Identification and Characterization of the Anticancer Potential of Indigenous Medicinal Plants of the Arabian Peninsula

Sameera Omar Mohammed Saeed Balhamar
IDENTIFICATION AND CHARACTERIZATION OF THE ANTICANCER POTENTIAL OF INDIGENOUS MEDICINAL PLANTS OF THE ARABIAN PENINSULA

Sameera Omar Mohammed Saeed Balhamar

This thesis is submitted in partial fulfillment of the requirements for the degree of Master of Medical Sciences (Biochemistry and Molecular Biology)

Under the Supervision of Dr. Farah Mustafa

October 2016
Declaration of Original Work

I, Sameera Omar Mohammed Saeed Balhamar, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "Identification and Characterization of the Anticancer Potential of Indigenous Medicinal Plants of the Arabian Peninsula", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Farah Mustafa, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Indigenous plant species historically used for their medicinal properties are a tremendous source for bringing newer and safer drugs to the market. A concerted effort is needed to characterize their medicinal potential and identify new molecules that could be exploited in modern medicine. The current study was undertaken to study the anticancer properties of several indigenous plants that are used by the local population of the Arabian Peninsula and beyond for various medicinal purposes. Towards this end, we acquired different plant extracts from five plants, namely *Boswellia sacra* (BS), *Cleome droserifolia* (CD), *Teucerium muscatensis* (TM), *Orchadenus arabicus* (OA), and *Acredocarpus orientalis* (AO) that were screened for their anticancer properties and mechanism of action. Of these, only the essential oil from *Boswellia sacra* has recently been shown to have anticancer activity.

Thirty-two different organic extracts were obtained from these native plants. Essential oil from *Boswellia sacra* was used as a control as well as to expand upon its anticancer activity profile. Initially, the essential oil and the extracts were screened for their anti-proliferation potential in two different human cancer cell lines, MCF-7 and HeLa, to ensure that their anticancer potential was not missed. The cell proliferation assay, MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), was used to study the effect of the oil and various extracts on cell viability in a time and dose-dependent manner. Once all the extracts had been screened, a select group of effective extracts were chosen from each plant for a more in-depth analysis in a full panel of human breast cancer cell lines comprising of MCF-7 and MDA-MB-231 and their normal counterpart, MCF-10A, as well as the prototypic human cervical cancer cell line, HeLa. MCF-7 represents a hormone-responsive breast cancer cell line, while MDA-MB-231 represents a non-hormone responsive (triple receptor negative) breast cancer cell line. The mechanism of cell death induced by these extracts was explored by testing the activities of various caspase enzymes induced during apoptosis, a major pathway of programmed cell death, using caspase-specific gel assays and western blot analysis. Finally, a few isolated compounds and their derivatives obtained from these extracts were acquired from our collaborators and tested to determine whether they could demonstrate the anti-
proliferative effects observed using the crude extracts and if so, whether it was due to apoptosis.

Test of the extracts using these assays revealed that some of the extracts exhibited anti-proliferative activity against the targeted cancer cell lines. The anti-proliferative effects of some of these extracts were found to be selectively more active against breast cancer cell line than HeLa cells. The ATP-dependent CellTiter-Glo® Luminescent Cell Viability Assay further confirmed the effect of the extracts on cell viability. Test of the activity of different caspase enzymes revealed that some of the effective extracts had the capability of inducing apoptosis. Western blotting was used to confirm the role of various caspases in the activation of apoptosis. Finally, isolated compounds from these extracts and their derivatives were screened with the MTT assay for their effect on cell viability. One of these isolated compounds has shown promising anti-proliferative activity and could induce a specific caspase, suggesting activation of caspase-dependent apoptosis. Thus, analyses from these multi-pronged approaches have resulted in the identification and characterization of extracts having anticancer potential from five indigenous plant species. We are further characterizing their mechanism(s) of action as well as exploring other cell death pathways that may be induced. Overall, our results demonstrate that traditional medicinal plants can provide an excellent source of natural raw material to isolate novel anticancer agents, enlarging the arsenal of new molecules available to fight cancer.

**Keywords:** Indigenous medicinal plants, Arabian Peninsula, *Boswellia sacra*, *Cleome droserifolia*, *Teuerium muscatensis*, *Orchadenus arabicus*, *Acredocarpus orientalis*, anticancer, anti-proliferation, apoptosis, caspases, anticancer drugs.
Title and Abstract (in Arabic)

تعريف و توصيف إمكانية مكافحة السرطان باستخدام النباتات الطبية المحلية في شبه الجزيرة العربية

الملخص

عرفت عبر التاريخ أنواع مختلفة من النباتات المحلية بالخداماتها الطبية و تعتبر هذه النباتات مصدرًا غنيًا يجب استثماره لتحويل أدوية جديدة تكون آمنة طبيًا في الدراسة الحالية تم جلب خمس من النباتات المحلية المستخدمة طبيًا في شبه الجزيرة العربية آلا و هي:

(Cleome droserifolia)
(Orchadenus arabicus (OA))
(Nebov dialog (ND))
(Boslvellia sacra)
(Teucrium muscatensis (TM))
(Acredoculis orientalis (AO))

32 مستخلصاً استخدم في التجربة بغرض تحديد ما إن كانت هذه المستخلصات ذات خصائص مكافحة للسرطان، بالإضافة إلى زيت اللبان الذي يعرف بخصائصه المكافحة للسرطان و الذي أضيف إلى الدراسة لتأكيد و تفصيل تأثيره على الخلايا السرطانية.

فعالية هذه المستخلصات في مكافحة السرطان تم اختبارها على نوعين من الخلايا السرطانية: 7-HeLa و MCF-7 بالتأكد من أن مفعولها المضاد للسرطان. باستخدام اختبار تكاثر الخلايا (MTT) مع تغيير جرعات هذه المستخلصات و وقت التعرض لها تمت دراسة تأثيرها على تكاثر الخلايا السرطانية. بعد التحقق من جميع المستخلصات تم اختبار مجموعة مستخلصات من كل نبتة و دراستها بعمق في أنواع مختلفة من خلايا الثدي السرطانية MCF-7، و نظائرها خلايا MCF-10A و نظيرها خلايا MDA-MB-231 و خلايا MCF-7، و antibodies NFMو MDA-MB-231 عبر نموذجاً يعتمد على الكرات بالهورمونات بينما .HeLa لا تستجيب للعلاج بالهرمونات. خلال هذه الدراسة استخدمت تقنيات متعددة لتحديد تأثير هذه المستخلصات على نمو و تكاثر الخلايا السرطانية إضافة إلى الآلات عملية أخيراً، تم استخراج بعض المركبات الفعالة و مشتقاتها من هذه المستخلصات بواسطة المتعاونين معنا لتحديد المكون المسؤول عن التأثير الذي لوحظ على تكاثر الخلايا السرطانية و ما إن كان له علاقة بموت الخلايا المبرمج.

نتائج هذه الدراسة أظهرت أن بعض المستخلصات أثرت على تكاثر و نمو الخلايا السرطانية و تسببت بخفض أعداد الخلايا. كما و أن قوة التأثير اختلفت من مستخلص لآخر.
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Special thanks go to my parents, brothers, and sisters who supported and helped me along the way. I am sure they suspected it was endless.
I dedicate this dissertation to my family and many friends. I have a special feeling of gratitude for my loving parents whose words of encouragement and push for tenacity rings in my ears. My sisters and brothers have never left my side and are very special.

I also dedicate this dissertation to my many friends who have supported me throughout the process of the entire Master Program. I will always appreciate all they have done.
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<tr>
<td>β2GPI</td>
<td>β2-glycoprotein 1</td>
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<td>annexin A1</td>
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<td>AP-1</td>
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<td>A1</td>
<td>Aromatase Inhibitor</td>
</tr>
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<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>AIF</td>
<td>Apoptosis Inducing Factor</td>
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<td>AKBA</td>
<td>Acetyl-11-Keto-beta-Boswellic Acid</td>
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<td>AO</td>
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<td>APBI</td>
<td>Partial Breast Irradiation</td>
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<td>Brain-specific Angiogenesis Inhibitor 1</td>
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<td>Colony Stimulating Factor</td>
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<td>Cleome droserifolia</td>
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<td>Epidermal growth factor receptor</td>
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<td>endoG</td>
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<td>FADD</td>
<td>Fas-Associated protein with Death Domain</td>
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<td>Growth arrest-specific 6</td>
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<td>Insulin-like growth factor</td>
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<td>MAE</td>
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<td>NCI</td>
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<td>NFKB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>OA</td>
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<td>Protein S</td>
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<td>Poly ADP ribose polymerase</td>
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<td>Platelet-derived growth factor</td>
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Chapter 1: Introduction

1.1 Herbal Medicine

Ethnomedicine, or more precisely “ethnobotanic medicine” is defined as the use of plants by humans as medicines [1, 2]. Ethnopharmacology, on the other hand, is transformation from using plants as medicine to drug discovery involving the observation, description, and experimental investigation of drugs and their biological activities [3]. This approach is dependent on botany, chemistry, biochemistry, pharmacology, and other factors such as anthropology, archaeology, history, and linguistics which have led to establishing the biological activity of natural products [4]. Humans have used plants as medicine since the earliest signs of civilization can be traced. For centuries, use of natural products with therapeutic properties has been recorded as medicine in almost all ancient civilizations, including Chinese, Indians, and North Africans, providing evidence of utilizing plants as a treatment of different diseases [5, 6].

Greeks were the first to classify and describe plants in order to identify them. Europe played an important role in the production of medicinal plants used to heal pain [7]. The 19th century saw the beginning of scientific investigation and isolation of active compounds from medicinal plants, for example, discovery of quinine from Cinchona bark by the French scientists Caventou and Pelletier [7]. This discovery opened the interest to find other plants from the New World in jungles and forests for new medicines. Explorers searched for a long time looking for new natural resources, while the well-equipped phytochemistry laboratory of Harbonney became the center for isolation of active principles of medicinal plants from the globe [7]. Numerous
flavonoids were isolated and expanded rapidly by pharmaceutical companies later on.

Many natural products isolated from plants before World War II (WWII) are still in clinical use today for example, morphine and codeine from *opium poppy*, digoxin from *Digitalis* leaves, atropine from *hyoscyamine* and hyoscine from *Solanaceae*. During and after WWII, many antibiotics were isolated from *Penicillium*, *Cephalosporium*, and *Streptomyces*. After WWII, other drug discoveries were made, such as reserpine from *Rauwolfia* (a pain-killer) and also vinblastine and vincristine from *Catharanthus roseus*, which were effective in cancer chemotherapy [7]. Later on, a lot of successful clinical agents were transferred from research teams to pharmacologists and synthetic chemists. For example, use of atenolol which is a beta-blocker and captopril (ACE-inhibitor) used for the treatment of hypertension, salbutamol (adrenergic receptor stimulant) used for asthma, and benzodiazepines (hypnotics and anxiolytics) used for insomnia and anxiety attacks [7].

Thus, plants have enormous medicinal properties, which make them a valuable source of new medicines. According to the World Health Organization (WHO), almost 65% of world population uses traditional medicines as a source of therapeutic agents for their healthcare [8]. Plants can be a source of bioactive compounds for direct use as drugs, as described above. Another importance of plants is to produce bioactive compounds of novel or known structures, leading to the synthesis of compounds which have high activity and lower toxicity. For example, metformin, nabilone, oxycodone, taxotere, teniposide, verapamil, and amiodarone are derived from plants [9]. Also the whole plant or part of it can be used as cure in our diet such as cranberry, echinacea, feverfew, garlic, etc. The numbers of plants on
the planet are estimated to be around 250,000 [10]. Only 6% of these plants have been screened for their biological activity and 15% evaluated for their phytochemical composition [11]. With advanced screening methods, the number of plants screened should change.

The importance of obtaining different therapies from nature used for different diseases cannot be underestimated. Around 25% of our current drugs come from plants. From 252 drugs considered basic and important by WHO, 11% are of plant origin. A majority of these drugs are obtained from plants. Natural compounds are the leading therapeutic agents, allowing the design and rational planning for new drug discovery, synthesis development, and discovery of new therapeutic properties not connected to known compounds [12]. Considering that in 1991 in the US, of every 10,000 pure compounds biologically evaluated, only 20 were tested in animal models, of those only 10 were evaluated clinically, and only one reached the US Food and Drug Administration (FDA) for approval for marketing through 10 years costing US $231 million [13], it is critical that new compounds should be continuously isolated and screened from plants.

1.2 Cancer Overview

Cancer is an uncontrolled growth of abnormal cells which results from alterations in DNA. These alterations change the genetic information and prevent the proper function of normal cells, leading them to divide without stopping and spread to surrounding tissues. All types of cancer can start anywhere in the human body made up of trillions of cells. It is one of the most common causes of morbidity and mortality worldwide [14]. Globally, the National Cancer Institute (NCI) reports that in 2012, there were 14 million new cases and 8.2 million deaths worldwide [15]. It is
expected to rise to 22 million new cases within the two next decades. In the US, between the years 2008 and 2012, there were about 454.8 cases and 171.2 deaths per 100,000 men and women per year. In 2015, there were ~1,658,370 new cases of cancer in the US and it is estimated that 589,430 will die from the disease [16]. By 2024, the new cases are expected to be around 19 million in numbers [16-18].

In the UAE, the most common cancers in men are colon and rectum cancers, while breast cancer is the most common in women. The overall incidence of cancer in men was 89.8, while the mortality was 76.1 per 100,000 population. On the other hand, the incidence of cancer in women was ~111.6-139.9 and mortality was 69.2 per 100,000 populations [19].

1.2.1 Causes of cancer

Cancer cells begin when the genes controlling basic cell function such as how they grow and divide are altered or mutated. These genetic changes can be inherited from the parents or modified by environmental factors such as tobacco smoke, radiation, and ultraviolet rays from the sun. In addition, they affect three main groups of genes, including proto-oncogenes, tumor suppressor genes, and DNA repair genes, which are also called drivers of cancer [20]. Proto-oncogenes play an important role in assuring regulated cell growth and division in normal cells, but in cancer cells they are altered or activated to become cancer causing genes (oncogenes), allowing cells to grow and survive when they should not. Tumor suppressor genes control cell growth and division in normal cells, whereas in cancer cells these genes are mutated, leading to dysregulated and uncontrolled cell division. DNA repair genes are involved in the repair of damaged DNA in normal cells, but these genes are altered in cancer, leading to the development of other mutations, ending up causing the cells to
become cancerous. These genes are important in the fidelity of DNA replication, assuring normal cell growth and division. Furthermore, cancer cells have biological capabilities that are required for the successful development and complexity of tumors in humans, including uncontrolled growth of cells, loss of cell differentiation, ability to ignore signals involved in normal cell growth and death, and the capability to influence normal cells, molecules, and blood vessels to supply the tumor with oxygen and nutrients (microenvironment) [20]. Also, they can evade the immune system that protects the body in order to hide from it or use the immune system to prevent it from killing the cancer cells. All of these factors make cancer a very successful disease.

1.2.2 Breast cancer

Breast cancer is the most common cancer in US women according to NCI with 124.8 diagnosed and 21.9 deaths per 100,000 women per year based on 2008-2012 data [16]. In 2012, there were 2,975,314 women living with breast cancer in the US alone [17]. Around 12% of women will be diagnosed in their lifetime based on 2010-2012 data [17]. It is estimated that 231,840 women will be diagnosed and 40,290 deaths will occur in women in the US in 2015, while 1% accounts of the breast cancer cases and deaths will occur in men [16-18]. In the UAE, the incidence of breast cancer was 33.9-45.8 per 100,000 populations, accounting for 31-43% of all cancer patients, while the breast cancer mortality was 10.1-13.1 per 100,000 populations, accounting for 8.2-12% of all cancer deaths in 2012 [19]. Recently, breast cancer incidence has declined compared to the last decade as a result of the development of better diagnostic techniques using mammography and treatment strategies that have reduced the number of women using postmenopausal hormonal therapy which had increased the incidence of invasive breast cancer as reported in
the UK and the US [16-18].

Genetic mutations in BRCA1 and BRCA2 genes [21-25] and the family history [26] are known as the most important risk factors for hereditary breast cancer. However, there are other risk factors associated with the sporadic form of breast cancer development such as increase in a women's age (ranging from 45-54 years [17], environmental factors [27, 28], hormonal therapy history [29], obesity [30, 31], and alcohol intake [32].

Breast tumor development begins once a genetic mutation arises which causes the mutated breast cell to divide and proliferate preferentially, leading to more cells with the same mistake [20]. Then, the uncontrolled growth of cancer cells leads to hyperplasia. After that, cancer cells proliferate too much as a result of further damage, leading to further abnormalities (atypia). The cells lose a sense of orientation and become blind to the surrounding cells and body’s signals to stop proliferation, a process called dysplasia. The progression of breast cancer involves pathological and clinical stages, starting with in situ carcinoma when cells become more abnormal in growth and appearance, but have not broken around the tumor through a boundary where the tumor is contained. Once the cancer cells break through the boundary and invade nearby tissues, the cancer can be life threatening and is called invasive cancer or primary tumor. Finally, when invasive cancer cells or primary tumor gain the ability to enter the blood stream and travel to distant areas (metastasize), it leads to secondary tumors (metastases) which is life threatening [20].
1.3 Cancer and Herbal Medicine

Many medicinal herbs have been used for the prevention and treatment of cancer. Around 60% of anti-tumor and anti-infectious drugs on the market are from natural origin under clinical trials and the number of new anticancer drugs are increasing [33]. It is presumed that natural compounds are safer than synthetic compounds as a result of their presence in diet, wide availability, and tolerability [34]. The bioactive compounds isolated from medicinal plants can be developed as anticancer agents. Many studies have demonstrated the anti-tumor properties of products isolated from plant sources. For example, *Camellia sinensis* (green tea) is the most popular beverage used in the world after water. It has a distinctive group of polyphenols called catechins [34]. There are four major bioactive components in green tea: epigallocatechin-3-gallate (EGCG), epicatechin (EC), epicatechin-3-gallate (ECG), and epicatechin (EC). Green tea has been shown to suppress cell growth and kill cancer after distinguishing tumor cells from normal cells [35]. Tea consumption has inhibitory carcinogenic effect at many organ sites. Green tea has the ability to inhibit the development of experimental rodent skin tumor [36], growth of tumor cells [37], invasion and metastasis [38, 39], and angiogenesis [40, 41]. Many studies suggest that people who drink more green tea have a lower risk of prostate [42, 43] and breast cancers [44].

*Curcuma longa* (Curcumin) is the yellow pigment in turmeric. It is a well-known spice in Southeast Asia [34]. Since the 1980s, curcumin has been shown as the chemopreventive agent known to suppress many biological factors important for proliferation of cancer cells [45-51]. Curcumin administration allows gradual decline of the tumor growth rate and increases animal survival significantly [35]. In addition, curcumin is able to suppress hemoglobin concentration in tumor cells, indicating
inhibition of angiogenesis. As a result of the poor absorption of curcumin in the blood stream, researchers are looking at its derivatives which may have enhanced bioavailability [52]. In a glioblastoma xenograft model system, curcumin has been shown to inhibit tumor growth as well as angiogenesis [53]. Other studies have shown that curcumin has an inhibitory effect on colon carcinogenesis as demonstrated in rodent models [54, 55]. It also inhibits the initiation and promotion of skin cancer [56]. Curcumin can inhibit the growth of cancer cells from multiple organs sites in vitro as tested in xenograft models by induction of cell cycle arrest and apoptosis [57-60]. Synergistic anticancer activity of curcumin has been observed with genistein [61], green tea [62], and embelin [63].

Vitis vinifera (red grape) is rich in resveratrol (3,4,5-trihydroxystilbene) which is the bioactive anticancer agent found in red grapes, red wine, and mulberries [34]. Resveratrol can prevent cancer by inhibiting cyclooxygenase enzymes and angiogenesis with modulation of drug metabolizing enzymes [35]. Experimentally, it has been shown to have protective effects against carcinogenesis [64-65, 66]. It is well known that it has antioxidant potential and anti-inflammatory activity [67]. It seems to affect cell cycle and apoptosis pathways involved in the process of preventing cancer [68]. In addition, it has effects against all stages of carcinogenesis of skin cancer in mice [69]. Another advantage of resveratrol is that it can be applied before or after UV exposure to decrease the incidence of skin cancer in mice [70]. The prophylactic use of resveratrol has been shown to reduce the number and size of tumors in esophageal, intestinal, and colon cancers [70, 71]. In addition, it is effective against cancer of other tissues such as liver, pancreas, gastrointestinal tract, lung, and soft tissues [57, 72, 73].
Other than these specific foods, daily consumption of vegetables develops the immune system and fights diseases like cancer [35]. Generally vegetables have sulforaphane and the flavonoid luteolin, especially in broccoli, celery, cabbage, spinach, green pepper, and cauliflower [34]. Sulforaphane and luteolin both have anti-inflammatory and anticancer activities [74]. Luteolin has been found to induce cell cycle arrest and apoptosis in various cancer cells, including oral squamous [75], esophageal [76], lung [77], colon [78], and liver [79]. In *in vitro* and *in vivo* studies, it has been shown to inhibit cell growth and induce apoptosis in prostate cancer [80]. Other studies have shown that luteolin increases the efficacy of chemotherapy (cisplatin) in gastric cancer [81]. It has the ability to decrease the development, initiation, and post-initiation stages of colon cancer in animal model systems [82].

Many other dietary and medicinal natural compounds are under investigation for their anticancer activity [34]. These compounds include ellagic acid, some triterpenes (such as lupeol, betulinic acid, ginsenosides, oleic acid) and ginkolide B. Ellagic acid is an antioxidant polyphenol found in many fruits and vegetables like grapes, strawberries, raspberries, pomegranate, and nuts [83, 84]. It has been shown to have chemopreventive activity against skin, lung, esophageal, colon, bladder, prostate, and breast cancers [83, 84]. Triterpenes, lupeol, and betulinic acid have been tested in cell culture and animal models for their chemopreventive activities and shown to have activity against multiple cancer types [85]. Ginkolide B from Ginkobiloba extracts has also been tested for its chemopreventive activities against many types of cancers [86, 87].

Many disorders such as atherosclerosis, arthritis, Alzheimer disease and cancers are due to increased concentrations of free radicals in the body as well as
from ultraviolet radiation and other environmental pollutants [88]. This results in disequilibrium in the antioxidant pro-oxidant balance [89, 90]. Antioxidants isolated from plants have well-known health promoting effects which donate an electron to compromised molecules to bring them back to proper function [91]. For example, many flavonoids (baicalein, baicalin, luteolin), monoterpenoids, triterpenoids (oleanolic and ursolic acids), glycosides, and phenolic compounds (caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid, and vanillic acid) in the *plantago* species have been used for treating different diseases such as inflammation, disorders of respiratory and digestive organs, reproductive system, blood circulation and cancer due to their immunomodulatory activities [92].

Flavonoids and phenolic acids are metabolites from plants with polyphenolic structure and high potential antioxidant effect. Flavonoids are classified into six major subgroups: flavones (luteonin, apigenin, tangeritin), flavonols (quercetin, kaemferol, myricetin, isorhamnetin, pachypodol), flavanones (hesteretin, naringenin, eriodictyol), flavan-3-ols (catechins and epicatechins), isoflavones (genistein, daidzein, glycine), and anthocyanidins compounds (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidin) [93]. Other flavonoid groups include aurones, xanthones, and tannins. Flavonoids are available in our daily diet [94, 95]. Almost 6000 flavonoids have been identified in plants [96, 97]. Flavonoid components that accumulate in the vacuoles of the plant cells are called glycosides. Differences in glycosides are due to the number and type of sugars involved in glycosylation [98]. Flavonoids are known for their antioxidant activity important for oxidative stress balance. Oxidative stress is involved in initiation and development of many pathological conditions such as inflammation, autoimmune diseases, cataract, cancer, Parkinson's disease, arteriosclerosis, and aging [99]. Thus, consumption of high
antioxidant rich fruits and vegetables should reduce the risk of many cancer types, suggesting that antioxidants are effective agents for inhibiting cancer [100].

Polyphenols from different plants have been considered as anticancer agents as well tested in many cancers cell lines at different stages of cancer growth. For example, kaempferol, quercetin, anthocyanins coumaric acid, and ellagic acid isolated from strawberry inhibit the growth of human cancers in breast (MCF-7), oral (KB, CAL-27), colon (HT-29, HCT-116), and prostate cancer cell lines (LNCaP, DU-145) [101,102]. Previous studies with similar effects have been reported from isolated polyphenols such as resveratrol, quercetin, catechin, and epicatechin from green tea polyphenols (epigallocatechin, epicatechin) [103].

It has been shown that flavonoids have anti-proliferative activity against several cancer cell lines [104]. It is reported that citrus flavonoids inhibit the growth of leukemia cells (HL-60) [105] and other cancer cell lines [106]. In addition, they are promising agents due to their safety, low toxicity, and general acceptance [107, 108]. Kaempferol is also a type of flavonoid, which has shown inhibition of the growth of ovarian cancer (91%) and breast cancer cell lines (94%) [109]. Epigallocatechin 3-gallate is an effective anti-angiogenic agent, inhibiting invasion and proliferation of tumor cells [110]. It also has been shown to inhibit the growth of bladder tumor cells and breast cancer cell lines [111]. Quercetin has anti-inflammatory activity, which inhibits the inflammation processes directly [112]. In addition, some tests have revealed anticancer properties of quercetin, including the inhibition of proliferation and migration of cancer cells [113]. In addition, quercetin is involved in mortality of prostate cancer showing 90% death within 48 hours and no mortality on normal cells [114]. Gallic acid, caffeic acid, and ferulic acid also
have been shown to have anticancer activities [115, 116]. Luteolin is a common flavonoid in many types of plants, including fruits, vegetables, and medicinal herbs. Studies have shown that luteolin has the potential for cancer prevention and therapy. It induces apoptosis cell death in many kinds of cancers [117-120] inhibits cancer cell proliferation [121-123], and suppresses angiogenesis of tumors [124].

Thus, phytochemicals isolated from the plants or natural compounds are promising anticancer agents due to their multiple inhibitory activities, especially for the various stages of cancer, including initiation, development, and angiogenesis.

1.4 Genes and Cancer

Human cancers are strongly linked to genetics. Approximately, 350 genes are thought to be involved directly or indirectly to cancer [125]. Accordingly, controlling genes could be a treatment or prevention of cancer. In order to improve the drugs used in treating cancer, the pharmaceutical industry has moved from mono-targeted to multi-targeted drugs, competing with natural compounds, which are naturally a multi-targeted therapy.

NF-kB and STAT 3 transcription factors are involved in expression of more than 400 genes crucial for prevention and treatment of cancer [126, 127]. They are responsible for transformation to cancer cells, their survival, proliferation, invasion, angiogenesis, and metastasis. Cancer cells are found to express the active forms of these transcription factors [126, 127]. Other studies have shown that environmental factors, high fat diet, stress, alcohol, and infections activate NF-κB and most growth factor receptors like EGFR, HER2, TNF also activate NF-κB [128]. Aggarwal and coworkers identified compounds from fruits and vegetables and other traditional medicine, which can suppress these pathways and their downstream gene products.
For example, curcumin can suppress both NF-κB and STAT3 pathways in various cancers, leading to inhibiting the survival of tumor cells, suppression of proliferation, and invasion [129]. Other natural compounds that have shown similar effect using cell culture and animal model systems, including capsaicin from red chili, thymoquinone (TQ) from black cumin, anethole from fennel, eugenol from cloves, and zerumbone from ginger. These natural compounds can activate the NF-κB signaling cascade, translocation of NF-κB to the nucleus, DNA binding of the dimers, or interactions with the basal transcriptional machinery. The inhibition of NF-κB results in inhibition of its target genes including Bcl-2, cyclin D1, matrix metalloproteases, VEGF, and others [128].

The tumor suppressor gene p53 controls the cell cycle, apoptosis, genomic integrity, and DNA repair [130, 131]. When p53 is activated, it binds to DNA regulatory sequences, resulting in cell cycle inhibition, apoptosis, genetic stability and inhibition of angiogenesis [132-134]. For example, EGCG in green tea induces the expression of p53 and its targets p21 and BAX in prostate and breast cancer cells [135, 136]. Induction of cell cycle arrest and apoptosis is observed by luteolin-activated p53 and its targets p21, BAX, and PUMA [81, 137]. The same effect has been observed for curcumin [138, 132] and soy isoflavone genistein in glioma cells [133].

Other regulatory elements can also be inhibited by phytochemicals such as activator protein AP-1 that activates transcription and mediates gene activation [134]. The target genes for AP-1 are involved in survival, metastasis, angiogenesis, proliferation, invasion, and differentiation [134]. Green tea (EGCG) [139], resveratrol [140], and curcumin [141] have been shown to suppress AP-1 activation.
Growth factors such as EGF, PDGF, FGF, tumor growth factor (TGF), insulin-like growth factor (IGF), and colony stimulating factor (CSF) are also very important in the development of cancer [141]. Once these factors are activated, they result in increased cell growth, suppression of apoptosis, and invasion by cancer cells. This activates the downstream signaling pathways such as PI3K-AKT and Ras-MAPK. Natural compounds have the ability to target these signaling pathways and inhibit cancer development [140-141]. For example, curcumin inhibits the EGFR and IGF receptor pathways and enhances the chemotherapeutic effects of oxaliplatin [142, 143]. EGCG increases the efficacy of erlotinib in head and neck cancer by inhibiting the activation of EGFR, AKT, and ERK [144, 145] and IGF-1 receptor in colon cancer cells [146]. Luteolin inhibits the IGF-1-induced activation of IGF-1R and AKT in prostate cancer cells and activates the suppression of EGFR and MAPK/ERK signaling pathways [147].

Angiogenesis is a very crucial step for cancer development, progression, and metastasis. It is also involved in the resistance to chemotherapy and radiation. EGCG inhibits cell viability, capillary tube formation, and migration of human umbilical vein endothelial cell (HUVEC), and in xenograft model of pancreatic cancer, inhibits the expression of angiogenic and metastasis markers (von Willebrand factor, VEGF, CD31, MMP-2, MMP-7, MMP-9, and MMP-12) [148]. Luotenol inhibits VEGF-induced angiogenesis and tumor growth in xenograft model [149]. The inhibition of angiogenesis in different studies has been suggested by curcumin [150, 151], genistein [152, 153], rutin, naringin, apigenin, and vitamin E [153].

Flavonoids have been shown to enhance several anticancer biological activities at nontoxic concentrations [154]. Many studies have shown the importance
of flavonoids in chemoprevention and chemotherapy. As mentioned earlier, they are also involved in the inactivation of carcinogens, have anti-proliferative action, cause cell cycle arrest, induce apoptosis and differentiation, inhibit angiogenesis, have antioxidative properties, and can reverse multidrug resistance or a combination of these mechanisms [154]. This shows that natural compounds such as flavonoids are promising agents in anticancer drug development [154].

1.5 Cancer Treatments

There are many types of cancer treatments. The type of treatment is dependent upon the type and stage of cancer diagnosed for the patient. Some people need only one type of treatment, while others may require a combination regimen to get rid of cancer cells. For example, surgery can be used to remove the tumor from the body along with chemotherapy (chemicals) to kill the cancer cells preferentially or stop their proliferation and/or radiation therapy to kill cancer cells and shrink the tumors. However, chemotherapy and radiation affect normal cells as well that are fast growing. Thus, these treatments are quite toxic to healthy cells as well. Thus, there are many new approaches being developed for treating cancer to specifically reduce and/or circumvent the side effects of the chemotherapy/ radiation treatments harming the healthy tissues and organs. Alternative treatments or targeted therapy could be the solution to give the highest effect against cancer cells and reduce the side effects of the traditional therapies, which can be discovered from nature [14].

1.5.1 Breast cancer treatment

Breast cancer treatment depends upon the type of tumor as well as the patient. In order to overcome these differences, breast cancer treatment plan is based on the patient’s overall health, biology of the tumor, and the stage of the tumor [14]. The
Effective treatment strategies are divided into two main areas, including the local and systemic therapy. Local therapy aims to remove the cancer from its origin or local area, helping to ensure that cancer will not come back to that area using surgery with or without radiation therapy to the breast area.

Systemic therapy (adjuvant therapy) depends on many factors such as stage, grade and molecular status of the tumor and whether the tumor is hormone receptor positive [14]. Tumor cells can express estrogen (ER), progesterone (PR), or human epidermal growth factor receptors (Her2/neu) alone or in combination. Thus, breast cancer can be categorized based on hormone receptor positivity status. Some tumors are completely devoid of these hormone receptors and are called “triple negative”. These tumors are the most difficult to treat. Depending upon the type of receptors expressed, the therapy is modified. Systemic treatment standards include chemotherapy, hormone therapy, and targeted therapy, which are used in addition to the basic local treatment (surgery with or without radiation) as a prevention to diminish the risk for metastasis. Systemic therapy includes estrogen antagonist tamoxifen, aromatase inhibitor (AI), ovarian function suppression, and chemotherapy, giving the best outcome to the patients [155-159].

The treatment strategies being currently used need improvement because of serious side effects of radiation and chemotherapy. Radiation therapy is associated with several toxic effects such as radiation pneumonitis, cardiac events, arm lymphedema, brachial plexopathy, contralateral breast cancer, and risk of second malignancy. The overall incidence of pneumonitis [160] and cardiac mortality [161, 162, 163] due to exposure at delivery has been minimized since the 1990s due to modern radiation therapy techniques such as brachytherapy or Accelerated Partial
Breast Irradiation (APBI). Similarly, other modifications to radiotherapy has reduced contralateral breast cancer incidence [164] and risk of second malignancy [165]. According to NCI, it reduces local recurrence [166]. However, it is strongly recommended to use whole breast radiation after surgery to reduce recurrence of the cancer within the breast. Radiation therapy after surgery results in significant reduction of recurrence in 10 years (19% for radiation therapy after surgery compared to 35% for surgery alone [167]). Chemotherapy has its own set of toxic effects that includes nausea, myelosuppression, alopecia, mucositis, heart failure, and thromboembolic events [168] as well as premature menopause [169]. Thus, it is important to modify these treatments to have the highest effect and lowest side effects for patient safety. In addition, searching for new targeted or alternative therapies against cancer from nature is essential.

Other more innovative therapeutic approaches are also being tried to improve treatment strategies for breast cancer such as “immunotherapy”. One such strategy is the use of monoclonal antibodies alone or along with radiation and chemotherapy as a guide to attack cancer cells [170]. The importance of these antibodies are based on the immune system, which recognizes and looks for cancer cells like an invader to fight off the cancer. For example, Trastuzumab is a monoclonal antibody against the HER2/neu receptor and can target tumors expressing high levels of this antigen. This FDA-approved “drug” is being used in combination with chemotherapy to improve patient survival and progression of cancer [170]. Another treatment design used in clinical trials is to reduce and block the proliferation and metastasis of cancer cells by using anti-angiogenic drugs which block the growth of new blood vessels. Cancer cells are capable of creating their own blood supply in order to grow and spread as in metastatic breast cancers. Similarly, cancer vaccines are being designed from cancer
cells or their parts to stimulate the immune system defenses to fight and kill cancer cells. These are still in clinical trials [170].

Another way to improve treatment choices is to create tumor profiling using the genes involved in causing cancer to predict the risk of recurrence in patients [170]. Accordingly, tumors with high score require aggressive treatment (chemotherapy along with hormonal therapy), but tumors with low scores may require only chemotherapy. In addition, doctors can help guide treatment options by predicting the response of the treatment. For example, circulating tumor cells of the patient can be studied to determine whether they will respond to certain chemotherapeutic drugs [170].

1.6 Herbal Extract Preparation

Natural products from medicinal plants, their extracts, or pure compounds may provide uncountable opportunities for new drugs because of their chemical diversity [171]. Many types of phytochemicals such as flavonoids and phenolics have been approved to have significant impact on health and cancer prevention [172]. Utilization of natural products from medicinal plants starts with pre-extraction and extraction of the medicinal plant compounds. Recently, it has become one of the main areas of research and development which is applied in the pharmaceutical, nutraceutical, and dermocosmetic sectors.

Pre-extract preparation of plant samples aims to preserve the biomolecules in the plants before the extraction. The samples are prepared fresh, dried, grounded or powdered from different parts of the plant such as leaves, bark, roots, fruits and flowers. Grinding and powdering methods are used to lower the size in order to increase surface contact between samples and extraction solvents. It is crucial for
high quality efficient extraction. Different preparation methods of plants affect the preservation of phytochemicals in the final extracts [173].

Extraction is the separation of medicinal active portions of plant by using selective solvents through known methods and procedures [174]. The aim of extraction is to separate the soluble metabolites from insoluble residues left behind. Crude extracts using these methods have complex mixtures of metabolites such as alkaloids, glycosides, phenolics, terpenoids, and flavonoids. Conventionally, solvent extraction and steam distillation are used for extraction of herbs in small research setting levels. Significant advances have been made in extraction methods which aim to increase the quality of outcome and lower the cost. These methods include microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and supercritical fluid extraction (SFE) [173].

MAE technology uses microwave energy to help in separating the analytes from sample into the solvent [175]. Depending upon absorption by the material, the energy of microwave is transferred to heat [176]. The radiation from microwave interacts with polarizable materials in the solvents and sample, heating the surface and transferring heat by conduction. MAE is simple, environment friendly, economical, and it reduces the extraction time and solvent volume. However, heat denaturation is expected if proper conditions are not used [177], resulting in drastic decrease in phenolics and flavanones caused by oxidation [175]. This method is considered as selective, favoring the small phenolic molecules.

UAE technology uses high intensity, high frequency sound wave interactions with materials. It does not require complex instruments and has lower cost. UAE has a lot of possible mechanisms for ultrasonic extraction, such as cell disruption.
improved penetration, swelling enhancement, and capillary effect [178]. The main advantage of this technology is that it uses shorter time, small amounts of material, minimum volume of solvents, and has higher extraction outcome. The disadvantage is the effect of the ultrasound energy on active compounds of medicinal plants, which could lead to affecting the active molecules through free radical formation [174].

SFE is considered a non-conventional technology of solvent extraction, but the application took time to take off due to sophisticated and expensive high pressure equipment required [179]. It is currently a well-known method of extraction using primarily CO₂ as a solvent because of its low toxicity and selective extraction properties [180]. At a specific critical temperature and pressure, CO₂ can adopt properties in between a gas and a liquid. At this stage, it can be used as a solvent to penetrate into a solid plant material efficiently and extract compounds in contrast to other conventional organic solvent methods. High pressure equipment is used during the extraction in batch or continuous manner, leading to selective extraction based on varied pressure. The solvent is put in contact with the plant material according to desirable separation of the product. For sample preparation, cylindrical extraction vessels are used [174]. For processing, the solid is placed in the extraction vessel and the solvent is fed in until the target extraction conditions are reached. An advantage of this technique is that the extraction co-solvent can be removed easily. Also, CO₂ is inexpensive, noncorrosive, odorless, and a clean solvent without residue left in the product. It is considered non-toxic and harmless, has high diffusivity, low viscosity and surface tension in pharmaceuticals [174, 181]. Other solvents can be used for SFE such as hexane, butane, and pentane. SFE can also be used for extracting
volatile compounds like essential oils without loss or degradation since extraction can be caused at ambient temperatures compared to other techniques mentioned.

Essential oil extraction can be through distillation, including water, water and steam, and steam distillation (hydrodistillation). Distillation has been used since ancient times (5000 years) [182]. During and beyond the middle ages, crude distillation was used to make floral or aromatic waters which means that distillation was used in perfumery, tonics, in cooking, and for trading. Nowadays, distillation is the most common process for extracting essential oils from plants. This procedure gives the ability to the volatile components to be distilled at lower temperatures, lower than the boiling points for their constituents, to be easily separated from condensed water. The plant material is placed inside a sill and then sealed once inside. Next, steam or water is used to break the plant material, removing its volatile components. The components then rise inside connecting pipes, leading into a condenser which cools the rising vapor giving the liquid form which is collected in vehicles below the condenser [183].

1.7 Cell Death Pathways

Cell death is a vital pathway in biological systems in order to maintain cellular and immunological balance in our bodies. Its disruption is involved in pathological conditions ranging from degenerative diseases to autoimmunity and cancer [184, 185]. It is considered a genetic program which is important in eliminating damaged or infected cells from the body to keep normal development and maintenance of tissue homeostasis in check, and an active immune system in place. There are three main pathways for cell death in humans, including necrosis, apoptosis, and autophagy (Fig. 1). Cell death modes have been studied for a long
time. The findings suggest that there is cross talk between apoptosis, necrosis, and autophagy, sharing similar initiator and effector molecules regulated by the same pathways [186]. These pathways depend on the cellular context of the death trigger, with apoptosis and necrosis working together in networks that involve autophagy.

1.7.1 Necrosis

Necrosis is characterized by unregulated and passive cellular explosion in response to trauma [187]. The morphological features of necrosis involve swelling of organelles (endoplasmic reticulum, mitochondria), rupture of plasma membrane, and lysis of the cell [187, 188] (Fig.1a). In contrast, in apoptosis, cells become distended and intact, while necrotic death is followed by inflammatory reactions [189]. Necrotic cells release factors to enhance inflammatory response [190] and activate proteins of inflammasome, leading to inflammasome activation and pro-inflammatory cytokine release. The activation of inflammasome is induced by ATP produced from mitochondria that are released from damaged cells [191]. It mediates cell death in response to damage or pathology but not through normal development [192, 193].
Figure 1: Types of cell death and their morphological hallmarks. Diagrammatic classification of different types of cell death. Morphological features of a) a healthy cell, b) a necrotic cell, c) an apoptotic cell, and d) an autophagic cell. PCD: programmed cell death [184].

1.7.2 Apoptosis

Apoptosis is also known as programmed cell death (PCD). It has been studied intensively and is considered as the main mechanism of regulated cell death [194]. It is activated during cell damage or stress, normal development, and morphogenesis. Apoptosis is triggered by extrinsic stimuli through cell surface death receptors such as Tumor necrosis factor (TNF)-α, Fas, and Tumor Necrosis Factor-Alpha-Related Apoptosis-Inducing Ligand (TRAIL), or by intrinsic stimuli through mitochondrial signaling pathways [195, 196]. Both the intrinsic and extrinsic stimuli lead to the activation of cysteine aspartyl proteases (caspases), which results in mitochondrial membrane permeabilization, chromatin condensation, and DNA fragmentation, ending in cellular destruction [197]. Thus, apoptosis is a caspase-dependent cell
death pathway, unlike necrosis which is a caspase-independent form of cell death. The morphological changes of apoptotic cells are shown in Fig. 1c involving cell shrinkage, chromatin condensation, cytoplasmic blebbing, apoptotic bodies, and vacuoles in cytoplasm.

1.7.3 Necroptosis

Necroptosis is an alternative programmed necrotic cell death pathway, which is triggered by the same death signals that induce apoptosis. It is common in vivo during physical trauma, neurodegeneration, and death caused by ischemia or infection (Fig. 1). The term apoptosis-like PCD is used to describe necroptotic death with apoptotic features; however, it activates cell death in a caspase-independent manner [198]. This type is controlled by mitochondria and the mitochondrial effector protein Apoptosis inducing factor (AIF) [199, 200]. AIF is considered as inducer of apoptosis-like cell death in experimental models [198]. The activity of AIF cleavage and transformation to a soluble form (tALF) is triggered by calpains or cathepsins, which are released from mitochondria and translocated into the nucleus. Their release is increased depending upon increased levels of intracellular calcium or extensive DNA damage [198]. Increased intracellular calcium levels trigger depolarization and loss of mitochondrial membrane, generation of reactive oxygen species (ROS), and poly ADP ribose polymerase (PARP). Activation of PARP is followed by activation and release of AIF from the mitochondria.

1.7.4 Autophagy

Autophagy (self-eating) is involved in self-engulfment of cytoplasmic material and intracellular organelles within double-membranated vesicles called autophagosomes (Fig.1d). When an autophagosome is formed, it is followed by
fusion with lysosomes to create autolysosomes, where the degradation takes place by acidic hydrolases [201]. Autophagy is considered as cytoprotective and cell death-promoting during normal development [202] and in disease [203]. It is important in the normal turnover of long-lived proteins and organelles which is crucial for maintaining healthy cells. The hemostasis is critical in cells such as neurons and cardiomyocytes. The activation of autophagy is dependent on many factors, including nutrition deprivation, growth factors depletion, and hypoxia [204, 205]. Cytoplasmic degradation is enhanced in response to stress and therapy-promoting survival.

1.8 Apoptosis

Since the 19th century, many observations have been made suggesting the role of cell death during physiological processes [206, 207]. The phrase “programmed cell death” was introduced in 1964, when cell death was proposed as not an accidental phenomenon but followed by controlled steps, leading to cellular self-destruction [208]. Apoptosis is from Greek origin, meaning “falling or dropping of”, such as when the leaves fall off trees or dropping off flowers. It emphasizes that death is necessary for the life cycle of organisms, required for the development of multicellular organisms, and regulation and maintenance of cells in physiological and pathological conditions. The term is based on the description of morphological processes that leads to controlled cellular destruction [192].

Apoptotic cells have special morphological changes mentioned earlier, including cells shrinkage, deformation and losing contact with neighboring cells, chromatin condensation and margination at the nuclear membrane, blebbing or budding of plasma membrane, and finally cell fragmentation into compact enclosed
structures called "apoptotic bodies" that contain cytosol, condensed chromatin, and organelles [209]. The activation of proteolytic enzymes activates the cleavage of DNA into oligonucleosomal fragments, while other specific proteins determine the integrity and shape of cytoplasm or organelles. Macrophages engulf the apoptotic bodies, which are removed from the tissues without causing inflammation [209].

The mechanism of apoptosis is highly complex and refined. Caspases are expressed in an inactive form (procaspases) in most of the cells and when activated, they can activate other procaspases, allowing initiation of a protease cascade [210]. Also, some procaspases can aggregate and activate automatically. This proteolytic cascade allows one caspase to activate other caspases, which amplifies the apoptotic signaling pathways, leading to rapid cell death [210]. Caspases are able to cleave proteins at aspartic acid residues as a result of their proteolytic activity. They vary in their ability to recognize different specificities of amino acids, and induce irreversible commitment towards cell death. Caspases serve various roles such as initiators (caspase 2, 8, 9, 10), executioners (caspase 3, 6, 7), and inflammatory caspases (caspase 1, 4, 5) [211, 212]. Apoptotic cells have extensive protein cross-linking as well, which is achieved by the expression and activation of tissue transglutaminase [213]. Also, DNA breakdown is induced by calcium and magnesium endonucleases, resulting in DNA fragmentation [214].

Another feature of apoptosis is that the apoptotic cells express cell surface markers required for phagocytic recognition by neighboring cells in order to permit quick phagocytosis with minimal compromise to surrounding tissues (Fig. 2). This is accomplished by the outward movement of the cytoplasmic phosphatidylserine (PS) lipid of the plasma membrane to the outer layer of the lipid bilayer [216]. The
externalization of phosphatidylserine is a recognition ligand for phagocytes on the surface of apoptotic cells. Recently, studies have shown that other proteins may be exposed on the cell surface during apoptosis, including Annexin I and calreticulin [216]. Annexin V is a calcium-dependent phospholipid binding protein that interacts with phosphatidylserine residues and is used for the detection of apoptosis [217]. Calreticulin is a protein bound to low density lipoprotein (LDL) receptor protein related to engulfing apoptotic cells and cooperates with phosphatidylserine as a signal for recognition. The two main pathways of apoptosis activation are described below:

Figure 2: Receptors and adaptor molecules involved in apoptotic cell recognition and engulfment.

Phosphatidylserine (PS) exposure enables the recognition of apoptotic cells by phagocytes. PS can be directly bound by either specific PS-receptors, such as brain-specific angiogenesis inhibitor 1 (BAI1), stabilin-2, and the T-cell immunoglobulin mucin (TIM) proteins (TIM1 and TIM4), or indirectly via bridging molecules like annexin A1 (anx A1), β2-glycoprotein 1 (β2GPI), the growth arrest-specific 6 (gas6), the milk-fat globule EGF-factor 8 (MFG-E8), and protein S (prot S) [215].
1.8.1 The intrinsic pathway

Mitochondria is the regulator of intrinsic apoptosis pathway. It plays a central role in the integration and propagation of death signals from the inside of the cell in response to DNA damage, oxidative stress, starvation, and chemotherapeutic drugs [218, 219]. Once the disruption of mitochondrial inner transmembrane and mitochondrial membrane permeability is initiated, the homeostasis of basic cell metabolic function is lost, such as ATP synthesis, oxidation of redox molecules (NADH, NADPH, and glutathione), increase in reactive oxygen species, etc. [220, 221]. Also, osmotic swelling of mitochondria is enhanced, leading to the rupture of outer mitochondrial membrane and release of pro-apoptotic proteins from mitochondria into the cytoplasm [222, 223] (Fig. 3). Cytochrome c is one of the proteins released to activate the apoptosome, caspase cascade, and other factors such as AIF (224), the endonuclease G (endoG) [225], Smac/Diablo [226], and Htr/Omi [227]. Intrinsic apoptotic pathway involves procaspase 9 activation, which activates downstream mitochondrial pro-apoptotic events at the apoptosome. It is a cytosolic death signaling protein complex formed when cytochrome c is released from the mitochondria [228]. Caspase 9 activation is dependent on the dimerization of the procaspase 9 molecules at Apaf-1 scaffold [229]. When the initiator caspase 9 is activated, the other effector proteolytic procaspases such as 3, 6, and 7 are activated and accordingly cleave specific protein substrates, resulting in mediation and amplification of death signal to execute the cell death with all the morphological features mentioned earlier [230].
1.8.2 The extrinsic pathway

The extrinsic apoptosis signaling is dependent upon the activation of death receptors [231]. They are cell surface receptors which transmit the death signals after ligation with specific ligands. Death receptors belong to tumor necrosis factor receptor (TNFR) gene superfamily, including TNFR-1, Fas/CD95, and TRAIL receptors DR-4 and DR-5 [232]. TNFR members contain extracellular subdomains consisting of cysteines for specific recognition of ligands, leading to trimerization and activation of death receptors [233]. The signaling is transmitted to the cytoplasmic part of the death receptor, which has conserved sequences, the death domain (DD). Adaptor molecules like FADD or TRADD also have a death domain that binds the DD of the receptors, leading to the formation of death-inducing signaling complex (DISC). The adapter molecules also contain death effector domain (DED) that is unmasked upon its interaction with the receptor, leading to the recruitment of procaspase 8 and 10 to the DISC. Recruitment of procaspases to the DISC results in autocatalytic cleavage, resulting in release of active caspase 8 and 10 which then activate other effector caspases, leading to the activation of the caspase...
cascade and eventually cell death [234]. The caspase-dependent extrinsic apoptosis pathway is classified as type 1 [234].

In type 2 extrinsic pathway, the signal coming from the activated receptor is not sufficient for activating the caspase signaling cascade for inducing cell death (Fig. 3). Thus, in type 2 extrinsic death signaling pathway, the signal is amplified through a mitochondrial apoptotic pathway provided by a Bcl-2 family member, Bid, that links it to the caspase signaling cascade. Bid is activated (cleaved) by caspase 8 and translocated into the mitochondria, where it acts with pro-apoptotic Bcl-2 family members, Bax and Bak, to induce cytochrome c release and mitochondrial pro-apoptotic factors into the cytosol [235]. Cytochrome c bound to Apaf-1 induces ATP-dependent changes to activate oligomerization of the apoptosome (a wheel structure with 7-fold symmetry). The wheel structure triggers the activation of procaspase 9 (initiator caspase) [236]. The activated caspase 9 then initiates the activation of other effector caspases such as caspase 3, 6, and 7, resulting in cell death [237].

1.9 Apoptosis and Cancer

Cancer is a result of dysregulated apoptosis. In the 1970s, pathologists observed morphological features of apoptosis following radiation and chemotherapy [238]. This suggested that anticancer agents induce apoptotic cell death after drug-target interaction. This observation had crucial impact on the development of anticancer agents [239-241]. Furthermore, the reduction of apoptosis or its resistance correlated with carcinogenesis. Anticancer agents inducing apoptosis reduced the treatment sensitivity [242]. Figure 4 shows the mechanisms by which apoptosis can be dysregulated in cancer:
1) defects in p53 tumor suppressor gene
2) imbalance of pro-apoptotic and anti-apoptotic proteins
3) reduced caspase function
4) increased expression of inhibitor apoptosis proteins (IAPs)
5) impaired death receptor signaling

Figure 4: Mechanisms contributing to evasion of apoptosis in carcinogenesis [243].

Anticancer agents primarily induce apoptosis through one of these mechanisms, while mutations in apoptotic programs may enhance drug resistance [239, 244]. For example, mutations in the tumor suppressor gene p53 reduced therapy-induced apoptosis in murine tumors [245, 246]. On the other hand, reintroduction of normal p53 in p53-mutant tumors and xenografts resulted in the induction of apoptosis and tumor regression during chemotherapy [247, 248]. Similarly, mutations in p53 have been linked to more than 50% of cancers [249]. P53 is required for drug-induced cell death through apoptosis, especially in tumors independent of p53 [247]. P53 contribution to drug-induced apoptosis is affected by
factors such as the chemical agent, the dose, tissue, and mutation in the tumor [247, 250, 251]. The degree of apoptosis contribution to treatment sensitivity in cancers is not clear, but some studies show correlation between p53 mutations and poor treatment response [242,252]. P53 knock-out mice exhibit reduced apoptosis following radiation or chemotherapy [253-258].

The Bcl-2 family of proteins comprise of both pro- and anti-apoptotic proteins important in regulating apoptosis (intrinsic pathway though the mitochondria) [259]. When expression of the anti- or pro-apoptotic members of the Bcl-2 family members are disrupted, this results in dysregulated apoptosis in the affected cells [243]. Similarly, abnormalities in death signaling pathways can lead to reduction of apoptosis through the extrinsic pathway of apoptosis. For example, a reduction of membrane expression of death receptors or abnormal receptor expression has been reported to be involved in death signaling pathways in cancers [260]. There are different strategies targeting apoptosis in cancer treatment as new treatment options, for example, targeting p53 for cancer treatment. They can be classified as gene therapy, drug therapy, and immunotherapy. The p53-based drug therapy has been investigated for targeting apoptosis through many mechanisms. For example, Phikan 083 is a small molecule and derivative of carbazole that has been predicted to bind and restore mutant p53 function in silico [261]. P53 vaccines (immunization) called p53-based immunotherapy has been tested in clinical trials for mice with advanced-stage cancer [262]. In another strategy, Bcl-2 antisense therapy has been tested in clinical trials as a targeted agent. It has shown good chemosensitization activity in combination with other conventional anticancer drugs, improving the survival in cancer patients [263, 264]. Caspase-based drug and gene therapy has also been used in inducing apoptosis. For example, apoptin and human
caspase 3 gene therapy are being used as targeted treatments for cancer. Many other gene-targeted therapies are in clinical trials to enhance the apoptotic pathways and develop novel treatment strategies for cancer.

1.10 Aims and Objectives of the Project

This project aimed to screen the anticancer activities of several indigenous medical plants of the Arabian Peninsula along with their mechanism of action. Towards this end, four to six different types of extracts were obtained from five indigenous medicinal plants made sequentially with various solvents. We hypothesized that since these plants have been used for their medicinal properties, the probability of their having anticancer activity was high. Thus, these were first screened in a breast cancer cell, MCF-7, and a cervical cancer cell line, HeLa, to determine if they had any anti-proliferation activity against these cell lines. Once screened, selected effective extracts from each plant were further tested in a more expanded range of breast cancer cell lines, including MCF-7 and MDA-MB-231, and a normal breast cell line, MCF-10A. The MTT cell viability assay was used to study the effect of the various extracts on the growth of the cancer cell lines in a dose-and time-dependent manner and the mechanism of action of cell death was explored by testing the activities of various caspase enzymes using Promega glo assays. Western blotting was used to confirm the results. Our studies not only characterized the anticancer potential of five indigenous medical plants, but they also characterized the anticancer potential of several isolated compounds and derivatives from these extracts. In future, we plan to characterize the anticancer potential of both the crude and some of the pure components further in depth to hopefully enlarge the pool of new molecules to fight cancer.
1.1.1 Introduction to the Indigenous Medicinal Plants Studied in this Thesis

Five different plants were investigated for their anticancer potential during the course of this thesis (Fig. 5). A brief introduction into the history and background on these plants is given below:

![Figure 5: Photographic images of the five plants used in this study. These plants included: *Boswellia sacra*, *Acredocarpus orientalis*, *Cleome droserifolia*, *Teucrium muscatensis*, and *Ochradenus arabicus* [339].](image)

1.1.1.1 *Boswellia sacra* (BS)

Sacred Frankincense as it is called, *Boswellia sacra* is the source of the famous aromatic resin obtained from the gums of genus *Boswellia* (*Burseraceae* family) (Fig. 5). *Boswellia* species includes *Boswellia sacra* from Oman and Yemen, *Boswellia carteri* from Somalia, and *Boswellia serrata* from India and China [265]. The resins are used in incense, fumigants, and fixative in perfumes. The aromas from the resins were valued for their qualities in religious customs since the ancient
Egyptians [266]. *Boswellia* species resins are considered as having a wealth of healing properties. They have been used as a treatment of rheumatoid arthritis [267] and inflammatory diseases like Crohn's disease [268]. Their anti-inflammatory activity has been associated with the regulation of immune cytokine production [269], and leukocyte infiltration [270, 271]. Extracts from *Boswellia* species have been shown to have anti-bacterial, anti-fungal [272], anti-carcinogenic [273], and anti-neoplastic properties [274, 275]. Extracts from *Boswellia* resins have been shown to reduce the peritumoral edema in glioblastoma patients [274] and reverse multiple brain metastases in breast cancer patients [276]. All the earlier mentioned results suggested that the resins from *Boswellia* species include active ingredients that modulate important biological and health activities.

Other than resin, essential oils are an important product of aromatic plants. Essential oils are called volatile or ethereal oils which are present as secondary metabolites in lower amounts in different parts of the plants. They are rich and concentrated hydrophobic liquids with aroma produced by the aromatic plants [277]. Other biological properties of essential oils are dependent on their constituents. These constituents are classified based on their chemical structures. The constituents are considered as less effective than essential oils because they have synergistic and selective effects [278]. The essential oil produced from *Boswellia* is characterized by a high content of monoterpenes, including piene, dipentene, phellandrene, and cadinene [279]. It includes acidic components among them boswellic acids, which are triterpenic acids such as alpha and beta boswellic acids [280], 11-alpha-hydroxy-beta-boswellic acid, 3-O-acetyl-11-hydroxy-beta-boswellic acid, and acetyl-11-keto-beta-boswellic acid (AKBA) [281]. Recent studies have identified ether-soluble gum fractions of sesquiterpenes, alcohols, esters, and boswellic acids. While the ether-
Egyptians [266]. *Boswellia* species resins are considered as having a wealth of healing properties. They have been used as a treatment of rheumatoid arthritis [267] and inflammatory diseases like Crohn’s disease [268]. Their anti-inflammatory activity has been associated with the regulation of immune cytokine production [269], and leukocyte infiltration [270, 271]. Extracts from *Boswellia* species have been shown to have anti-bacterial, anti-fungal [272], anti-carcinogenic [273], and anti-neoplastic properties [274, 275]. Extracts from *Boswellia* resins have been shown to reduce the peritumoral edema in glioblastoma patients [274] and reverse multiple brain metastases in breast cancer patients [276]. All the earlier mentioned results suggested that the resins from *Boswellia* species include active ingredients that modulate important biological and health activities.

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insoluble gum fractions include polysaccharides [282, 283]. The non-volatile components include diterpenoids, while the volatile oil components include monoterpenes as hydrocarbons, alcohols, and ketones, sesquiterpenes, and diterpenes [284]. The composition of the *Boswellia* essential oil differs in different climate, harvesting conditions, and geographical source. For example, boswellic acids isolated are different from resins derived from Indian, Somalian and Arabian regions [283-289].

Thus, since ancient times, essential oil from *Boswellia* and its components have been used as perfumes and flavoring agents in food products as a result of their specificity of aromas. In addition, they have been used in aromatherapy to improve health because they have sedative and stimulant properties. For example, they have traditionally been used for massages, baths, and for inhalation and relaxation [290]. Essential oils are described as antioxidants [291, 292], and antimicrobial [293]. They have been used for the management of severe diseases such as cardiovascular disease [294], diabetes [295], and Alzheimer’s disease [296]. For example, asthmatic patients have used it traditionally as it eases their breathing and has a soothing action in colds, cough, bronchitis, and laryngitis [297]. It can also remove scars and stretch marks [298].

The pharmacological activities of *Boswellia sacra* as a crude extract or an essential oil, or the isolated compounds have been shown to have anti-bacterial, antifungal, and immunomodulatory activities [285, 299-304]. Other studies have shown that *B. sacra* has anti-inflammatory, anti-leukotriene, anti-acetylcholinesterase, and anticancer activities, especially due to the boswellic acids derivatives [305-312]. Tetra and pentacyclic triterpene acids such as tirucallic, lupeolic, and roburic acids
(boswellic acids) have anti-inflammatory activity as a result of microsomal prostaglandin E2 synthase-1 inhibition ability [313]. AKBA has been considered as an anti-inflammatory agent which blocks leukotriene synthesis by inhibiting activity of lipoxygenase [309]. AKBA has a protective effect in an induced mouse colitis model [314].

Essential oils from *Boswellia* species are reported to have anticancer properties [291, 315, 265]. They can be used to improve chemotherapy and radiotherapy when combined by decreasing the side effects of the drugs. As a result, they are considered as a source of novel anticancer compounds since the nature of the essential oils allows them to cross the membranes of the cells and reach inside the cells. Clinical studies have shown that boswellic acids can inhibit the growth of cancer cells *in vivo* and *in vitro*, indicating cytotoxicity on cancer cells by multiple molecular mechanisms involving transcription factors, signal transducers, and growth regulating genes [280]. It has been proposed that AKBA and boswellic acids have anti-neoplastic activity via anti-proliferation and pro-apoptotic activities in several human cancer cell lines, including meningioma [316], leukemia [310], hepatoma [317], melanoma, fibrosarcoma [318], colon cancer [319], and prostate cancer [320-322]. Triterpenoid acids such as ursolic and oleanolic acid can inhibit tumor initiation and promotion [323]. In addition, they have anti-inflammatory, hepatoprotection, analgesia, cardiotonic, sedative, and tonic effects. Purified compounds of *Boswellia sacra* triterpenoids acids, especially boswellic acids (BAS), are the bioactive ingredients, which are responsible for the anti-neoplastic activities. These compounds have chemopreventive effects in prostate [321, 324], cervical [322], breast [265], colorectal [325], pancreatic [326], and bladder cancer cell lines [327]. *Boswellia sacra* essential oil has been studied in human breast cancer cell
lines and it was able to induce tumor-specific apoptosis and suppress cancer cell proliferation by inducing Akt and ERK1/2 activation, cyclin D1 and cdk4 expression, and caspases activation. [265]. It has also been shown to have anti-proliferative activity against basal cell carcinoma of the skin [328]. In addition, it induces human pancreatic cancer cell death in a xenograft murine model [329].

1.11.2 *Orchadenus arabicus* (OA)

*Orchadenus arabicus* is a medicinal plant from the genus *Orchadenus* belonging to family *Resedaceae* [330] (Fig. 5). It is found mostly in the desert and dry regions from 0-2000 meters above sea level of Saudi Arabia, Oman, UAE, and Yemen [331-333]. Plants of *Orchadenus* have complicated branching systems with deciduous leaves and occasional polygamous flowers with or without ephemeral corolla. These characteristics are crucial for the plants in order to adapt to the climate [334]. *Orchadenus arabicus* has medicinal properties and traditionally has been used as a therapy for different ailments as a result of its anti-inflammatory, antioxidant, immune stimulating, and hypocholestrimic activities as well as its ability to reduce the risk of getting cancer and heart disease because of the presence of bioactive compounds within this plant [335].

*Orchadenus arabicus* has several bioactive compounds such as flavonoid [336]. These phyto-constituents accumulate in the aerial parts of the plant affected by the biotic and abiotic factors [336]. The constituents include quercetin-3-O-beta-glucosyl (1-2)-alpha-rhamnoside-7-O-alpha-rhamnoside, quercetin glycosides, quercetin-3-O-p-coumaryl(1-6)-beta-glucosyl(1-6)-beta-glucoside-7-O-alpha-rhamnoside, quercetin-3-gentiobioside, isoquercitrin, and other known kaempferol glycosides, afzelin and astragalin [337]. Analysis of the nutritional composition of
Orchadenus arabicus has shown that the fruits and leaves have higher levels of carbohydrates, crude fats, fibers, proteins, moisture, ash, and energy values than other parts of the plant [338]. Antioxidant analysis of Orchadenus arabicus extracts show higher total phenolic and flavonoid contents, resulting in higher radical scavenging activity compared to ascorbic acid, which is known for free radical scavenging properties [338]. These observations make Orchadenus arabicus a good candidate for the search of new types of anticancer molecules.

1.1.3 Acridocarpus orientalis (AO)

Acridocarpus orientalis is a medicinal plant of the genus Acridocarpus and the family Malpighiaceae (Fig. 5). Locally it is called qafas. It is located in the wadis and mountain foothills of UAE [339] growing in rocky areas up to 1500 meters in height [340]. It has been found in the foothills of the Jebel Hafeet Mountain between Oman and UAE [341, 342]. It has also been found to grow in the northern and southern mountains of Oman [342] especially surrounding Jabal Shams [343], and in Somalia [339] at elevations between 100-700 meters [344]. Acridocarpus orientalis is a small, highly branched shrub, having stems with hairy, and yellow flowers in clusters [344, 339]. Its yellow flower petals are not joined to each other [344, 339]. The flowers are bisexual, including male and female reproductive organs [345]. Often, the leaves are evergreen [345]. The young leaves are covered in reddish brown hair, which are lost upon growth, creating smooth leathery leaves with prominent veins [344, 339]. It is used locally as a pain relief medicine. The crushed seeds from a crude extract and the oil produced from this plant are massaged onto the forehead and joints [339, 342] to relieve pain from chronic headaches, paralyzed limbs, and for muscle and tendon pain [346]. The seeds are used as a source of yellow dye in Oman [341], but the reddish hair of the young leaves are used as a
tanning agent and to treat udder inflammation in cattle [339]. Many reports indicate that several plant species in the genus Acridocarpus are used as a medicine in Africa for gastro-intestinal disorders, paralysis, and skin blisters (pemphigus) [346-349]. In Oman, it is used as a topical application for treating paralysis [347]. The oil is also applied as a moisturizer on face and body to make the skin softer [350].

Acridocarpus genus species have several phytochemicals, including five triterpenes: beta-sitosterol [351], stigmasterol [352], friedelin [348], oleanolic acid [353], ursolic acid [354] and five falvonoids: apigenin [355], luteolin [356], vitexin [349], kaempferol [357], and quercetin [358]. Extracts of Acridocarpus orientalis have been analyzed and screened for their phytochemicals. Phytochemical screening has revealed the availability of primary and secondary metabolites including: carbohydrates, phenolic compounds and tannins, flavonoids, and saponins [359]. The free radical scavenging activity estimated for Acridocarpus orientalis extracts has shown that the antioxidant activity increases with increasing concentration of the extract [360]. Antioxidant-based drugs are used for treating and preventing diseases including atherosclerosis, stroke, diabetes, Alzheimer’s disease, Parkinson’s disease, cancer, and other diseases [361, 362]. Thus, Acridocarpus has great potential as an important drug for various illnesses, including cancer.

1.11.4 Cleome droserifolia (CD)

Cleome droserifolia is a medicinal plant of the genus Cleome and family Cleomaceae (Fig. 5). Locally Cleome droserifolia is called samwa [363]. Other terminologies include rem el bard, afeen, and mashta in Arabic, while frossk in English [364]. In addition, it has other names like “spider flower” and “mountain bee plant” [365]. All of the Cleome species grow at similar locations with different soil
types. Moist places and rocky regions are favored for some species, while others grow in black fertile soil and rainy season, regions with waste water, and some in shaded areas in red soil which develops in warm, temperate, and moist climate during rainy season [365]. It includes 150 species in tropical and subtropical countries in the Old and New Worlds [366]. Cleomaceae family is also found in North Africa [367] and India [365].

*Cleome drosorifolia* is used traditionally for improving stomachics and rubefacients, as well as treating many ailments like scabies, rheumatic fever, inflammation, etc. [368]. It has antimicrobial, analgesic, antipyretic, and anti-inflammatory activities [369]. It has immediate effect on abdominal and rheumatic pain, diabetes, inflammation, and from snake and scorpion bites [364]. In addition, it improves carbohydrate metabolism [370], fights obesity [371], and has hypoglycemic effects or is antidiabetic for treating diabetes mellitus [363, 372-375]. It is reported that it also has antimalarial effect [374] and antioxidant activity in diabetic rats [376-380]. Though not much is known about its anticancer potential, a related species, *Cleome gynandra*, has been shown to be effective when injected in Swiss albino mice using Ehrlich’s ascites carcinoma cells [381, 382].

Several bioactive constituents have been isolated from *Cleome drosorifolia*, including three sesquiterpenes (bucharol, teucladiol, daucosterol) [383] and a flavonoid derivative [375]. Other studies of *Cleome drosorifolia* indicate the presence of glycosides, carbohydrates, cardenolides, saponins, sterols, triterpenes, tannins, catechins, and falvonoids [365, 384] and several triterpenoids [385-387]. These constituents are thought to be responsible for the hypoglycemic effect of *Cleome* in rats [388-392] as well as its liver-protective properties [393]. In addition,
Cleome species contain 13 flavonoids glycosides [394, 395], kaempferol 3-glucuronide from roots [396], new naringenin glycoside [397], and three new coumarino lignoids from seeds [398]. Furthermore, Cleome species have antioxidant properties [367]. Thus, a combination of antioxidant/phytonutrients present in Cleome makes it an attractive candidate for further drug discovery [362].

1.11.5 Teucrium muscatensis (TM)

Teucrium muscatensis is a medicinal plant of the genus Teucrium belonging to Lamiaceae or Labiatae family (Fig. 5) [399]. All of the Teucrium species grow in rocky limestone areas, dry mountain meadows and pastures, and at the edge of sparse oak and pine forests up to 1000 meters above sea level. The genus includes 300 species distributed in central Europe, the Mediterranean region, Western Asia and North of Africa [400-402]. Members of Lamiaceae family are highly aromatic, durable grass plants, have small fleshy leaves, and are multi-branched stems with callous white exterior [402, 338]. Teucrium species have traditional medical uses since the times of Socrates and Jalinous [402]. They have been used in traditional and modern medicine because of their bioactive constituents [403-406]. Species of Teucrium are rich in phenolic compounds with strong biological activities. The medicinal properties of the Teucrium genus includes antibacterial, anti-inflammatory, and antipyretic activities [400]. Other species from the same family in areas like Sardinia and Baronia of Siniscola has been used for its antimalarial activities [407]. Also, other species are reported that have hypolipidemic activities [408]. Research shows that Teucrium species have anti-diabetic, anti-spasmodic, anti-inflammatory, analgesic, and antioxidant effects [409-415]. Other species of genus Teucrium in Europe are used for the treatment of digestive disorders, abscesses, gout, conjunctivitis, and stimulation of fat and cellulite breakdown [416, 417].
*Teucrium muscatensis* and *Teucrium species* in general are known to contain diterpenoids and triterpenoids in the aerial parts of the plant such as [418-421]. The secondary metabolites from *Teucrium* species are glycosides, terpenoids, steroids, tannins, and phenolic compounds [422]. These metabolites have general protective functions in the human body involving antioxidant, anti-allergic, antibiotic, and hypoglycemic effects [423-424].
Chapter 2: Materials and Methods

2.1 Cell Lines and Tissue Culture

The hormone responsive human breast cancer cell line, MCF-7 and the triple negative human breast cancer cell line, MDA-MB-231, were obtained from Prof. Samir Attoub, Department of Pharmacology, College of Medicine and Health Sciences, UAE University, Al Ain, UAE. The normal breast epithelial cell line, MCF-10A, was obtained from ATCC (USA). The human cervical cancer cell line, HeLa, was a gift of Prof. Tahir A. Rizvi, Department of Microbiology and Immunology, College of Medicine and Health Sciences, UAE University, Al Ain, UAE.

MCF-7 and MDA-MB-231 were grown in DMEM/high glucose medium (Hyclone, USA) supplemented with 10% FBS (Hyclone), 10,000 units/ml penicillin/streptomycin (Hyclone), and 50 μg/ml gentamicin (Hyclone). The HeLa cell line was grown in DMEM/high glucose medium (Hyclone) supplemented with 7% fetal calf serum (FCS) (Hyclone), 10,000 units/ml penicillin-streptomycin (Pen/Strep) (Hyclone), and gentamicin (Hyclone). The MCF-10A cell line was grown in DMEM/F12 medium (Dulbecco’s Modified Eagles Medium/Hams nutrient mixture) (Sigma, USA) and 5% Horse serum (Sigma) supplemented with 10,000 units/ml of Pen/Strep 10 μg/ml, Insulin (Sigma), 20 ng/ml epidermal growth factor (EGF) (Sigma), 0.5 mg/ml hydrocortisone (Sigma), and 100 ng/ml cholera toxin (Sigma). MCF-7 and MDA-MB-231 cells were maintained by removing the old medium, washing with 7 ml phosphate buffered saline (PBS) (Hyclone), detaching cells from the surface by adding 2 ml of 0.05% trypsin (GIBCO), and adding 4 ml of fresh DMEM complete medium (Hyclone). HeLa cells were maintained similarly to MCF-7 and MDA-MB-231, but by using 0.025% trypsin instead of 0.05%. MCF-
10A cells were maintained similarly, except that after trypsinization in 0.05% trypsin, 2 ml of resuspension media (DMEM/F12 media supplemented with 20% horse serum and 10,000 units/ml Pen/Strep) was added and cells were spun down at 1500 rpm for 5 minutes to remove the trypsin and resuspended in complete media prepared freshly. The other cancer cell lines were cultured in the ATCC-recommended media as per ATCC instructions. All cells were maintained at 37°C in a 5% CO2 incubator. For conducting experiments, cells were plated a day earlier and seeded at the cell density mentioned in the experiments, depending upon the plate and well size used for the experiment. All MTT assays were conducted in 96-well plates using 5,000 cells per well, unless otherwise stated. This was followed by treatment of the cell cultures with different concentrations of the tested extracts along with appropriate dilutions of the control solvent (normally dimethyl sulfoxide (DMSO)).

2.2 Plant Extract Preparation

Plant extracts from the 5 different plants included those from *Boswellia sacra* (BS), *Cleome droserifolia* (CD), *Teucrium muscatensis* TM), *Orchadenus arabicus* (OA), and *Acredocarpus orientalis* (AO) were obtained. These extracts were prepared and supplied by our collaborators Profs. Ahmed Al Harrasi and Javid Hussain, University of Nizwa, Sultanate of Oman. The extracts were solubilized in DMSO or water to prepare stock solutions at 12.5 mg/ml, 25 mg/ml, and 50 mg/ml concentrations, depending upon the form and solubility of each extract, then stored at -20°C (Table 1). From the stock solutions, different extract treatments were prepared at twice the final concentration (50, 100, 250, and 500 mg/ml) in the media.
depending upon the cell line used, since 100 µl of treatment was added to cells plated in 100 µl media on the day of treatment.

Table 1: Description of the extracts, their stock concentrations, and solvent used for solubilization

<table>
<thead>
<tr>
<th>S. No</th>
<th>Extract</th>
<th>Plant</th>
<th>Concentration</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Essential Oil (EO)</td>
<td><em>Boswellia</em></td>
<td>Pure distilled oil</td>
<td>DMSO</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>sacra</em></td>
<td>10 mg/ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(BS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CH3Cl Extract (CH)</td>
<td>CD</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>3</td>
<td>MTOH Extract (MT)</td>
<td>TM</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>4</td>
<td>Hexane Extract (HX)</td>
<td>TM</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>5</td>
<td>Aqueous Extract (AQ)</td>
<td>OA (Whole)</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>6</td>
<td>CD Butanol Extract (BT)</td>
<td>OA AQ</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>7</td>
<td>CD CH</td>
<td>OA CH</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>8</td>
<td>CD Ethylacetate Extract (ET)</td>
<td>OA MT</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>9</td>
<td>CD HX</td>
<td>OA HX</td>
<td>12.5 mg/ml</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>TM ET</td>
<td>OA ET</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>11</td>
<td>TM MT</td>
<td>OA BT</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>12</td>
<td>TM BT</td>
<td>OA HX</td>
<td>12.5 mg/ml</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>TM HX</td>
<td>AO (Leaves)</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>14</td>
<td>TM CH</td>
<td>AO MT</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>15</td>
<td>TM AQ</td>
<td>AO AQ</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>16</td>
<td>OA CH</td>
<td>AO BT</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>17</td>
<td>OA AQ</td>
<td>AO MT</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>18</td>
<td>OA MT</td>
<td>AO HX</td>
<td>12.5 mg/ml</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>OA ET</td>
<td>AO (Leaves)</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>20</td>
<td>OA BT</td>
<td>AO (Stem)</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>21</td>
<td>OA HX</td>
<td>AO MT</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>22</td>
<td>AO CH</td>
<td>AO AQ</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>23</td>
<td>AO MT</td>
<td>AO MT</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>24</td>
<td>AO HX</td>
<td>AO HX</td>
<td>12.5 mg/ml</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>AO AQ</td>
<td>AO (Leaves)</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>26</td>
<td>AO ET</td>
<td>AO MT</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>27</td>
<td>AO BT</td>
<td>AO BT</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>28</td>
<td>AO BT</td>
<td>AO BT</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>29</td>
<td>AO MT</td>
<td>AO MT</td>
<td>50 mg/ml</td>
<td>DMSO</td>
</tr>
</tbody>
</table>
The DMSO control contained the same concentration of DMSO as present in the tested extracts as a control for cell death induced by DMSO alone. The extracts were prepared as shown in Table 2 to be tested in the MTT assay in 1 ml or 1.4 ml media, depending upon the cell line used and the volume needed in the experiment.

Table 2: Recipes of the extract stock solution used in this study

<table>
<thead>
<tr>
<th>12.5mg/ml Stock</th>
<th>DMSO/extract in 1ml media</th>
<th>DMSO/extracts in 1.4 ml media</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µg/ml</td>
<td>4µl</td>
<td>5.6µl</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>8µl</td>
<td>11.2µl</td>
</tr>
<tr>
<td>125 µg/ml</td>
<td>20µl</td>
<td>28µl</td>
</tr>
<tr>
<td>250 µg/ml</td>
<td>40µl</td>
<td>56µl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>25mg/ml Stock</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µg/ml</td>
<td>2µl</td>
<td>2.8µl</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>4µl</td>
<td>5.6µl</td>
</tr>
<tr>
<td>125 µg/ml</td>
<td>10µl</td>
<td>14µl</td>
</tr>
<tr>
<td>250 µg/ml</td>
<td>20µl</td>
<td>28µl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>50mg/ml Stock</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µg/ml</td>
<td>1µl</td>
<td>1.4µl</td>
</tr>
<tr>
<td>50 µg/ml</td>
<td>2µl</td>
<td>2.8µl</td>
</tr>
<tr>
<td>125 µg/ml</td>
<td>5µl</td>
<td>7µl</td>
</tr>
<tr>
<td>250 µg/ml</td>
<td>10µl</td>
<td>14µl</td>
</tr>
</tbody>
</table>
2.3 Essential Oil Preparation

The steam-distilled essential oil extracted from *Boswellia sacra* was solubilized using molecular biology grade DMSO at a 1:2 dilution which was used in various experiments at different dilutions prepared in the appropriate media.

2.4 MTT Cell Viability Assay

MTT is a colorimetric assay for cell viability based on the cellular cleavage of the yellow tetrazolium salt, [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), into purple formazan crystals that is soluble in cell culture medium and is measured at 560 nm using the plate reader directly in 96-well assay plates. Absorbance is directly proportional to the number of living cells in the culture. The reagents needed to conduct the MTT assay are listed in Table 3.

Table 3: List of reagents required for the MTT assay

<table>
<thead>
<tr>
<th>Item</th>
<th>Company/Catalogue No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96 well plate for tissue culture&lt;br&gt;Nunc Microwell 96 wells w/lid Nunclon DSi</td>
</tr>
<tr>
<td>2</td>
<td>MTT Reagent&lt;br&gt;[3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]</td>
</tr>
<tr>
<td>3</td>
<td>DMSO Dimethyl Sulfoxide (Molecular Biology Grade), &gt; 99.9%</td>
</tr>
<tr>
<td>4</td>
<td>DMSO Dimethyl Sulfoxide ≥ 99.5%</td>
</tr>
<tr>
<td>5</td>
<td>Plate Reader</td>
</tr>
<tr>
<td>6</td>
<td>Plate Reader</td>
</tr>
</tbody>
</table>

To conduct the assay, the MTT reagent was dissolved in 1 x PBS at a concentration of 5 mg/ml PBS, depending on the number of plates, as shown below:
<table>
<thead>
<tr>
<th>No. of Plates</th>
<th>MTT</th>
<th>1 X PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13 mg</td>
<td>2.6 ml</td>
</tr>
<tr>
<td>2</td>
<td>26 mg</td>
<td>5.2 ml</td>
</tr>
<tr>
<td>3</td>
<td>39 mg</td>
<td>7.8 ml</td>
</tr>
<tr>
<td>4</td>
<td>52 mg</td>
<td>10.4 ml</td>
</tr>
</tbody>
</table>

After mixing (MTT + PBS) well using the vortex, the mixture was heated at 37°C water bath for 2 minutes to make sure that all the MTT particles were dissolved, spun down at 1000 rpm for 1 minute in a table top centrifuge to collect the contents, followed by filtration using a syringe and 0.2 μm filter, and spun down again to be ready for adding to individual wells.

Cells were cultured in 96-well plates at a density of 5 x 10³ cells/well/100 μl media. The cell number was determined empirically after testing the proliferation capacity of MCF-7 and HeLa cells in MTT assays using increasing numbers of cells (Fig. 1S in the appendix). Using this data, a cell number range was used which provided a linear signal for either MCF-7 or HeLa cell lines. After 24 hours, cells were treated with 100 μl of the different concentrations of the plant extracts, essential oil, DMSO (as a negative control), or culture media alone for various time points. Stock solution of MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (5 mg/ml) was added at 25 μl/well and the plates were incubated for 3-4 hours in a 37°C incubator. Culture media was decanted from the plates by a firm but swift hand movement and residual liquid cleaned by wiping the plates on tissues. The formazan crystals thus formed were dissolved by adding 200 μl of DMSO using a multichannel pipette. Absorbance was measured at 560 nm using the plate reader directly in the 96-well assay plates. Cell viability was calculated as a percentage of cells treated with DMSO alone as a control using the same concentrations of DMSO that were used to dissolve the extracts. This ranged from 0.2-1%. All experiments
were carried out at a minimum three times having all samples in triplicates or more. Results were analyzed using MS Excel and GraphPad Prism v5 software.

2.5 Cell Titer-GLO Luminescent Cell Viability Assay

Appropriate cell lines were cultured in 96-well, opaque white well plates at a density of 5 x 10^3 cells/well/100 μl. After 24 hours, 50 μl of the cell culture was removed and treated with 50 μl of the different extract concentrations at 37°C. After 48 hours, cells were assayed for cell viability using the Cell Titer-GLO Luminescent Cell Viability Assay (Promega, USA) as per manufacturer’s directions. Briefly, the substrate kit was thawed at room temperature. Fifty μl of the culture media was removed from each well and 50 μl of the viability substrate was added. After that, the plate was incubated for 10 minutes on a shaking platform (in the dark or covered with aluminum foil) at room temperature. Luminescence was measured using the Infinite M200 Pro Tecan plate reader. Details of the reagents used is provided in Table 4.

Table 4: List of reagents required for Promega cell viability & caspase assays

<table>
<thead>
<tr>
<th>Item</th>
<th>Company/Catalogue No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96 well opaque white plates for tissue culture and luminometer</td>
</tr>
<tr>
<td>2</td>
<td>Cell titer GLO cell viability assay kit 100ml</td>
</tr>
<tr>
<td>3</td>
<td>Caspase 3/7 GLO assay kit 10ml</td>
</tr>
<tr>
<td>4</td>
<td>Caspase 8 GLO assay kit 10ml</td>
</tr>
<tr>
<td>5</td>
<td>Caspase 9 GLO assay kit 10ml</td>
</tr>
<tr>
<td>6</td>
<td>Plate Reader</td>
</tr>
</tbody>
</table>
Data were presented as percent cell viability of experimental groups compared to DMSO-treated cells, the viability of which was taken as 100%. Each sample was tested in triplicates. The essential oil and effective extracts were tested in two independent experiments except AO (S).

2.6 Caspase-GLO 3/7, 8 and 9 Assays

Appropriate cells were cultured in 96-well, opaque, white-well plates at a density of 5 x 10^3 cells/well/100 µl. After 24 hours, 50 µl of the supernatant was removed from each well and then cells were treated with 50 µl different concentrations of the desired extracts and incubated at 37°C incubator. After 48 hours, cells were assayed for the activity of different caspase enzymes using the Promega caspase GLO assays as per manufacturer’s directions by thawing the kits at room temperature. This was followed by removal of 50 µl of the culture media which was replaced by 50 µl of the various caspases substrates. The plates were incubated for 3 hours at room temperature with shaking in the dark (or covered with aluminum foil). Luminescence was measured using the Infinite M200 Pro Tecan plate reader. The data was reported as percentage of increase compared to control and DMSO-treated cells. All samples were tested in triplicates for each extract (treatment) and the experiments were repeated 2-3 times. A representative experiment from these is shown in the results which were analysed by MS Excel and GraphPad Prism 5 as a measure of mean ±SD and t-test. Details of the reagents used are provided in Table 4.

2.7 Protein Extraction and Estimation

To determine the mechanism of cell death being utilized by the effective extracts, western blot analyses were conducted of protein lysates. First, cells treated
with various concentrations of the effective extracts were harvested by trypsinization as described, washed with 1 x PBS, and lysed using 100 μl of RIPA lysis buffer (10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) per million cells supplemented with 50 μl of β-mercaptoethanol/ml RIPA and 1 mM of the serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF). Nuclei were separated from the lysed cells by spinning at 14,000 rpm for 10 minutes at 4°C. The cell lysates were separated from the pellets and either used immediately or stored at -80°C.

Protein amount in the cellular extracts was quantified using the Bio-Rad Bradford colorimetric assay. A sample of the protein lysate was diluted in 50 mM Tris-Cl, pH 8, to a final volume of 800 μl followed by addition of 200 μl of the dye concentrate. A series of protein standards were made in parallel using bovine serum albumin to estimate the amount of protein present in each sample by measuring absorption at the visible wavelength of 595 nm using a spectrophotometer.

2.8 Western Blot Analysis

Total cellular protein lysates (40 μg per lane) were loaded onto 8-12% SDS-polyacrylamide mini gels (Bio-Rad) and blotted onto nitrocellulose membranes using standard protocol. After wet transfer, the membranes were blocked with 5% low fat dried milk in 1 x PBS and 0.1% Tween-20 (PBST) for 1 hour. The membranes were then incubated at 4°C overnight with the following primary antibodies in 1% milk in PBST. Details of the antibodies used are provided in Table 5.
Table 5: List of antibodies used in the western blot assays

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Antibody</th>
<th>Company</th>
<th>Catalogue No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PARP Antibody (1:1000)</td>
<td>Cell Signaling Technology</td>
<td>Cat. No 9542</td>
</tr>
<tr>
<td>2</td>
<td>Actin (1:1000)</td>
<td>Sigma</td>
<td>Cat. No A-3854</td>
</tr>
<tr>
<td>3</td>
<td>Caspase 3 Antibody (1:1000)</td>
<td>Cell Signaling Technology</td>
<td>Cat. No 9668</td>
</tr>
<tr>
<td>4</td>
<td>Caspase 7 Antibody (1:1000)</td>
<td>Cell Signaling Technology</td>
<td>Cat. No 9492</td>
</tr>
<tr>
<td>5</td>
<td>Caspase 8 Antibody (1:1000)</td>
<td>Cell Signaling Technology</td>
<td>Cat. No 9746</td>
</tr>
<tr>
<td>6</td>
<td>Caspase 9 Antibody (1:1000)</td>
<td>Cell Signaling Technology</td>
<td>Cat. No 9502</td>
</tr>
<tr>
<td>7</td>
<td>Anti-mouse antibody (1:2000)</td>
<td>Sigma</td>
<td>Cat. No A-0412</td>
</tr>
<tr>
<td>8</td>
<td>Anti-rabbit antibody (1:2000)</td>
<td>Sigma</td>
<td>Cat. No A-6154</td>
</tr>
</tbody>
</table>

The blots were then incubated with the appropriate secondary antibodies (anti-mouse or anti-rabbit) for 1 hour and 30 minutes at room temperature followed by detection using the Pierce™ ECL Plus Western Blotting Substrate and visualized using Typhoon reader. Details of the reagents and instruments used in the western blot procedure are provided in Table 6.

Table 6: List of reagents used in the western blot assays

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Reagent Name</th>
<th>Company/Catalogue No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tris-Cl</td>
<td>Sigma/Cat. No T5941</td>
</tr>
<tr>
<td>2</td>
<td>Acrylamide 30%</td>
<td>Bio-Rad/Cat. No 161-0158</td>
</tr>
<tr>
<td>3</td>
<td>SDS</td>
<td>Bio-Rad/Cat. No 161-0302</td>
</tr>
<tr>
<td>4</td>
<td>Ammonium persulfate</td>
<td>Sigma/Cat. No A3678</td>
</tr>
<tr>
<td>5</td>
<td>TEMED</td>
<td>Sigma/Cat. No T9281</td>
</tr>
<tr>
<td>6</td>
<td>NaCl</td>
<td>Sigma/Cat. No S7653</td>
</tr>
<tr>
<td>7</td>
<td>KCl</td>
<td>Sigma/Cat. No P9541</td>
</tr>
<tr>
<td>8</td>
<td>Na₂HPO₄</td>
<td>Sigma/Cat. No S0876</td>
</tr>
<tr>
<td>9</td>
<td>KH₂PO₄</td>
<td>Sigma/Cat. No P-0662</td>
</tr>
<tr>
<td>10</td>
<td>Tris-base</td>
<td>Sigma/Cat. No T6066</td>
</tr>
<tr>
<td>11</td>
<td>Glycine</td>
<td>Sigma/Cat. No G8898</td>
</tr>
</tbody>
</table>
2.9 Morphological Studies

Cell cultures treated with different extracts were followed for morphological changes by observation via an inverted light microscopic attached to a CCD camera. The treated cells were compared with cells treated with similar DMSO concentrations and untreated cells. Images were saved at various magnifications at different times after treatment.

2.10 Purified Compounds and their Derivatives

Purified compounds from some of the crude extracts were made available by our collaborator, Prof. Javid Hussain, University of Nizwa, Oman, and his team as well as their derivatives. They were successful in isolating one compound from AO (FTZ) and one from TM plant (IM-60) (Table 7). Three different derivatives were also prepared from the FTZ compound. A description of the natural and synthetic derivatives is provided in Table 7. Each of these were solubilized in DMSO and tested for their anticancer activity using the MTT assay using the breast cancer cell
line MCF-7. Effective compounds were further tested in the caspase assays to
determine if they could induce caspase enzymatic activity.

Table 7: List of purified compounds and synthetic derivatives tested in this study

<table>
<thead>
<tr>
<th>S. No</th>
<th>Code</th>
<th>Source (Plant or Synthetic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FTZ</td>
<td>Isolated from <em>Acridocarpus orientalis</em></td>
</tr>
<tr>
<td>2</td>
<td>F-OTF(1)</td>
<td>Synthetic derivative of FTZ</td>
</tr>
<tr>
<td>3</td>
<td>F-OTF-SS1</td>
<td>Synthetic derivative of FTZ</td>
</tr>
<tr>
<td>4</td>
<td>TZ-SM3</td>
<td>Synthetic derivative of FTZ</td>
</tr>
<tr>
<td>5</td>
<td>1M-60</td>
<td>Isolated from <em>Teucrium mascatensis</em></td>
</tr>
</tbody>
</table>

2.11 Statistical Analysis

Statistical analysis of the presented data was accomplished by using either
Microsoft Excel or Graphpad Prism v5 software. Average values were calculated for
each of the samples in replicates of three or more followed by calculation of their
standard deviations that were plotted as error bars on the column graphs. Paired,
two-tailed student’s t-test was used to determine significant differences between any
two groups. The extent of significance was shown as either one to three stars,
depending upon the value obtained (* = p <0.05; ** p <0.01; *** = p <0.001). The
dose- and time-dependent graphpad figures were generated by normalizing the
values obtained for the test groups with that of their DMSO control since a specific
DMSO control was used for each extract tested, whether it was the MTT assay or the
Promega glo assays.
Chapter 3: Results

3.1 Description of Plant Extracts Tested

One essential oil and thirty-two different plant extracts were obtained from our collaborators, Prof. Ahmed Al Harrasi and Dr. Javid Hussain, University of Nizwa, Sultanate of Oman. The essential oil was prepared by steam distillation, while the extracts were prepared using organic solvents based on their polarity from low to high, as shown in the Figure 6.

![Types of Extracts](image)

Figure 6: Organic solvents used for plant extract preparation in the order of increasing polarity.

The description of the extracts obtained from the five medicinal plants is given below:

1. *Boswellia sacra* (BS). Steam-distilled essential oil, three resin extracts made from hexane, chloroform, methanol, and the residual aqueous extract.

2. *Cleome droserifolia* (CD). Four extracts prepared by extraction with hexane, chloroform, ethyl acetate, butanol.

3. *Teucrium muscatensis* (TM). Five extracts prepared by extraction with hexane, chloroform, ethyl acetate, butanol, methanol and the residual aqueous extract.
4. *Orcadenus arabicus* (OA). Five whole plant extracts prepared by extraction with hexane, chloroform, ethyl acetate, butanol, methanol, and the residual aqueous extract.

5. *Aridocarpus orientalis* (AO).

a. Leaf (L). Five leaf extracts prepared by extraction with hexane, chloroform, ethyl acetate, butanol, methanol, and the residual aqueous extract.

b. Stem (S). Five stem extracts prepared by extraction with hexane, chloroform, ethyl acetate, butanol, methanol, and the residual aqueous extract.

The rationale of obtaining extracts made by different solvents was to ensure that any bioactive compound that may be present in the plants being tested was not missed due to the extraction procedure since some solvents may be better than others in extracting the various biochemical activities found in plants.

A typical serial exhaustive extraction procedure used to prepare the extracts is shown in Figure 7 [425]. It involves the use of sequential solvents with increasing polarity to ensure that bioactive molecules of a wide polarity range are successfully extracted from the crude plant material. Briefly, the process started with either the whole plant or its parts that were dried in the dark, chopped and ground to a coarse powder. The powdered plant material was extracted with methanol three times for two weeks at room temperature. Then, the methanol extract was evaporated under pressure, leaving a greenish syrup residue. The methanol extract was suspended in water and then hexane was added with vigorous shaking in a separating funnel. The mixture was allowed to separate into two layers; the hexane upper layer was removed and the extraction with hexane was repeated twice. All hexane layers were combined and evaporated on a rotary evaporator, resulting in the hexane fraction (Fig. 7). Next, the other lower layers were taken in a separating funnel where chloroform was
added and separated as mentioned earlier. The chloroform lower layer was collected and evaporated on a rotary evaporator, giving rise to the chloroform fraction. The upper layer of chloroform was taken into a separating funnel and ethyl acetate was added, separated and evaporated with rotary evaporator, giving rise to the ethyl acetate fraction. The same procedure was applied for butanol, giving rise to the butanol extract, while the remaining lower layer was collected and evaporated, giving rise to the aqueous fraction.
Ochradenus arabicus (28.4 kg)

- Soaked in methanol for two weeks and filtered

Methanol Extract (437.4 g)

- n-hexane + water

$n$-hexane + water

Evaporated through Rotary

Hexane Soluble Fraction (68.1 g)

Crude Extract

- Extracted with Chloroform

Crude Extract

Evaporated through Rotary

Extracted with Ethyl Acetate

Chloroform Fraction (85.8 g)

Evaporated through Rotary

Crude Extract

Ethyl acetate (27.8 g)

Aquous Fraction (145.3 g)

Evaporated through Rotary

n-butanol extract (87.5 g)

Figure 7: Illustration of a typical extraction procedure used for preparing the extracts tested in this study.

Figure courtesy of Dr. Javid Hassan, University of Nizwa, Oman.
3.2 Experimental Design

To test the anticancer potential of the extracts described above, we started our investigations with the *Boswellia sacra* essential oil, an oil that has already been shown to have anti-proliferative effects in several cancer systems, including breast cancer [265]. We wanted to confirm some of these observations using the steam distilled oil preparation that was obtained from our collaborators. This was with the intention to extend this observation to other cancer cell model systems to determine the breadth and effectiveness of this oil as well as study its mechanism of action and interaction with other chemotherapeutic drugs (not part of this thesis). This was achieved by testing the oil in a human cancer cell model system that included a breast and cervical cancer cell line. This also allowed us to establish the assays needed to carry out the main aim of the thesis, to screen the *Boswellia sacra* and other novel plant extracts for their anticancer potential in a more efficient manner. Once established, the other plant extracts were then screened for their anticancer potential. Due to the large number of extracts obtained, this screening was initially limited to only two different cell lines: the breast cancer cell line MCF-7 and the cervical cancer cell line, HeLa. Once the effective extracts from each plant were short-listed for their effectiveness on cell proliferation, one representative extract with anti-proliferative effects was chosen from each plant and characterized further in a more expanded cancer model system that included the following cell lines:

1. MCF-10A. A normal human breast cell line used to test the extracts on normal human cells [426].

2. MCF-7. A hormone-responsive human invasive breast ductal carcinoma cell line that expresses estrogen and progesterone receptors (ER and PR), but no HER2/neu gene amplification has been observed [427].
3. MDA-MB-231. A triple negative human breast cancer cell line that does not express any of the hormone receptors mentioned above [428].

4. HeLa. A human cervical cancer cell line [429] chosen to determine the breadth of anti-proliferation activity being observed.

Once the anti-proliferative activity of the chosen extracts had been confirmed, their mechanism of action was explored using either the MCF-7 or HeLa cell lines using various biochemical and cellular assays.

### 3.3 Test of *Boswellia sacra* Essential Oil on Human Cancer Cell Lines

We started our screening efforts with the essential oil from *Boswellia sacra* to confirm the anti-proliferative effects being reported in literature [265], establish the various cell assays, and determine the effective dose and time characteristics of the oil obtained from our collaborators. Using the MTT assay, we screened the steam distilled *Boswellia sacra* essential oil on the normal (MCF-10A) and three cancer cell lines (MCF-7, MDA-MB-231, and HeLa) in a dose- and time-dependent manner. The MTT assay is based on the cellular conversion of the tetrazolium dye MTT into purple formazan crystals by the mitochondrial NADPH-dependent oxido-reductase enzymes that are easily detected using a 96-well plate reader. The ability to convert the tetrazolium salt into the formazan product varies among different cell lines, depending upon their metabolic capacity and the absorbance reading is directly proportional to the number of living cells.

As can be seen from Figure 8, test of various dilutions of the *Boswellia sacra* essential oil (1:500, 1:800, 1:1000, 1:1600, 1:2000, 1:3200, 1:4000) in individual cell lines at 24 and 72 hours post treatment revealed that strong anti-proliferative effects could be observed in these cell lines, including the normal cells. The column graphs
in Figure 8, panels A-D show the dose- and time-dependent results of *Boswellia sacra* treatment as a percent of control DMSO-treated cells. The essential oil was solubilized in DMSO at a ratio of 1:1 and the same concentration of DMSO was used in the DMSO control-treated cells to ensure that we were observing the effects of the essential oil on cell viability and not the toxicity induced by DMSO alone. Our results revealed that MCF-10A and MCF-7 cells were equally sensitive to 1:500 and 1:800 dilutions of the essential oil with cell viability decreasing by > 95% by 72 hours. Panels E-F show the same data but combined for all the cell lines in one graph separately for the 24 and 72 hours, allowing easy comparison between the effects of the oil on the different cell lines. As can be seen, the triple-negative MDA-MB-231 cells were the most sensitive to killing and even a 1:1000 dilution led to ~80% killing by 72 hours. At this dilution, neither the MCF-10A or MCF-7 cells were sensitive to killing, showing essentially 100% cell viability (Fig. 8, panels E and F). HeLa were a lot less sensitive to the essential oil than the breast cancer cell lines, killing ~ 50% of the cells at a dose of 1:500. Expanded versions of the MTT column graphs A-D of Figure 8 are reproduced in Fig. 2S of the appendix for better visualization.
Figure 8: MDA-MB-231 is the most sensitive cell line to cell death by *Boswellia sacra* essential oil. Various dilutions of the essential oil were tested using the MTT assay on A) MCF-10A, B) MCF-7, C) MDA-MB-231, and D) HeLa cells after 24 and 72 hours. Dose-dependent effect of *Boswellia sacra* on cell viability as a percentage of untreated cells for E) the 24 hour and F) 72 hour time points for all four cell lines tested. * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** p<0.01; *** = p<0.001). The significance shown in panels A-D is between the DMSO-control and essential oil-treated samples, while the significance...
shown in panel E-F is between the normal and cancer cell lines. This figure shows a representative experiment of several experiments conducted using this oil in the four cell lines. Expanded versions of the MTT column graphs A-D are reproduced in Fig. 2S of the appendix for better visualization.

Analysis of the time-dependent response of the essential oil in the individual cell lines showed that the killing profiles for the various cell lines were identical at 24 and 72 hours (Fig. 9), suggesting that the oil was very efficient at killing cells, accomplishing its cytopathic effects by 24 hours. Together, these data show that *Boswellia sacra* essential oil can efficiently kill both breast cancer and cervical cancer cells, though its effects on cell viability are more specific for breast cancer than cervical cancer cells. Furthermore, these data show that the MDA-MB-231 cells are more sensitive to this oil than the normal cells, suggesting that *Boswellia sacra* essential oil could be an option to treat triple negative breast cancer which historically has been more difficult to treat, without much cytotoxicity for normal cells at the same dose and time exposures. Suhail and colleagues also observed cytotoxic effects of the essential oil on breast cancer cells, but much lower cytotoxicity for normal MCF-10A cells than what we observed [265], which could be due to difference in essential oil preparations used.
Figure 9: *Boswellia sacra* essential oil has early kinetics of cell death induction in human normal and cancer cell lines.

Time-dependent effect of *Boswellia sacra* essential oil different cell lines. A) MCF-10A, B) MCF-7, C) MDA-MB231, and D) HeLa cells 24 and 72 hours post treatment.

3.4 Test of *Boswellia sacra* Extracts on Human Cancer Cell Lines

Next, we analyzed the extracts of *Boswellia sacra* to determine their anti-proliferation potential using the MTT assay in comparison to the essential oil. This was conducted to determine whether the anti-proliferation activity being observed could be observed in the sequentially-prepared extracts or was it unique to the essential oil. Figure 10 shows the results of the test of the different *Boswellia sacra* extracts, including chloroform, methanol, and the remaining aqueous extract on MCF-7 and HeLa cells at different concentrations (10, 100, 250, 500 μg/ml). Since the hexane extract could not be weighed due to its sticky and semi-fluid nature, the entire amount was solubilized in DMSO and was tested at two different dilutions (1:200 and 1:400). As can be seen, all three organic extracts, but not the aqueous
extract of *Boswellia sacra* resin, exhibited strong anti-proliferative activity against the two tested cancer cell lines. Both methanol and chloroform extracts showed very similar effects on the viability of MCF-7 and HeLa cells, inducing ~80% killing in MCF-7 cells and about 90% in HeLa cells, all in a statistically significant manner (Fig. 13, panels A and C). The hexane extract also showed very similar effects on the two cell lines at 1:200 dilution killing > 80% of the cells; however, it lost the cytotoxic effect on HeLa cells at the 1:400 dilution, while still killing the MCF-7 cells by ~75%, suggesting that MCF-7 cells were more sensitive to hexane extract than HeLa cells (Fig. 13, panels B and D). Interestingly, the aqueous extract enhanced the proliferation rates of the two cell lines tested rather than causing cell killing or having no effect (Fig. 13, panels A and C), an observation that suggests that the anti-proliferative compounds within the crude extracts keep the growth of cells in check. Furthermore, it suggests that these compounds are soluble in the organic solvents and have been successfully removed from the aqueous extracts by the sequential extraction process.
Figure 10: Effect of *Boswellia sacra* plant extracts on cancer cell viability. Organic (chloroform, methanol, & hexane) and aqueous extracts of *Boswellia sacra* were tested using MTT assay on A) & B) MCF-7 (72 hours), and C) & D) HeLa cells (48 hours) post treatment. * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** p<0.01; *** = p<0.001)

3.5 Morphological Changes of Cell Treated with *Boswellia sacra*

Apoptotic cell death can be defined by various morphological changes that distinguish it from other forms of cell death. To determine whether the organic extracts of *Boswellia sacra* could cause apoptosis, we next analyzed the MCF-7 and HeLa cells treated with different concentrations of chloroform extract of *Boswellia sacra* for morphological changes compared with DMSO control. As shown in Figure 11, the most noticeable changes observed included cell shrinkage and rounding, followed by detachment of the cells. These changes are characteristics of apoptotic cell death, and were absent in the control DMSO-treated cells. The changes became more remarkable with increasing time of treatment, suggesting that MCF-7 and HeLa may be undergoing apoptosis post treatment.
Figure 11: Morphological effects of *Boswellia sacra* on human cancer cell lines. Photomicrographs of *Boswellia sacra* chloroform extract-treated MCF-7 and HeLa cells at 14 hours post treatment at 100 and 250 μg/ml, respectively. The photographs were taken at the same magnification for all the four panels (100X).

### 3.6 *Boswellia sacra* Induces DNA Fragmentation

Apoptotic cell death is also accompanied by various biochemical changes unique to this form of cell death. One of the main features of apoptosis is DNA fragmentation when the genomic DNA is cleaved by endonuclease, which breaks apart the chromatin into nucleosomal-sized units that appear as a DNA ladder when run on agarose gel [430, 431]. Thus, we tested the ability of *Boswellia sacra* essential oil to fragment DNA of MCF-7 and HeLa cells at 24 and 48 hours post treatment compared to media and DMSO control. As apparent in Figure 12, *Boswellia sacra* essential oil was able to induce DNA fragmentation within treated cells, consistent with results obtained with the MTT assay and morphological changes observed under the light microscope (Fig. 12).
Figure 12: *Boswellia sacra* essential oil can induce DNA fragmentation in human cancer cell lines. Agarose gel electrophoresis showing DNA laddering of MCF-7 and HeLa cells after treatment with *Boswellia sacra* essential oil (1:400) after 24 and 48 hours. The white arrows point to the DNA ladders being observed in the gel, this experiment was conducted once.

### 3.7 *Boswellia sacra* Activates Apoptosis

Next, we confirmed the ability of *Boswellia sacra* essential oil to induce apoptosis by the activation of caspase enzymes, as has been reported earlier, but with our preparation of the essential oil [265]. During apoptosis, a group of cysteine aspartyl proteases, caspases, are activated in a cascade fashion, leading to the activation of the initiator caspases (such as caspase-8 and 9) that cleave the executioner caspases (such as caspase-3 and 7), enzymes that play a critical role in programmed cell death. Activation of caspases results in mitochondrial membrane
permeabilization, chromatin condensation, and DNA fragmentation, ending in cell destruction.

Thus, the activation of the caspase enzymes was monitored using the Promega Caspase-Glo assays for four different caspase enzymes, including caspase-3/7, 8, and 9. These assays provide a pro-luminescent caspase substrate containing caspase-specific tetrapeptide sequences that can be recognized and cleaved by the targeted caspase enzymes, thus cleaving the substrate of luciferase and releasing a glow-type light that can be captured and quantitated by the appropriate instruments. These results were normalized to the viability of the cultures as assessed by the Promega CellTiter-Glo® Luminescent Cell Viability Assay to ensure accurate results were obtained reflective of the enzyme activation on a per cell basis. This assay is a very sensitive and rapid method to assess the number of viable cells in culture based on the quantitation of ATP as an indirect measure of metabolically active cells. It is similar to MTT, but provides another independent measure of cell viability in a quantitative manner.

As revealed by Figure 13, *Boswellia sacra* essential oil was able to induce the activity of caspase enzymes in MCF-7 breast cancer cells in a statistically-significant manner. Specifically, strong activation of caspase 7, 8, and 9 all could be observed, suggesting that *Boswellia sacra* essential oil was inducing apoptosis in the breast cancer cells through the activation of both the intrinsic and extrinsic pathways. These results also confirmed the cytotoxic effect on breast cancer cells being induced by the essential oil, as observed by the MTT assay.

Together, our results confirm the anticancer activity of *Boswellia sacra* essential oil on breast cancer cells and extend it to cervical cancer cells. They also
suggest that the essential oil and organic extracts of its resin have the ability to induce apoptosis by the activation of both the intrinsic and extrinsic pathways. Further studies are being conducted to determine how *Boswellia sacra* extracts can be used to enhance the anticancer potential of other cancer chemotherapeutic drugs currently in use in different cancer cell line systems.

Figure 13: *Boswellia sacra* essential oil can induce activation of caspase enzymes. Cell viability and caspase activity assay of *Boswellia sacra* essential oil (1:200 dilution) on MCF-7 at 48 hours treatment. * indicates statistically significant differences between the DMSO control and *Boswellia sacra*-treated samples (* = p <0.05; ** p <0.01; *** = p <0.001). This experiment was conducted once.

### 3.8 Screening of Medicinal Plant Extracts for Anticancer Activity

Next we proceeded with screening the other 28 extracts from the four different shrubs *Cleome droserifolia*, *Teucrium muscatensis*, *Orchadenus arabicus*, and *Acredocarpus orientalis* provided by our collaborators. As mentioned earlier, these plants had been targeted for studying their anticancer activity due to their well-known medicinal properties.

Due to the large number of extracts to be tested, we first performed an initial screening of all the extracts using the MTT assay with the breast cancer cell line.
MCF-7, and the cervical cancer cell line, HeLa in a dose- and time-dependent manner. All extracts were tested at different concentrations (25, 50, 125, 250 μg/ml) at two time points (24 and 72 hours) post treatment. Table 8 summarizes the results obtained from the first screening.

As can be seen, all spectrum of anti-proliferative activities could be observed in the tested extracts. Some could kill both cell lines (extracts shaded in green), while some could not kill either of the cell lines (extracts not shaded and marked by x). Interestingly, several extracts could selectively kill HeLa cells without killing MCF-7 cells (extracts highlighted in blue and yellow), while none of the extracts was found to kill MCF-7 specifically. However, there were some extracts that were more “MCF-7-specific” and could kill these cells at lower doses and early on, while HeLa cells only at higher doses and later on (for example, extracts shown in pink).

Overall, 11 out of 28 (39%) of the extracts showed anti-proliferative effects on the two cancer cells, while 17 out of 28 (60.7%) extracts were ineffective in killing either cell line. Interestingly, at least one effective extract was identified from each of the plants screened. This is an important result and suggests that plants that have been used for medicinal purposes historically provide a valuable source of raw material for screening of anticancer compounds.
Table 8: Summary of the first screening of plant extracts using the MTT assay

<table>
<thead>
<tr>
<th>Extracts</th>
<th>MCF-7 (24/72 HRS)</th>
<th>HeLa (24/72 HRS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD BT</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CD CH</td>
<td>24HR: 125/250</td>
<td>24HR: 7250</td>
</tr>
<tr>
<td></td>
<td>72HR: 50 onwards</td>
<td>72HR: 125/250</td>
</tr>
<tr>
<td>CD ET</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CD HX</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>TM CH</td>
<td>24HR: 250</td>
<td>24HR: None</td>
</tr>
<tr>
<td></td>
<td>72HR: 125/250</td>
<td>72HR: 250</td>
</tr>
<tr>
<td>TM MT</td>
<td>24HR: 250</td>
<td>24HR: None</td>
</tr>
<tr>
<td></td>
<td>72HR: 125/250</td>
<td>72HR: 250</td>
</tr>
<tr>
<td>TM HX</td>
<td>24HR: 250</td>
<td>24HR: None</td>
</tr>
<tr>
<td></td>
<td>72HR: 125/250</td>
<td>72HR: 250</td>
</tr>
<tr>
<td>TM AQ</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>TM BT</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>TM ET</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>OA ET</td>
<td>X</td>
<td>24HR: None</td>
</tr>
<tr>
<td></td>
<td>72HR: 125/250</td>
<td>72HR: 250</td>
</tr>
<tr>
<td>OA MT</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>OA HX</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>OA AQ</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>OA CH</td>
<td>24HR: 250</td>
<td>24HR: None</td>
</tr>
<tr>
<td></td>
<td>72HR: 125/250</td>
<td>72HR: 250</td>
</tr>
<tr>
<td>OA BT</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>AO BT(L)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>AO BT(S)</td>
<td>X</td>
<td>24HR: None</td>
</tr>
<tr>
<td></td>
<td>72HR: 250</td>
<td></td>
</tr>
<tr>
<td>AO ET(L)</td>
<td>X</td>
<td>24HR: None</td>
</tr>
<tr>
<td></td>
<td>72HR: 125/250</td>
<td></td>
</tr>
<tr>
<td>AO ET(S)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>AO MT(L)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>AO MT(S)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>AO AQ(L)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>AO AQ(S)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>AO CH(L)</td>
<td>Beginning to kill at 72 hours with 250</td>
<td>24HR: 250</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72HR: 125/250</td>
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<tr>
<td>AO CH(S)</td>
<td>X</td>
<td>24HR: None</td>
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<tr>
<td></td>
<td>72HR: 125/250</td>
<td></td>
</tr>
<tr>
<td>AO HX(L)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>AO HX(S)</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

X = Not effective in either cell line
Green = Effective in both cell lines
Pink = Effective in MCF-7 cells
Blue & Yellow = Effective in HeLa cells only

A closer look at Table 8 reveals that for each individual plant, only a few of the extracts had effective cytotoxic activity and most were ineffective in killing the cancer cells. A summary of effective extracts from each plant is listed below:
CD → CH only
TM → CH, MT, HX
OA → CH, ET (HeLa only)
AO (L) → CH, ET (HeLa only)
AO (S) → CH (HeLa only), BT (HeLa only)

As can be seen, the chloroform (CH) extracts were uniformly able to induce cytotoxic effects in the cancer cell lines, although in one case (AO (S)), it was not effective on MCF-7 cells. The remaining effective extracts were prepared by other solvents that included two by ethyl acetate (ET), and one each by hexane (HX), methanol (MT), and butanol (BT). None of the extracts aqueous extracts (AQ) were found to be effective. These results suggest that chloroform may have the best ability, among the solvents tested, to extract bioactive molecules that have anticancer activity, though other solvents can extract such activities as well.

3.9 Cytotoxic Effect of Organic Solvents used for Extraction

It is possible that the cytotoxic activity of the extracts being observed was due not to compounds within the extracts but due only to any remaining organic solvents used for the extraction purposes. Therefore, we tested the cytotoxicity of the organic solvents used for the extraction process using the MTT assay on the three cancer cell lines used: MCF-7, MDA-MB-231, and HeLa.

As can be seen, testing of the pure solvents starting with a 1:500 dilution on the three cell lines revealed that there was no cytotoxic effect of the organic solvents on the cell lines tested (Fig. 14), confirming that any residual solvent that may have remained in the extracts despite days of evaporation (sometimes over a month or more) was not responsible for the anti-proliferative effects that were being observed.
Furthermore, the extracts were tested by our collaborators directly using nuclear magnetic resonance (NMR) at the University of Ṣīwā, Oman to determine whether physical traces of the solvents could actually be detected. Their tests confirmed that no residual organic solvents could be detected in the extracts (data not shown).

Next, five effective extracts from Table 8 were short-listed for further analysis of their anticancer properties. At least one extract was chosen from each plant or part thereof. For example, CD CH was chosen from *Cleome droserifolia* since it represented a “more MCF-7-specific” extract. OA CH was chosen from *Orchadenum arabicus* and TM CH from *Teucrium muscatensis* since they could kill both MCF-7 and HeLa cancer cell lines. AO BT(S) and AO CH(L) were chosen from *Acredocus orientalis* since AO BT(S) was HeLa-specific, while AO CH(L) from the leaves could kill MCF-7 as well, though less efficiently. These extracts were tested in a more expanded series of cell lines, including MCF-10A, the normal breast cell line and MDA-MB-231, the prototypic triple negative breast cancer cell line along with MCF-7 and HeLa cells.
Various organic solvents used for extract preparation were tested on MCF-7, MDA-MB-231, and HeLa cells using the MTT assay at 24 and 72 hours post treatment, this experiment was done once.

First, the MTT assay was repeated in all these cell lines in a dose-dependent manner using three different concentrations (50, 125, 250) μg/ml) after 24, 48, and 72 hours treatment. This allowed us to compare the effects of the extracts in both the normal and cancer cell lines at the same time in the same manner.

Figure 15 shows the results of the test of Cleome droselifolia extract on the tested cell lines. As can be seen, this extract could induce cell death in all the three breast cell lines in a comparable fashion in both a dose- and time- dependent manner. MCF-10A and MCF-7 were equally sensitive to this extract, while MDA-MB-231 was slightly less sensitive as detected by the treatment with the lower doses (Fig. 15.
By 250 µg/ml dose, all three cell lines were equally sensitive, leading to >90% death of the culture compared to cell growth in the presence of DMSO alone by 72 hours. When we compared all the four cell lines in a time-dependent manner, HeLa cells were less sensitive to this extract in the beginning, but by 72 hours, had nearly killed similar percentages of cells as the breast cells (Fig. 15, panel F). This revealed that *Cleome drosirifolia* crude extract affects cell proliferation of both normal mammary and breast cancer cells equally, while the HeLa cells were somewhat less sensitive to this extract. Expanded versions of the MTT column graphs A-D of Figure 15 are reproduced in Fig. 3S of the appendix for better visualization.
Figure 15: *Cleome droserifolia* extracts can induce potent cell death in human normal and cancer cell lines. Cytotoxic effect of different concentration of *Cleome droserifolia* using MTT assay on A) MCF-10A, B) MCF-7, C) MDA-MB-231, and D) HeLa cells after 24, 48, and 72 hours post treatment. Panel E) shows dose-dependent effect of *Cleome droserifolia* on cell viability as a percentage of untreated cells for the 72 hour time point. Panel F) shows the time-dependent effect of *Cleome droserifolia* on cell viability as a percentage of untreated cells for the 250 µg/ml treatment. * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** p<0.01; *** = p<0.001). The significance shown in panels A-D is between the DMSO-control and extract-treated groups, while the significance shown in panels E and F is between the normal and cancer cell lines. This is a representative experiment done two to three times. Expanded versions of the MTT column graphs A-D are reproduced in Fig. 3S of the appendix for better visualization.
Figure 16 reveals the results of *Teucrium muscatensis* on the normal and cancerous cell lines. It could induce cytotoxic effects on the three cancer cell lines in a statistically significant manner. However, the normal MCF-10A cells were the most sensitive to this extract than the cancer cells. The MCF-7 and MDA-MB-231 cells showed quite comparable cytotoxicity profiles (Fig. 16, panel E), while like CD, the HeLa cells were slightly less susceptible, as observed by the time course of cell killing (Fig. 16, panel F). Thus, our results reveal that *Teucrium muscatensis* has significant effect on not only breast and HeLa cancer cell viability, but also normal breast cells. Since it can kill cancer cells effectively, though not specifically, it is still worth to explore this extract further by isolating the active biomolecules to determine if they may have more specific anticancer effects without affecting normal cells. Expanded versions of the MTT column graphs A-D in Figure 16 are reproduced in Fig. 4S of the appendix for better visualization.
Figure 16: *Teucrium muscatensis* extracts can induce potent cell death in human normal and cancer cell lines.

Cytotoxic effects of different concentration of *Teucrium muscatensis* using MTT assay on A) MCF-10A, B) MCF-7, C) MDA-MB-231, and D) HeLa cells after 24, 48, and 72 hours post treatment. Panel E) shows dose-dependent effect of Teucrium muscatensis on cell viability as a percentage of untreated cells for the 72 hour time point. Panel F) shows the time-dependent effect of *Teucrium muscatensis* on cell viability as a percentage of untreated cells for the 250 µg/ml treatment. * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** p<0.01; *** = p<0.001). The significance shown in panels A-D is between the DMSO-control and extract-treated groups, while the significance shown in panels E and F is between the normal and cancer cell lines. This is a representative experiment done two to three times. Expanded versions of the MTT column graphs A-D are reproduced in Fig. 4S of the appendix for better visualization.
Figure 17 shows the results of effects of *Orchadenus arabicus* on the normal and cancerous cell lines. The triple-negative breast cancer cell line, MDA-MB-231, was the most sensitive to *Orchadenus arabicus*, causing > 90% cell death within 72 hours of treatment with 250 μg/ml extract (Fig. 17, panels E and F). The normal cells, MCF-10A, and MCF-7 breast cancer cells were more intermediately affected by this extract. HeLa cervical cancer cells were the least affected by this extract with ~80% of the cells still viable after treatment with 250 μg/ml for 72 hours. Thus, this data suggests that the OA extract has specific anti-proliferative activity against breast cancer cells, in particular the hormone non-responsive cells MDA-MB-231, that could be exploited further for therapy. Expanded versions of the MTT column graphs A-D of Figure 17 are reproduced in Fig. 5S of the appendix for better visualization.
Figure 17: *Orchadenus arabicus* extracts can induce cell death in human normal and cancer cell lines. Cytotoxic effect of different concentrations of *Orchadenus arabicus* using MTT assay on A) MCF-10A, B) MCF-7, C) MDA-MB-231, and D) HeLa cells after 24, 48, and 72 hours post treatment. Panel E) shows dose-dependent effect of *Orchadenus arabicus* on cell viability as a percentage of untreated cells for the 72 hour time point. Panel F) shows the time-dependent effect of *Orchadenus arabicus* on cell viability as a percentage of untreated cells for the 250 μg/ml treatment. * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** p<0.01; *** = p<0.001). The significance shown in panels A-D is between the DMSO-control and extract-treated groups, while the significance shown in panels E and F is between the normal and cancer cell lines. This is a representative experiment done two to three times. Expanded versions of the MTT column A-D are reproduced in Fig. 5S of the appendix for better visualization.
Figures 18 and 19 show the results of the test of *Acredocarpus orientalis* on the normal and cancerous cell lines. For this plant, we had separate extracts available from the leaves (AO (L)) and the stems (AO (S)). Test of AO (L) on the four cell lines revealed that the leaf extract could efficiently kill all the four cell lines equally well, including the normal breast and cervical cancer cells (Fig. 18). Thus, it could kill >75-80% of the four cell lines compared to DMSO control treated cells by 72 hours at the highest dose tested (250 μg/ml) (Fig. 18, panel E). This result suggests that the leaf extract from *Acredocarpus orientalis* has strong cytotoxic activity and may have a number of different bioactive components with individual characteristics that could be explored further separately as specific anticancer agents. Expanded versions of the MTT column graphs A-D of Figures 18 and 19 are reproduced in Fig. 6S and 7S, respectively, of the appendix for better visualization.
Figure 18: 

*Acredocarpus orientalis* (L.) extracts can induce cell death in human normal and cancer cell lines. 

Cytotoxic effect of different concentrations of *Acredocarpus orientalis* (L.) using MTT assay on A) MCF-10A, B) MCF-7, C) MDA-MB-231, and D) HeLa cells after 24, 48, and 72 hours post treatment. Panel E) shows dose-dependent effect of *Acredocarpus orientalis* (L.) on cell viability as a percentage of untreated cells for the 72 hour time point. Panel F) shows the time-dependent effect of *Acredocarpus orientalis* (L.) on cell viability as a percentage of untreated cells for the 250 µg/ml treatment. * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** p<0.01; *** = p<0.001). The significance shown in panels A-D is between the DMSO-control and extract-treated groups, while the significance shown in panels E and F is between the normal and cancer cell lines. This is a representative experiment done two to three times. Expanded versions of the MTT column graphs A-D are reproduced in Fig. 6S of the appendix for better visualization.
Test of *Acredocarpus orientallis* (S), the stem extract from AO plant, on the other hand revealed a very different profile compared to the leaf extract and had only limited anti-proliferative activity against the cell lines tested (Fig. 19). Whereas AO (L) caused nearly 70-80% killing of the four cell lines tested by 72 hours, the proliferation of the normal breast cells or the two breast cancer cell lines was not affected at all by AO (S) (Fig. 19, panels A-C). In fact, statistically significant proliferation of the treated normal breast cells (MCF-10A) and the two breast cancer cell lines (MCF-7 and MDA-MB-231) was observed after 24 hours that persisted with time for all except MCF-7 cells (Fig. 19, panel F). We believe that the increase in conversion of the tetrazolium salt to formazan by the dehydrogenase enzymes is due to increase in cell numbers and not enzyme per cell since we used cell numbers in the range that reflects a linear increase in tetrazolium conversion with increasing number of cells (Fig. 1S in the appendix).

HeLa cells, on the other hand, showed some anti-proliferative effect starting 48 hours post treatment (Fig. 19, panels D-F). This was very different from the broadly-cytotoxic activity observed with the leaf extract from the same plant. These results suggest that extracts from *Acredocarpus orientalis* have multiple activities with the potential of providing variable anticancer activities. Thus, exploration of isolated components from this plant would be valuable since it may lead to compounds that could kill the cancer cells specifically over the normal cells, and lead to the proliferation of normal cells at the same time.
Figure 19: *Acredocarpus orientallus* (S) induces cell death in normal and human cancer cell lines to a much lower extent.

Cytotoxic effect of different concentrations of *Acredocarpus orientallus* (S) using MTT assay on A) MCF-10A, B) MCF-7, C) MDA-MB231, and D) HeLa cells after 24, 48, and 72 hours post treatment. Panel E) shows dose-dependent effect of *Acredocarpus orientallus* (S) on cell viability as a percentage of untreated cells for the 72 hour time point. Panel F) shows the time-dependent effect of *Acredocarpus orientallus* (S) on cell viability as a percentage of untreated cells for the 250 μg/ml treatment. * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** p<0.01; *** = p<0.001). The significance shown in panels A-D is between the DMSO-control and extract-treated groups, while the significance shown in panels E and F is between the normal and cancer cell lines. This is a representative experiment done two to three times. Expanded versions of the MTT column graphs A-D are reproduced in Fig. 7S of the appendix for better visualization.

Next, we asked how effective were each of the five extracts in each cell line individually in terms of their comparative ability to induce cell death. Thus, the dose-and time-dependent effect of each extract on cell viability as a percentage of
DMSO-control-treated cells was plotted for all extracts on each cell line separately and is shown in Figures 20 and 21. Analysis of the effectiveness of the extracts in MCF-10A normal cells revealed that CD and TM were the most cytopathic extracts for normal mammary cells, killing over 90% of the cells by 72 hours (Figs. 20 and 21, panel A). This was followed by OA and AO (L) that showed intermediate levels of cytotoxicity, leading to 70% cell death by 72 hours. This was in stark contrast to AO (S) stem extract that was not cytotoxic for the normal cell at all, and in fact had a proliferative effect on the growth of these normal cells in a dose-dependent manner (Fig. 20, panel A).

A comparison of the effect of the five extracts on MCF-7 cells revealed that all were effective at killing this cell line in a dose- and time-dependent manner, leading to 70-90% death of these cells compared to the DMSO-control treated cells (Figs. 20 and 21, panel B). CD and TM were more cytotoxic at lower doses than OA and AO (L), while AO (S) could not kill these cells at the same dose and time. In fact, it led to a proliferation of the MCF-7 early on at lower doses in a statistically significant manner (Figs. 20 and 21, panel B).
Figure 20: Comparative effect of different plant extracts on normal and cancerous human cell lines with dose. A) MCF-10A, B) MCF-7, C) MDA-MB231, and D) HeLa cell lines using the MTT assay in a dose-dependent manner. * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** p<0.01; *** = p<0.001). This figure shows replotting of data shown in Figs. 15-19.

Similar results were obtained for MDA-MB-231 where all the extracts could kill >75-90% of the cells at the highest dose tested at 72 hours, except for AO (S) that once again led to a proliferation of these cancer cells at even low doses early on (Figs. 20 and 21, panel C). CD and AO (L) extracts were slightly more toxic for these cells than OA and TM (Fig. 20, panel C), though this was not apparent from the time-dependent graphs (Fig. 21, panel C).
Figure 21: Comparative effect of different plant extracts on normal and cancerous human cell lines with time. 
A) MCF-10A, B) MCF-7, C) MDA-MB231, and D) HeLa cell lines using the MTT assay in a time-dependent manner. * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** p<0.01; *** = p<0.001). This figure shows replottting of data shown in Figs. 15-19.

Finally, the cervical cancer cell line HeLa was found to be most sensitive to the anti-proliferative effects of CD and AO (L) followed by TM, similar to the normal and cancerous MCF-7 mammary cells; however, it was barely sensitive to the cytotoxic effects of OA (Fig. 20, panel D). AO (S) was the most effective at killing HeLa cells among the four cell lines tested, though only to a limited extent by about 20-25% (Fig. 20, panel D). It thus could be termed as an extract that affects HeLa cells primarily compared to the mammary gland cell lines tested, but does not seem to have the level of cytotoxicity observed with the other extracts.

The variable effects of the tested extracts on the four cell lines suggests that each plant extract has a different set of bioactive components in different
concentrations, resulting in variable effects on the cell lines tested, making them a valuable source of biomolecules as anticancer compounds.

3.10 Effect of Medicinal Plant Extracts on Cell Morphology

We also studied the ability of the extracts to induce morphological changes in the breast cancer cells, MCF-7 compared to untreated cells. Differences in cell morphology were observed by light microscopy after 29 hours of treatment, as shown in Figure 22. The changes observed included cell rounding, shrinkage, and detachment from other cells as well as the plate. Interestingly, each extract showed different types of morphological changes. Some of these changes are reminiscent of apoptotic cell death, which was absent in untreated cells, suggesting that MCF-7 may partly be dying by the process of apoptosis.

Figure 22: All effective plant extracts could induce cytopathic effects in MCF-7 breast cancer cell line. Photomicrographs of MCF-7 cells treated with 250 µg/ml of the effective extracts including CD (Cleome droserifolia), TM (Teucrium muscatensis), OA (Orchaderus arabicus), AO Acredocarpus orientallis leaf (L) and stem (S), 29 hours after treatment. All panels show 400X magnification.
3.11 Effect of Medicinal Plant Extracts on Apoptosis

To explore the potential mechanism of action of the effective extracts, the ability of the various extracts to induce apoptosis was explored since apoptosis is the most widely induced type of cell death pathway observed. Towards this end, as with the *Boxwellia saera*, the ability of the effective plant extracts to affect cell viability as well as induce enzymatic activity of the caspase 3, 7, 8, and 9 enzymes, the key regulators of the process of apoptosis were determined using the Promega glo assays.

Two independent experiments were carried out, as shown in Figure 23, in which MCF-7 cells were treated with four of the effective extracts (CD, TM, OA, and AO (L)) for 48 hours. AO (S) was tested in the HeLa cells only (Fig. 23) since our data suggested that this extract was more HeLa-cell specific for inducing cell death. As can be seen from Experiment 1 (Fig. 23), CD and TM induced the most potent anti-proliferative effects in MCF-7 cells (> 80% in Experiment I and > 98% in Experiment II), and were also able to induce the highest levels of caspase 3, 7, 8 and 9 in a statistically significant manner (Fig. 23). This observation correlated well with the results of the MTT assays that were obtained earlier. All results obtained for the induction of caspases were normalized to the number of viable cells in culture (see Materials & Methods). Since MCF-7 cells do not express caspase 3 [432], any induction of caspase 3/7 in this system is most likely due to the induction of caspase 7.
Figure 23: The tested medicinal plant extracts are able to induce various caspase enzymes in MCF-7 cells. MCF-7 cells were treated with 250 μg/ml of the stated plant extracts for 48 hours and then assayed for cell viability and induction of different caspase enzyme activities (3/7, 8, and 9). CD (*Cleome drosertifolia*), TM (*Teucrium muscatensis*), OA (*Orchadenus arabicus*), AO *Acredocardus orientallus leaf* (L). * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** = p<0.01; *** = p<0.001).

OA was observed to induce high levels of cytotoxic effects as well in the MCF-7 cells (~85-95% of DMSO control); however, it was less efficient in the induction of the caspases compared to CD and TM, especially caspase 8, which was statistically not significant in Experiment II (Fig. 23). The reason for the increased cytopathicity observed by OA in the glo assays is not clear, but it was consistently observed in two independent experiments. Much lower levels of cytopathicity were observed for this extract in the MCF-7 cells by the MTT assays (Figs. 20 and 21). The fact that lower levels of caspase induction was also observed suggests that the cell viability readings may be over reported by the Promega Glo assays. This
observation is being investigated further to determine which assay is more accurate to determine cell viability.

AO (L) was less efficient in inducing cell death compared to the other extracts overall, leading to a much lower induction of caspases in the two experiments consistently. It was able to activate caspase 8 and 9 in a statistically significant manner; however, induction of caspase 3/7 was inconsistent with statistically significant levels in the first experiment and no induction in the second experiment (Fig. 23). The inconsistent results observed regarding activation of certain caspases by OA and AO (L) extracts is probably due to the low levels of caspases induced, especially by AO (L), which may be at the threshold of detection of these assays.

Overall, these results suggest that extracts CD and TM primarily contain bioactive molecules that induce apoptosis as their main mechanism of cell death, while OA and AO (L) can induce apoptosis, but probably contain other bioactive molecules that may activate other cell death pathways as their primary death-inducing mechanism.

Finally, the AO (S) extract was tested in the HeLa cells to study its mechanism of action since it could cause significant cell death only in this cervical cancer cell line and not the mammary cells. As observed in Figure 24, AO (S) was able to induce death of HeLa cells; however, it was unable to induce either caspase 3 or 7 enzymatic activities. Interestingly, it was able to activate caspases 8 and 9 in a statistically significant, but less efficiently when compared to CD or TM and more like OA and AO (L). This suggests that even though AO (S) can stimulate apoptosis, similar to AO (L) in MCF-7 cells, AO (S) is probably activating other cell death
mechanism(s) for its cytotoxic effects in HeLa cells. Once again, AO (§) was able to induce higher toxicity for HeLa cells as assessed by the glo assay compared to MTT (Figs. 20 and 21) the reason for which is not clear.

Figure 24: *Acredocarpus orientallus* stem (S) is able to induce caspase 8 and 9 in HeLa cells. HeLa cells were treated with 250 µg/ml of the effective extract for 48 hours and then assayed for cell viability and induction of different caspase enzyme activities (3/7, 8, and 9). * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** = p<0.01; *** = p<0.001). This experiment was conducted once.

### 3.12 Effect of Medicinal Plant Extracts on Key Apoptotic Proteins

To confirm whether the extracts tested in the Promega glo enzyme assays for caspase activation could indeed induce apoptosis, our next aim was to confirm the activation of caspases associated with apoptosis using western blot analyses. MCF-7 or HeLa cells were treated with 250 µg/ml of the appropriate extracts for various time points, cell lysates were prepared and tested for the expression and cleavage of various caspases and Poly ADP-ribose polymerase (PARP). PARP is one of the enzymes that is cleaved during the execution phase of apoptosis by activated caspases [433]. Thus, activation of caspases and PARP are two major hallmarks of a
cell undergoing apoptosis. Cell lysates (40 µg protein) from the treated cell cultures were separated by 10-12% SDS-PAGE, immunoblotted onto nitrocellulose membranes, and incubated with the appropriate antibodies. The western blots for each extract were conducted multiple times (2-3 times) using different time points to ensure that cleavage of the apoptotic proteins could be observed efficiently in at least one instance, if not all.

Figure 25 shows the western blot analysis of CD, TM, OA, and AO (L) extracts on MCF-7 cells 24 hours post treatment. Treated cell lysates were tested for the expression of caspase 7, 8, 9, and PARP proteins and actin was used as a loading control. As can be seen, we could not detect a cleaved band for any of the caspase proteins and in fact, the antibody reactivity for caspase 8 and 9 was very poor for these proteins from MCF-7 cells; however, diminished amounts of pro-caspases 7, 8, and 9 could be observed at 24 hours, suggesting that the cleavage reaction had taken place (Fig. 25, panels A-D). Test of the PARP protein revealed that a cleaved product could be observed for OA and CD (Fig. 25, panels A and C), while the full length PARP band intensity had slightly reduced in AO (L)-treated cultures, once again suggesting the activation of PARP (Fig. 25, panel D). The PARP band had completely disappeared in TM-treated cultures as well (Fig. 25, panel B), also suggesting PARP activation, but lack of actin band more likely suggests either protein degradation, or most likely complete cell death.
Figure 25: All effective extracts could induce cleavage of one or more proteins of the apoptotic pathway.

Test of the ability of extracts of *Orchadenum arabicus* (OA), *Cleome drosorifolia* (CD), *Teucrium muscatensis* (TM), and *Acredocarpus orientalis* (AO) leaf (L) to induce caspase-dependent apoptosis in MCF-7 cells. MCF-7 cells were treated with 250 µg/ml of the specified extracts for 24 hours. Western blots were carried out on the untreated, DMSO-treated, and extract-treated cellular lysates for the specified caspases or poly ADP ribose polymerase (PARP) proteins. Actin antibody was used as a loading control. FL PARP, full length PARP.

To verify the results, MCF-7 cells were once again treated with the four extracts, but for shorter time periods. Figure 26 shows the results for MCF-7 cells treated for < 15 mins, 12hrs, and 24 hrs with OA and CD. Both extracts were able to induce the cleavage of caspase 7 and PARP starting from around 15 minutes to 12
hours. The cleaved product decreased or disappeared by 24 hours, as had been observed earlier (Fig. 25). No such cleavage or reduced proteins were observed in either the untreated or DMSO-treated cell lysates. This observation confirms that these two plant extracts have the potential to induce apoptosis in MCF-7 cells. We also tested for the expression and cleavage of caspase 8 and 9 proteins; however, unfortunately, we were unable to detect even the uncleaved bands in the MCF-7 cell lysates with these antibodies.

![Figure 26: Orchadenum arabicus (OA) and Cleome droserifolia (CD) induce caspase-dependent apoptosis in MCF-7 cells. MCF-7 cells were treated with 250 μg/ml of the specified extract for the time frame indicated. Western blots were carried out on the untreated, DMSO-treated, and extract-treated cellular lysates for the specified caspases or poly ADB ribose polymerase (PARP) proteins. Actin antibody was used as a loading control.](image-url)

To ensure that the results we were getting were truly due to the ability of these extracts to cause apoptosis, we took one of the CD extracts (ethyl acetate extract, E) that had shown an inability to cause cell death in MCF-7 cells (Table 8) and treated the cells in a similar manner (Fig. 27). Results of the western blot
analysis of these extracts revealed a complete lack of either cleavage of the procaspases 7, 8, and 9, or PARP, or decrease in their expression in these extracts, confirming that the cleavage of the procaspases or PARP was truly due to the ability of these extracts to induce apoptosis.

Figure 27: *Cleome droserifolia* (CD) ethyl acetate extract (E) is unable to induce caspase-dependent apoptosis in MCF-7 cells. MCF-7 cells were treated with 250 µg/ml of CD E for the time frame indicated. Western blots were carried out on the untreated, DMSO-treated, and extract-treated cellular lysates for the specified caspases or poly ADB ribose polymerase (PARP) proteins. Actin antibody was used as a loading control.

Since the test of the TM extract suggested complete degradation of cellular proteins, we tested the TM extract using the early time points as shown in Figures 26 and 27; however, we observed similar results with complete degradation of the cell lysates (data not shown). Therefore, we changed our strategy to determine whether
TM could induce cleavage of procaspases and PARP. Figure 28 reveals the results of the test of TM extract on MCF-7 cells. In the new design, different concentrations of the extract were tested at one time; thus a dose-responsive approach was used rather than a time-dependent approach. As can be seen in Figure 28, using this methodology, we were able to successfully detect the expression and cleavage of caspase 7 and PARP proteins, confirming that TM extract has the potential to induce apoptosis in MCF-7 cells via a caspase-dependent mechanism.

Figure 28: *Teucrium muscatensis* (TM) induces caspase-dependent apoptosis in MCF-7 cells.

MCF-7 cells were treated with various concentrations of the extract for 24 hours. Western blots were carried out on the untreated, DMSO-treated, and extract-treated cellular lysates for the specified caspases or poly ADP ribose polymerase (PARP) proteins. FL PARP, full length PARP. Actin antibody was used as a loading control.

Finally, Figure 28 shows the results of the test of *Acacocarpus orientalis* (AO) stem (S) and leaf (L) extracts on HeLa cells. As can be seen, both extracts
were able to induce cleavage of PARP in a time-dependent manner. However, we could not observe cleavage of caspase 7, but only a time-dependent disappearance of the full length band (Fig. 28). Based on the previous results obtained, this can be considered as an indication of activation of the caspases. In the HeLa cell lysates, the pro-caspase 8 band was weakly detectable, similar to how it was being observed in the MCF-7 cells; however, pro-caspase 9 could be detected much more strongly (Fig. 29). Yet, the cleavage of neither pro-caspase 8 or 9 could be clearly observed and only a decrease in the pro-caspase band with time could be detected. Together with a clear cleavage of PARP, we take these observations as suggestive of the induction of apoptosis in these cell lysates.

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<th>Untreated</th>
<th>DMSO-treated</th>
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<tr>
<td></td>
<td></td>
<td>&lt;15 mins 12 hrs 24 hrs</td>
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<td>AO (S)</td>
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<td>AO (L)</td>
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Figure 29: Acredocarpus orientalis (AO) stem (S) and leaf (L) extracts induce caspase-dependent apoptosis in HeLa cells. HeLa cells were treated with 250 µg/ml of the specified extract for the time frame indicated. Western blots were carried out on the untreated, DMSO-treated, and extract-treated cellular lysates for the specified caspases or poly ADB ribose.
polymerase (PARP) proteins. FL PARP, full length PARP. Actin antibody was used as a loading control.

Medicinal plants and their metabolites have an important role in cancer treatment [462] and their synthetic derivatives could play an important role in preventing, slowing, or reversing cancer development [463, 464]. In the last part of this thesis, we screened two purified compounds isolated from the organic extracts as well as their synthetic derivatives provided by our collaborators to determine their anticancer activity.

3.13 Description of Purified Compounds and their Derivatives for their Anti-proliferation Activity against Cancer Cells

Table 9 lists the description and characteristics of each compound. As can be seen, the two purified compounds included FTZ from Acridocarpus orientalis (AO) and IM-60 from Teucrium mascatensis (TM). Screening of the stem (S) and leaf (L) extracts from AO had revealed divergent effects on normal and cancer cell lines. While AO (L) was observed to be cytotoxic for all four cell lines tested, the AO (S) extract was found to have mitogenic activity for the normal MCF-10A cells, minor to none for the MCF-7 and MDA-MB-231 cells, and intermediate cytotoxic effects for HeLa cervical cancer cells (Figs. 21 and 22). The TM extract was similar to AO (L) and observed to be cytotoxic for all four cell lines (Figs. 21 and 22). In addition to the purified compounds, three synthetic derivatives of FTZ were also obtained as shown in Table 9: F-OTF-1, F-OTF-SS1, and FTZ-SM3 (Table 9).
Table 9: List of compounds from the plant extracts and their derivatives

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<tr>
<th>Name</th>
<th>Source (Plant or Synthetic)</th>
<th>Molecular Formula (MW)</th>
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<tbody>
<tr>
<td>FTZ</td>
<td>Isolated from <em>Acridocarpus orientalis</em></td>
<td>C$<em>{21}$H$</em>{20}$O$_{12}$ (464.376)</td>
</tr>
<tr>
<td>F-OTF-1</td>
<td>Synthetic derivative of FTZ</td>
<td>C$<em>{20}$H$</em>{15}$F$<em>{17}$O$</em>{17}$S$_{5}$ (962.550)</td>
</tr>
<tr>
<td>F-OTF-SS$_{1}$</td>
<td>Synthetic derivative of FTZ</td>
<td>C$<em>{26}$H$</em>{12}$F$<em>{12}$O$</em>{15}$S$_{4}$ (920.609)</td>
</tr>
<tr>
<td>FTZ-SM3</td>
<td>Synthetic derivative of FTZ</td>
<td>C$<em>{50}$H$</em>{40}$O$_{7}$ (752.848)</td>
</tr>
<tr>
<td>IM-60</td>
<td>Isolated from <em>Teucrium muscatensis</em></td>
<td>C$<em>{15}$H$</em>{26}$O (222.366)</td>
</tr>
</tbody>
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The anti-proliferative potential of the listed compounds was tested by performing the MTT assays, as described earlier, using the MCF-7 breast cancer cell line. Figure 30 reveals the results of IM-60 and FTZ. As can be seen, IM-60, initially showed a spike in cell proliferation, but this was followed by > 90% cell death at the highest concentration tested (1.7 mM), compared to the DMSO-treated cells at all the three time points tested (24, 48, and 72 hours; Fig. 30). On the other hand, FTZ showed a statistically significant proliferative effect on MCF-7 cells throughout the concentrations tested, similar to what has been observed in its mother extract AO (S) (Figs. 21, 22 compared to Fig. 30).
Figure 30: IM-60 induces cytotoxic effects in MCF-7 cells. The MTT assay was used to test different concentrations of purified compounds A and B) FTZ and C & D) IM-60 on MCF-7 at 24, 48, and 72 hours post-treatment. Analysis of the effect on cell viability of the purified compounds in a dose-dependent manner at 72 hours post-treatment. * indicates statistically significant differences between the control and treated samples (\(* = p<0.05; \,** = p<0.01; \,** *= p<0.001\)). These compounds were tested 2 times in the MTT assay.
Next we tested the ability of the various molecular derivatives of FTZ to induce cell death in MCF-7 cells. As can be seen in Figure 31, none of the three derivatives of FTZ, F-OTF-1, F-OTF-SS1, TZ-SM3, showed any cytotoxic effect on MCF-7 cell proliferation, and if at all, like the original compound FTZ, demonstrated a slight proliferative effect at the tested doses (Fig. 31).

Figure 31: FTZ derivatives do not affect viability of MCF-7 cells. Effect of different concentrations of FTZ derivatives on MCF-7 cells at 24, 48, and 72 hours post treatment. using the MTT assay. A) F-OTF-1. B) F-OTF SS1. C) TZ-SM3. D) Comparative analysis of the three FTZ derivatives on MCF-7 cell viability at 72 hours post treatment. * indicates statistically significant differences between the control and treated samples (\( * = p<0.05; ** p<0.01; *** = p<0.001 \)). The derivatives were tested only once in the MTT assay.
The cytotoxic effects of IM-60 observed in Figure 30 were at only one concentration which was also the highest dose tested, suggesting toxic effects of the compound rather than a specific mechanistic effect on cell proliferation. To determine whether a more dose-dependent response could be observed, the MTT assay was repeated using different dilutions closer to the effective dose and an earlier time point. Figure 32 shows the results of the MTT assay on MCF-7 cells 6 and 24 hours post treatment. As can be seen, a more effective dose response could be observed of the IM-60 cell killing. The compound was quite effective, killing > 90% of the MCF-7 cells by six hours post treatment at concentrations starting with 425 µM.

Figure 32: IM-60 is highly efficient in killing MCF-7 cells in a dose-dependent manner within six hours of treatment.

Test of IM-60 using MTT assay on MCF-7 A) 6 and B) 24 hours post treatment. C) Dose-dependent effect of IM-60 on cell viability as a percentage of untreated cells. * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** p<0.01; *** = p<0.001). IM-60 was tested twice in the MTT assay.
3.14 Effect of IM-60 on Cell Morphology

Next, we analyzed the morphological changes being induced by IM-60 on MCF-7 cells compared to DMSO-treated and untreated cells (Fig. 33). Differences in cell morphology could be observed by light microscopy as early as 3 hours post treatment. The changes noticed included cell shrinkage and detachment of the cells from other cells. These changes suggest apoptotic cell death could be a possible mechanism used by this purified compound which was isolated from TM. The crude extract of TM itself was observed to induce these changes as well in MCF-7 cells (Fig. 22).

Figure 33: IM-60 induces cytopathic effects in MCF-7 cells within 3 hours post treatment. Photomicrographs of MCF-7 cells treated with IM-60 compound at 0.425 mM 3 hours post treatment (Magnification 400X).
3.15 IM-60 can Activate Caspases

To confirm whether IM-60 had the potential to induce apoptosis, the Promega Cell Titer and caspase glo assays were used to study its ability to activate caspase enzymes, as shown previously, on MCF-7 cells using 425 µM concentration for six hours. Figure 34 reveals the effects of IM-60 on cell viability as well as the activation of caspase enzymes. As can be seen, > 90% effect on cell viability was observed in a statistically significant manner (p value < 0.002), confirming the cytotoxic effects of IM-60 on MCF-7 observed by the MTT assay earlier on (Figs. 30 and 32). Among the four caspase enzymes tested (3/7, 8 and 9), no significant activation of the initiator caspases 8 or 9 was observed (Fig. 34).

However, a statistically significant activation of caspase 3/7 was observed (p value <0.03). This data suggests that IM-60 has the ability to induce caspase-3/7 enzymes, thereby leading to caspase-dependent apoptosis in MCF-7 cells. Studies are in progress to confirm these results and study the mechanism of action of this compound further.
Figure 34: IM-60 purified compound is able to induce caspase 3/7 in MCF-7 cells. MCF-7 cells were treated with 425 μM of the compound for 6 hours and then assayed for cell viability and induction of different caspase enzyme activities (3/7, 8, and 9). * indicates statistically significant differences between the control and treated samples (* = p < 0.05; ** = p < 0.01; *** = p < 0.001). This experiment was conducted once.
Chapter 4: Discussion and Future Directions

4.1 Discussion

The aim of this thesis was to characterize the anticancer potential of select medicinal plants from the Arabian Peninsula. Towards this end, 32 organic extracts were obtained from five medicinal plants from the University of Nizwa, Oman along with one essential oil that has already been shown to have anticancer potential (Table 1). Specifically, a breast (MCF-7) and cervical cancer cell line (HeLa) were used for the initial screening. The extracts were tested for their anticancer potential by first conducting cell proliferation assay, MTT, to determine whether anti-proliferation activity could be observed (Table 8). This was followed by confirming this activity in a select group of extracts chosen from each plant in a wider panel of breast (MCF-10A, MCF-7, and MDA-MB-231) and cervical (HeLa) cell lines (Figs. 15-22), where MCF-10A normal breast cell line served as a control to determine the effect of the extracts on normal cells. Once confirmed, enzyme activity and western blot assays were conducted to determine the apoptosis-inducing potential of the short-listed extracts from each plant, revealing that apoptosis could be induced by them in a caspase-dependent manner (Figs. 23-29). However, it was apparent that in some extracts, the primary cell death mechanism may be other than apoptosis, such as necrosis, necroptosis, autophagy, or a combination thereof (Figs. 23-24).

Furthermore, a few isolated compounds and derivatives were obtained from these extracts from our collaborators and tested for their anti-proliferation effect on the MCF-7 cell line (Table 7). One of these isolated compounds was found to be effective in inducing inhibition of MCF-7 cell proliferation (Figs. 30 and 32), and activation of caspases (Fig. 34), suggesting that it has specific anticancer activity that uses a caspase-dependent pathway of apoptotic cell death (Fig. 31). Thus, this work
has resulted in the characterization of the anticancer potential of five indigenous medicinal plants and led to the identification of a possible novel isolated compound with good apoptotic activity against breast cancer cells. Together, this work reveals that medicinal plants provide a valuable source for the search of new anticancer agents since we were able to observe such activity in all the five plants tested in this study.

An additional outcome of this study was that it shed light on the effectiveness of various organic solvents in successfully extracting the anticancer activity within the plant extracts. Briefly, the 32 plant extracts tested used five different organic solvents, including hexane, chloroform, ethyl acetate, butanol, and methanol, that were used in a sequential manner [425]. The aim of this type of exhaustive sequential extraction was to separate the active constituents of plants in a selective manner based on polarity (charge). The products of these extractions consist of complex mixtures of metabolites such as alkaloids, glycosides, terpenoids, flavonoids, lignans, etc. [429, 434]. Thus, this process allows one to start with the crude plant parts and finally to get the desired portion of various metabolites, while excluding the unwanted material when treated with selective solvents.

The quality of the extract is influenced by many variables, such as the origin of the plant and the season of harvest, the plant part used, the solvent, and the extraction procedure used [435]. Additionally, other variables affecting the quantity and type of secondary metabolites of the extract depend upon the temperature of extraction, the nature, concentration, type and polarity of solvent [435]. Furthermore, the choice of the solvent has a direct effect on the quantity of phytochemicals of the extract, the rate of extraction, variety of compounds extracted.
ease of the handling procedure of the extracts, toxicity of the solvent for cells, and the health hazard associated with the extraction [436].

During the extraction, the solvents are diffused into the solid plant part and the active biological components solubilized through similar polarity. There is a strong relationship between the biological activity observed and the solvent used in the extraction process, resulting in the extraction of different potential biological activities with different polarities [437]. Table 10 shows the type of biologically active molecules that can be preferentially extracted using different types of solvents. As can be seen, methanol seems to have the most versatile ability to extract different types of biological molecules, while chloroform and acetone seem the most limited. Different solvents can extract the same biological molecules as well. For example, terpenoids can be extracted by nearly all the solvents listed in Table 10 other than acetone, while alkaloids can be extracted only by ethanol and ether, and polypeptides are water soluble only due to their polar nature.

Table 10: Solvents used for active component extraction [438]

<table>
<thead>
<tr>
<th>Water</th>
<th>Ethanol</th>
<th>Methanol</th>
<th>Chloroform</th>
<th>Ether</th>
<th>Acetone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthocyanins</td>
<td>Tannins</td>
<td>Anthocyanins</td>
<td>Terpenoids</td>
<td>Alkaloids</td>
<td>Phenol</td>
</tr>
<tr>
<td>Starches</td>
<td>Polyphenols</td>
<td>Terpenoids</td>
<td>Flavonoids</td>
<td>Terpenoids</td>
<td>Flavonols</td>
</tr>
<tr>
<td>Tannins</td>
<td>Polyacetylenes</td>
<td>Saponins</td>
<td></td>
<td>Coumarins</td>
<td></td>
</tr>
<tr>
<td>Saponins</td>
<td>Flavonol</td>
<td>Tannins</td>
<td></td>
<td>Fatty acids</td>
<td></td>
</tr>
<tr>
<td>Terpenoids</td>
<td>Terpenoids</td>
<td>Xanthoxylines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polypeptides</td>
<td>Sterols</td>
<td>Totarol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lectins</td>
<td>Alkaloids</td>
<td>Quassinoids</td>
<td>Lactones</td>
<td>Flavones</td>
<td>Phenones</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Polyphenols</td>
<td></td>
</tr>
</tbody>
</table>
Interestingly, we observed that chloroform was the most effective solvent for the isolation of anti-proliferation activity for cancer cells among the five organic solvents tested. Figure 35 plots the number of different types of extracts tested and whether they could induce anti-proliferation activity in the MTT assay against cancer cells. As can be seen, 100% of the chloroform extracts were effective for inducing cell death in either MCF-7 or HeLa cancer cell line, and this activity was not due to any residual chloroform remaining in the effective extracts (Fig. 14 and NMR data not shown). Ethyl acetate and methanol were the next best solvents, followed by hexane, and butanol. The aqueous content remaining, on the other hand, was devoid of any anti-proliferation activity, suggesting that the biochemical molecules that induce anti-proliferation are primarily non-polar (hydrophobic) in nature. Chloroform has been reported to be effective in the extraction of anticancer activity of several medicinal and other plants, such as *Nepeta deflersiana* [439], *Solanum nigrum* [440], *Angelica archangelica* [435]. As can be seen from Table 10, it primarily purifies terpenoids and flavonoids, compounds that have been shown to have anticancer activity in cell lines [441]. This suggests that some of the compounds in our extracts may be either terpenoids and/or flavonoids. Thus, the nature of the solvent is critical for the successful extraction of anticancer activity from plants and chloroform seems the best among the ones tested in our study.
Figure 35: Chloroform is the best solvent for the successful extraction of anti-proliferation activity from plant extracts. The graph shows the percent of extracts that were effective in inducing cell death in MCF-7 or HeLa cell lines based on the solvents that were used for their extraction.

4.1.1 *Boswellia sacra* essential oil and extracts

*Boswellia sacra* essential oil is known for its medicinal properties locally and its anticancer activity has been tested by other research groups [265, 328, 329]. We confirmed these observations and have shown that the anti-proliferative effect of *Boswellia sacra* essential oil was the highest on MDA-MB-231 cell line compared to the other breast cell line tested, MCF-7, the cervical cancer cell line HeLa, or even the normal breast cells, MCF-10A. This observation suggests that *Boswellia sacra* essential oil may be a good candidate for the treatment of triple negative types of cancers that are much harder to treat than the hormone responsive breast cancers due to their resistance to the treatment options. Thus, cancer cell sensitivity to the essential oil opens up new strategies (such as combined therapy with classical
chemotherapeutics drugs) to reduce their side effects for treating cancer, even the types where their normal cells are sensitive to the essential oil.

In addition to the distilled essential oil of *Boswellia sacra* resin, we also tested a number of extracts (both organic and aqueous) of the *Boswellia sacra* resin and showed that they could also induce death of cancer cells like the essential oil (Figs. 10 and 11), except for the aqueous extract that showed no such activity. This observation suggests that the biomolecules responsible for the anticancer activity of these extracts are hydrophobic in nature and can be successfully extracted using either chloroform, methanol, or hexane solvents. Furthermore, the chloroform and methanol extracts induced nearly identical cell death profiles in a dose-dependent manner in the two cell lines; however, MCF-7 cells were more sensitive to the hexane extract than HeLa cells as observed by the reduction in cell death with increasing dilutions of the extract in HeLa but not MCF-7 cells (Fig. 10). This observation suggests that the constituents of the hexane extract are different from those of the chloroform and methanol extracts which seem to be more similar. The residual aqueous extract, on the other hand, led to a proliferation of both cancer cell lines in a significant manner, suggesting that most of the anti-proliferation activity from the resin had been successfully removed by the sequential extraction procedure.

4.1.2 Anticancer activity of *Cleome droserifolia*, *Teucrium muscatensis*, *Orchadenus arabicus*, and *Acredocarpus orientalis* crude organic extracts

We also screened 28 organic extracts from four regional medicinal plants, including *Cleome droserifolia*, *Teucrium muscatensis*, *Orchadenus arabicus*, and *Acredocarpus orientalis*. We observed *Cleome droserifolia* to be the most cytotoxic of the four plants tested in the four cell lines tested, with similar cytotoxic activities for both normal and cancer cell lines (Figs. 20 and 21).
As mentioned in the Introduction, Cleome drosérfolía has anti-diabetic, anti-pyretic, analgesic, antimicrobial, liver protective, anti-rheumatic fever, and anti-inflammatory properties, and has been shown to have high levels of antioxidants and many phytochemicals (see Introduction for references). In terms of anticancer effects, Cleome gynandra has been observed to have therapeutic effects in the Swiss albino mouse model of Ehrlich’s ascites carcinoma as well [361, 362]. An extensive literature search while writing this thesis found one study from Egypt (published in a German journal) that has shown that aqueous and ethanolic extracts of Cleome drosérfolía variety from Egypt and its four subfractions (hexane, chloroform, ethyl acetate, and butanol fractions) could induce significant cytotoxic effects on MCF-7 and HCT116, a human colon carcinoma cell line [442]. They isolated six terpenoids and two flavonol glycosides from the more active chloroform and ethyl acetate fractions and observed some of these isolated compounds to have cytotoxic effects similar to those observed with the anticancer drug, doxorubicin [442]. Similarly, in another recent study, the triterpene from Cleome arabica has been shown to have cytotoxic effects against the mouse leukemia cell line, P388 [443]. These studies confirm our findings that Cleome drosérfolía has strong anticancer activity. Even though as a crude extract, Cleome drosérfolía has cytotoxic effects against normal cells, one may be able to isolate bioactive molecules that may have differential ability to kill normal versus cancer cells, making this medicinal plant valuable for the exploration of novel anticancer agents.

Test of Teucríum muscatensis extracts overall showed a more moderate anti-proliferative activity against cancerous than normal cells of the four plants tested (Fig. 10). Similar to Cleome drosérfolía, the genus Teucríum has been shown to have anti-bacterial, anti-inflammatory, analgesic, anti-pyretic, hypolipidemic, anti-
diabetic, and many other activities [419]. Not much is known about the anticancer potential of *Teucrium muscatensis*. Most of the scientific studies on this plant have been done on *Teucrium polium*. In one early study from 2008, *Teucrium polium* has been shown to be an effective and safe chemosensitizing agent for cancer therapy, as demonstrated by potentiating the anti-proliferation and apoptotic potential of several chemotherapeutic drugs [444]. It may have anticancer properties due to the presence of flavonoids and diterpenoids [445-446]. A recent study using decoctions of *Teucrium polium* in rats has shown significant anticancer activity in rats against hepatocellular carcinomas [447]. *Teucrium* species secondary metabolites have toxicity against cancer [420-422].

Interestingly, the one effective pure compound identified in this thesis, IM-60, was isolated from *Teucrium muscatensis* crude extracts. It was not only able to induce anticancer cell proliferation (Figs. 30 and 32), but also activate caspase 7 activity in MCF-7 cells, suggesting that it has the potential to cause caspase-dependent cell death (Fig. 34). Further tests of IM-60 revealed that it could induce rapid cell death (within 3 hours) with morphological changes reminiscent of apoptosis as observed by microscopy at a concentration of 425 μM (Fig. 33). We would like to investigate IM-60 further once more product has been made available to us.

Like *Teucrium muscatensis*, the *Ochradenus arabicus* extracts were observed to have a more moderate anti-proliferation activity on breast cancer cells (Figs. 20 and 21). However, HeLa cells were quite resistant with only ~20% of the cells sensitive to this plant (Figs. 20 and 21). A recent paper from our collaborators has analyzed the types of biological activities present in *Ochradenus arabicus* extracts
and were shown have a rich source of many different types of bioactivities, including antibacterial and antifungal activities [448]. These extracts included methanol, hexane, ethyl acetate, chloroform, butanol, and water. They were further analyzed for their anticancer activities by another laboratory in Korea on several different cell lines, including human colon adenocarcinoma cell lines HT29 and HCT116, human liver cancer cell line HepG2, and MCF-7. In these analyses, only the ethyl acetate, butanol, and aqueous extracts were observed to cause suppression of the growth of these cancer cell lines, except HepG2 cells [448]. Interestingly, we got very different results where we observed anti-proliferation activity primarily with the chloroform extract in both MCF-7 and HeLa cell lines, and some activity against HeLa cells with the ethyl acetate extract. In contrast to the anti-proliferation activity observed in the butanol and aqueous extracts, we did not observe cell death with the butanol or aqueous extracts in our cell line system (Table 8). In fact none of the aqueous extracts tested by us, including those from *Boswellia sacra*, showed any cytotoxic effects (Table 8).

A closer look at the data presented in the paper suggests that proper controls were not included in the analysis to compare the rate of proliferation of the treated cells with cells when exposed to the test extracts [448]. For example, in our case, all treated samples were compared to the cells treated with the same amount of DMSO to ensure that the cytotoxic effect of the solvent used to solubilize the extracts was taken into account. In the published study, no information is provided about how the extracts were solubilized before addition to the cells. Furthermore, no dose-response is observed in the treated cells whether 1 μg or 100 μg/ml of the extract is used (Fig. 36). In fact, based on the data provided, not much cytotoxicity is observed in any of the cell lines since the cell viability rates were well above 80% for all the treated
cells (Fig. 36). Finally, no details are given as to how the percent of viable cells were calculated. Thus, based on these observations, we believe that their conclusions regarding these extracts may not be reflective of what the data suggests.

Figure 36: Reproduction of data claiming to show induction of cell death in various human cancer cell lines using AO extracts. Anticancer activities of various concentrations of the extracts against HT29, HCT116, HepG2 and MCF-7 cell lines. The error bars shows the standard deviation of the mean values. Figure copied from reference [448].

Finally, the last plant tested was Acredocarpus orientalis for which we tested the leaf and stem extracts separately. Interestingly, most of the cytotoxic activity of the plant was observed in its leaf extracts (Figs. 20, 21, and Table 8). In fact, the AO (S) extracts led to increased proliferation of the breast cancer and normal breast cells (Figs. 20 and 21, panels A-C). The AO (L) extracts, on the other hand, resulted in nearly 80-90% decrease in cell viability of all the four cell lines tested (Figs. 20 and 21). Of the four effective AO extracts (ET and CH for AO (L) and BT and CH for AO (S)), only one (AO (L) CH) was able to kill both MCF-7 and HeLa cell lines, while the rest showed cytotoxic effects for HeLa cells only (Table 8). This suggests
that metabolites from *Acredocarpus orientalis* are more efficient at targeting HeLa than breast cancer cells.

We further tested one isolated compound from *Acredocarpus orientalis*, FTZ, and three of its derivatives for effects on cancer cell proliferation using the MTT assay. However, FTZ and its derivatives in fact induced cell proliferation (Figs. 30 and 31). In retrospect, being an AO isolate, a plant that showed a more HeLa-specific cell death activity, it is possible that we may have missed observing its anticancer potential by using MCF-7 cells. Thus, it would be interesting to test FTZ in HeLa cells to determine if some anti-proliferative effects can be observed. Two different flavonoids have recently been isolated from *Acredocarpus orientalis* that have been tested for cytotoxic activity against three cancer cell lines HT29, HCT116, and HepG2 [449]. Though the MTT cell viability assay has been conducted by the same laboratory that tested the *Ochradenus arabicus* extracts above [448], these results are more convincing since a control is shown against which the comparison is being made. One of the flavonoids isolated, morin, seems to have a 40-55% inhibitory effect on the three cell lines mentioned, while the other compound, morin-3-O-β-D glucopyranoside, has a more drastic effect with 60-90% effect on control cell viability [449]. These compounds were isolated from the “aerial” part of the plants. Thus, it is possible that they came from the leaves and our data shows that leaves have more anticancer activity than the stems. This may also explain why we did not find FTZ or its derivatives to have any anti-proliferation activity. In fact, since they were observed to be causing “cell proliferation” just like our AO (S) extracts, they may represent isolates from the stem part of the plant.
Overall, these results suggest that *Acrocarpus orientalis* has compounds that can both inhibit and activate cancer cell proliferation, and its active anticancer activity may be more concentrated in the leaves of the plant.

**4.1.3 Assessment of apoptosis induced by the crude extracts**

Once the anti-proliferation profile of the effective extracts had been confirmed, multiple assays were used to determine whether these extracts were able to induce apoptosis. These included: 1) morphological observation of the treated cells, 2) enzymatic activity analysis of specific caspase enzymes using Promega Glo assays, and 3) analysis of the cleavage of the pro-caspase and other apoptosis-related protein PARP. The morphological analysis of the treated cells revealed that the various extracts could lead to cell shrinkage and detachment of the treated cells as well as their rounding off, which are characteristics of apoptotic cells (Figs. 11 and 22). Analysis of different caspase enzyme activities revealed that many of the caspases were significantly activated by the test extracts (Figs. 13, 23, and 24). *Boswellia sacra* essential oil had the most robust activation of the caspases (Fig. 13) compared to the other extracts (Figs. 23 and 24) with nearly a 10-20-fold higher activation than that induced by the extracts. This may reflect the concentration of the anti-apoptotic biological activity in the essential oil compared to the complex composition of the crude extracts. Additionally, BS, TM, and CD could activate all the caspases tested (3/7, 8, and 9) significantly (Figs. 13 and 23). OA showed better activation in the first experiment compared to the second, while AO (L) and AO (S) were both more limited in their activation of the caspase enzymes, suggesting that different combination of cell death activities were present in the various crude extracts and apoptosis is probably not the main cell killing mechanism being
induced, leaving the room open for the exploration of the activation of other cell death pathways by these extracts.

Next, western blotting was used to determine whether cleavage of procaspase proteins could be observed as well as PARP, which are hallmarks of apoptosis induction. Indeed, cleavage of both caspase 7 and PARP could be detected by OA, CD, and TM in MCF-7 cells in a reproducible manner (Figs. 26 and 28). However, OA (L) could only reduce the levels of procaspase 7 in MCF-7 cells, while in HeLa cells, OA (L) and OA (S) extracts showed only cleavage of PARP with a similar reduction of procaspase 7 observed in MCF-7 cells, suggesting activation of procaspase 7. Overall, these results suggest that the tested extracts have the ability to kill cancer cells by enhancing apoptosis in MCF-7 and for some, in HeLa cells.

4.2 Conclusions

In summary, our work has resulted in the characterization of the anticancer potential of one essential oil and 32 crude extracts from five medicinal plant species that have traditionally been used by the locals of the Arabian Peninsula for therapy. Many of the tested extracts were able to induce suppression of breast and cervical cancer cell lines and induce apoptosis, which is the most common cell death mechanism observed in successful anticancer drugs. Furthermore, we were able to identify one lead compound from Teucrium muscatensis, IM-60, with promising anticancer activity for breast cancer cells. Thus, active biomolecules from these plants, such as IM-60, could be a source of promising treatments for various types of cancers in near future. Overall, our results reveal that traditional medicinal plants could be an excellent source of natural anticancer agents available to fight cancer.
4.3 Future Directions

Based on the work outlined above, several lines of investigations can be carried out in the future. They range from characterizing the extracts further for their mechanism of action, to exploring the anticancer potential of IM-60 further. Some of the future experiments that can be conducted are listed below:

- Characterize IM-60 and FTZ further to determine their effect on normal cells as well as other cancer cell lines, especially the effect of FTZ on HeLa cells.

- Explore the mechanism of action of IM-60. Only caspase 7 was induced without affecting levels of caspase 8 or 9. Could it be inducing necrotosis?

- Test the derivatives of IM-60 to see if lower concentrations can be used along with lower toxicity for normal cells (if it has any toxicity for normal cells).

- Expand the cell lines to be tested with the essential oil and the effective extracts (ovarian cancer cell line, for example because of connections between breast and ovarian cancer).

- Test other purified compounds from these effective extracts for anticancer activity and mechanism of action, and compare different extracts from the same plant to determine if different mechanism of cell death can be observed that could suggest the presence of different types of bioactive molecules alters the potential of the extracts for various biochemical processes.

- Test the essential oil and select effective isolated compounds directly in animal model system such as mice to determine their efficacy and toxicity in animals.
• Explore other cell death pathways activated by the crude extracts, especially extracts which are not clearly showing apoptosis, such as autophagy

• Test the potential anticancer activity of isolated compounds from these extracts in combination with chemotherapeutics drugs being used clinically to reduce their side effects in treating cancer

• Conduct phytochemical screening of the effective extracts to determine the phytochemical constituents and antioxidant capacity within the effective extracts which is strongly associated with anticancer activity

4.4 Manuscripts in preparation

1. Induction of caspase-dependent apoptosis by *Boswellia sacra* essential oil and its organic extracts in human cancer cells.

2. *Cleome draserifolia* can induce apoptosis in a caspase-dependent manner in a human cancer cell lines.


5. Organic extracts of *Acridocarpus orientalis* can induce cell death in human breast and cervical cancer cells, but this activity lies primarily in its leaves and not stems.

6. Chloroform is a robust solvent for the extraction of anticancer activity from medicinal plants.
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Figure 1S: The proliferative capacity of MCF-7 cells. Increasing numbers of A) MCF-7 and B) HeLa cells as measured by the MTT assay. Increasing numbers of cells were plated in microtiter plates in triplicates and allowed to grow for 72 hours followed by analyzing their proliferative capacity in the MTT assay. As can be seen, a linear prolife could be observed for both cell lines between 1562 and 25,000 for MCF-7 and and between 1562 and 50,000 cells per well for HeLa cells. Based on this data, 5,000 cells per well was chosen to analyze cell viability of treated and untreated cells in the thesis.
Figure 2S: MDA-MB-231 is the most sensitive to the killing by *Boswellia sacra* essential oil. Various dilutions of the essential oil were tested using the MTT assay on A) MCF-10A, B) MCF-7, C) MDA-MB-231, and D) HeLa cells after 24 and 72 hours. Dose-dependent effect of *Boswellia sacra* on cell viability as a percentage of untreated cells for E) the 24 hour and F) 72 hour time points for all four cell lines tested. * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** p<0.01; *** = p<0.001). The significance shown in panels A-D is between the DMSO-control and essential oil-treated samples. The full figure is shown in Figure 8 of the thesis.
**Figure 3S:** Cytotoxic effects of different concentrations of *Cleome droserofolia*.

MTT assay on A) MCF-10A, B) MCF-7, C) MDA-MB-231, and D) HeLa cells after 24, 48, and 72 hours post treatment. * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** p<0.01; *** = p<0.001). The significance shown in panels A-D is between the DMSO-control and extract-treated groups. The full figure is shown in Figure 15 of the thesis.
**Figure 4S**: Cytotoxic effects of different concentrations of *Teucrium muscatensis*.

MTT assay on A) MCF-10A, B) MCF-7, C) MDA-MB-231, and D) HeLa cells after 24, 48, and 72 hours post treatment. * indicates statistically significant differences between the control and treated samples (\( * = p<0.05; ** = p<0.01; *** = p<0.001 \)). The significance shown in panels A-D is between the DMSO-control and extract-treated groups. The full figure is shown in Figure 16 of the thesis.
Figure 4S: continued
Figure 5S: Cytotoxic effects of different concentrations of *Orchadenus arabicus*. MTT assay on A) MCF-10A, B) MCF-7, C) MDA-MB-231, and D) HeLa cells after 24, 48, and 72 hours post treatment. * indicates statistically significant differences between the control and treated samples (\* = p<0.05; ** p<0.01; *** = p<0.001). The significance shown in panels A-D is between the DMSO-control and extract-treated groups. The full figure is shown in Figure 17 of the thesis.
**Orchadenus arabicus** on MDA-MB-231

Absorbance at 560nm

**Orchadenus arabicus** on HeLa

Absorbance at 560nm

Figure 5S: continued
Figure 6S: Cytotoxic effect of different concentrations of *Acredocarpus orientallus* (L.).

MTT assay on A) MCF-10A, B) MCF-7, C) MDA-MB-231, and D) HeLa cells after 24, 48, and 72 hours post treatment. * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** p<0.01; *** = p<0.001). The significance shown in panels A-D is between the DMSO-control and extract-treated groups. The full figure is shown in Figure 18 of the thesis.
Figure 6S: continued
Figure 7S: Cytotoxic effect of different concentrations of *Acredocarpus orientallus* (S).

MTT assay on A) MCF-10A, B) MCF-7, C) MDA-MB231, and D) HeLa cells after 24, 48, and 72 hours post treatment. * indicates statistically significant differences between the control and treated samples (* = p<0.05; ** p<0.01; *** = p<0.001). The significance shown in panels A-D is between the DMSO-control and extract-treated groups. The full figure is shown in Figure 19 of the thesis.
Figure 7S: continued