

Additionally, reactive oxygen species (ROS) are among the characteristics of apoptotic cell death. Excessive levels of ROS are known to result in oxidative damage to DNA, proteins, RNA and lipids, leading to the activation of apoptosis (Meng et al., 2018). However, this current study revealed a significant decrease of ROS level in MCF-7 cells due to SbBEE treatment. Thus, while these results may not support ROS being inducers of apoptosis in this case, they highlight the antioxidant effect of SbBEE on MCF-7 cells. In fact, much of previous literature has shown extracts of different Sternbergia species to be a good antioxidant source (Iscan et al., 2017) although others have found them to have a low antioxidant activity profile (Can Ağca et al., 2021). However, the latter could be justified by the procedure in which extraction took place. In both cases, though, ethanolic extracts of Sternbergia exhibited more significant antioxidant capacity than aqueous extracts. Nonetheless, the presence of antioxidants could explain the reduction in ROS since antioxidants stand as an ROS defense mechanism in which the aforementioned oxidative damages are mitigated (Das & Roychoudhury, 2014). On the other hand, a number of antioxidant phytochemicals have been proven to be responsible for downregulating the expression of genes related to cancer such as cyclooxygenase 2 (COX-2), which is often observed in breast cancer among other types of cancer as well (Kumar et al., 2012). Thus, if found in SbBEE, these antioxidants could contribute to SbBEE’s anticancer activity on MCF-7 cells through the inhibition of cell proliferation, cell cycle arrest and the induction of apoptosis (Gloria et al., 2014).

Therefore, further pro-apoptotic mechanisms caused by SbBEE may also be attributed to the extract’s composition, which was proven to be rich in compounds of
potential cell death inducing properties. Among the well-known compounds detected in SbBEE in this study is lycorine. This was expected since lycorine was previously proven to be present in *Sternbergia clusiana* (G. Çitoğlu et al., 1998). Lycorine present in SbBEE is thought to have contributed to a great extent in inducing apoptosis in MCF-7 cells especially because it was found to have cell death activating ability in these cells by prior research (Ying et al., 2017). That is in addition to its capability to induce apoptosis in leukemia cells and anti-proliferation activity in multiple myeloma cell line ARH-77 and ovarian cancer Hey1B cells (Ren et al., 2014); (Luo et al., 2015); (Z. Cao et al., 2013).

9S,13R-12-Oxophytodienoic acid (OPDA) is another compound found in great amounts in SbBEE. OPDA was proven by previous studies to inhibit breast cancer cells proliferation via degrading the protein cyclin D1 and nuclear β-catenin and, hence, arresting the G1 cell cycle phase (Altıok et al., 2008). Moreover, a beta-oxidation product of this acid, jasmonic acid, revealed an ability at millimolar concentrations to inhibit cell growth as well as cause apoptosis in human leukemia cells, B-lymphoma cells and lung cancer cell lines (Rotem et al., 2003); (Fingrut et al., 2005); (Yeruva et al., 2006). Therefore, OPDA might be partly responsible for the induction of apoptosis in MCF-7 cells upon treatment with SbBEE.

Adding to the above, SbBEE was also found to be rich in Fumaritine N-oxide. Fumaritine N-oxide is an alkaloid belonging to the class isoquinoline (PubChem, n.d.-b). Studies revealing Fumaritine N-oxide’s anticancer properties are yet to be found. However, research around other isoquinoline alkaloids has shown promising anticancer effects, including mechanisms of apoptosis, cell cycle arrest and autophagy, hence, causing cell death (Yun et al., 2021). The latter was proven in
different types of cancer and not only breast cancer. Thus, this could explain the induced apoptosis seen on MCF-7 cells in this study.

Furthermore, 4-Indolecarbaldehyde was revealed to be highly abundant in SbBEE as it occupied a large area within it. It is known as a heteroarene carbaldehyde as well as an indole (PubChem, n.d.-c). Research has shown 4-Indolecarbaldehyde to be a promising anticancer agent for cancer therapy. For instance, a compound made up of 4-Indolecarbaldehyde and 4,5,6-trimethoxy indanone was found to play a significant antiproliferative and apoptosis-inducing role; it resulted in a G2/M cell cycle arrest, thus, interfering with mitosis in five different human cancer cell lines: HeLa, A549, PC-3, Bel-7402 and K562 (Chen et al., 2015). In fact, indole derivatives in general have been proven over the years to be associated with pro-apoptotic activities, including in breast cancer cells (Kaur & Jaitak, 2019); (Jia et al., 2020). Indole scaffolds have also been revealed in previous literature to contribute to the inhibition of DNA topoisomerase, protein kinase, histone deacetylase and tubulin, thus, showing results effective against lung cancer (Dhuguru & Skouta, 2020).

Additional compounds detected within SbBEE include rhamnetin, which has been praised for its ability to induce apoptosis by suppressing MCF-7 cells’ viability and activating caspase-3/9 (Lan et al., 2019). Ferulic acid, associated previously with anticancer activity in cervical cancer cells via cell cycle arrest and autophagy (J. Gao et al., 2018), was also located in SbBEE. Adenosine was proven to be another component of SbBEE, and it leads to apoptosis through the mitochondrial intrinsic pathway and induces the activity of caspase-3 and -9 via the PI3K/Akt/mTOR signaling pathway according to past research on FaDu human pharyngeal squamous carcinoma cells(Choi et al., 2018).
SbBEE’s composition further extends to include a number of other pro-apoptotic and anti-proliferative compounds, suggesting that they act together to mitigate the development of breast cancer in MCF-7 cells. However, a number of the compounds detected in this study have not been addressed by previous literature, and their anticancer properties remain unknown. For that, this should be accounted for when explaining SbBEE’s apoptosis-promoting activity in MCF-7 breast cancer cells.
Chapter Five

Conclusion

Despite the continuous advancements in cancer therapeutics, plants remain a safe and promising source of bioactive components capable of effectively targeting cancer cells. The current study displayed an anti-proliferative and pro-apoptotic activity of ethanolic extracts of Sternbergia clusiana bulbs (SbBEE) on breast cancer cell line MCF-7. A cell cycle arrest via evident cellular and DNA fragmentation, an antioxidant effect, an activation of the mitochondrial pathways through an increase in Bax/Bcl-2 ratio and the cleavage of PARP have all been identified throughout this study and supported the induction of apoptosis. The latter is in addition to the pro-apoptotic impact of compounds identified to be present in the extract via chemical analysis. While the findings suggest SbBEE as a potential candidate for novel therapeutic drugs against MCF-7 cells, future research should consider exploring SbBEE’s anti-proliferative and pro-apoptotic activity on cell lines belonging to other types of cancer in order to investigate whether *Sternbergia clusiana* is cancer-type specific. Studies should also confirm the extract’s promising results in vivo.

Moreover, compounds should be classified according to the extent of their biological anticancer activity via extract fractionation. Additionally, Sternbergia has been associated with anti-acetylcholinesterase (AChE) or AChE inhibitory (AChEI) activity (Calderón et al., 2010); (Rhee et al., 2001), which is essential for mitigating the destruction of acetylcholine in the brain and, hence, Alzheimer’s disease. Thus, further investigations on *Sternbergia clusiana*’s potential in treatments differing from cancer therapy (e.g. treatment of Alzheimer’s disease) is also recommended.
References


