THERAPEUTIC EFFECTS OF HPLC-ISOLATED SUBFRACTIONS FROM ETHANOLIC *MORINGA OLEIFERA* LEAF EXTRACTS ON CANCER AND INFLAMMATORY DISEASES

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by
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Abstract

THERAPEUTIC EFFECTS OF HPLC-ISOLATED SUBFRACTIONS FROM ETHANOLIC *MORINGA OLEIFERA* LEAF EXTRACTS ON CANCER AND INFLAMMATORY DISEASES

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The nutrient-dense and medicinal plant, *Moringa oleifera*, has a variety of reported therapeutic applications, which makes it a popular nutraceutical on the natural product market. However, scientists have only recently started to verify the therapeutic potential of *M. oleifera* products. Previous studies in our labs have shown potent anti-inflammatory and anticancer effects associated with ethanolic *M. oleifera* whole-leaf extracts. To further investigate potential applications of these *M. oleifera* extracts, our current project uses high performance liquid chromatography (HPLC) to separate bioactive compounds from the whole extract into distinct subfractions. We hypothesized that these HPLC-isolated *M. oleifera* subfractions could exhibit therapeutic effects in a cancer microenvironment by decreasing cancer cell viability and attenuating inflammatory cytokine production. Our results indicated that several of the subfractions significantly decreased the viability of HeLa and SiHa cervical cancer cells in a dose and time dependent manner. Additionally, these subfractions decreased macrophage secretion of the pro-inflammatory cytokines, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα), in a lipopolysaccharide (LPS)-induced

model of inflammation. Overall, these data indicate that chemical compounds in *M. oleifera* may be explored as an alternative treatment option for some cancers and inflammatory disorders. Further studies in our labs will seek to identify the bioactive compounds in our subfractions using time-of-flight mass spectrometry and other analytical techniques.

Acknowledgments

I would like to extend a special "thank you" to my mentors and chairpersons, Dr. Maryam Ahmed and Dr. Nathan Mowa, whose guidance and support has made this thesis project possible. As mentors, they have cultivated my critical-thinking skills, urged me to explore novel concepts, counselled my project through difficulties, and shaped my understanding of what it means to be an academic researcher. I would also like to thank my thesis committee members, Dr. Dru Henson and Dr. Darren Seals, whose astute insights have further refined my thesis project. Together, my thesis committee has instilled confidence within me and given me the tools needed to be successful, thus I am exceedingly grateful for all of their endorsements as I continue on my journey as an emerging research scientist.

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Finally, I would like to thank Dr. Jennifer Cecile, from Appalachian State University's chemistry department, for her continued assistance with our *M. oleifera* projects, as well as Appalachian State University's Office of Student Research and the Student and Faculty Excellence Fund for financial support throughout my project.

Dedication

I would like to dedicate this thesis in memory of my parents, Mr. James Raymond Slate and Mrs. Kimberly Sutherland Slate. The lessons they taught me, and the values they instilled within me, have guided me to success throughout my undergraduate and graduate career. I love and miss them both, but I hope this project would have made them proud!

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Chapter One

1. Introduction

The nutrient-dense and medicinal plant, *Moringa oleifera*, has long been utilized by various tropical and subtropical countries for its reported therapeutic effects and health benefits (Abdull Razis et al., 2014). While *M. oleifera* is native to the sub-Himalayan regions of Asia (including parts of India, Pakistan, Bangladesh, and Afghanistan), it is now globally dispersed and considered to be indigenous to regions of Southeast Asia, Africa, and South America (Abdull Razis et al., 2014; Kasolo et al., 2010). There are at least 12 other species in the *Moringa* genus, but *M. oleifera* is by far the most widely cultivated species and goes by a plethora of regional names such as Mulangay, Marango, Benzolive, Drumstick tree (to describe its seed pods), Horseradish tree (to describe its root flavor), and the "miracle tree" (due to its bountiful nutritional, medicinal, and economical uses) (Abdull Razis et al., 2014; Melo et al., 2013). This so-called miracle tree has become a staple crop for many rural communities due to its fast growth, drought resistance, high-yield of edible matter (including roots, seed pods, flowers, and leaves), and its applications in phytomedicine (Abdull Razis et al., 2014; Melo et al., 2013). Since many of these rural communities are isolated from primary healthcare facilities, the phytomedicinal and nutritive properties of *M. oleifera* products are offered as reasonably priced alternatives to western medicines (Abdull Razis et al., 2014). Several of the reported uses for *M. oleifera* products include antiseptic applications, managing inflammatory diseases (like asthma and arthritis), treating microbial infections (i.e. bacterial, viral, and fungal), controlling diabetes, preventing cardiovascular disease, and even mitigating some types of cancer (Abdull Razis et al., 2014; Kasolo et al., 2010).

1.1 Moringa oleifera's Applications as a Natural Product

<u>Nutritional Benefits</u>:

Generations of *M. oleifera* consumers have proclaimed the health benefits and applications of this medicinal plant; however, the scientific community has only recently started to investigate and verify some of these claims. One claim that has gained substantial backing from the scientific community states that consuming various parts of the plant (*e.g.* roots, leaves, flowers, or seeds) can provide bountiful nutritional benefits. When this claim was tested, analytical chemists determined that *M. oleifera* leaf powder, or leaf dry matter (d.m.), can contain 25 times more iron than spinach, 17 times more calcium than milk, 15 times more potassium than bananas, and 9 times more protein than yogurt (Jaroszewska et al., 2012). Additional chemical analysis has shown that *M. oleifera* leaf products contain significant quantities of essential micronutrients like iron (54.7 mg/kg d.m.), phosphorus (5,700 mg/kg d.m.), potassium (37,300 mg/kg d.m.), calcium (12,800 mg/kg d.m.), magnesium (9,600 mg/kg d.m.), manganese (25.5 mg/kg d.m.), and zinc (17.4 mg/kg d.m.) (Jaroszewska et al., 2012). It is also worth noting that many of these analyzed leaf products were very low in toxic heavy metals (like lead, nickel, or cadmium), and all previously listed micronutrients were present in concentrations deemed safe by the World Health Organization (Jaroszewska et al., 2012).

While numerous studies have reported large quantities of micronutrients to be found in *M. oleifera* leaves, other studies have shown that a variety of factors can impact the nutritive value of *M. oleifera* products (Jaroszewska et al., 2012). For example, the type of edible plant matter (flowers, seedpods, leaves, roots, etc.), the regional water and soil conditions, or even the preparation techniques (raw, cooked, dried, extracted, etc.) used to

produce *M. oleifera* products can significantly impact micronutrient concentrations (Kumssa et al., 2017; Jaroszewska et al., 2012). Most studies on *M. oleifera* nutrition only focus on the benefits that leaf products offer, but newer studies are looking at the nutritional benefits of the other edible plant parts. For instance, *M. oleifera* leaves are known to contain high levels of calcium (18,300 mg/kg d.m.) and moderate amounts of iron (202 mg/kg d.m.), while *M. oleifera* flowers contain lower amounts of calcium (3,650 mg/kg d.m.) but significantly higher amounts of iron (253 mg/kg d.m.) (Jaroszewska et al., 2012). It is also pertinent to consider the regional soil environments that *M. oleifera* products are grown in, as there is a significant positive correlation between soil elemental concentrations and the elemental concentrations in harvested plant matter (Kumssa et al., 2017).

As previously stated, the way *M. oleifera* products are prepared can greatly affect the nutritional value and bioactivity of the plant matter. While consuming fresh *M. oleifera* products may be optimal for managing micronutrient deficiencies, it is often more practical to dry and store the harvested plant matter for later use when fresh products are limited (Jaroszewska et al., 2012). Human micronutrient deficiencies in rural sub-Sahara African communities are known to spike in dry seasons, when nutrient-dense vegetables become scarce (Kumssa et al., 2017). However, there is a growing body of research that postulates increased production, consumption, and preservation of drought-resistant *M. oleifera* crops could stabilize these seasonal spikes in micronutrient deficiencies (Kumssa et al., 2017). While dried products may lose some of their bioactivity when stored for long periods of time, the general scientific consensus seems to indicate that drying leaf and other plant matter is an effective way to preserve most nutritional components and prevent spoilage due to microbial growth (Jaroszewska et al., 2012).

Antimicrobial Activity:

While performing chemical analyses on *M. oleifera* extracts, some researchers have observed potent antimicrobial effects associated with extracts prepared from the leaves, roots, bark, and seeds of the plant (Mangundayao and Yasurin, 2017; Ugwoke et al., 2017). One study found that a hydroethanolic leaf extract had stronger inhibitory capabilities than tetracycline (a common broad-spectrum antibiotic) on the following bacterial species: *Pseudomonas aeruginosa, Shigella sonnei, Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Bacillus megaterium,* and *Sarcina lutea* (Rahman et al., 2009). A similar study looked at the antimicrobial potential of hydromethanolic leaf and root extracts, and they found that it had comparable antibacterial effects to Gentamicin on *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Salmonella typhi* (Ugwoke et al., 2017). These bactericidal properties found in *M. oleifera* extracts are being investigated by biomedical and food-production researchers for the development of new antimicrobials (Mangundayao and Yasurin, 2017). Overuse of commercial antibiotics in these industries has led to the rise of drug resistant pathogens, and thus an ensuing need to develop alternative treatments to deal with these pathogenic threats to the general public and agricultural animals (Mangundayao and Yasurin, 2017).

The studies indicated previously have predominantly shown that *M. oleifera* leaf extracts have potent antibacterial effects, but more promising broad-spectrum antimicrobial effects have been observed from seed and husk extracts. Extracts made from *M. oleifera* seeds, pods, and husks encompass notable bacteriostatic, bactericidal, fungicidal, and water coagulative properties (Mangundayao and Yasurin, 2017; Arora and Onsare, 2014). In a recent study, one research group tested an organic *M. oleifera* husk extract against a variety

of bacterial species as well as a few fungal species; the results of their experiments showed that their extract had comparable microbicidal effects to Ciprofloxacin for tested bacterial strains and Amphotericin B for tested fungal strains (Arora and Onsare, 2014). These potent microbicidal effects were even observed on Methicillin-resistant *Staphylococcus aureus* (MRSA), which is known to be unaffected by many common antibiotics (Arora and Onsare, 2014). These results further indicate that some *M. oleifera* extracts may have broad-spectrum antimicrobial activity, which could be developed into future nutraceuticals (Arora and Onsare, 2014).

While nutraceutical companies are looking to develop new *M. oleifera* products for the diverse pharmacological potential of seed and husk extracts, these extracts are already being used by rural communities to purify drinking water due to their antimicrobial activity (Araújo et al., 2013). Extracts made from the seeds and roots of *M. oleifera* are believed to clarify drinking water in these communities through two main mechanisms. First, these extracts contain compounds which can disrupt microbial membranes (Arora and Onsare, 2014; Mangundayao and Yasurin, 2017). Second, lectins (proteins capable of binding carbohydrates) extracted from the plant material are able to flocculate contaminants and microbes in the water for removal in subsequent filtration (Araújo et al., 2013; Mangundayao and Yasurin, 2017). The combination of these two mechanisms has made *M. oleifera* a popular natural product to treat drinking water, and follow up studies have shown that these products are safe for human consumption in concentrations commonly used for water purification (Araújo et al., 2013).

Anti-inflammatory and Anticancer Properties:

One of the most common uses of *M. oleifera* products is treating acute and chronic inflammatory illnesses. This is due to the plant material containing potent immunomodulatory compounds. More specifically, leaf extracts that contain higher concentrations of phytochemicals, like phenols and flavonols, appear to have the most potent anti-inflammatory capabilities when tested *in vitro* and *in vivo* (Abdull Razis et al., 2014). Initial research with *M. oleifera* extracts has shown that bioactive compounds in the plant were able to reduce the secretion of pro-inflammatory cytokines by ubiquitous immune cells, like macrophages (Araújo et al., 2013). Interestingly, successive studies have shown that extracted compounds from *M. oleifera* can concurrently increase anti-inflammatory immune-cell signaling while decreasing inflammatory signaling (Fard et al., 2015). Furthermore, researchers have shown that these bioactive compounds can modulate several different inflammatory pathways, like NF-κB and STAT, to achieve their efficacious results (Fard et al., 2015; Qin et al., 2017).

These immunomodulatory and anti-inflammatory properties of *M. oleifera* extracts have therapeutic potential for a plethora of diseases, many of which are chronic due to self-perpetuating overactive immune responses. In fact, immunomodulatory natural products have recently been suggested as novel therapeutic options to treat some types of cancer, a classification of diseases in which inflammation has recently been specified as hallmark of tumor progression (Qin et al., 2017). While acute inflammation initially plays a role in tumor regression, it has been shown that chronic inflammation creates tumor microenvironments that can lead to compounding cellular mutations, initiate cancerous tumor growth, and increase metastatic capabilities of the tumor cells (Qin et al., 2017). Consequently, some

researchers have looked at the ability of anti-inflammatory compounds, like those found in *M. oleifera* extracts, to attenuate inflamed tumor environments and halt the progression of different types of cancer (Qin et al., 2017).

A large focus of my project has been exploring *M. oleifera* extracts as anticancer agents with tumoricidal activity. In addition to controlling inflammation in the tumor microenvironment, several studies have identified antiproliferative and apoptotic compounds in *M. oleifera* extracts; these anticancer compounds include phytochemicals, like quercetin and kaempferol, which have induced cancer cell apoptosis in previous *in vitro* experiments (Sreelatha et al., 2011). One such study found that an aqueous *M. oleifera* leaf extract was able to decrease cell viability of KB tumor cells in a dose-dependent manner upon application (Sreelatha et al., 2011). Further investigations from this laboratory group showed that the extract was inducing apoptosis, or programmed cell death (normally seen in healthy cells), in these cancerous cells (Sreelatha et al., 2011).

1.2 Overview of Inflammation, Anti-Inflammatory Drugs, and Therapeutic Natural Products

Many of the communities that have traditionally consumed medicinal *M. oleifera* products are isolated from primary healthcare facilities (Abdull Razis et al., 2014). Therefore, people in these areas tend to heavily rely on phytomedicines, or nutraceuticals, to treat a variety of diseases and chronic conditions (Arulselvan et al., 2016; Abdull Razis et al., 2014). Chronic illnesses often disrupt homeostasis and can create imbalanced or overactive immune responses, so the immunomodulatory and anti-inflammatory properties of *M. oleifera* extracts are well suited to treating these conditions. Typically, anti-inflammatory extracts from *M. oleifera* are often more affordable, easier to access, and potentially less toxic for

prolonged use when compared to standard anti-inflammatory drugs (*e.g.* steroids) from primary healthcare facilities (Arulselvan et al., 2016). Subsequently, this has led to *M. oleifera* products becoming a staple on the natural product market and in drug discovery research facilities (Arulselvan et al., 2016; Abdull Razis et al., 2014).

Although inflammation is often thought of as problematic, it is an integral part of the immune system's defense response and can be beneficial. When properly regulated, inflammation functions to protect the body from pathogens and foreign objects by localizing damage and alerting the immune system into action (Xu and Larbi, 2017). Molecules shed by pathogens or released by damaged tissues can recruit local macrophages, or other immune cells, to the immediate area and initiate an immune response (Xu and Larbi, 2017). These recruited immune cells will begin secreting early pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and interferon gamma (INF-γ), that stimulate the production of vasodilators and draw additional immune cells to the area (Xu and Larbi, 2017). As the acute inflammatory response progresses, many of the secreted cytokines further polarize the gathering immune cells into an activated state (Mack, 2017). Once activated, these immune cells will secrete additional inflammatory cytokines, like interleukins 1 and 6 (IL-1 and IL-6) (Mack, 2017).

As inflammatory cytokines accumulate in the area, they trigger cascading pathways that further exacerbate the inflammatory response. Inflammatory cytokine signaling induces an enzyme, cyclooxygenase 2 (COX-2), to convert arachidonic acid (AA) into the central prostaglandin precursor, prostaglandin H2 (PGH2) (Aoki and Narumiya, 2012). This precursor can then be converted into various forms of prostaglandins (potent vasodilators) including, PGD2, PGE2, PGF2, PGI2, and thromboxane-A2 (Aoki and Narumiya, 2012).

PGE2 plays an important role in creating an inflammatory positive feedback loop by binding to a receptor that increases the production of the transcription-regulating protein NF-κB (Aoki et al., 2011). Once produced in appreciable quantities, NF-κB accumulates in the nucleus and induces the expression of pro-inflammatory genes, including COX-2 (Aoki et al., 2011). The succeeding production of COX-2 completes the loop by converting more AA into PGH_2, and then PGH_2 into PGE_2 (Aoki et al., 2011).

The NF-κB pathway is a crucial part of the acute inflammatory response, as phosphorylated NF-κB proteins increase the expression of inflammatory genes in immune cells. Moreover, this pathway has been directly linked to the pathogenesis of several chronic inflammatory disorders, including cancer, when it remains in an upregulated and activated state (Aoki et al., 2011; Tilborghs et al., 2017). The NF-κB protein is generally thought of as heterodimer consisting of the p65 and p50 subunits, both of which remain bound to inhibitory IkB proteins before an inflammatory response (Tilborghs et al., 2017). However, pro-inflammatory molecules like microbial lipopolysaccharides (LPS), TNFα, and IL-1 can activate IkB kinases (IKK1 and IKK2), which subsequently degrade the inhibitory IkB proteins bound to the NF-κB subunits, ultimately leading to the activation of the NF-κB pathway (Tilborghs et al., 2017).

Inflammatory pathways, such as the NF-κB pathway, are prime targets for conventional drugs like glucocorticoids and nonsteroidal anti-inflammatory drugs (NSAIDs). Glucocorticoids are a common class of steroidal drugs administered for attenuating immune responses and decreasing inflammation (Press et al., 2016). They are able to easily pass through cell membranes due to their lipophilic nature, and once inside a cell they readily bind to glucocorticoid receptors in multiple signaling pathways (Press et al., 2016). Once bound to

their receptors, glucocorticoids exhibit pleiotropic anti-inflammatory effects, which have been shown to alleviate inflammation through genomic and non-genomic mechanisms (Press et al., 2016). On the genomic level, receptor-bound glucocorticoids are known to translocate into a cell's nucleus, promote transcription of anti-inflammatory genes, and subsequently increase the production of anti-inflammatory cytokines (*e.g.* IL-4, IL-10, and IL-13) (Press et al., 2016). Outside of the nucleus, glucocorticoids can bind to membrane receptors, many of which are linked to rapid signaling systems, and therefore release cytosolic proteins to quell inflammatory signaling (Press et al., 2016).

Steroidal drugs are highly effective at treating inflammatory conditions, but even short term use can induce unwanted side effects such as high blood pressure, osteoporosis, cataracts, insulin resistance, and increased susceptibility to infections. Thus, it is far more common for primary health care providers to recommend NSAIDs (*e.g.* aspirin, ibuprofen, naproxen, etc.), many of which are readily accessible without a prescription (Dwivedi et al., 2015). As their name implies, NSAIDs are not steroids, but they share therapeutic mechanisms with steroids like blocking the synthesis of prostaglandins (Dwivedi et al., 2015). Most NSAIDs inhibit COX-1 and COX-2 enzymes, which limits the amount of AA available to be converted into various prostaglandins (Dwivedi et al., 2015). While NSAIDs represent favorable temporary treatments for inflammation, prolonged use may also result in medical complications (Dwivedi et al., 2015). Many NSAIDs are non-selective in their inhibitory-binding to COX like proteins, which can result in adverse complications for the cardiovascular system, the kidneys, the liver, and the gastrointestinal (GI) tract (Dwivedi et al., 2015). For example, COX-1 is important for maintaining the protective GI mucosa, but

prolonged NSAID consumption can wear away the GI mucosa and leave the GI tract susceptible to infection (Dwivedi et al., 2015; Gudis and Sakamoto, 2005).

Overall, both steroidal drugs and NSAIDs are generally regarded as safe when taken as prescribed by physicians, but they are not always accessible by rural communities. Nevertheless, the prevalence of natural products can provide comparable treatment options for inflammatory disorders (Abdull Razis et al., 2014). In fact, many of these natural products contain potent bioactive compounds that are pharmacologically similar to conventional anti-inflammatory drugs (Gautam and Jachak, 2009). Several studies have found that bioactive compounds isolated from *M. oleifera* extracts act on a variety of inflammatory pathways including the AA pathway, the inducible nitric oxide synthase (iNOS) pathway, and the NF-κB pathway (Gautam and Jachak, 2009). A prominent bioactive compound that is frequently isolated from *M. oleifera* leaf extracts is kaempferol, which inhibits inflammatory signaling in the NF-κB pathway and limits the production of several inflammatory cytokines like IL-1, IL-6, and TNFα (Rajendran et al., 2014). Of course, there are many other anti-inflammatory compounds in *M. oleifera* that can attenuate inflammatory processes by preventing the secretion of inflammatory cytokines, deactivating COX enzymes that produce prostaglandins, or inhibiting the expression of inflammatory response genes (Gautam and Jachak, 2009).

1.3 Extraction and Identification of Bioactive Compounds in Moringa oleifera

Although it is not uncommon for natural products to be consumed raw or minimally processed, herbalists and nutraceutical researchers alike often develop extracts from natural products to concentrate their bioactive compounds (Sultana et al., 2009). These extractions

often use a variety of solvents including water, methanol, ethanol, acetone, ethyl acetate, as well as many other organic solvents (Sultana et al., 2009). Numerous researchers, using a variety of solvents, have shown that hydro-organic solvents (generally in concentrations ranging from 50% to 70% organic solvent) appear to be the most effective at recovering phenolic and flavonoid compounds; since these are the major classifications of bioactive compounds found in the *M. oleifera* plant, many *M. oleifera* products are extracted with aqueous organic solvent mixtures (Sultana et al., 2009; Vongsak et al., 2013). These aqueous organic mixtures typically increase the solubility of bioactive compounds within the chosen solvent, which ultimately results in larger extraction yields of phenolic compounds (Rodríguez-Pérez et al., 2015).

After choosing an appropriate solvent, there are a number of extraction techniques utilized to isolate bioactive compounds from natural product plant matrices such as "squeezing", maceration, decoction, percolation, Soxhlet extraction, and ultrasound-assisted extraction (Rodríguez-Pérez et al., 2015; Vongsak et al., 2013). Squeezing is a rudimentary extraction technique where plant material is first dampened with a desired solvent, and then the wet mixture is pressed or squeezed through a filter before collection (Vongsak et al., 2013). Maceration (commonly referred to as solid-liquid extraction) and decoction are very similar techniques that involve fully soaking the finely ground plant material in a solvent for a short amount of time (usually less than an hour or two); the main difference between these two techniques is that the solvent for decoction is usually kept heated or close to boiling (Rodríguez-Pérez et al., 2015; Vongsak et al., 2013). Percolation and Soxhlet extraction are techniques that involve heating the solvent, similar to decoction, but the boiling solvent does not remain in constant contact with the plant material (Vongsak et al., 2013). In these

extractions the solvent is heated until boiling, and then rising vapor condense back down onto the plant material before being filtered back into the heating flask (Vongsak et al., 2013). Finally, several of the aforementioned techniques can be enhanced using machines to increase agitation during the extraction process such as placing the apparatus on a shaker, or using a sonicator to disrupt the mixture with ultrasonic waves (Rodríguez-Pérez et al., 2015; Sultana et al., 2009).

Overall, choosing an appropriate solvent and efficient technique is an important first step in the extraction process, but the reaction conditions under which the extraction takes place are also important to consider. The extraction temperature has a pronounced positive effect on extraction yield, yet it can negatively affect the bioactivity of the extracted compounds (Sultana et al., 2009). When researchers quantified the extraction yields (grams of final product divided by grams of initial plant material) of several natural products, including a *M. oleifera* extract, they saw a significant increase in yield when the plant material was extracted under reflux conditions (Sultana et al., 2009). Yet, when they analyzed the total phenolic and flavonoid content of these extracts, they saw significant decreases in both classifications of bioactive compounds (Sultana et al., 2009). They therefore concluded that increasing the reaction temperature of an extraction likely decreases its potency, because it degrades the thermolabile bioactive compounds, like phenols and flavonoids (Sultana et al., 2009).

Following the extraction process, the resulting mixture of solvent and plant chemicals is referred to as a whole extract, and there is a substantial amount of scientific reports that detail the therapeutic effects associated with *M. oleifera* whole seed and leaf extracts. However, the focus of the scientific community has largely been shifting away from whole

extract studies in favor of analytical studies that seek to isolate and identify extracted compounds. Subsequently, more researchers are using analytical chemistry techniques to characterize the bioactive compounds in their extracts (Rodríguez-Pérez et al., 2015). High-performance liquid chromatography (HPLC), which is often paired with mass spectrometry, is probably the most common analytical technique for separating and identifying compounds in complex extraction samples (Rodríguez-Pérez et al., 2015). Nevertheless, there are many other separation and identification techniques in use, such as gas chromatography (GC), thin layer chromatography (TLC), counter current chromatography (CCC), and nuclear magnetic resonance (NMR) spectroscopy to name a few (Yang et al., 2016; Sreelatha et al., 2011).

HPLC instruments have been a staple in industrial research laboratories since the 1980s, and HPLC separation techniques are frequently used in pharmaceutical research facilities due to their high-resolution capabilities and relatively fast run times (Wirtz, 2014). Other advantages to using HPLC separation include low run temperatures (which decreases thermolabile sample degradation), reduced solvent waste, variable solid-phases, and the ability to couple the instrument with a multitude of detectors (like time-of-flight mass spectrometers) for analyte identification (Wirtz, 2014). As the name suggests, HPLC separates compounds in a liquid mixture using column chromatography. This means that a liquid sample is injected into the HPLC system (usually < 100 microliters), where it will mix with the mobile-phase solvent(s) that transports the sample to the column (Wirtz, 2014). Common solvents used for HPLC mobile-phases include tetrahydrofuran (THF), trifluoroacetic acid (TFA), formic acid, acetic acid, ammonium acetate, acetonitrile, and methanol (Wirtz, 2014).

After mixing with the mobile-phase, the sample will travel to the HPLC column where it undergoes separation by interacting with the stationary-phase (Wirtz, 2014). The inside of the column (or stationary phase) is packed with tiny silica-gel particles (particles < 2 micrometers in diameter), which are engineered to have varying degrees of separation selectivity (Wirtz, 2014). The selectivity of the column packing material is dependent on optimal run conditions though, so pH and temperature ranges must be taken into account when choosing a suitable stationary-phase (Wirtz, 2014). As the sample runs through the column, analytes with a higher affinity for the packing material will stay in the column longer, while analytes with a lower affinity will quickly move through the column and into the detector, effectively separating sample matrix.

Finally, once the analytes have been separated by their run through the HPLC column, they are transported to a detector which will generate a chromatograph and help identify chemical compounds in the sample. Mass spectrometer (MS) coupled detectors are commonly implemented in natural product research, and can characterize ionized analytes based on their mass-to-charge ratio (*m/z*) (Wirtz, 2014). Inside the MS, ionized analytes are ejected into a high vacuum environment (10^{-5} to 10^{-7} torr) where magnetic and electrical fields separate the ions based on their masses (Wirtz, 2014). Of course, there are several different types of mass analyzers that can quantify an ionized analyte's mass-to-charge ratio, but time-of-flight (TOF) mass analyzers have become exceedingly commonplace in the biomedical sciences (Wirtz, 2014). In TOF MS, ionized analytes are accelerated with an electrical field through a drift tube, where lighter ions will travel faster than heavier ions; thus, the ion's mass-to-charge ratio can be determined by measuring the time that it takes for an ion to travel from the ionization source to the detector (Wirtz, 2014).

To truly understand the therapeutic potential of *M. oleifera* natural products, extracted bioactive compounds must be isolated, identified, and empirically tested to show their contribution in medicinal whole extracts. Current studies attribute *M. oleifera's* numerous medicinal properties to the large yields of phytochemicals that can be extracted from the plant matter, especially secondary metabolites like alkaloids, tannins, flavonoids, quinones, phenols, and phenolic acids (Gupta et al., 2017; Brilhante et al., 2017). While extracted chemical composition varies greatly depending on aforementioned factors (plant material, extraction solvent, extraction technique, etc.), commonly extracted bioactive compounds with known health benefits include caffeolyquinic acids, flavonoids (kaempferol and quercetin), glucosinolates, and glycosides (Brilhante et al., 2017). These groupings of bioactive compounds have exhibited salutary effects when tested for antimicrobial, anticancer, and anti-inflammatory/ immunomodulatory capabilities (Gupta et al., 2017; Brilhante et al., 2017).

As mentioned earlier, overuse of antibiotics has led to the rise of drug-resistant microbial strains, so researchers are trying to isolate bioactive compounds that may be used to generate new antimicrobial agents (Brilhante et al., 2017). Some antimicrobial compounds isolated from *M. oleifera* extracts include gallic acid, sapponins, phenolic alkaloids, thymol, tannins, glucomoringin, glucosinolates, and kaempferol derivatives (Gupta et al., 2017; Brilhante et al., 2017). Certain antimicrobial compounds, like benzyl isothiocyanate, have been shown to suppress bacterial growth (by disrupting bacterial membranes) with results comparable to standard antibiotics (Brilhante et al., 2017). *M. oleifera* extracts also appear to have antiviral chemicals, like isocyanates and niaziminin, which were able to inhibit viral

replication in several strains of herpesvirus *in vitro* (Gupta et al., 2017; Brilhante et al., 2017).

Researchers are also looking at *M. oleifera* extracts for their anticancer effects. Some extracts contain antitumor and anti-proliferative compounds, which are being proposed for use in new combinatorial cancer therapies. Interestingly, two of the previously mentioned antimicrobial compounds, niaziminin and benzyl isothiocyanate, have been shown to have tumorcidal capabilities *in vitro* (Brilhante et al., 2017). Other isolated compounds that have shown *in vitro* antitumor potential include 3-O-(6'-O-oleoyl-β-D-glucopyranosyl)-β-sitosterol, β-sitosterol-3-O-β-D-glucopyranoside, kaempferol, quercetin, and derivatives of both phenylacetonitrile and quinic acid (Gupta et al., 2017; Brilhante et al., 2017). It is important to note that many of these bioactive compounds have the desirable property of exhibiting cytotoxic effects in several cancerous cell lines, but do not appear to harm healthy cells (Brilhante et al., 2017).

Finally, analytical scientists are elucidating the chemicals that give *M. oleifera* extracts their potent anti-inflammatory and immunomodulatory capabilities. Numerous publications have reported on the *in vitro* and *in vivo* immunomodulatory effects of whole extracts, and several of these studies have compared *M. oleifera* extracts to common NSAIDs (Brilhante et al., 2017). Impressively, these plant extracts exhibited similar or better anti-inflammatory effects compared to non-prescription anti-inflammatory drugs (Brilhante et al., 2017). These anti-inflammatory effects are attributed to extracted compounds inhibiting inflammatory signaling from immune cells and also decreasing inflammatory gene expression (Gupta et al., 2017; Brilhante et al., 2017). For example, quercetin and kaempferol have been shown to inhibit the activation of the NF-κB pathway, decrease the

release of inflammatory cytokines (IL-6 and TNFα), and regulate the expression of some proteins (like iNOS, IFN-γ, and C-reactive protein) (Rajendran et al., 2014; Brilhante et al., 2017). Other extracted anti-inflammatory compounds believed to work through similar mechanisms include linoleic acid, oleanolic acid, thymol, thymoquinone, and resveratrol (Gautam and Jachak, 2009; Brilhante et al., 2017).

1.4 Current Project Overview

Previous studies have shown potent anti-inflammatory and anticancer effects associated with ethanolic *Moringa oleifera* whole-leaf extracts. To further investigate the therapeutic properties and potential applications of these ethanolic extracts, this current project used high performance liquid chromatography (HPLC) to separate bioactive compounds from the whole ethanolic extract into distinct subfractions. We hypothesized that these HPLC-isolated *M. oleifera* subfractions might exhibit therapeutic effects in a cancer microenvironment by decreasing cancer cell viability and attenuating inflammatory cytokine production. In order to test this hypothesis, our project had three main goals including 1) isolating bioactive compounds from the whole ethanolic extract into discrete subfractions using HPLC separation techniques, 2) evaluating the anti-inflammatory and immunomodulatory properties of these subfractions, and 3) assessing the ability of these subfractions to decrease cervical cancer cell viability and exploring the mechanisms contributing to any anticancer effects. From our assessments, we hope to explore therapeutic applications for these concentrated subfractions in various cancers and inflammatory diseases, as well as characterize each subfraction's bioactive chemical profile.

Chapter Two

2. Materials and Methods

2.1 Cell Culture

THP-1 cells, a human monocytic leukemia cell line, were cultured at 37°C and 5% CO_2 in enriched RPMI-1640 media. This RMPI media was further supplemented with 0.3 g/L L-glutamine and sodium bicarbonate, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% vitamins, 10 mM HEPES, and 0.05 mM 2-mercaptoethanol. HeLa and SiHa cervical cancer cells were also cultured at 37°C and 5% CO_2 in DMEM media supplemented with 10% FBS. HeLa cells were the first immortalized cell line to be grown in culture, and they were acquired in 1952 from a 31 year old African American female (Rahbari et al., 2009). HeLa cells have recently been characterized as a rare adenocarcinoma (formed in epithelial glandular structures) and been shown to contain human papillomavirus (HPV)-18 DNA (Rahbari et al., 2009). SiHa cervical cancer cells are classified as a squamous cell carcinoma that have been shown to contain HPV-16 DNA; these cells were originally isolated from a 55 year old Asian female (Meissner, 1999). All cell lines were acquired from the American Type Culture Collection (ATCC).

2.2 Whole Ethanolic Moringa Oleifera Extract

Previous studies in our labs have shown potential anti-inflammatory and anticancer effects associated with whole ethanolic extracts from *Moringa oleifera* leaves (Brown et al., 2015). Therefore, we used the following extraction method to obtain our whole extract that was subsequently separated and analyzed with high performance liquid chromatography (HPLC). Dried *M. oleifera* leaf powder was obtained by Drs. Jahangir Emrani and Joshua

Idassi (North Carolina Agricultural and Technological State University). The dried leaf powder was extracted using maceration techniques with 200 proof ethanol as the solvent; each batch of leaf powder was extracted twice, both times with ethanol as the solvent, and the extraction mixtures were compiled together before filtration and concentration. Each batch of extraction mixture was filtered through double layered cheese-cloth, and then concentrated using rotary evaporation. Then, the concentrated whole ethanolic *M. oleifera* extract was either injected into the HPLC system, or it was further concentrated and reconstituted in deionized water to a final concentration of about 1 gram of original leaf powder (d.m.) per 1 milliliter of water. These whole extracts were stored at –20°C until use.

2.3 HPLC Generated Subfractions from Whole Ethanolic Moringa oleifera Extract

As mentioned in the previous section, part of the whole ethanolic *M. oleifera* extract would be concentrated and injected into an HPLC system for separation. The HPLC system that was used was a Varian-ProStar Model HPLC, equipped with a ProStar 410 AutoSampler and injection unit, two 210/215/218 SD-1 Pumps for solvent delivery, a ThermoFisher Hypersil GOLD C18 Selectivity Liquid Chromatography Column (Model Number 25012-159070), and a ProStar 325 LC Detector that used a single channel for detecting ultraviolet (UV) radiation at 330 nanometers. For each separation run, 100 microliters of concentrated whole ethanolic *M. oleifera* extract would be injected into the HPLC system and separated over a run time of 55 minutes. During a 55 minute run, the solvent being pumped through the system gradually changed from a solution of 0.1% formic acid in water to a solution of 0.1% formic acid in acetonitrile. The separated effluent mixture was collected in several intervals to generate 8 total subfractions: subfraction 1 (0-6 minutes), subfraction 2 (6-11 minutes),

subfraction 3 (11-17 minutes), subfraction 4 (17-25 minutes), subfraction 5 (25-34 minutes), subfraction 6 (34-42 minutes), subfraction 7 (42-48 minutes), and subfraction 8 (48-55 minutes). These subfractions were compiled over several runs and subsequently concentrated and reconstituted in deionized water. While most of the subfractions were thoroughly tested for immunomodulatory and anticancer properties, subfraction 1 was not able to be concentrated to useable levels and was regarded as HPLC forerun (mostly containing acidified water). The concentrated and reconstituted subfractions were stored at –20°C until needed.

2.4 Collection of THP-1 Cell Supernatants for Anti-inflammatory Assays

THP-1 monocytes were pretreated with *M. oleifera* whole extract and subfractions, and then challenged with lipopolysaccharides (LPS) to determine if the extracted solutions had any immunomodulatory capabilities. First, THP-1 monocytes were collected and centrifuged at 1400 rpms for 4 minutes, and the supernatant was discarded. The centrifuged pellet was suspended in fresh RPMI media, and then 750,000 cells in fresh media were seeded into the wells of 12-well plates. Subsequently, each subfraction was added to an individual well until the final concentration ranged from 0-200 µg (d.m.)/mL. Alternatively, reconstituted whole ethanolic extract could be added to an individual well until the final concentration ranged from 0-200,000 µg (d.m.)/mL. The THP-1 cells and *M. oleifera* solutions were incubated for 4 hours at 37°C with 5% CO_2 before being challenged with 675 ng/mL of LPS. Following LPS challenge, the supernatant of the wells were collected in microcentrifuge tubes at 24 hour and 48 hour time-points. The collected supernatant was then centrifuged and stored at –80°C until needed.

2.5 Inflammatory Cytokine Quantification using ELISAs

Inflammatory cytokine levels (IL-6 or TNFα) from supernatants obtained from *M. oleifera* treated THP-1 cells were measured using enzyme-linked immunosorbent assays (ELISAs) as detailed in the manufacturer's (BD OptEIA) protocol. First, inflammatory cytokine capture antibody was coated onto high-affinity binding ELISA plates overnight at 4°C. Excess antibody was washed off, then plates were blocked with blocking buffer for 1 hour at room temperature, followed by additional washes to remove excess blocking buffer. Next, *M. oleifera* pretreated THP-1 supernatants were added to the wells and incubated for 2 hours at room temperature. The plate was thoroughly washed, and detection antibody was incubated with the plate for 1 hour at room temperature. After a final series of washes, substrate solution was added to the ELISA plate at room temperature for 30 minutes in the dark. Finally, stop solution was added to each well, and the ELISA plate was immediately read using a spectrophotometer set to measure absorbance at 450 nm with a wave correction set to 570 nm.

2.6 HeLa and SiHa Cervical Cancer Cell Viability Assays

HeLa or SiHa cells were treated with various concentrations of *M. oleifera* whole extract or subfractions for 24 or 48 hours. Cell viability was determined by a MTT cell viability assay according to the manufacturer's (Roche Diagnostics) protocol. Cancer cells suspended in DMEM were seeded into 96-well plates and incubated at 37°C with 5% CO_2 until the wells were around 70% confluent. Upon reaching confluency, *M. oleifera* subfractions or whole ethanolic extract was added until the final concentration ranged from 0-200 μg (d.m.)/mL (or 0-200,000 μg (d.m.)/mL for the whole ethanolic extract). These

plates were placed back in the incubator for 24 or 48 hours, and then each well received MTT labeling reagent. After 4 hours of incubation with the MTT reagent, a solubilizing reagent was added to each well and further incubated overnight at 37°C. Finally, the plate was read with a spectrophotometer set to measure absorbance at 570 nm.

Additionally, the cervical cancer cell viability was assessed after the application of combinations of *M. oleifera* subfractions. The following subfraction combinations were applied to HeLa or SiHa cancer cells at 70% confluency to determine their impact on cell viability: subfractions 2&3; 2&5; 2&7; 2&8; 3&5; 3&7; 3&8; 5&7; 5&8; 7&8; 2, 3&5; 2, 5&7; 3, 5&7; 3, 5&8. The subfraction combination MTT assay followed a similar procedure as described above.

2.7 Collection of HeLa Cell Lysates for Western Blot Analysis

HeLa cervical cancer cells were treated with *M. oleifera* whole extract and subfractions to determine their anticancer properties. HeLa cells were suspended in DMEM and seeded into 6-well plates; these plates were incubated at 37°C with 5% CO_2 until the wells were around 70% confluent. Upon reaching confluency, *M. oleifera* subfractions or whole ethanolic extract was added until the final concentration ranged from 0-100 µg (d.m.)/mL. These plates were placed back into the incubator for 24 hours. The supernatant was subsequently removed and RIPA lysate solution (containing aprotinin) was added to each well for 3 minutes while being kept cool on ice. The resulting lysates were then sheared using a 20 gauge syringe, and the protein concentration of each lysate was determined using a Pierce BCA Protein Assay Kit following the manufacturer's (Thermo Scientific) protocol. The remaining lysates were quickly transferred and stored at –80°C until needed.

2.8 Western Blot Analysis of Anticancer Pathways

The lysates mentioned previously were used in Western blot analysis to determine if the *M. oleifera* solutions would alter any of the common inflammatory or proliferative pathways in cancer cells. Approximately 15 μg of protein from each subfraction or whole extract lysate were loaded into a precast polyacrylamide gel and separated using electrophoresis. The separated proteins were then transferred to a nitrocellulose membrane. Immunoblots were generated with antibodies against phosphorylated and total NF-κB, as well as Bax and Bcl-xL, with β-actin being the internal standard (all from Cell Signaling Technology). These blots were subsequently incubated with horseradish peroxidase (HRP) linked secondary antibodies, and then Dura (Thermo Scientific) luminol was used to visualize the signal in combination with a gel imager. Finally, ImageQuant TL v2005 was used to quantify relative band intensities.

Chapter Three

3. Results

3.1 HPLC Chromatograph and Generation of Subfraction Groups

Previous results on the anti-inflammatory and anticancer effects of various *Moringa oleifera* whole leaf extracts led us to further investigate the therapeutic potential of the ethanolic *M. oleifera* leaf extract (Brown et al., 2015). Following the previously established protocol, an ethanolic *M. oleifera* leaf extract was generated, concentrated using rotary evaporation, and subsequently separated through our high performance liquid chromatography (HPLC) system (Brown et al., 2015). A detector set to the 330 nm wavelength channel was used to generate a HPLC chromatograph, and the effluent sample-solvent mixture from the 55 minute HPLC run was collected in test tubes during 1 minute cycles. Using the newly generated chromatograph (Figure 1), the 55 test tubes of effluent solution were combined into 8 distinct subfraction groupings. The test tubes were combined to generate the following subfraction groups: subfraction 1 (0-6 minutes), subfraction 2 (6-11 minutes), subfraction 3 (11-17 minutes), subfraction 4 (17-25 minutes), subfraction 5 (25-34 minutes), subfraction 6 (34-42 minutes), subfraction 7 (42-48 minutes), and subfraction 8 (48-55 minutes).

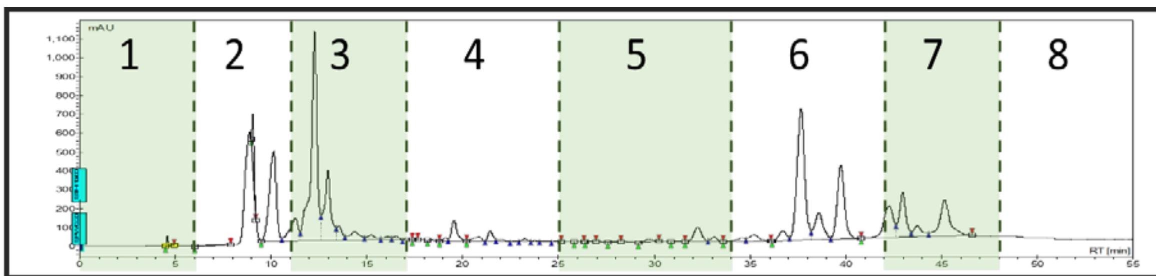


Figure 1. HPLC Chromatograph and Subfraction Groupings. Ethanolic *M. oleifera* leaf extract was separated using HPLC techniques and the chromatograph above was generated over a 55 minute run with a UV-Vis detector set at 330 nm. A total of 8 subfraction groupings were established based on observed gaps in analyte peaks.

After generating the HPLC chromatograph (Figure 1), it was observed that there were two main time frames in which detectable analytes were eluting through the column. The first region was early in the run, when the primary solvent moving through the column was acidified water, which occurred between 7 and 17 minutes. The second region of detectable analytes occurred much later in the run, when the primary solvent had switched over to acidified acetonitrile, which occurred between 34 and 48 minutes. While 8 total subfraction groupings were generated using natural breaks in the chromatograph, it is worth noting that subfraction 1 was left out of subsequent analysis after being deemed as forerun (mostly aqueous solvent with no appreciable amounts of analytes) from the HPLC system.

3.2 Immunomodulatory Effects of M. oleifera Ethanolic Subfractions

Several studies have shown the anti-inflammatory potential of *M. oleifera* extracts, including ethanolic *M. oleifera* leaf extracts, which have been shown to alter inflammatory cytokine secretion (IL-1, IL-6, TNFα, etc.) from lipopolysaccharide (LPS)-stimulated macrophages (Fard et al., 2015). Since the subfractions we generated were isolated from an ethanolic *M. oleifera* leaf extract, we hypothesized that some of these subfractions should

also exhibit therapeutic anti-inflammatory effects. To determine the immunomodulatory properties of our subfractions, we used a similar LPS challenge model with THP-1 monocytes, which were generously supplied to us from Dr. Darren Seals at Appalachian State University. THP-1 cells are frequently used as a model system for human monocytes as they mimic the inflammatory behavior of peripheral blood derived mononuclear cells after stimulation with LPS (Vasanth et al., 2015). We suspected that pretreatment with our HPLC-isolated subfractions would decrease IL-6 and TNFα inflammatory cytokine secretion from THP-1 cells following LPS challenge

Approximately 750,000 THP-1 cells were seeded into the wells of a 12-well plate and were then pretreated with one of the HPLC generated subfractions (2-8) at concentrations of either 100 or 200 µg/mL. Additionally, the ethanolic *M. oleifera* whole leaf extract (W), from which the subfractions were derived, was applied at 100,000 and 200,000 µg/mL. The THP-1 cells were incubated with the extracts for 4 hours before being challenged with 675 ng/mL of LPS, which stimulates potent immune responses and activates monocytes into inflammatory macrophages. The cells were then incubated for 24 hours, and supernatants were collected for enzyme-linked immunosorbent assays (ELISAs) to determine the effect of *M. oleifera* ethanolic subfractions on LPS-induced secretion of inflammatory cytokines, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα). ELISAs were performed according to the manufacturer's protocol, and relative amounts of inflammatory cytokine production were compared to the LPS-only positive control sample as seen in Figures 2A and 2B.

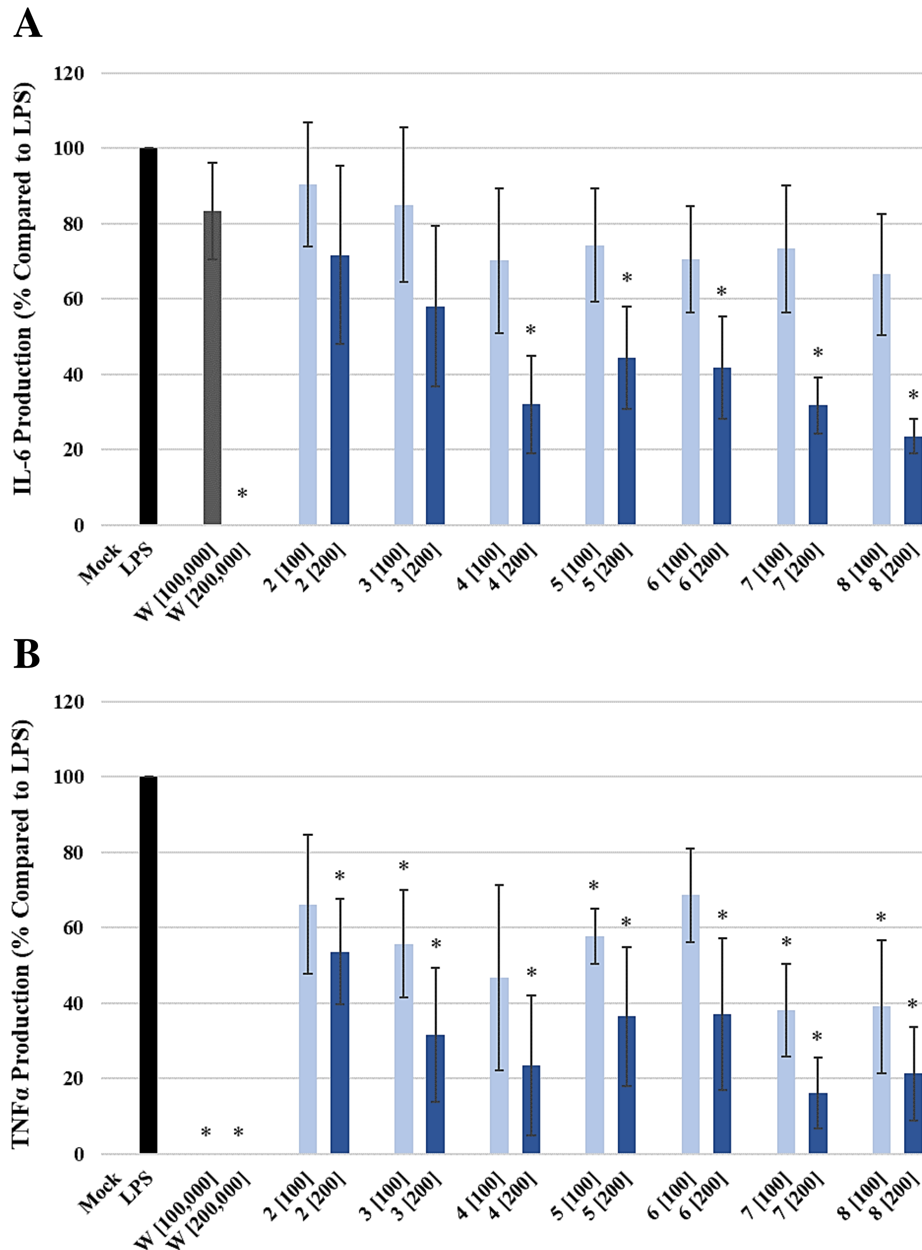


Figure 2. Decrease in Inflammatory Cytokine Production from LPS-Stimulated THP-1 Cells Pretreated with *M. oleifera* Subfractions. THP-1 monocytes were pretreated with ethanolic *M. oleifera* leaf extract (W) or a subfraction (2-8) for 4 hours. The cells were subsequently challenged with 750 ng/mL of LPS, and supernatants were collected after 24 hours. A standard ELISA protocol was used to determine the amount of A) IL-6 or B) TNFα produced, and all values were normalized as a percentage of the LPS-only positive control. The concentrations for each extract are recorded in micrograms of original leaf powder per milliliter [µg/mL]. The data shown are an average of three experiments ± standard error. Asterisks indicate a significant reduction as compared to the LPS positive control (P = <0.05) using one-tailed Student's t-tests.

Data from the IL-6 ELISA (Figure 2A) showed that subfractions 4, 5, 6, 7, and 8 were all able to significantly reduce LPS-stimulated production of IL-6 when applied 4 hours before LPS challenge at a concentration of 200 µg/mL. Interestingly, the ethanolic *M. oleifera* whole leaf extract (W) was not able to significantly alter IL-6 production at 100,000 µg/mL (a 1,000-fold higher concentration than any of the subfractions), but it did decrease IL-6 production similar levels of the mock negative control (DI water added instead of LPS) when applied at 200,000 µg/mL. Subfraction data from the TNFα ELISA (Figure 2B) showed similar reductions in cytokine production. However, all subfractions (2-8) were able to significantly reduce TNFα production when THP-1 cells were pretreated at a concentration of 200 µg/mL. Subfractions 3, 5, 7, and 8 also reduced TNFα production at the lower concentration of 100 µg/mL. Unlike the IL-6 data, the whole extract was able to significantly reduce TNFα production at both the 100,000 µg/mL and 200,000 µg/mL concentrations, with both treatments reducing TNFα cytokine production to levels comparable to the negative control.

3.3 Anticancer Effects of Subfractions

Previous studies have shown that *M. oleifera* extracts have anti-proliferative and apoptotic effects on some cancer cell lines both *in vitro* and *in vitro*, and recent research has shown that modulating apoptotic pathways has promising therapeutic applications for a variety of cancers (Sreelatha et al., 2011). Building upon this information, previous studies in our labs have looked at the anti-proliferative and apoptotic potential of *M. oleifera* whole extracts in several cervical cancer cell lines, and data from these studies has shown that ethanolic whole leaf extracts are able to alter cervical cancer cell proliferation and induce

apoptosis (Brown et al., 2015). Thus, it was hypothesized that subfractions derived from ethanolic *M. oleifera* leaf extract may also induce anticancer effects in a HeLa and SiHa cervical cancer cell lines.

To test this hypothesis, HeLa (Figures 3A and 3B) and SiHa (Figures 4A and 4B) cervical cancer cells were seeded into the wells of 96-well plates at or near confluence (≈ 70% of surface area covered), allowed to adhere for approximately 4 hours, and then treated with either an individual subfraction or the ethanolic whole extract. Similar volumes of deionized water were added to wells to serve as negative controls (mock-treatment group). The cells were left to incubate with the extracts for 24 hours (Figures 3A and 4A) or 48 hours (Figures 3B and 4B), after which the cancer cell viability was determined using a MTT assay and reported as a percent of the negative control.

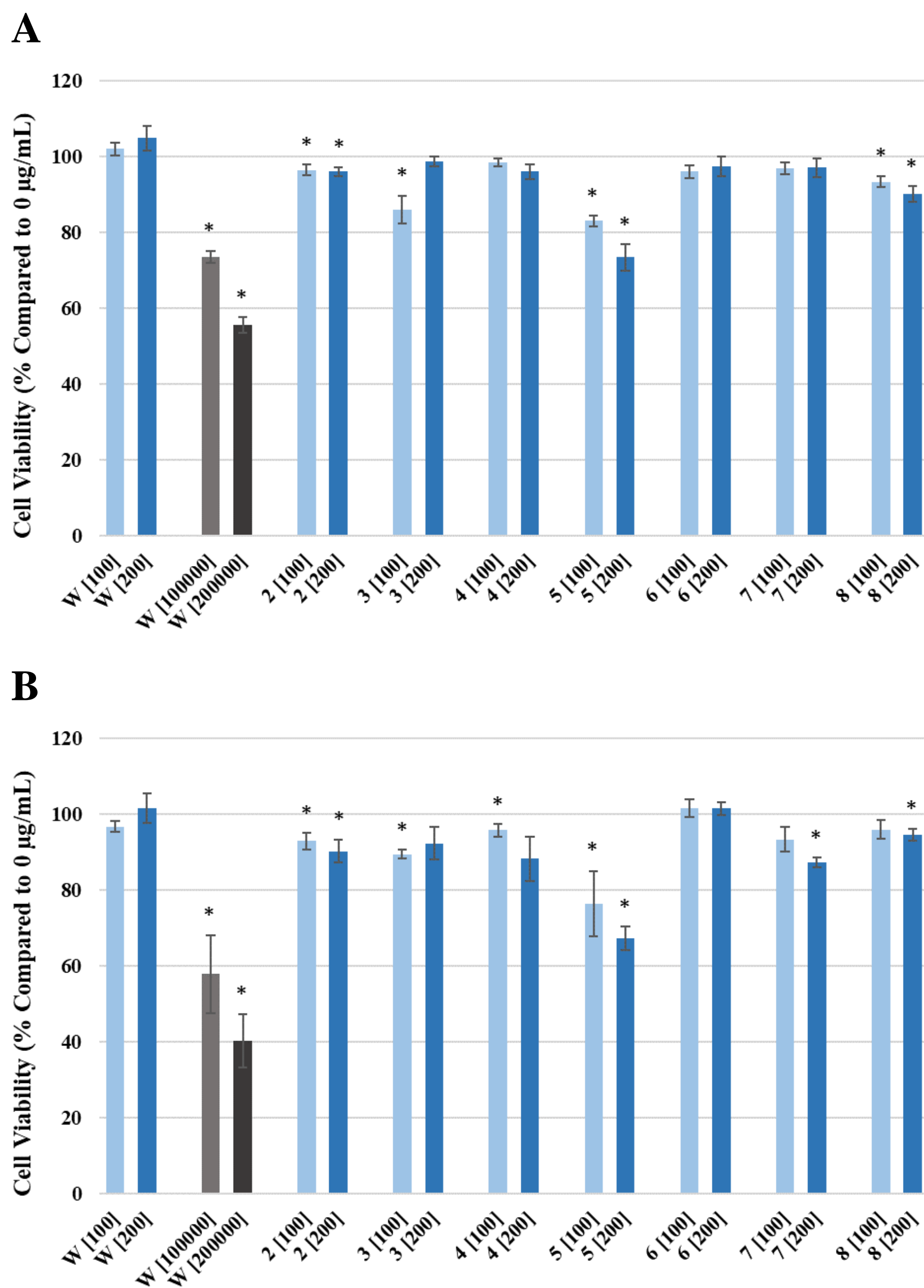


Figure 3. Changes in Viability of HeLa Cervical Cancer Cells after Treatment with *M. oleifera* Whole Extract and Subfractions. HeLa cervical cancer cells were treated with ethanolic *M. oleifera* leaf extract (W) or an individual subfraction (2-8) for 24 or 48 hours. A MTT assay was used determine the cell viability after 24 hours of treatment (3A) or 48 hours after treatment (3B). The data were then normalized as a percentage of the mock-treated negative control, which received water in place of extract. The concentrations for each extract are recorded in micrograms of original leaf powder per milliliter [µg/mL]. The data shown are an average of three to five experiments ± standard error. Asterisks indicate a significant change in cell viability as compared to the mock-treated negative control (P = <0.05) using one-tailed Student's t-tests.

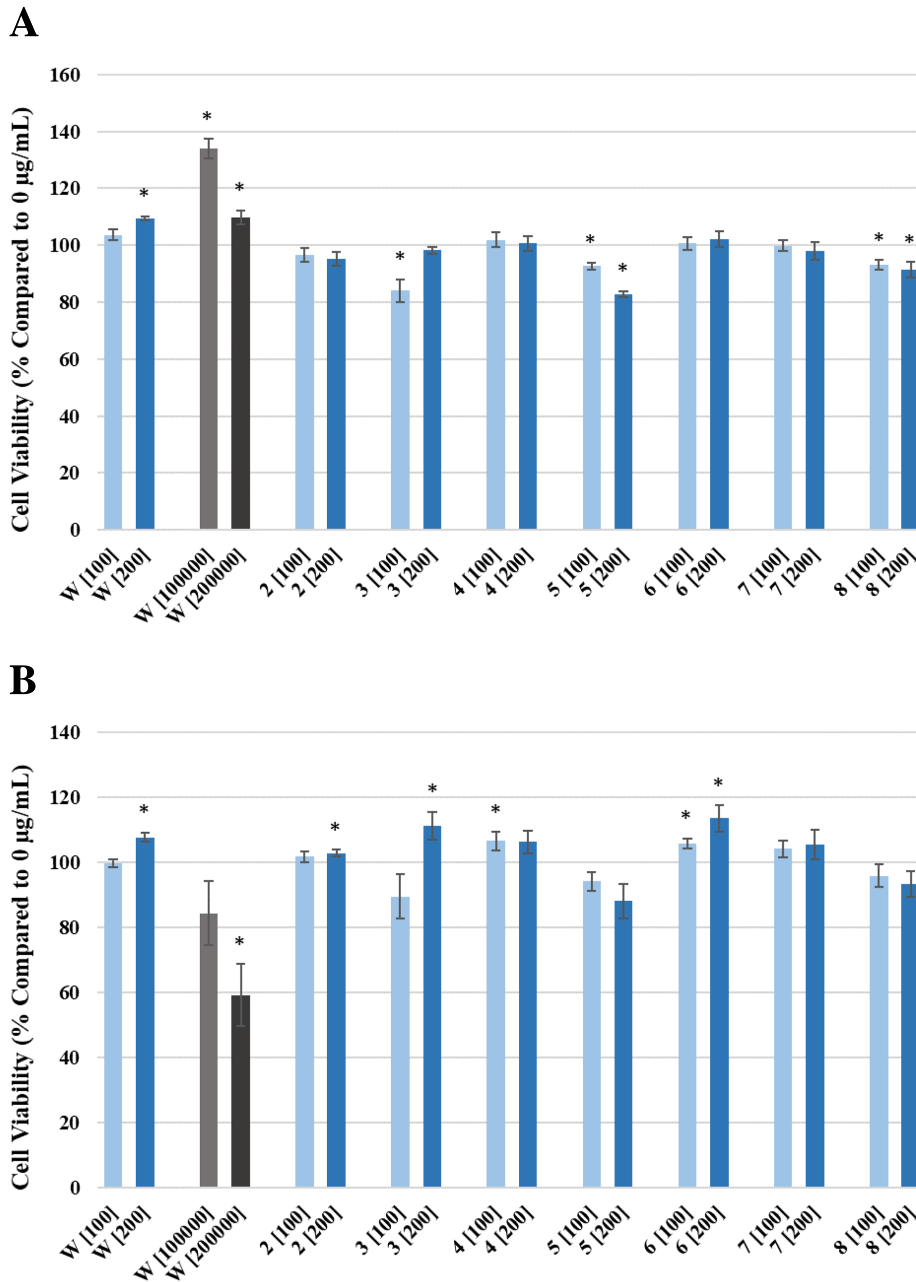


Figure 4. Changes in Viability of SiHa Cervical Cancer Cells after Treatment with *M. oleifera* Whole Extract and Subfractions. SiHa cervical cancer cells were treated with ethanolic *M. oleifera* leaf extract (W) or an individual subfraction (2-8) for 24 or 48 hours. A MTT assay was used determine the cell viability after 24 hours of treatment (4A) or 48 hours after treatment (4B). The data were then normalized as a percentage of the mock-treated negative control, which received water in place of extract. The concentrations for each extract are recorded in micrograms of original leaf powder per milliliter [µg/mL]. The data shown are an average of three to five experiments ± standard error. Asterisks indicate a significant change in cell viability as compared to the mock-treated negative control (P = <0.05) using one-tailed Student's t-tests.

The MTT data corroborated previous results from our lab, showing that ethanolic whole extract (W) was able to significantly decrease cervical cancer cell viability at high treatment concentrations of 100,000 and 200,000 µg/mL; there was a notable exception to this reduction in cell viability in the SiHa cells measured after 24 hours, in which case both the 100,000 and 200,000 µg/mL treatment concentrations caused a significant increase in SiHa cell viability. While the whole extract exhibited promising anticancer effects at high concentrations, there were no decreases in HeLa or SiHa cancer cell viability when cells were treated with the whole extract at lower concentrations of 100 or 200 µg/mL. In fact, treatment with 200 µg/mL of ethanolic whole extract caused a significant increase in SiHa cell viability at both the 24 hour and 48 hour time point.

In contrast to results obtained from the whole extract, individual subfractions at the lower concentrations did influence the viability of both HeLa and SiHa cells. Overall, the MTT data showed that HeLa cell viability decreased more significantly than SiHa cell viability when individual subfractions were added. HeLa cell viability generally decreased from treatment with 100 µg/mL to 200 µg/mL of subfractions, and there were also less viable cells after 48 hours compared to data from 24 hours. The subfractions that significantly decreased HeLa cell viability include subfractions 2, 3, 4, 5, 7, and 8, with subfraction 5 causing the greatest decrease in cell viability.

As mentioned above, SiHa cancer cells responded much differently to treatment with individual subfractions as compared to HeLa cells. At the 24 hour time point, subfractions 3, 5 and 8 significantly reduced SiHa cell viability (Figure 4A), but viability did not continue to decrease over time (Figure 4B). In fact, none of the individual subfractions caused a significant decrease in SiHa cell viability after 48 hours. Rather, treatment with subfractions

2, 3, 4, and 6 actually significantly increased cell viability by 48 hours. These data indicate that there are individual cancer cell differences that may influence the effects of *M. oleifera* subfractions on cellular viability, even when cells are derived from the same tissue.

Seeing the therapeutic potential of several of the HPLC-isolated subfractions, we decided to test if combinations of these subfractions could further reduce HeLa and SiHa cell viability. Subsequently, we tested treatments with two and three subfraction combinations using the most promising subfractions (2, 3, 5, 7, and 8) according to data obtained in Figure 3. Using a similar protocol for testing the individual subfractions, we generated a cancer cell viability heatmap to visually show the effects of subfraction combinations on HeLa (Figure 5) and SiHa (Figure 6) cervical cancer cells.

	24hr [100]	24hr [200]	48hr [100]	48hr [200]
W	102.0	104.8	96.7	101.6
2	**96.4**	**96.0**	**92.8**	**90.2**
3	**86.0**	98.7	**89.4**	92.3
4	98.4	96.0	**95.8**	88.2
5	**83.0**	**73.4**	**76.5**	**67.3**
6	96.0	97.3	101.5	101.5
7	96.9	97.0	93.3	**87.3**
8	**93.3**	**90.1**	95.9	**94.4**
2 & 3	96.8	91.2	**91.5**	**79.8**
2 & 5	89.4	**76.0**	**74.3**	**59.2**
2 & 7	**93.8**	**88.8**	**78.3**	**71.3**
2 & 8	88.2	**88.0**	**80.1**	**76.3**
3 & 5	92.4	88.3	**75.7**	**65.6**
3 & 7	**94.3**	92.7	**79.1**	**77.3**
3 & 8	**93.4**	92.7	**77.9**	**75.5**
5 & 7	94.0	**85.2**	**78.5**	**63.2**
5 & 8	97.4	87.8	**83.9**	**66.7**
7 & 8	96.7	92.8	**81.7**	**80.6**
2 & 3 & 5	92.3	83.3	**79.3**	**57.5**
2 & 5 & 7	91.9	83.2	**69.6**	**53.9**
3 & 5 & 7	91.4	87.5	**72.3**	**58.0**
3 & 5 & 8	92.8	85.1	**75.1**	**62.9**

105 -115

95 - 105

85 - 95

75 -85

65 - 75

55 - 65

45 - 55

Figure 5. Changes in HeLa Cervical Cancer Cell Viability after Treatment with Individual Subfractions and Combinations of subfractions. HeLa cervical cancer cells were treated with ethanolic *M. oleifera* leaf extract (W) or subfraction(s) (2-8) for 24 or 48 hours, and a MTT assay was used determine the cell viability following treatment. The data were then normalized as a percentage of the mock-treated negative control. The darker blue samples indicate a greater decrease in cell viability relative to the mock-control, while yellow indicates an increase in cell viability. The concentrations for each extract are recorded in micrograms of original leaf powder per milliliter [µg/mL]. The data shown are an average of three to six experiments. Bolded numbers indicate a significant change in cell viability (P = <0.05) using one-tailed Student's t-tests.

	24hr [100]	**24hr [200]**	**48hr [100]**	**48hr [200]**
W	103.7	**109.5**	99.7	**107.7**
2	96.7	95.3	101.7	**102.8**
3	**84.1**	98.2	89.5	**111.2**
4	101.9	100.6	**106.6**	106.3
5	**92.7**	**82.9**	94.1	88.0
6	100.7	102.2	**105.8**	**113.5**
7	99.9	98.1	104.2	105.3
8	**93.2**	**91.5**	95.9	93.3
2 & 3	98.3	**88.7**	**90.0**	**80.2**
2 & 5	**82.4**	**60.1**	**75.8**	**51.9**
2 & 7	97.5	**93.7**	**91.6**	**82.0**
2 & 8	**97.2**	93.8	**90.1**	**84.4**
3 & 5	**75.9**	**54.8**	**77.0**	**53.8**
3 & 7	96.7	**90.6**	**89.6**	**80.6**
3 & 8	96.1	**91.0**	**89.8**	**80.0**
5 & 7	**79.0**	**58.2**	**83.0**	**58.7**
5 & 8	**78.2**	**56.1**	**82.1**	**55.0**
7 & 8	**91.5**	**84.4**	**92.2**	**81.2**
2 & 3 & 5	**77.8**	**51.1**	**80.1**	**50.2**
2 & 5 & 7	**78.2**	**56.1**	**76.7**	**50.5**
3 & 5 & 7	**80.7**	**56.2**	**79.1**	**52.1**
3 & 5 & 8	**79.4**	**54.0**	**79.6**	**51.7**

Legend
105 -115
95 - 105
85 - 95
75 -85
65 - 75
55 - 65
45 - 55

Figure 6. Changes in SiHa Cervical Cancer Cell Viability after Treatment with Individual Subfractions and Combinations of subfractions. SiHa cervical cancer cells were treated with ethanolic *M. oleifera* leaf extract (W) or subfraction(s) (2-8) for 24 or 48 hours, and a MTT assay was used determine the cell viability following treatment. The data were then normalized as a percentage of the mock-treated negative control. The darker blue samples indicate a greater decrease in cell viability relative to the mock-control, while yellow indicates an increase in cell viability. The concentrations for each extract are recorded in micrograms of original leaf powder per milliliter [µg/mL]. The data shown are an average of three to six experiments. Bolded numbers indicate a significant change in cell viability ($P = <0.05$) using one-tailed Student's t-tests.

As indicated in the results section of Figure 3, a temporal effect of individual subfractions becomes apparent when looking at HeLa cell viability in Figure 5, where there was a greater reduction in cell viability at the 48 hour time as compared to 24 hour time point

data. The visual data from Figure 5 also suggests that treatment with two or three subfraction combinations potentially decreases HeLa cell viability more than treatment with individual subfractions, especially when applied for 48 hours.

The temporal effect seen in HeLa cells does not appear to apply when looking at SiHa cell viability in Figure 6. Rather, there is a more pronounced effect on the treatment concentration of subfraction combinations when looking at SiHa cell viability. Data from Figure 6 shows considerable decreases in SiHa cell viability when subfraction combinations were treated at a concentration of 200 µg/mL instead of 100 µg/mL. It is also worth noting that in contrast to results seen for individual subfractions, no treatment combinations caused an increase in SiHa cell viability. Somewhat surprisingly, treatment with three subfraction combinations does not appear to drastically reduce SiHa cell viability any more than treatment with two subfraction combinations.

Overall, treatment with subfraction combinations appears to significantly reduce cervical cancer cell viability. However, the MTT data from the subfraction combinations presented in Figures 5 and 6 do not directly compare combination treatments to their individual subfraction constituents. Therefore, we further analyzed the previously generated cell viability data in order to determine if subfraction combination treatments further reduced cell viability than an individual subfraction that made up the combination treatment. HeLa cell viability treatment differences were analyzed for significance in Tables 1 and 2 below, while SiHa cell viability treatment differences were analyzed for significance in Tables 3 and 4.

	Cell Viability (%)	2 [100]	3 [100]	5 [100]	7 [100]	8 [100]	2 [200]	3 [200]	5 [200]	7 [200]	8 [200]
Cell Viability (%)		96.4	86.0	83.0	96.9	93.3	96.0	98.7	73.4	97.0	90.1
2 & 3 [100]	96.8	0.9301	0.0849								
2 & 3 [200]	91.2						0.5377	0.3523			
2 & 5 [100]	89.4	0.2277		0.2641							
2 & 5 [200]	76.0						0.1418		0.8230		
2 & 7 [100]	93.8	0.2695			0.2002						
2 & 7 [200]	88.8						0.1650			0.1377	
2 & 8 [100]	88.2	0.2208				0.4208					
2 & 8 [200]	88.0						0.0955				0.6433
3 & 5 [100]	92.4		0.2711	0.0768							
3 & 5 [200]	88.3							0.2212	0.1130		
3 & 7 [100]	94.3		0.1347		0.3283						
3 & 7 [200]	92.7							0.2345		0.4100	
3 & 8 [100]	93.4		0.1694			0.9839					
3 & 8 [200]	92.7							0.1855			0.5653
5 & 7 [100]	94.0			**0.0236**	0.4099						
5 & 7 [200]	85.2								0.1349	0.1241	
5 & 8 [100]	97.4			**0.0313**		0.4002					
5 & 8 [200]	87.8								0.1610		0.7974
7 & 8 [100]	96.7				0.9462	0.3544					
7 & 8 [200]	92.8									0.3817	0.5400
2 & 3 & 5 [100]	92.3	0.3962	0.3001	0.1011							
2 & 3 & 5 [200]	83.3						0.1931	0.1346	0.2998		
2 & 5 & 7 [100]	91.9	0.3430		0.1049	0.2980						
2 & 5 & 7 [200]	83.2						0.2060		0.3181	0.1808	
3 & 5 & 7 [100]	91.4		0.3920	0.1585	0.3177						
3 & 5 & 7 [200]	87.5							0.2554	0.1756	0.3251	
3 & 5 & 8 [100]	92.8		0.2489	0.0714		0.9060					
3 & 5 & 8 [200]	85.1							0.2126	0.2758		0.6109

Table 1. Treatment with Subfractions Combinations Compared to Individual Subfraction Constituents applied to HeLa cells for 24 hours. HeLa cervical cancer cells were treated with combinations of subfractions (2-8) over 24 hours, and cancer cell viability (CV) was measured using an MTT Assay. The data were then normalized as a percentage of the mock negative control, which received water in place of extract. Statistical analyses were performed to determine which subfraction combinations were significantly different from their individual subfraction constituents. The concentrations for each extract are recorded in micrograms of original leaf powder per milliliter [µg/mL]. The data shown are an average of three to six experiments ± standard error. Bolded numbers indicate a significant difference in cell viability ($P = <0.05$) using two-tailed Student's t-tests. P-values highlighted in blue show combinations exhibiting significant reductions compared to their individual constituents, while yellow indicates a significant increase.

	Cell Viability (%)	2 [100]	3 [100]	5 [100]	7 [100]	8 [100]	2 [200]	3 [200]	5 [200]	7 [200]	8 [200]
Cell Viability (%)		92.8	89.4	76.5	93.3	95.9	90.2	92.3	67.3	87.3	94.4
2 & 3 [100]	91.5	0.7326	0.5697								
2 & 3 [200]	79.8						**0.0447**	0.0744			
2 & 5 [100]	74.3	**0.0006**		0.8222							
2 & 5 [200]	59.2						**0.0008**		0.1826		
2 & 7 [100]	78.3	**0.0049**			**0.0116**						
2 & 7 [200]	71.3						**0.0086**			**0.0156**	
2 & 8 [100]	80.1	0.0674				**0.0356**					
2 & 8 [200]	76.3						0.0662				**0.0322**
3 & 5 [100]	75.7		**0.0086**	0.9328							
3 & 5 [200]	65.6							**0.0061**	0.7664		
3 & 7 [100]	79.1		0.0811		**0.0383**						
3 & 7 [200]	77.3							0.0518		0.0789	
3 & 8 [100]	77.9		0.0911			**0.0223**					
3 & 8 [200]	75.5							**0.0340**			**0.0064**
5 & 7 [100]	78.5			0.8368	**0.0132**						
5 & 7 [200]	63.2								0.4197	**0.0014**	
5 & 8 [100]	83.9			0.4567		**0.0084**					
5 & 8 [200]	66.7								0.9179		**0.0012**
7 & 8 [100]	81.7				**0.0361**	**0.0072**					
7 & 8 [200]	80.6									0.1891	**0.0220**
2 & 3 & 5 [100]	79.3	0.0609	0.1281	0.7917							
2 & 3 & 5 [200]	57.5						**0.0004**	**0.0018**	0.0967		
2 & 5 & 7 [100]	69.6	**0.0028**		0.5058	**0.0030**						
2 & 5 & 7 [200]	53.9						**0.0001**		**0.0152**	**0.0000**	
3 & 5 & 7 [100]	72.3		**0.0040**	0.6689	**0.0026**						
3 & 5 & 7 [200]	58.0							**0.0030**	0.0656	**0.0001**	
3 & 5 & 8 [100]	75.1		**0.0185**	0.8880		**0.0033**					
3 & 5 & 8 [200]	62.9							**0.0038**	0.3990		**0.0004**

Table 2. Treatment with Subfractions Combinations Compared to Individual Subfraction Constituents applied to HeLa cells for 48 hours. HeLa cervical cancer cells were treated with combinations of subfractions (2-8) over 48 hours, and cancer cell viability (CV) was measured using an MTT Assay. The data were then normalized as a percentage of the mock negative control, which received water in place of extract. Statistical analyses were performed to determine which subfraction combinations were significantly different from their individual subfraction constituents. The concentrations for each extract are recorded in micrograms of original leaf powder per milliliter [µg/mL]. The data shown are an average of three to six experiments ± standard error. Bolded numbers indicate a significant difference in cell viability ($P = <0.05$) using two-tailed Student's t-tests. P-values highlighted in blue show combinations exhibiting significant reductions compared to their individual constituents, while yellow indicates a significant increase.

	Cell Viability (%)	2 [100]	3 [100]	5 [100]	7 [100]	8 [100]	2 [200]	3 [200]	5 [200]	7 [200]	8 [200]
Cell Viability (%)		96.7	84.1	92.7	99.9	93.2	95.3	98.2	82.9	98.1	91.5
2 & 3 [100]	98.3	0.7498	**0.0403**								
2 & 3 [200]	88.7						0.2208	0.0871			
2 & 5 [100]	82.4	**0.0042**		**0.0144**							
2 & 5 [200]	60.1						**0.0002**		**0.0041**		
2 & 7 [100]	97.5	0.8307			0.4689						
2 & 7 [200]	93.7						0.6202			0.2860	
2 & 8 [100]	97.2	0.8461				0.0863					
2 & 8 [200]	93.8						0.7559				0.6557
3 & 5 [100]	75.9		0.1263	**0.0001**							
3 & 5 [200]	54.8							**0.0000**	**0.0004**		
3 & 7 [100]	96.7		**0.0452**		0.4290						
3 & 7 [200]	90.6							0.1115		0.1731	
3 & 8 [100]	96.1		**0.0478**			0.3688					
3 & 8 [200]	91.0							0.1670			0.9265
5 & 7 [100]	79.0			**0.0094**	**0.0010**						
5 & 7 [200]	58.2								**0.0008**	**0.0000**	
5 & 8 [100]	78.2			**0.0037**		**0.0028**					
5 & 8 [200]	56.1								**0.0002**		**0.0000**
7 & 8 [100]	91.5				**0.0408**	0.6209					
7 & 8 [200]	84.4									**0.0192**	0.1489
2 & 3 & 5 [100]	77.8	**0.0042**	0.3007	**0.0137**							
2 & 3 & 5 [200]	51.1						**0.0000**	**0.0001**	**0.0005**		
2 & 5 & 7 [100]	78.2	**0.0042**		**0.0147**	**0.0021**						
2 & 5 & 7 [200]	56.1						**0.0011**		**0.0083**	**0.0006**	
3 & 5 & 7 [100]	80.7		0.5319	**0.0181**	**0.0020**						
3 & 5 & 7 [200]	56.2							**0.0006**	**0.0043**	**0.0002**	
3 & 5 & 8 [100]	79.4		0.4401	**0.0310**		**0.0263**					
3 & 5 & 8 [200]	54.0							**0.0001**	**0.0009**		**0.0001**

Table 3. Treatment with Subfractions Combinations Compared to Individual Subfraction Constituents applied to SiHa cells for 24 hours. SiHa cervical cancer cells were treated with combinations of subfractions (2-8) over 24 hours, and cancer cell viability (CV) was measured using an MTT Assay. The data were then normalized as a percentage of the mock negative control, which received water in place of extract. Statistical analyses were performed to determine which subfraction combinations were significantly different from their individual subfraction constituents. The concentrations for each extract are recorded in micrograms of original leaf powder per milliliter [µg/mL]. The data shown are an average of three to six experiments ± standard error. Bolded numbers indicate a significant difference in cell viability (P = <0.05) using two-tailed Student's t-tests. P-values highlighted in blue show combinations exhibiting significant reductions compared to their individual constituents, while yellow indicates a significant increase.

	Cell Viability (%)	**2 [100]**	**3 [100]**	**5 [100]**	**7 [100]**	**8 [100]**	**2 [200]**	**3 [200]**	**5 [200]**	**7 [200]**	**8 [200]**
Cell Viability (%)		101.7	89.5	94.1	104.2	95.9	102.8	111.2	88.0	105.3	93.3
2 & 3 [100]	90.0	**0.0035**	0.9486								
2 & 3 [200]	80.2						**0.0000**	**0.0035**			
2 & 5 [100]	75.8	**0.0005**		**0.0043**							
2 & 5 [200]	51.9						**0.0000**		**0.0018**		
2 & 7 [100]	91.6	**0.0129**			**0.0093**						
2 & 7 [200]	82.0						**0.0000**			**0.0096**	
2 & 8 [100]	90.1	**0.0041**				0.2062					
2 & 8 [200]	84.4						**0.0009**				0.1140
3 & 5 [100]	77.0		0.1623	**0.0054**							
3 & 5 [200]	53.8							**0.0006**	**0.0064**		
3 & 7 [100]	89.6		0.9934		**0.0033**						
3 & 7 [200]	80.6							**0.0016**		**0.0061**	
3 & 8 [100]	89.8		0.9712			0.1706					
3 & 8 [200]	80.0							**0.0038**			**0.0287**
5 & 7 [100]	83.0			**0.0203**	**0.0005**						
5 & 7 [200]	58.7								**0.0075**	**0.0008**	
5 & 8 [100]	82.1			**0.0145**		**0.0116**					
5 & 8 [200]	55.0								**0.0032**		**0.0001**
7 & 8 [100]	92.2				**0.0112**	0.3561					
7 & 8 [200]	81.2									**0.0075**	**0.0375**
2 & 3 & 5 [100]	80.1	**0.0001**	0.2628	**0.0098**							
2 & 3 & 5 [200]	50.2						**0.0000**	**0.0002**	**0.0036**		
2 & 5 & 7 [100]	76.7	**0.0000**		**0.0031**	**0.0001**						
2 & 5 & 7 [200]	50.5						**0.0000**		**0.0032**	**0.0004**	
3 & 5 & 7 [100]	79.1		0.2247	**0.0059**	**0.0002**						
3 & 5 & 7 [200]	52.1							**0.0002**	**0.0036**	**0.0004**	
3 & 5 & 8 [100]	79.6		0.2425	**0.0067**		**0.0054**					
3 & 5 & 8 [200]	51.7							**0.0003**	**0.0044**		**0.0002**

Table 4. Treatment with Subfractions Combinations Compared to Individual Subfraction Constituents applied to SiHa cells for 48 hours. SiHa cervical cancer cells were treated with combinations of subfractions (2-8) over 48 hours, and cancer cell viability (CV) was measured using an MTT Assay. The data were then normalized as a percentage of the mock negative control, which received water in place of extract. Statistical analyses were performed to determine which subfraction combinations were significantly different from their individual subfraction constituents. The concentrations for each extract are recorded in micrograms of original leaf powder per milliliter [µg/mL]. The data shown are an average of three to six experiments ± standard error. Bolded numbers indicate a significant difference in cell viability (P = <0.05) using two-tailed Student's t-tests. P-values highlighted in blue show combinations exhibiting significant reductions compared to their individual constituents, while yellow indicates a significant increase.

While data analysis from Tables 1 – 4 does show that many subfraction combination treatments did not perform significantly better than some of their individual subfraction constituents, numerous other combination treatments significantly outperformed their

individual constituent subfractions in reducing cervical cancer cell viability. Thus, Table 5 below was constructed to summarize which subfraction combination treatments were significantly more effective at reducing cell viability than individual subfractions.

A

	2 [100]	3 [100]	5 [100]	7 [100]	8 [100]	2 [200]	3 [200]	5 [200]	7 [200]	8 [200]
24 Hours			5 & 7 [100]							
			5 & 8 [100]							
48 Hours	2 & 5 [100]	3 & 5 [100]		2 & 7 [100]	2 & 8 [100]	2 & 3 [200]	3 & 5 [200]	2 & 5 & 7 [200]	2 & 7 [200]	2 & 8 [200]
	2 & 7 [100]	3 & 5 & 7 [100]		3 & 7 [100]	3 & 8 [100]	2 & 5 [200]	3 & 8 [200]		5 & 7 [200]	3 & 8 [200]
	2 & 5 & 7 [100]	3 & 5 & 8 [100]		5 & 7 [100]	5 & 8 [100]	2 & 7 [200]	2 & 3 & 5 [200]		2 & 5 & 7 [200]	5 & 8 [200]
				7 & 8 [100]	7 & 8 [100]	2 & 3 & 5 [200]	3 & 5 & 7 [200]		3 & 5 & 7 [200]	7 & 8 [200]
				2 & 5 & 7 [100]	3 & 5 & 8 [100]	2 & 5 & 7 [200]	3 & 5 & 8 [200]			3 & 5 & 8 [200]
				3 & 5 & 7 [100]						

B

	2 [100]	3 [100]	5 [100]	7 [100]	8 [100]	2 [200]	3 [200]	5 [200]	7 [200]	8 [200]
24 Hours	2 & 5 [100]	**2 & 3 [100]**	2 & 5 [100]	5 & 7 [100]	5 & 8 [100]	2 & 5 [200]	3 & 5 [200]	2 & 5 [200]	5 & 7 [200]	5 & 8 [200]
	2 & 3 & 5 [100]	**3 & 7 [100]**	3 & 5 [100]	7 & 8 [100]	3 & 5 & 8 [100]	2 & 3 & 5 [200]	2 & 3 & 5 [200]	3 & 5 [200]	7 & 8 [200]	3 & 5 & 8 [200]
	2 & 5 & 7 [100]	**3 & 8 [100]**	5 & 7 [100]	2 & 5 & 7 [100]		2 & 5 & 7 [200]	3 & 5 & 7 [200]	5 & 7 [200]	2 & 5 & 7 [200]	
			5 & 8 [100]	3 & 5 & 7 [100]			3 & 5 & 8 [200]	5 & 8 [200]	3 & 5 & 7 [200]	
			2 & 3 & 5 [100]					2 & 3 & 5 [200]		
			2 & 5 & 7 [100]					2 & 5 & 7 [200]		
			3 & 5 & 7 [100]					3 & 5 & 7 [200]		
			3 & 5 & 8 [100]					3 & 5 & 8 [200]		
48 Hours	2 & 3 [100]		2 & 5 [100]	2 & 7 [100]	5 & 8 [100]	2 & 3 [200]	2 & 3 [200]	2 & 5 [200]	2 & 7 [200]	3 & 8 [200]
	2 & 5 [100]		3 & 5 [100]	3 & 7 [100]	3 & 5 & 8 [100]	2 & 5 [200]	3 & 5 [200]	3 & 5 [200]	3 & 7 [200]	5 & 8 [200]
	2 & 7 [100]		5 & 7 [100]	5 & 7 [100]		2 & 7 [200]	3 & 7 [200]	5 & 7 [200]	5 & 7 [200]	7 & 8 [200]
	2 & 8 [100]		5 & 8 [100]	7 & 8 [100]		2 & 8 [200]	3 & 8 [200]	5 & 8 [200]	7 & 8 [200]	3 & 5 & 8 [200]
	2 & 3 & 5 [100]		2 & 3 & 5 [100]	2 & 5 & 7 [100]		2 & 3 & 5 [200]	2 & 3 & 5 [200]	2 & 3 & 5 [200]	2 & 5 & 7 [200]	
	2 & 5 & 7 [100]		2 & 5 & 7 [100]	3 & 5 & 7 [100]		2 & 5 & 7 [200]	3 & 5 & 7 [200]	2 & 5 & 7 [200]	3 & 5 & 7 [200]	
			3 & 5 & 7 [100]				3 & 5 & 8 [200]	3 & 5 & 7 [200]		
			3 & 5 & 8 [100]					3 & 5 & 8 [200]		

Table 5. Subfraction Combinations that Significantly Changed Cancer Cell Viability Compared to Individual Constituents. The statistical analyses comparing treatment with subfraction combinations to individual constituents were summarized for HeLa (A) and SiHa (B) cervical cancer. Significant increases in cell viability from combination treatment are highlighted in yellow, while all other combinations exhibited significant decreases. The concentrations for each extract are recorded in micrograms of original leaf powder per milliliter [µg/mL].

Table 5A shows that the treatment combination of subfractions 5 & 7, as well as subfractions 5 & 8, actually increased HeLa cell viability compared to treating HeLa cells with subfraction 5 only (with a treatment concentration of 100 µg/mL after 24 hours). Similarly, the treatment combinations of 2 & 3, 3 & 7, and 3 & 8 increased SiHa cell

viability (Table 5B) compared to subfraction 3 alone (with a treatment concentration of 100 μg/mL after 24 hours). Therefore, these treatment combinations would not be used over some of their individual subfraction constituents alone.

Despite these outliers, Table 5 shows that most treatment combinations outperformed their subfraction constituents alone. As mentioned previously, Table 5A supports a temporal effect on HeLa cell viability after treatment with subfraction combinations. There were no subfraction combinations that outperformed individual subfraction constituents at a 24 hour time point. Yet, several subfraction combinations significantly reduced HeLa cell viability when compared to individual subfraction constituents at a 48 hour time point. Looking at the collection of SiHa data (Table 5B), we can see that most treatment combinations applied to SiHa cells are able to further decrease cell viability compared to any individual subfraction constituents. This is in line with the results from Figure 6; results from Figure 6 indicate that individual subfractions applied to SiHa cells typically increased cell viability, so cell viability reductions from the combinations are likely to be significant compared to the cell viability increases of single subfractions.

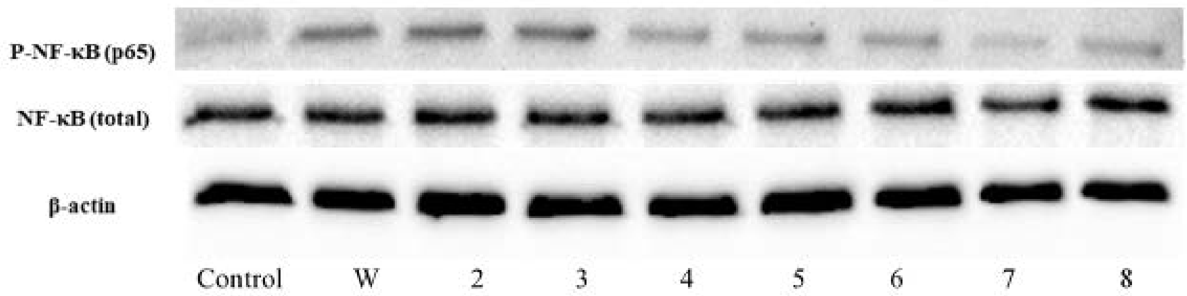
3.4 Potential Anticancer Molecular Mechanisms Altered by Subfraction Treatment

Results from our MTT assays support our hypothesis that HPLC-isolated subfractions from ethanolic *M. oleifera* leaf extracts would exhibit anticancer effects, but the MTT data cannot explain how or why HeLa and SiHa cervical cancer cell viability is decreasing. One possibility is that these subfractions are altering apoptotic pathways inside the cancer cells, which could account for the observed decrease in cell viability. While the NF-κB pathway has been linked to increased cancer cell growth, metastasis, and proliferation, as well as

increased proliferation of immune cells and their subsequent inflammatory responses, it is also associated with inducing apoptosis through induction of the pro-apoptotic protein, Bax. Consequently, we hypothesized that subfraction treatments might be altering the NF-κB pathway to induce apoptosis of these cervical cancer cell lines.

In order to determine if the NF-κB pathway was changing in response to subfraction treatment, we applied subfraction treatments at a concentration of 100 µg/mL to HeLa cells for 24 hours. Following the 24 hour incubation, the media was removed, the cells were lysed using a detergent buffer, and cell lysates were collected for Western blot analysis. Levels of phosphorylated NF-κB (p65) and total NF-κB were determined by immunoblotting, and the proteins were compared back to mock-treated negative control as shown in Figure 7.

A



B

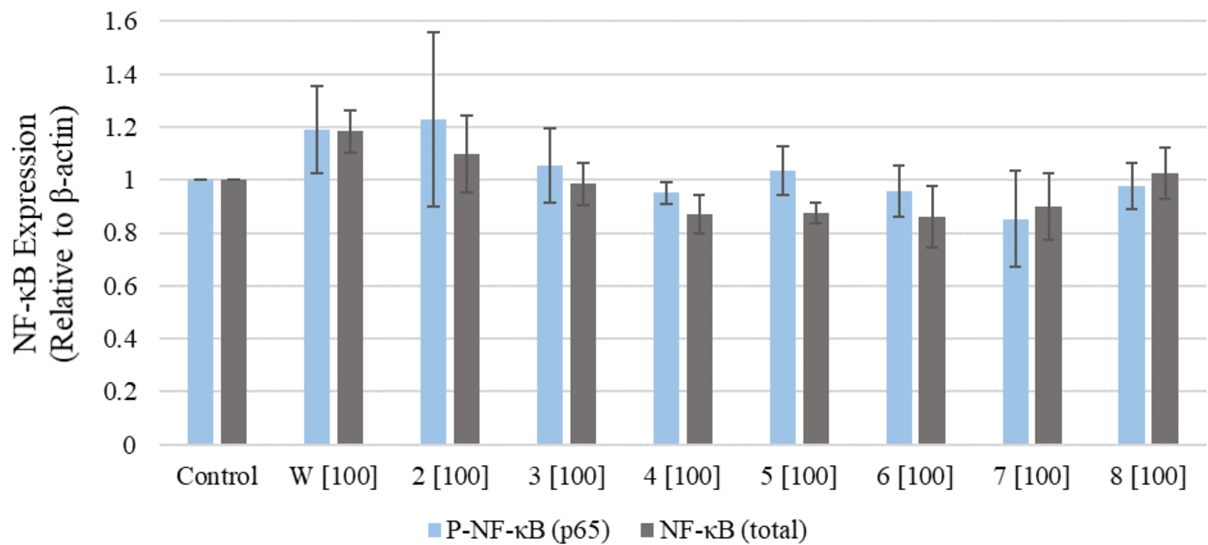


Figure 7. Subfraction Effects on NF-κB Expression and Activation in HeLa Cells. HeLa cervical cancer cells were treated with ethanolic *M. oleifera* leaf extract (W) or an individual subfraction (2-8) for 24 hours. Cell lysates were collected and p-NF-κB and Total NF-κB protein expressions were determined via Western blotting. Representative images from bands subjected to Western blot analysis are shown (A). Protein levels were normalized to β-actin and then compared to the mock negative control. The concentrations for each extract are recorded in micrograms of original leaf powder per milliliter [µg/mL]. The data shown are an average of three experiments ± standard error. Asterisks indicate a significant change in protein levels compared to the negative control (P = <0.05) using two-tailed Student's t-tests.

Figure 7B did not show any significant changes in NF-κB protein expression, although there might be a slight, but insignificant, decrease in p-NF-κB and Total NF-κB protein levels with subfractions 4, 5, 6, and 7. The data might also be indicating a slight, but insignificant, increase in p-NF-κB and Total NF-κB protein levels after treatment with whole extract (W) and subfraction 2.

While the results from Figure 7 were not conclusive in showing NF-κB protein expression was being altered, we decided to further investigate the alteration of the NF-κB pathway by looking at two downstream proteins, Bax and Bcl-xL. These proteins have an antagonistic relationship where Bax induces apoptosis through mitochondrial stress, while Bcl-xL inhibits these apoptotic processes. Based on our previous data, we expected the Western immunoblotting data in Figure 8 to show the subfractions decreasing Bcl-xL levels (the inhibitor of apoptosis) and increasing Bax protein levels.

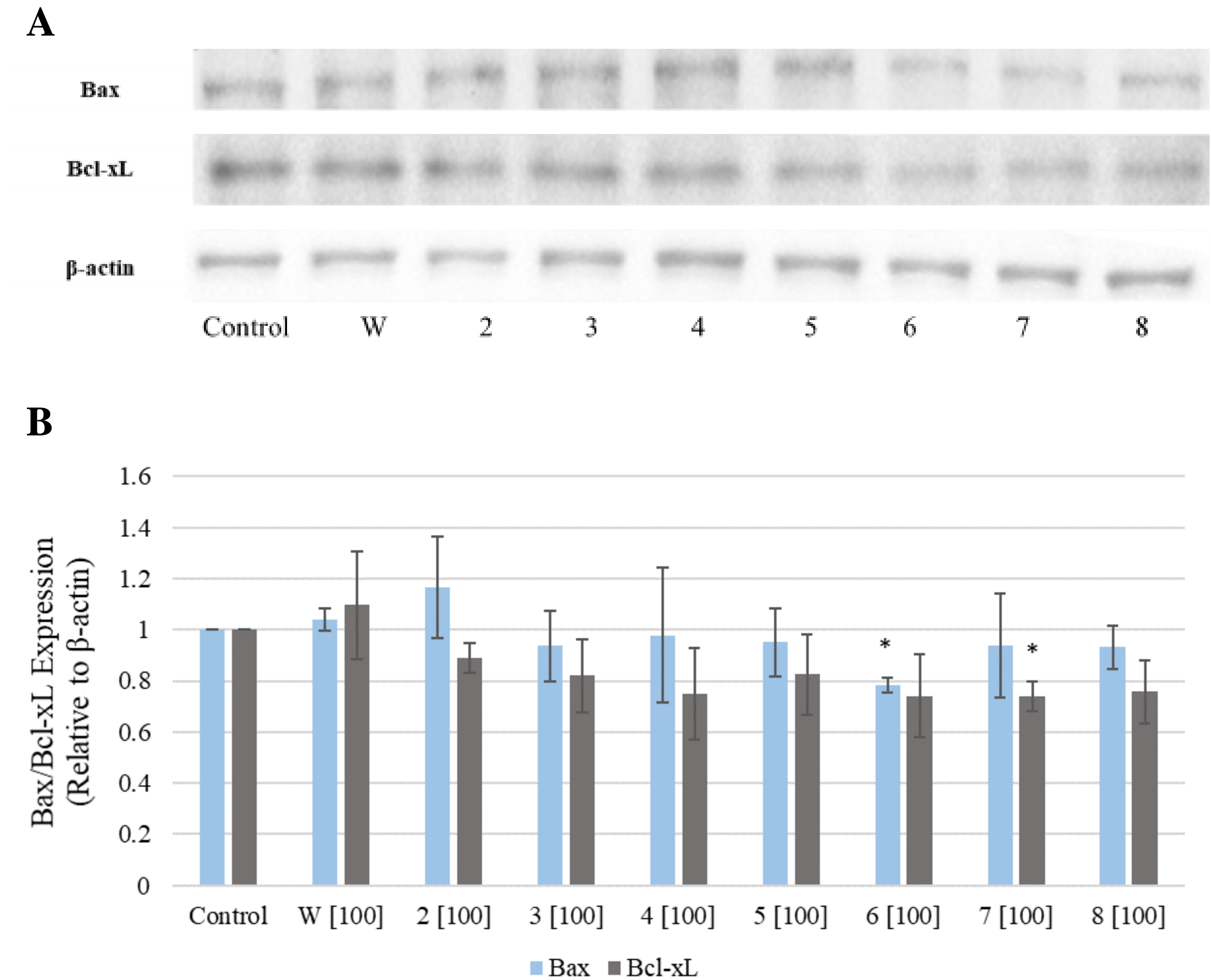


Figure 8. Subfraction Effects on Bax/Bcl-xL Expression in HeLa Cells. HeLa cervical cancer cells were treated with ethanolic *M. oleifera* leaf extract (W) or an individual subfraction (2-8) for 24 hours. Cell lysates were collected and Bax and Bcl-xL protein expression was determined via Western blotting. Representative images from bands subjected to Western blot analysis are shown (A). Protein levels were normalized to β-actin and then compared to the mock negative control. The concentrations for each extract are recorded in micrograms of original leaf powder per milliliter [µg/mL]. The data shown are an average of three experiments ± standard error. Asterisks indicate a significant change in protein levels as compared to the negative control (P = <0.05) using two-tailed Student's t-tests.

Similar to the previous NF-κB Western blot data, we did not see great changes in Bax or Bcl-xL protein levels. The two exceptions to this were the significant decrease in Bax after treatment with subfraction 6 and the significant decrease in Bcl-xL in subfraction 7. Interestingly, the whole extract and subfraction 2 may increase Bax protein levels, which could be a sign of increased apoptosis from those treatments. In general, subfractions 2-8 did show increased levels of Bax while simultaneously exhibiting lower levels of Bcl-xL, which further supports our hypothesis that these subfraction treatments are increasing apoptosis in HeLa cells.

Chapter Four

4. Discussion and Conclusions

While there are numerous applications of the medicinal plant, *Moringa oleifera*, the data presented in our study highlights the therapeutic potential of HPLC-separated leaf extracts in controlling inflammation and treating some types of cancer. HPLC separation of an ethanolic *M. oleifera* leaf extract generated 8 chemically unique subfractions, several of which had potent anti-inflammatory and anticancer properties associated with them. While most of these subfractions were able to alter inflammatory cytokine levels, subfractions 4, 5, 6, 7, and 8 were able to significantly reduce the secretion of IL-6 and TNFα from THP-1 cells in a lipopolysaccharide (LPS)-induced model of inflammation. Additionally, a handful of these HPLC-isolated subfractions were able to significantly reduce the cell viability of HeLa and SiHa cervical cancer cells. Furthermore, cervical cancer cell viability markedly decreased when treated with combinations of subfractions 2, 3, 5, 7, and 8, and a general temporal effect was observed when subfractions were applied for extended durations. These anti-proliferative effects of the subfractions on these cancer cell lines may tentatively be explained by alterations of the NF-κB pathway post treatment. While not statistically significant, treatment with some of the subfractions indicated a potential decrease in the expression of phosphorylated and total NF-κB protein. Western blot analysis also indicated that some subfraction treated cells produced an imbalanced ratio of pro-apoptotic Bax proteins compared to the antagonistic Bcl-xL protein.

Based on our results, we believe that these HPLC-isolated subfractions have therapeutic potential to control dysregulated inflammatory processes that can contribute to tumor progression, as well as directly decrease the cell viability of some cancerous cell types.

Finally, we believe these subfractions, separated from the whole ethanolic *M. oleifera* leaf extract, can be further investigated through analytical techniques to identify the chemical constituents responsible for the extract's bioactivity.

Several research groups have reported that *M. oleifera* extracts are able to reduce inflammation and induce deleterious effects on cancer cells *in vitro*. Of course, these extracts can be prepared many ways, but even relatively simple extraction techniques and solvents can yield potent *M. oleifera* products. For example, one research group was able to characterize several potent anti-inflammatory compounds from an aqueous *M. oleifera* extract, and they even reported that many of these compounds had naturally been converted into stable bioactive forms (Waterman et al., 2014). They found that naturally myrosinase in their extract was converting moringa glucosinolates into isothiocyanates, which are "shelf" stable and potentially useful for nutraceutical development. Using an *in vitro* murine neoplastic mononuclear cell model of LPS-induced inflammation, these extracted isothiocyanates were shown to substantially alter inflammatory cytokine gene expression (including iNOS, IL-1β, IL-6, and TNFα) (Waterman et al., 2014).

Other research groups have looked at comparable *M. oleifera* products and found various anticancer compounds are prevalent in extracts made from the leaves and bark of the plant. Even more promising, many of these extracts appear to be effective at killing or inhibiting cell growth across different cancerous cell lines (Al-Asmari et al., 2015). Extract made from both the leaves and bark were able to drastically reduce MDA-MB-231 breast cancer cell viability, as well as significantly reduce HCT-8 colon cancer cell viability, colony formation, and cell motility (Al-Asmari et al., 2015). Isothiocyanates were also found in these *M. oleifera* leaf and bark extracts, as reported by the previously mentioned research

group, and were believed to be exhibiting anticancer properties along with several other known anticancer compounds like eugenol and D-allose (Al-Asmari et al., 2015).

In addition to these recent reports, previous studies in our labs have shown that the ethanolic *M. oleifera* leaf extract can modulate the secretion of inflammatory cytokines, decrease cervical cancer cell viability, and alter proliferative pathways inside some cervical cancer cell lines. Therefore, to further investigate these therapeutic properties, we decided to separate the complex whole extract using HPLC and isolate the bioactive compounds in distinct subfractions. As seen in Figure 1, we divided the effluent mixture into 8 subfractions using natural divisions in the chromatograph. There were two discernable regions of detectable analytes in our chromatograph, an early region from 7 to 17 minutes and a later region between 34 and 48 minutes. While these regions were initially useful for dividing the whole extract into manageable subfractions, it is worth noting that the gap in analyte peaks between 18 and 33 minutes is most likely due to the limits of the detector and not lack of discernable analytes. As several studies have identified a wide range of phytochemicals in *M. oleifera* leaf extracts, it is reasonable to postulate that using a shorter wavelength channel (< 330 nm) for the detector could have produced more analyte peaks in this region of the chromatograph. In fact, one our most prominent subfractions, Subfraction 5, was isolated from this region of the HPLC run, indicating there are bioactive compounds not being detected from the 330 nm channel.

The time frame of subfraction isolation is indicative of the types of chemical constituents that can be expected to be found inside each separated grouping due to our unique 2-solvent elution. In the beginning of the run, the primary solvent is acidified water which most effectively carries polar inorganic compounds through the column. However,

during the course of the HPLC run, the solvent system slowly incorporates acidified acetonitrile into the elution mixture until it is only organic solvent after 40 minutes of run time. This 2 step elution system not only helps to efficiently separate the whole ethanolic extract, but it also provides reasonable insights to the types of bioactive compounds that might be identified in the subfractions. For example, both quercetin and kaempferol are known phytoconstituents in *M. oleifera* extracts and both are polar/protic organic compounds. Therefore, it is likely that these bioactive compounds would be isolated from a subfraction with a mix of water and acetonitrile, where polar water molecules could help elute the polar organic analytes that aren't completely miscible with the aprotic acetonitrile.

Dysregulated inflammatory responses from the immune system can lead to self-perpetuated cell signaling, increased immune cell recruitment, and subsequent activation of other localized immune cells. While inflammatory positive feedback loops may be advantageous for mounting an effective immune response in the early stages of an infection, this prolonged cell signaling, and immune response can have damaging effects (both locally and systemically) when it becomes chronic. Chronic inflammation is a contributing factor to a plethora of diseases and more recently has been identified as a hallmark of cancer pathogenesis. The increased immune cell signaling in chronically inflamed tissues can lead to angiogenesis which provides additional nutrients to a growing tumor. Further activation of these immune cells leads to the production of reactive oxygen species, which can cause additional damage to DNA and lead to multiple cancer cell mutations. Interestingly, data from our experiments suggest that several of our subfractions could have therapeutic effects in inflammatory diseases due to their ability to decrease immune cell secretions of pro-inflammatory cytokines. As seen in Figures 2A and 2B, THP-1 monocytes pretreated with *M.*

oleifera subfractions 4, 5, 6, 7, and 8 before LPS challenge significantly reduced levels of the inflammatory cytokines, IL-6 and TNFα. When only looking at IL-6 data (Figure 2A), it is interesting to note that our subfractions were able to significantly reduce the secretion of this inflammatory cytokine at markedly lower concentrations than the ethanolic whole extract from which they were derived. The subfractions were applied at a concentration of 200 µg/mL, versus the whole extract (W) which was applied at a concentration of 200,000 µg/mL, or three orders of magnitude higher. While these subfractions were clearly effective at reducing IL-6 secretion at concentrations of 200 µg/mL, none of the extracts were able to decrease cytokine secretion to levels comparable with our mock challenged (negative control) except for the whole extract at 200,000 µg/mL.

The results from the TNF data (Figure 2B) were even more auspicious, as they showed that pretreatment with subfractions 3, 5, 7, and 8 could significantly reduce levels of TNFα at low concentrations of 100 µg/mL. Equally promising, all tested subfractions (2-8) significantly reduced levels of secreted TNFα when treated at concentrations of 200 µg/mL. However, similar to the IL-6 data, pretreatment with the ethanolic whole extract was the only treatment type that showed results comparable to mock. Although, it is worth mentioning that treatment with subfractions 7 and 8 at 100 µg/mL were producing comparable effects to W at 100,000 µg/mL.

Overall, our data indicate that pretreatment with HPLC-isolated subfractions is able to considerably inhibit the secretion of IL-6 and TNFα inflammatory cytokines from THP-1 monocytes in an LPS-challenge model. Both of these inflammatory cytokines are used as biomarkers for inflammation, and their relative abundance has been directly linked to the severity of local inflammation (Bernecker et al., 2013; Del Giudice and Gangestad, 2018).

These immune signaling proteins accumulate in areas of tissue damage, where they stimulate a protective immune response and recruit immune cells like neutrophils and monocytes (which later differentiate into macrophages) (Del Giudice and Gangestad, 2018). Initially, the nonspecific inflammatory response triggered by these cytokines is helpful for clearing infections and containing local damage; however, as these immune responses become chronic and dysregulated, the presence of these cytokines can lead to excess tissue damage, swelling, pain, increased metabolic rates, and various other harmful side effects that can become systemic (Del Giudice and Gangestad, 2018). Thus, the capability of our subfractions to inhibit the secretion of these inflammatory cytokines could be useful in creating novel nutraceuticals for acute and chronic inflammatory conditions.

That being said, our data cannot attest to the effects of the subfractions if they are applied to THP-1 cells after exposure to LPS. Although, there is at least one study that used an ethanolic extract (analogous to our whole *M. oleifera* extract) to treat CFA-induced arthritis in rats, in which they saw significant reductions in swelling and other signs of inflammation post-challenge (Mahdi et al., 2018). Additionally, while our THP-1 monocytes behave comparably to normal human monocytes, they are cancerous in nature being a monocytic leukemia cell line. While there are numerous studies using THP-1 monocytes as analogues for human immune cells in LPS-induced models of inflammation, it cannot be ruled out that the immunomodulatory effects seen in our data may be attributable to the *M. oleifera* extracts altering, or potentially killing off, these cells. While cell viability assays were not performed in tandem with our experiments, it seems unlikely that cell death was the principle factor for the decreasing cytokine levels. During the ELISA supernatant collection process, pellets of cells were often observed after centrifugation, and many other cells were

seen to be adherent on the plate after challenge with LPS. The increased propagation of cells in our dishes and supernatant, along with changes in media conditions, is highly indicative of metabolically active cells being present over the course of the experiments.

The immunomodulatory properties of our extracts offer promising therapeutic potential for inflammatory diseases, but it is also conceivable that these properties could be impactful in a tumor microenvironment, where immune cell signaling is often chronic and supplemental to tumor progression. There is also a growing body of evidence that indicates *M. oleifera* products may have anticancer properties associated with them, and several studies have successfully isolated and identified known anticancer compounds from their extracts. As discussed earlier, a couple of research groups have found a class of anticancer compounds known to induced apoptosis in *M. oleifera* extracts called isothiocyanates (Al-Asmari et al., 2015; Giacoppo et al., 2017). Other research groups have identified and pursued follow-up studies with two potent antiproliferative compounds, quercetin and kaempferol, both of which have shown to be efficacious across several cell lines (Li et al., 2016; Rajendran et al., 2014).

Prior studies in our lab have generated data that indicates ethanolic *M. oleifera* extract has *in vitro* anticancer effects in a cervical cancer cell model. Therefore, we predicted that some our HPLC-isolated subfractions, which were derived from the ethanolic whole leaf extract, would also exhibit anticancer effects when applied to cervical cancer cells. Indeed, our results did show that some subfraction treatments could reduce HeLa and SiHa cervical cancer cell viability in both a concentration and time dependent manner. Furthermore, combination treatments with our most potent subfractions were able to induce significant decreases in cervical cancer cell viability.

Initially, we wanted to see if any of these subfractions could significantly reduce cervical cancer cell viability in a comparable way to the ethanolic whole leaf extract from which they were derived (data presented in Figures 3 and 4). We chose to use two cervical cancer cell lines, HeLa and SiHa, to get a broader understanding of any potential anticancer effects, since cancerous cells are known to be highly variable (even in the same types of cancer). While both cell lines are classified as cancerous cells from the cervix, HeLa cells have been classified as a rare form of adenocarcinoma, while SiHa cells are classified as a squamous cell carcinoma (Rahbari et al., 2009; Meissner, 1999). Additionally, the strain of HPV that infected these cells and led to subsequent cancerous mutations was also different, with HeLa cells containing HPV-18 DNA and SiHa cells containing HPV-16 DNA (Ristriani et al., 2009). Both of these strains of HPV are indicated as "high risk" strains due to their ability to express two viral oncoproteins, E6 and E7 (Nominé et al., 2006). In particular, this E6 oncoprotein is known to ubiquinate tumor suppressor p53, ultimately resulting in its degradation and leading to tumorigenesis (Nominé et al., 2006; Ristriani et al., 2009).

Previous experiment from our labs, also using whole *M. oleifera* extracts, has revealed that these two cell lines respond quite differently to treatment with the same extract and treatment concentrations; thus, comparing the results between these two cell lines might help us gauge the potency of our subfractions across similar cell lines of the same type of cancer. Ultimately, we did observe subfractions eliciting different results in one cell line compared to the other, but both cell types did show some degree of susceptibility to treatment with our HPLC-isolated subfractions.

Looking at the data for the HeLa cells (Figures 3A and 3B), we can see that treatment with some subfractions significantly reduced cancer cell viability. Subfraction 6 was the only

subfraction to not induce any significant changes after 48 hours. It is worth noting that more subfractions exhibited a significant impact on HeLa cell viability after 48 hours (3B) compared to the 24 hour time point (3A). We believe this observation is indicative of a temporal relationship between cell viability and subfraction treatments. In addition to this temporal relationship, we also observed what appears to be a general decrease in cell viability with an increase in treatment concentration. However, one notable exception to this would be subfraction 3, where the 100 µg/mL concentration decreased cell viability more than the 200 µg/mL at both the 24 and 48 hour time points. While cell viability did not change significantly with whole extract (W) treatment at concentrations of 100 or 200 µg/mL, we observed the most significant changes to HeLa cell viability when W was applied at concentrations of 100,000 and 200,000 µg/mL. Excluding treatment with the highest concentrations of W, we can say that HeLa cell viability was most significantly reduced by subfractions 2, 3, 5, 7, and 8, with subfraction 5 being the most prominent subfraction to reduce cell viability.

The SiHa cell viability data (shown in Figures 4A and 4B) is noticeably different than the HeLa cell viability, as there were several subfractions that appeared to significantly increase the cancer cell viability. Subfractions 2, 3, 4, and 6 all significantly increased cell viability by 48 hours, and while some subfractions (3, 5, and 8) initially decreased cell viability at the 24 hour time point, these reductions were no longer significant at the 48 hour time point (Figure 4B). In fact, the general temporal trend we observed in the HeLa cell viability data no longer appears in the SiHa cell viability data. The effects from most of our subfraction treatments applied to SiHa cells seems to either stagnate after 24 hours or cause

an undesirable increase in SiHa cell viability from the 24 hour time point to the 48 hour time point.

Clearly, our subfraction data is comparable in some ways to our labs' previously published data, where some treatments that work for one cancer cell line may not work for another. Tentatively, a couple of explanations for why the SiHa cell viability might be increasing over time could involve the cells degrading harmful bioactive compounds in the extracts, using compounds in the extracts for nourishment, or even a combination of both. If all bioactive compounds have been degraded by 24 hours, we wouldn't expect to see noticeable differences in cell viability after 48 hours. Additionally, if the cancerous cells have neutralized or degraded the bioactive compounds by 24 hours, their ensuing proliferation would be uninhibited and potentially increased by other extract constituents, leading to increased cell viability at the 48 hour time point. Other researchers have also commented on the potential negative effects of phytates or other antinutrients commonly found in *M. oleifera* extracts; these phytoconstituents can obstruct the absorption or alter the activity of other bioactive compounds (Mahdi et al., 2018).

So far, most of our cancer cell viability analysis has focused solely on the effect of individual HPLC-isolated subfractions, but it is interesting to compare the effects of the subfractions to those of the whole extract. In HeLa cells, treatment with the lower concentrations of W (100 and 200 µg/mL) had minimal impacts on cell viability. However, SiHa cell viability actually increased significantly when the lower concentrations of W were applied. These observations further attest to the greater potency of the subfractions, where our subfractions were efficacious at low concentrations but the whole extract was not. Conversely, when higher concentrations of the whole extract (100,000 and 200,000 µg/mL)

were applied to the cancer cells, both cell types had strikingly significant reductions in cell viability. As it was observed, both cell types exhibited temporal and dose-dependent relationships between cancer cell viability and whole extract application. Overall, the reduction in cancer cell viability from the 200,000 µg/mL concentration of W was noticeable, but it might not offer a viable therapeutic options due to impractical application volumes this concentration would require.

As a whole, we did observe underlying anticancer effects from our cervical cancer MTT assays, although none of our subfractions produced exceptional cell viability reductions on their own. Therefore, we decided to test combinations of the most effective subfractions to determine if they might have synergistic effects with one another. Thus, we tested combinations of two and three distinctive subfractions to determine if complimentary bioactive compounds could more effectively decrease cell viability when applied to HeLa (Figure 5) and SiHa (Figure 6) cervical cancer cells. Overall, we noticed considerably lower reductions in cell viability when subfractions were applied in combination than on their own; additionally, the previously mentioned temporal and dose-dependent trends were further elucidated by these results.

Focusing on the data for subfraction combinations being applied to HeLa cells (Figure 5), our results further support a temporal and dose-dependent relationship between cancer cell viability and subfraction treatment. Additionally, we see that most combinations of subfractions seemed to further decrease HeLa cell viability, with the three subfraction combinations showing the most striking differences (indicated by the darker shades of blue). However, several subfraction combinations containing subfraction 5 do not appear statistically different than subfraction 5 alone. Ultimately, this data seems to indicate that

most of the subfractions have synergistic effect with one another, except for subfraction 5, which appears to be most effective when applied alone or in combination with 2 other subfractions.

Treatment with subfraction combinations in SiHa cells (Figure 6) resulted in pronounced reductions in cancer cell viability, which was not commonly observed with individual subfraction treatments. As stated previously, several of the individual subfractions applied to SiHa cells resulted in significant increases in cancer cell viability; however, none of the subfraction combination treatments increased cell viability. In fact, SiHa cell viability was significantly decreased with most combination treatments at the 24 hour time point, and all subfraction combinations caused a significant reduction in SiHa cell viability when applied for 48 hours. This data also suggests there may be a slight temporal effect when applying the subfraction combinations, but there does not seem to be an obvious difference between the 24 hour and 48 hour data. In every case, increasing the subfraction concentration resulted in a subsequent decrease in SiHa cell viability, which is indicative of a strong negative correlation between treatment concentration and cancer cell viability. By and large, this SiHa cell viability heat map indicates that many of these subfractions have synergistic effects when applied together, including several of the combinations containing subfraction 5 (contrary to the HeLa cell results).

After examining the general trends in the cervical cancer cell viability heat map, it is apparent that some combinations of subfractions have additive effects; yet, other combinations do not seem to noticeably change cell viability, and some may actually dampen the therapeutic effects of a single subfraction alone.

Therefore, we used a series of Student's t-tests to determine if there were any significant differences between subfraction combination treatments and their individual subfraction components in reducing cell viability. The results of all of these t-tests can be seen in Tables 1 – 4. Clearly there were numerous comparisons to be made, but the most promising comparisons were those that showing significant changes in cell viability from treatment with subfraction combinations compared to their individual components. Subsequently, all of the combination treatments that significantly changed cell viability compared to the individual subfraction treatments alone were compiled into a condensed table, with HeLa cell data in Table 5A and SiHa cell data in Table 5B.

Looking at the HeLa cell viability data from Table 5A, we can see that most of the subfraction combination treatments did have some additional effects compared to their constitutive subfractions alone. Although, as mentioned previously, treatment with subfraction 5 alone seemed to be highly effective a reducing HeLa cell viability. Thus, the only significant reduction we see from a combination treatment, compared to treatment with subfraction 5 alone, was at the 48 hour time point with subfraction 2, 5, and 7 applied at 200 µg/mL. Across the board, subfraction combination treatments only significantly reduced HeLa cell viability after 48 hours, which further falls in line with the previously noted temporal effect seen when treating HeLa cells. While observing synergistic effects between the combination treatments offers some additional therapeutic potential to these subfractions, the data seem to indicate subfraction 5 alone as an exemplary subfraction to begin chemical analysis and identify potential anticancer compounds from.

The SiHa cell viability data (from Table 5B) looks noticeably different than the HeLa cell viability data. Arguably, there are two prominent differences, the first being the lack of a

temporal effect. As Table 5B shows, a multitude of combinations treatments resulted in significant decreases compared to their individual constitutive subfractions at both the 24 hour and 48 hour time points. Yet, this may not be too surprising considering that our heat map data (Figure 6) suggests that treatment concentration is far more important than treatment duration at reducing SiHa cell viability. The second distinct difference is that more subfractions appear to work synergistically to reduce cell viability, including combination treatments including subfraction 5, which was not the case when these combination treatments were applied to HeLa cells. Looking back at the heat map data, SiHa cells did not appear to be susceptible to individual subfraction treatments alone, with subfractions 3 & 5 as a partial exception; however, several subfraction combination treatments were able to significantly reduce SiHa cell viability where their constitutive subfractions could not (as highlighted in Table 5B).

Considering all of the cell viability data together, several conclusions can be made for the therapeutic potential of our *M. oleifera* HPLC-isolated subfractions when applied to cervical cancer cells *in vitro*. First off, our data suggest that treatment efficacy depends on the cell type to which a subfraction is applied to. When looking at individual subfraction treatments (Figures 3 and 4), we can see that SiHa cells seem to be far more resistant to treatment than HeLa cells, which was previously noted in (Brown et al., 2015). While both cell lines originated from patients with cervical cancer, they have each acquired unique mutations during their cancerous development, which could drastically alter the way they respond to different treatments. Similarly, we know from the HPLC chromatograph (Figure 1) that our subfractions have been thoroughly separated, and that each subfraction has its own unique chemical profile of various bioactive compounds. Therefore, due to the unique

nature of each cell line and subfraction, it is not surprising that the therapeutic potential of an individual subfraction might change even when applied to different cervical cancer cells (let alone cells from another type of cancer).

A second general conclusion we can make is that subfraction treatment efficacy is time-dependent. In both HeLa and SiHa cell lines, we observed that subfraction treatment further decreased cell viability over time, and these temporal effects particularly notable in the SiHa cell viability data (Figure 6). We also observed a prominent dose-dependent reduction in cell viability with the HeLa cell line, but this connection was not as evident in our results for the SiHa cell line. One possible explanation for this might be that a plateau effect was occurring, particularly with the combination treatments. As commented on previously, there appeared to be additional therapeutic synergy when combinations of subfractions were applied to the SiHa cells. So, it seems plausible that some of the combination constituents may be promoting SiHa cell death through similar mechanisms. Subsequently, we may be seeing a treatment plateau, where multiple subfractions are working in comparable ways to reduce SiHa cell viability; therefore, the resulting deleterious effects are more dependent on time rather than ample exposure to bioactive compounds. In other words, there are sufficient amounts of bioactive compounds to instigate SiHa cell death, but treatment time may be the limiting factor to see further reductions in cell viability.

While the cell viability assays show promising potential for our HPLC-isolated *M. oleifera* subfractions to treat some types of cervical cancer, it is important to consider the scope of our experimental results. Stated once more, our experiments have only looked at the effects of subfraction treatment when applied to cervical cancer cells, and even within different types of cervical cancer cell lines we observed varying treatment efficacy. Ergo,

these auspicious results are not directly applicable for treating other types of cancers without further investigation. Furthermore, it would be insightful to confirm that treatment with our subfractions does not cause pernicious effects in healthy cell types. There is a large body of research supporting the safety and efficacy of *M. oleifera* extracts, but supplemental cell viability assays on healthy cervical cells would be an important follow-up step before considering *in vitro* experiments. As a final counterpoint to our conclusions thus far, the presented cell viability data does not provide any insightful information as to how our subfraction treatments are affecting cancer cell viability. To help elucidate these mechanisms of action, we decided to investigate how known proliferative pathways may be altered with subfraction treatment using a series of Western blotting techniques.

One such pathway is the NF-κB pathway, which is a key pathway in regulating both cell survivability as well inflammatory responses. Activation of the NF-κB protein complex leads to the upregulation of several inflammatory and proliferative genes, and several cancerous cell lines are known to have dysregulated NF-κB pathways which enhances their survival and proliferative capabilities. Additionally, the upregulation of inflammatory genes from this pathway can promote tumor progression by creating chronically inflamed tumor microenvironments, which can further foster the proliferation, survivability, migration, and inevitable metastasis of the cancerous cells (Coussens and Werb, 2002). Taking into account the broad implications this pathway has on cancer development, we used Western blotting techniques to determine if our subfractions might be altering the levels of phosphorylated NF-κB (the transcriptionally active form) or total NF-κB in our HeLa cervical cancer cells.

Reviewing the Western blot data for the NF-κB proteins did not show any significant changes in expression levels. However, upon further examination there are some potential

differences between the treatments. Protein expression could potentially be increasing with whole extract and subfraction 2 treatments, while treating HeLa cells with subfractions 4, 5, 6, and 7 could be causing subtle differences in expression levels. Subfractions 4, 5, and 6 appear to have steady levels of the active phosphorylated NF-κB, but these treatments may be reducing the total amount of NF-κB protein inside the cancer cells. Although this reduction in total NF-κB protein is not large, it could be the precursor of more significant and impactful changes given higher treatment concentrations or increased treatment time. Treatment with subfraction 7 may be having similar effects, where total NF-κB levels may be declining, and even phosphorylated NF-κB may be decreasing as well. Yet, this data is hard to discern due to the large range in uncertainty, so additional Western blot analysis would be required to make more definitive claims.

Despite our NF-κB protein expression data being somewhat inconclusive, it is worth considering that the aforementioned subtle changes are in line with our cell viability data. Our cell viability data for treating HeLa cells with individual subfractions over 24 hours (Figure 3A) did not show much change in the cancer cell viability. The only prominent decreases in HeLa cell viability at this treatment concentration and time point occurred with subfraction 5 treatment, which might also be the treatment that caused the most reduction in total NF-κB expression levels. Due to the ambiguous Western blot data for NF-κB expression, future experiments could be executed at a 48 hour time point to give ample time for expression levels to change in response to subfraction treatment (presumably at both the 100 and 200 μg/mL concentrations). Since our investigation into subfraction treatments directly altering NF-κB protein expression was inconclusive, we decided to look at other

proteins commonly linked to cell death mechanisms that our HPLC-isolated *M. oleifera* subfractions may be altering.

A critical hallmark in cancerous cell progression is the capacity for cells to evade apoptosis (Hanahan and Weinberg, 2011). Apoptosis, or programmed cell death, is a highly regulated process in healthy cells that insures old or damaged cells are removed without damaging healthy cells and provoking inflammatory responses (Pucci et al., 2000). Taking this into consideration, it is not only desirable for cancer treatments to kill abnormal tumor cells, but also induce to apoptosis in the cancerous cells which will mitigate tissue damage and additional local inflammation. Two protein groups known to tightly regulate apoptosis are Bax, a pro-apoptotic protein, and the Bcl-2 family proteins (including Bcl-xL), that act antagonistically to Bax and prevent apoptosis. Interestingly, some researchers have reported that the consumption of *M. oleifera* products is able to induce colon cancer cell death by upregulating the ratio of Bax to Bcl family proteins (Kraiphet et al., 2018). Subsequently, we decided to see if our subfraction treatments might be altering the ratio of Bax to Bcl-xL proteins in our HeLa cervical cancer cell model, and linking some of the aforementioned decrease in HeLa cell viability to apoptotic pathways.

Similar to our results on NF-κB protein expression, Western blot analysis for Bax and Bcl-xL proteins isolated from HeLa cells treated with 100 μg/mL of subfractions over 24 hours did not yield many statistically significant changes in protein expression. Although, due to the interconnected and antagonistic nature of these two proteins, it may be more important to consider how the expression ratio of these two proteins changed rather than how each individual protein's expression changed. It has been shown that although Bax and Bcl-xL act antagonistically to each other, Bax is dependent on Bcl-xL to efficiently translocate to

the mitochondrial outer membrane, where it can initiate endogenous apoptosis (Renault et al., 2017). Cancer cells are often known to overexpress Bcl-xL proteins, so decreasing expression of Bcl-xL in these cells is certainly key in reinstating standard endogenous apoptosis (Renault et al., 2017). Yet, this recent study also indicates that having some Bcl-xL proteins present allows for more effective recruitment of Bax molecules to come together and form pores in the mitochondrial outer membrane (Renault et al., 2017). Therefore, an expression ratio of these proteins shifted towards Bax expression, but not completely lacking in Bcl-xL expression, may be the most effective at restoring endogenous apoptosis to cancer cells (assuming there are no additional mutations in Bax rendering it ineffective).

With that in mind, treatment using subfraction 2 may have drastically altered this ratio, in which we see increased levels of pro-apoptotic Bax and decreased levels of anti-apoptotic Bcl-xL. This imbalanced ratio between Bax and Bcl-xL protein expression is observable to some extent from treatment with subfractions 3, 4, 5, 7, and 8, albeit not as drastic as subfraction 2. Further support that our subfraction treatments might be altering the ratio of Bax/Bcl-xL proteins comes from looking back at the cell viability data in Figure 3A. There does appear to be some overlapping effects in our cell viability data and apoptotic protein data. Subfraction 2 did significantly reduce HeLa cell viability, and our Western blot analysis showed it created a large imbalance in the Bax/Bcl-xL ratio. Additionally, treatment with subfractions 3, 5, and 8 also significantly reduced HeLa cell viability, and potentially shifted Bax/Bcl-xL protein expression towards pro-apoptotic Bax proteins. Nonetheless, the small experimental sample size ($n = 3$) and relatively large variation between experimental trials makes it hard to definitively conclude that these subfractions are favorably altering the Bax/Bcl-xL balance towards the pro-apoptotic Bax protein.

While there were not many statistically significant changes in Bax or Bcl-xL protein expression from our Western blot analysis, two subfraction treatments did significantly alter the expression of these antagonistic proteins. Treatment with subfraction 6 actually significantly reduced the expression pro-apoptotic Bax, but also appeared to reduce the expression of anti-apoptotic Bcl-xL. Looking back at the cell viability data from Figure 3A again, we can see that this subfraction treatment did not cause any significant changes in cell viability. Thus it stands to reason that while this subfraction is lowering Bax and Bcl-xL protein expression, it's likely not altering the balance between the two proteins. Subfraction 7 treatment also significantly decreased anti-apoptotic Bcl-xL expression. Unfortunately, there was a lot of variation in our Western blot analysis of subfraction 7's effect on Bax protein expression, so there may not be a large imbalance between Bax expression and Bcl-xL expression.

As mentioned within the discussion of our NF-κB Western blot analysis, we might observe more significant changes in Bax/Bcl-xL expression if we increase treatment concentrations or allow additional time for cumulative effects on intracellular apoptotic protein expression. Another limitation to our study was that we only looked at one cervical cancer cell line thus far (HeLa cells), but future experiments could examine changes in other cervical cancer cell lines like SiHa cells. While our Western blot data is not entirely conclusive, we did see that our subfractions have the potential to alter apoptotic protein expression in HeLa cells. Treatment with subfraction 7 caused a significant reduction in expression of the anti-apoptotic protein, Bcl-xL, which warrants further investigation. Conversely, treatment with subfraction 2 appeared to drastically increase the expression of pro-apoptotic Bax, although increase was not statistically significant. While no Western blot

analysis was done on subfraction combination treatments, it is interesting to think about the therapeutic potential of combining the observed effects of subfractions 2 and 7, where there might be a significant increase in Bax and a significant decrease in Bcl-xL. This potential is underpinned by the results of Figure 5, which shows this subfraction combination treatment did significantly reduce HeLa cell viability. Overall, our Western blot analysis of apoptotic proteins seems to indicate that some of our HPLC-isolated *M. oleifera* subfractions may be inducing apoptosis in HeLa cancer cells, but further investigation is required.

In summary, the results from this project suggest that our HPLC-isolated subfractions partitioned from an ethanolic *M. oleifera* leaf extract have therapeutic properties that could be developed into treatments for inflammatory disorders and some types of cancers. We saw that pretreatment with our HPLC-isolated subfractions was able to significantly decrease the secretion of IL-6 and TNFα inflammatory cytokines from THP-1 monocytes in an LPS-challenge model. Several of our HPLC-isolated subfractions were able to reduce HeLa and SiHa cervical cancer cell viability in a dose and time-dependent manner, and they were efficacious at applied concentrations much lower than the original whole extract from which they were derived. Additionally, we observed synergistic effects when combining our subfractions treatments in groups of two or three, which resulted in substantial reductions to HeLa and SiHa cell viability. While our Western blot data was somewhat inconclusive, there were underlying favorable trends in the protein expression ratios of Bax/Bcl-xL, which could be indicating that our subfractions have the potential to induce apoptosis in HeLa cancer cells. As a final note, our HPLC chromatograph did reveal that our subfractions contained distinct chemical profiles, and we hope to use this knowledge (coupled with analytical

chemistry techniques) to identify the bioactive compounds in these subfractions that hold therapeutic potential.

References

Abdull Razis, A.F., Ibrahim, M.D., and Kntayya, S.B. (2014). Health benefits of Moringa oleifera. Asian Pac. J. Cancer Prev. *15*, 8571–8576.

Al-Asmari, A.K., Albalawi, S.M., Athar, M.T., Khan, A.Q., Al-Shahrani, H., and Islam, M. (2015). Moringa oleifera as an Anti-Cancer Agent against Breast and Colorectal Cancer Cell Lines. PLOS ONE *10*, e0135814.

Aoki, T., and Narumiya, S. (2012). Prostaglandins and chronic inflammation. Trends in Pharmacological Sciences *33*, 304–311.

Aoki, T., Nishimura, M., Matsuoka, T., Yamamoto, K., Furuyashiki, T., Kataoka, H., Kitaoka, S., Ishibashi, R., Ishibazawa, A., Miyamoto, S., et al. (2011). PGE $_2$ -EP $_2$ signalling in endothelium is activated by haemodynamic stress and induces cerebral aneurysm through an amplifying loop via NF-κB: PGE $_2$ -EP $_2$ induce cerebral aneurysm via NF-κB. British Journal of Pharmacology *163*, 1237–1249.

Araújo, L.C.C., Aguiar, J.S., Napoleão, T.H., Mota, F.V.B., Barros, A.L.S., Moura, M.C., Coriolano, M.C., Coelho, L.C.B.B., Silva, T.G., and Paiva, P.M.G. (2013). Evaluation of Cytotoxic and Anti-Inflammatory Activities of Extracts and Lectins from Moringa oleifera Seeds. PLoS ONE *8*, e81973.

Arora, D.S., and Onsare, J.G. (2014). In vitro antimicrobial evaluation and phytoconstituents of Moringa oleifera pod husks. Industrial Crops and Products *52*, 125–135.

Arulselvan, P., Tan, W., Gothai, S., Muniandy, K., Fakurazi, S., Esa, N., Alarfaj, A., and Kumar, S. (2016). Anti-Inflammatory Potential of Ethyl Acetate Fraction of Moringa oleifera in Downregulating the NF-κB Signaling Pathway in Lipopolysaccharide-Stimulated Macrophages. Molecules *21*, 1452.

Bernecker, C., Scherr, J., Schinner, S., Braun, S., Scherbaum, W.A., and Halle, M. (2013). Evidence for an exercise induced increase of TNF-α and IL-6 in marathon runners: TNF-α, IL-6 and leptin expression post-marathon. Scandinavian Journal of Medicine & Science in Sports *23*, 207–214.

Brilhante, R.S.N., Sales, J.A., Pereira, V.S., Castelo-Branco, D. de S.C.M., Cordeiro, R. de A., de Souza Sampaio, C.M., de Araújo Neto Paiva, M., Santos, J.B.F. dos, Sidrim, J.J.C., and Rocha, M.F.G. (2017). Research advances on the multiple uses of Moringa oleifera : A sustainable alternative for socially neglected population. Asian Pacific Journal of Tropical Medicine *10*, 621–630.

Brown, A., Mowa, N., and Ahmed, M. (2015). The Effect Of Moringa Oleifera On The Oncolytic Activity Of Vesicular Stomatitis Virus In Cervical Cancer Cells.

Coussens, L.M., and Werb, Z. (2002). Inflammation and cancer. Nature *420*, 860–867.

Del Giudice, M., and Gangestad, S.W. (2018). Rethinking IL-6 and CRP: Why they are more than inflammatory biomarkers, and why it matters. Brain, Behavior, and Immunity *70*, 61–75.

Dwivedi, A.K., Gurjar, V., Kumar, S., and Singh, N. (2015). Molecular basis for nonspecificity of nonsteroidal anti-inflammatory drugs (NSAIDs). Drug Discovery Today *20*, 863–873.

Fard, M., Arulselvan, P., Karthivashan, G., Adam, S., and Fakurazi, S. (2015). Bioactive extract from moringa oleifera inhibits the pro-inflammatory mediators in lipopolysaccharide stimulated macrophages. Pharmacognosy Magazine *11*, 556.

Gautam, R., and Jachak, S.M. (2009). Recent developments in anti-inflammatory natural products. Medicinal Research Reviews *29*, 767–820.

Giacoppo, S., Rajan, T.S., De Nicola, G.R., Iori, R., Rollin, P., Bramanti, P., and Mazzon, E. (2017). The Isothiocyanate Isolated from *Moringa oleifera* Shows Potent Anti-Inflammatory Activity in the Treatment of Murine Subacute Parkinson’s Disease. Rejuvenation Research *20*, 50–63.

Gudis, K., and Sakamoto, C. (2005). The Role of Cyclooxygenase in Gastric Mucosal Protection. Digestive Diseases and Sciences *50*, S16–S23.

Gupta, S., Jain, R., Kachhwaha, S., and Kothari, S.L. (2017). Nutritional and medicinal applications of Moringa oleifera Lam.—Review of current status and future possibilities. Journal of Herbal Medicine.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of Cancer: The Next Generation. Cell *144*, 646–674.

Jaroszewska, A., Jaroszewska, A., Biel, W., and Łysoń, E. (2012). Nutritional quality and safety of moringa (Moringa oleifera Lam., 1785) leaves as an alternative source of protein and minerals. Journal of Elementology.

Kasolo, J., Bimenya, G., Ojok, L., Ochieng, J., and Ogwal-Okeng, J. (2010). Phytochemicals and uses of Moringa oleifera leaves in Ugandan rural communities. Journal of Medicinal Plants Research *4*, 753–757.

Kraiphet, S., Butryee, C., Rungsipipat, A., Budda, S., Rattanapinyopitak, K., and Tuntipopipat, S. (2018). Apoptosis induced by Moringa oleifera Lam. pod in mouse colon carcinoma model. Comparative Clinical Pathology *27*, 21–30.

Kumssa, D.B., Joy, E.J., Young, S.D., Odee, D.W., Ander, E.L., and Broadley, M.R. (2017). Variation in the mineral element concentration of Moringa oleifera Lam. and M. stenopetala (Bak. f.) Cuf.: Role in human nutrition. PLOS ONE *12*, e0175503.

Li, Y., Yao, J., Han, C., Yang, J., Chaudhry, M., Wang, S., Liu, H., and Yin, Y. (2016). Quercetin, Inflammation and Immunity. Nutrients *8*, 167.

Mack, M. (2017). Inflammation and fibrosis. Matrix Biology.

Mahdi, H.J., Khan, N.A.K., Asmawi, M.Z.B., Mahmud, R., and A/L Murugaiyah, V. (2018). In vivo anti-arthritic and anti-nociceptive effects of ethanol extract of Moringa oleifera leaves on complete Freund's adjuvant (CFA)-induced arthritis in rats. Integrative Medicine Research *7*, 85–94.

Mangundayao, K., and Yasurin, P. (2017). Bioactivity of Moringa oleifera and its Applications: A Review. Journal of Pure and Applied Microbiology *11*, 43–50.

Meissner, J.D. (1999). Nucleotide sequences and further characterization of human papillomavirus DNA present in the CaSki, SiHa and HeLa cervical carcinoma cell lines. Journal of General Virology *80*, 1725–1733.

Melo, V., Vargas, N., Quirino, T., and Calvo, C. (2013). Moringa oleifera L. An underutilized tree with macronutrients for human health. Emirates Journal of Food and Agriculture *25*, 785.

Nominé, Y., Masson, M., Charbonnier, S., Zanier, K., Ristriani, T., Deryckère, F., Sibler, A.-P., Desplancq, D., Atkinson, R.A., Weiss, E., et al. (2006). Structural and Functional Analysis of E6 Oncoprotein: Insights in the Molecular Pathways of Human Papillomavirus-Mediated Pathogenesis. Molecular Cell *21*, 665–678.

Press, R., Hiew, F.L., and Rajabally, Y.A. (2016). Steroids for chronic inflammatory demyelinating polyradiculoneuropathy: evidence base and clinical practice. Acta Neurologica Scandinavica *133*, 228–238.

Pucci, B., Kasten, M., and Giordano, A. (2000). Cell cycle and apoptosis. Neoplasia *2*, 291–299.

Qin, J., Wang, W., and Zhang, R. (2017). Novel natural product therapeutics targeting both inflammation and cancer. Chinese Journal of Natural Medicines *15*, 401–416.

Rahbari, R., Sheahan, T., Modes, V., Collier, P., Macfarlane, C., and Badge, R.M. (2009). A novel L1 retrotransposon marker for HeLa cell line identification. BioTechniques *46*, 277–284.

Rahman, M., Sheikh, M., Sharmin, S., Islam, S., Rahman, A., Rahman, M., and Alam, M. (2009). Antibacterial Activity of Leaf Juice and Extracts of Moringa oleifera Lam. against Some Human Pathogenic Bacteria. CMU. J. Nat. Sci. *8*, 219–227.

Rajendran, P., Rengarajan, T., Nandakumar, N., Palaniswami, R., Nishigaki, Y., and Nishigaki, I. (2014). Kaempferol, a potential cytostatic and cure for inflammatory disorders. European Journal of Medicinal Chemistry *86*, 103–112.

Renault, T.T., Dejean, L.M., and Manon, S. (2017). A brewing understanding of the regulation of Bax function by Bcl-xL and Bcl-2. Mechanisms of Ageing and Development *161*, 201–210.

Ristriani, T., Fournane, S., Orfanoudakis, G., Travé, G., and Masson, M. (2009). A single-codon mutation converts HPV16 E6 oncoprotein into a potential tumor suppressor, which induces p53-dependent senescence of HPV-positive HeLa cervical cancer cells. Oncogene *28*, 762–772.

Rodríguez-Pérez, C., Quirantes-Piné, R., Fernández-Gutiérrez, A., and Segura-Carretero, A. (2015). Optimization of extraction method to obtain a phenolic compounds-rich extract from Moringa oleifera Lam leaves. Industrial Crops and Products *66*, 246–254.

Sreelatha, S., Jeyachitra, A., and Padma, P.R. (2011). Antiproliferation and induction of apoptosis by Moringa oleifera leaf extract on human cancer cells. Food and Chemical Toxicology *49*, 1270–1275.

Sultana, B., Anwar, F., and Ashraf, M. (2009). Effect of Extraction Solvent/Technique on the Antioxidant Activity of Selected Medicinal Plant Extracts. Molecules *14*, 2167–2180.

Tilborghs, S., Corthouts, J., Verhoeven, Y., Arias, D., Rolfo, C., Trinh, X.B., and van Dam, P.A. (2017). The role of Nuclear Factor-kappa B signaling in human cervical cancer. Critical Reviews in Oncology/Hematology *120*, 141–150.

Ugwoke, C., Eze, K., Tchimene, K., and Anze, S. (2017). PHARMACOGNOSTIC EVALUATION AND ANTIMICROBIAL STUDIES ON MORINGA OLEIFERA LAM. (MORINGACEAE). IJPSR *8*, 88–94.

Vasanth, K., Minakshi, G.C., Ilango, K., Kumar, R.M., Agrawal, A., and Dubey, G.P. (2015). Moringa oleifera attenuates the release of pro-inflammatory cytokines in lipopolysaccharide stimulated human monocytic cell line. Industrial Crops and Products *77*, 44–50.

Vongsak, B., Sithisarn, P., Mangmool, S., Thongpraditchote, S., Wongkrajang, Y., and Gritsanapan, W. (2013). Maximizing total phenolics, total flavonoids contents and antioxidant activity of Moringa oleifera leaf extract by the appropriate extraction method. Industrial Crops and Products *44*, 566–571.

Waterman, C., Cheng, D.M., Rojas-Silva, P., Poulev, A., Dreifus, J., Lila, M.A., and Raskin, I. (2014). Stable, water extractable isothiocyanates from Moringa oleifera leaves attenuate inflammation in vitro. Phytochemistry *103*, 114–122.

Wirtz, A. (2014). Ultra-High Performance Liquid Chromatography and Its Applications Quanyun AlanXu, Ed. John Wiley & Sons, Inc. 2013. ISBN 978-0-470-93842-3. Hardcover, 304 pages. €95.90. Journal of Separation Science *37*, 187–187.

Xu, W., and Larbi, A. (2017). Immunity and Inflammation: From Jekyll to Hyde. Experimental Gerontology.

Yang, Z., Wu, Y., and Wu, S. (2016). A combination strategy for extraction and isolation of multi-component natural products by systematic two-phase solvent extraction- 13 C nuclear magnetic resonance pattern recognition and following conical counter-current chromatography separation: Podophyllotoxins and flavonoids from Dysosma versipellis (Hance) as examples. Journal of Chromatography A *1431*, 184–196.

Vita

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