CANNFLAVINS A AND B EXERT ANTI-CANCER EFFECTS IN CHEMOTHERAPEUTIC RESISTANT BREAST CANCER CELL LINES

by

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Dalhousie University is located in Mi'kma'ki,
the ancestral and unceded territory of the Mi'kmaq.
We are all Treaty people

Dedication

To my family for their unconditional love and support, and for reminding me that everything will always work out the way it is supposed to.

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Abstract

1 in 8 women in Canada will be diagnosed with breast cancer in their lifetime and although survival rates have increased due to increased screening and improved therapies chemotherapeutic resistance can limit treatment options and lead to a poorer prognosis. There is a clear need for novel treatment options to help patients who have reduced survival rates due to chemotherapeutic resistance. Studies have shown many compounds found in *Cannabis sativa* can exert anti-cancer effects *in vitro and in vivo*. Interestingly little is known about cannflavin A and B, two flavonoids present in Cannabis, and their role in chemotherapeutic breast cancer. This study showed that cannflavin A and B reduced the cell viability of taxol-resistant breast cancer cell lines in a dose-dependent manner while not affecting the viability of a non-tumorigenic breast cell line. Cannflavins A and B induced apoptosis, promoted autophagy, and reduced invasiveness of the chemotherapeutic resistant breast cancer cells. When combined with the cannabinoid THC or the chemotherapeutic agent paclitaxel Cannflavin A and B produced variable responses–from antagonistic to additive, and even synergistic, depending on the concentrations used. Some combinations of cannflavins and THC or paclitaxel significantly reduced cell viability and acted synergistically. Results indicate that cannflavin A and B, two lesser characterized compounds from Cannabis, can reduce the viability of taxol-resistant breast cancer cells. These compounds can act synergistically with cannabinoids and paclitaxel. Future studies should be completed in *in vivo* models to confirm the anti-cancer effects of cannflavin A and B.

List of Abbreviations and Symbols Used

ABC	ATP-binding cassette
AC4	Adriamycin and cyclophosphamide
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATG	Autophagy related gene
ATP	Adenosine triphosphate
BCRP	Breast cancer resistance protein
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CBD	Cannabidiol
CMF	Cyclophosphamide, methotrexate, and 5-fluorouracil
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
ECS	Endocannabinoid system
ER	Estrogen receptor
FBS	Fetal bovine serum
HER2	Human epidermal growth factor receptor 2
IsoB	Isocannflavin B
kDA	Kilodaltons
MDR	Multidrug resistant

MeOH	Methanol
MMP	Matrix metalloproteinase
MRP1	Multidrug resistant protein 1
NBD	Nucleotide binding domain
NK	Natural killer
PBS	Phosphate buffered saline
P-gp	P-glycoprotein pump
PR	Progesterone receptor
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction
SEM	Standard error of the mean
Tax-AC	Anthracycline and taxane
TC4	Docetaxel and cyclophosphamide
THC	Delta-9-tetrahydrocannabinol
TME	Tumor microenvironment
TNBC	Triple-negative breast cancer
IC_{50}	Half maximal inhibitory concentration
2D	2-dimensional
3D	3-dimensional
Δ	Delta
µL	microlitre
µM	micromolar
*****	Indicates statistical significance between specified groups
%	Percent

±	Plus minus
°C	Degrees Celsius

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Ashley, thank you for you unwavering support throughout the last two years. Even though you weren't here with me physically you were always only a phone call away to listen to my concerns, give advice, and share our love of Jeopardy. Your drive and dedication to others inspires me to work harder and be the best version of myself.

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Chapter 1: Introduction

1.1 Breast Cancer

Breast cancer is currently the most commonly diagnosed cancer in women, accounting for 25% of all diagnoses (Brenner et al., 2022). Presently, 1 in 8 Canadian women will be diagnosed with breast cancer in their lifetime. Breast cancer is the cause of 14% of all cancer related deaths in Canadian women, the second leading cancer cause of death following only lung cancer. It has an overall net five-year survival rate of 89% and this rate has improved over the last 30 years due to increased mammography screening and advancements in treatment options (Canadian Cancer Statistics Advisory Committee et al., 2021). Unfortunately, the survival rate for individual patients is variable and depends on patient age, tumor grading and staging at time of diagnosis, and receptor status of the tumor. The majority of breast cancer is diagnosed in post-menopausal women however 20% of cases occur in patients under 50 years of age. These pre-menopausal cases are more aggressive and difficult to treat because they are often diagnosed at a later stage and are more likely to be triple-negative or hormone receptor negative which results in a poorer prognosis due to limited treatment options (Heer et al., 2020). Additionally, approximately 30% of breast cancers have spread to regional lymph nodes such as the axillary lymph nodes at time of diagnosis and approximately 6% are considered distantly metastatic at time of diagnosis (Waks & Winer, 2019). Metastasis renders treatment options like surgery ineffective, causing the patient to rely on systemic forms of therapy and is responsible for 90% of cancer deaths (Ganesh & Massagué, 2021). Although more treatment options exist for patients with non-metastatic breast cancer other limitations to therapy can arise. Resistance to chemotherapy, whether innate

or acquired over the course of treatment, often occurs and results in a poorer prognosis for patients due to treatment limitations (Bukowski et al., 2020). Metastasis, chemotherapeutic resistance and cellular subtypes lacking targetable markers all reduce patient prognosis and reveal the need to discover novel treatment options to be used in these cases.

Traditionally the presence and number of axillary lymph nodes metastases was the most important prognostic marker for breast cancer, in addition to tumor size and tumor grade the – later having limitations due to the lack of reproducibility (Barzaman et al., 2020). The shift to using biomarkers as a prognostic and predictive factor has occurred due to the emergence of personalized treatment options. Many multigene signature tests used clinically are expensive and not widely available in many countries; however the nuclear protein involved in cell proliferation Ki-67, the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) are inexpensive biomarkers to assess breast cancer prognosis and are commonly used (Barzaman et al., 2020; Dai et al., 2015). Breast cancer can be divided into numerous types depending on their receptor status (Figure 1.1)(Tsang & Tse, 2020). The first type is hormone positive breast cancer where the ER, PR, or both are present on the cancer cells. This type can be further divided into luminal A, luminal B, or normal-like breast cancer. Luminal A breast cancer is ER and/or PR positive, HER2 negative. and expresses low levels of the protein Ki-67 resulting in slower growing cells and a better prognosis. Luminal B breast cancer is ER and/or PR positive, may be HER2 positive and has high levels of Ki-67 resulting in a poorer prognosis (Tsang & Tse, 2020). Normal-like breast

cancer is ER and/or PR positive and negative for HER2 with low levels of ki-67 and is less prevalent than the luminal A or luminal B subtypes. Breast cancer cells expressing HER2 are classified as HER2-enriched breast cancer. This type is negative for ER and PR and the presence of HER2 helps the tumor to grow more quickly than cells without it, however this receptor can be targeted during treatment and often helps prognosis. The last breast cancer type is triple-negative breast cancer (TNBC). This type is negative for the ER, PR, and HER2 rendering targeted treatment to any of these receptors through endocrine or molecular targeted therapy ineffective. TNBC is also prone to recurrence and metastasis; this in combination with limited treatment options results in a poor prognosis for patients (Tsang & Tse, 2020). Approximately 15-20% of breast cancer diagnoses are TNBC. Metastasis of TNBC unfortunately results in a five-year survival rate of less than 30% which is far lower than the overall net five-year survival rate for breast cancer at 89% (Canadian Cancer Statistics Advisory Committee et al., 2021; Lehmann et al., 2011).

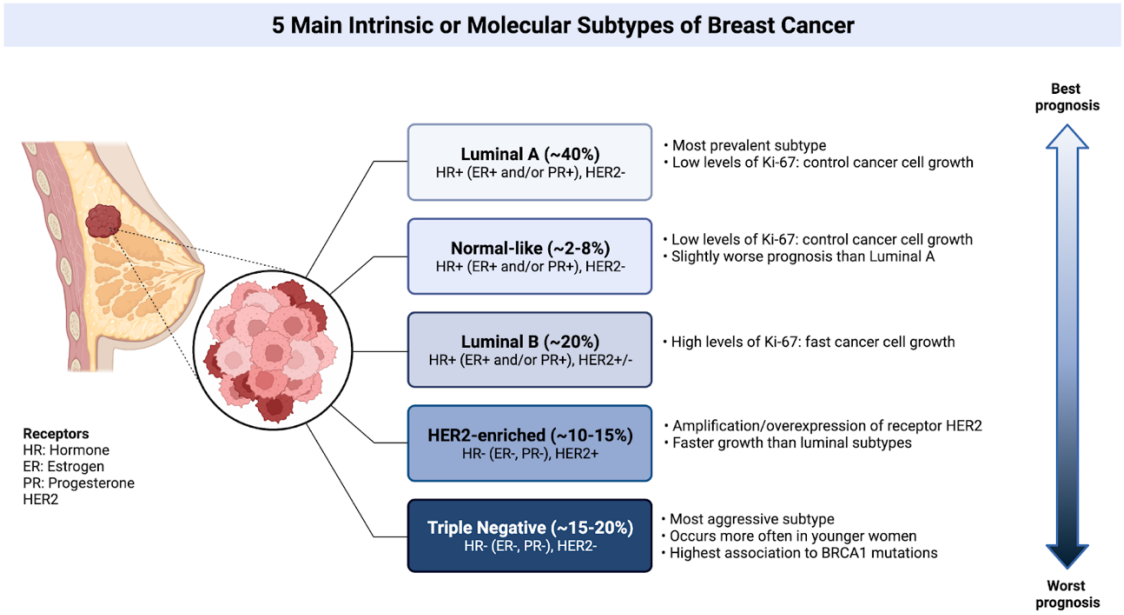

Figure 1.1 The five main intrinsic subtypes of breast cancer and their molecular phenotypes. Luminal A breast cancer has the best prognosis and triple-negative breast cancer has the worst prognosis. Luminal A is the most common breast cancer subtypes followed by luminal B, triple-negative, HER2-enriched, and normal-like. Adapted from "Intrinsic and Molecular Subtypes of Breast Cancer", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

TNBC can be further divided into six subtypes based on gene expression profiling due to its heterogeneity (Figure 1.2)(Lehmann & Pietenpol, 2014). Basal-like 1 TNBC has alterations in cell cycle and proliferation related genes and deletions of genes related to deoxyribonucleic acid (DNA) damage repair. The Basal-like 2 subtype has abnormal activation of growth factor related signaling pathways, alterations in glycolysis and gluconeogenesis, and altered expression of myoepithelial markers. Mesenchymal-like TNBC, also referred to as metaplastic breast cancer, is more likely to develop chemotherapeutic resistance. This subtype has overactive cell migration and differentiation pathways and increased growth factor signaling related pathways leading to sarcoma or squamous epithelial cell-like characteristics (Lehmann & Pietenpol, 2014). The mesenchymal stem-like subtype is similar to the mesenchymal subtype however it possesses high expression of stemness related genes and low expression of cell proliferation and differentiation related genes. The immunomodulatory subtype of TNBC has been shown to overexpress genes associated with immune cells and pathways. These upregulated immune related pathways can include T or B lymphocyte related signaling, interleukin pathways, the natural killer (NK) cell pathways among others (Lehmann & Pietenpol, 2014). The final subtype of TNBC is the luminal androgen receptor subtype. This cellular subtype is much different than the previous five subtypes and although it is negative for the hormone receptors (ER/PR) it does express the androgen receptor and has highly active hormone related pathways such as steroid synthesis and androgen and estrogen metabolism (Lehmann & Pietenpol, 2014; Yin et al., 2020). The luminal androgen receptor subtype has a better prognosis than other TNBC subtypes likely due to the ability to use targeted therapies to the androgen receptor or androgen synthesis

(Gerratana et al., 2018). Differences in breast cancer types and subtypes at the cellular level can inform physicians and patients about their prognosis, treatment courses available and their likelihood of effectiveness.

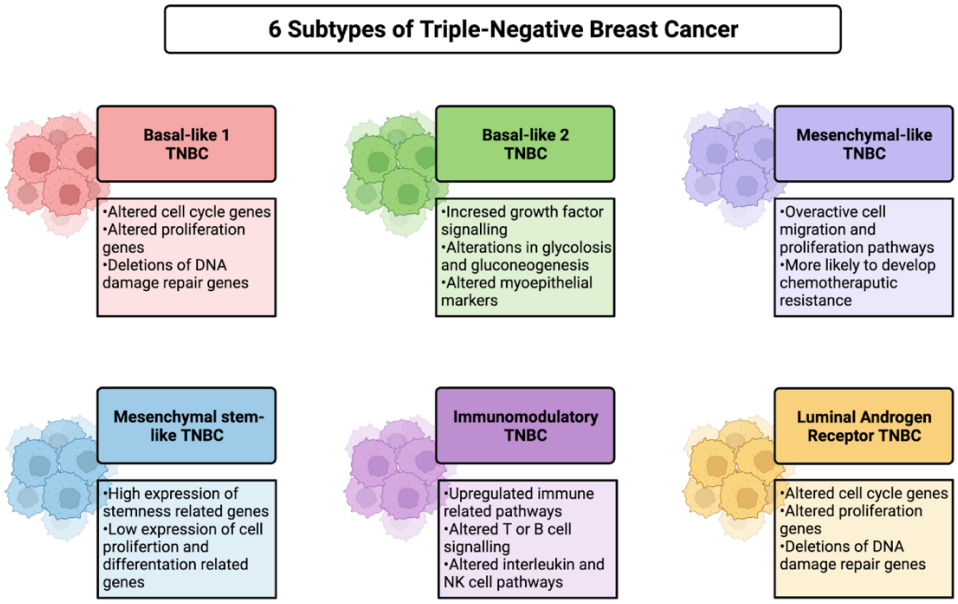

Figure 1.2 Triple-negative breast cancer subtypes. TNBC can be divided into six molecular subtypes based on gene profiling. These include basal-like 1, basal-like 2, mesenchymal-like, mesenchymal stem-like, immunomodulatory, and luminal androgen receptor subtypes. Created using BioRender.com.

1.2 Breast Cancer Therapy

1.2.1 Surgery, Radiation, Endocrine Therapy

For patients diagnosed with non-metastatic breast cancer many treatment options are available, one of which being surgery (Waks & Winer, 2019). Typically, two approaches are used surgically, either a totally mastectomy where the entire breast is removed including the breast tissue, areola, and nipple, or an excision plus radiation approach. In this second approach a lumpectomy is performed to remove only the cancer cells within the breast and a small amount of surrounding tissue to ensure a healthy margin is achieved, this is then often followed by radiation to ensure all cancer cells have been destroyed. The lumpectomy results in similar survival rates and recurrence rates to a total mastectomy (Waks & Winer, 2019). Radiation is also a viable option for patients with non-metastatic breast cancer. Radiation following lumpectomy is delivered to a portion of or the whole breast and following mastectomy it is delivered to the entire chest wall. Additional radiation may be applied to the regional lymph nodes and is associated with significantly improved disease-free survival but not overall survival and is unfortunately associated with an increase in radiation toxicities. For these reasons lymph node radiation is not universally prescribed and only considered in higher risk patients (Waks & Winer, 2019).

In addition to surgery and radiation, non-metastatic breast cancers can also be treated with systemic therapy delivered throughout the body such as chemotherapy, immunotherapy, and endocrine (hormone) therapy. Endocrine therapy is the primary systemic therapy used for endocrine receptor positive tumors (ER/PR) whether non-

metastatic or metastatic (Trayes & Cokenakes, 2021). This regimen is used to reduce estrogen-promoted tumor growth and consists of anti-estrogen medication taken for up to 5 years. Tamoxifen is a widely used endocrine therapy that binds to the estrogen receptor and inhibits its activation and binding by estrogen. It can be used in pre and post-menopausal women and can reduce the patient's relative breast cancer reoccurrence rate by 50% in the first five years compared to patients who did not receive tamoxifen (Trayes & Cokenakes, 2021). Another form of endocrine therapy is aromatase inhibitors. These compounds, such as anastrozole or exemestane, function by preventing the conversion of androgens to estrogens and therefore decrease circulating estrogen levels in the body. Aromatase inhibitors are only effective in post-menopausal women but are somewhat more effective in reducing breast cancer reoccurrence rates than tamoxifen (Joshi 2018). Although these therapies are effective, aromatase inhibitors are limited to post-menopausal patients reducing the viable options for pre-menopausal breast cancer patients who often have more aggressive tumors. Additionally, they have unpleasant side effects such as hot flashes and joint stiffness and discomfort which can reduce a patient's quality of life while undergoing treatment (Trayes & Cokenakes, 2021; Waks & Winer, 2019).

1.2.2 Immunotherapy

The role of the immune system in the development and treatment of cancers is complex. Acute inflammation occurs early in the development of mammary tumors and activates the innate immune response. There is then a shift to a chronic inflammatory state resulting in a complex tumor microenvironment (TME). This complex TME

consists of suppressive immune cells, fibroblasts, and endothelial cells and results in immune suppression within the breast TME allowing the tumor to evade the immune system and progress (Emens, 2018).

Immune checkpoints allow proteins on T-cells to bind with associated proteins on other cells such as cancer cells and relay an "off" signal to the T-cells. This prevents the immune system from killing the cancer cell. Immune checkpoint inhibitors are a type of immunotherapy used to disrupt this interaction between immune cells and cancer cells and allow the T-cell to kill the cancer cells (Barzaman et al., 2021). Immune checkpoints such as cytotoxic T-lymphocyte-associated antigen-4, programmed cell death receptor-1, programmed cell death receptor ligand-1 and their inhibitors have been used to treat breast cancer (Lipson et al., 2015). The programmed cell death receptor-1 on activated T-cells is able to interact with the programmed cell death receptor ligand-1 on the surface of tumor cells and reduce T-cell activation, dampening the immune response. Monoclonal antibodies that target programmed cell death receptor ligand-1 (avelumab and atezolizumab) and programmed cell death receptor-1 (pembrolizumab) are able to block this interaction and allow for enhanced immune priming or decreased immunosuppressive signals in the TME (Emens, 2018; Lipson et al., 2015).

Other common breast cancer therapies are immune targeting agents such as Trastuzumab and Pertuzumab that are monoclonal antibodies that bind to the HER2 receptor. These agents are key therapies for patients with HER2 positive breast cancer and are effective in prolonging survival in patients with metastatic breast cancer and

reducing recurrence in non-metastatic tumors by approximately 50% (Lipson et al., 2015). In addition to the specific monoclonal antibodies mentioned, other monoclonal antibodies have been used to target cell surface receptors leading to cancer cell death, to deliver cytotoxic compounds to cells, to activate immune cells, and to disrupt vascularization and are considered promising therapies for breast cancer treatment (Emens, 2018). Although immunotherapy shows promise in treating breast cancer including metastatic and TNBC, because of tumor heterogeneity some patients do not respond to check-point inhibitors and mutations in tumor cells can lead to monoclonal antibody resistance. This renders immunotherapy ineffective for certain patients depending on the tumor grading and cellular sub-type (Barzaman et al., 2021).

1.2.3 Chemotherapy

Chemotherapy is a form of systemic therapy used to treat metastatic and non-metastatic breast cancer and is used to prevent recurrence in patients. Despite its negative side effects and long-term risks chemotherapy remains an essential treatment option for many patients in addition to other types of therapy. Hormone receptor positive tumors can receive chemotherapy in addition to endocrine therapy and clinical benefit is determined by tumor grade, stage, and other genomic factors (Emens & Middleton, 2015). For patients with HER2 positive breast cancer chemotherapy can also be used in combination with immunomodulatory therapy and has been shown to improve immune therapy effects by altering the TME and improving efficacy of immune targeting agents (Emens & Middleton, 2015). Patients who receive surgical resection of their tumor may receive chemotherapy as a neoadjuvant (before surgery) or adjuvant (after surgery)

therapy to reduce the risk of tumor recurrence and axillary lymph node involvement (Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2011). TNBC patients typically receive chemotherapy in addition to surgery if their tumor is larger than 5 mm due to their poorer prognosis (Denkert et al., 2017). In patients with metastatic breast cancer chemotherapy is used either alone, in the case of TNBC, or in combination with aromatase inhibitors for hormone receptor positive patients, or trastuzumab in HER2 positive patients (Cardoso et al., 2009; Waks & Winer, 2019).

For patients with early stage breast cancer there are a number of chemotherapy regimens used consisting of two or more compounds. For late stage metastatic breast cancer in particular TNBC, single agent chemotherapy is still used however there is not a particular agent recommended (Waks & Winer, 2019). The rationale underlying combination therapy is that it allows clinicians to reduce the dose needed of individual agents while maintaining or increasing efficacy or the treatment regimen due to additive or synergistic effects. The reduction in dose of individual compounds achieved by combining agents allows for reduced toxicity and negative side effects experienced by patients improving their quality of life. In addition, reducing the dose of individual agents reduces the risk of the patient developing chemotherapeutic resistance rendering the chemotherapy less effective or ineffective altogether (Fisusi & Akala, 2019). Common chemotherapeutic regimens for lower risk patients have greater considerations for toxicity and include docetaxel and cyclophosphamide (TC4); Adriamycin and cyclophosphamide (AC4); and cyclophosphamide, methotrexate, and 5-fluorouracil (CMF). In high-risk patients with a higher tumor grade or stage a chemotherapy regimen containing an

anthracycline (Adriamycin) and a taxane (docetaxel/paclitaxel) (Tax-AC) remain the best choice (Blum et al., 2017; Emens, 2018; Jones et al., 2009; Martín et al., 2010).

Anthracyclines are a class of chemotherapeutic agent typically used in treating high-risk breast cancer patients which accounts for up to 32% of all breast cancer diagnoses. The most commonly used anthracycline and chemotherapeutic used to treat breast cancer is doxorubicin (brand name Adriamycin) and was introduced into chemotherapeutic regimens for the first time in the 1970s (McGowan et al., 2017). There are multiple mechanisms by which doxorubicin exerts cytotoxicity in cancer cells. The main proposed mechanisms of action include DNA intercalation; inhibition of topoisomerase II and production of reactive oxygen species resulting in DNA strand breaks; and inducing sphingolipid metabolism that compromises cellular membrane integrity. All of these mechanisms eventually lead to apoptosis and cancer cell death (McGowan et al., 2017; Nicoletto & Ofner, 2022). Unfortunately, anthracyclines like doxorubicin exhibit serious adverse effects including tissue necrosis, severe myelosuppression, and cardiotoxicity as a result of accumulation of doxorubicin in cardiomyocytes. These cardiotoxic effects may have a delayed onset and not appear for 4-20 years following end of therapy (Nicoletto & Ofner, 2022).

Another important class of chemotherapeutics for treating breast cancer are taxanes which include paclitaxel and docetaxel. Taxanes are microtubule stabilizing agents and prevent them from breaking down following cell division. By stabilizing the microtubules taxanes prevent cancer cells from dividing and resulting in cell death

through apoptosis, mitotic catastrophe or other mechanisms thus slowing the cancer growth (Willson et al., 2019). Paclitaxel has side effects including alopecia, myelosuppression, gastrointestinal symptoms and febrile neutropenia. Peripheral neuropathy is associated with paclitaxel administration and symptoms worsen as cumulative dose increases (Abu Samaan et al., 2019). Docetaxel is a second generation taxane and shares the same common side effects of paclitaxel however differs in its pharmacokinetics. Despite their differing pharmacokinetic properties both compounds improved overall survival and disease-free survival in breast cancer patients, and these were further improved when combined in anthracycline-containing regimens (Fisusi & Akala, 2019).

The negative systemic side effects associated with chemotherapy due to their non-specific action make them undesirable for patients despite their excellent cytotoxic activity in cancer cells. Patients often need to take additional drugs to overcome the negative side effects of chemotherapy and those drugs themselves can have their own unwanted side effects further reducing the patient's quality of life. The patients' response to chemotherapy can vary greatly due to inter-tumor heterogeneity (differences in tumors between patients), intra-tumor heterogeneity (differences between cells within one tumor) as well as previous exposure to chemotherapy. This can render certain chemotherapy regimens less effective, requiring over 8 cycles of chemotherapy in some patients and only 2 in others (Alfarouk et al., 2015; Lainetti et al., 2020)(Prihantono & Faruk, 2021). Ultimately some patients have innate resistance or acquire resistance to chemotherapy over time rendering their current chemotherapy regimen less effective or ineffective all

together. The negative side-effects and variable response rate associated with chemotherapy, immunotherapy, and endocrine therapy highlights the need for novel treatment options for patients to be used alone or to complement current available therapies and overcome the challenges associated with current therapeutic options.

1.3 Chemotherapeutic Resistant Breast Cancer

Due to the fact that almost all treatment regimens for breast cancer include some form of chemotherapy whether alone or in combination with other treatment options, chemotherapeutic resistance can negatively affect patient outcomes. Chemotherapeutic resistance is defined as either the innate or acquired ability of cancer cells to evade the effects of one or multiple chemotherapy drugs resulting in the low efficacy and efficiency of chemotherapy to produce a beneficial response (Alfarouk et al., 2015; Lainetti et al., 2020). Often cells will develop resistance to more than one chemotherapeutic agent resulting in multidrug resistance (MDR) and can explain why some patients do not respond to a particular therapy even though they have not previously been exposed to it. Innate chemotherapeutic resistance occurs when a patient does not respond or has a lower response than expected to a particular dose of chemotherapy regimen at the initial treatment point. Acquired chemotherapeutic resistance can either be induced during or following treatment with a chemotherapeutic regimen. In the former case, resistance to a specific dose of chemotherapy could develop during the course of treatment requiring an increased dose over the course of the treatment plan. Alternatively, in the latter case, acquired resistance to chemotherapy could present itself upon breast cancer recurrence and subsequent administration of chemotherapy (Lainetti et al., 2020).

Chemotherapeutic resistance can be a result of many different mechanisms, all of which render the compound being administered and potentially other future compounds less effective or completely ineffective. These can be the result of cell to cell interactions within the TME, tumor and cell heterogeneity, and cancer stem cells (Lainetti et al., 2020). There are many resistance mechanisms at occur at the cellular level such as changes in drug uptake and efflux, altered drug metabolism, inactivation of the drug, changes in DNA repair and apoptosis rates, and alteration of drug targets and their expression (Lainetti et al., 2020; Mansoori et al., 2017).

1.3.1 ABC Transporters

ATP-binding cassette (ABC) transporters are membrane proteins found on the plasma membrane or the membranes of vesicles within cells and hydrolyze adenosine triphosphate (ATP) to transport substrates such as chemotherapy and other xenobiotics across these membranes (Figure 1.3)(Mansoori et al., 2017). Typical ABC transporters have four domains: two transmembrane domains (TMD) containing 6 transmembrane helices each which are present in the lipid bilayer of the membrane and two ABCs or nucleotide binding domains (NBD) present in the cytoplasm (Rees et al., 2009). NBDs are highly conserved motifs across the family of transporters and bind and hydrolyze ATP to transport molecules across the membrane regardless of the concentration gradient (Rees et al., 2009). The TMDs are heterogenous allowing them to bind different substances and transport them across the membrane. Briefly, the mechanism of transport by the ABC transports is as follows. The substrate binds in the binding pocket of the

TMD, two molecules of ATP bind to the two binding sites in the NBDs, the first molecule of ATP is hydrolyzed to induce a conformational change allowing the substrate to be transported and released from the protein, finally the second molecule of ATP is hydrolyzed to induce a conformational change and reset the transport to the original state where it can bind another substrate (Mansoori et al., 2017)

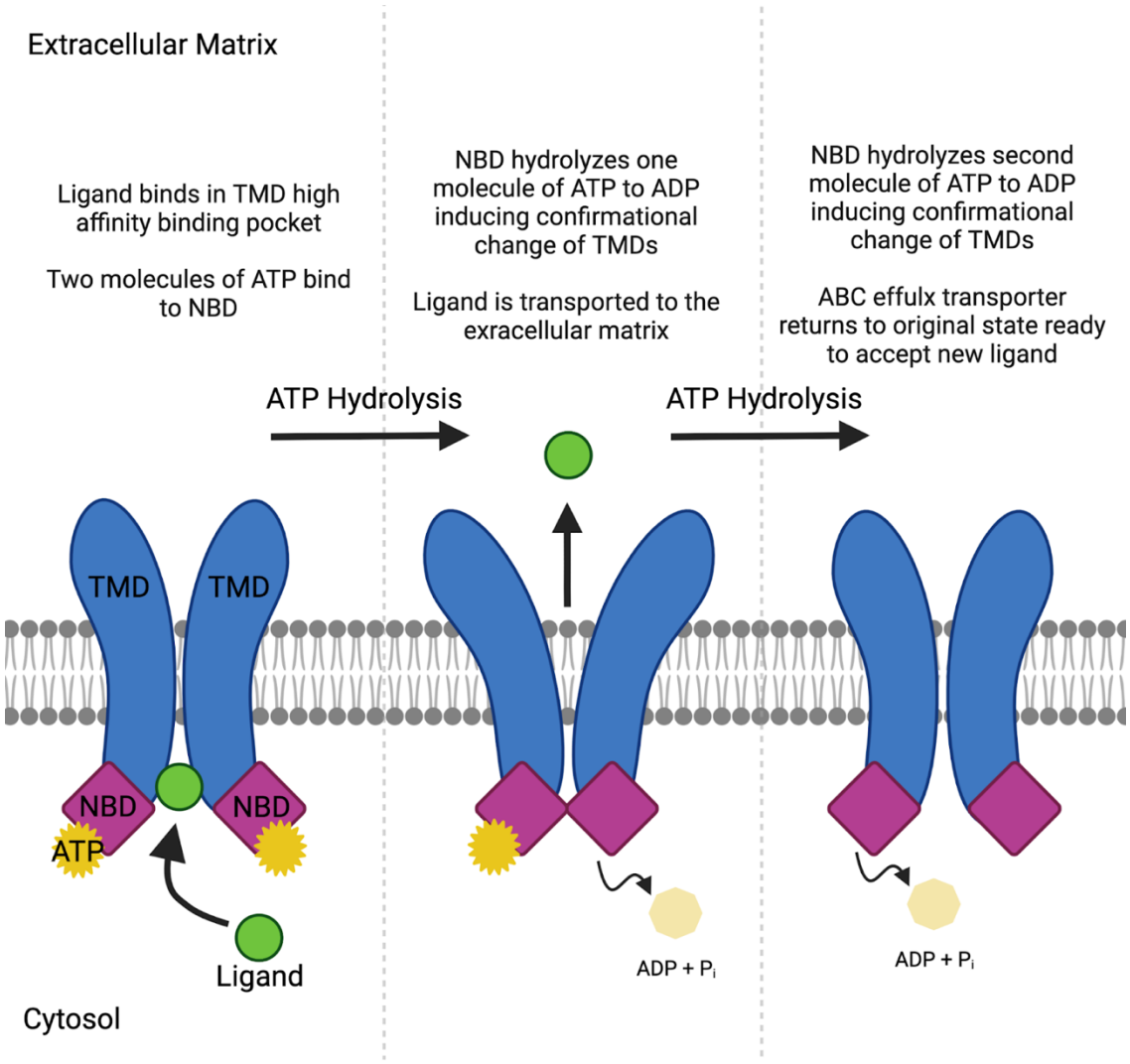

Figure 1.3 ATP binding cassette transporter efflux of ligand from the cytosol to the extracellular matrix. The ligand binds the high affinity binding pocket of the transmembrane domains along with two ATP molecules to the nucleotide binding domains. The NBD hydrolyzes one molecule of ATP inducing a conformational change in the TMDs and releasing the ligand into the extracellular space. The second molecule of ATP is hydrolyzed by the NMD to induce another conformational change in the TMDs and return them to their original state, ready to accept a new ligand. Figure created with BioRender.com

Although some transporters within the ABC family have very specific substrates, some have a wide specificity allowing them to bind to many different molecules and are particularly implicated in MDR (Robey et al., 2018). Many chemotherapeutic agents are substrates of ABC transporters and result in their efflux from the intracellular to extracellular environment. This reduces the amount of chemotherapy able to accumulate within the cancer cells and reduces their anti-cancer effects. The over expression of ABC transporters in cancer cells can be acquired over the course of treatment resulting in MDR and reduced survival for patients. Three ABC transporters have been shown to account for the majority of MDR in cancer patients: multidrug resistance protein-1 (MRP1), breast cancer resistance protein (BCRP), and p-glycoprotein pump (p-gp) (Sun et al., 2012).

MRP1 is overexpressed in many types of cancers including breast cancer and has substrate specificity for multiple types of anti-cancer agents implicating it at least partially in the development of chemotherapeutic resistance in these types of cancer. General MRP1 substrates include hydrophobic compounds, organic anion and anionic conjugates, glutathione, heavy metal oxyanions, and glutathione and glucuronate conjugates (Choi & Yu, 2014). Chemotherapeutic agents such as vinca alkaloids, camptothecins, and anthracyclines, like doxorubicin, are substrates of MPR1 but taxanes like paclitaxel are not (Sun et al., 2012). BCRP is another ABC transporter present in many different cancer types including glioblastoma, ovarian cancer, non-small cell lung cancer, and importantly here, breast cancer (Sun et al., 2012). It is present mainly in the plasma membrane of cancer cells and results in drug efflux but its presence in

cytoplasmic vesicles can also sequester drugs within the cell and prevent them from reaching intracellular targets and having an effect (Natarajan et al., 2012). The substrate specificity for BCRP is broad and includes many physiological compounds, dietary xenobiotics, and anticancer agents such as doxorubicin, 5-fluorouracil, and several tyrosine kinase inhibitors (Natarajan et al., 2012). P-gp was the first ABC transporter discovered to be responsible for chemotherapeutic resistance in cancer. It is expressed throughout the body in many different tissue types and can be found to be overexpressed in both solid and blood cancers that have developed MDR (Sun et al., 2012). Like the other ABC transporters p-gp has a wide variety of substrates including anticancer drugs such as vinca alkaloids, anthracyclines (doxorubicin), and taxanes (paclitaxel) (Choi & Yu, 2014).

Many of the chemotherapeutic agents that are substrates of ABC transporters are structurally unrelated and exert their effects through different mechanisms. The broad substrate specificity of ABC transporters provides an explanation for how MDR can develop and present in cancer patients, and why resistance to seemingly unrelated chemotherapeutic agents can occur. Inhibitors of these transporters have been discovered and many of them inhibit more than one of these transporters (Choi & Yu, 2014; Cui et al., 2015). However, problems in use including toxicity, poor solubility, alterations in pharmacokinetics and pharmacodynamics of the anti-cancer agent of choice, have limited the use of many ABC transport inhibitors to chemosensitize tumors. In cases where inhibitors have been shown to sensitize cells to previously resistant chemotherapeutics clinical trials have often failed due to tumor heterogeneity, unknown drug distribution to

the tumor site, and co-expression of ABC efflux transporters resulting in compensation by remaining transporters unaffected by the inhibitor and MDR remains (Choi & Yu, 2014). Although there are challenges in addressing MDR by inhibiting ABC transporters, novel approaches can be used to overcome the previously noted challenges including more targeted approaches for reaching these proteins (W. Li et al., 2016).

1.3.2 Cell Death Inhibition

Cell death is an important regulatory process for cells and can occur through apoptosis or autophagy. Many chemotherapeutic agents function by ultimately inducing cell death in cancer cells; however, the development of chemotherapeutic resistance can indicate the cancer cells have found a way to evade these typical cell death mechanisms (L. Chen et al., 2018). Apoptosis leads to programmed cell death resulting in the controlled removal of unwanted or malfunctioning cells and can occur through two different pathways: the caspase-dependent pathway which includes the intrinsic and extrinsic pathways and the caspase-independent pathway. The intrinsic pathway is mediated by the mitochondria and is initiated by the release of cytochrome C from the mitochondria into the cytosol triggering the activation of caspase-9 and downstream caspase signaling eventually resulting in caspase-3 activation and cell death (L. Chen et al., 2018; Housman et al., 2014). The extrinsic pathway is mediated by death-receptors on the cell surface that activate caspases and cell death protein complexes that ultimately result in the formation of a mitochondrial apoptosis-induced channel (MAC). Cytochrome C is again released and caspase signaling results in caspase-3 activation and cell death.

There are a number of pro- and anti-apoptotic proteins involved in apoptosis and their expression can determine whether a cell undergoes apoptosis. Proteins such as Bcl-2 and Bcl-XL, Akt, and Mcl-1 exert anti-apoptotic effects and inhibit apoptosis. Proteins such as Bax, Bak, Bad among others exert pro-apoptotic effects and push the cell towards the apoptosis pathways and induce cell death (Chen et al., 2018; Housman et al., 2014). Increased expression of anti-apoptotic proteins and reduced expression of pro-apoptotic proteins can be exaggerated in cancer cells resistant to chemotherapy. In these cells the chemotherapeutic agent is no longer able to induce apoptosis as the cell has altered its protein expression in response to exposure to the agent to prevent cell death from occurring. Novel compounds to induce apoptosis or compounds that evade or alter apoptosis pathways could provide a solution to patients who have chemotherapeutic resistance.

Autophagy is a cellular degradation process that under normal conditions, functions to help the cell degrade non-functional proteins or organelles maintain survival and homeostasis. Autophagy begins when an external stimulus or cellular stress triggers a membrane called a phagophore to expand and engulf intracellular components like organelles or proteins in a double membraned autophagosome. The autophagosome then fuses with the lysosome where lysosomal proteases degrade its contents. The resulting amino acids are returned to the cytoplasm where they are reused to maintain the cell (Glick et al., 2010). In cancer, autophagy has complex contrasting roles. In some instances, autophagy can be seen as pro-tumorigenic because it functions to promote cell survival in nutrient-deprived and stressful conditions, things that are beneficial to cancer

cell survival. In other cellular settings autophagy can provide an alternative cell death pathway and can be linked to apoptosis (Maiuri et al., 2007). Proteins that are the result of autophagy related genes 5, 9, and 12 (ATG5)(ATG9)(ATG12) interact with pro-apoptotic proteins and promote formation of MAC and cytochrome c release, inducing apoptosis. Additionally, the interaction between ATG12 and Bcl-2 increases caspase activation. Crosstalk between autophagy and apoptosis under normal conditions helps to maintain homeostasis however in cancer cells abnormal apoptosis-autophagy crosstalk can help cells evade apoptosis through the dysregulation of the previously mentioned interactions (L. Chen et al., 2018). Additionally, alterations in autophagy-apoptosis crosstalk in cancer cells resistant to chemotherapy along with alterations in apoptosis pathways themselves and can promote cancer cell survival rather than cell death following chemotherapy treatment. The interaction between autophagy and apoptosis is complex and context dependent and can result in pro or anti-tumorigenic effects.

By outlining a few of the cellular mechanisms related to chemotherapeutic resistance relevant to this project it is clear that chemotherapeutic resistance is a multifactorial problem. Multiple cellular transporters are responsible for resistance to a number of anticancer agents and many different molecular pathways can be altered in cells resistant to chemotherapy. The interactions between these mechanisms is complex and it is unlikely that there is one solution to overcome this problem. For these reasons combinations of therapies are thought to provide the most benefit when avoiding or overcoming chemotherapeutic resistance. Options include i) combinations of multiple chemotherapies to induce multiple different signaling pathways and hopefully evade

ABC transporters, ii) combinations of chemotherapeutic agents and inhibitors have shown limited clinical promise, and iii) the use of targeted nanomedicine is being investigated to improve chemotherapy delivery and overcome resistance mechanism (Ji et al., 2019; Waks & Winer, 2019). No single solution is available for patients who have chemotherapeutic resistance, and unfortunately, patients who have resistance have lower survival rates than other patients due to limited treatment options. For these reasons novel treatment options need to be investigated to help patients where resistance has made chemotherapy less effective or ineffective altogether.

1.4 Cannabis and Breast Cancer

Cannabis sativa is a plant comprising over 500 chemically and biologically active compounds which can be categorized into three major categories: cannabinoids, terpenes, and flavonoids (Kisková et al., 2019; A. M. Tomko et al., 2020). These compounds are able to exert a multitude of effects in nature including anti-bacterial, anti-inflammatory, anti-parasitic, anti-cancer just to name a few. Clinically, cancer patients may turn to medical cannabis to alleviate negative side effects of their current cancer treatment regimens or to be used palliatively due to the properties the compounds can provide. Cannabis has been shown to provide antiemetic effects, promote appetite, and ameliorate cancer-related pain including neuropathic pain. Additionally, cannabis has the potential to induce sleepiness and reduce anxiety and depression overcoming some problematic symptoms associated with a cancer diagnosis (Abrams & Guzman, 2015). Dronabinol, nabilone, and Sativex are synthetic cannabinoids or whole plant extracts containing Δ9-tetrahydrocannabinol (THC) and/or cannabidiol (CBD) currently approved for medical

use by Health Canada. In addition to these prescription compounds patients may also consume medical cannabis or recreational cannabis since its legalization in Canada in 2018 to alleviate their cancer related symptoms and side-effects and improve quality of life (Abrams & Guzman, 2015; Kisková et al., 2019). Unfortunately, consuming whole plant extracts or synthetic versions THC can be problematic. THC has psychoactive effects due to its ability to pass through the blood brain barrier and produce psychoactive effects that may be undesirable to perform day to day activities (Amin & Ali, 2019).

1.4.1 Individual Effects in Breast Cancer

In addition to the potential to alleviate side effects and symptoms, some compounds in cannabis have been shown to exhibit anti-cancer effects on breast cancer themselves. In some of these cases whole plant extracts have shown anti-cancer effects but, in many instances, isolated compounds other than THC have shown anticancer effects alleviating the psychoactive component from consideration. Although all compounds in cannabis that have shown anticancer effects in breast cancer will not be mentioned here, a comprehensive review has been completed by our lab (Tomko et al., 2020). Here, general categories of compounds and notable findings to date will be highlighted.

Cannabinoids are the first group of compounds within Cannabis sativa that will be discussed and comprise the common phytocannabinoids THC, CBD and the lesser known and less abundant phytocannabinoids: cannabigerol, cannabichromene, cannabinol, cannabivarin, cannabidivarin, and tetrahydrocannabivarin (Figure 1.4). In breast cancer

models, the primary phytocannabinoid THC has been shown to reduce cell growth and proliferation through the inhibition of the cell cycle and induction of apoptosis (Caffarel et al., 2010). Notably THC has been shown to inhibit p-gp and BCRP in *in vitro* breast cancer models suggesting a potential role in chemotherapeutic resistance (Tournier et al., 2010). CBD, another abundant cannabinoid, has also exhibited anti-cancer effects in *in vitro* and *in vivo* models of breast cancer by reducing proliferation, inducing apoptosis, and inhibiting the epithelial to mesenchymal transition (Elbaz et al., 2015; Shrivastava et al., 2011). CBD was also able to increase sensitivity to anticancer agents in vitro by downregulating resistance protein expression (García-Morales et al., 2020) The lesser known cannabinoids have been evaluated previously in this lab and have been shown to exert anti-proliferative and anti-invasive effects in cellular models of breast cancer including MDR breast cancer (Whynot, 2021). Currently there have not been any clinical trials conducted investigating the anti-cancer of cannabinoids in breast cancer patients, however, there are promising results in glioblastoma multiforme patients. In 2016 a two part clinical trial investigated the combination of Sativex (1:1 THC:CBD) and temozolomide and found that the combination increased the rate of 1-year survival by 39% (NCT01812603 and NCT01812616)(on behalf of the GWCA1208 study group et al., 2021).

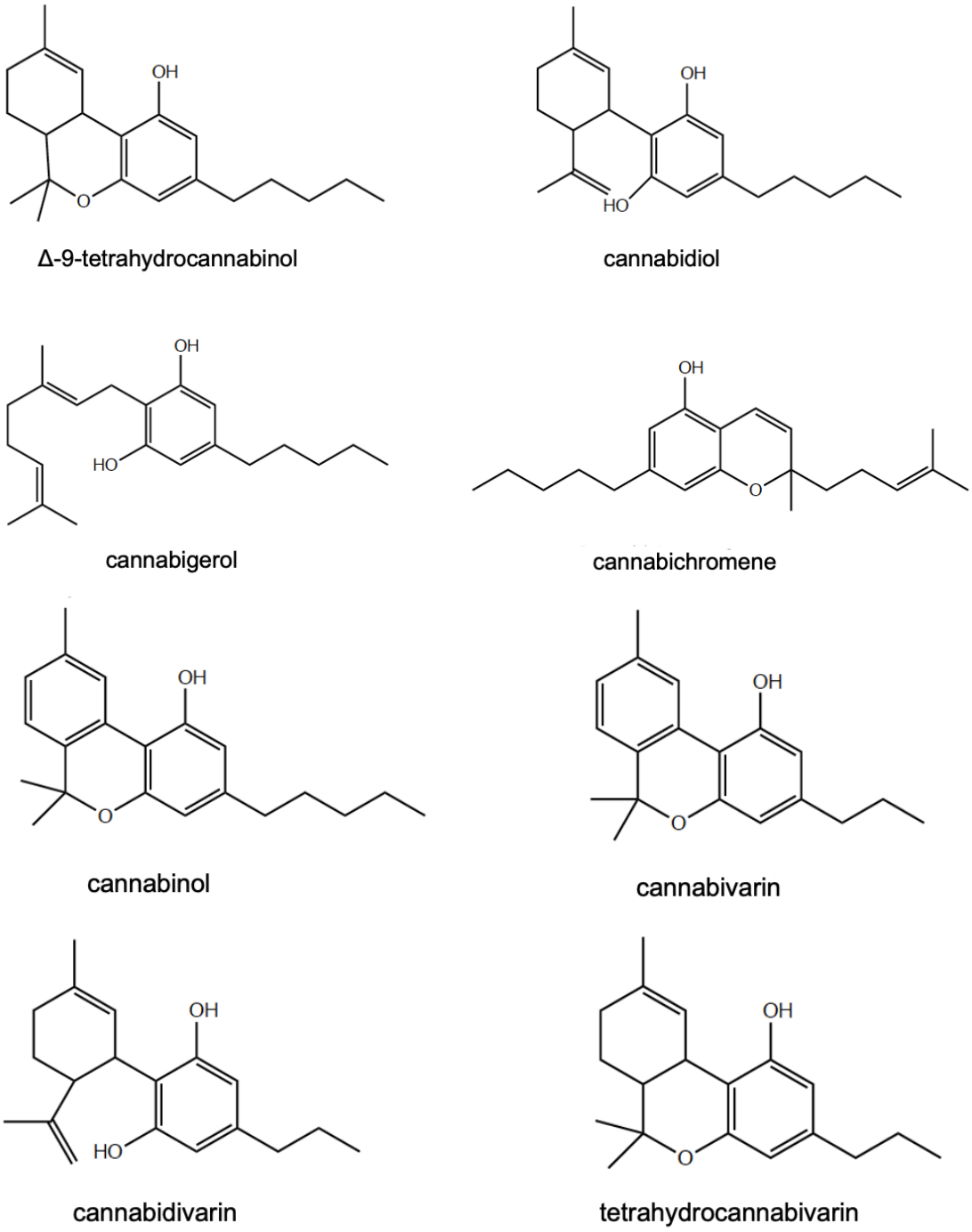

Figure 1.4 Phytocannabinoid structures. Major phytocannabinoids are Δ9-tetrahydrocannabinol and cannabidiol, minor phytocannabinoids include cannabigerol, cannabichromene, cannabinol, cannabivarin, cannabidivarin, and tetrahydrocannabivarin.

Terpenes are a group of compounds found in *Cannabis sativa* among other plants and are found at a much lower percent per weight (ranging from 1-5%) within the plant compared to cannabinoids (Lewis et al., 2018). These compounds are found in various combinations depending on the strain of cannabis and have been shown to exert many different anticancer effects (Booth & Bohlmann, 2019; Giese et al., 2015). A number of the terpenes present in cannabis are able to reduce breast cancer cell proliferation, reduce invasiveness, and induce apoptosis in *in vitro* and *in vivo* models (Hanušová et al., 2017; Miller et al., 2015; Ravizza et al., 2008). While many of these compounds may exhibit anti-tumoral effects, they will not all be discussed here as they were not the subject of this study, but are reviewed in Tomko et al 2020 and have been evaluated in previous studies in our lab (Tomko et al., 2020). In an unpublished study done by our lab we found that the terpenes nerolidol and b-caryophyllene were able to exert concentration dependant inhibitory effects of cell viability of paclitaxel resistant breast cancer cell lines. Their effects where exerted through the induction of apoptosis and the compounds reduced the invasiveness of the cell.

The last category of compounds in cannabis to be discussed and the focus of this project are flavonoids (Figure 1.5). Flavonoids account for roughly 10% of the compounds found in cannabis and can be divided into 6 subclasses: flavones, flavanols, flavanone, flavanols, isoflavones, and anthocyanidins (Wen et al., 2020, p. 202). Flavonoids are present in many plants and are responsible for the pigment in leaves in flowers, however, much of the current anti-cancer knowledge surrounding flavonoids does not focus on flavonoids specific to cannabis. Flavonoids present in cannabis that are

also present in many other plants have been studied extensively. The flavonoids kaempferol, silymarin, luteolin, and quercetin are found in cannabis and other plants and have shown anti-cancer effects in *in vitro* and *in vivo* breast cancer models (L. Chen et al., 2018; Q. Li et al., 2019; Prieto-Vila et al., 2020; Schomberg et al., 2020; Wang et al., 2019). Anti-cancer effects exhibited include anti-proliferative and anti-invasive effects, promotion of apoptosis, cell-cycle arrest among others. Flavonoids exclusive to *Cannabis sativa* will be discussed later in this chapter.

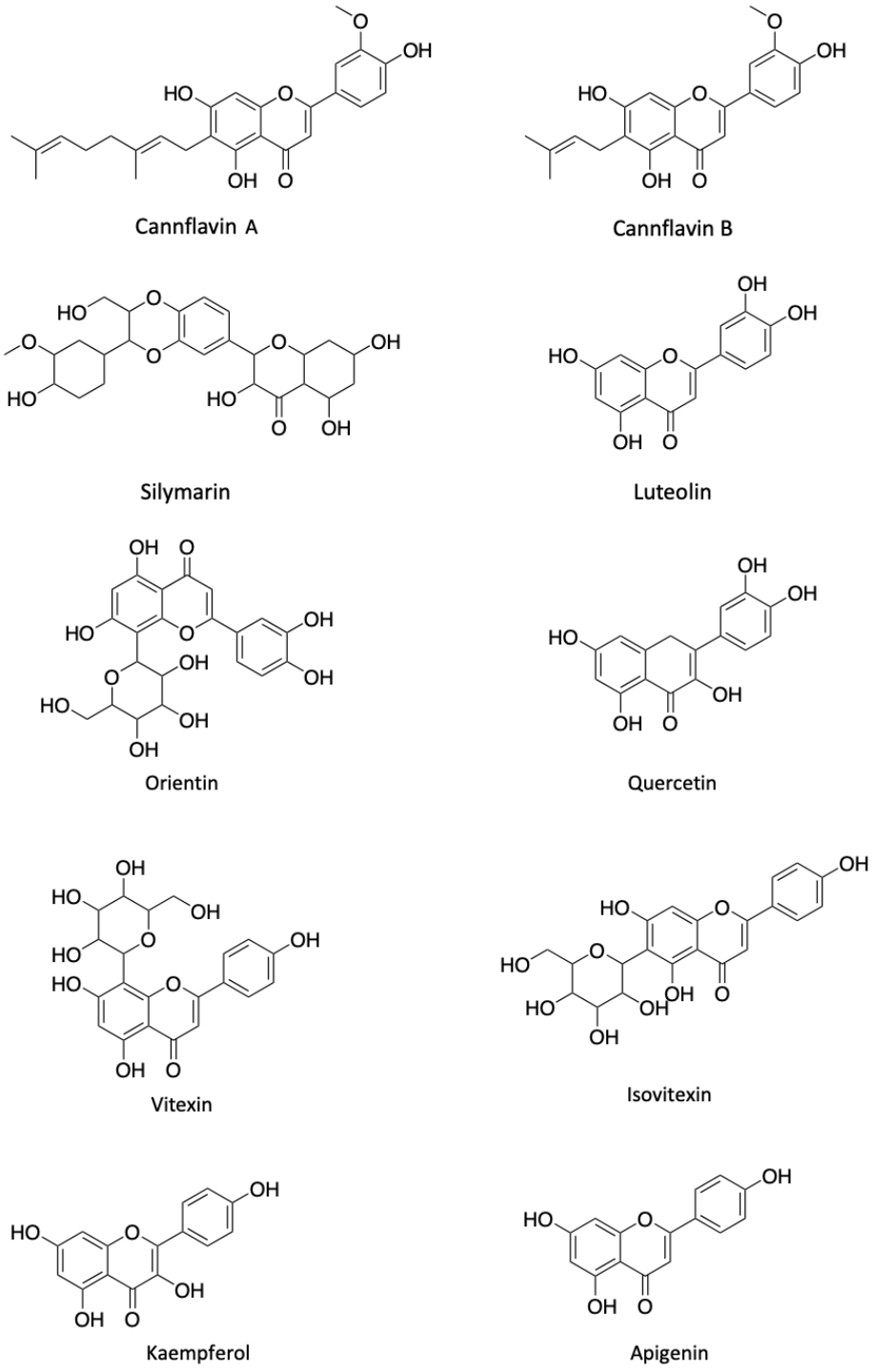

Figure 1.5 Flavonoid structures. Cannabis flavonoids investigated in this study: cannflavin A, cannflavin B, silymarin, luteolin, orientin, quercetin, vitexin, isovitexin, kaempferol, and apigenin.

1.4.2 Cannabis Compound Combinations

As mentioned previously, many isolated compounds found in cannabis have been shown to exert anti-proliferative and anti-invasive properties *in vitro* and *in vivo* as well as in models of chemotherapeutic resistant breast cancer on their own. Interestingly, botanical extracts can exert greater effects than isolated compounds alone, however, the mechanism by which this effect occurs is not well characterized or understood. A study by Blasco-Benito in breast cancer models showed that a whole botanical extract of cannabis had improved anticancer effects compared to isolated THC alone (Blasco-Benito et al., 2018). The results indicated that the improved effect was not due to the five most abundant terpenes present suggesting that other compounds, potentially other cannabinoids, terpenes, or flavonoids were contributing to this increased effect (Blasco-Benito et al., 2018). Except for the main cannabinoids present in cannabis, many of the exact mechanisms by which the compounds in the plant act, including their receptor binding and downstream signaling pathways, are unknown. Classes of compounds within the plant; cannabinoids, terpenes, and flavonoids, often share structural similarities. This could result in similar receptor binding or downstream signaling for multiple compounds explaining benefits observed when compounds are used in combination.

In addition, some common chemotherapeutic agents such as paclitaxel, which was derived from plants and has a structure from the diterpene class, share structural similarities with compounds found in cannabis (Roberts, 2007). The structural similarities between compounds found in cannabis and chemotherapeutic agents could allow these compounds to bind to similar receptors/targets or alter signaling pathways leading to

improved results when the compounds are combined. Results have shown that combinations of compounds found in cannabis with chemotherapeutic agents can restore sensitivity to chemotherapeutics in resistant models and provide additive or synergistic effects compared to either compound (Holland et al., 2008; Riahi-Chebbi et al., 2019; G.-N. Zhang et al., 2015) alone . These interactions and improved effects could have implications for patients who have chemotherapeutic resistance and need novel treatment options. As mentioned previously THC was able to inhibit p-gp and BCRP providing a potential mechanism by which the improved effects seen with combinations of cannabinoids are occurring (Tournier et al., 2010). CBD nanoparticle co-administration with paclitaxel or doxorubicin had synergistic antiproliferative activity in breast cancer cells (Fraguas-Sánchez et al., 2020). Terpenes also provided improved effects when combined with chemotherapeutic agents in breast cancer cells along with other cancer models (Ambrož et al., 2015, 2019; Di Giacomo et al., 2019; Hanušová et al., 2017; Meng et al., 2018; G.-N. Zhang et al., 2015).

Flavonoids found in cannabis also showed improved effects when combined with anticancer agents. In MDR breast cancer cells the combination of doxorubicin and quercetin reduced cell viability compared to the compounds alone and re-sensitized docetaxel resistant breast cancer cells by acting synergistically with the chemotherapeutic (S. Liu et al., 2020). In addition, quercetin acted *in vitro* and *in vivo* to inhibit BCRP in a cervical cancer model suggesting the potential mechanism behind its chemotherapeutic interactions (Song et al., 2020). In non-breast cancer models other flavonoids also acted

synergistically with chemotherapeutics or re-sensitized resistant cells to chemotherapy (J. H. Lee et al., 2020a; Q. Li et al., 2019; Zhou et al., 2020a).

1.4.3 Cannflavins

Cannflavins A, B, and C belong to the subclass of flavonoid known as flavones. The biosynthetic pathway of the flavone class of compounds has been elucidated in other plants however their synthesis in *Cannabis sativa* has only recently been proposed (Rea et al., 2019). Cannflavins A, B, and C are thought to be distinct to cannabis and have two modifications that separate them from other similar flavones. The first is prenylation at the 6' ring of the flavone A-ring which increases lipophilicity of the compounds and improves bioavailability. The second being methoxylation at the 3' position of the flavone B ring which further increases the lipophilicity of the compounds. The order in which these modifications occur in the synthesis pathway is unknown. Rea et al. have proposed a synthesis pathway for cannflavins A and B based on the known flavone pathway, phylogenetic, and biochemical approaches that is presented here (Figure 1.6).

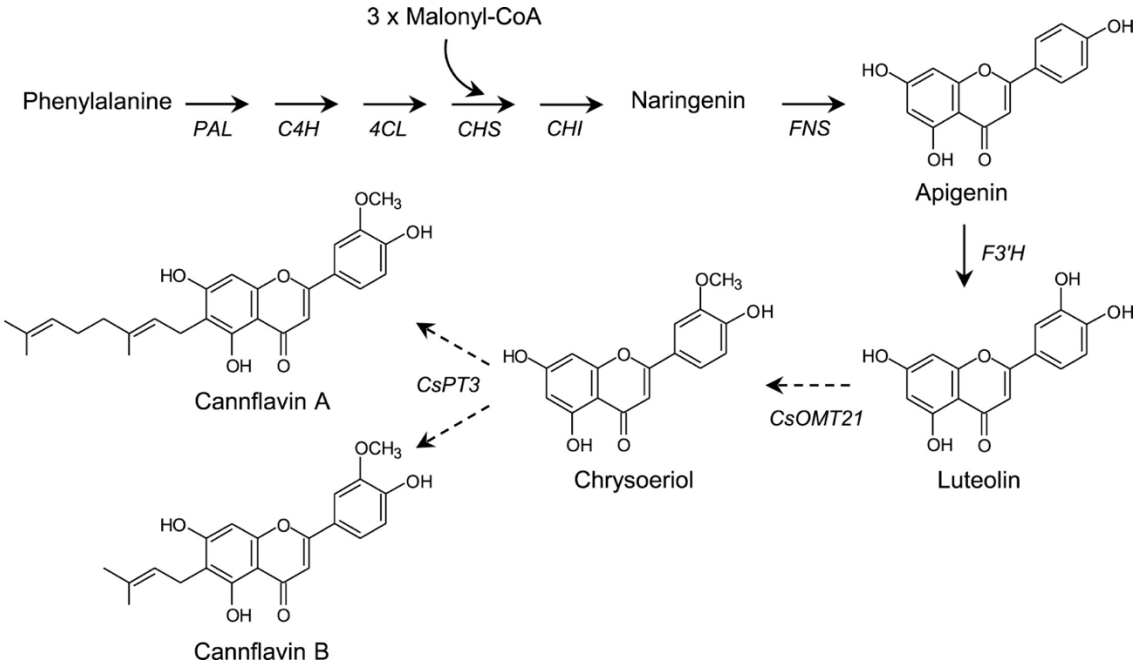

Figure 1.6 Proposed biosynthesis of cannflavins A and B. Solid arrows represent established flavonoid biosynthesis pathways steps. Dashed arrows represent proposed cannflavin biosynthesis steps by Rea et al. (2021). Luteolin is converted to chrysoeriol through a methylation reaction by C. *sativa* O-methyltransferase 21 (CsOMT21). Chrysoeriol is then prenylated by C. *sativa* prenyltransferase 3 to produce cannflavin A and cannflavin B. Utilized with permission from Elsiver: Phytochemistry (Rea et al., 2019), Copyright© 2021: https://creativecommons.org/licenses/by/4.0/.

As of now little is known about the actions of Cannflavins A and B despite their isolation in 1980 (Erridge et al., 2020). Their known molecular targets include 5-lipoxygenase and microsomal prostaglandin E2 synthase where they act as an inhibitor and have been shown to have anti-inflammatory properties through COX-1 and COX-2 inhibition (Barrett et al., 1985; Werz et al., 2014). In neuronal PC-12 cells cannflavin A induced proliferative effects in concentrations ranging from 1-10 μM but neurotoxic effects in concentrations ranging from 10-100 μM (Eggers et al., 2019). Conflicting evidence about the antioxidant properties of cannflavin A have been shown using a 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay (Radwan et al., 2008; Werz et al., 2014). Cannflavin A has also been shown to have anti-parasitic properties against *Leishmania donovani* and had high docking energies when screened as an antiviral agent against zika virus, human immunodeficiency virus and dengue virus (Byler et al., 2016; Erridge et al., 2020).

Only two studies have been done to date involving cannflavins and cancer. ER positive T47-D and ER negative MDA-MB-231 breast cancer cells were treated with concentrations ranging from 1-100 μM of isocannflavin B (isoB), an unnatural regioisomer of cannflavin B. Results showed that in ER positive and negative cells isoB had caused cell growth arrest at concentrations greater than 1 μM and toxicity at concentrations greater than 25 μM (Brunelli et al., 2009). At concentrations greater than 25 μM isoB reduced Akt phosphorylation and inhibited cyclin dependent kinase inhibitor 1. Results showed that isoB could induce autophagic cell toxicity in the ER positive T47-D cells but not the ER negative MDA-MB-231 cells suggesting a role of the ER in its

effects (Brunelli et al., 2009). The second study involving cancer also used the compound isoB (named FBL-0G3 in this study) and investigated its role in *in vitro* and *in vivo* models of metastatic pancreatic cancer. FBL-0G3 reduced survival of two pancreatic cancer cell lines when combined with a radiation dose. *In vivo* when delivered with a smart radiotherapy biomaterial isocannflavin B significantly reduced local pancreatic tumor size when co-administered with radiotherapy and without co-administration however significant overall increases in surinvivovival were only seen with the combination therapy (Moreau et al., 2019). FBL-0G3 was granted orphan drug status by the United States Food and Drug Administration for the treatment of pancreatic cancer under the name Caflanone in 2019 (U.S. Food & Drug Administration, n.d.). Clinical trials with the drug have yet to be initiated and it is not currently approved by the Food and Drug Administration for use. These studies show promise for the use of cannflavins in cancer treatment however further investigation is required to understand their anticancer potential, and interaction with chemotherapeutics and other compounds found within cannabis.

1.5 The Endocannabinoid System and Cancer

In humans the endocannabinoid system (ECS) is comprised of the cannabinoid-1 (CB1) and cannabinoid-2 (CB2) receptors, endogenous ligands anandamide and 2-arachidonoylglycerol, and enzymes responsible for their production, metabolism and transport (Braile et al., 2021). The dysregulation of the ECS has been reportedly associated with cancer and endocannabinoid modulation has been linked to cancer aggressiveness (Braile et al., 2021). The immune system and the ECS have an intertwined

relationship. Almost all immune cells interact with the ECS in some way either through receptor expression and activation or cellular mediators and it has been suggested that the ECS is the gatekeeper and regulator of the immune system (Braile et al., 2021).

The TME contains a variety of immune cells responsible for producing cellular mediators that impact the proliferation, angiogenesis, and metastasis of cancer cells. Because both CB1 and CB2 receptors are expressed on the surface of immune cells and may be expressed on cancer cells, the ECS and its interaction with the immune system could play an important role in tumor progression and provide a therapeutic target for novel treatment options (Braile et al., 2021). Cannabinoids have been shown to modulate the function of T cells, macrophages, monocytes, NK cells, dendritic cells, mast cells, neutrophils, and eosinophils. Importantly for cancer, cannabinoid receptor activation on macrophages inhibits angiogenic and lymphangiogenic factor release inhibiting cancer cell growth and metastasis. This suggests that not only can compounds found in cannabis directly exert cytotoxic effects, but they can also modulate the TME through their interaction with the ECS and reduce proliferative and invasive properties of the tumor indirectly (Braile et al., 2021).

1.6 Rationale, Objectives, Hypothesis

Although breast cancer survival rates have improved over the last 30 years due to the emergence of more targeted therapies and increased surveillance measures, treatment options can be limited for certain patients which negatively impacts their prognosis (Heer et al., 2020; Lehmann & Pietenpol, 2014). Patients who have metastatic or TNBC –

forms of breast cancer that can be the most aggressive – often rely on chemotherapy as one of the only available treatment options. Chemotherapy acts systemically and is associated with negative side effects such as hair loss, nausea, weight loss, and neuropathy due to its effects on non-target tissues (Abu Samaan et al., 2019). These side effects negatively impact the patient's quality of life in addition to the symptoms associated with the cancer itself. When chemotherapeutic resistance occurs in these patients they are left with a worse prognosis and even more limited treatment options. Novel treatment options need to be investigated in order to improve prognosis and survival in patient populations with chemotherapeutic resistance with limited available treatment options. These novel options could also benefit all patient options undergoing chemotherapy as they could have the ability to reduce the required dose of chemotherapy if combined with another substance. Combinations of compounds with chemotherapy could allow the same anticancer effect to be achieved while lowering the dose of chemotherapy required and minimizing adverse side effects (Fisusi & Akala, 2019).

Compounds found in cannabis including cannabinoids, terpenes, and flavonoids have been shown to exert anti-proliferative and anti-invasive properties in preclinical breast cancer models including in some models resistant to chemotherapy (A. M. Tomko et al., 2020). Some of these compounds including flavonoids found in cannabis, among other plants, have demonstrated the ability to act synergistically with chemotherapy or resensitize resistant cells to chemotherapy (A. M. Tomko et al., 2020). Although the effects of many compounds found in cannabis have been investigated, little is known about the anticancer effects of cannflavins specific to cannabis: cannflavin A and

cannflavin B. The objective of the project is to investigate and characterize the anti-cancer effects of cannflavins A and B in *in vitro* chemotherapeutic resistant breast cancer cells. This objective can be broken down into the following aims:

1. Determine the anti-cancer effects of individual flavonoids in *in vitro* models focusing on their anti-proliferative and anti-invasive effects and their role in inducing apoptosis and autophagy.
2. Determine the interaction (additive, synergistic, antagonistic) between flavonoids and the cannabinoids THC and CBD.
3. Determine the interaction (additive, synergistic, antagonistic) between flavonoids and paclitaxel.

It was hypothesized that cannflavins A and B would exert anti-proliferative and anti-invasive effects in chemotherapeutic resistant breast cancer cells. These effects were hypothesized to be increased when combined with paclitaxel and THC or CBD. This project aims to uncover the potential of cannflavins to exert anticancer effects in chemotherapeutic resistant breast cancer models and reveal if their combination with other compounds should be further investigated for the development of new therapeutic strategies.

Chapter 2: Materials and Methods

2.1 Flavonoids, Cannabinoids, and Anti-Cancer Agents

Paclitaxel was obtained from Millipore-Sigma. Δ-9-tetrahydrocannabinol was purchased from Cayman Chemical and cannabidiol was purchased from Toronto Research Chemical. Flavonoids were purchased from a mixture of Millipore-Sigma and Cayman Chemical. Flavonoids and paclitaxel were dissolved in dimethyl sulfoxide (DMSO), cannabinoids were dissolved in methanol (MeOH)

2.2 Cell lines

Paclitaxel resistant (PR) MDA-MB-231 cells were derived from sensitive MDA-MB-231 cells and were obtained from Drs. Kerry Goralski, David Hoskin, and Anna Greenshields (Dalhousie University). Paclitaxel resistant (PR) MCF-7 cells were generated by serial passage of MCF-7 cells with increasing concentrations of paclitaxel up to 470 nM and were provided by Dra. Robbery Robey and Susan Bates (National Cancer Institute, Bethesda, MD). MCF-10A cells and appropriate medium were obtained from Dr. Yassine El Hiani (Dalhousie University).

2.3 Cell Culture

PR Human Breast Adenocarcinoma MDA-MB-231 and MCF-7 cells (470 nM) were cultured in Dulbecco's Modified Eagle's Medium-high glucose (DMEM: Sigma-Aldrich) with 1% penicillin-streptomycin containing 10% fetal bovine serum (FBS: Gibco, Life Technologies) and 470 nM paclitaxel to maintain resistance. Non-tumorigenic breast epithelial MCF-10A cells were cultured in DMEM/Ham's Nutrient

Mixture F-12 (Gibco, Life Technologies) containing 5% horse serum, 0.5mg/ml hydrocortisone, 1 ng/mL cholera toxin, 10 μg/mL epidermal growth factor and 10 μg/mL insulin. All cells were incubated at 37°C in 5% CO_2.

2.4 Cytotoxicity Assays

PR MDA-MB-231 and PR MCF-7 cells were seeded in black 96-well plates (ThermoFisher Scientific) at a density of 10,000 cells/well and allowed to grow overnight. Cells were treated with DMEM containing 1% FBS or DMEM/F12 complete with the following flavonoids found in cannabis: Cannflavin A, Cannflavin B, Silymarin, Luteolin, Orientin, Quercetin, Vitexin, Isovitexin, Kaempferol, Apigenin. Treatments of increasing concentrations were done alone or in combination with 470 nM paclitaxel for 24 hours. AlamarBlue® (Bio-Rad Laboratories) was added to each well equal to 10% of the total volume per well and plates were incubated for 3 hours. Fluorescence was measured using a Biotek Cytation 3 plate reader at 560 nm excitation and 590 nm emission as a measurement of cell viability. Cell viability was calculated as a percent relative to the vehicle control normalized to 100% and presented as mean ± standard error of the mean (SEM). A minimum of 3 independent trials performed in quintuplicate were conducted.

2.5 Apoptosis Assay

PR MDA-MB-231 and PR MCF-7 cells were seeded at 3,000 cells/well in DMEM with 10% FBS in clear 96 well plates and allowed to grow overnight. Cells were treated with 2.5 μM of cannflavin A, cannflavin B, or vehicle (DMSO) in DMEM with

1% FBS for 24 hours. An Annexin V apoptosis detection kit (Santa Cruz Biotechnology) was used to detect apoptosis. Cells were resuspended in annexin V assay buffer and incubated in the dark with propidium iodide (PI) and annexin V–fluorescein isothiocyanate–conjugated stain for 20 minutes. Cells were observed by fluorescence microscopy, and a minimum of five fields of view were counted manually using an Olympus IX81 microscope with a Photometrics coolSNAP HQ2 camera and an Excite series 120Q light source. The annexin V stain was excited at 488 nm and imaged at 525 nm. PI was excited at 535 nm and imaged at 617 nm. Rates of apoptosis were calculated by dividing the number of cells that stained positive for annexin V or PI divided by the total number of cells.

2.6 Cell Lysis and Western Blotting

PR MDA-MB-231 and PR MCF-7 cells were seeded at 100,000 cells/well in 6-well plates (Corning) and allowed to grow overnight. Cells were treated with 2.5 μM cannflavin A, cannflavin B, or vehicle (DMSO) in DMEM with 1% FBS for 24 hours. Following treatment cells were detached with phosphate buffered saline (PBS), pelleted by centrifugation, and lysed using 150 μl RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and Roche's complete™ EDTA-free protease inhibitor cocktail). Cells were lysed further by subjecting samples to 15 seconds of sonication. Bovine serum albumin–coated Protein A-Sepharose beads and 10% DNase I were added to remove nucleic acid and organellar material from the sample. Lysates were mixed 50:50 with Laemmli buffer (BioRad Laboratories) containing 5% 2-mercaptoethanol. Samples were run on a sodium dodecyl

sulfate–polyacrylamide electrophoresis gel, transferred to a nitrocellulose membrane and blocked in a PBS solution containing 10% skim milk powder for 60 minutes. Primary antibodies (anti-alpha-tubulin [2125s], anti-Bax [2772S], anti-Bcl-2 [4223S](Cell Signaling Technology), were added to the milk solution (1:1000) and incubated overnight at 4°. Membranes were washed with TBS-Tween and incubated in anti-rabbit [7074s] secondary antibodies (Cell Signaling Technology) for 1 hour (1:1000). Membranes were washed again with TBS-Tween to remove any unbound antibody. Chemiluminescence was performed using Western Lightning® Plus-ECL Enhanced Chemiluminescence Substrate (PerkinElmer) and then membranes were developed for 1-10 minutes using a BioRad Chemidoc imaging system.

2.7 Autophagy Inhibitor Assay

PR MDA-MB-231 and PR MCF-7 cells were seeded at 10,000 cells/well in black 96-well plates and allowed to grow overnight. Cells were treated with 2.5 μM cannflavin A, cannflavin B, or vehicle (DMSO) in DMEM and 1% FBS with or without inhibitors of autophagy (1 mM 3-methyladenine, 100nM Bafilomycin A or the combination of 10 μg/ml E-64d and 10 μg/ml pepstatin A) for 6 hours. AlamarBlue® was added to each well equal to 10% of the total volume per well and incubated for 3 hours. Fluorescence was measured using a Biotek Cytation 3 plate reader at 560 nm excitation and 590 nm emission as a measurement of cell viability. Cell viability was calculated as a percent relative to the vehicle control normalized to 100% and presented as mean ± SEM. A minimum of 3 independent trials performed in quintuplicate were conducted.

2.8 Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

PR MDA-MB-231 cells were seeded at a density of 100,000 cells/well in 6-well plates. Cells were treated with 2.5 μM of cannflavin A, cannflavin B, or vehicle (DMSO) in DMEM with 1% FBS for 6 hours or 24 hours. Total RNA was extracted using the Aurum Total RNA kit (Bio-Rad Laboratories) following the spin protocol as per the manufacturer's instructions. Following elution, the concentration of RNA was estimated spectrophotometrically on the SPECTROstar Nano spectrophotometer (BMG Labtech, Mandel Scientific Company). Reverse transcription was performed with the iScript cDNA synthesis kit (Bio-Rad Laboratories) using 1 μg of RNA as template for each sample. Real-Time qPCR was performed with the SsoFast EvaGreen Supermix kit (Bio-Rad Laboratories) with GAPDH as the reference gene (Table 1). The individual genes were optimized for both annealing temperature and conditions. PCR cycling conditions were: (95˚C for 30 sec) + (95˚C x 5 sec + 60˚C x 5 sec + fluorescence read) x 40 cycles + melt curve analysis. The melting curve program was a 2 sec hold time with plate readings for every 0.5˚C increase from 65˚C to 95˚C. PCR was performed with the Bio-Rad CFX96 real-time system C1000 touch thermal cycler (Bio-Rad Laboratories). Data was analyzed using the CFX Maestro software (Bio-Rad Laboratories) using the Cq method. Statistical comparisons were performed using the average value of triplicate technical replicates for all experiments. Four independent samples were analyzed for each condition for each gene of interest. Target genes quantified in RT-qPCR include ATG5, ATG7, ATG12, and MAP1LC3b2 (LC3b). PrimeTime® qPCR primers were used for all genes (Integrated DNA Technologies). The primer sequences are shown in Table 1.

Table 2.1 Primer sequences of target genes in RT-qPCR. Primers are presented in the 5' to 3' direction.

Target Genes	Forward	Reverse
GAPDH	ACATCGCTCAGACACCATG	TGTAGTTGAGGTCAATGAAGGG
ATG5	TGTCCTTCTGCTATTGATCCTG	CAGATGTTCACTCAGCCACT
ATG7	TCCAAGGTCAAAGGACGAAG	GGAAACTGCTACTCCATCTGT
ATG12	GCGAACACGAACCATCCAA	CACTGCCAAAACACTCATAGAG
LC3b	ACCTCTCGGGAGTGCAG	GCATGGTGTGGGGATCTG

2.8 Matrigel Invasion Assay

Growth factor reduced 8.0-µm Matrigel Invasion Chambers (Corning), and Cell Culture Inserts with an 8.0-µm membrane pore (Corning) were added to a 24-well plate. Matrigel Invasion Chambers were hydrated with 250 µL of DMEM containing 0.2% FBS, and 5 µM of cannflavin A, cannflavin B, or vehicle (DMSO) and incubated for 1 hour at 37°C. Following incubation, 700 µL of DMEM containing 10% FBS was added to the lower chamber of Matrigel Invasion Chambers and Cell Culture Inserts and 250 µl of DMEM containing 0.2% FBS DMSO was added to the upper chamber of cell culture inserts. Two hundred and fifty microliters of PR MDA-MB-231 cells at a concentration of 100,000 cells/mL were added to each cell culture insert and Matrigel invasion chamber resulting in a final cell concentration of 25,000 cells/well and final drug concentration of 2.5 µM. Cells were incubated for 24 hours. After incubation, cells that did not invade through the Matrigel or migrate were removed from the inside of the insert using a cotton swab dampened with PBS. Wells were fixed in methanol for 10 minutes and then stained with 3.5 g/L crystal violet in 2% ethanol solution for 10 minutes. Following staining, wells were rinsed with H_2O and left to dry overnight. Cells that migrated or invaded through the membranes or matrigel were counted using an Olympus CKX41 light microscope. The number of cells invaded for each condition were represented as a percentage relative to the number of cells migrated when exposed to the vehicle control.

2.9 Assessment of synergism, additivity or antagonism

Synergies between cannflavin A, cannflavin B, and paclitaxel were studied using a checkerboard assay in PR MDA-MB-231 and MR MCF-7 cells. Synergy was also

assessed between cannflavin A, cannflavin B and THC or CBD. PR MDA-MB-231 cells were seeded at a density of 10,000 cells/well in black 96-well plates and allowed to grow overnight. Cells were treated with combinations of compounds in DMEM with 1% FBS for 24 hours. Drug concentrations ranged from 0-10 μM for the cannabinoids, 0-100 μM for the cannflavins, and 0-60 μM for paclitaxel. AlamarBlue® was added to each well equal to 10% of the total volume per well and incubated for 3 hours. Fluorescence was measured using a Biotek Cytation 3 plate reader at 560 nm excitation and 590 nm emission as a measurement of cell viability. Cell viability was calculated as a percent relative to the vehicle control normalized to 100% and presented as mean ± SEM. A minimum of 3 independent trials were conducted. Further analysis was performed using SynergyFinder 2.0 (Ianevski et al., 2020), where the Bliss independence drug interaction model was used. Drug combination responses were also plotted as 3-dimensional (3D) synergy maps to assess the potential synergy, antagonism or additive behaviors of the drug combinations. These maps provide visual representations of synergy and identified the concentrations at which the drug combinations had maximum effects on cell viability. The synergy scores represent the average excess response due to drug interactions. A synergy score of <-10 was considered as antagonistic, a range from -10 to +10 as additive and >+10 as synergistic (Ianevski et al., 2020). The data was then further visualized graphically and plotted as dose-response curves on an x-y axis to assess for statistical significance between combinations with cannflavins and either paclitaxel or cannabinoids alone.

2.10 Statistical Analysis

Statistical analysis was completed using GraphPad Prism Software. All error bars are representative of mean ± SEM. Unpaired student's t-tests were performed for analysis of two independent groups. One-way ANOVA with Tukey's post-hoc test was used to assess multi-group comparisons. *p* values are reported as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Cell viability curves were fitted using Graph Pad Prism's nonlinear regression function: [inhibitor] vs. normalized response (variable slope). To compare the IC_{50} values of two cell viability curves the extra-sum-of-squares F test was used to determine if IC_{50} differences were statistically significant.

Chapter 3: Results

Two cell lines were used throughout this project to model chemotherapeutic resistant breast cancer. The cell lines used were paclitaxel resistant (PR) MDA-MB-231 cells and PR MCF-7 cells. PR MDA-MB-231 cells are human epithelial breast adenocarcinoma cells that are triple negative for the estrogen, progesterone, and human epidermal growth factor (HER2) receptors. PR MCF-7 cells are another human breast adenocarcinoma cell line that is positive for the estrogen and progesterone receptors and negative for the HER2 receptor. These two cell lines were chosen due to their varying receptor profiles to ensure results were not cell line specific. Initially, ten flavonoids found in cannabis were screened for their cytotoxic effects and to determine appropriate concentration ranges. The most promising flavonoids (cannflavin A and cannflavin B) were then selected for further assays to evaluate their ability to induce apoptosis, promote autophagy, and reduce invasiveness. Cannflavins were then combined with various concentrations of the chemotherapeutic paclitaxel or cannabinoids THC or CBD to assess for synergistic cytotoxic effects following co-administration of these compounds.

3.1.1 Flavonoids exerted dose-dependent cytotoxicity in PR MDA-MB-231 cells

The first step in this study was to evaluate the effects of individual flavonoids on cell viability in PR cell lines. Cells were treated with varying concentrations of ten flavonoids (Cannflavin A, cannflavin B, silymarin, luteolin, orientin, quercetin, vitexin, isovitexin, kaempferol, and apigenin) for 24 hours and cell viability was measured by fluorescence using an AlamarBlue® assay. In PR MDA-MB-231 cells cannflavin A, cannflavin B, silymarin, luteolin, quercetin, and apigenin exerted dose-dependent

cytotoxic effects on cell viability and relative IC_{50} values were calculated (Figure 3.1A,B,C,D,F,J). IC_{50} values for flavonoids are as follows: 5.97 μM (cannflavin A), 5.69 μM (cannflavin B), 9.30 μM (silymarin), 16.8 μM (luteolin), 3.70 μM (quercetin), and 0.643 μM (apigenin)(Table 3.1). In PR MDA-MB-231 cells orientin, vitexin, isovitexin, and kaempferol did not exert as strong effects on cell viability or in a dose-dependent manner and IC_{50}s could not be calculated (Figure 3.1E,G,H,I).

3.1.2 Paclitaxel did not reduce the IC_{50} for cannflavin curves in PR MDA-MB-231 cells

In addition to evaluating the effects of the flavonoids alone, flavonoids were combined with 470 nM paclitaxel to determine if the combination could lower the relative IC_{50} compared to each flavonoid alone. This concentration was chosen because the PR MDA-MB-231 cells are resistant to 470 nM paclitaxel. Co-treatment of silymarin and luteolin with paclitaxel resulted in similar relative IC_{50}s (Figure 3.1 C,D) while cotreatment of cannflavin A, cannflavin B, quercetin, and apigenin resulted in slightly lower IC_{50}s when combined with paclitaxel (Figure 3.2 A,B,F,J). IC_{50} values for flavonoids combined with 470 nM paclitaxel are as follows: 2.59 μM (cannflavin A), 3.27 μM (cannflavin B), 10.7 μM (silymarin), 17.1 μM (luteolin), 3.38 μM (quercetin), and 0.661 μM (apigenin)(Table 3.1). As mentioned previously, in PR MDA-MB-231 cells orientin, vitexin, isovitexin, and kaempferol did not exert as strong effects on cell viability or in a dose-dependent manner and IC_{50}s could not be calculated (Figure 3.1E,G,H,I). For any of the six flavonoids where IC_{50}s were calculated there was no

significant difference between IC_{50}s when the PR MDA-MB-231 cells were treated with flavonoid alone or with flavonoid and paclitaxel co-treatment.

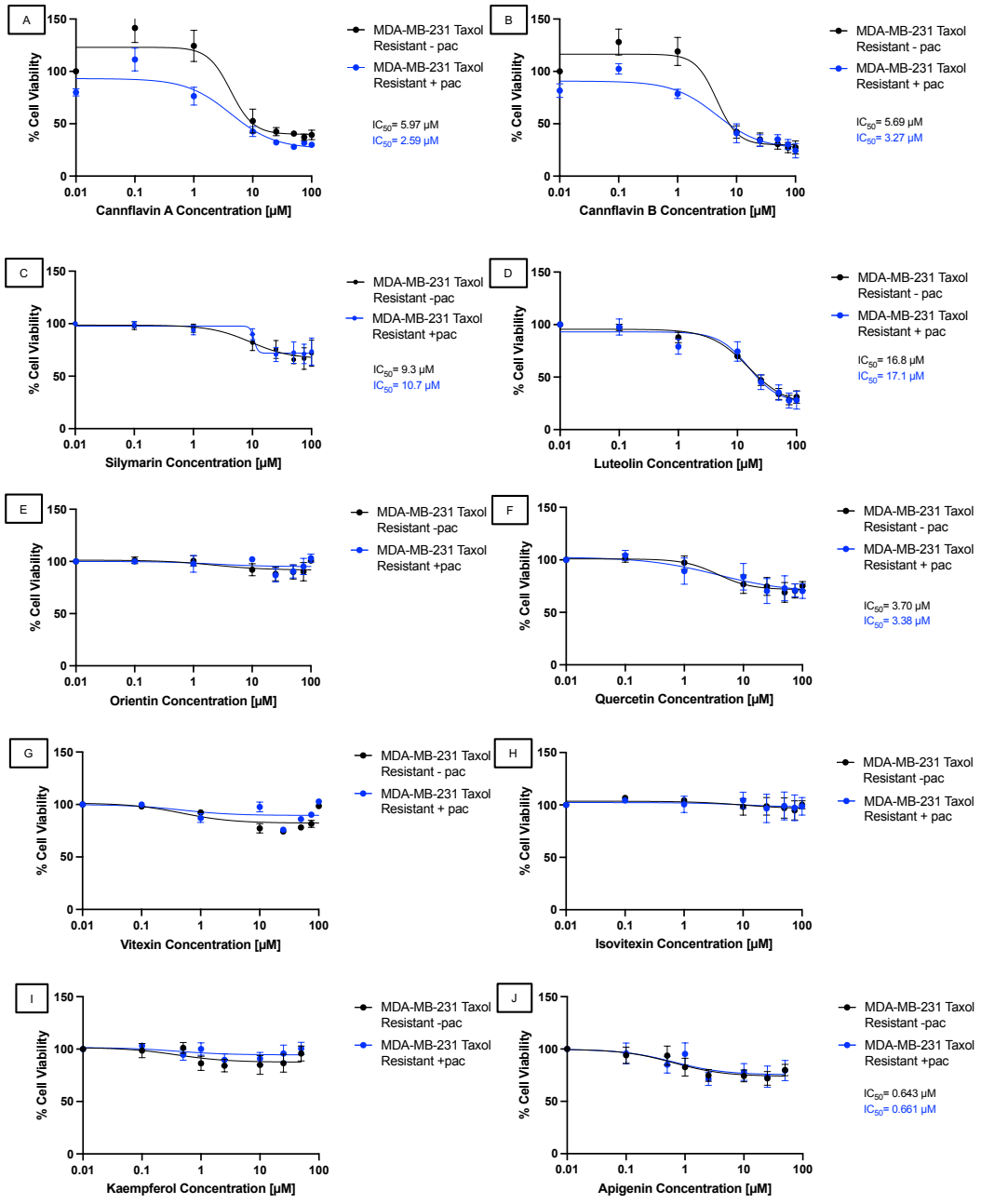

Figure 3.1 Dose-response curves generated for ten flavonoids with or without paclitaxel in PR MDA-MB-231 cells. Cells were treated with 10 flavonoids for 24 hours with or without 470 nM of paclitaxel (-pac)(+pac) and cell viability was measured with an AlamarBlue assay. Effects in PR MDA-MB-231 of (A) cannflavin A, (B) cannflavin B, (C) silymarin, (D) luteolin, (E) orientin, (F) quercetin, (G) vitexin, (H) isovitexin, (I) kaempferol, (J) apigenin. Data presented as mean ± SEM of at least 3 independent experiments.

3.2.1 Cannflavins exerted dose-dependent cytotoxicity in PR MCF-7 cells

Similarly, the effects of individual flavonoids on cell viability in PR MCF-7 cells were evaluated. Cells were once again treated with varying concentrations of ten flavonoids (cannflavin A, cannflavin B, silymarin, luteolin, orientin, quercetin, vitexin, isovitexin, apigenin, and kaempferol) for 24 hours and cell viability was measured by fluorescence using an AlamarBlue assay. In PR MCF-7 cells cannflavin A, cannflavin B, silymarin, and luteolin exerted dose-dependent cytotoxic effects on cell viability and relative IC_{50} values were calculated (Figure 3.2A,B,C,D). IC_{50} values for flavonoids are as follows: 3.44 μM (cannflavin A), 3.35 μM (cannflavin B), 7.96 μM (silymarin), 28.0 μM (luteolin). In PR MDA-MB-231 cells orientin, quercetin, vitexin, isovitexin, kaempferol, and apigenin did not exert as strong effects on cell viability or in a dose-dependent manner and IC_{50}s could not be calculated (Figure 3.2 E-I).

3.2.1 Paclitaxel did not reduce the IC_{50} for cannflavin curves in PR MCF-7 cells

Flavonoids were again combined with 470 nM paclitaxel to determine if the combination could lower the relative IC_{50} compared to each flavonoid alone. Co-treatment of cannflavin B and silymarin with paclitaxel resulted in slightly higher relative IC_{50}s (Figure 3.2 B,C) while cotreatment of cannflavin A and luteolin resulted in lower IC_{50}s when combined with paclitaxel (Figure 3.2 A,D). IC_{50} values for flavonoids combined with 470 nM paclitaxel are as follows: 2.21 μM (cannflavin A), 3.39 μM (cannflavin B), 13.6 μM (silymarin), 24.6 μM (luteolin). As mentioned previously, in PR MCF-7 cells orientin, quercetin, vitexin, isovitexin, kaempferol, and apigenin did not exert as strong effects on cell viability or in a dose-dependent manner and IC_{50}s could not

be calculated (Figure 3.2E-I). For any of the four flavonoids where IC_{50}s were calculated there was no significant difference between IC_{50}s when the PR MCF-7 cells were treated with flavonoid alone or with flavonoid and paclitaxel co-treatment.

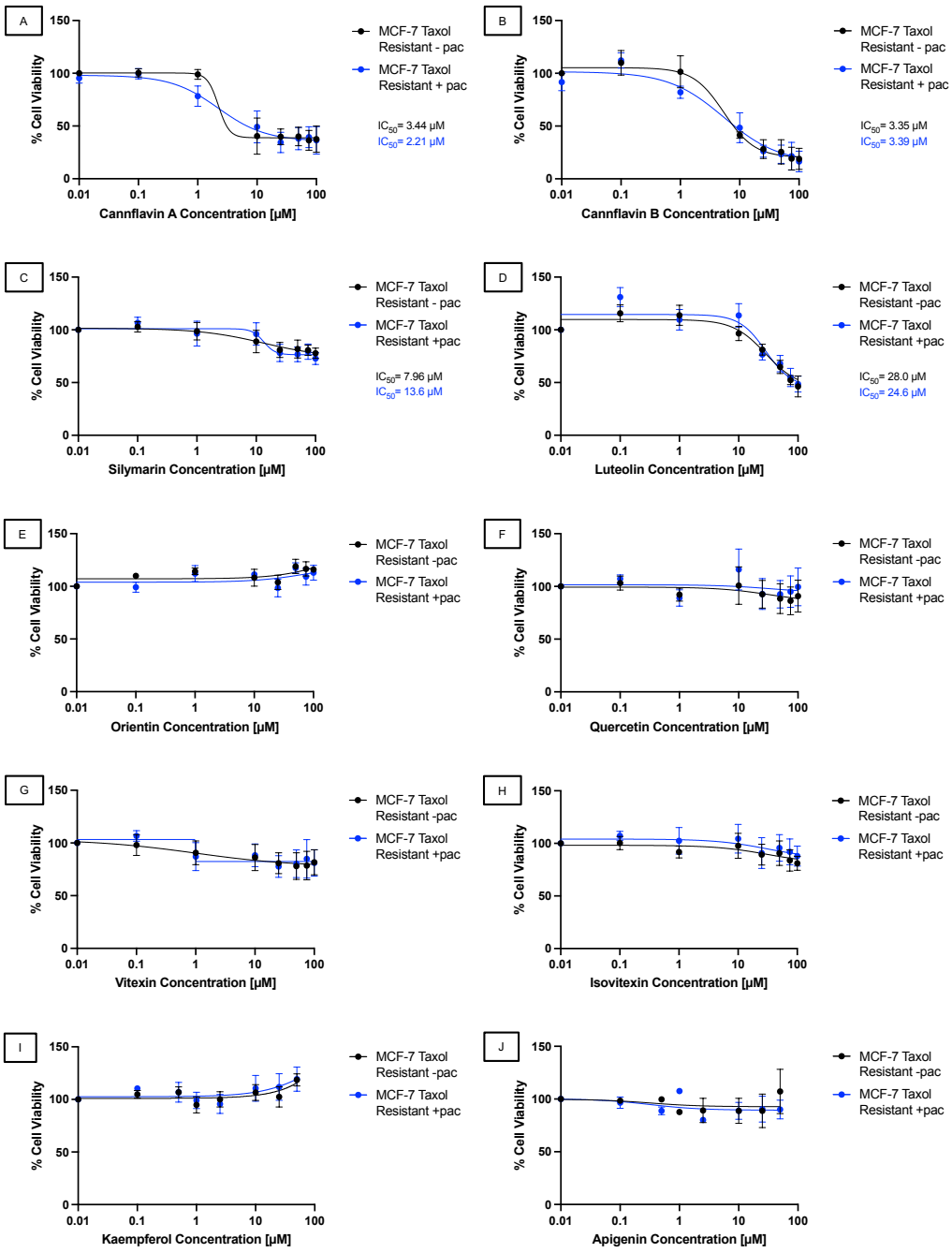

Figure 3.2 Dose-response curves generated for ten flavonoids with or without paclitaxel in PR MCF-7cells. Cells were treated with 10 flavonoids for 24 hours with or without 470 nM of paclitaxel (-pac)(+pac) and cell viability was measured with an AlamarBlue assay. Effects in PR MCF-7 cells of (A) cannflavin A, (B) cannflavin B, (C) silymarin, (D) luteolin, (E) orientin, (F) quercetin, (G) vitexin, (H) isovitexin, (I) kaempferol, (J) apigenin. Data presented as mean ± SEM of at least 3 independent experiments.

Table 3.1 Relative IC_{50}s of flavonoids ± 470 nM paclitaxel in PR MDA-MB-231 and PR MCF-7 cells. IC_{50} values as determined in Figure 1.1-1.2. ND (not determined) indicates IC_{50} value could not be calculated.

	PR MDA-MB-231		PR MCF-7	
	-paclitaxel	*+paclitaxel*	*-paclitaxel*	*+paclitaxel*
Cannflavin A	5.97 ± 1.2 μM	2.59 ± 1.0 μM	3.44 ± 2.0 μM	2.21 ± 0.6 μM
Cannflavin B	5.69 ± 0.8 μM	3.27 ± 1.4 μM	3.35 ± 1.1 μM	3.39 ± 2.1 μM
Silymarin	9.30 ± 2.1 μM	10.7 ± 1.9 μM	7.96 ± 1.5 μM	13.6 ± 7.8 μM
Luteolin	16.8 ± 2.8 μM	17.1 ± 4.7 μM	28.0 ± 4.4 μM	24.6 ± 3.4 μM
Orientin	ND	ND	ND	ND
Quercetin	3.70 ± 1.1 μM	3.38 ± 1.5 μM	ND	ND
Vitexin	ND	ND	ND	ND
Isovitexin	ND	ND	ND	ND
Kaempferol	ND	ND	ND	ND
Apigenin	0.643 ± 0.2 μM	0.661 ± 0.3 μM	ND	ND

3.3 Cannflavins do not exert cytotoxicity in non-tumorigenic MCF10-A cells

Typical chemotherapeutic agents often have negative systemic side effects due to their actions on non-cancerous cells. It is important to consider whether the flavonoids being investigated for this project have cytotoxic effects specific to cancer cells or if they also act on non-cancer cells. MCF-10A cells are a non-tumorigenic human mammary epithelial cell line often used in breast cancer research to model normal breast cells in two dimensional *in vitro* models (Qu et al., 2015) Previous research in our lab has demonstrated that cannabinoids, terpenes, and some synthetic cannabinoids are able to preferentially exert their cytotoxic effects in breast cancer cells lines while not affecting MCF-10A cells (A. Tomko et al., 2019; A. M. Tomko, Whynot, O'Leary, et al., 2021; Whynot, 2021). After determining which flavonoids had dose-dependent cytotoxic effects in PR breast cancer cell lines we wanted to determine if these compounds were cytotoxic in non-tumorigenic MCF-10A breast epithelial cells. MCF-10A cells were treated with the maximal concentration of a flavonoid used in the previous cell viability assays ranging from 50-100 μM for 24 hours. Cell viability was measured using an AlamarBlue® assay. Cannflavin A, cannflavin B, and apigenin did not produce significant cytotoxic effects in MCF-10A cells following treatment however silymarin, quercetin, and luteolin significantly reduced MCF-10A cell viability compared to vehicle control treated cells (Figure 3.3). Cell viabilities measured following flavonoid treatment are as follows: 89.1 ± 13.2 (cannflavin A), 86.7 ± 8.28 (cannflavin B), 85.5 ± 10.2 (apigenin), 36.7 ±6.86 (silymarin), 66.0 ± 9.62 (quercetin), 22.5 ± 8.45 (luteolin)(Figure 3.3).

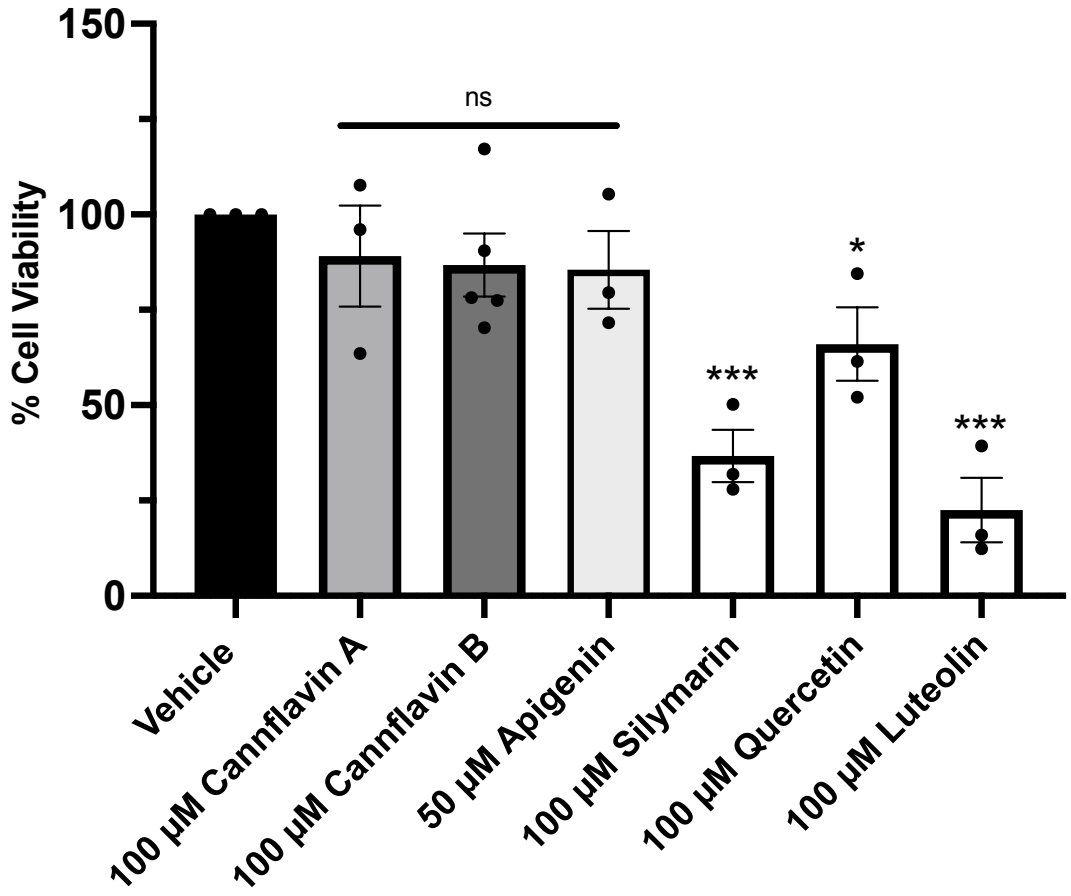

Figure 3.3 Effects of flavonoids on non-tumorigenic MCF-10A cell viability. Cells were treated with maximal concentrations of flavonoids used in this study for 24 hours and cell viability was measured using an AlamarBlue assay. Data presented is a mean ± SEM of at least 3 independent experiments. Unpaired student's t-tests were used to assess differences between flavonoid treatments and vehicle control *$p<0.05$, ***$p<0.001$.

3.4 Cannflavins induced apoptosis in PR MDA-MB-231 cells

After determining which flavonoid exerted dose-dependent cytotoxic effects in PR breast cancer cell lines and which flavonoids did not have cytotoxic effects in the non-tumorigenic MCF-10A cells two compounds were chosen to undergo further characterization. Cannflavin A and cannflavin B were chosen due to their dose-dependent cytotoxic effects achieving the lowest cell viabilities and low relative IC_{50}s in both PR breast cancer cell lines and because they did not have cytotoxic effects in the MCF-10A cell line. Although apigenin also showed dose-dependent cytotoxic effects and was not cytotoxic in the MCF-10A it was not chosen. This is because cannflavins are more novel and there is limited information available about their anti-cancer effects. In contrast the anti-tumoral effects of apigenin have been more extensively documented in breast cancer as well as other cancer types (A. M. Tomko et al., 2020).

We further characterized these flavonoids by looking at the induction of apoptosis, first using an annexin V apoptosis assay. Under normal conditions, phosphatidylserine is present on the intracellular leaflet of the cell membrane. Upon induction of apoptosis, phosphatidylserine flips to the extracellular leaflet of the cell membrane. Fluorescent annexin V binds to phosphatidylserines when present on the extracellular leaflet and the fluorescence's presence is an indicator of early apoptosis. Propidium iodide is a nuclear stain that enters the cell when the cell membrane is compromised during late apoptosis or necrosis. Cells were treated with 2.5 μM of cannflavin A or cannflavin B for 24 hours and then stained with annexin V and propidium iodide. This concentration of cannflavins was chosen because it was able to

slightly kill the breast cancer cells while not reducing cell viability to an extent where it would be difficult to accurately determine rates of apoptosis due to low numbers of cells left following treatment. This concentration was lower than the relative IC_{50} for both compounds in PR MDA-MB-231 cells. Both cannflavin A and cannflavin B induced apoptosis in PR MDA-MB0231 cells (Figure 3.4.1). In vehicle-treated cells, 9.3 ± 1.9 percent of cells stained positive with annexin V and 4.5 ± 1.1 percent stained positive for propidium iodide. In cannflavin A treated cells, 37.7 ± 2.6 percent of cells stained positive for annexin V and 11.8 ± 5.8 percent stained positive for propidium iodide. In cannflavin B treated cells, 23.8 ± 3.3 percent of cells stained positive for annexin V and 6.5 ± 2.0 percent of cells stained positive with propidium iodide (Figure 3.4.1). Cells treated with cannflavin A and B had a significantly greater number of cells stained with annexin V compared to vehicle control while there was no significant difference in the number of cells stained with propidium iodide between treatment groups.

To investigate what apoptotic pathway and proteins may be involved in cannflavin A and B's apoptotic effects western blotting for Bax and Bcl-2 was performed. PR MD-MB-231 cells were treated with 2.5 μM of cannflavin A, cannflavin B, or vehicle for 24 hours. SDS-PAGE, western blotting, and chemiluminescence was performed. Results showed that following cannflavin treatment there was an increase in Bax expression seen at 21 kilodaltons (kDa) compared to vehicle treated cells (Figure 3.4.2, 3.4.3). In addition, there was a decrease in Bcl-2 expression seen at 26 kDa following cannflavin treatment compared to vehicle control. Tubulin expression at 55 kDa was used as a loading control to ensure equal protein concentration in each well and its expression stayed relatively constant (Figure 3.4.2, 3.4.3).

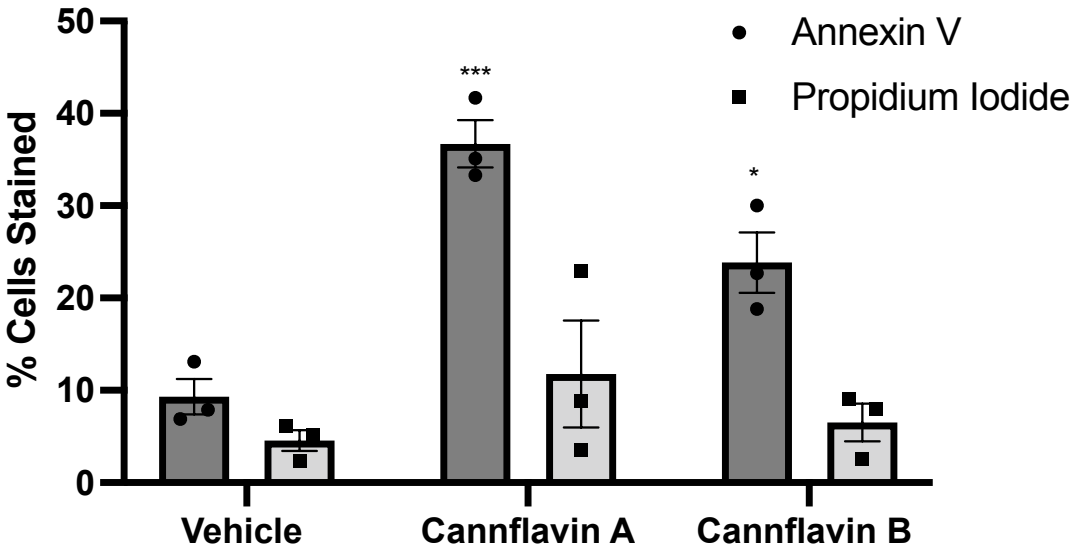

Figure 3.4.1 Apoptosis induction by cannflavin A and cannflavin B in PR MDA-MB-231 cells. Cells were treated with 2.5 μM of either cannflavin A or B for 24 hours. Cells were stained with annexin V to assess for early apoptosis and propidium iodide to detect for late apoptosis or necrosis. Stained cells were counted using a fluorescence microscope and expressed as a percentage of the total number of cells counted. Data presented is a mean ± SEM of at least 3 independent experiments. Unpaired student's t-tests were used to assess differences between flavonoid treatments and vehicle control *$p<0.05$, ***$p<0.001$.

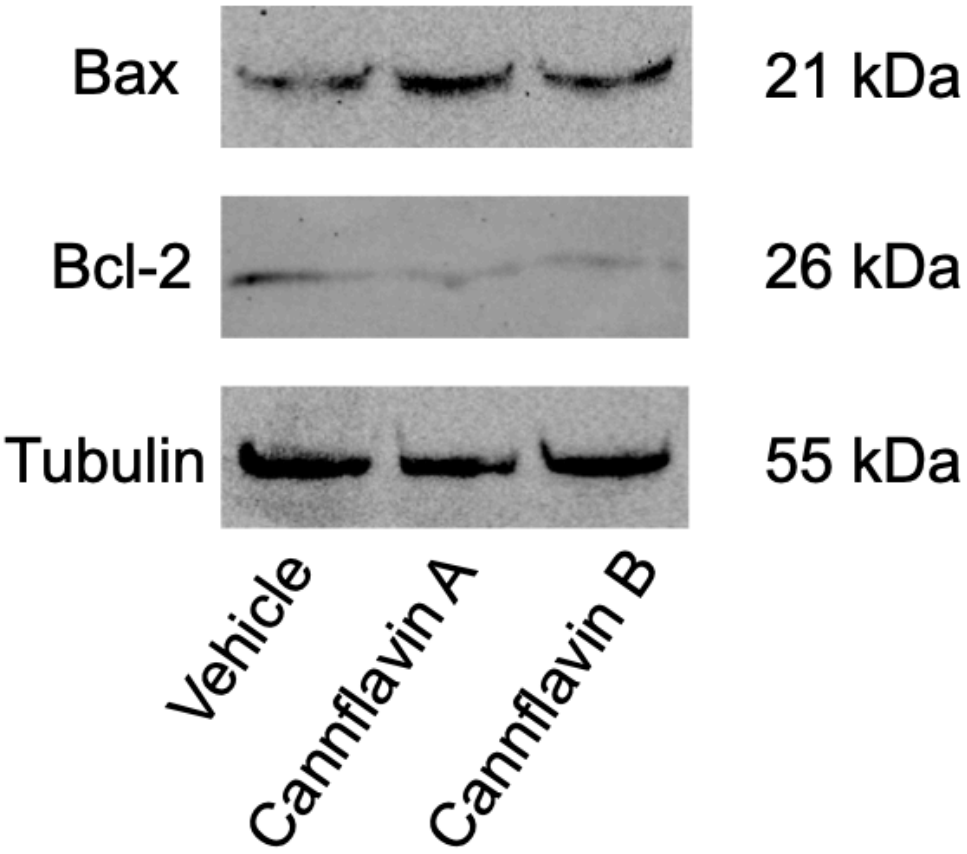

Figure 3.4.2 Western blotting for apoptotic proteins in PR MDA-MB-231 cells following cannflavin A or cannflavin B treatment. Cells were treated with 2.5 μM cannflavin A or cannflavin B for 24 hours. Samples were run on an SDS-PAGE, western blotting and chemiluminescence was then performed. Apoptotic proteins Bax and Bcl-2 and a control protein tubulin were targeted.

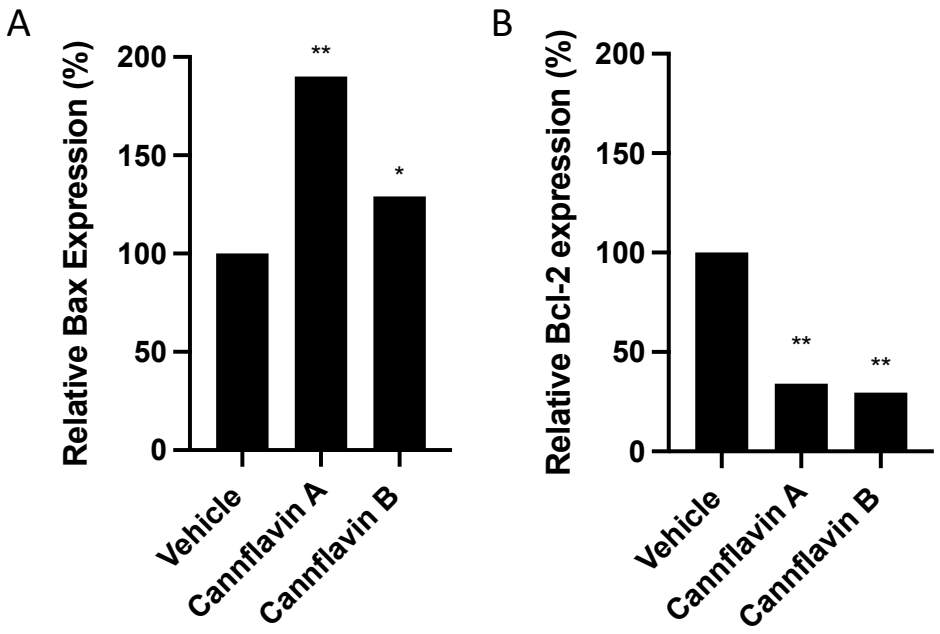

Figure 4.3.3 Protein quantification from western blots in PR MDA-MB-231 cells following cannflavin A or cannflavin B treatment. Western blots were quantified using imageJ software. Protein expression of (A)Bax and (B) Bcl-2 was normalized to the tubulin loading control and expressed relative to cells treated with vehicle. Data presented is a mean of at least 3 independent western blots. Unpaired student's t-tests were used to assess differences between flavonoid treatments and vehicle control *$p<0.05$, **$p<0.01$.

3.5 Cannflavins promoted autophagy in PR MDA-MB-231 cells

To further characterize the anti-cancer effects of cannflavin A and cannflavin B their role in the induction of autophagy was explored. Bafilomycin, 3-methyladenine, E-64d, and pepstatin A are known inhibitors of autophagy at different points within the autophagy pathway. Bafilomycin is an inhibitor of vacuolar H+-ATPase, 3-methyladenine inhibits class III phosphatidylinositol 3 kinase, and E-64d and pepstatin A inhibit lysosomal proteases (Dong et al., 2019; Mauvezin & Neufeld, 2015; Nakamura et al., 2021). PR MDA-MB-231 cells were treated with a combination of 2.5 μM of cannflavin A or cannflavin B and either 100nM Bafilomycin A, 1 mM 3-methyladenine, or the combination of 10 μg/ml E-64d and 10 μg/ml pepstatin A for 6 hours to determine if the autophagy inhibitors could eliminate the cytotoxic effects seen with cannflavin treatment. Treatment with 3-methyladenine, E-64d and pepstatin A, or bafilomycin alone did not significantly reduce cell viability of PR MDA-MB0231 cells (Figure 3.5.1). Treatment with cannflavin A and cannflavin B significantly reduced cell viability compared to vehicle control to 65.8 ± 11.1 and 65.5 ± 8.1 percent respectively. The combination of cannflavin A and E-64d and pepstatin A significantly increased cell viability compared to cannflavin A treatment alone returning cell viability to 101 ± 4.2 percent, which is not statistically different from the vehicle control treated cells (Figure 3.5.1). Similarly, the combination of cannflavin B and E-64d and pepstatin A significantly increased cell viability compared to cannflavin B treatment alone returning cell viability to 93.0 ± 4.6 percent, which is not statistically different from the vehicle control treated cells (Figure 3.5.1). 3-methyladenine or bafilomycin when combined with

either cannflavin did not significantly increase cell viability compared to cannflavin treatment alone.

The next step was to evaluate if cannflavin A or cannflavin B altered the expression of several genes related to autophagy. Gene expression of autophagy related proteins 5, 7, and 12 were assessed along with LC3b. PR MDA-MB-231 cells were treated for 6 or 24 hours with either cannflavin A or cannflavin B, RNA was extracted, cDNA was made, and RT-qPCR was performed. Following 6 hours of treatment cannflavin A significantly increased the expression of ATG5 (1.3 ± 2.2), ATG12 (1.2 ± 0.16), and LC3b (0.99 ± 0.10) genes compared to vehicle controls (0.68 ± 0.20, 0.44 ± 0.23, 0.37 ± 0.22 respectively), cannflavin B significantly increased the gene expression of ATG12 (1.6 ± 0.24) compared to vehicle control (0.44 ± 0.23)(Figure 3.5.2). Following 12 hours of treatment cannflavin A significantly increased the gene expression of LC3b (1.6 ± 0.23) compared to vehicle control (0.56 ± 0.20), cannflavin B did not alter the gene expression of any of the genes investigated at 24 hours (Figure 3.5.2).

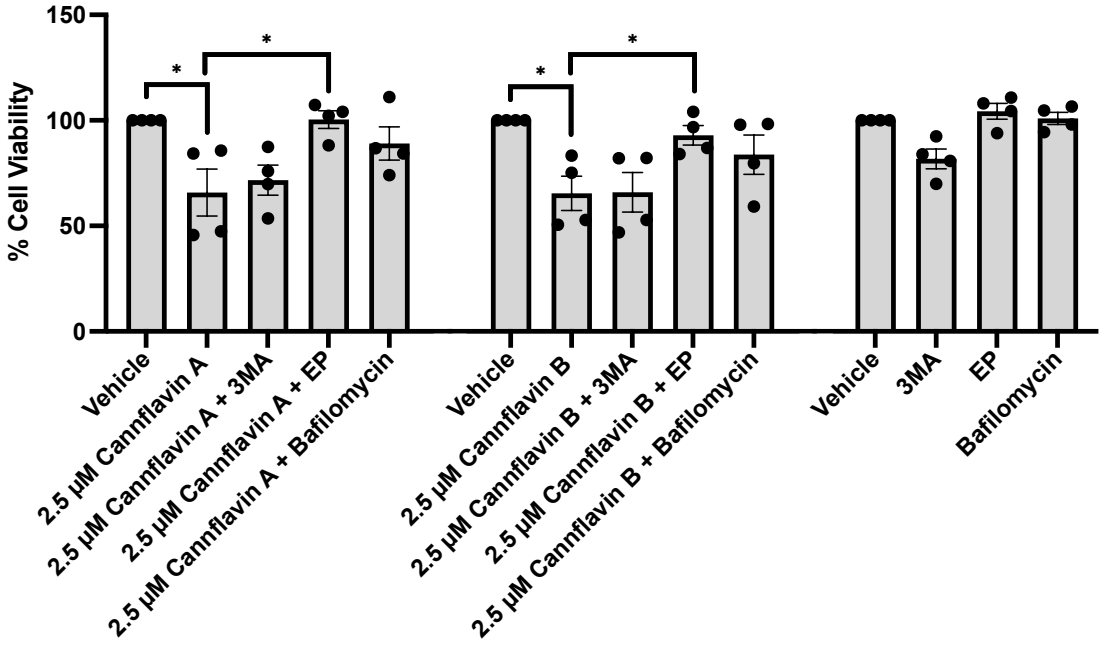

Figure 3.5.1 Effects of combinations of cannflavin A, cannflavin B, and autophagy inhibitors on PR MDA-MB-231 cell viability. Cells were treated with 2.5 µM of cannflavin A or cannflavin B and either 100nM Bafilomycin A, 3-methyladenine (3MA), or the combination of 10 µg/ml E-64d and 10 µg/ml pepstatin A for 6 hours. Cell viability was measured using an AlamarBlue assay. Data presented is a mean ± SEM of at least 3 independent experiments. Unpaired student's t-tests were used to assess differences between flavonoid treatments and vehicle control *$p<0.05$.

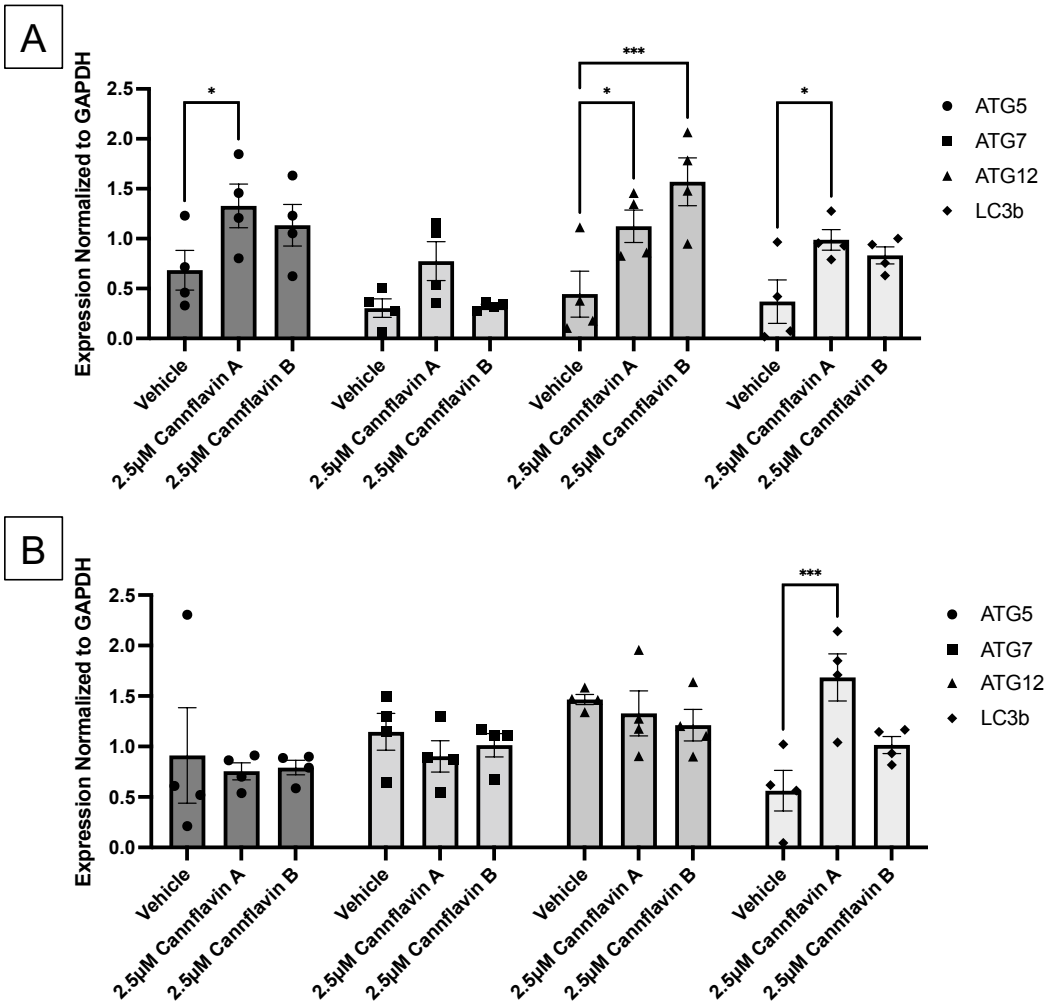

Figure 3.5.2 Effect of cannflavin A and cannflavin B on expression of genes related to autophagy in PR MDA-MB-231 cells. PR MDA-MB-231 cells were treated with 2.5 μM cannflavin A or cannflavin B for 6 or 24 hours. RNA was extracted, cDNA was prepared, and RT-qPCR was performed to quantify the expression of autophagy related gene 5 (ATG5), autophagy related gene 7(ATG7), autophagy related gene 12 (ATG12), and microtubule-associated proteins 1A/1B light chain 3B (LC3b). Expression is normalized to the housekeeping protein GAPDH. Data presented is a mean ± SEM of at least 4 independent samples. Unpaired student's t-tests were used to assess differences between flavonoid treatments and vehicle control *$p<0.05$, ***$p<0.001$.

3.6 Cannflavins reduced the invasive property of PR MDA-MB-231 cells

Highly invasive forms of breast cancer can cause invasion of the primary tumor cells to neighboring tissues and metastasis resulting in tumors located distant to the primary tumor site. Metastatic breast cancer is more difficult to treat because surgery is typically no longer effective, and patients must rely on systemic forms of therapy like hormone therapy, immunotherapy, and chemotherapy. The first step in metastatic cascade is invasion, this is when cells use both individual and collective invasion to penetrate the basement membrane where they are located, cells then migrate through the extracellular matrix to the blood vessels where they then travel to other sites within the body (Novikov et al., 2021). This study will focus on the effects of cannflavin A and cannflavin B on invasion as it is the first step in the metastatic cascade. Triple negative breast cancer is highly aggressive and invasive so the PR MDA-MB-231 cells will serve as a model to assess the ability of cannflavin A and cannflavin B to reduce their invasive ability.

A Matrigel invasion assay was used to determine the invasive ability of PR MDA-MB-231 cells following cannflavin treatment. Matrigel is a matrix-like substance that is used *in vitro* to model the basement membrane. Migration wells lacking the matrigel were used as a control to determine the number of seeded cells that had the ability to migrate. PR MDA-MB-231 cells were treated with 2.5 μM of cannflavin A or cannflavin B for 24 hours in the Matrigel invasion wells. Cells that invaded were stained and counted manually using a light microscope. The number of cells that invaded through the Matrigel in the vehicle and treatment conditions were expressed as a percentage relative to the number of cells that migrated through the vehicle control treated well lacking the

Matrigel. Following vehicle treatment 25.1 ± 1.72 percent of cells were able to invade the Matrigel. Cannflavin A and cannflavin B were able to significantly reduce the number of cells that invaded through the matrigel wells compared to vehicle control to 15.4 % ± 1.55 and 13.7 % ± 0.714 respectively (Figure 3.6).

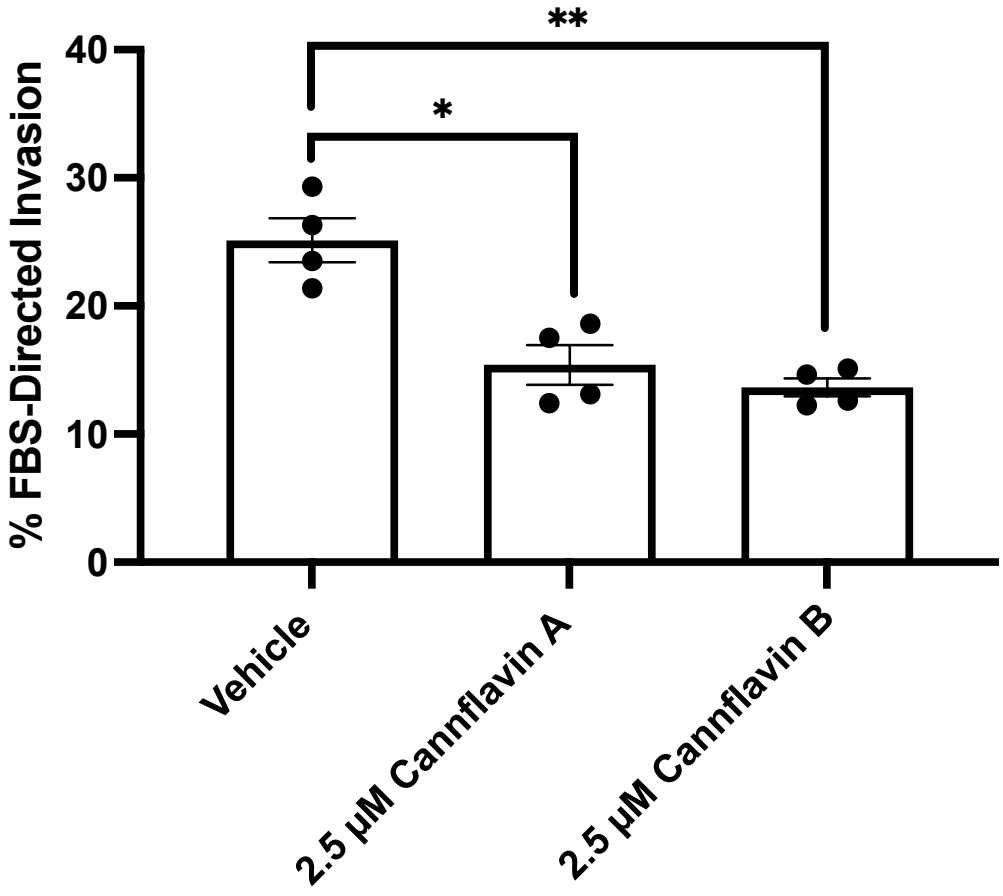

Figure 3.6 Effect of cannflavin A and cannflavin B on invasive ability of PR MDA-MB-231 cells. PR MDA-MB-231 cells were treated with 2.5 µM of cannflavin A or cannflavin B for 24 hours in matrigel invasion wells or migration wells. Cells that migrated or invaded through the wells were stained and counted using a light microscope and represented relative to the number of cells that migrated through vehicle-treated wells and expressed as a percent. Data presented is a mean ± SEM of at least 3 independent experiments. Unpaired student's t-tests were used to assess differences between flavonoid treatments and vehicle control *$p<0.05$, **$p<0.01$.

3.7 Cannflavins combined with THC elicited synergistic and additive inhibitory effects on cell viability in PR MDA-MB-231 cells

Currently, those seeking complementary therapies typically consider the main cannabinoids THC and CBD due to their beneficial effects on patient quality of life. THC or CBD alone may have beneficial effects but what would happen if these compounds were combined with other compounds that have anti-cancer effects? After characterizing the effects of cannflavin A and cannflavin B alone the next step was to assess their effects in combination with other compounds in cannabis and chemotherapeutic agents. Previous studies have shown that whole botanical extracts have shown greater anti-cancer effects and other therapeutic benefits than cannabinoids alone, suggesting other compounds such as flavonoids in the plant are acting with these compounds (Blasco-Benito et al., 2018).

To assess the effects of cannflavin A and cannflavin B in combination with other compounds a comprehensive dose-response matrix assay was performed. This assay allows a large number of concentration combinations between two compounds to be examined. A comprehensive explanation will be provided here for how this assay was performed and analyzed; however, all future combinations follow the same protocol and analysis. Cells were treated in 96-well plates for 24 hours with compounds and cell viability was measured using an AlamarBlue® assay. Cell viability data was input into the Synergy Finder (2.0) online tool (Ianevski et al., 2020) to assess for potential synergistic, additive, or antagonist concentration combinations. This tool provides visual data in the form of mountain plots and heat maps as well as synergy scores ± SEM for all possible

combinations to aid in screening for combinations that show promising synergistic effects.

The reference model chosen to assess for synergy was the Bliss independence model (Q. Liu et al., 2018). This model assumes that the two compounds being combined are acting independently of one another. This assumption was made for our combination of compounds because cannabinoids and chemotherapeutic agents have known molecular targets and pathways while less is known about the mechanism of action of cannflavins A and B regarding their cytotoxicity. This model compares expected responses with observed responses and produces a synergy score for each combination within the matrix. This model uses the following equation to calculate expected response: $Y_{ab} = y_a + y_b - y_a y_b$, where Y_{ab} is the predicted response and y_a and y_b are the observed response with drug A at dose a and drug B at dose b, respectively (Q. Liu et al., 2018). The set-up of the matrix in the 96-well plates allowed the individual drug responses to be evaluated which were then used in the equation to calculate the expected response. This matrix also allowed the cell viability following combination treatment to be evaluated which was then used as the observed response in the equation (Figure 3.7.1). Synergy scores >10 indicate that the observed response is greater than the expected response and corresponds to synergy. Synergy scores <10 and >-10 indicate that the observed response is similar to the expected response and the interaction is additive (a synergy score of zero would indicate that the observed response is equal to the expected response). Synergy scores <-10 represent antagonism because the observed response is lower than the expected

response. Using this model, a synergy score of 40 would indicate a 40% greater response than expected based on the combination of the drug responses individually.

The combination of THC and cannflavin A in PR MDA-MB-231 cells provided an overall inhibitory synergistic response with some dose combinations also providing additive scores (Figure 3.7.2, Table 3.2). The highest synergistic scores were 62.8 (1.25 μM THC + 11.11 μM cannflavin A), 60.7 (1.25 μM THC + 33.33 μM cannflavin A), and 46.7 (1.25 μM THC + 0.41 μM cannflavin A) as summarized in Table 3.2 that provides the highest and lowest synergy scores for the combination of THC and cannflavin A.

As mentioned previously Synergy Finder 2.0 was used as a tool to screen for potential combinations of compounds to be further investigated and to determine what combinations were acting synergistically. To further investigate these combinations, data from the cell viability assays was also plotted as dose-response curves and statistically analyzed. By doing this we could determine if there were combinations that significantly reduced cell viability compared to both compounds alone. This data was then combined with the synergy scores from Synergy Finder (2.0) to determine the most promising combinations of compounds. These curves represent overall cytotoxic effects represented as a decrease in cell viability. Hollow data points on the right side of the graph represent cell viability for cannflavin alone at various concentrations. Combinations that are statistically significant from both drugs individually are marked with an *. Red boxes represent combination data points that are both statistically significant based on their

ability to reduce cell viability and synergistic as determined with the bliss independence model.

In PR MDA-MB-231 cells the combination of 1.25 μM THC and 3.7 μM cannflavin A was found to be acting synergistically and reduced cell viability significantly more than either compound alone. The combination reduced cell viability to 39.3% ± 16.1 compared to 89.7% ± 16.5 (1.25 μM THC) and 55.3% ± 6.95 (3.7 μM cannflavinA) (Figure 3.7.3A). In cells treated with the combinations of THC and cannflavin B, 0.625, 1.25, and 2.5 μM THC combined with 0.411 μM cannflavin B were both synergistic and significantly reduced cell viability compared to either compound alone. The combination of 0.625 μM THC and 0.411 cannflavin B reduced cell viability to 59.4% ± 9.27 compared to 98.5% ± 13.1 and 83.8% ± 2.06 alone respectively. The combination of 1.25 μM THC and 0.411 cannflavin B reduced cell viability to 49.6% ± 9.27 compared to 82.3% ± 11.4 and 83.8% ± 2.06 alone respectively. The combination of 2.5 μM THC and 0.411 cannflavin B reduced cell viability to 38.9% ± 9.60 compared to 69.8% ± 7.72 and 83.8% ± 2.06 alone respectively (Figure 3.7.3B)

	Drug B				
	0μM A, 0μM B	**1μM B**	**2μM B**	**5μM B**	**10μM B**
	1μM A	1μM A, 1μM B	1μM A, 2μM B	1μM A, 5μM B	1μM A, 10μM B
Drug A	**2μM A**	2μM A, 1μM B	2μM A, 2μM B	2μM A, 5μM B	2μM A, 10μM B
	5μM A	5μM A, 1μM B	5μM A, 2μM B	5μM A, 5μM B	5μM A, 10μM B
	10μM A	10μM A, 1μM B	10μM A, 2μM B	10μM A, 5μM B	10μM A, 10μM B

Figure 3.7.1 Example matrix set-up in 96-well plates for synergy assessment. Drug A concentrations increase on the y-axis. Drug B concentrations increase on the x-axis.

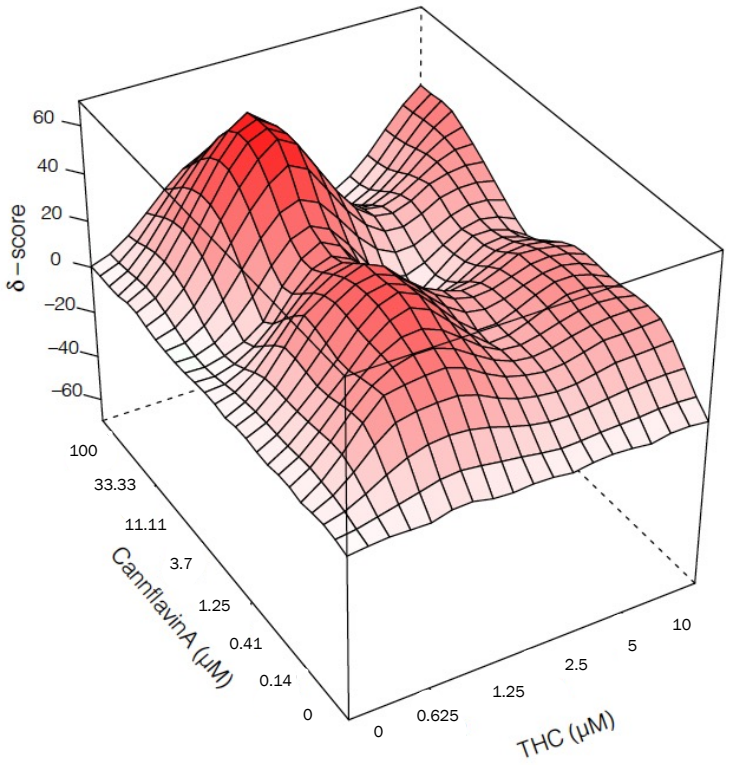

Figure 3.7.2 Assessment of synergy between cannflavin A and THC in PR MDA-MB-231 cells. Cells were treated with combinations of 0-10 μM THC and 0-100 μM cannflavin A for 24 hours and cell viability was measured using an AlamarBlue assay. Data was entered into Synergy Finder (2.0) to generate the mountain plot providing a visual representation of synergy scores for each combination treatment across the range of concentrations. Data presented is a mean of 3 independent experiments.

Table 3.2 Summary of synergy score for the combination of cannflavin A and THC in PR MDA-MB-231 cells.

Synergy Score	[THC] µM	[Cannflavin A] µM
62.8	1.25	11.11
60.7	1.25	33.33
46.7	1.25	0.41
5.81	33.33	2.5
0.561	100	2.5
-4.99	2.5	3.7

Scores in red are synergistic, black are additive, and green are antagonistic.

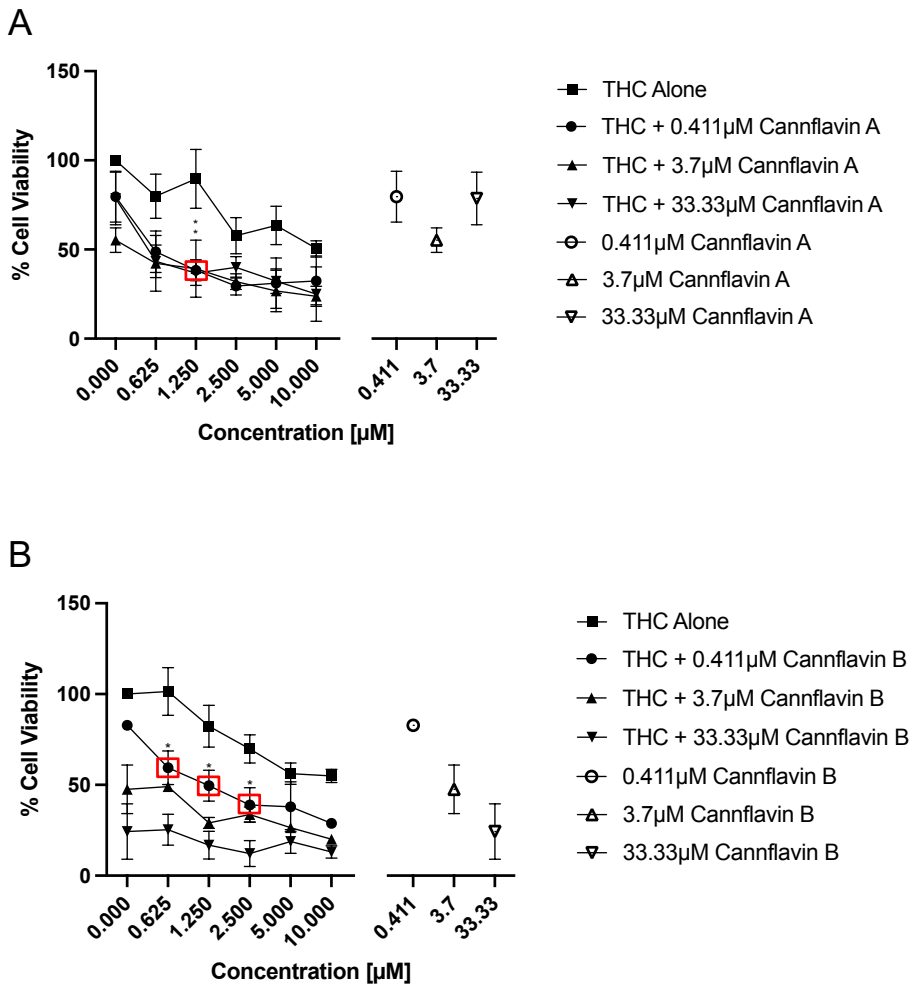

Figure 3.7.3 Effects of cannflavin A and cannflavin B co-treatment with THC on PR MDA-MB-231 cell viability. Cells were treated with 0-10 µM of THC and 0-100 µM of (A) cannflavin A or (B) cannflavin B in a matrix assay for 24 hours. Cell viability was measured using an AlamarBlue assay. Dose-response curves of THC with 3 different concentrations of cannflavins selected from the matrix are presented. Hollow points represent cannflavin effects alone. Red boxes indicate concentration combinations that are synergistic as determined by the bliss independence model using SynergyFinder2.0 software and that are significantly different from THC or cannflavin treatment alone. Data presented is a mean ± SEM of at least 3 independent experiments. One-way ANOVA was used to assess differences between flavonoid or THC treatments alone and combination treatments *$p<0.05$.

3.8 Cannflavins combined with CBD did not elicit significant synergistic inhibitory effects on cell viability in PR MDA-MB-231 cells

To assess whether cannflavin A and cannflavin B acted synergistically with CBD to reduce PR MDA-MB-231 cell viability a dose-response matrix assay was performed. PR MDA-MB-231 cells were treated with combinations of 0-10 μM CBD and 0-100μM of cannflavin A or cannflavin B, cell viability was measured using an AlamarBlue® assay. Data was entered into Synergy Finder (2.0) to determine if synergistic combinations were present and presented graphically to allow for statistical analysis. Following analysis, it was determined that some synergistic combinations were present and were mostly found at low concentrations of cannflavins and CBD. Additional combinations resulted in additivity and antagonism. There were no combinations of compounds that significantly lowered cell viability compared to each compound alone (Figure 3.8A,B).

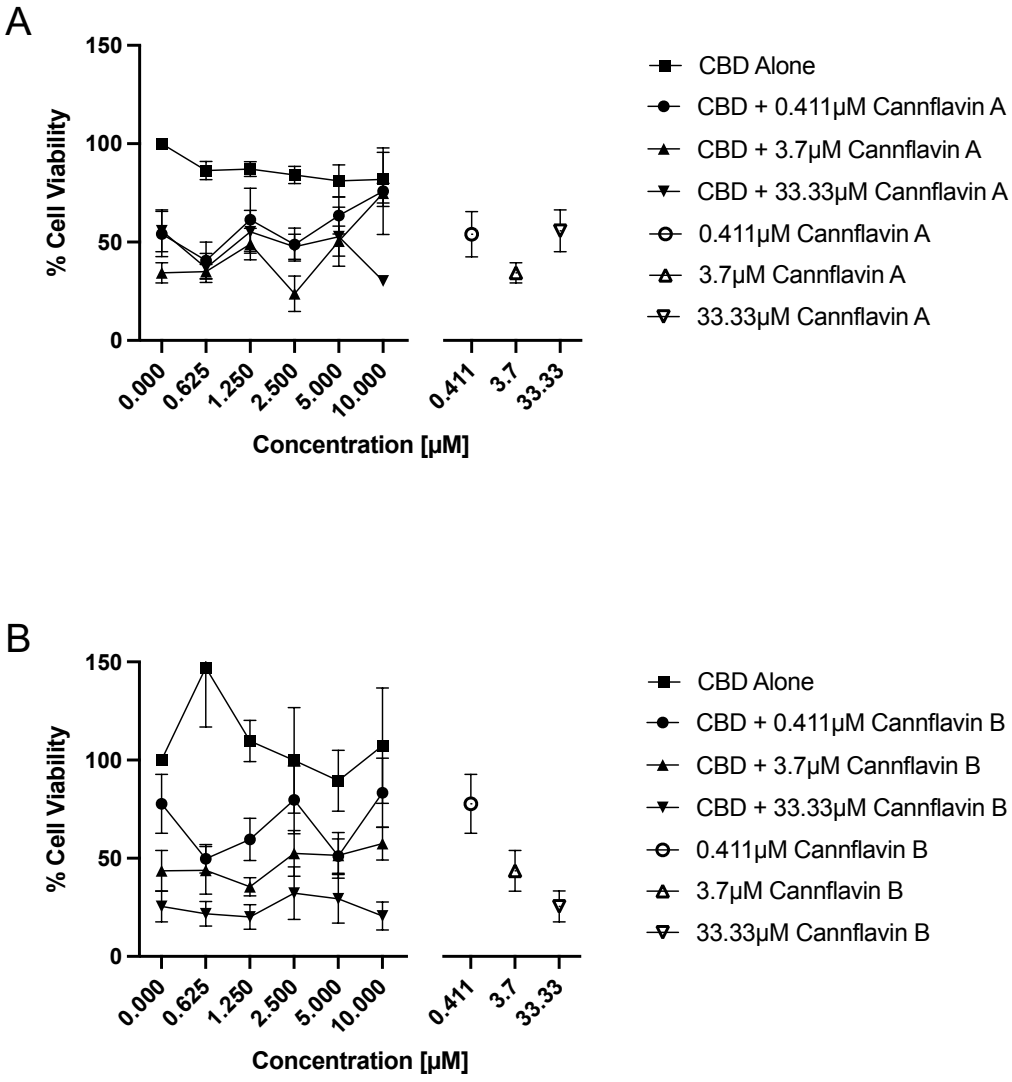

Figure 3.8 Effects of cannflavin A and cannflavin B co-treatment with CBD on PR MDA-MB-231 cell viability. Cells were treated with 0-10 µM of CBD and 0-100 µM of (A) cannflavin A or (B) cannflavin B in a matrix assay for 24 hours. Cell viability was measured using an AlamarBlue assay. Dose-response curves of CBD with 3 different concentrations of cannflavins selected from the matrix are presented. Hollow points represent cannflavin effects alone. Data presented is a mean ± SEM of at least 3 independent experiments. One-way ANOVA was used to assess differences between flavonoid or CBD treatments alone and combination treatments.

3.9 Cannflavins combined with THC did not elicit significant synergistic effects on cell viability in PR MCF-7 cells

To assess whether cannflavin A and cannflavin B acted synergistically with THC to reduce PR MCF-7 cell viability a dose-response matrix assay was performed. PR MCF-7 cells were treated with combinations of 0-10 μM THC and 0-100μM of cannflavin A or cannflavin B, cell viability was measured using an AlamarBlue® assay. Data was entered into the Synergy Finder (2.0) tool to determine if synergistic combinations were present and was presented graphically to allow for statistical analysis. Following analysis, it was determined that some synergistic combinations were present with the highest scores ranging in the 30-40 range for the combinations with cannflavin A and cannflavin B. There were no combinations of compounds that significantly lowered cell viability compared to each compound alone (Figure 3.9A,B).

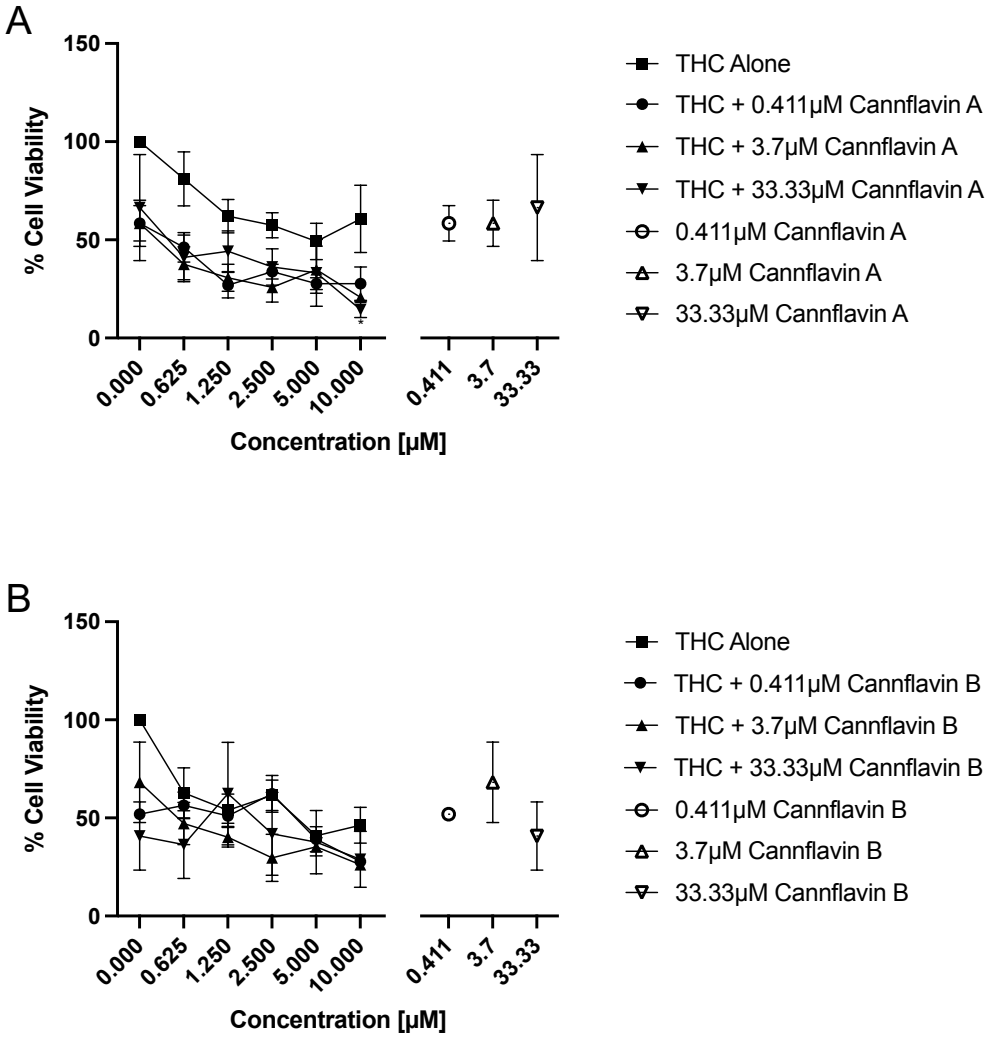

Figure 3.9 Effects of cannflavin A and cannflavin B co-treatment with THC on PR MCF-7 cell viability. Cells were treated with 0-10 μM of THC and 0-100 μM of cannflavin A or cannflavin B in a matrix assay for 24 hours. Cell viability was measured using an AlamarBlue assay. Dose-response curves of THC with 3 different concentrations of cannflavins selected from the matrix are presented. Hollow points represent cannflavin effects alone. Data presented is a mean ± SEM of at least 3 independent experiments. One-way ANOVA was used to assess differences between flavonoid or THC treatments alone and combination treatments.

3.10 Cannflavins combined with CBD did not exert synergistic effects on cell viability in PR MCF-7 cells

To assess whether cannflavin A and cannflavin B acted synergistically with CBD to reduce PR MCF-7 cell viability a dose-response matrix assay was performed. PR MCF-7 cells were treated with combinations of 0-10 μM CBD and 0-100μM of cannflavin A or cannflavin B, cell viability was measured using an AlamarBlue assay. Data was entered into the Synergy Finder (2.0) tool to determine if synergistic combinations were present and presented graphically to allow for statistical analysis of cell viability. Following analysis, it was determined that some synergistic combinations were present when CBD and cannflavin B were combined, with the highest scores ranging from 50-60. The combinations of CBD and cannflavin A resulted in mostly additive or antagonistic interactions. There were no combinations of compounds that significantly lowered cell viability compared to each compound alone (Figure 3.10A,B).

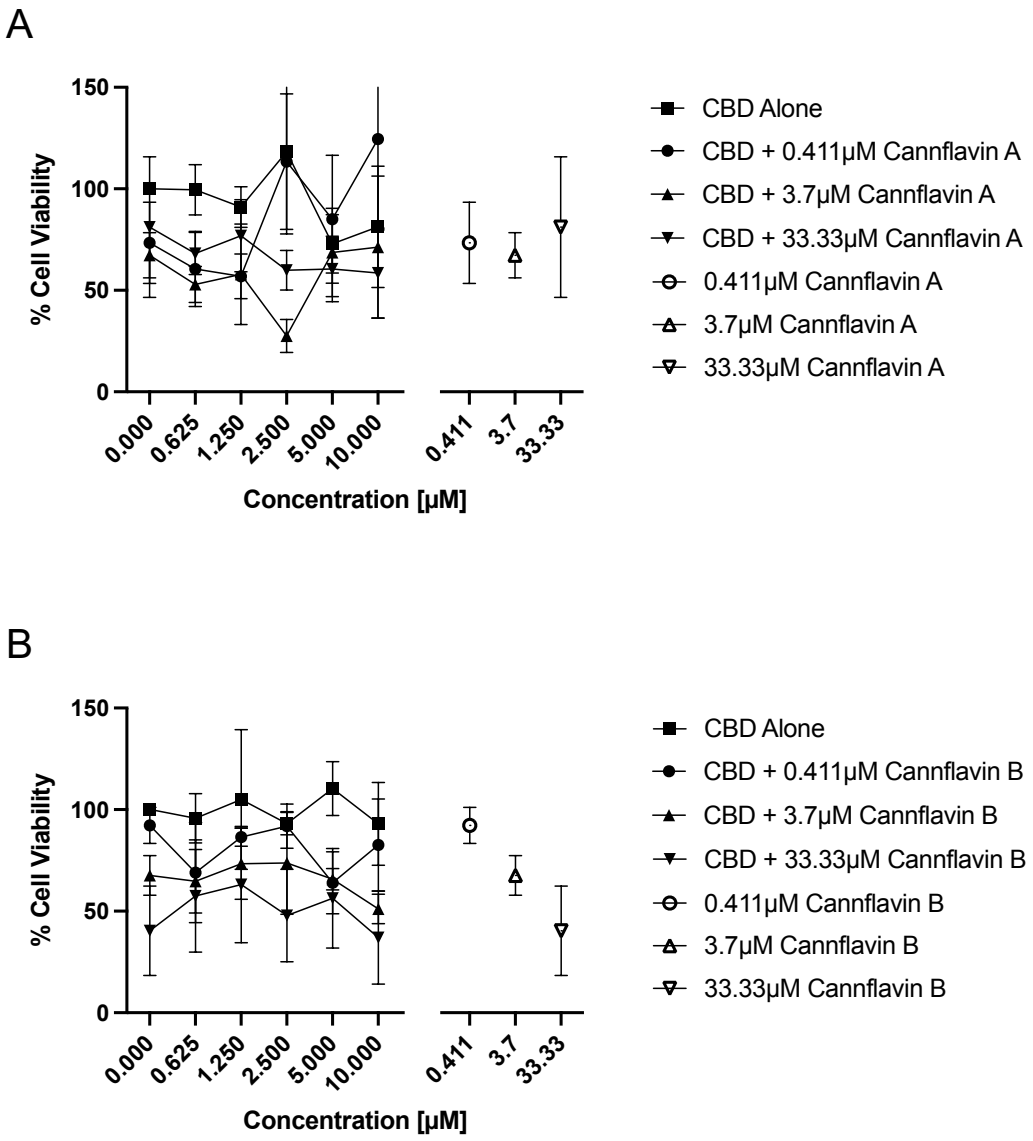

Figure 3.10 Effects of cannflavin A and cannflavin B co-treatment with CBD on PR MCF-7 cell viability. Cells were treated with 0-10 µM of CBD and 0-100 µM of (A) cannflavin A or (B) cannflavin B in a matrix assay for 24 hours. Cell viability was measured using an AlamarBlue assay. Dose-response curves of CBD with 3 different concentrations of cannflavins selected from the matrix are presented. Hollow points represent cannflavin effects alone. Data presented is a mean ± SEM of at least 3 independent experiments. One-way ANOVA was used to assess differences between flavonoid or CBD treatments alone and combination treatments.

3.11 Cannflavins combined with paclitaxel elicited synergistic and additive inhibitory effects on cell viability in PR MDA-MB-231 cells

This study previously explored whether the combination of cannflavin A and B and a constant 470 nM concentration of paclitaxel reduced the IC_{50} of cannflavins in PR MDA-MB-231 cells (Figure 1). Results showed that there was no significant difference in IC_{50} following co-treatment with a constant dose of paclitaxel. To further evaluate the interaction between paclitaxel and cannflavins A and B the dose-response matrix assay was performed. This allowed more concentrations of paclitaxel to be assessed and determine if synergy was occurring between the compounds at concentrations lower or higher than 470 nM. Being able to potentially lower a dose of the chemotherapeutic agent could help reduce adverse effects and promote better compliance and continuation of treatment regimens. PR MDA-MB-231 cells were treated with combinations of 0-100 µM of cannflavin A or cannflavin B and 0-60 µM of paclitaxel, cell viability was measured using an AlamarBlue® assay. Data was entered into Synergy Finder (2.0) to determine if synergistic combinations were present and presented graphically to allow for statistical analysis. Results showed that some synergistic combinations were present particularly at low concentrations of cannflavins and across a range of paclitaxel concentrations (Figure 3.11). In cells treated with the combinations of paclitaxel and cannflavin A, 0.81 µM paclitaxel and 0.41 µM cannflavin A were both synergistic and significantly reduced cell viability compared to either compound alone. The combination reduced cell viability to 63.5% ± 7.44 compared to 94.0% ± 7.28 (paclitaxel) and 98.4% ± 2.31 (cannflavin A) alone (Figure 3.11 A). Additionally, the combination of 1.625, 3.25, 7.5, 15, 30, and 60 µM paclitaxel with 3.7µM cannflavin A acted both synergistically and significantly

reduced cell viability compared to either compound alone. The combinations of 3.25, 7.5, 15, 30, and 60 μM paclitaxel with 33.3 μM cannflavin A produced synergistic effects and significantly reduced cell viability compared to either compound alone (Figure 3.11A). In cells treated with the combinations of paclitaxel and cannflavin B, 0.051, 0.41, 0.81, 1.625, 3.25, 7.5, 15, 30, 60 μM paclitaxel and 0.41 μM cannflavin B were both synergistic and significantly reduced cell viability compared to either compound alone (Figure 3.11 B).

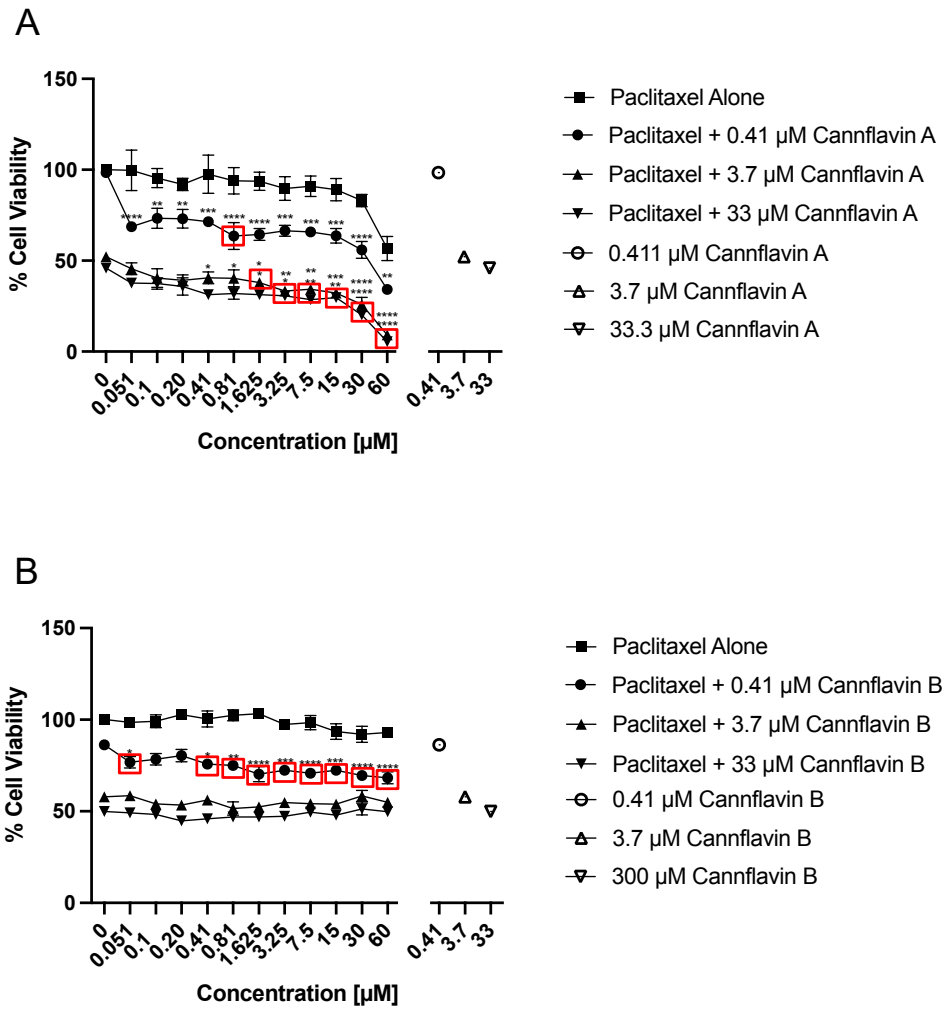

Figure 3.11 Effects of cannflavin A and cannflavin B co-treatment with paclitaxel on PR MDA-MB-231 cell viability. Cells were treated with 0-60 μM of paclitaxel and 0-100 μM of (A) cannflavin A or (B) cannflavin B in a matrix assay for 24 hours. Cell viability was measured using an AlamarBlue assay. Dose-response curves of paclitaxel with 3 different concentrations of cannflavins selected from the matrix are presented. Hollow points represent cannflavin effects alone. Red boxes indicate concentration combinations that are synergistic as determined by the bliss independence model using SynergyFinder 2.0 software and that are significantly different from paclitaxel or cannflavin treatment alone. Data presented is a mean ± SEM of at least 3 independent experiments. One-way ANOVA was used to assess differences between flavonoid or paclitaxel treatments alone and combination treatments *p<0.05, **P<0.01, ***p<0.001, ****p<0.0001.

3.12 Cannflavins combined with paclitaxel did not elicit synergistic or additive inhibitory effects on cell viability in PR MCF-7 cells

To assess whether cannflavin A and cannflavin B acted synergistically with paclitaxel to reduce PR MCF-7 cell viability a dose-response matrix assay was performed. PR MCF-7 cells were treated with combinations of 0-60 μM CBD and 0-100 μM of cannflavin A or cannflavin B, cell viability was measured using an AlamarBlue® assay. Data was entered into the Synergy Finder (2.0) tool to determine if synergistic combinations were present and presented graphically to allow for statistical analysis of cell viability. Following analysis, it was determined that some synergistic combinations were present when paclitaxel and cannflavin A and cannflavin B were combined, with the highest scores ranging from 30-40. There were no combinations of compounds that significantly lowered cell viability compared to each compound alone (Figure 3.12A,B).

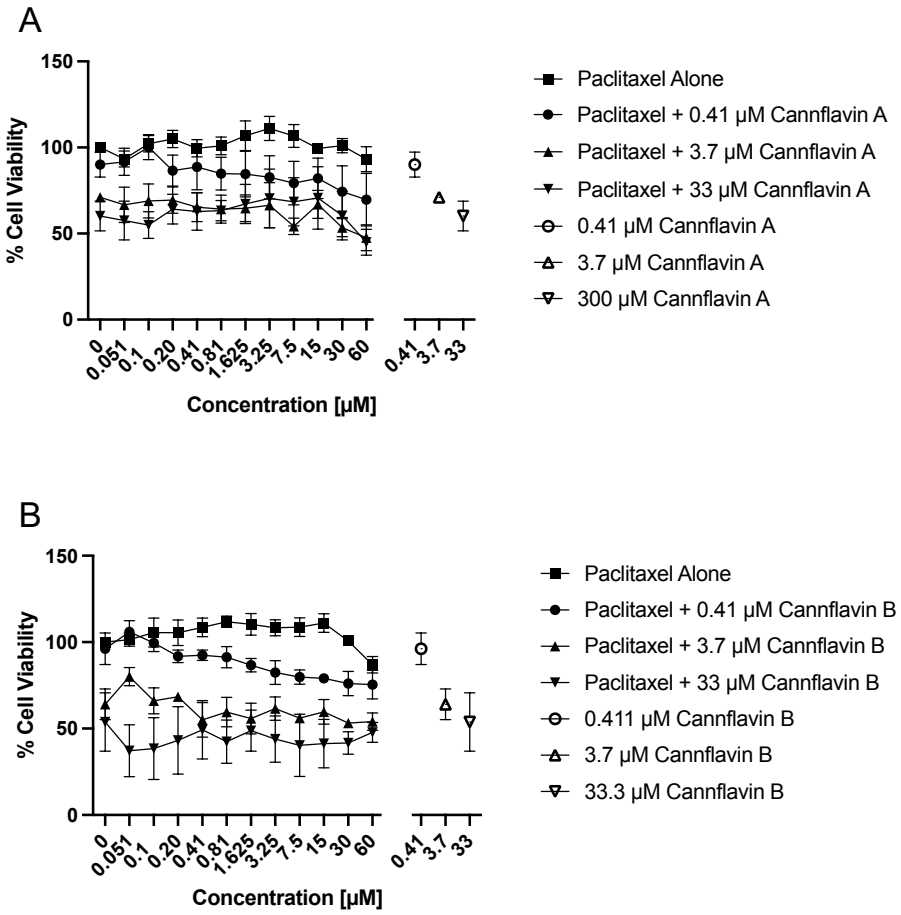

Figure 3.12 Effects of cannflavin A and cannflavin B co-treatment with paclitaxel on PR MCF-7 cell viability. Cells were treated with 0-60 µM of paclitaxel and 0-100 µM of (A) cannflavin A or (B) cannflavin B in a matrix assay for 24 hours. Cell viability was measured using an AlamarBlue assay. Dose-response curves of paclitaxel with 3 different concentrations of cannflavins selected from the matrix are presented. Hollow points represent cannflavin effects alone. Red boxes indicate concentration combinations that are synergistic as determined by the bliss independence model using SynergyFinder 2.0 software and that are significantly different from paclitaxel or cannflavin treatment alone. Data presented is a mean ± SEM of at least 3 independent experiments. One-way ANOVA was used to assess differences between flavonoid or paclitaxel treatments alone and combination treatments *$p<0.05$.

Chapter 4: Discussion

4.1 Overview

Breast cancer is the most commonly diagnosed cancer in women, impacting 1 in 8 Canadian women in their lifetime. Although survival rates have increased due to improved screening, quicker diagnosis, and therapeutic advances some patients still face a poor prognosis and will ultimately die as a result of their diagnosis (Canadian Cancer Statistics Advisory Committee et al., 2021). Particularly, patients diagnosed with metastatic breast cancer or TNBC have limited treatment options. In patients with metastatic breast cancer surgery to remove the tumor is no longer an option as the cancer has migrated to sites distant to the primary tumor site. For patients with TNBC, a highly aggressive subtype of breast cancer, targeted therapies such as hormone therapy or immunotherapy are not feasible due to the receptor status of the tumor (ER, PR, and HER2 negative). For both of these patient groups, chemotherapy may be the only treatment option (Waks & Winer, 2019).

For high-risk patients a chemotherapy regimen (Tax-AC) consisting of an anthracycline such as doxorubicin and a taxane such as paclitaxel or docetaxel is often used (Waks & Winer, 2019). These compounds produce negative systemic side-effects including cardiotoxicity, myelosuppression, tissue necrosis, and febrile neutropenia to name a few. These can reduce the quality of life of patients and may require other drugs to overcome them which each come with their own set of side-effects (Nicoletto & Ofner, 2022). Unfortunately, chemotherapy may not work at all in some patients. Chemotherapy has a variable response rate in patients due to tumor heterogeneity and previous

chemotherapy exposure, which means that not all treatments will have the same efficacy and efficiency in each patient, and some may require higher doses or more rounds of treatment (Prihantono & Faruk, 2021). Innate or acquired chemotherapeutic resistance can occur. Resistance to more than one chemotherapeutic can occur, known as multidrug resistance, leaving patients with even fewer treatment options particularly if it occurs in patients with metastatic breast cancer or TNBC (Lainetti et al., 2020).

As previously mentioned, chemotherapy is associated with negative side effects and cannabis may be used by patients to help overcome some of these unwanted side-effects or ones associated with the tumor itself (Abrams & Guzman, 2015). The endocannabinoid system plays a role in pain sensation, mood, and appetite, which are three concerns cancer patients often have. Cannabinoids, including THC, CBD, and to some extent CBG have been shown to mediate cancer related pain, anxiety, increase appetite, improve sleep, and reduce chemotherapy related nausea and vomiting (Sexton et al., 2021). Other compounds found in cannabis such as terpenes and flavonoids have also been shown to have effects. The terpenes beta-caryophyllene which binds the CB2 receptor and linalool that potentiates GABAa have both been shown to reduce anxiety in *in vivo* models and in human studies respectively (Klauke et al., 2014; Linck et al., 2010; Malcolm & Tallian, 2017). Flavonoids luteolin and apigenin have been shown to reduce neuropathic pain and reduce gastrointestinal inflammation and have been suggested to alleviate some gastrointestinal problems related to chemotherapy respectively (Fernández et al., 2021).

In addition to improving patients' quality of life by mediating negative side effects, compounds found in cannabis have been shown to exert anti-cancer effects themselves in preclinical models. The cannabinoids THC and CBD have been studied the most extensively in a variety of cancer types including breast cancer and exert anti-proliferative effects. In our lab we have previously demonstrated that synthetic cannabinoids, other minor phytocannabinoids, and terpenes are able to exert cytotoxic effects, induce apoptosis, and reduce invasiveness of breast cancer cells and these results have been shown in *in vitro* and *in vivo* models of other cancer types as well (A. Tomko et al., 2019; A. M. Tomko et al., 2020; A. M. Tomko, Whynot, O'Leary, et al., 2021; Whynot, 2021). Flavonoids found in cannabis and other plants have also shown anti-cancer effects however, not all flavonoids in cannabis have been studied to the same extent and there is a lot left to learn about these compounds (Kabała-Dzik et al., 2018). Flavonoids' role in mediating chronic inflammation, anti-proliferative and pro-apoptotic effects, anti-metastatic properties, and potential to chemosensitize cancer cells highlights their potential to act as a novel breast cancer treatment (A. M. Tomko et al., 2020).

Chemotherapeutic resistance, negative side effects associated with chemotherapy, and the variable response rate of chemotherapy between patients highlight the need for novel therapies to be investigated to help treat breast cancer. This study's objective was to investigate the anti-cancer effects of flavonoids found in cannabis in preclinical *in vitro* models of paclitaxel-resistant breast cancer. We aimed to determine what flavonoids in cannabis exerted dose-dependent cytotoxicity and found that cannflavin A and cannflavin B were cytotoxic. Minimal studies have investigated their anti-cancer effects – due to

their novelty and cytotoxicity these compounds were evaluated further. We then determined cannflavin A and B's effects on apoptosis and autophagy induction, and invasion. Following their characterization individually the cannflavins were combined with the cannabinoids THC and CBD and evaluated for their potential synergistic effects on cell viability as other compounds in cannabis have been shown to act synergistically together (Whynot, 2021). Cannflavins were then combined with the chemotherapeutic paclitaxel to determine if they could chemosensitize the resistance cells to the agent or if they could act synergistically with a chemotherapeutic as other flavonoids have been shown to do in the literature (J. H. Lee et al., 2020b; S. Liu et al., 2020; X. Zhang et al., 2020a; Zhou et al., 2020b).

4.2 Cytotoxicity *in vitro*

4.2.1 Chemotherapeutic resistant breast cancer cells

The first step in evaluating novel compounds for cancer therapeutics is determining their effects on cell viability. The ten flavonoids chosen were assessed for their cytotoxic effects using an AlamarBlue® cell viability assay that measures the reducing ability of cells. The AlamarBlue® reagent contains the active compound resazurin which begins as a non-fluorescent blue dye and is reduced to a highly fluorescent pink dye (Rampersad, 2012). This allowed us to determine what compounds were exerting dose-dependent cytotoxic effects and what concentration range these effects were demonstrated. Both PR MDA-MB-231 and PR MCF-7 cells were used, and concentration ranges of flavonoids assessed were between 0-100 μM. These

concentration ranges were chosen based on the literature available at the onset of this study (Kabała-Dzik et al., 2018; J. Lee et al., 2019; Vrhovac Madunić et al., 2018).

We assessed whether the dose-dependent cytotoxicity of the flavonoids seen in our studies was shown in known studies in the literature and compared IC_{50} values when available. We will first discuss the flavonoids only assessed in the initial portion of this study. In PR MDA-MB-231 cells, silymarin, luteolin, quercetin, and apigenin all induced dose-dependent cytotoxic effects and in PR MCF-7 cells silymarin and luteolin exerted dose-dependent cytotoxic effects, these results are consistent with the literature where these compounds were effective in reducing cell viability in breast and other types of cancer cells (Bektur Aykanat et al., 2020; Imran et al., 2019; Magura et al., 2021; Tang et al., 2020) Outside of our study, these compounds have not yet been evaluated in a chemotherapeutic resistant model of breast cancer.

Silymarin reduced cell viability in a dose-dependent manner in colorectal cancer cells, melanoma and epithelial carcinoma cells, and breast cancer cells however IC_{50}s were not reported (Kim et al., 2021; Ramakrishnan et al., 2009; Vaid et al., 2015; Zi et al., 1998). In breast cancer cell lines greater effects were seen in MCF-7 cells than MDA-MB-231 cells. This is in contrast to what we saw in our study where cell viability was reduced to 68% in MDA-MB-231 cells and 77% in MCF-7 cells however the differences between cell lines seen were much smaller in our results than those reported in the literature. Previous studies have shown that luteolin is able to reduce cell viability in numerous cancer types *in vitro* (Magura et al., 2021; Potočnjak et al., 2020). In breast

cancer cells lines IC_{50}s reported in the literature in MDA-MB-231 cells were two times greater, at 31 and 27 μM, than those found in our study (Monti et al., 2020; Wu et al., 2021).

Quercetin was a flavonoid that had dose-dependent effects only in PR MDA-MB-231 cells with an IC_{50} of 3.7 μM. This compound has been shown to reduce breast cancer cell viability throughout the literature however reported IC_{50}s were approximately 30 times higher. Additionally we reported a 25% decrease in cell viability following 10 μM treatment but similar reductions were not shown until 60 μM treatment in the literature (Kabała-Dzik et al., 2018; Safi et al., 2021). In PR MDA-MB-231 cells we saw that apigenin reduced the cell viability to approximately 70% in a concentration-dependent fashion. A study by Madunic et al. has shown that apigenin could reduce cell viability in both MDA-MB-231 cells and MCF-7 cells to approximately 40% cell viability with IC_{50}s of 55 and 38 μM in each cell line respectively – significantly higher than reported in our assay. One difference between the studies was that our cells were treated for 24 hours while Madunic et al. treated the cells for three times as long at 72 hours (Vrhovac Madunić et al., 2018). This could explain why greater reductions in cell viability were seen.

Both cannflavin A and B reduced cell viability in a dose-dependent manner and the addition of 470 nM paclitaxel had no significant effect on the IC_{50} in either cell line (Figure 3.1.1-3.1.2). To date no studies have been done to assess the effects of cannflavin A in cancer cells however one study has assessed its neurotoxic effects in PC-12 cells. In

the PC-12 cells cannflavin A reduced cell viability in a dose-dependent manner to a maximum of 50% viability with a 100 μM treatment however no IC_{50} value was reported (Eggers et al., 2019). This is comparable to what was seen in the PR MDA-MB-231 cells and a slightly higher cell viability than was seen in the PR MCF-7 cells (Figure 3.1.1-3.1.2). Although cannflavin B has not yet been evaluated for its effects on cell viability, a derivative of cannflavin B called isocannflavin B has been evaluated. IsoB reduced the cell viability of T47-D breast cancer cells at concentrations greater than 25 μM but this effect was not seen in MDA-MB-231 breast cancer cells unlike in our study (Brunelli et al., 2009). It is possible that this disparity between our results and the results found by Brunelli et al. is due to the structural difference between isocannflavin B and cannflavin B at the C-8 location which could alter its receptor interactions or lipophilicity. Additionally, isoB reduced survival of two pancreatic cancer cell lines when combined with a radiation dose. *In vivo* when delivered with a smart radiotherapy biomaterial isocannflavin B significantly reduced local pancreatic tumor size when co-administered with radiotherapy and without co-administration (Moreau et al., 2019).

One potential reason for the discrepancy between IC_{50}s found in our study and those in the literature was the way the IC_{50} was calculated. In the cell viability assay concentrations up to 100 μM were unable to reduce cell viability all the way to zero and in some cases did not reduce cell viability to 50%, because of this an absolute IC_{50} was not calculated. Absolute IC_{50} is the concentration when 50% cell viability is achieved. Instead a relative IC_{50}, the concentration at which 50% of the maximal achieved effect was observed, was calculated. This allowed comparisons between cells treated with

flavonoid alone or flavonoid plus paclitaxel and helped determine what concentrations to use for future experiments but made it difficult to compare between our compounds or to the literature. For example, in PR MDA-MB-231 cells silymarin alone had a relative IC_{50} of 9.3 μM and luteolin had a relative IC_{50} of 16.8 μM however their maximal inhibition on cell viability achieved were very different. Silymarin reduced cell viability to 67% meaning 9.3 μM achieved 50% of this effect while luteolin reduced cell viability to 23% meaning 16.8 μM achieved 50% of this effect. In the literature when cell viability is not reduced to zero and an absolute IC_{50} is not calculated it is not possible to compare these values, rather comparing concentration ranges used and effects on cell viability at specific concentrations is a better way to evaluate our results in the context of the literature.

4.2.2 Effects of flavonoids in non-cancerous breast epithelial cells

A key consideration when evaluating novel compounds for cancer therapy is their effects on non-cancerous cells. Chemotherapy is known for having negative side-effects throughout the body due to its non-specific action. Typically, chemotherapy acts on rapidly dividing cells which include the cells within the tumor but also hair follicles, and cells in the gastrointestinal tract, reproductive system, and bone marrow resulting in the unwanted side effects such as hair loss, myelosuppression, and nausea, vomiting and diarrhea (Abrams & Guzman, 2015). When evaluating new drugs, non-tumorigenic cell lines are often used *in vitro* to evaluate whether the drug is cytotoxic in normal cells in addition to cancer cells. In breast cancer studies the most commonly used cell line to model 'normal' breast cells is the MCF-10A cell line (Qu et al., 2015). These cells are estrogen receptor negative and are considered non-tumorigenic although they have been

immortalized like many cancerous cell lines. A 2015 study by Qu et al. evaluated their strength as a representative model for non-tumorigenic breast cells and concluded that in 2D *in vitro* conditions they possessed a basal-like phenotype similar to non-cancer breast cells (Qu et al., 2015).

It has been documented in the literature as well as in previous studies in our lab that some compounds found in cannabis are able to have cytotoxic effects on cancer cell lines while being non-toxic to non-tumorigenic cell lines. We have previously found that some synthetic cannabinoids, phytocannabinoids, and some terpenes were able to exert cytotoxic effects in MDA-MB-231 and MCF-7 cells while not significantly reducing the cell viability of non-tumorigenic MCF-10A cells (A. Tomko et al., 2019; A. M. Tomko, Whynot, O'Leary, et al., 2021; Whynot, 2021). Selectivity of cytotoxicity towards breast cancer cells has been reported in a number of flavonoids (Bartmańska et al., 2018; Razak et al., 2019). A flavone eupatorine has been shown to exert cytotoxic effects in MDA-MB-231 and MCF-7 cells with IC_{50}s six times lower than those required in non-tumorigenic MCF-10A cells to achieve similar effects demonstrating the compounds selective nature towards cancer cells (Razak et al., 2019). The prenylated flavonoid isoxanthohumol found in hops also exerted anti-proliferative effects in breast, prostate, and colon cancer cell lines. When breast cancer and MCF-10A cell lines were compared, isoxanthohumol possessed a selectivity between 5 and 10 times greater for the cancer cells compared to the non-tumorigenic cells (Bartmańska et al., 2018).

In this study MCF-10A cells were treated with maximal concentrations (either 50 or 100 μM) of flavonoid shown to exert dose-dependent cytotoxic effects in the cell viability assays with PR MDA-MB-231 and MCF-7 cells. Cannflavin A, cannflavin B, and apigenin did not significantly reduce the cell viability of MCF-10A cells while silymarin, quercetin, and apigenin did significantly reduce MCF-10A cell viability (Figure 3.3). This supports that at least some flavonoids are capable of preferentially killing cancer cells compared to non-tumorigenic cells. It is unclear why some flavonoids are able to preferentially kill cancer cells and what mechanisms are underlying this result. Interestingly other flavonoids like silymarin appear to preferentially reduce MCF-10A cell viability for example: silymarin reduces cell viability to 37% in MCF-10A cells compared to 72% in the PR MDA-MB-231 cells.

Cancer cells have differential expression of receptors compared to non-cancerous cells and metabolic pathways may be upregulated or downregulated resulting in their tumorigenicity. These differences between cancer and non-cancer cells could provide an explanation for why some flavonoids tested exert cytotoxicity preferentially in MCF-10A cells. 5-lipoxygenase is an enzyme responsible for producing signaling molecules known as eicosanoids and their metabolites have been shown to be mediators of inflammation in cancer. The enzyme 5-lipoxygenase is often constitutively overexpressed in many cancer types including breast cancer and has been suggested as a regulator of the tumor microenvironment during tumor progression (Jiang et al., 2006). Inhibitors of 5-lipoxygenase have demonstrated anti-proliferative and pro-apoptotic effects in several cancer types *in vitro* including prostate and pancreatic cancer and have been a focus of

anti-cancer research due to their preferential effects on cancer cells (Ding et al., 1999; Sarveswaran et al., 2011). Interestingly both cannflavin A and cannflavin B have known inhibitory actions on 5-lipoxygenase resulting in their reported anti-inflammatory effects (Werz et al., 2014). Their inhibitory action on 5-lipoxygenase could provide an explanation for why cannflavins A and B are cytotoxic in PR MDA-MB-231 and PR MCF-7 cells but non-toxic in MCF-10A cells.

Another known inhibitory target of cannflavin A and B is microsomal prostaglandin E2 synthase, an enzyme responsible for producing prostaglandin E2 (Barrett et al., 1985). COX enzymes convert arachidonic acid to a precursor molecule prostaglandin H2 which is then converted to prostaglandin E2 and 3 other prostaglandin subtypes by prostaglandin E2 synthase implicating upstream COX enzymes (COX-1 and COX-2) in prostaglandin E2 effects. COX-2 plays an important role in the inflammatory response related to cancer and has been shown to be upregulated in breast cancer subtypes associated with poor prognosis and is essentially undetectable in normal tissues. Due to its upstream relation to prostaglandin E2 synthase and prostaglandin E2 it suggests their increased expression as well (Reader et al., 2011). Prostaglandin E2 is also a mediator of inflammation and has been shown to play a role in colorectal and breast cancer cell tumorigenesis, however its exact action is unknown (Mizuno et al., 2019; Reader et al., 2011).Prostaglandin E2 also interacts with immune cells in the tumor microenvironment to promote a pro-cancer phenotype in macrophages and neutrophils and suppress T cell and NK cell responses (Mizuno et al., 2019). Inhibition of prostaglandin E2 synthase would reduce the amount of prostaglandin E2 produced,

reducing its pro-cancer effects. Cannflavin A and cannflavin B's inhibitory effect on prostaglandin E2 synthase and the enzymes' potential increased expression in cancer cells could provide another explanation for why cannflavins exerted anticancer effects in cancer cell lines but were not cytotoxic in MCF-10A cells.

4.3 Cannflavins induce apoptosis and promote autophagy in PR breast cancer cells

4.3.1 Apoptosis

After determining that some of the flavonoids tested were inducing dose-dependent cytotoxicity in PR breast cancer cells and at what concentration ranges, and which compounds were not cytotoxic in MCF-10A cells we wanted to determine how these compounds were killing the PR breast cancer cells. Cannflavin A and cannflavin B were chosen to proceed with further investigation for several reasons. First, these two compounds produced some of the greatest reductions in cell viability. They reduced PR MDA-MB-231 cell viability to 37% and 27% following cannflavin A and cannflavin B treatment respectively (Figure 3.1). In PR MCF-7 cells cannflavin A reduced cell viability to 38% and cannflavin B reduced cell viability to 19% (Figure 3.2). Secondly, they did not produce significant cytotoxic effects in non-tumorigenic MCF-10A cells (Figure 3.3). Lastly, cannflavin A and cannflavin B are novel compared to the other compounds investigated in this study. These flavonoids are unique to cannabis and limited research has been done about their potential anti-cancer effects. For these three reasons cannflavin A and cannflavin B were chosen for further investigation.

Apoptosis is a controlled form of cell death within the cell and is a desired outcome for anti-cancer agents. Flavonoids found in cannabis have been shown to induce

apoptosis *in vitro* however little is known about the ability of cannflavin A and cannflavin B to induce apoptosis (A. M. Tomko et al., 2020). This study aimed to determine if cannflavins A and B were reducing the cell viability of PR breast cancer cells through the induction of apoptosis. To evaluate this PR MDA-MB-231 cells were treated with 2.5 μM of cannflavin A or cannflavin B for 24 hours and an annexin V apoptosis assay was used to determine if these compounds were increasing rates of apoptosis compared to cells treated with vehicle alone. The concentration 2.5 μM was chosen based on the results from the cell viability assay. This concentration was able to slightly reduce cell viability but was lower than the relative IC_{50}s for both compounds. This would allow some effect to be seen without killing too many cells making determining rates of apoptosis difficult. Following treatment staining, cells that stained positive for annexin V were determined to be going through early apoptosis and cells that stained positive for propidium iodide were determined to be going through late apoptosis or necrosis. Higher rates of cells were stained with annexin V than propidium iodide suggesting that apoptosis is occurring following cannflavin A and cannflavin B treatment (Figure 3.4.1).

Similar to other flavonoids found in cannabis we demonstrated that cannflavin A and cannflavin B were able to induce apoptosis in breast cancer cells. Following vehicle treatment 9% of cells were Annexin V positive compared to 38% of cannflavin A treated cells and 24% of cannflavin B treated cells. Low levels of propidium iodide positive cells were present and similar across all treatment conditions indicating these are basal levels unrelated to the treatment. As previously mentioned, other flavonoids found in cannabis

have been shown to induce apoptosis in a variety of cancer cells. Kaempferol induced apoptosis in breast, ovarian, and cervical cancer cells, and in leukemia and glioma cells (Abotaleb et al., 2018). In ovarian carcinoma cells apoptosis was mediated by an upregulation in expression of proteins including p53, Bax, and caspase-3 and a downregulation in Bcl-2 expression (Luo et al., 2011). In another study MCF-7 breast cancer cells treated with quercetin underwent apoptosis and the expression of Bax was increased and Bcl-2 was decreased (L. Zhang, 2012). Our study revealed similar alterations in apoptosis related protein expression following cannflavin A and B treatment through western blotting. We saw an increase in expression of the pro-apoptotic protein Bax and a decrease in the expression of the anti-apoptotic protein Bcl-2 compared to vehicle-treated cells (Figure 3.4.2). These two proteins are involved in the intrinsic mitochondrial mediated apoptosis pathway suggesting cannflavins A and B are inducing apoptosis through this pathway, however, their exact mechanism is still unclear (Pistritto et al., 2016).

4.3.2 Autophagy

Autophagy is a cellular process that can be involved in tumor suppression or tumor promotion (Yun & Lee, 2018). Basal levels of autophagy have been shown to act as a tumor suppressor to manage damaged cells and regulate homeostasis. Studies have shown that the knockdown or knockout of core autophagy related proteins (ATGs) are associated with oncogenesis indicating the important role autophagy plays in tumor suppression (Yun & Lee, 2018). We evaluated the effects of cannflavin A and cannflavin B on autophagy using two different methods. The first was assessing cell viability of PR

breast cancer cells when co-treated with cannflavins and known inhibitors of different parts of the autophagy pathway. We found that co-treatment of 2.5 μM cannflavin A or B with a combination of E-64d and pepstatin A significantly reversed cannflavin induced cytotoxicity (Figure 3.5.1). E-64d and pepstatin A are both inhibitors of autophagy that act by suppressing lysosomal proteases, specifically lysosomal cathepsins which participate in the degradation of autophagic bodies within the cell following fusion with the lysosome (Yang et al., 2013). Because these inhibitors prevent the autophagosome from being degraded following fusion with the lysosome, the last step in the autophagy pathway, this suggests that autophagy must already be occurring following cannflavin treatment. This could implicate autophagy in cannflavins' cytotoxic effects.

Several studies have shown that cancer cells upregulate cathepsin-D (a lysosomal protease) following treatment with cytotoxic agents. This upregulation leads to an increase in apoptosis and subsequently a decrease in cell viability. A study by Beaujoine et al. demonstrated that the lysosomal protease inhibitor pepstatin A had no effect on the increase in apoptosis seen. They then suggested that the overexpression of cathepsin-D did not have an effect on the catalytic activity of the enzyme rather that it was interacting with apoptosis related proteins (Beaujouin et al., 2006). A more recent study by Zhang et al. found that a polyphenol increased autophagy related apoptosis through increased cathepsin-D release from the lysosome in drug resistant leukemia cells (Z. Zhang et al., 2018) Following pre-treatment with the autophagy inhibitor 3-methyladenine, a reduction in apoptosis related cell death was observed. Additionally, results showed that apoptosis was induced through the intrinsic pathway as demonstrated by increased expression of

Bax and decreased expression of Bcl-2 (Z. Zhang et al., 2018). These results could provide an explanation for the role of cannflavins in apoptosis and autophagy. It is possible that cannflavin A and cannflavin B are increasing cathepsin-D expression and its release into the cytosol through increased lysosomal membrane permeability and that this is promoting the increased rates of apoptosis observed in our study. In contrast to the previously mentioned studies, our study showed that lysosomal protease inhibitors reduced the cytotoxic effects of cannflavins. This suggests that it may be more likely that it is an increase in the catalytic activity of cathepsin-D that is causing the increase in cell death following cannflavin treatment. The increase in Bax and decrease in Bcl-2 expression implicating the intrinsic apoptosis pathways seen in the study by Zhang et al. is similar to what our study showed following cannflavin treatment. This suggests that cannflavin A and cannflavin B could be promoting autophagy-dependent apoptosis and that it is possibly mediated by cathepsin-D.

The second method used to determine if autophagy was involved in the cytotoxicity of cannflavins A and B was RT-qPCR. We quantified the expression of four genes related to the autophagy pathway (ATG5, ATG7, ATG12, and LC3b) to determine if cannflavins A and B altered their expression compared to cells treated with vehicle control at two different treatment time points, 6 hours and 12 hours. These time points were chosen because 6 hours was used for the autophagy inhibitor assay and results indicated autophagy was occurring at that time point, 12 hours was also chosen to help further determine what effects cannflavins were having on later parts of the autophagy pathway. Our results revealed that following 6 hours of treatment cannflavin A increased

the gene expression of ATG5, ATG12, an LC3b and cannflavin B treatment increased the expression of only ATG12. Following 12 hours of treatment cannflavin A increased the expression of LC3b (Figure 3.5.2).

Autophagy consists of four main steps, initiation, formation of the phagophore, membrane expansion, and maturation to the autophagosome which will then fuse with the lysosome (Yu et al., 2018). As previously mentioned, a number of proteins are involved in different steps of this process. In the membrane expansion phase the four proteins investigated in this study play a role in creating an autophagosome. ATG5 and ATG12 form a complex with several other autophagy related proteins that is mediated by ATG7. The downstream effects of these proteins and their interactions is the conjugation of LC3b with phosphatidylethanolamine known as LC3 lipidation contributing to the autophagosome membrane (Hurley & Young, 2017). Increased expression of ATG5, ATG12 and LC3b following 6-hour cannflavin A treatment and even greater LC3b expression following 12 hours of treatment could indicate that cannflavin A is inducing autophagy. Following 6 hours of treatment early membrane expansion may be occurring and autophagosome maturation may occur following 12 hours of treatment due to the increased expression of LC3b. Following 6-hour cannflavin B treatment only ATG12 gene expression was significantly increased and at 12 hours there were no significant increases in gene expression compared to vehicle control. This does not necessarily mean that autophagy is not occurring because although the changes are not significant there are similar increases to cannflavin A in ATG5 and LC3b expression following 6-hour treatment and increases in LC3b expression following 12 hour treatment (Figure 3.5.2).

Many studies have shown that flavonoids are capable of inducing autophagy resulting in apoptosis as we have suggested here in our study. Apigenin induced autophagy in colorectal cancer cells resulting in apoptosis by targeting the m-TOR pathway (X. Chen et al., 2019). In breast cancer cells the flavonoid baicalein has been shown to induce autophagic cell death (Yan et al., 2018). Our study demonstrates the first observation of potential autophagy-mediated apoptosis by cannflavin A and cannflavin B however more investigation is needed to fully understand the mechanism underlying these effects.

4.4 Cannflavins reduce invasiveness of PR breast cancer cells

Metastasis is defined as the development of secondary tumor growths at a location distant to the primary tumor site (Kozłowski et al., 2015). Approximately 90% of cancer related deaths occur due to metastasis and it can develop even decades after the initial tumor occurs. Certain breast cancer subtypes including the highly aggressive TNBC have a higher risk of metastasis occurring which can lower five-year survival rates to about 25%, significantly lower than patients with non-metastatic breast cancer (Kozłowski et al., 2015). Unfortunately, metastasis and its available treatment options remain fairly misunderstood because it is a complex, multistep event. Five main steps contribute to the development of metastatic cancer: invasion into tissue surrounding the primary tumor, intravasation, circulation, extraversion, and growth at a new tissue site (Steeg, 2006). Following the first step, invasion, it is difficult to stop the process of metastasis, therefore preventing invasion pharmacologically may provide a pathway to prevent metastasis and improve patient survival. Flavonoids have been shown to reduce invasiveness in cancer cells including ovarian, esophageal, prostate, colorectal, bladder, breast, and glioblastoma

(X. Chen et al., 2019; Han et al., 2016; Imran et al., 2019; Kollareddy & Martinez, 2021; S. Liu et al., 2020; Tavsan & Kayali, 2019).

Little is known about the anti-invasive effects of flavonoids in chemotherapeutic resistant cancer cells or about the anti-invasive properties of cannflavin A or cannflavin B. We aimed to assess the anti-cancer potential of cannflavin A and cannflavin B in our PR breast cancer cell lines. To do this we used a Matrigel invasion assay and cannflavins at a concentration of 2.5 μM to determine this *in vitro.* This concentration was used for the same reason as the apoptosis assay, slight cytotoxic effects were seen at this concentration allowing enough cells to live to detect differences between treatment conditions. We found that both cannflavin A and cannflavin B had anti-invasive properties and significantly reduced the number of cells invaded through the Matrigel compared to vehicle control (Figure 3.6). Under vehicle conditions approximately 25% of cells invaded through the matrigel, while both cannflavins reduced the percentage of cells invading through the Matrigel to approximately 15%. This is the first report of cannflavins A and B reducing the invasive capability of cancer cells *in vitro*.

The mechanism underlying the anti-invasive properties of flavonoids is not entirely known. Proteins associated with the extracellular matrix degradation such as matrix metalloproteinases (MMP) have been implicated in the invasion of cancer cells (Castro-Castro et al., 2016). The flavonoid quercetin decreased protein expression of MMP9 and MMP2 in esophageal carcinoma cells and decreased expression of vascular endothelial growth factor (VEGF) and MMP9 and MMP2 in osteosarcoma cells (Lan et

al., 2017; S. Liu et al., 2020). VEGF is a signaling protein responsible for the growth of new blood vessels and plays a role in epithelial to mesenchymal transition (EMT). EMT is the process by which cancer and other cells lose their epithelial properties and gain mesenchymal properties. Cancer cells then lose their polarization and have increased motility often leading to metastasis (L. Chen et al., 2020). Other flavonoids have been shown to inhibit EMT through inhibition of MMPs and VEGF and could provide an underlying mechanism to cannflavin A and B's anti-invasive properties however more research needs to be done to evaluate these mechanisms (Magura et al., 2021).

4.5 Potential for synergy between cannabinoids in cannflavins

Compounds found in cannabis have been previously shown to exert greater cytotoxic effects in cancer models compared to individual compounds alone (Blasco-Benito et al., 2018). Combinations of cannabinoids with other cannabinoids were shown to produce synergistic anti-cancer effects and combinations of terpenes with cannabinoids have also produced enhanced anti-cancer effects (Russo, 2016). Additionally, a study under review in our lab has shown that cannflavin A combined with THC or CBD produced synergistic and additive anti-proliferative effects in bladder cancer cell lines (A. M. Tomko, Whynot, & Dupré, 2021). We wanted to evaluate the potential synergistic effects of cannflavins A and B when combined with THC or CBD in PR breast cancer cell lines. These compounds were chosen because they are the most extensively studied cannabinoids and previous studies in our lab have demonstrated their synergistic potential when combined with the lesser studied phytocannabinoids (Whynot, 2021).

We used a matrix assay in PR MDA-MB-231 and PR MCF-7 cells to evaluate the following combinations: cannflavin A + THC, cannflavin A + CBD, cannflavin B + THC, and cannflavin B + CBD. Synergy scores were calculated using the Bliss Independence model in Synergy Finder (2.0) software. Synergy scores greater than 10, indicating inhibitory cell viability response 10% or greater than expected were seen for all combinations. However, not all synergistic combinations resulted in statistically significant reductions in cell viability compared to either compound alone. An explanation for this phenomenon is the fact that synergy scores are relative – a score of 20 means a 20% greater effect than expected if the effects of individual compounds were combined. When synergistic scores occur at low concentrations, they often result in minimal inhibitory effects on cell viability that are unlikely to be significantly different that either compound alone at those concentrations. Additionally, combinations can be synergistic and not statistically significant when one compound appears to be responsible for the majority of the effect. Often the combination may show greater effects than expected based on their combination indicating synergy however the combination is not statistically different from one of the individual compounds. In this case although synergy is occurring the combination does not seem to be necessary to achieve the reduction in cell viability and a single drug treatment would often achieve an almost identical result.

In PR MDA-MB-231 cells combinations of 1.25 μM THC with 0.41 μM cannflavin A produced synergistic and statistically significant reductions in cell viability. Combinations of 0.625, 1.25, and 2.5 μM THC with 0.41 μM cannflavin B also resulted in synergistic and statistically significant reductions in cell viability (Figure 3.7.2).

Combinations of cannflavins with CBD did not produce any synergistic and statistically significant reductions in cell viability (Figure 3.7.3). In PR MCF-7 cells no combinations of cannflavins and cannabinoids resulted in synergistic and statistically significant reductions in cell viability (Figure 3.7.4-Figure 3.7.5). Interestingly, synergy was observed following low concentrations of cannflavin and low to moderate concentrations of THC however combinations with CBD were ineffective. It is unfortunate that combinations with CBD are not synergistic and statistically significant because its lack of psychoactive effects would make its use more tolerable than THC. It is still unclear what mechanism underlies cannflavin and THC synergy in cancer cells however our results indicated that the response is variable depending on the concentrations used.

4.6 Potential for synergy between paclitaxel and cannflavins

The combination of flavonoids and known chemotherapeutic agents have been evaluated in numerous studies. Kaempferol was shown to have improved effects when combined with 5-fluorouracil in two studies in colorectal cancer cells (Q. Li et al., 2019; Riahi-Chebbi et al., 2019). When combined with paclitaxel, quercetin exerted improved cytotoxic effects in a prostate cancer model and enhanced the effects of paclitaxel in murine prostate cancer models without inducing additional side effects (X. Zhang et al., 2020b). Quercetin has also shown improved effects when combined with doxorubicin in colon cancer cells and breast cancer cells (S. Liu et al., 2020; Zhou et al., 2020b). Additionally, quercetin was able to resensitize chemotherapeutic resistant cells to docetaxel and inhibited BCRP *in vivo* (Prieto-Vila et al., 2020; Song et al., 2020). Due to the promising effects of flavonoids combined with chemotherapeutic agents in the

literature we wanted to further evaluate the effects of cannflavins A and B when combined with paclitaxel in PR resistant breast cancer cells.

Our initial study that evaluated the combinations of flavonoids with 470 nM paclitaxel revealed no significant difference between the IC_{50}s for cells treated with flavonoid alone or flavonoid combined with paclitaxel (Figure 3.1-Figure 3.2). We wanted to explore whether a range of different paclitaxel concentrations could act synergistically with cannflavins and therefore performed the matrix dose-response assay as was previously done to assess cannflavin and cannabinoid combinations in PR MDA-MB-231 and PR MCF-7 cells. We found that the combination of paclitaxel with cannflavin A and cannflavin B produced synergistic and statistically significant reductions in cell viability in PR-MDA-MB-231 cells however these effects were not observed in PR MCF-7 cells (Figure 3.11-Figure 3.12). Combinations of 0.81 μM paclitaxel with 0.41 μM cannflavin A resulted in synergistic and statistically significant reductions in cell viability. Additional combinations of 1.625-60 μM paclitaxel with 3.7 and 33.3 μM cannflavin A resulted in synergistic and statistically significant reductions in cell viability (Figure 3.11). Combinations of 0.051 and 0.41-60 μM paclitaxel with 0.41 μM cannflavin B resulted in synergistic and statistically significant reductions in cell viability (Figure 3.12).

At this time there are no other published studies assessing the anti-proliferative effects of cannflavins when combined with paclitaxel and our study shows that there is promise for cannflavin and chemotherapeutic combinations. Our results show that with

the exception of one combination of cannflavin B and paclitaxel, promising synergistic and statistically significant combinations occur at paclitaxel concentrations greater than 470 nM. A key concern when treating patients with chemotherapy is the unwanted negative side effects. Understandably, as chemotherapeutic doses increase side effects can worsen and reduce a patient's quality of life. The ability to reduce the dose of chemotherapy while maintaining the desired cytotoxic effects though combination therapy would benefit the patient and the potential for paclitaxel to be combined with cannflavins had been shown in our study in difficult to treat chemotherapy resistant cells.

4.7 Limitations and future directions

A few key limitations are present in our study and need to be considered before conclusions about our results can be made. First, our cell viability assays did not use concentrations of flavonoids that reduce cell viability to 0%. This was done because we determined that concentrations above 100 μM were not pharmacologically relevant or did not correspond with concentration ranges found in the literature. This resulted in the inability to calculate absolute IC_{50} values and relative IC_{50} values had to be calculated instead based on pharmacological guidelines (Sebaugh, 2011). These results should be interpreted and compared to results outside of this study with caution, however, the relative IC_{50}s calculated provided beneficial information within this study itself by aiding in evaluating concentrations to use for apoptosis, autophagy, and invasion studies, and deciding to assess for synergy between cannflavins and paclitaxel using a matrix dose-response assay.

During our evaluation of apoptosis using the Annexin V assay cell counting using a fluorescence microscope was used. An alternative method to determine rates of apoptosis would have been to use fluorescence activated cell sorting (FACS) to minimize the potential for human error when counting; however, this method would be more expensive than the method we employed (Wallberg et al., 2016). Additionally, FACS would have been unlikely to change our overall result determined from the Annexin V experiment. We were less concerned about the exact rates of apoptosis that were occurring and simply wanted to assess whether apoptosis was occurring or not, similar results would have been found using either method. Another limitation to assessment of apoptosis was the number of proteins assessed using western blotting. Additional proteins including caspase-3 and p53 could provide a more definitive determination about a more precise apoptotic signaling cascade involved. Although our autophagy assay suggested that autophagy was involved in the cytotoxicity following cannflavin A treatment and RT-qPCR showed upregulations of several autophagy related genes western blotting is needed to confirm whether this upregulation translated to protein expression.

Ultimately, *in vitro* studies alone do not provide an accurate analysis of the anti-cancer effects of cannflavins A and B. The TME is an integral component of cancer progression that cannot be entirely replicated in 2D *in vitro* models. To improve this a 3D spheroid model could be used *in vitro* to model more realistic cell-cell interactions or an air-liquid interface organoid model that more accurately represents the TME and immune cell interactions with the cancer could be used (Feder-Mengus et al., 2008; Neal et al., 2018). Furthermore, *in vivo* studies are required to sufficiently evaluate the anti-cancer

potential of cannflavin A and B alone or in combination with cannabinoids or chemotherapeutics. *In vivo* zebrafish models provide a cost-effective, high-throughput model for screening potential drugs at a variety of concentrations and our lab has employed this method to assess the anti-cancer potential of synthetic cannabinoids previously (A. Tomko et al., 2019). The zebrafish model allows for toxicity and behavioral effects to be assessed before moving to a more costly and time consuming model such as a murine model (Brown et al., 2017). In addition, xenografts of cancer cells can be assessed in zebrafish models allowing the assessment of the compounds of proliferation and migration to be preliminarily assessed. To more accurately assess proliferation and migration/invasion a murine model using patient derived xenograft (PDX) could be evaluated. The murine model allows for a more accurate evaluation than the zebrafish model because the xenograft can be done in murine breast tissue rather than the yolk sac of the zebrafish embryos (Shi et al., 2020). Currently no *in vivo* studies have assessed the anti-cancer effects of cannflavin A or cannflavin B alone or in combination with other compounds and based on the results of our study could be a promising area of research.

One frequent concern that arises when discussing the anti-cancer potential of compounds found in cannabis is whether these effective concentrations are achievable *in vivo* or clinically? By demonstrating the anti-cancer of cannflavins we are not suggesting that patients should consume cannabis whether through inhalation or orally to achieve these anti-cancer effects. Therefore, another route of administration is needed to deliver these compounds to the target breast cancer tissue. One study where rats ingested 213

mg/kg of the flavonoid naringenin twice daily for nine days found that following 5 days a steady state was achieved and a plasma concentration of 17 μM was achieved in tumor bearing rats (Silberberg et al., 2006). This concentration is higher than the IC50 for both cannflavins A and B found *in vitro* in our study. Although delivery of cannflavins to breast tumor tissue may prove difficult, one area to explore could be the use of nanoparticles. Nanoparticles can be designed to specifically target different cell or tissue types throughout the body and to release the encapsulated drug at a specific rate, they also can increase drug solubility and improve the pharmacokinetic and pharmacological properties of the compounds they encapsulate (Jain, 2020). Many preclinical studies have assessed the potential for flavonoid delivery via nanoparticles in *in vitro* and *in vivo* models of melanoma, breast, lung, and liver cancer (Dobrzynska et al., 2020). Different types of nanoparticles including liposomes, micelles, and polymers containing chemotherapeutic agents are currently undergoing clinical trials in cancer types including breast cancer (Z. Li et al., 2017). Although nanoparticles present their own unique challenges that need to be addressed, there is potential for these drug delivering nanoparticles in clinical trials to be modified in the future to encapsulate other compounds such as cannflavins or combinations of compounds that were shown to be beneficial in this study.

4.8 Conclusions

This research could potentially contribute to a greater understanding and the development of novel treatment options for patients with chemotherapeutic resistant breast cancer. Chemotherapeutic resistance and metastasis limit treatment options for

patients and new discoveries that overcome these barriers could help improve patient survival and quality of life. Importantly, this research evaluated the use of cannflavins in combination with other compounds such as chemotherapy, these combinations may allow the dose of chemotherapy to be lowered yet achieve the same beneficial effects. This could reduce the likelihood of chemotherapeutic resistance developing and reduce adverse events as a result of chemotherapy treatment. This study showed that cannflavins A and B were able to reduce the viability of PR breast cancer cell lines in a dose-dependent manner while leaving non-tumorigenic breast cells unaffected. These compounds exerted their effects by inducing apoptosis and autophagy and reducing the invasive properties of the cells *in vitro*. Additionally, specific dose combinations of cannflavins A and B with THC and paclitaxel produced synergistic effects that significantly reduced PR breast cancer cell viability. Although extensive research must be done *in vivo* to further characterize these compounds and make stronger conclusions, this study provides initial insight into the promise of the anti-cancer effects cannflavins A and B and chemotherapeutic resistant breast cancer and their potential improved effects when combined with other compounds.

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