PROFILING PHYTOCHEMICALS FOUND IN LEAF EXTRACTS OF THE APONOGETON MADAGASCARIENSIS AND INVESTIGATING THEIR ANTICANCER EFFECTS IN TRIPLE-NEGATIVE BREAST CANCER CELLS

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.

"We know what we are, but know not we may be"

(Hamlet 4.5.43-44)

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ABSTRACT

Triple negative breast cancer (TNBC) accounts for 10 – 20 % of all breast cancer diagnoses. TNBC is a subtype of cancer that does not have targeted therapies available. Increasing amount of evidence has shown that plant phytochemicals possess anticancer activities. Previous work has demonstrated that phytochemicals extracted from *A. madagascariensis* (lace plant) exhibit apoptotic characteristics in TNBC. The following work has therefore investigated the anticancer activity of leaf extracts from the lace plant in TNBC, MDA-MB-231 cells and profiled phytochemicals in the extracts. As such, this body of work discusses the specific goals, methods, results and an interpretation of the findings in detail including (i) profiling the lace plant metabolome to investigate possible bioactive compounds (ii) investigating the biological effects of the lace plant leaf extracts in preventing TNBC cell migration and (iii) understanding the mechanism of action of cancer cell death. For this, lace plant extracts were prepared from leaves taken from sterile lace plant cultures. The phytochemical profile of the extracts was then analyzed through untargeted and targeted metabolomics using liquid chromatography with tandem mass spectrometry. Anticancer activity was assessed using MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5)-diphenyltetrazolium bromide), CyQUANT® NF cell and wound healing assay to measure cell viability, cell proliferation and cell migration respectively. Intra-cellular ROS was measured using the CM-H_2DCF-DA fluorescent assay. The results of this work show that phytochemicals present in lace plant extracts show promising anticancer activity in the MDA-MB-231 cell line. This work is the first to profile the phytochemical metabolome of the lace plant and test endpoints that showcase the anticancer potential of lace plant extracts. As such, the findings of this work will be relevant in improving our understanding of botanical natural products and their potential to aid in developing novel drugs for the treatment of TNBC.

LIST OF ABBREVIATIONS USED

a.u	absorbance units
AAF	Agriculture and Agri-Food Canada
A.D	Anno Domini
AF4	Apple peel fraction 4
ANOVA	Analysis of variance
AUC	Area Under the Curve
CO_2	Carbon dioxide
Da	Daltons
DAD	Diode array detector
DEG	Differentially expressed genes
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
ESI	Electrospray ionization
FA	Formic acid
FBS	Fetal Bovine serum
GC	Gas chromatography
HPLC	High pressure liquid chromatography
KRDC	Kentville Research and Development Center
LC	Liquid chromatography
MRDC	Morden Research and Development Center

MS	Mass Spectrometry
MS2	Tandem mass spectrometry
NAC	N-acetylcysteine
PCA	Principal Component Analysis
PCD	Programmed Cell Death
PSM	Plant Secondary Metabolites
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RT	Retention Time
SLE	Solid-Liquid Extraction
TNBC	Triple negative breast cancer
UPLC	Ultra-pressure liquid chromatography

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CHAPTER 1: INTRODUCTION

1.1 PLANT METABOLITES

As sessile organisms, plants produce and utilize diverse groups of chemical compounds to maintain growth and development and to increase their chances of survival in challenging environmental conditions (Teoh., 2015; Fernie and Pichersky., 2015). These chemical compounds can be broadly classified into: plant hormones, primary metabolites and secondary metabolites (Elshafie et al., 2023; Erb and Kliebenstein., 2020). Plant hormones are compounds that regulate the production of other plant metabolites through interactions with receptors (Erb and Kliebenstein., 2020). Also referred to as phytohormones, plant hormones play a significant role in plant growth and development including morphogenesis, cell division, sex determination, fruiting, germination and elongation (Pal et al., 2023). Phytohormones such as gibberellin, auxin, abscisic acid, ethylene, cytokinin, jasmonic acid and salicylic acid have all been identified as important regulators of plant growth and development (Pal et al., 2023). Primary metabolites are highly conserved, low molecular weight compounds that play an integral and direct role in the growth, development, and reproduction of plants (Fernie and Pichersky., 2015). Primary metabolites are formed when energy is used for the synthesis of essential constituents needed for cellular functions such as proteins, lipids carbohydrates and DNA/RNA (Fernie and Pichersky., 2015). Plant secondary metabolites (PSMs), also referred to as phytochemicals, are specialized compounds responsible for the adaptation of plants to their environment by regulating symbiosis, seed germination, stress tolerance, flowering and defence against herbivores and pathogens (Makkar et al., 2007; Guerriero et al., 2018). Over 200,000 plant secondary metabolites have been

identified *in planta* to date, making them some of the most chemically diverse compounds found in nature (Aftab and Hakeem., 2021). In addition to their defensive role, most PSMs can also modulate growth and development through various mechanisms, making them almost indistinguishable from primary metabolites and plant hormones (Erb and Kliebenstein., 2020). For example, epigallocatechin-3-gallate (EGCG) is a type of PSM found in tea plants known to increase the tea plant's ability to manage environmental conditions such as salinity, shading, light intensity, temperature, and drought. However, EGCG has also been shown to play an important role in the plant's physiology by mediating hormone crosstalk, reactive oxygen species signalling and homeostasis (Ahammed et al., 2023). The ability of PSMs to perform developmental roles, in addition to ancillary roles, has raised the validity of previous claims that PSMs are just by-products of primary metabolism and thus do not play an essential role in the growth of the plant (Theis and Lerdau., 2003).

The 200,000 plant secondary metabolites can be broadly classified into two groups based on the presence of nitrogen (Nawrot-Chorabik et al., 2022; Aftab and Hakeem., 2021). Nitrogen-containing secondary metabolites are termed alkaloids while nitrogen-free secondary metabolites can be further divided into three important classes: terpenoids, steroids and phenolics. **Figure 1** shows the classification of plant chemical compounds into their respective classes and subclasses.

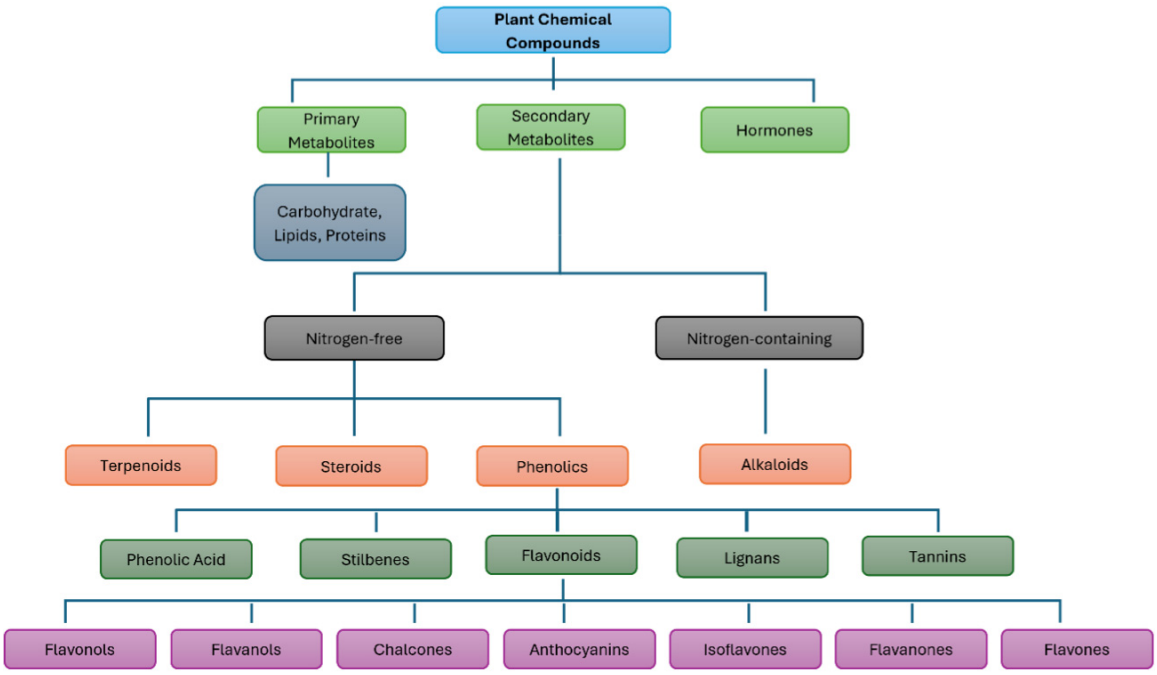

Figure 1. The classification of plant chemical compounds. Plant chemical compounds can be classified into primary metabolites, secondary metabolites, and hormones. Secondary metabolites are a large group of simple and complex molecules that can be subdivided into nitrogen-free compounds: terpenoids, steroids and phenolics and nitrogen-containing compounds: alkaloids. Phenolics are one of the largest groups of secondary metabolites and can be further grouped into phenolic acids, flavonoids, stilbenes, lignans and tannins. Figure created with information derived from Li et al., 2010; Lin et al., 2016; Jamwal et al., 2018; Nicolas-Garcia et al., 2021; Kumar et al., 2024.

1.1.1 SECONDARY METABOLITES: PHENOLICS

Phenolics are one of the largest classes of phytochemicals defined by a hydroxyl group (-OH) bonded to an aromatic hydrocarbon group (Li et al., 2010). They are distributed throughout the plant and fulfill specific advantageous functions including attracting pollinators through the expression of colour and odour, deterring herbivores or pathogens and mediating the plant's response to their environments through allelopathy (Li et al., 2010; Lin et al., 2016). Structurally, phenolics vary from simple compounds to

complex polymers (polyphenolics) and can be further classified into phenolic acids, flavonoids, stilbenes, lignans and tannins (Lin et al.,2016; Nicolas-Garcia et al., 2021; Jamwal et al., 2018). Of primary interest to this work are flavonoids which constitute over 10,000 compounds broadly divided into flavonols, flavanones, chalcone, flavones, isoflavones, flavanols and anthocyanins.

1.1.2 ANTHOCYANINS

Anthocyanins (From the Greek *Anthos* meaning = flower and *kianos* meaning = blue) are commonly found in fruits, flowers, bulbs, and/or stems and are one of the largest and most important classes of glycosylated polyphenolics belonging to the flavonoid subgroup (Hatier & Gould, 2009; Yousuf et al., 2016). They are water-soluble pigments containing a positive charge (flavylium ion) at the oxygen atom of the ring labelled "C" in **Figure 2** (Khoo et al., 2017; Liu et al.,2018). Anthocyanins are found in plant cell vacuoles, and their distribution within different tissues is highly influenced by their functional roles, for example, some plant leaves express higher amounts of anthocyanins during growth, stress, and senescence (Liu et al., 2018; Silva et al., 2017). This is due to the strong antioxidant ability of anthocyanins that can be attributed to the glycosylated B-ring at the R3' and R4' positions (**Figure 2)** as well as their ability to donate electrons to free radicals. This antioxidant property balances the oxidative stress generated during growth or stress (Khoo et al., 2017; Kong et al., 2003). In addition to anthocyanins maintaining redox homeostasis, they are also responsible for producing colours in petals to attract animals for pollination and seed dispersal (Echegaray et al., 2022). Anthocyanins are responsible for orange, pink, red, violet and blue coloration in leaves and flower petals. The visual expression of anthocyanins varies greatly depending on the pH of the environment, appearing red in acidic conditions and blue in alkaline conditions (Khoo et al., 2017). To

date, six anthocyanidins (aglycones) are reported as being commonly expressed in plants: cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin (Echegaray et al., 2022). Sugars such as arabinose, galactose, glucose, rhamnose and xylose may occur at the 3', 4' or 5'-positions **(Figure 2)** of the anthocyanidins to form over 700 distinct anthocyanins in nature (Echegaray et al., 2022; Wallace and Giusti., 2019).

In addition to the valuable role anthocyanins play in plants, epidemiological studies have shown health-promoting benefits of anthocyanins in humans (Khoo et al., 2017; de Arruda Nascimento et al., 2022). Anthocyanins such as pelargonidin-3-glucoside, cyanidin-3-glucoside and delphinidin-3-glucoside are scavengers of reactive oxygen species and therefore, have been linked to anti-inflammatory, anti-diabetic and anti-cancer properties and reduced cardiovascular incidence (de Arruda Nascimento et al., 2022; Yousuf et al., 2016; Mattioli et al., 2020; Khoo et al., 2017). Cyanidin-3-glucoside and delphinidin-3-glucoside from various fruit sources, for example, have been shown to successfully stimulate the secretion of insulin from rodent pancreatic β-cells *in vitro* (Jayaprakasam et al., 2005). While cyanidin-3-arabinose, cyanidin-3-galactoside and cyanidin-3-xyloside from chokeberry plants were shown to reduce the cell viability in the human colon cancer cell line CACO2 (Wei et al., 2020). Extracts from the same plant also showed antiproliferative activity against glioblastoma cells (Wei et al., 2020). These various therapeutic activities of anthocyanins have made them candidates for the discovery and development of novel agents within pharmaceutical and health industries globally (Yousef et al., 2016).

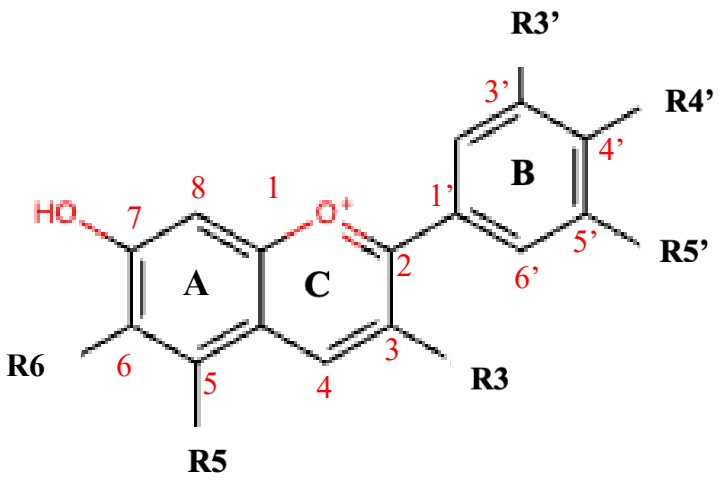

Figure 2. The basic structure of an anthocyanin. Anthocyanins are glycosides of anthocyanidins that possess two benzene rings joined by a three-carbon chain. The positive charge at the oxygen atom (in the C-ring) is called the flavylium ion. Image created using Chemspace (Chemical Search - Draw Chemical Structure | Chemspace, n.d.).

1.2 MEDICINAL PLANTS

The earliest written record of plants used for medicinal purposes dates back to 5000 years ago, in India, describing recipes utilizing over 250 different plants (Petrovska., 2012). In China, Emperor Shen Nung (2599 BC) writes about the use of the ginseng and cinnamon as treatments for various ailments (Petrovska., 2012). The Ebers Papyrus (1550 BC) refers to 700 medicinal plants including pomegranate, castor oil plant, aloe, fig, juniper, etc (Petrovska., 2012). Dioscorides known as the "father of pharmacognosy" kept a written record of medicinal plants, their preparations, and their therapeutic effects as he travelled with the Roman army in 77 AD. Since then, the discovery of various medicinal plants and their therapeutic benefits have continued to fill the pages of medical journals and texts. At present, much of our knowledge of medicinal plants derives from their traditional and historical use in ayurvedic, traditional Chinese, Unani and Indigenous medicinal practices.

For example, a gummy material collected from the leaves of the camelthorn plant (*Alhagi maurorum*) was used in Ayurvedic medicines for the treatment of constipation, fever and leprosy while in Israel, the plant was boiled and used to treat diarrhea (Ahmad et al., 2015). More recently, 14 flavonoids isolated from *Alhagi maurorum* were found to have potent antioxidant activity and strong hepatoprotective activity against carbon tetrachloride-induced hepatotoxicity corroborating the early use of this plant in Ayurvedic medicine (Al-Saleem et al., 2019). As such, the use of medicinal plants in concoctions, poultices, rubs, oils and essences still persists today, with the World Health Organization estimating that 70 – 95 % of populations in developing countries still use traditional medicines and medicinal plants for the treatment of ailments such as asthma, constipation, fever and skin conditions (Palhares et al., 2015;). Despite facing significant competition from other drug discovery methods, bioactive compounds isolated from plants continue to contribute to the development of new drugs and drug candidates (Atanasov et al., 2021; Nasim et al., 2022). In fact, study conducted on the contribution of traditional medicines to the development of modern medicines and pharmaceutical drugs found that 80% of the 122 compounds studied can be traced back to their use in folk medicines with compounds originating from over 94 plant species (Fabricant and Farnsworth., 2001).

1.2.1 PHYTOCHEMICALS IN DRUG DISCOVERY

Salicin is a well-known example of how chemical, pharmacological and clinical studies of bioactive phytochemicals can lead to the development of a successful therapeutic agent. Salicin is an alcoholic β-glycoside isolated from the bark of a willow tree *Salix alba,* the discovery of salicin contributed to the synthesis of acetylsalicylic acid (aspirin) used in the treatment of pain and fever (Rey-Ladino et al., 2011). Likewise, digitoxin (isolated from fox glove, *Digitalis purpurea* L.) and paclitaxel (isolated from Pacific Yew, *Taxus*

brevifolia) are two plant constituents used in congestive heart failure and breast cancer respectively (Dias et al., 2012; Yuan et al., 2016). Another famous example is the discovery of morphine (alkaloid), the active ingredient in opium, a dark brown resin produced by *Papaver somniferum* (Opium Poppies). Since its discovery, morphine has been one of the most used drugs in the treatment of severe pain and is considered the world's first true drug (Brook et al., 2017). Quinine is yet another well-known example of a plant secondary metabolite used in the discovery and development of a pharmaceutical drug. An alkaloid extracted from the bark of *Cinchona* trees whose traditional use in Indigenous medicines led to the use of quinine in treating malaria (Woodland and Chibale., 2022).

Many other plant bioactive compounds in isolation or extracts have since been found to elicit therapeutic effects against diseases including diabetes, inflammation, and cancer in pre-clinical studies (Boudreau et al., 2013; Maroyi., 2021). For example, an evaluation of the medicinal uses of crude extracts of cornflower veronica (*Linzia glabra*) showed its widespread use in the treatment of burns, abdominal pains, sores, wounds, gonorrhea, syphilis and diabetes (Maroyi., 2021). Likewise, a review of the medicinal properties of various phytoconstituents present in brown beech (*Litsea glutinosa*) showed analgesic, antioxidant, wound healing, antimicrobial and anti-inflammatory properties (Chawra et al., 2021). Ranasinghe et al (2012) conducted a systematic review several studies that evaluated the pharmacological and therapeutic effects of cinnamon (*Cinnamomum zeylanicum*) in treating diabetes. Additionally, water and methanol extracts of dried nelli fruit (*Phylanthus embelica*), and methanol extracts of ranawara (*Cassia auriculata*) were found to inhibit α-glucosidease, an enzyme important in catalyzing the conversion of glucose from disaccharides, an important target in treating diabetes

(Abesundara et al., 2004). **Table 1** shows a select few examples of the biological and therapeutic activity of phytochemicals in isolation or as extracts.

Table 1. Medicinal plants, their extracts and biological activities.

Species name	Part of plant used	Extract	Biological activity	Reference
Calendula officinalis	flowers	ethanol crude extracts	wound healing	Nicolaus et al., 2016
Cinnamomum zeylanicum	bark	crude extracts	anti-diabetic	Ranasinghe et al., 2012
Crocus sativus	flower	various crude extracts	anti-nociceptive and anti-inflammatory	Gohari et al., 2013
Ginko biloba	leaves	leaf extracts containing ginkgetin	anti-inflammatory	Son et al., 2005
Linzia glabra	leaves	crude extracts	antibacterial	Maroyi., 2021
Linzia glabra	leaves	aqueous crude extracts	antifungal activity	Maroyi., 2022
Litsea glutinosa	leaves	crude leaf extracts	antimicrobial	Chawra et al., 2021
Litsea glutinosa	Bark	crude bark extracts	antibacterial	Chawra et al., 2022
Moringa oleifera	leaves, seeds, pods	leaf, seed and pod extracts	antioxidant, anticancer, hepaprotective	Ma et al., 2020
Tinospora cordifolia	leaves	leaf and stem extracts	antioxidant, immunomodulatory, anticancer	Rachana et al., 2022

1.2.2 EXTRACTION TECHNIQUES FOR BIOACTIVE PHYTOCHEMICALS

Given the potential of phytochemicals in the discovery and development of drugs, establishing effective extraction and profiling methods for these biologically active components remains a critical initial step. Two of the most common and traditional techniques to extract phytochemicals are solid-liquid extraction (SLE) and soxhlet extraction. However, newer methods including supercritical fluid extraction, ultra-sound-assisted extraction and microwave-assisted extraction are also used frequently (Ligor et al., 2018; Altemimi et al., 2017). Of the various methods available, the SLE extraction method remains the most conventional method of extraction due to its straightforward application, sustainability and cost-effectiveness (Ligor et al., 2018). Briefly, in this method fresh, frozen or dried plant resultant is manually or mechanically homogenized, solvent extracted, and the extracts then filtered to remove unwanted plant debris (Ligor et al., 2018, Dai and Mumper., 2010). The yield, physical characteristics, and biological activity of the samples can be influenced by several parameters during the extraction process including solvent type, temperature and extraction time (Escriche and Juan Borras., 2018; Dai and Mumper et al., 2010). Janani et al. (2022) demonstrated this by testing three extraction techniques to evaluate differences in 1-(2, 6-dimethylphenoxy)-2-(3, 4-dimethoxyphenylethylamino) propane hydrochloride (DPPH) free radical scavenging activity of Propolis, a resin-like material made by bees from constituents collected from parts of plants, buds and exudates. The study found significant differences between each extraction method and their DPPH scavenging activity which highlighted the importance of selecting the appropriate method for the extraction of bioactive compounds from plant materials.

1.2.3 PROFILING THE PLANT METABOLOME

Plant extracts contain various phytochemicals that often require extensive and rigorous analyses to identify the components present. Metabolomics can offer a glimpse of the compositions of small molecules found in a complex sample such as in a plant extract (Climaco Pinto et al., 2022). Metabolomics is defined as the comprehensive analysis of metabolites in a complex biological sample. The most common approach in metabolomics is liquid chromatography-high-resolution mass spectrometry (LC-HR) (Chen et al., 2021; Bjarnholt et al., 2014). LC-MS analysis is useful in measuring thousands of ion peaks associated with metabolites present in plant samples with or without chromophores. Anthocyanins, for example, contain chromophores and can be detected using high-pressure liquid chromatography when attached to a diode-array detector (HPLC-DAD), which acquires data based on the selected ultraviolet and wavelength information (Lee et al., 2008). As technological advancements continue to enhance quantitative and qualitative metabolite profiling, the goal of plant metabolomics is to reliably detect and identify every metabolite in a plant extract to better elucidate bioactive plant secondary metabolites (Hall et al., 2002).

Though a highly complex task, this can be approached through the meticulous performance of untargeted and targeted metabolomic analysis of plant extracts. Untargeted metabolomics can analyze all detectable metabolites generating a greater number of peaks which can be annotated by comparing the exact mass and retention time (RT) or tandem mass spectrometry (MS-MS or MS2) fragment spectra to standards in structural databases (Chen et al., 2021; Dührkop et al., 2021). Targeted metabolomics focuses on a select few categories of compounds with more precision and often run in tandem with the standards of the compounds of interest (Han et al., 2023). Employed

together, untargeted and targeted analyses can provide a comprehensive profile of metabolites detected in a sample with more selectivity and sensitivity (Roberts et al., 2012).

1.2.4 MEDICINAL PROPERTIES OF APONOGETONS

Certain plants belonging to the *Aponogeton* genus of the family Aponogetonacea have been shown to display medicinal properties (Chowdhury et al., 2019; Chougule et al., 2022). Reports from Africa, Asia, and Australia, where many species of the *Aponogeton* genus are found, highlight their use in traditional medicinal practices to treat cuts, wounds, coughs, acne, dysentery, and snake bites (Chowdhury et al., 2019). The phytochemical profiles of the *Aponogeton* species have shown that they are rich sources of polyphenolics, flavonoids, flavanols, alkaloids and tannins (Chowdhury et al., 2019). An evaluation of the pharmacological activity of *Aponogetons* in several *in vitro* models showed that extracts from diverse *Aponogeton* species had antidiabetic, antioxidant, wound healing, anti-inflammatory and anticancer activity (Chowdhury et al., 2019). For example, methanol and chloroform extracts from *A. natans* showed potent wound healing activity while various extracts from *A. appendiculatus* and *A. undulates* showed anti-inflammatory and anti-tumor activities respectively (**Table 2,** Dash et al., 2018; Chowdhury et al., 2019).

`Table 2. *Aponogeton spp*, their extracts and biological activities.

Species name	Part of plant used	Extract	Biological activity	Reference
Aponogeton appendiculatus	leaves	crude ethanol extracts	antioxidant	Jyothi and Sunil., 2018
Aponogeton undulatus	leaves	organic fractions	antioxidant	Rahman et al., 2017
Aponogeton undulatus	leaves	crude extracts	antitumor	Islam et al., 2015
Aponogeton natans	leaves	crude extracts	wound healing activity	Dash et al., 2018
Aponogeton madagascariensis	leaves	crude extracts	anticancer	Gunawardena et al., 2021

1.3 CANCER

Cancer is second only to cardiovascular disease as the leading cause of death worldwide (Bradbury., 2007; Siegel et al., 2023). In 2022, the World Health Organization (WHO)'s cancer agency estimated 20 million new cancer diagnoses and 9.7 million deaths from cancer (WHO, 2022). Of total cancer cases, lung cancer remains the leading cause of death (18.7 % of total cancer deaths), followed by colorectal cancer (9.3 %), liver cancer (7.8 %), breast cancer (6.9 %) and stomach cancer (6.8 %). However, lung cancer (12.4 % new cases) and breast cancer (11.5 %) rank as the first and second most commonly occurring cancers worldwide, respectively.

The term cancer/cancerous is used to describe an uncontrollable growth of cells that progresses and spreads from a primary site to other parts of the body (National

Cancer Institute, 2021). There are over 100 types of cancers, typically named after the organ or tissue they originate from. Regardless of the type of cancer, the ability of cancer cells to divide and proliferate uncontrollably remains one of the most common features shared between all cancers (Feitelson et al., 2015). Apart from unabated cellular proliferation, cancers also display additional characteristics that distinguish them from healthy, non-cancerous cells. These are known as the hallmarks of cancer. Hanahan (2022) describes the 8 hallmarks of cancer: 1) Sustaining proliferative signalling 2) Evading growth suppressors 3) Enabling replicative immortality 4) Tumor promoting inflammation 5) Activating invasion and metastasis 6) Inducing angiogenesis 7) genome instability and mutation 8) Resisting cell death and two enabling characteristics: 1) Avoiding immune destruction 2) Deregulating cellular metabolism.

1.3.1 BREAST CANCER

Breast cancer is the most diagnosed cancer among women worldwide (CCS, 2024). It is a highly heterogeneous disease that can be divided into subtypes based on several factors including histological appearance, immunohistochemical expression of hormone receptors and molecular markers (Saunders et al., 2019). There are four main subtypes of breast cancer: Luminal A, Luminal B, HER2 and triple-negative breast cancer (TNBC). The Luminal A subtype accounts for about 50 % of breast cancers and tests positive for estrogen receptors (ER), and progesterone receptors (PR) but negative for human epidermal growth factor receptor (HER2). Luminal B accounts for 15 % of cases and is ER and PR positive and, like Luminal A, negative for HER2. However, Luminal B tumors are of higher grade and worse prognosis than Luminal A (Orrantia-Borunda et al., 2022). The HER2 subtype accounts for 20 % of cases and is HER positive (and ER and PR positive in some cases). Triple negative breast cancer (TNBC) subtype,

on the other hand, accounts for 15 % of breast cancer cases and is devoid of any receptors i.e. ER, PR and HER2 negative (Irvin & Carey, 2008)

TNBC is highly metastatic, prone to early relapse and is considered one of the most aggressive subtypes of breast cancer (Orrantia-Borunda et al., 2022). Given that most available breast cancer therapies are hormone receptor-dependent (for example Tamoxifen) the lack of ER, PR and HER2 receptors in TNBC limits treatment options available for this subtype. This often results in poor patient prognosis and limited options for treatments (Waks and Winer, 2017). Cytotoxic chemotherapies are currently the most used treatments for TNBC; however, they are known to present unfavourable adverse effects to the patient (Obidiro et al., 2023). As such, natural products have been proposed as alternative chemotherapeutic agents due to their low toxicity and selectivity toward cancer cells (Seca et al., 2018; Hasanpourghadi et al., 2017).

1.3.2 MECHANISMS OF ACTION OF PLANT METABOLITES IN CANCER

One of the hallmarks of cancer is the evasion of cell death. In cancer cells, this is achieved through the downregulation of tumour suppressor genes and the upregulation of pro-survival proteins such as p53 and bcl2 respectively (Hanahan & Weinberg, 2011). Typically, chemotherapeutic agents induce cell death in cancer cells by targeting the apoptotic pathways to counter existing pro-survival mechanisms (Fulda and Debatin, 2013). The extrinsic pathway and the intrinsic mitochondrial pathway are two of the most common apoptotic pathways by which PSMs/phytochemicals exert their cell death mechanisms (Huang et al., 2012; Kim et al., 2021). In brief, the extrinsic pathway involves the binding of the ligand or "death signal" (FAS/TRAIL; **Figure 3**) to the cell surface receptors (FADD) leading to the formation of a death inducing signaling complex

initiating the activation of pro-caspases such as caspase-8 and caspase-10 (Jan & Gul-e-Saba., 2019). However, elevated levels of reactive oxygen species (ROS) have also been linked to the activation of the extrinsic apoptotic pathway in triggering caspase-8 mediated cleavage of caspase-3 (NavaneethaKrishnan et al., 2019). The mitochondrial intrinsic pathway, on the other hand, is initiated when elevated levels of ROS depolarize the mitochondrial membrane, triggering the release of cytochrome C from the mitochondria. The release of cytochrome C then activates the proapoptotic protein caspase-9 (Jan & Gul-e-Saba., 2019). The intrinsic and extrinsic pathways converge on the activation of executioner protein caspase-3 leading to DNA damage and eventual cell death (**Figure 3**; Wani et al., 2023; NavaneethaKrishnan et al., 2019).

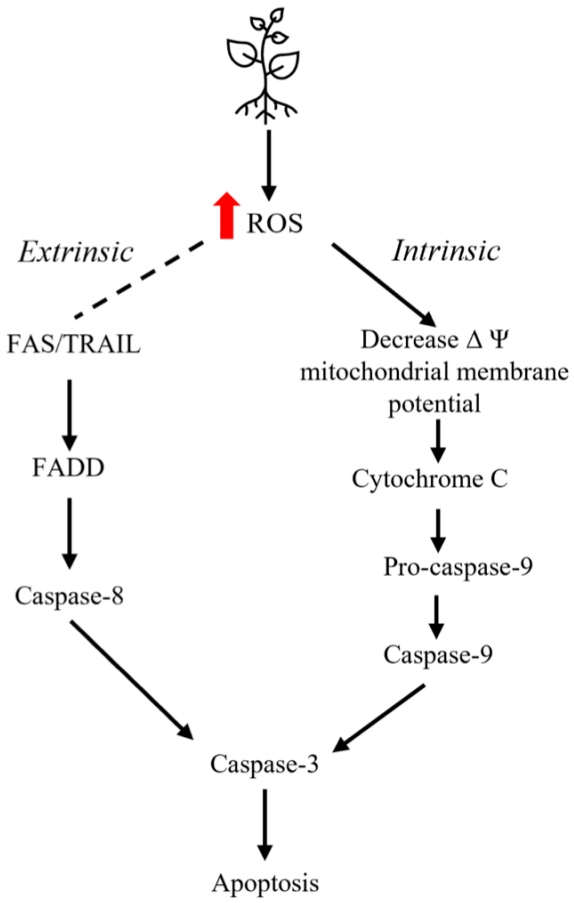

Figure 3. The intrinsic and extrinsic apoptotic pathways are triggered by phytochemicals found in plants. FAS: FS-7-associated surface antigen ligand, TRAIL: TNF-related apoptosis-inducing ligand, FADD: Fas-associated death domain. Both the intrinsic and extrinsic pathways converge in the cleavage of caspase-3 leading to apoptosis. Increase ROS has also been implicated in the triggering of the extrinsic apoptotic pathway, represented by the broken arrow (NavaneethaKrishnan et al., 2019).

1.4 LACE PLANT

Aponogeton madagascariensis, commonly known as the lace plant **(Figure 4A)**, is an aquatic monocot native to freshwater river systems of Madagascar and the Comoros Islands and is one of the 60 *Aponogeton* species belonging to the family Aponogetonaceae (van Bruggen., 1985). During leaf morphology, the leaves of the lace plant develop a unique pattern of perforations that occurs via a process known as developmentally regulated programmed cell death or PCD (Gunawardena et al., 2004). In addition to lace plants, the only other species known to form perforations during leaf development belongs to the family Araceae (Van bruggen., 1985). Though the advantage of perforation formation in the lace plant is unclear, many speculate that it might play a role in deterring herbivores or protecting against drag forces typical in fast-flowing rivers or streams that lace plants are often found in (Gunawardena and Dengler., 2006).

1.4.1 LACE PLANT AS A MODEL ORGANISM FOR PCD

The predictable perforation formation in the leaves of the lace plant provides an excellent opportunity for the study of developmentally regulated PCD (Gunawardena et al., 2008; Rowarth et al., 2023). Due to the thin translucent leaves that facilitate live-cell imaging, established sterile cultures for whole plant propagation (**Figure 4B**) and the predictability of perforation formation, the lace plant is considered an emerging novel model organism for the study of plant PCD (Gunawardena et al., 2008; Rowarth et al., 2023). Gunawardena et al. (2004), characterize the developmental stages of the lace plant into five stages: pre-perforate, window, perforation formation, perforation expansion and mature. Leaves in the pre-perforation stage are tightly furled and express high amounts of anthocyanins. **Figure 4C** shows leaves at the mature and window stages of development.

At the window stage, the visible loss of anthocyanins marks the onset of PCD in the leaves. During the perforation formation stage, the loss of cells starts from the center of the areole extending outwards while at the perforation expansion stage, the loss of cells continues until it stops approximately 4-5 cells away from the leaf vein. At full maturity, leaves lose all visible signs of anthocyanins with a fully formed perforation in the center of the areole. **Figures 4D and 4E** show a single areole at the mature and window stages of development respectively.

Figure 4. *Aponogeton madagascariensis* (Lace plant). The lace plant is an emerging model organism to study the predictability of programmed cell death (PCD) during leaf morphogenesis. (A) whole lace plant showing juvenile and adult leaves emerging from the corm, its underground energy storage unit. (B) whole lace plant growing in a culture vessel. (C) mature (left) and window (right) leaves show differences in leaf size and coloration. (D) micrograph of an areole in a mature leaf that shows a fully formed perforation. (E) micrograph of a window leaf showing an areole saturated with anthocyanin during leaf morphogenesis.

1.4.2 ANTICANCER ACTIVITY OF LACE PLANT EXTRACTS IN VITRO

Previously, our lab group reported on the *in vitro* anticancer activity of crude anthocyanin extracts from window and mature leaves on breast and ovarian cancer cells (Gunawardena et al., 2021). The results from this work showed that both window and mature lace plant leaf extracts were cytotoxic against the MDA-MB-231 TNBC cancer cell line and OVCAR-8 and SKOV-3 ovarian cancer cell lines. At 48 h post-treatment, the extracts were shown to reduce the cell viability of MDA-MB-231, OVCAR-8 and SKOV-3 cells as well as display apoptotic-like characteristics and cause DNA fragmentation in MDA-MB-231 cells at concentrations ranging from 25 – 250 µg/mL. Following these findings, the study raised two important questions: (i) which phytochemical compounds are responsible for biological activity and (ii) what pharmacological mechanisms of action are involved in inducing this anticancer effect post-treatment. These questions prompted the objectives outlined in this thesis.

1.5 HYPOTHESIS AND OBJECTIVES OF THE STUDY

It was previously reported that extracts from the lace plant induce apoptotic characteristics in MDA-MB-231 TNBC cells *in vitro*. However, the exact bioactive phytochemical constituents and the pharmacological processes involved were unknown. This work aims to expand our understanding of the plant constituents present in lace plant extracts and analyze the anticancer effects exerted by the phytochemicals present in the extracts. As such, it was hypothesized that **the lace plant extracts contain a diverse array of phytochemicals between window and mature extracts and that the phytochemicals present in window and mature extracts will reduce cell viability, cell**

proliferation and cell migration in human TNBC cells and that the cytotoxic effect will be mediated through a ROS-dependent pathway.

The overall goals of the project were to create a metabolomic profile and investigate the various anticancer activities that phytochemicals in lace plant leaf extracts have and to determine their pharmacological mechanisms of cell death in TNBC cells *in vitro*. The following is a detailed list of specific objectives followed to achieve these goals:

1) Revise the extraction protocol for an efficient method of extracting compounds from window and mature leaves of the lace plant while retaining the bioactivity of extracted compounds (In collaboration with Dr. Shawna MacKinnon, Kentville Research and Development Center, Agriculture and Agri-Food Canada (KRDC-AAFC))

2) Conduct (i) an untargeted metabolomic analysis to investigate the phytochemicals found in lace plant leaf extracts and identify the most abundant compounds found within window and mature leaf extracts (in collaboration with Dr. Thusi Rupasinghe, SCIEX, New South Wales Australia and Dr. Kosala Ranathunga, University of Western Australia) (ii) conduct a targeted analysis to identify any anthocyanin compounds present in window and mature extracts (In collaboration with Dr. Srinivas Sura, Morden Research and Development Center, Agriculture and Agri-food Canada (MRDC-AAFC)).

3) Investigate the anticancer properties of window and mature leaf extracts from lace plants in MDA-MB-231 cells by (i) test the effect of extracts on TNBC cell viability,

cell proliferation and cell migration (ii) evaluate the specificity of extracts on cell viability of TNBC cells versus normal breast epithelial cells (iii) investigate the pharmacological mechanisms of action by which the extracts are exerting their anticancer effects.

CHAPTER 2: MATERIALS AND METHODS

2.1 TESTING EVAPORATION TECHNIQUES

2.1.1 LACE PLANT PROPAGATION

Aponogeton madagascariensis cultures were propagated according to established protocols described by Gunawardena et al. (2006). Lace plant cultures showing signs of advanced senescing or those which had outgrown their culture vessel were selected for further subculturing. Any dead, dying or decaying plant material was carefully excised from the corm under sterile conditions. Corms were then thoroughly cleaned by shaving off dead tissue material and cut into 1 cm^3 cubes. Approximately 0.5 cm of healthy shoot apical meristem was left intact to ensure successful propagation of the plant. The freshly cultured corms were placed into sterile Magenta GA-7 boxes **(Figure 5A and B)** or 946 mL autoclave-safe glass culture vessels **(Figure 5C and D)** (PhytoTechnology Laboratories, USA) containing solid Murashige and Skoog medium. Liquid Murashige and Skoog medium was then carefully poured into the vessel to fully submerge the corm. Liquid MS media was prepared in-house using 30 g/L sucrose (BioS hop, Canada), 2.15 g/L MS basal salts (PhytoTechnology Laboratories), 0.1 g/L Myo-inositol (Sigma-Aldrich, Oakville Canada) and 0.1 mg/L thiamine HCl (Sigma-Aldrich). Solid MS media was prepared using liquid MS media and 1 % micropropagation grade agar (Phytotechnology Laboratories). The newly prepared cultures were wrapped in sealing film and placed under 125 umol/m^2/s daylight fluorescent light bulbs (Philips, Daylight Delux, F40T12/DX, Markham, Canada) on a 12-h light/dark cycles at 24 °C. Lace plant cultures were carefully monitored for 8 weeks (2 months) and leaves were harvested as needed.

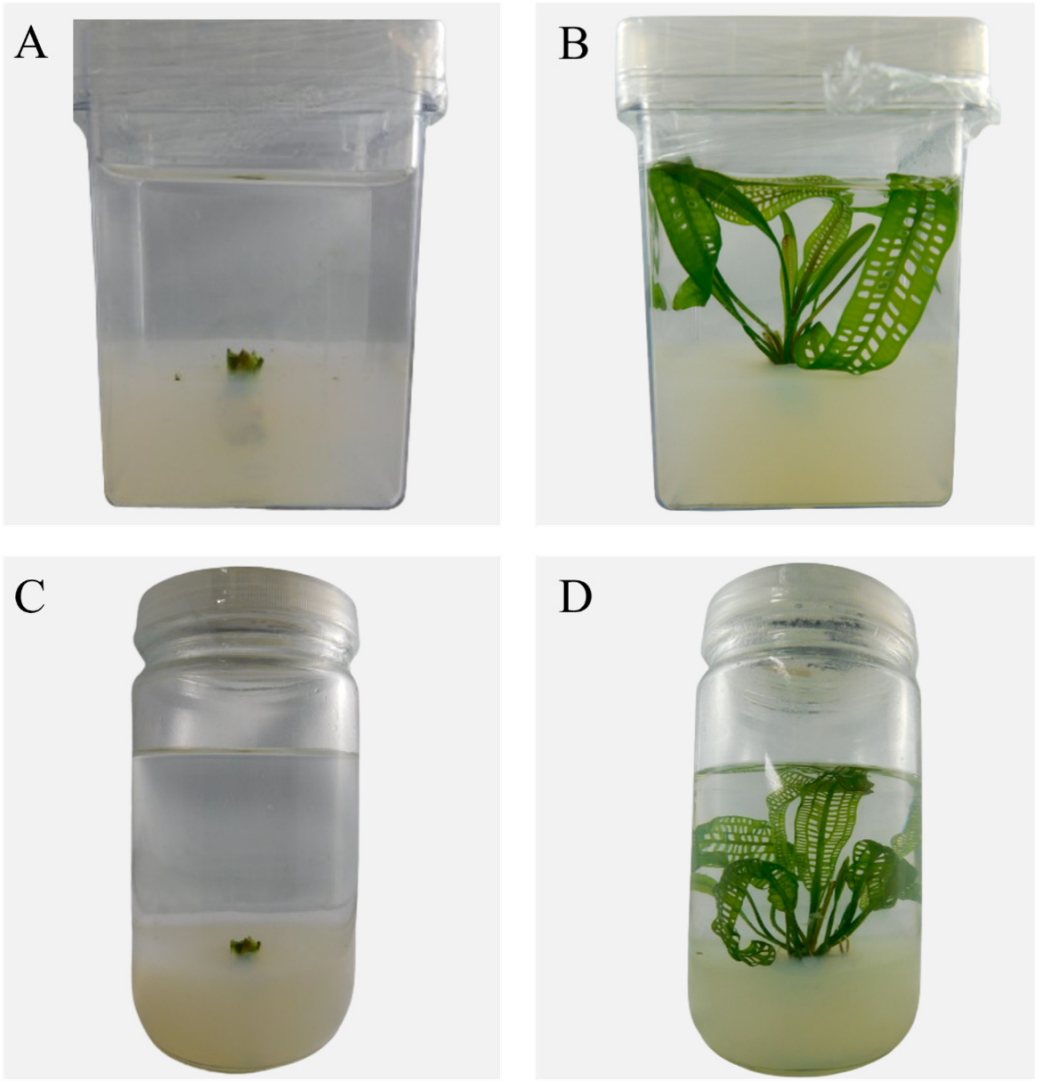

Figure 5. Propagation of lace plant cultures under sterile conditions. Freshly cleaned lace plant corms were placed in magenta GA7 boxes containing 150 mL of liquid Murashige and Skoog (MS) medium and 100 mL of solid MS medium substrate (A) until ready to be subcultured (B). To increase growth space, clean corms were placed in glass culture vessels containing 450 mL of liquid MS medium and 200 mL of the solid medium substrate (C) until ready to be subcultured (D). Following 2 months of growth, window and mature leaves were harvested for crude anthocyanin extractions (B, D).

2.1.2 EXTRACTION OF CRUDE LACE PLANT LEAF EXTRACTS

Crude leaf extracts were prepared as described in Dauphinee et al. (2017) with slight modifications. Briefly, window and mature leaves from the lace plant were harvested from sterile cultures, blot-dried, weighed, and frozen at -80 ºC until extraction (**Figure 6 a-d**). Previously frozen lace plant leaves (no older than 3-4 months) were placed in a mortar and pestle, flash-frozen with liquid nitrogen, and macerated until finely powdered (**Figure 6 e-h**). An extraction solvent consisting of 95 % methanol and 5 % formic acid (FA) (v/v) was added to the freshly macerated leaves (1 mL per gram of leaf tissue) and left to incubate in the dark on ice for 50 – 60 mins **(Figure 6 i-l**). Following incubation, the solvent mixture was filtered through an 80 µm mesh filter and centrifuged at 10,000 g for 15 mins at 4 ºC **(Figure 6 m – p)**. The supernatant was then carefully decanted and filtered again through the 80 µm mesh and poured into glass dishes left to dry in a temperature-controlled chamber overnight at 25 ºC (**Figure 6 q-r**) or poured into scintillation vials and evaporated using a stream of nitrogen (**Figure 6 s-t**). The dry residue was then collected and stored at –80 ºC until further analysis (**Figure 6 u-x**). Dry extracts from both extraction techniques were then delivered to the MacKinnon lab (RDC-AAFC) for UPLC-DAD analysis. Dry extracts from the temperature-controlled dry technique were freeze-dried and shipped for plant metabolomics analyses via LC-MS to Dr. Thusi Rupasinghe (SCIEX, New South Wales, Australia), Dr. Kosala Ranathunga (School of Biological Sciences, University of Western Australia), Dr. Srinivas Sura (RDC-AAFC).

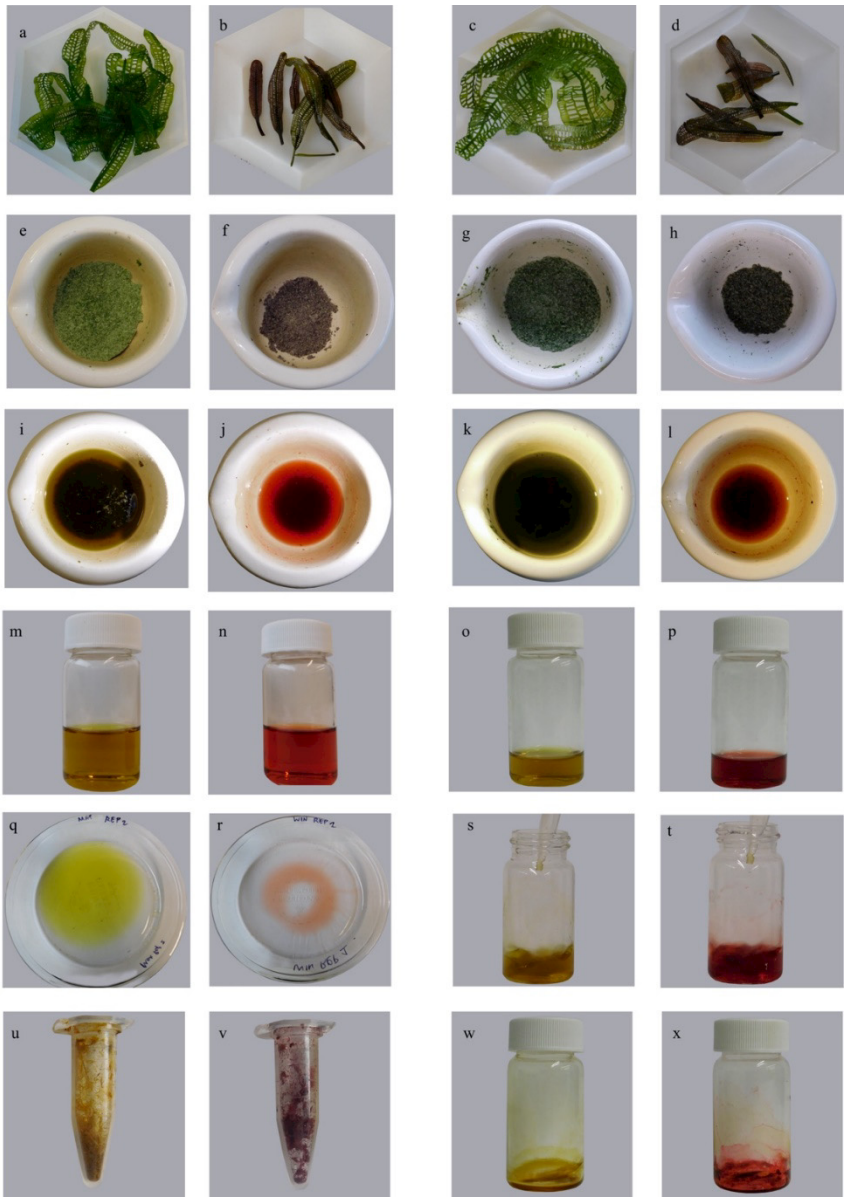

Figure 6. Two extraction techniques used in the extraction of crude anthocyanins from window and mature leaves of the lace plant. Leaves were harvested from lace plants grown in sterile cultures (a- d), macerated under liquid nitrogen (e-h) and incubated in a methanolic extraction solvent for 50 – 60 mins (i-l). The solvent was then filtered, centrifugated and decanted (m-p). For the incubator dry technique, the filtrate was poured into flat pyrex dishes and left to evaporate unassisted at 25 ºC (q,t). In the nitrogen stream evaporation technique, the filtrate was poured into scintillation vials left under a steady stream of nitrogen directed into the vial until most of the solvent was evaporated and freeze-dried. The dried extracts from each technique were collected and stored as needed (u-x).

2.1.3 ULTRA-HIGH PRESSURE LIQUID CHROMATOGRAPHY – DIODE ARRAY DETECTOR ANALYSIS

This work was done in collaboration with Dr. Shawna MacKinnon (KRDC-AAFC). Lace plant crude anthocyanin extracts were prepared for UPLC analysis by dissolving in 2 % formic acid in water and filtered through a 0.22 μm syringe filter. After which, 10 μL of the dissolved sample was injected into an Agilent 1290 Infinity II LC system (Agilent Technologies Inc., Santa Clara, CA, USA) consisting of a PFP Kinetex 2.6 μm column (Phenomenex, Torrance, CA, USA), an Agilent binary pump system, diode array detector (DAD), autosampler and column heater. The following gradient method was used to elute the sample from the column: decreasing proportions of mobile phase A (2% FA in water) to mobile phase B (2 % FA in acetonitrile). Decreasing proportions of mobile phase A to mobile phase B (0-3 mins 1.1 % B; 3 - 34 min, 1.1 - 20.7 % B; 34 - 40 min, 20.7 - 90.2 % B; 40 - 43 min, 90.2 % B; 43 - 47 min, 90.2-1.1 % B and 47 - 50 min, 1.1 % B). A flow rate of 1.0 mL/min was maintained with the column temperature at 55 ºC. The resulting eluant was monitored at 520 nm. UPLC parameters used for lace plant crude anthocyanin samples are given in **Table 3**.

Table 3. Ultra-pressure liquid chromatography parameters for the analysis of crude lace plant extracts.

UPLC parameters	Profiling settings
Sample diluent	2 % formic acid in water
Temperature	55 ºC
Volume injected	10 µL
Analytical column dimensions	50 x 2.1 mm, 2.6 µm
Mobile phase A	2% formic acid in water
Mobile phase B	2 % formic acid in acetonitrile
Flow rate	1.0 mL/min
Full run time	50 mins

2.2 LACE PLANT PHYTOCHEMICAL PROFILING

2.2.1 UNTARGETED ANALYSIS

Untargeted LC-MS-MS analysis for this work was done in collaboration with Dr. Thusi Rupasinghe (SCIEX, New South Wales, Australia) and Dr. Kosala Ranathunga (University of Western Australia). Briefly, lace plant crude anthocyanin extracts were prepared for HPLC-MS-MS analysis by resuspending the dry extract in 500 µL of 50 % acetonitrile/water (50:50 v/v). Samples were vortexed for 5 mins at room temperature and centrifuged (16,000 g) before being transferred to HPLC vials. Next, 10 µL of the dissolved sample was injected into a Shimadzu Prominence LC system containing a Phenomenex F5 column maintained at 40 ºC. Separation was achieved using an LC gradient of mobile phase A (0.1 % FA in water) and mobile phase B (0.1 % FA in

acetonitrile). The gradient was set up from 0 % B to 5 % B in the first 3 mins, from 5 % to 60 % B in the next 3 mins, from 60 % to 70 % from 6 mins to 8 mins, from 70 % B to 89 % B from 8 mins to 9.1 mins, 89 % to 92 % from 9.10 to 10.50 mins, from 92 % B to 100 % B from 10.50 to 10.90 mins and kept at 100 % B for one min and held at 5 % B for 3 mins until equilibration. The total run time amounted to 15 mins for the analysis. The parameters used in the analysis are summarized in **Table 4**.

Following the LC analysis, the data was collected in an untargeted manner using information-dependent acquisition with a SCIEX ZenoTOF 7600 system (SCIEX) with a Duospray Turbo V ion source and an electrospray ionization (ESI) probe at the Sydney Mass Spectrometry facility, Charles Perkins Center, Camperdown, University of Sydney, New South Wales, Australia. The instrument was calibrated every 5 samples using an automated calibrant delivery system using an ESI calibration solution. Data was then collected using a data-dependent acquisition method in positive and negative modes. MS/MS was acquired in ESI mode with a mass range of 30 – 650 and a 100 msec accumulation time. The collision energy was set to 12 and electron kinetic energy as 10 eV with an electron beam current as 5500 nA for electron activation dissociation fragmentation. The Zeno threshold was set as 10,000 cps.

To improve plant metabolite identification, data was analyzed using MS-DIAL 5.1 software with metabolomics libraries containing collision-induced dissociation and electron-activated dissociation MS/MS fragments. The MS/MS spectra was compared to the following libraries: Riken PlaSMA bio-MS/MS from plant tissues, CASMI2016, Fiehn HILIC, MetaboBASE, RIKEN PlaSMA authentic standards, ReSpect, and Fiehn/Vaniya natural product. Statistical analysis was performed using MetaboAnalyst 5.0 Software.

Table 4. High-pressure liquid chromatography parameters for the analysis of metabolites detected in crude lace plant extracts.

HPLC parameters	Profiling settings
Sample diluent	50% acetonitrile in water
Temperature	40 ºC
Volume injected	10 µL
Analytical column dimensions	100 x 4.6 mm, 1.8 µm particle size
Mobile phase A	0.1 % formic acid in water
Mobile phase B	0.1 % formic acid in acetonitrile
Flow rate	200 µL/min

2.2.2 TARGETED METABOLOMICS

Targeted metabolomic analysis was conducted in collaboration with Dr. Srinivas Sura (AAFC, Morden) using an ultra-high-pressure liquid chromatography coupled with a high-resolution mass spectrometer (UHPLC-HRMS; Vanquish UHPLC, ID-X Tribrid Orbitrap, Thermo Fisher Scientific, Mississauga, Canada) as described in Kodikara et al. (2024). Dry lace plant crude leaf extracts were prepared for analysis by resuspending the extracts in ethanol: water (80:20 v/v) followed by vertexing. Resuspended extracts (10 µL) were then injected onto a reverse phase biphenyl column (2.6 µm particle size, 100x 2.1, Kinetex, Phenomenex) maintained at 35 ºC. Elution was achieved using a LC gradient of mobile phase A (water with 0.1 % formic acid) and mobile phase B

(acetonitrile with 0.1 % formic acid at a flow rate of 0.25 mL/min. Analyte mass spectra detection was carried out using TraceFinder (v.4.1, ThermoFisher Scientific, Canada). Run parameters are summarized in **Table 5**.

Table 5. Ultra-high-pressure liquid chromatography parameters for the analysis of metabolites detected in crude lace plant extracts.

UHPLC parameters	Profiling settings
Sample Diluent	80 % ethanol in water
Temperature	35 ºC
Volume Injected	10 µL
Analytical column dimensions	Biphenyl 100 x 2.1 mm, 2.6 µm particle size
Mobile phase A	0.1 % formic acid in water
Mobile phase B	0.1 % formic acid in acetonitrile
Flow rate	0.25 mL/min

A total of 66 phenolic compounds (14 anthocyanins and 52 non-anthocyanin phenolics) were targeted and analytical standards were compared for retention times, accurate masses, fragment ions (**Tables 6.1 and 6.2**). All 66 standards were selected based on their common occurrence in berry fruits.

Table 6.1. Anthocyanin standards used in the targeted LC-MS analysis of crude lace plant leaf extracts.

Anthocyanin standards used in targeted LC-MS-MS		
Cyanidin chloride	Delphinidin-3-glucoside	Peonidin
Cyanidin-3-arabinoside	Malvidin	Peonidin-3-glucoside
Cyanidin-3-O-glucoside	Malvin	Petunidin
Cyanidin-3-rutinoside	Malvidin-3-glucoside	
Delphinidin	Pelargonidin	

Table 6.2. Phenolic (non-anthocyanin) standards used in the targeted LC-MS analysis of crude lace plant leaf extracts.

Non-anthocyanin phenolic standards used in targeted LC-MS-MS		
Afzelin	Gallic acid	Polydatin
Apigenin	Genistein	Procyanidin A2
Arbutin	Glycitin	Procyanidin B2
Aromadendrin	Herbacetin	Procyanidin C2
Caffeic acid	Hesperetin	Protocatechuic acid
Caftaric acid	Isoquercetin	Quercetin
(+)Catechin	Isorhamnetin	Quercetin-3-galactoside
Chlorogenic acid	Isovitexin	Resveratrol
Daidzein	Kaempferol	Rhapontin
Daidzin	Kaempferol-3-glucoside	Rutin
Ellagic acid	Liquiritingenin	Sinapic Acid
(-)Epicatechin	Luteolin	Syringic Acid
Epicatechin Gallate	Myricetin	Taxifolin
(-)Epigallocatechin	Naringin	Vanillic Acid
Ferulic acid	Nicotiflorin	Vicenin-2
Fisetin	Okanin	Vitexin
	Orientin	Vitexin-2--rhamnoside
	Para coumaric acid	Zeaxanthin
	Piceatannol	

2.3 INVESTIGATING THE ANTICANCER EFFECTS OF CRUDE LACE PLANT LEAF EXTRACTS

2.3.1 CELL LINES AND CELL CULTURE

Triple-negative human breast cancer MDA-MB-231 and non-cancerous human epithelial breast cells MCF-10A were kindly provided by Drs. David Hoskin and Anna Greenshields (Dalhousie University, Halifax, NS, Canada). The MDA-MB-231 cell line was used as the representative cell line for TNBC and the MCF-10A cell line was used as a healthy control cell line for this work. MDA-MB-231 cells were cultured in 75 cm^2 tissue culture flasks (Corning Inc, Corning, NY) in phenol-free Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 IU/mL penicillin and 250 µg/mL streptomycin and 1mM sodium pyruvate (complete medium). MCF-10A cells were cultured in 75 cm^2 tissue culture flasks (Corning Inc, Corning, NY) in phenol free F12/DMEM supplemented with 10 % horse serum, 0.02 µg/mL epidermal growth factor, 0.05 µg/mL hydrocortisone, 10 µg/mL bovine insulin and 100 U/mL penicillin/ 100 µg/mL Streptomycin. Cells were maintained in a 37 °C humidified incubator supplied with 5% CO_2 (standard conditions) with the growth medium changed every 3 - 4 days. Upon reaching 80 – 90 % confluence, cells were passaged by removing the old medium, rinsing with PBS (1X) and adding 0.25 % trypsin diluted in PBS. The cells were then resuspended in complete medium and centrifuged for 6 mins at 740 x g. The medium and trypsin mixture were then carefully aspirated leaving the cell pellet undisturbed. The cell pellet was resuspended in 5 mL of fresh, complete medium and distributed into a new culture flask. Cell counts were conducted using a TC20 Automated

cell counter (Bio-Rad Laboratories, Mississauga, ON) when seeding multi-well plates (Corning Inc.) for experimentation.

2.3.2 MTT VIABILITY ASSAY

MTT (Thiazolyl blue methyl tetrazolium bromide) assays were used to measure the cell viability of MDA-MB-231 and MCF10A cells following crude lace plant anthocyanin extract treatments. MTT is one of the most common ways to assess changes in mitochondrial cell viability as a measure of cytotoxicity. The assay constitutes a reagent that passes through the mitochondrial inner membrane of viable cells, that gets reduced to formazan by metabolically active cells. This chemical reaction provides a colorimetric-based measure of cell viability in a colony of cells (Ghasemi et al., 2021). Briefly, cells were seeded in a 96 well plate at 5000 cells/well in 100 µL of complete medium and left to adhere for 24 h under standard conditions. Following incubation, the cells were treated with crude lace plant extracts. A stock concentration (100,000 µg/mL) of window and mature extracts were prepared by dissolving the extracts in DMSO. The stock concentration was then further diluted to 25, 50, 100, 250, 500 µg/mL prior to treatments. Cells were then treated with window and mature extracts with 25, 50, 100, 250, 500 µg/mL extracts, vehicle control or media control for 48 h. After 48 h had lapsed, 50 µL of MTT solution (5 mg/mL) in PBS was added to each well and left to incubate for 2 h under standard conditions. The media and MTT mixture were then aspirated and 100 µL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. The optical density of the formazan crystals was measured at 550 nm in quintuplicates using a BioTek Synergy HT plate reader (BioTek, Winooski, VT, USA) using Gen5 v2.01 software (Agilent Technologies, Mississauga, Canada). Readings from blank cells were subtracted from treated wells and percent cell viability was calculated by dividing the

absorbance of each well divided by the average of control wells (vehicle), multiplied by 100. A total of 4 biological replicates were performed.

$$\% \text{ Cell viability} = \frac{\text{Average absorbance in test wells}}{\text{Average absorbance in control wells}} \times 100$$

2.3.3 CELL PROLIFERATION ASSAY

The proliferation of MDA-MB-231 cells was measured using CyQUANT® NF cell proliferation assay kit (Thermofisher Scientific, Canada) as per the manufacturer's instructions. CYQUANT ® proliferation assay which uses a green, fluorescent dye that exhibits a strong fluorescent signal when bound to cellular nucleic acids. Briefly, cells were seeded in a 96-well plate at 5000 cells/well in 100 µL of complete medium and left to adhere for 24 h under standard conditions. Following incubation, the cells were treated with crude lace plant anthocyanin extracts at concentrations of 25, 50, 100, 250, and 500 µg/mL, vehicle control or media control for 24 or 48 h. After 24 or 48 h, plates were retrieved from the incubator and the medium was removed by aspiration. Next, 100 µL of the reagent was added to each well and left to incubate under standard conditions for 60 minutes. The resulting fluorescence intensity was measured at EX 485 nm and EM 530 nm in quintuplicates using a BioTek Synergy HT plate reader using Gen5 v2.01 software (Agilent Technologies, Mississauga, Canada). Readings from blank wells were subtracted from treated or control wells. Each experiment was repeated at least 4 times. Percent cell proliferation was calculated by dividing the fluorescence intensity of each well divided by the average of the control wells (vehicle), multiplied by 100. A total of 3 biological replicates were performed.

$$\% \text{ Cell Proliferation} = \frac{\text{Average fluorescence intensity in test wells}}{\text{Average fluorescence intensity in control wells}} \times 100$$

2.3.4 WOUND HEALING ASSAY

MDA-MB-231 cells were seeded in a 96-well plate at a concentration of 60,000 cells/well in 100 µL of complete medium and left to adhere for 24 h under standard conditions. Following incubation, a sterile 10 µL pipette tip was used to make a vertical scratch in the middle of each well. The detached cells and media were then aspirated from each well and replaced with vehicle control, media control or crude lace plant anthocyanin extracts in 1% FBS DMEM at concentrations of 25, 50, 100, 250, 500 µg/mL. The plate was then placed under a Zeiss Axio Observer with a monochrome camera in a 37 ºC, humidified chamber supplied with 5% CO_2. Each well was imaged every 1 h for 14 h with a 10X objective. Three biological replicates were performed. The scratch area was determined using the *Wound Healing Size Tool,* an ImageJ ® plugin. The % wound closure was determined according to the following equation:

$$\% \text{ Wound closure} = \frac{(\text{average of initial wound area} - \text{ average of final wound area})}{(\text{average of initial wound area})} \times 100$$

2.3.5 REACTIVE OXYGEN SPECIES ASSAY

Intracellular reactive oxygen species in MDA-MB-231 cells were measured using the ROS-sensitive dye 5-(and-6)-chloromethyl-20 ,70 -dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA). Briefly, MDA-MB-231 cells were seeded in a black-sided clear-bottomed plate at a concentration of 20,000 cells/well in 100 µL of

complete medium. Cells were left to adhere for 24 h under standard conditions. After 24 h, the medium was removed, and cells were rinsed with PBS (1X). 100 µL of 7.5 µmol/L of CMDCFH_2-DA in FluoroBrite DMEM media (Thermofisher Scientific, Canada) was added to each well excluding blank wells and left for 1 h under standard conditions. After incubation, CM-H_2DCFDA was removed, and cells were rinsed in PBS (1X). Each well was then treated with a vehicle control, media control or crude lace plant anthocyanin extracts at concentrations of 25, 50, 100, 250, and 500 µg/mL. After 24 h, fluorescence was read at EX 485/20 nm and EM 528/20 nm using a BioTek Synergy HT plate (BioTek, Winooski, VT, USA) in quintuplicate. Three biological replicates were performed. Background fluorescence from blank wells were subtracted and fold change ROS activity was calculated using the following equation:

$$\text{Fold change in ROS activity} = \frac{\text{Average fluorescence intensity in test wells}}{\text{Average fluorescence intensity in control wells}}$$

2.3.6 REACTIVE OXYGEN SPECIES ASSAY CO-TREATED WITH NAC

MDA-MB-231 cells were seeded in a black-sided clear-bottomed plate at a concentration of 20,000 cells/well in 100 µL of complete medium. Cells were left to adhere for 24 h under standard conditions. After 24 h, the media was removed, and cells were rinsed with PBS (1X). 100 µL of 7.5 µmol/L of CMDCFH_2-DA in FluoroBrite DMEM media (Thermofisher Scientific, Canada) was added to each well excluding blank wells and left for 1 h under standard conditions. After incubation, CMDCFH_2-DA was removed by aspiration, and cells were rinsed in PBS (1X). Each well was then treated with 80 µL of N-acetylcysteine (NAC) at 10 mM and left for 1 under standard conditions. After 1 h, 20 µL of vehicle control, media control or crude lace plant anthocyanin

extracts at a final concentration of 25, 50, 100, 250, or 500 µg/mL were added to each well. After 24 h, fluorescence was read at EX 485/20 nm and EM 528/20 nm using a BioTek Synergy HT plate (BioTek, Winooski, VT, USA) in quintuplicate. Three biological replicates were performed. Background fluorescence from blank wells was subtracted and fold change ROS activity was calculated using the following equation:

$$\text{Fold change in ROS activity} = \frac{\text{Average fluorescence intensity in test wells}}{\text{Average fluorescence intensity in control wells}}$$

2.3.7 STATISTICAL ANALYSIS

All data are expressed as mean ± standard error of means of at least three biological replicates. A two-way ANOVA was performed for multiple comparisons with 2 independent variables with a Bonferroni *post-hoc* test for the analysis of significant ANOVA results. A difference in means was considered to be significant when $P \leq 0.05$. All statistical tests were conducted using GraphPad Prism 9 (GraphPad Software).

CHAPTER 3: RESULTS

3.1 EVAPORATION TECHNIQUES TESTED ON WINDOW AND MATURE LEAVES OF THE LACE PLANT

3.1.1 COMPARISON OF CYTOTOXIC EFFECTS BETWEEN NITROGEN STREAM AND DRYING TECHNIQUE

Previously our research group used an extraction protocol described by Dauphinee et al., (2017) to prepare crude extract from window and mature leaves of the lace plant. However, to improve the quality of the lace plant leaf extracts, we experimented with two evaporation techniques: temperature-controlled dry technique (Technique A) and nitrogen stream technique (Technique B). To determine if there were any significant differences in the bioactivity of the extracts prepared from each technique, a MTT assay was used. MDA-MB-231 cells were exposed to extracts prepared from Technique A and Technique B at concentrations of 25, 50 and 100 µg/mL for 48 h. Significant differences in percent cell viability were observed in MDA-MB-231 cells treated with mature and window extracts prepared using Technique A at 50 and 100 µg/mL. In cells treated with mature extracts derived from Technique A, a reduction of 15 – 20 % at 50 and 100 µg/mL in cell viability was measured. No significant reduction in cell viability was measured for cells treated with mature extracts from Technique B. Similarly, in cells treated with window extracts derived from Technique A, a reduction of 30 – 35 % at 50 and 100 µg/mL in cell viability was observed while no significance was measured in cells treated with window extracts derived from Technique B (**Figure 7A**). In fact, no significant dose-dependent reduction in percent cell viability was measured for cells

treated with window or mature extracts compared to vehicle controls in cells exposed to extracts prepared using Technique B (**Figure 7B**).

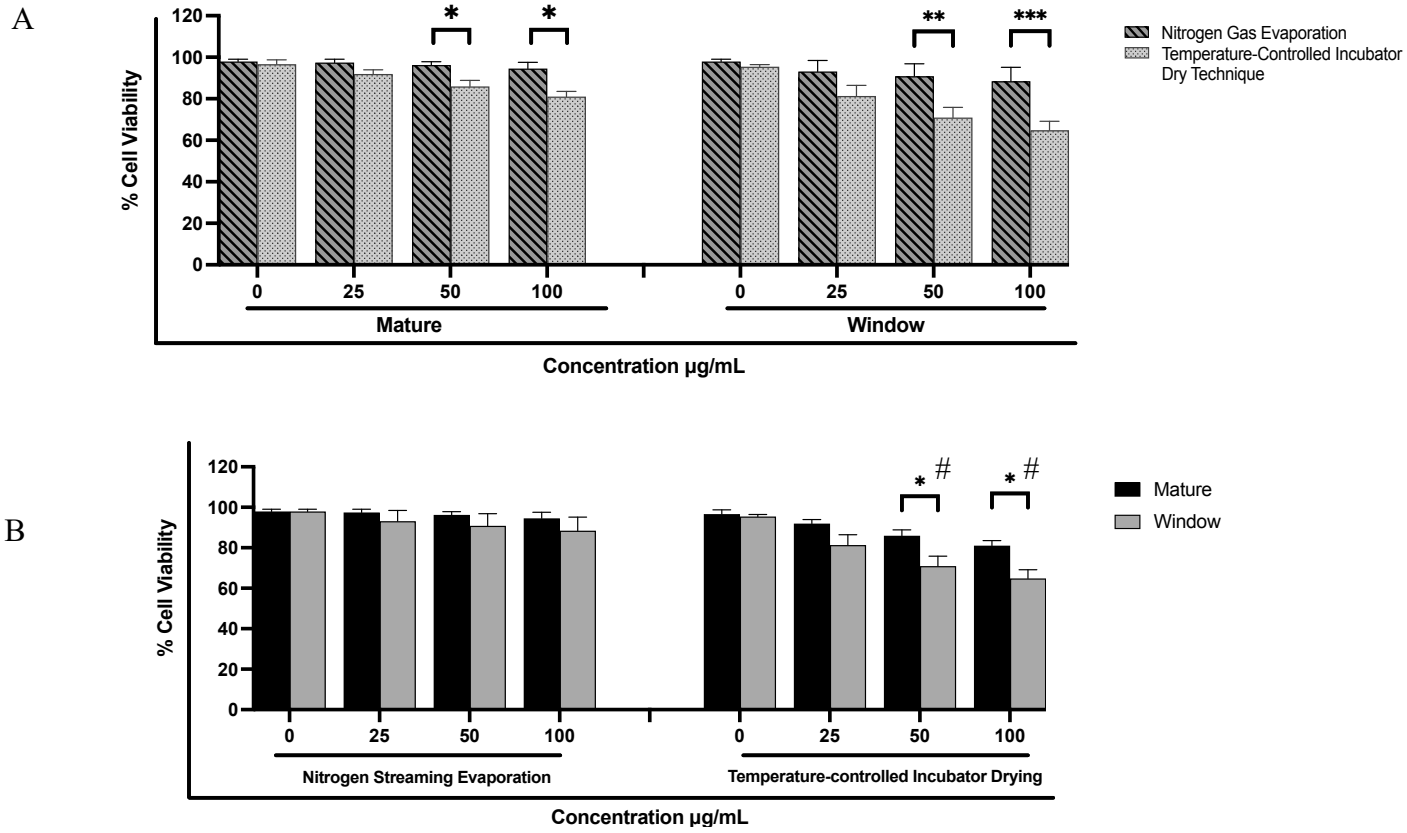

Figure 7. Comparison of bioactivity between temperature-controlled evaporation technique and nitrogen stream evaporation technique. The cell viability of cells treated with window or mature extracts derived from Technique A had a significant reduction in % cell viability at 50 and 100 µg/mL 48 h post-treatment (A). MDA-MB-231 cells treated with extracts derived from Technique A also showed a concentration-dependent reduction in percent metabolism after 48 h compared to vehicle controls denoted by # (B). Absorbance was measured at 550 nm and % cell viability was expressed as means ± SEM of 3 biological replicates. Differences among means were analyzed using a 2-way ANOVA and Bonferroni's test; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.1.2 UPLC-DAD ANALYSIS OF LACE PLANT EXTRACTS BETWEEN NITROGEN AND DRY EVAPORATION TECHNIQUE

UPLC-DAD analysis was performed to determine the differences in the relative abundance of anthocyanin compounds present in the window and mature extracts derived from each evaporation technique. Six anthocyanin standards were used in the analysis including delphinidin-3-glucoside, cyanidin 3-glucoside, pelargonidin-3-glucoside, petunidin-3-gucoside, peonidin-3-glucoside and malvidin-3-glucoside. The RTs of each standard are listed in **Table 7**.

Table 7. UPLC-DAD retention times for six common anthocyanin standards were used in the analysis.

	Anthocyanin standard	RT (min)
1	Delphinidin-3-glucoside	2.98
2	Cyanidin 3-glucoside	4.55
3	Pelargonidin-3-glucoside	5.82
4	Petunidin-3-gucoside	6.55
5	Peonidin-3-glucoside	7.89
6	Malvidin-3-glucoside	9.69

A comparison of the chromatograms showed no significant differences in the overall metabolic profiles or the RT values at the wavelength tested (520 nm) between technique A and B **(Figure 8)**. There were, however, differences in metabolic profiles between mature and window extracts. For example, window extracts had more peaks than mature extracts at 520 nm. The most significant peak (RT ~15.5 mins) was more prominent in window extracts than in mature extracts. The RT times of the compounds detected differed from the RTs of the standards used in this work.

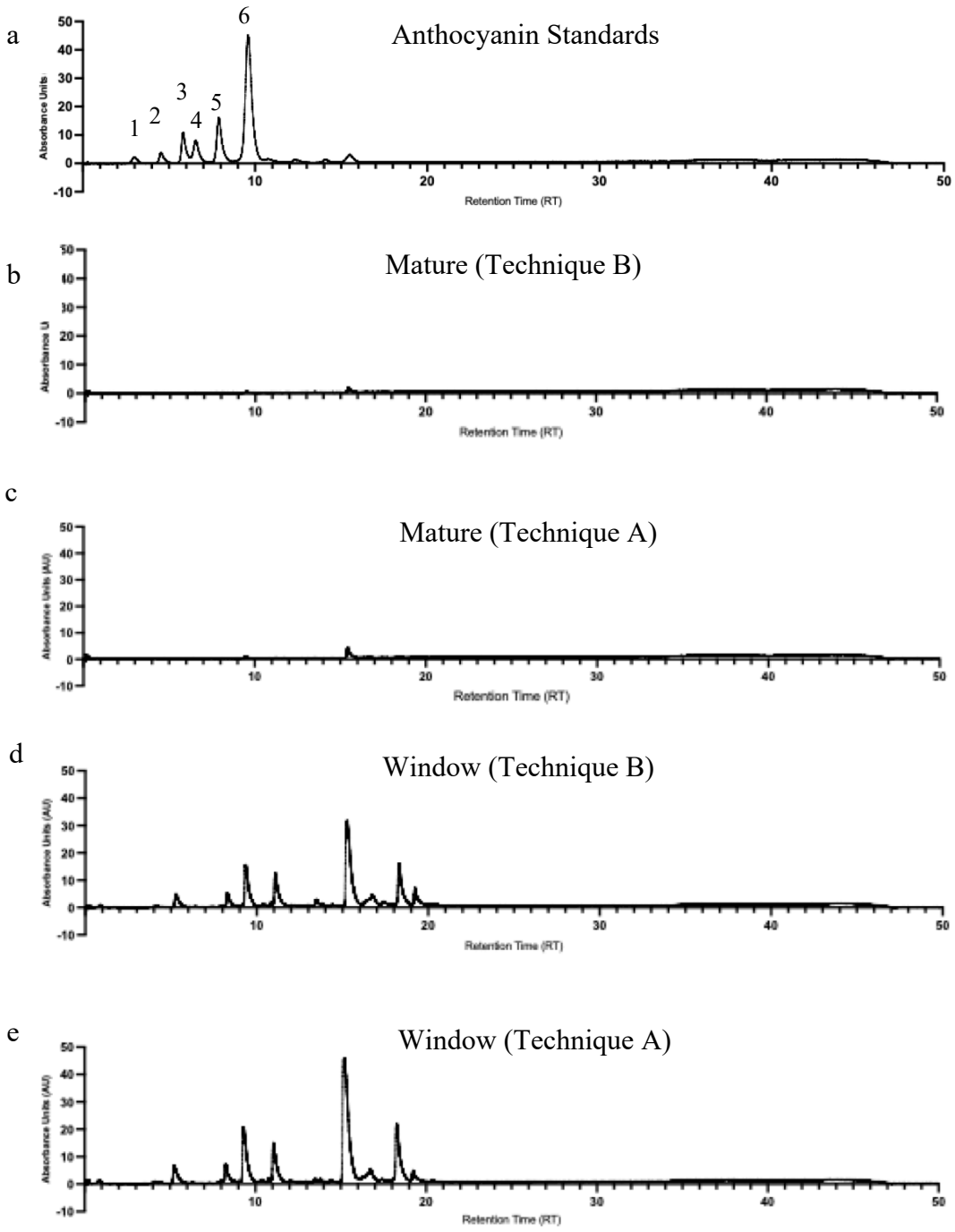

Figure 8. UPLC-DAD assessed the anthocyanins in samples collected using the two evaporation techniques. Six anthocyanin standards consisting of the most common anthocyanins in plants (a in figure) were compared against mature extracts from Technique B (b in figure) mature extracts from technique A (c in figure). Window extracts from Technique B (d in figure) window extracts from Technique A (e in figure). The metabolic profiles do not differ significantly between Techniques A and B

3.2 PHYTOCHEMICAL PROFILING OF LACE PLANT EXTRACTS

3.2.1 UNTARGETED ANALYSIS

The metabolic profile of metabolites present in crude anthocyanin extracts from window and mature extracts from lace plants was analyzed through untargeted LC-MS-MS. The goal of untargeted LC-MS is to create a metabolic profile for the phytochemicals present within the leaves of the lace plant. For this, three biological replicates were analyzed via LC-MS-MS and the resulting data was processed using MS-DIAL5.0 and MetaboAnalyst 5.0.

A Principal Component Analysis (PCA) is useful when determining the general metabolite profile among sample groups and the variability between the sample groups being analyzed (Wang et al., 2022). A PCA plot was used to show the distribution of each replicate in the window and mature sample sets. The three biological replicates for the mature sample clustered together, while the biological replicates for the window sample clustered together. The overall clustering patterns displayed in the PCA plot show a clear distinction between the window and mature stages across their respective replicates indicating that the two stages of the lace plant have different metabolic profiles (**Figure 9**).

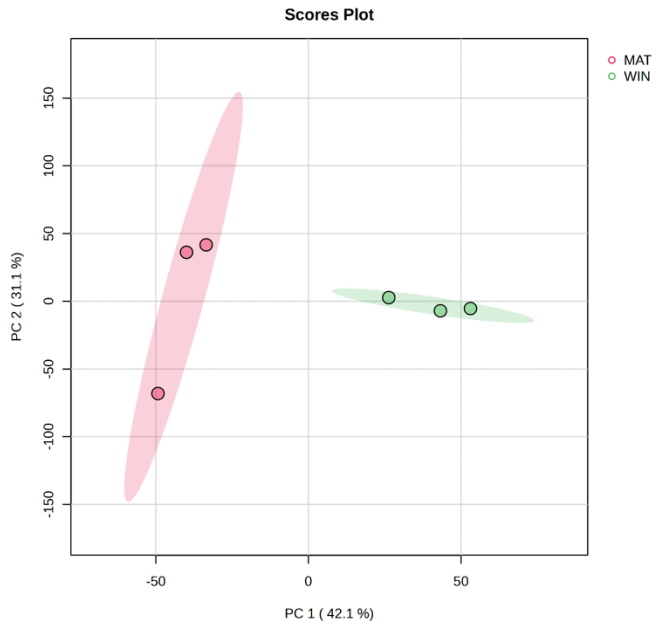

Figure 9. PCA scores plot for window and mature extracts across three biological replicates. The clustering patterns for window and mature replicates indicate distinct clustering for the biological replicates for window and mature extracts.

By compounding the untargeted LC-MS data, a total of 20,646 features were detected in the window and mature extracts of the lace plant **Figure 10**. The term "features" in this analysis is used for bounded, two-dimensional LC/MS signals with a mass charge ratio (m/z) and RT. The total ion chromatogram (TIC) of all the features detected in window and mature extracts across three replicates are shown in **Figure 11**. A PCA plot was conducted to evaluate the biological replicates.

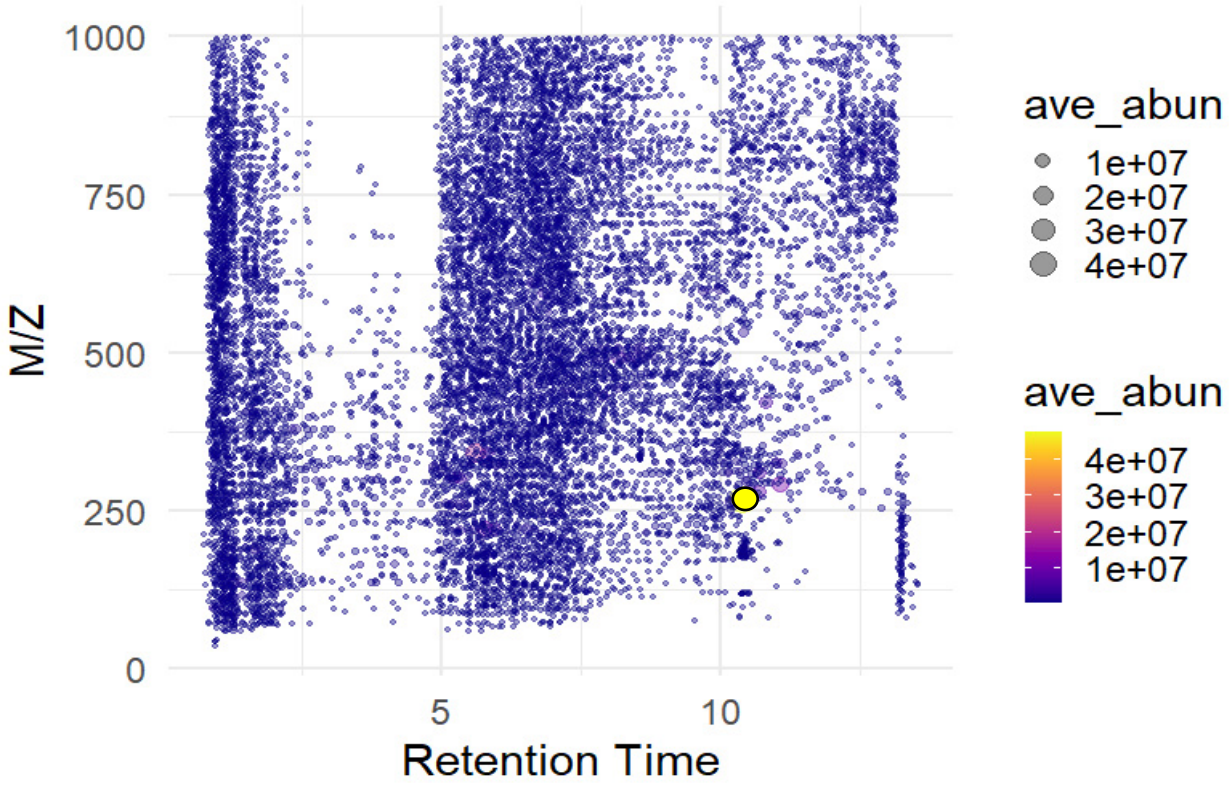

Figure 10. Feature plot for all the features detected in window and mature extracts of the lace plant analyzed by untargeted LC-MS. Each dot represents a single feature and the difference in size corresponds to the relative abundance of the feature within the samples. The most abundant feature detected in window and mature extracts is indicated by the large yellow dot.

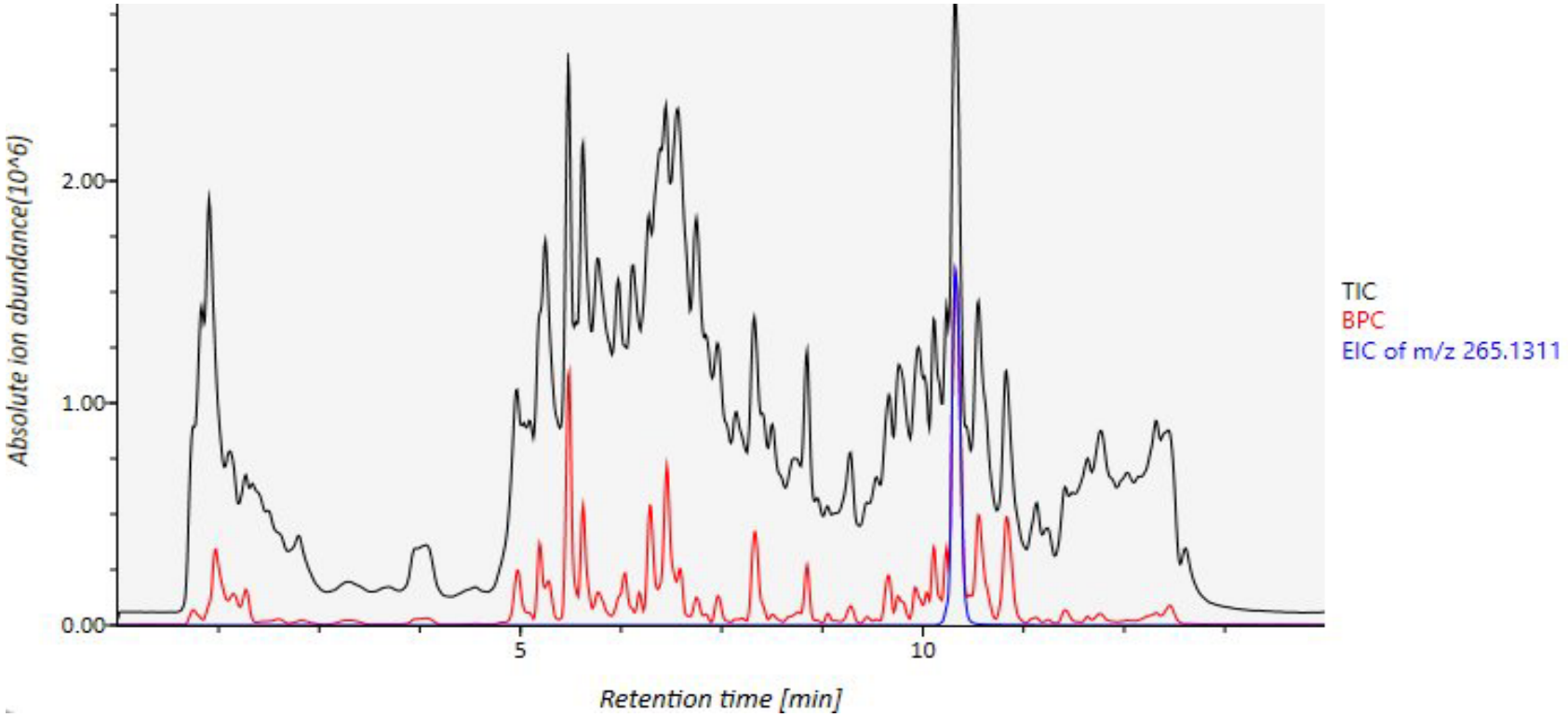

Figure 11. Shows the chromatogram of all the features detected in window and mature extracts of the lace plant analyzed through untargeted LC-MS. The total ion chromatogram of window and mature extracts of the lace plant (TIC, black) against the base ion chromatogram (BPC, red) and the extracted ion chromatogram of the most abundant feature (EIC, blue).

All features detected in window and mature extracts were compared to spectral libraries (as described in section 2.2.1) and were tentatively identified based on the mass charge ratio and fragmentation patterns of the ions. Of the total 20,646 features that were detected, 6,232 features were tentatively identified while 14,414 features remained unmatched. Fifteen features were found unique to the mature stage while only 3 features were unique to the window stage. Features that were assigned an identification through MS-DIAL are referred to as compounds. The summary of these findings is presented in **Table 8**.

Table 8. Summary of untargeted analysis of window and mature extracts via MS-DIAL

	Number of features detected	**Percent of total features (%)**
Total features detected in sample	20,646	100
Tentatively identified features	6,232	30.18
Unmatched/Unknown features	14,414	69.81
Features unique to mature stage	15	0.0007
Features unique to window stage	3	0.0001

MS-DIAL analysis of the data revealed a compound tentatively identified as Honokiol, a biphenolic, to be the most relatively abundant compound in both window and mature samples. The top 10 most abundant features in mature and window extracts are shown in **Table 9.1** and **Table 9.2.** Of the top 10 features detected in the mature extracts, 4 were unmatched with no identification made through the spectral libraries used for the

analysis. Of those tentatively identified, both primary and secondary compounds were detected. Similarly, of the top 10 features detected in the window extracts, 2 features remained unknown but of those tentatively identified, both primary and secondary compounds were detected.

Table 9.1. Top 10 most abundant metabolites detected in the untargeted analysis of mature extracts of the lace plant.

Tentative compound name	Average RT (min)	Average m/z ratio	Average peak area
Honokiol	10.47	265.13	34733892.07
Unknown	5.95	222.06	14859640.65
Coumachlor	5.58	341.07	13427861.63
Unknown	5.76	222.06	12134497.52
Thymol-beta-D-glucoside	10.67	311.15	10317049.78
Unknown	8.19	491.18	10079271.50
Koumidine	11.04	293.16	10060433.11
Unknown	10.78	421.20	8387674.02
Unknown	8.42	491.18	8282700.97
[5-acetyloxy-3-(hydroxymethyl)-2-oxo-6-propan-2-ylcyclohex-3-en-1-yl] 3-methylbutanoate	11.03	325.16	7732961.57

Table 9.2. Top 10 most abundant metabolites detected in the untargeted analysis of window extracts of the lace plant

Tentative compound name	Average RT (min)	Average m/z ratio	Average peak area
Honokiol	10.40	265.13	42114217.44
Coumachlor	5.61	341.07	26359461.20
Koumidine	11.04	293.16	11926570.57
Unknown	5.79	222.06	10243912.58
kampferol-7-O-hexosyl(1-2)deoxyhexoside	6.82	593.11	8617518.03
Flavone base + 3O, 1MeO	5.25	299.06	7590181.46
Mianserin-N-Oxide	10.69	279.15	7512064.20
Unknown	7.93	431.07	7267539.12
C21H34O11	8.58	507.21	7102435.24
Malic acid	1.21	133.01	7078509.73

3.2.2 THE MOST ABUNDANT FEATURE DETECTED IN THE UNTARGETED ANALYSIS

The untargeted analysis via MS-DIAL tentatively identified honokiol as the most abundant feature detected in both window and mature extracts of the lace plant. Because this tentative identity is based only on m/z values, it was necessary to perform additional analyses to confirm the identity of the compound in question. For this, fragmentation patterns observed in the MS/MS spectra between a commercial standard of honokiol were compared to the compound found in window and mature extracts. The MS/MS spectra of the honokiol standard (with the parent ion 267.1392 Da), the "honokiol" compound found in window extracts (with the parent ion 267.1856 Da) and the "honokiol" compound in mature (with the parent ion 267.1754 Da) are shown in **Figures 12 A, B** and **C** respectively. A comparison of their fractionation patterns in the MS/MS spectra shows that the fragmentation patterns of the parent ions are not comparable between the

honokiol standard, and the compound found in window and mature extracts. Specifically, the main product ion detected in the honokiol standard was 239.1074 Da (circled black in the figure) while the main product ions detected in window and mature extracts was 235.1688 Da and 235.2031 Da respectively (circled red in the figure). The findings of this work indicate that the compound identified in the window and mature extracts detected via the untargeted analysis is not honokiol.

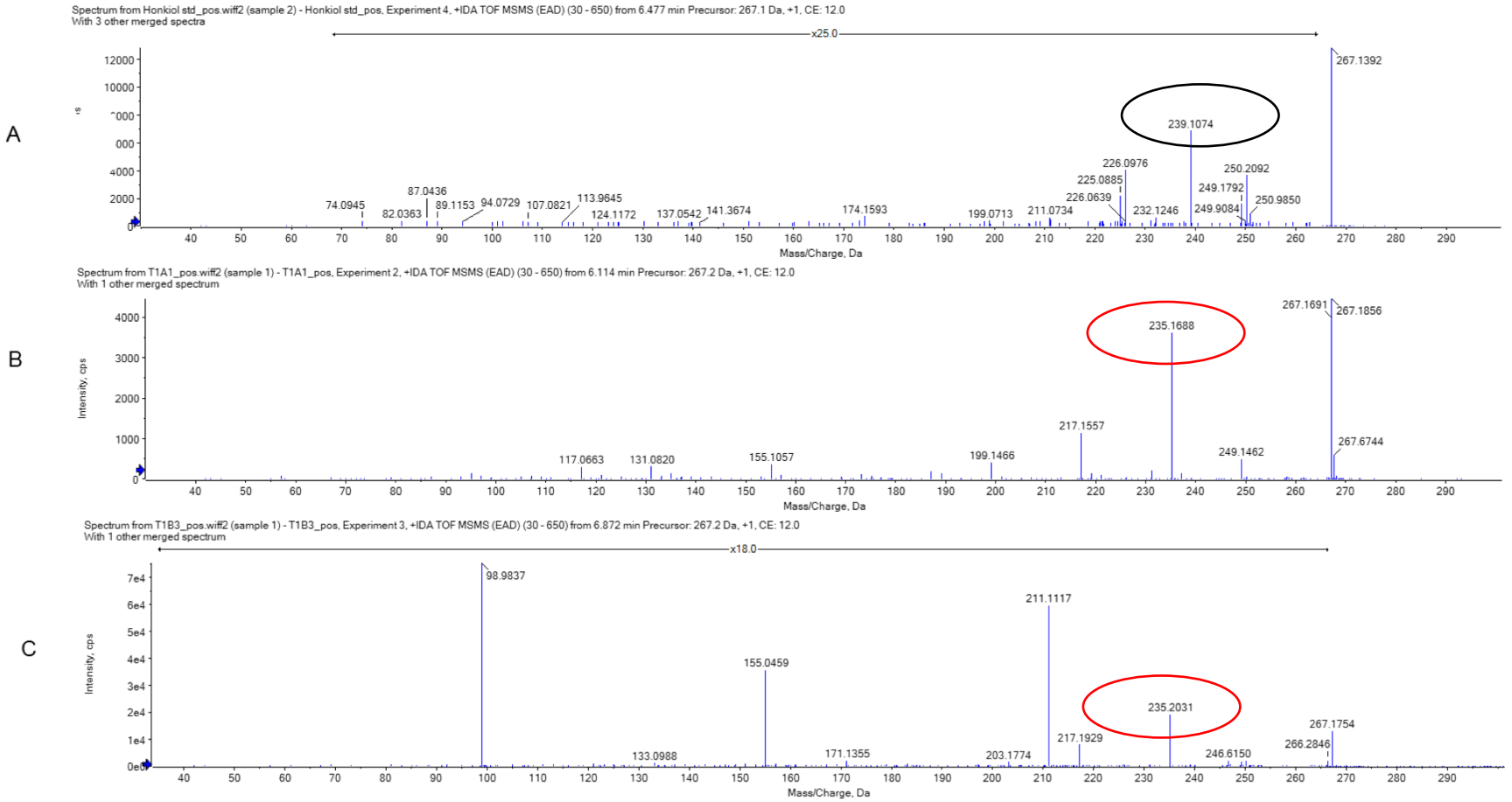

Figure 12. MS/MS fragmentation spectra of the honokiol standard vs lace plant extracts. MS/MS spectra of the honokiol standard (A) and the MS/MS spectra of the compound in window extracts (B) and mature extracts (C) show different fragmentation patterns and are therefore not comparable. Notably, the main production of the honokiol standard (239.1 Da), circled in black, is absent in the compound of interest in both the window and mature fragmentation spectra. The main product in the compound detected in the leaf extracts is circled in red (235.2 Da).

3.2.3 TARGETED METABOLOMIC ANALYSIS

Following the untargeted analysis of window and mature extracts of the lace plant, three biological replicates of a targeted LC-MS analysis were conducted to detect phenolic compounds that are either anthocyanin or non-anthocyanin-based in window and mature extracts of the lace plant. **Table 10.1** presents the complete list of anthocyanins and non-anthocyanin phenolics detected. Thirty-four phenolic compounds (anthocyanins and non-anthocyanins) were detected in window extracts and thirty phenolic compounds (anthocyanin and non-anthocyanins) were detected in mature extracts. The most commonly detected anthocyanin was delphinidin-3-glucoside at 472.54 µg/g in window and 91.85 µg/g in mature extracts. Followed by the non-anthocyanin phenolic compound, rutin at 303.27 µg/g and 50.57 µg/g in window and mature extracts respectively. Non-anthocyanins such as gallic acid, Okanin and vitexin-2-O-rhamnoside anthocyanins such as malvidin and peonidin were only present in window extracts. Whereas the non-anthocyanin, sinapic acid, was exclusively present in mature extracts. Twenty-three non-anthocyanins and eight anthocyanins were not detected (ND) in either window or mature extracts **(Table 10.2)**.

Table 10.1 Concentration of phenolics (anthocyanin and non-anthocyanin) compounds detected in window and mature extracts through targeted LC-MS analysis. Values are expressed as concentration µg per gram of dry leaf weight. Compounds below the limit of detection are denoted ND (not detected).

Name of Standard	Concentration (µg/g)	
	Window	Mature
Afzelin	0.73	0.06
Arbutin	1.20	0.08
Aromadendrin	0.23	0.03
Caffeic Acid	23.97	5.40
Catechin	56.55	13.88
Chlorogenic Acid	0.45	0.05
Epicatechin	0.71	40.26
Gallic Acid	0.58	ND
Isovitexin	0.67	0.06
Kaempferol	0.05	0.001
Kaempferol-3-glucoside	0.23	0.03
Luteolin	0.12	0.01
Naringin	2.12	0.06
Nicotiflorin	5.08	4.72
Okanin	0.02	ND
Orientin	0.36	0.17
Para-coumaric Acid	0.21	0.03
Polydatin	0.11	0.01
Procyanidin C	9.60	0.14
Protocatechuic Acid	2.75	0.25
Quercetin	0.81	0.10
Quercetin-3-galactoside	1.12	0.48
Rutin	303.27	50.57
Sinapic Acid	ND	0.10
Syringic Acid	3.72	0.19
Taxifolin	1.05	0.92
Vicenin-2	5.19	4.78
Vitexin	0.67	0.06
Vitexin-2-O-rhamnoside	1.38	ND
Cyanidin Chloride	15.30	0.34
Cyanidin-3-O-glucoside	3.80	0.04
Cyanidin-3-rutinoside	0.63	0.075
Delphinidin-3-glucoside	472.54	91.85
Malvidin	0.58	ND
Malvidin-3-glucoside	ND	ND
Peonidin	0.745	ND

Table 10.2 List of phenolic compounds below the limit of detection (not detected, ND) in window or mature extracts via targeted LC-MS analysis.

Name of standard	Concentration (µg/g)	
	Window	Mature
Apigenin	ND	ND
Caftaric Acid	ND	ND
Daidzein	ND	ND
Daidzin	ND	ND
Ellagic Acid	ND	ND
Epicatechin Gallate	ND	ND
Epigallocatechin	ND	ND
Ferulic Acid	ND	ND
Fisetin	ND	ND
Genistein	ND	ND
Glycitin	ND	ND
Herbacetin	ND	ND
Hesperetin	ND	ND
Isoquercetin	ND	ND
Isorhamnetin	ND	ND
Liquiritingenin	ND	ND
Myricetin	ND	ND
Piceatannol	ND	ND
Procyanidin A	ND	ND
Procyanidin B	ND	ND
Resveratrol	ND	ND
Rhapontin	ND	ND
Vanillic Acid	ND	ND
Zeaxanthin	ND	ND
Cyanidin-3-arabinoside	ND	ND
Delphinidin	ND	ND
Malvin	ND	ND
Oenin	ND	ND
Pelargonidin	ND	ND
Peonidin-3-O-glucoside	ND	ND
Petunidin	ND	ND

3.2 CYTOTOXIC EFFECTS OF CRUDE EXTRACTS FROM LACE PLANT LEAVES IN TRIPLE-NEGATIVE BREAST CANCER CELLS

3.3.1 PERCENT METABOLIC REDUCTION IN TRIPLE NEGATIVE CELLS VS HEALTHY BREAST EPITHELIAL CELLS

Previous work by our research group showed that crude anthocyanin extracts display apoptotic characteristics in triple-negative MDA-MB-231 cells (Gunawardena et al., 2021). Thus, once an appropriate evaporation technique was established, the cytotoxicity of crude anthocyanin extracts from lace plants in MDA-MB-231 cells was measured and compared to healthy breast epithelial cells via MTT viability assay at 48 h. Data from the MTT assays shows that crude anthocyanin extract from mature and window leaves of the lace plant reduced the percent cell viability of MDA-MB-231 cells in a dose-dependent manner at 48 h (**Figure 13A**). In MDA-MB-231 cells treated with mature extracts, significant effects ($P < 0.05$) were observed at 50, 100 and 250 µg/mL with a percent metabolic reduction of 15 -30 %. Likewise, significant effects ($P < 0.05$) were observed in MDA-MB-231 cells treated with window extracts across all four concentrations tested with a percent metabolic reduction of 15 – 40 %. Healthy breast epithelial MCF10A cells did not show a dose-dependent decrease in percent cell viability in cells treated with either mature or window extracts at any concentration tested (**Figure 13B**).

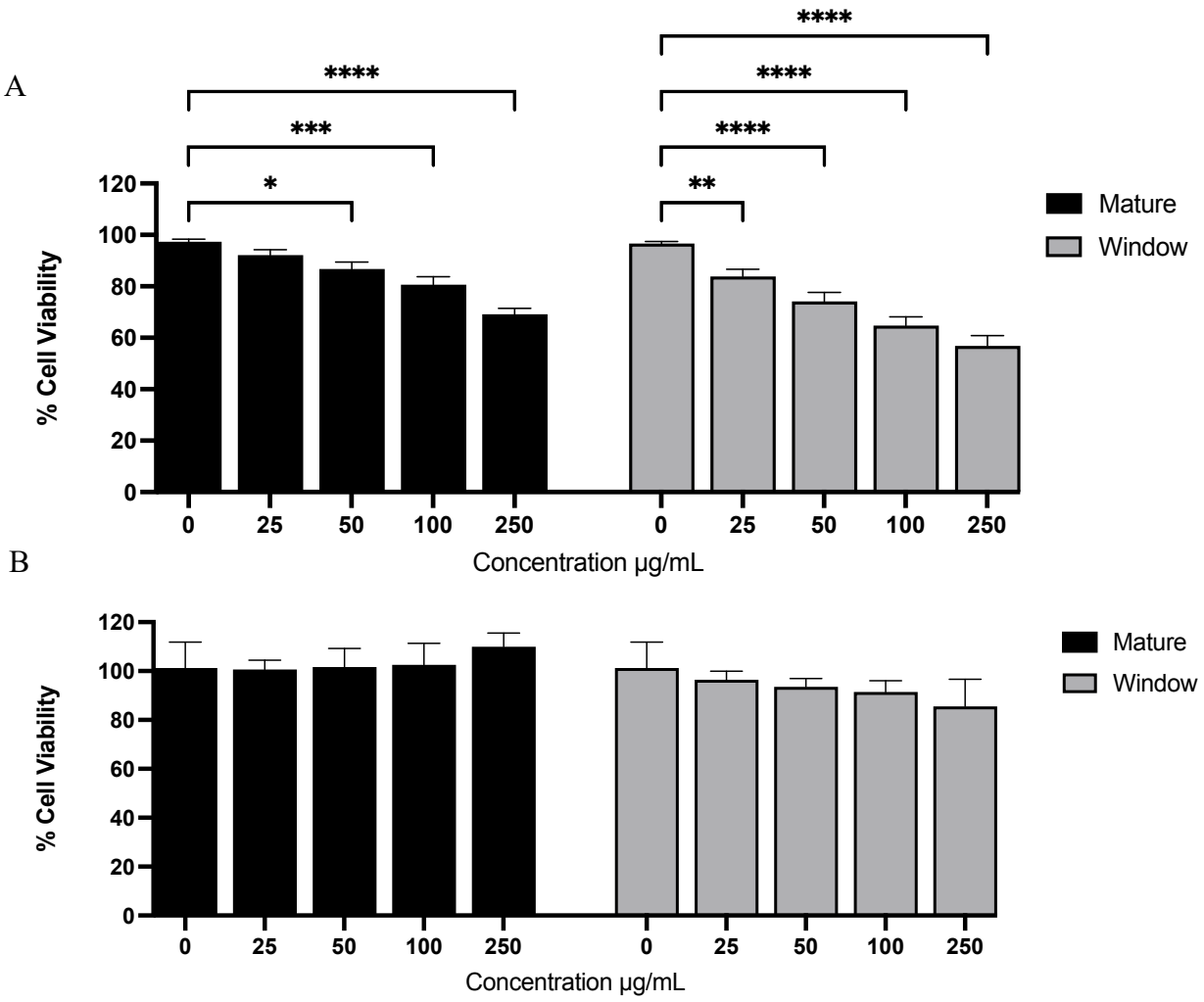

Figure 13. Comparison of bioactivity between MDA-MB-231 Triple negative breast cancer cells and MCF-10A, healthy breast epithelial cells. The % cell viability of MDA-MB-231 breast cancer cells treated with 0, 25, 50, 100 and 250 µg/mL treated with window and mature extracts of *A. madagascariensis* was determined using an MTT assay after 48 h (A). The % cell viability of MCF-10A healthy breast cells treated with 0, 25, 50, 100 and 250 µg/mL treated with window and mature extracts of *A. madagascariensis* was determined using an MTT assay after 48 h (B). MDA-MB-231 cells treated with window and mature extracts showed a concentration-dependent reduction in percent metabolism after 48 h while no cytotoxic effect was measured for MCF-10A cells. Absorbance was measured at 550 nm and % cell viability was expressed as means ± SEM of 4 biological replicates. Differences among means were analyzed using a 2-way ANOVA and Bonferroni's test; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.3.2 EFFECTS OF CRUDE LACE PLANT EXTRACTS ON TNBC CELL PROLIFERATION

Following cell metabolic measurements in triple-negative cancer cells, the next approach was to test whether window and mature lace plant extracts reduced cell proliferation in MDA-MB-231 cells at 24 and 48 h. Data from this work, shows that mature and window extracts of the lace plant reduced cell proliferation in MDA-MB-231cells in a dose-dependent manner 24 h and 48 h post-treatment. At 24 h, both window and mature extracts reduce MDA-MB-231 cell proliferation across all concentrations tested (0, 25 - 250 µg/mL) (**Figure 14A**). After 24 h, at the highest concentration tested, there was a 52 % reduction in cell proliferation in MDA-MB-231 cells treated with window extracts, while only a 20 % reduction in cells treated with mature extracts was measured. At 48 h post-treatment, significance ($p < 0.05$) in cell proliferation was only observed at the highest concentration (250 µg/mL) for mature extract treatments, while window extracts sustained antiproliferative effects at all concentrations tested (**Figure 14B**). At 48 h post-treatment, at 250 µg/mL, there was a 44 % reduction in cell proliferation in cells treated with window extracts while there was a 31 % reduction in cell proliferation in cells treated with mature extracts. A stronger dose-dependent effect on reducing cell proliferation in MDA-MB-231 is observed in cells treated with window extracts for 48 h.

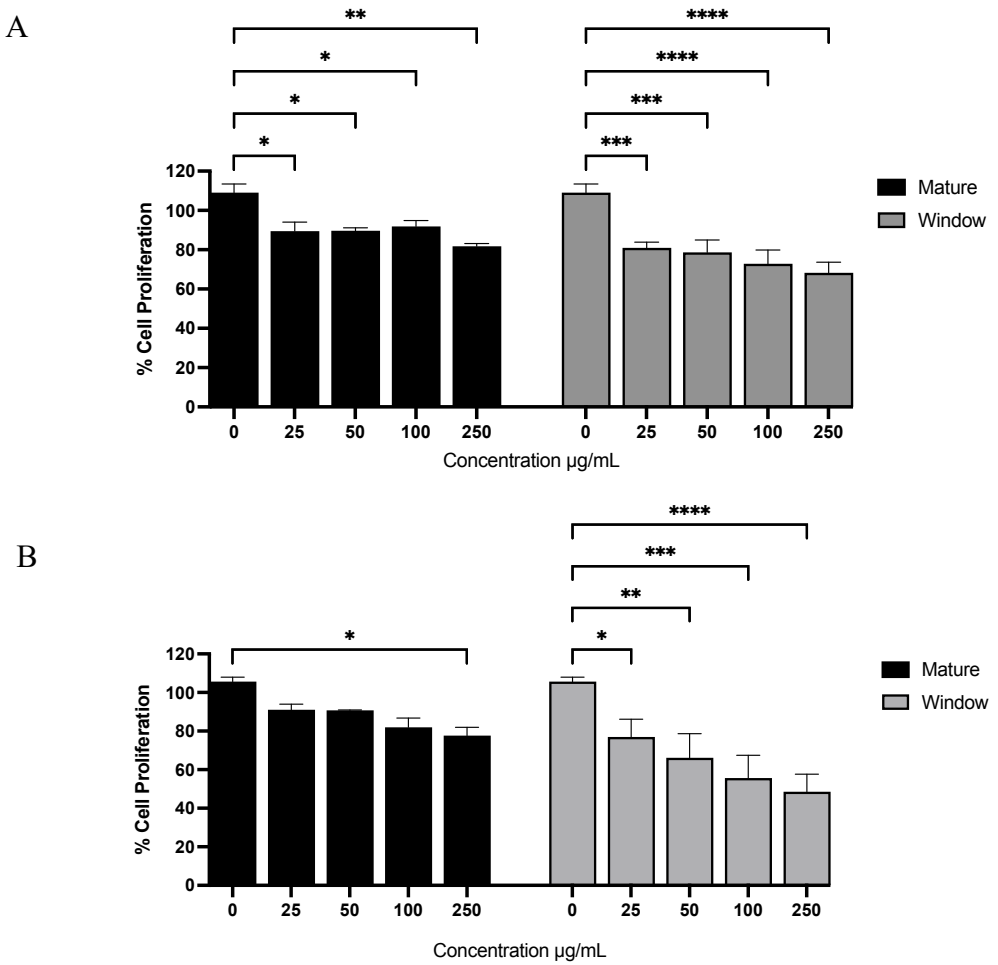

Figure 14. Percent cell proliferation between MDA-MB-231 triple negative breast cancer cells at 24 h (A) and 48 (B) post-treatments. The % cell proliferation of MDA-MB-231 breast cancer cells treated with 0, 25, 50, 100 and 250 µg/mL treated with window and mature extracts of *A. madagascariensis* was determined using CyQuant proliferation assay at 24 h (A). The % cell proliferation of MDA-MB-231 with 0, 25, 50, 100 and 250 µg/mL treated with window and mature extracts of *A. madagascariensis* at 48 h (B). MDA-MB-231 cells treated with window and mature extracts showed a concentration-dependent reduction in percent proliferation after 24 h. Fluorescence was measured at EX 480 nm/EM 520 nm and % cell proliferation was expressed as means ± SEM of 3 biological replicates. Differences among means were analyzed using a 2-way ANOVA and Bonferroni's test; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3.3.3 EFFECTS OF LACE PLANT EXTRACTS ON TNBC CELL MIGRATION

To understand if lace plant extracts prevent cell migration of MDA-MB-231 cells, a wound healing assay was performed. A wound healing assay entails a manually induced vertical "scratch" in a monolayer of adherent cells followed by the exposure of the cells to the treatment. Treatments are followed by image acquisition of the scratch/gap in the beginning and at regular intervals for a defined period. The images acquired were used to capture the migration of cells towards the gap over time. If the motility of the cells is not affected, a gradual closure of the gap will be observed. However, if treatments have impeded the cells' motility, the gap area will remain unchanged. Images of the scratch for MDA-MB-231 cells following treatments of lace plant window and mature extracts are shown in **Figure 15**. Analysis using the image J plugin *wound_healing_size_tool* showed no significant reduction/arrest in the migration in MDA-MB -231 cells exposed to window or mature extracts over 14 h post-treatment. Though an overall decrease in wound area across all treatments including in media and vehicle **(Figure 16A)**, window and mature extracts at 25, 50 and 100 µg/mL **(Figure16B, C and D respectively)** are measured, the effects are not significant. This was further confirmed when the area under the curve was calculated for the changes in wound area and no significant changes in the AUC between treated and non-treated cells was observed (**Figure 17**).

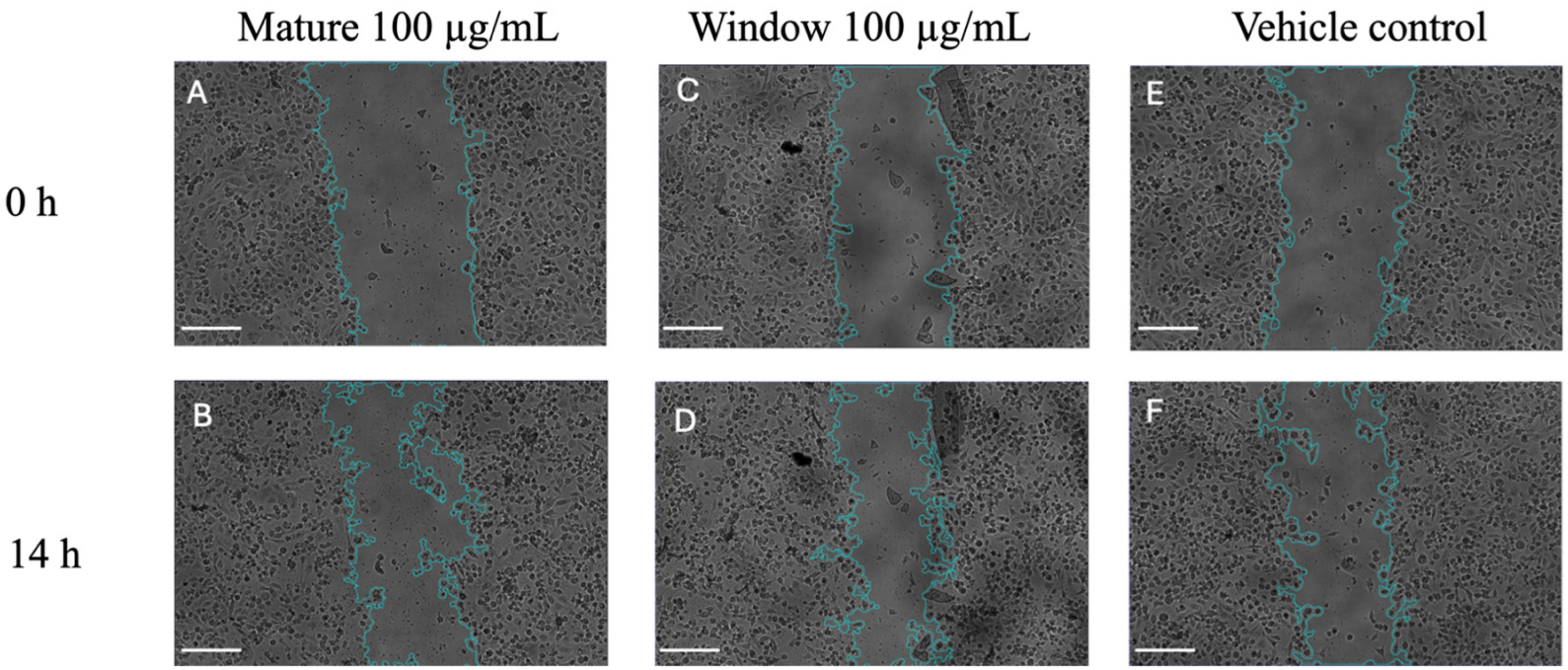

Figure 15. Wound closure of MDA-MB-231 cells following treatments of extracts from lace plants leaves 14 h post-treatment. The area of the horizontal wound for cells treated with mature extracts from lace plant (**A,B**) window extracts from the lace plant (**C,D**) and vehicle control (**E,F**) was measured using the wound healing assay and analyzed using the Wound Healing Sizing Tool on Image J. Scale bar: A- F = 200 µM

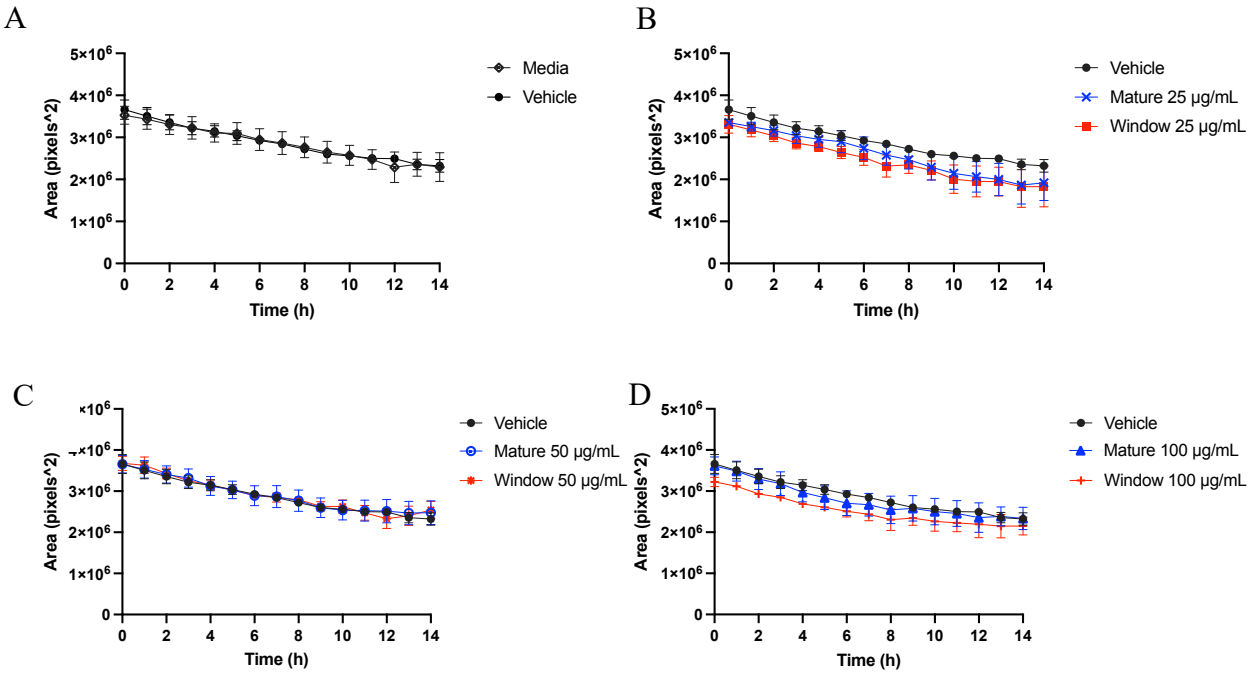

Figure 16. The reduction in the scratch area over 14 h post-treatment. A vertical scratch was induced on a monolayer of MDA-MB-231 cells to mimic a wound and the migration of cells into the gap left by the scratch was imaged at regular intervals for 14 h post-treatment. The area reduction of the scratch was measured using Image J and analyzed by the *wound healing size tool.* Three concentrations of the window and mature extracts were tested, alongside a vehicle and media controls. The graphs represent the reduction in wound area in media and vehicle controls (A), mature and window extracts at 25 µg/mL (B), mature and window extracts at 50 µg/mL (C) and mature and window extracts at 100 µg/mL (D). The results were collected from 3 biological replicates.

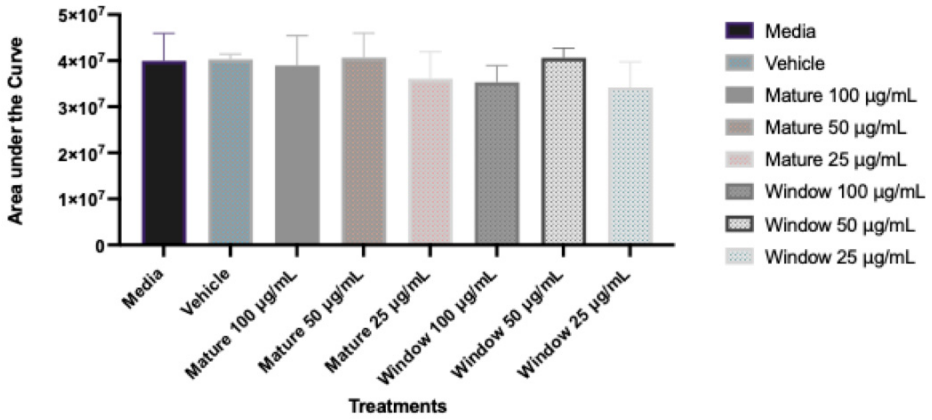

Figure 17. Area under the curve of the scratch area in MDA-MB-231 cells. AUC was calculated for the reduction in the scratch area after cells were exposed to media, vehicle and window/mature extracts at concentrations of 25, 50 and 100 µg/mL. Reduction in wound area across 14 h post-treatment with window and mature extracts of the lace plant (A) and the AUC of the wound area for each treatment concentration tested (B).

3.3.4 ROS CHANGES IN IN TNBC CELLS POST TREATMENTS OF CRUDE EXTRACTS

To determine if extracts from the lace plant induce their anticancer effects in MDA-MB-231 cells through the intrinsic mitochondrial pathway, fold change in ROS activity was measured using the $CMDCFH_2$-DA, oxidative stress indicator. $CMDCFH_2$-DA functions by passively diffusing into the cell, where it is de-esterified to CMDCF. Once de-esterified, CMDCF is restricted within the cell and in the presence of ROS forms a fluorescent complex that can be measured and quantified as an indicator for ROS ("Reactive Oxygen Species (ROS) Detection Reagents," n.d.). The results from this assay, show a dose-dependent increase in ROS activity in cells treated with mature extracts 24 h post-treatment, with 100 and 250 µg/mL showing the highest fold change in ROS activity. The fold increase in cells treated with mature was higher as compared to cells treated with window extracts **(Figure 18)**. MDA-MB-231 cells co-treated with N-acetylcysteine treatments (10 mM) showed a reduction in ROS activity compared to cells

that were not co-treated with NAC. This effect was significant for 50, 100 and 250 µg/mL for mature and 250 µg/mL for window extract-treated cells (**Figure 19**).

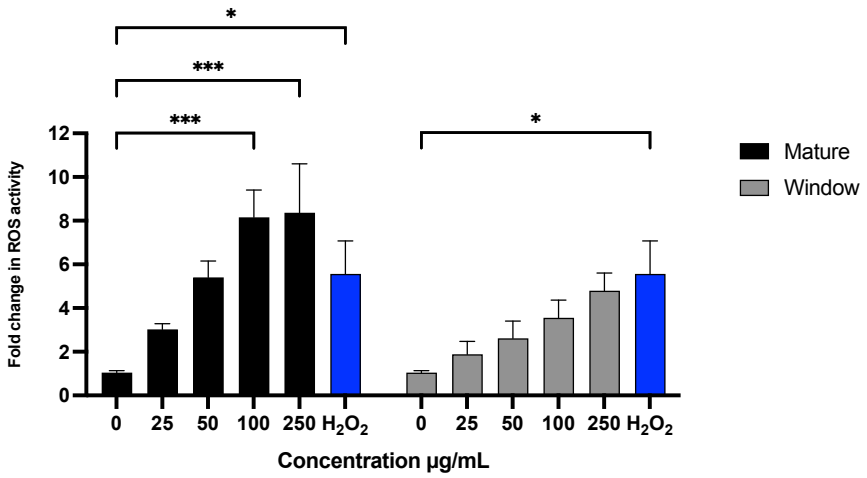

Figure 18. Fold change in reactive oxygen species activity in MDA-MB-231 cells following treatments of window and mature extracts from lace plant. Fluorescence was measured at EX 485/EM 530 and fold change was expressed as means ± SEM of 3 biological replicates. Differences among means were analyzed using a 2-way ANOVA and Bonferroni's test; *P < 0.05, **P < 0.01, º***P < 0.001, ****P < 0.0001

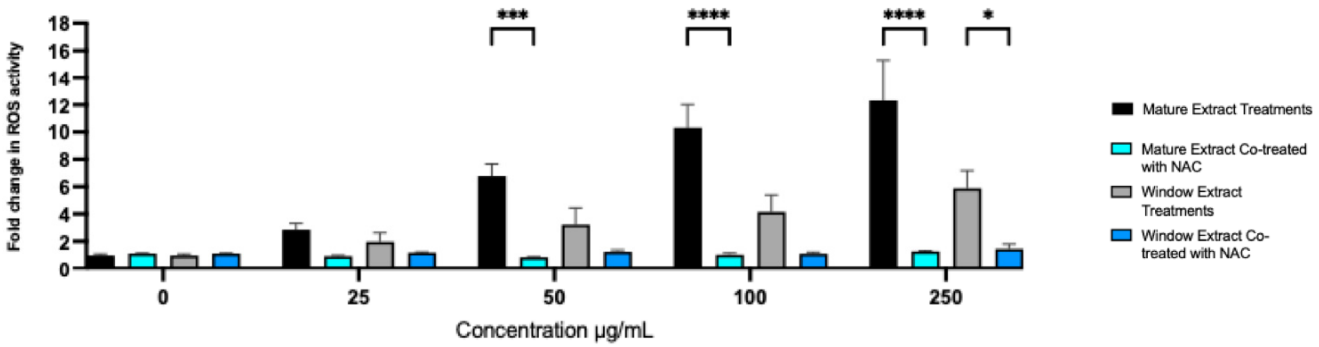

Figure 19. ROS measurements after MDA-MB-231 cells were co-treated with N-acetylcysteine. Fluorescence was measured at EX 485/EM 530 and fold change was expressed as means ± SEM of 3 biological replicates. Differences among means were analyzed using a 2-way ANOVA and Bonferroni's test; *P < 0.05, **P < 0.01, º***P < 0.001, ****P < 0.0001.

CHAPTER 4: DISCUSSION

Following the selection of the most optimal solid-liquid evaporation technique in the extraction of phytochemicals from window and mature leaves of the lace plant, we performed a series of biological assays in the TNBC cell line, MDA-MB-231, to evaluate the anticancer properties of leaf extracts from the lace plant. This was to validate previous findings that window and mature leaf extracts from the lace plant generated morphological changes consistent with apoptosis in MDA-MB-231 cells post-treatment (Gunawardena et al., 2021).

The results reported in the current study successfully demonstrate that phytochemicals found within window and mature lace plant extracts have anticancer activity in MDA-MB-231 cells, including a reduction in cell viability and cell proliferation. This work also supports our hypothesis that lace plant leaves will increase oxidative stress in MDA-MB-231 cells post-treatment by showing an increase in ROS in extract-treated MDA-MB-231 cells post-treatment. While the exact metabolites responsible for the bioactivity are still under investigation, this study details possible metabolite candidates for the reported bioactivity by listing the most abundant phytochemicals found in window and mature extracts detected via targeted and untargeted LC-MS-MS analysis. The subsequent sections will discuss the findings related to each objective outlined in this thesis.

4.1 EVAPORATION TECHNIQUE A YIELDS BETTER CYTOTOXICITY IN TNBC CELLS

The first objective of this work was to select a simple evaporation technique that can be used in the extraction of phytochemicals from lace plant leaves. Two evaporation techniques with different were evaluated to fulfill this. In brief, Technique A involved the incubation of macerated window or mature leaves in a methanolic extraction solution followed by centrifugation, filtration and removal of the extraction solvent using a temperature-controlled incubator at 25 ºC. Technique B consisted of incubating the macerated window or mature leaves in a methanolic extraction solution followed by centrifugation, filtration and removal of the extraction solution by directing a stream of nitrogen gas into the vial containing the solution. Extracts from each method were subsequently tested in MDA-MB-231 cells to evaluate their efficacy *in vitro*.

The first key finding of this work is that cells treated with extracts derived from Technique A had a statistically significant percent reduction of cell viability in MDA-MB-231 cells compared to cells treated with extracts derived from Technique B as measured by MTT assay 48 h post-treatment **(Figure 7)**. Extraction techniques and operating conditions used in the preparation of plant extracts can greatly influence the yield of individual active ingredients present and corresponding biological activities (Naviglio et al., 2023). Technique A was selected as the more favourable extract preparation method as it exhibited the highest biological activity on MDA-MB-231 cells.

Despite the differences in the biological activity of the extracts between the two techniques, our HPLC data shows no significant differences between the metabolic profiles, or the RT of the compounds detected at 520 nm between the two techniques (**Figure 8**). One reason for this contradictory finding between the results from the

biological assay and the HPLC analysis of anthocyanins detected could be that the main active ingredient(s) responsible for the bioactivity is not an anthocyanin compound, but another PSM not detected at the wavelength tested in this analysis (520 nm). At the time of this writing, two additional HPLC replicates were collected to replicate these initial findings. Again, no difference in metabolic profiles or their RT times were observed between the two evaporation techniques. This data is presented in **Appendix 1.** It is important to note, however, that there are marked differences in peak intensities between the two leaf stages, mature and window, in both techniques tested. For example, window extracts generally have more peaks and higher peak intensities than mature extracts as exemplified by the highest most abundant peak (~ 15 min RT). Although, this trend appears to be independent of the evaporation technique and more in support of previous findings from our research group that window leaves of the lace plant have higher anthocyanin content than mature leaves (Dauphinee et al., 2017).

Based on the bioactivity of the extracts in MDA-MB-231 cells *in vitro,* window and mature extracts derived from technique A were used for all subsequent biological and analytical experiments conducted in this research work.

4.2 PHYTOCHEMICAL PROFILING OF THE LACE PLANT VIA UNTARGETED AND TARGETED LC-MS ANALYSIS

This work is the first to report a comprehensive phytochemical profile for window and mature leaf extracts from the lace plant via untargeted and targeted metabolomics. Metabolomics is an evolving field generally defined as “a comprehensive, high-throughput measurement of all metabolites and low-molecular-weight molecules in a complex metabolite mixture” (Hall et al., 2002; Clish., 2015). Hence, plant metabolomics

is becoming an increasingly attractive tool for researchers whose goal is to profile large numbers of metabolites that are present in a mixture as is typical of plant extracts (Salam et al., 2023). Both untargeted and targeted analyses were applied when profiling the metabolites found in the lace plant's window and mature leaf extracts.

4.2.1 UNTARGETED METABOLOMICS REVEALS A COMPLEX METABOLITE PROFILE FOR LACE PLANT EXTRACTS

The untargeted metabolomic analysis conducted for this work showed separate clustering patterns for the metabolites detected between window and mature samples as presented in **Figure 9**. This suggests that the biological replicates for window and mature extracts have distinct differences that distinguish them from each other. Previously our research group conducted an RNA-Seq analysis on the lace plant and identified distinct patterns in differentially expressed genes (DEGs) between window and mature leaves. In window leaves, higher expression of genes associated with the biosynthesis of anthocyanins and plant proteases were upregulated. In mature leaves, DEGs associated with chlorophyll development and photosynthesis were more common (Rowarth et al., 2021). The variations in DEGs suggest that different metabolic processes are involved in leaf morphogenesis and perforation formation across the two leaf stages. For example, window leaves that are actively undergoing PCD will require metabolites that both facilitate and guard against oxidative stress, whereas mature leaves will only require metabolites necessary for routine cellular functions. This is corroborated by the clustering patterns displayed by the PCA plot that separates window and mature metabolite profiles.

Overall, the untargeted analysis detected a total of 20, 646 features in window and mature extracts of the lace plant **(Figure 10 and Figure 11).** The top 10 most abundant features/metabolites were then sorted by average peak area for window and mature

extracts **(Table 9.1 and 9.2 respectively).** Extensive structural databases were used to identify the features detected via LC-MS-MS which resulted in the tentative identification of approximately 6,232 features which accounted for about ~30 % of the total detected. A total of 14,414 features accounting for 69.81 % of the total detected remained unmatched. Though a greater number of peaks were left unidentified, this is not unusual and remains a challenge in the field of metabolomics (Chen et al., 2021). Unidentified/unmatched features can be a result of limited information, adducts, fragments or isotopes of metabolites or due to features being of non-biological origin. Additionally, it is also possible that these unidentified features are beyond the scope of structural databases due to incomplete natural product libraries (Climaco Pinto et al., 2022; Chen et al., 2021; Strutz et al., 2022).

When only considering the compounds that were tentatively identified, the most abundant metabolite was tentatively identified as "honokiol" accounting for 6.6 % and 5.8 % of the total peak area in window and mature extracts respectively. Honokiol is a phenyl-proponoid secondary metabolite found in *Magnolia* species *M. obovata, M. officinalis, grandiflora* and *M. dealbata* (Rauf et al., 2018). Though it is not entirely understood what role honokiol plays in the magnolia plant, honokiol and the structurally similar; magnolol and obovatol have previously been demonstrated to have pharmacological and neuroprotective properties (Rauf et al., 2018; McKeown et al., 2014). For example, honokiol has been proven to be efficacious against lung, prostate, gall bladder, colon and breast cancers (Banik et al., 2019). Its isomer magnolol was shown to cause cell cycle arrest and interact with insulin-like growth hormones in human prostate cancer cells (McKeown et al., 2014). To the best of our knowledge, honokiol and the related magnolol have not been detected in other plant species. Hence, the tentative

identification of honokiol as the most abundant metabolite in lace plant leaf extracts was unexpected. It is important to note that though untargeted metabolomics reveals an accurate mass charge/ratio (m/z) of compounds and their molecular formula, it does not provide the structural information of the compound. As such, all identifications are tentative unless otherwise confirmed through additional methods (Strutz et al., 2022). Therefore, the initial analysis of the untargeted metabolomics data prompted additional follow-up work to compare the tentatively identified "honokiol" metabolite against a commercial honokiol standard. **Figure 12** shows the ion fragmentation patterns displayed in the MS/MS spectra of the honokiol standard (A), window extracts (B) and mature extracts (C). While the precursor ion of the honokiol standard and the compound of interest (tentatively identified as "honokiol") in window and mature extracts had similar mass to charge ratios at 267.1392 Da, 267.1856 Da and 276.1754 respectively, the ion fragmentation patterns did not match. This is evident especially when comparing the product ions between the standard (239.1074 Da) and window extracts (235.1688 Da) and mature extracts (235.2031 Da). This indicates that the compound tentatively identified as honokiol is, in fact, not honokiol but a compound that is chemically very similar to honokiol and, henceforth, will be referred to as honokiol-like. Considering the similarity, it is likely that this honokiol-like compound has a similar bioactivity as honokiol and its isomer magnolol in cancerous cells *in vitro*.

4.2.2 TARGETED METABOLOMICS REVEALS THE PRESENCE OF DIVERSE PHENOLIC COMPOUNDS

Following the untargeted analysis of window and mature extracts, a targeted metabolomic analysis was performed to detect anthocyanin and non-anthocyanin phenolic compounds. Targeted metabolomics is designed to measure a pre-defined subset

of the plant metabolome that is based on *a priori* knowledge of the possible metabolites present in the sample (Roberts et al., 2012). Based on the significance of anthocyanins to the initiation of PCD in the lace plant, the goal of this work was to explore the expression of phenolic compounds (both anthocyanin and non-anthocyanin). To achieve this, a series of anthocyanin and non-anthocyanin phenolic compounds, commonly detected in dietary plants, were used as references in the targeted metabolomics of lace plant extracts.

The analysis detected 34 anthocyanin and non-anthocyanin compounds out of 67 total compounds screened. The concentrations detected (expressed as µg/g of dry leaf material) are shown in **Table 10.1 and 10.2**. The most common non-anthocyanin compound detected in both mature and window extracts was rutin, at a concentration of 50.57 µg/g and 303.27 µg/g respectively. Rutin, a flavonoid, is found abundantly in plants like passion flowers, tea plants and apples and is known extensively for its biological activities including anticonvulsant, anti-Alzheimer, antarthritic, antidiabetic, chemotherapeutic activities and anticancer (Ganeshpurkar and Saluja., 2017; Satari et al.,2021). The most common anthocyanin detected in both window and mature was delphinidin-3-glucoside at 472.54 µg/g and 91.85 µg/g respectively. Delphinidin-3-glucoside is abundant in various pigmented fruits, vegetables, flowers or leaves and is known for its antioxidant and anticancer activity (Yang et al., 2016). Due to the ubiquitous expression of rutin and delphinidin-3-glucoside in other plants, their detection in lace plant extracts is expected. It is also important to note that delphinidin-3-glucoside, was detected at a higher concentration in window than mature extracts, supporting previous findings that window leaves have more anthocyanins than mature leaves to facilitate the initiation of PCD (Dauphinee et al., 2017; Rowarth et al., 2021).

Though this is the first time phytochemicals have been profiled in the *A. madagascaiensis*, attempts have previously been made to identify bioactive constituents in other members of the Aponogeton genus. A review by Chougule et al (2022) looked at the phytochemicals isolated from various *Aponogeton* species and their biological activities *in vitro* and *in vivo*. In *A. appendiculatus*, the most abundant bioactive phenolics were phen-1,4-diol, 2,3- dimethyl-5- trifluoromethyl, naphthalene, cyclopentadecanone and benzoic acid. The flavonoids kaempferol and quercetin were the most bioactive compounds detected in *A. desertorum* and *A. natans*. Other non-phenolic compounds such as quinones, steroids, terpenes and terpenoids were also found in *A. appendiculatus* and *A. natans* and were identified as being bioactive in various disease models (Chougule et al., 2022). Though this work does not report on the expression of non-phenolic secondary metabolites or their respective bioactivities, it does not suggest that lace plants do not express these compounds. The preliminary targeted metabolomics conducted in the lace plant was to elucidate the family of phenolic compounds, such as anthocyanin, previously found to be important for PCD in the lace plant. Therefore, at the time of this writing, our research group had not screened for other classes of secondary metabolites such as alkaloids, steroids and terpenoids.

4.3 EVALUATION OF ANTICANCER ACTIVITY OF CRUDE LACE PLANT EXTRACTS IN TNBC CELLS

4.3.1 CYTOTOXICITY AND SELECTIVITY OF LACE PLANT EXTRACTS IN TNBC CELLS

Phytochemicals present in crude leaf extracts of the *A. madagascariensis* plant were previously reported to induce cell death in human TNBC cells via apoptosis

(Gunawardena et al., 2021). One of the stated subsequent future directions from this study was to assess the selectivity of window and mature extracts toward MDA-MB-231 TNBC cells as compared to healthy control MCF-10A breast epithelial cells. This comparison is important because as chemotherapeutics exert their effects on cancerous cells, they also cause unintended harm in other rapidly dividing cells in the body leading to adverse effects. As such, though anticancer therapies have significantly improved in recent decades, toxicity and side effects from chemotherapies resulting from poor specificity to cancerous cells, remain a challenge (Riedl et al., 2011). Although most dietary phytochemicals such as anthocyanins are perceived to be safe, the rise in interest in phytochemical-based anticancer treatments necessitates the evaluation of their selectivity towards cancerous cells over healthy non-cancerous cells. The results presented in this work (**Figure 13)** demonstrate that window and mature extracts reduced cell viability of TNBC cells but not in breast epithelial cells when tested at equivalent concentrations. This suggests that neoplastic cells have an increased sensitivity to phytochemicals in lace plant leaf extracts. These findings are consistent with those previously reported by Solowey et al (2014) who evaluated the anticancer activity of *Urtica membranacea* (Urticaceae), *Artemesia monosperma* (Asteraceae), and *Origanum dayi post* (Labiatae) in human hematopoietic tumours such as T cell lymphoma and leukemia against normal human lymphoblasts. They found that the plant extracts showed specificity toward the tumor cells (SU-DHL-1, Karpas 299, HUT 102) over normal human lymphoblasts (OSTRA and YC cells). Chao-Yu et al (2019), also showed cytotoxicity in MDA-MB-231 cells over MCF-10A cells following treatments of apple-peel fraction (AF4). They reported that the cytotoxicity is partially due to the accumulation of intracellular ROS. Because cancer cells have higher baseline ROS levels compared to normal cells, they are potentially more susceptible to oxidative stress-

induced cell death via ROS production (Reczek and Chandel., 2017). Therefore, elevating ROS levels could be more cytotoxic to cancer cells when compared to normal healthy cells. As such, the selectivity reported in this work for window and mature extracts for TNBC cells over normal breast epithelial cells could be due to similar mechanisms.

4.3.2 ANTIPROLIFERATIVE EFFECTS OF LACE PLANT EXTRACTS IN TNBC CELLS

The next experiment evaluated the anti-proliferative effects of window and mature extracts in MDA-MB-231 cells. Uncontrolled proliferation is one of the fundamental hallmarks of cancer (Hanhan and Weiberg., 2011). While normal tissues firmly maintain the homeostasis of cell function by controlling growth-promoting signals, cancer cells function by suppressing or deregulating these signals (Liang et al., 2023). The unregulated growth of cancer cells can consequently lead to increased disease severity and progression if left untreated. Hence, developing novel agents that can induce antiproliferative signalling is an effective way to improve the clinical outcomes of patients undergoing treatments. The present work shows that crude lace plant extracts have antiproliferative effects at 24 h across all concentrations tested (**Figure 14)**. Our observations of the anti-proliferative effects of window and mature extracts on MDA-MB-231 cells, however, contrast what was previously reported by our research group (Gunawardena et al., 2021). There could be several reasons for this disparity. First, the current work assessed changes in proliferation at earlier time points at 24 h and 48 h post-treatment than the previous study at 72 h. In a highly proliferative cell line such as MDA-MB-231, testing multiple time points when measuring proliferation is beneficial to capture the full effect of the treatment. This is clearly demonstrated in the results of the current study, where mature extracts showed greater potency at 24 h compared to 48 h. Another advantage of the current work is that all four concentrations (25, 50, 100 and 250 μg/mL) were evaluated compared to the previous

study where only 2 concentrations were assessed (25 and 50 μg/mL). As demonstrated in **Figure 14**, antiproliferative effects were more prevalent at high concentrations in cells treated with mature extracts at 48 h. Finally, the present work was conducted using a CyQUANT ® assay which binds to cellular DNA via a fluorescent dye. This is typically seen as a more sensitive and reliable measure of cell count compared to other fluorescent-based assays (Smilkstein et al., 2004). Therefore, the measure of cell proliferation in the work presented in this thesis may be more sensitive than the Oregon Green ® phospholipid-binding assay used in the previous study.

4.3.3 MIGRATION EFFECTS OF LACE PLANT EXTRACTS IN TNBC CELLS

Cell migration is another hallmark of cancer that contributes to the aggressiveness of the disease (Hanhan and Weinburg., 2011). In a tumor environment, cells can migrate and invade other organs via the lymphatic circulation leading to cancer metastasis. TNBC especially is known for its highly metastatic nature and often results in poor patient prognosis. Hence, the ability of a therapeutic agent to impair the migration of TNBCs can be useful in reducing its severity. A simple wound-healing assay was used to demonstrate the effects of the extracts on MDA-MB-231 cell migration. The results from this work show that window and mature extracts did not inhibit MDA-MB-231 cell migration **(Figure 15)**. This finding is unexpected as other phytochemical studies have shown a reduction in the migration of cancerous cells *in vitro* post-treatment. For example, a study by Thakuri et al., (2020) showed that 9 TNBC cell lines treated with the phenolic compounds, fisetin and quercetin, inhibited the migration of cells. It is possible that the inconsistency could be due to the shorter treatment exposure times tested in the current study (14 h) compared to 48 h time course followed by Shahi Thakur et al., (2020).

Another explanation is that the active compound responsible for the inhibition of migration in TNBC is either not present in lace plant extracts or is not present at high enough concentrations. For example, although quercetin is detected in the targeted analysis of this work, if quercetin is important in the inhibition of cancer cell migration, even at the highest concentration used in the migration assay (100 µg/mL) only 0.26 µM will be available to the cells. Thakuri et al., (2020) found that 200 µM of quercetin is needed to elicit an inhibitory effect on TNBC migration.

4.3.4 MECHANISMS OF CYTOTOXICITY

To determine the mechanism by which crude extracts from lace plants induce cytotoxicity, MDA-MB-231 cells were evaluated by measuring changes in ROS activity. Reactive oxygen species are formed through the reduction of oxygen during metabolism with levels fluctuating depending on internal and external stimuli or stressors (Sarmiento-Salinas et al., 2021). At baseline, ROS can be a useful signalling molecule to carry out various cellular functions including cell proliferation, survival and differentiation (Fernando et al., 2019). However, an excess accumulation of ROS can cause oxidative stress leading to DNA damage and molecular disruptions via the PI3K/Akt pathway (Fernando et al., 2019). The PI3K-Akt is a known survival pathway that regulates various cellular functions including protein synthesis, proliferation, motility, growth, autophagy, cell survival and apoptosis (Kumar and Bansal., 2022). The ability of phytochemicals and other naturally occurring compounds to exhibit oxidative stress in cancer cells *in vitro* has been explored by several studies (Chao-Yu et al., 2019; Hall et al., 2015; Ediriweera et al., 2016). Chao-Yu et al (2019) reported that phytochemicals found in AF4 caused an increased ROS activity in MDA-MB-231 cells. Ediriweera et al (2016) reported that treatments of mango peel extracts (*M. Zeylanica*) increased ROS at the highest dose (50

μg/mL) in MCF-7 cells. Interestingly, other naturally occurring compounds that are not plant-based also reported increased levels of ROS in breast cancer cell lines post-treatment. For instance, Hall et al (2015) found that Jadomycin B, a natural bioactive compound found in soil bacteria *Streptomyces venezuelae*, increased ROS in MCF-7 cells. This effect was reversed upon co-treatments with NAC suggesting that jadomycin cytotoxicity was mediated via oxidative stress-induced cell death pathway.

The results from this work demonstrate that cells stained with the ROS-sensitive dye CM-H2DCFDA and exposed to the crude extracts at the cytotoxic concentrations of 25, 50, 100 and 250 μg/mL for 24 h showed an increase in ROS activity **(Figure 18)**. Though cells treated with window extracts did not have a statistically significant increase in ROS levels, cells treated with mature extracts, on the other hand, exhibited a significantly higher fold change in ROS activity when compared to vehicle controls. This suggests that the cytotoxicity of crude mature extracts could be due to the accumulation of intracellular ROS that cause oxidative stress-induced cell death. Cell death via ROS-dependent intrinsic apoptotic pathway is likely **(Figure 3)**. As window extracts did not show an appreciable increase in levels of ROS post-treatment, cell death via a ROS-independent pathway is the likely mechanism at play, in this instance. The different cell death mechanisms could be attributed to the different phytochemical constitutions between window and mature extracts.

While phytochemicals are generally thought to be antioxidants, they are also recognized to have prooxidant properties; a phenomenon better known as hormesis (Eghbaliferiz and Irnshasi., 201; Fernando et al., 2019). Hormesis explains the bi-phasic behaviour of natural compounds, such as phytochemicals, that elicit a non-toxic or positive response at low concentrations (antioxidant activity) and a toxic response at high

concentrations (prooxidant activity), depending on the microenvironment (Eghbaliferiz and Irnshasi., 2016; Calabrese et al., 2010). This prooxidant activity is based on a redox complex that phytochemicals like phenolics can form with transition metal ions (Eghbaliferiz and Irnshasi., 2016). Typically, cancer cells express high amounts of transition metal ions like copper ions which can be adjuvants to phenolics to increase ROS and initiate a cytotoxic effect (Eghbaliferiz and Irnshasi., 2016). Hence, the ability of lace plant mature extracts to increase ROS and elicit a cytotoxic effect could be, in part, due to the prooxidant activities of phenolics found in the extracts. To understand the link between ROS generation and cytotoxicity, extract-treated MDA-MB-231 cells were also exposed NAC. NAC is a cysteine precursor that, when converted to cysteine, becomes the rate-limiting compound in the synthesis of glutathione. Glutathione is an important component in the antioxidant defence system in the cell (Dodd et al., 2008). As such, in the absence of NAC, an increase in fold change in ROS is observed in MDA-MB-231 cells treated with window and mature extracts. In the presence of NAC, which acts as an inhibitor of ROS, a reduction in the fold change in ROS activity is observed **(Figure 19)**. This work, however, will need to be followed up with cell viability assays to confirm ROS mechanisms and subsequent links to cytotoxicity.

Interestingly, honokiol was shown to cause apoptosis in human hepatocellular carcinoma SMMC-7721 cells which was attributed to changes in membrane potentials in mitochondria which was accompanied by an increase in ROS (Han et al.,2009). Since, the most abundant compound, detected via the untargeted analysis, in the lace plant is honokiol-like, it may be partially responsible for the increase in ROS in MDA-MB-231 cells. It is important to note, however, that there could be other mechanisms (both ROS-dependent and ROS-independent pathways) associated with the cytotoxicity, especially,

given the complexity of the crude extract and the structural diversity of the phytochemicals present that allow for other cellular interactions.

4.4 LIMITATIONS AND CONCLUSIONS

For the first time, this work shows a phytochemical profile for crude window and mature extracts of the lace plant in addition to anticancer activity exhibited in TNBC cells. Though the findings of this work are promising, several limitations need to be addressed. First, several of the most abundant compounds detected via targeted and untargeted analysis (honokiol-like compound, rutin or delphinidin-3-glucoside) were not tested for their bioactivity in isolation in TNBC cells to see if they elicit the same effect as the crude extracts. This will be important to know if these compounds can elicit the same effect as the crude extracts or if the anticancer activity of the crude extracts is a product of the synergistic effects between several different phytochemicals. Second, cell viability assays to confirm ROS mechanisms (with and without NAC treatments) were not conducted. Therefore, conducting cell viability assays could further enhance our understanding of the putative mechanisms at play and confirm if the increase in ROS levels in the cells is linked to the cytotoxicity measured. Third, we only tested one TNBC cell line, therefore, at the time of this writing, we are not certain if the cytotoxic effects of window and mature extracts are cell-line selective. As such, it will be important to know if other TNBC cell lines display similar anticancer activities following treatments of window and mature extracts. Another general limitation to working with phytochemicals as a potential therapeutic agent is the insufficient availability of the compounds. Therefore, as this work progresses and specific bioactive compounds are identified, it will be important to either commercially purchase these compounds (if commercially

available) or synthesize these compounds (if novel). Lastly, because phytochemicals are generally known to have poor oral bioavailability, it will be important to conduct bioavailability studies with the compound(s) of interest found in the lace plant extracts. This will be especially important to increase the replicability and applicability of the work conducted and ensure a translation of findings from *in vitro* to *in vivo* to clinical studies

To conclude this work, our research group previously showed that extracts from lace plants at two stages of leaf growth: window and mature, induced apoptosis in TNBC MDA-MB-231 cells. However, the exact phytochemicals present or the full extent of the anticancer activity of these extracts remained unknown. The present work was conducted to revise the extraction protocol, profile the phytochemicals in the lace plant and investigate the anticancer activities. The data collected indicate that the evaporation Technique A yielded a greater decrease in cell viability in MDA-MB-231 cells while showing no significant differences in the HPLC analysis in the RTs of the compounds detected at the wavelength used for the analysis. An untargeted metabolomics analysis was used to profile the phytochemicals present in window and mature extracts of the lace plant. The analysis identified honokiol as the most abundant compound which on further analysis, was shown not an exact match and therefore only honokiol-like. Alternative methods, as discussed in section 4.4.1, should be considered to further analyze this honokiol-like compound for more accurate identification as discussed in section 4.4.1. Additionally, a targeted metabolomic analysis was conducted to screen for the expression of phenolic compounds (anthocyanin and non-anthocyanins). The decision to focus on anthocyanins was based on their previous identification as an important group of compounds in the induction of PCD in lace plant leaves during peroration formation.

Although, it is unclear if the phytochemicals that elicit the anticancer effects in TNBC cells are the same as those that induce PCD in the lace plant. The analysis showed there are differences in the concentrations of anthocyanins present between the two stages. Window extracts, for example, had higher concentrations of delphinidin-3-glucoside compared to mature extracts. This corroborates previous work showing that although mature leaves express anthocyanins, window leaves have higher concentrations of anthocyanin.

Extracts from window and mature leaves were also assessed for their anticancer activity. The results show that extracts reduce cell viability and proliferation in TNBC cells. This supports previous findings of anticancer activity from phytochemicals extracted from species of the *Aponogetoncea* family such as in the *Aponogeton undulatus*. This work for the first time shows that cells treated with the extracts increase ROS, which may be one of the mechanisms by which the phytochemicals present in the extracts elicit its cytotoxicity. However, additional work to confirm this mechanism with purified extracts or isolated compounds should be conducted to better understand the cellular signalling pathways and downstream regulators responsible for apoptosis in TNBC cells. There is, however, one advantage to using crude extracts because cancers adopt multiple mechanisms to escape growth regulators and evade cell death and the presence of several different compounds may be advantageous to bind to several different intracellular targets to elicit a beneficial therapeutic effect.

Table 11. Summary of research findings between mature and window extracts.

Experimental condition	Window	Mature
TNBC % cell viability between Technique A and B	Extracts from Technique A yielded better cytotoxicity in TNBC cells	Extracts from Technique A yielded better cytotoxicity in TNBC cells
HPLC (Evaporation Techniques A and B)	No difference between metabolic profiles for window extracts between Technique A and B	No difference between metabolic profiles for mature extracts between Technique A and B
LC-MS-MS (Untargeted)	Honokiol-like compound detected as most abundant	Honokiol-like compound detected as most abundant
LC-MS-MS (Targeted)	Significantly higher concentrations of Delphinidin-3-glucoside and Rutin	Lower concentrations of Delphinidin-3-glucoside and Rutin
TNBC % cell viability	Cytotoxic from 25 – 250 µg/mL	Cytotoxic from 50 – 250 µg/mL
TNBC % cell proliferation	Anti-proliferative effects across all concentrations at both 24 and 48 h	Anti-proliferative effects across all concentrations at 24 h but not 48 h
TNBC cell migration	No effect on TNBC cell migration	No effect on TNBC cell migration
ROS levels	No significant increase in ROS	Significant increase in ROS at 100 and 250 µg/mL

4.4.1 FUTURE DIRECTIONS

The untargeted analysis indicates that honokiol is the most abundant phytochemical in window and mature lace plant extracts. However, MS/MS fragmentation spectra between the compound in question and a honokiol standard, show differences in the fragmentation patterns. These differences suggest the compound of interest is not honokiol but has a similar chemical formula with a different chemical structure. Therefore, alternative methods such as nuclear magnetic resonance analysis should be considered to characterize this compound and verify its identity. To facilitate this, we have established a collaboration with the Biological Mass Spectrometry Core facility at Dalhousie University to fractionate the honokiol-like compound. This compound, in isolation, can be used to investigate the exact pharmacological mechanisms that are responsible for the anticancer measured in TNBC cells. This will be useful in understanding what cellular mechanisms are employed in the induction of cytotoxicity in TNBC cells post-treatment. It will also be valuable to replicate these findings in other TNBC cell lines such as MDA-MB-468 to understand if the anticancer effect is cell-line specific.

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APPENDIX

Appendix 1. UPLC-DAD assessed the anthocyanins in samples collected using the two extraction techniques.