THE ANTI-INFLAMMATORY ACTIVITY AND BIOAVAILABILITY OF MORINGA ISOTHIOCYANATES

BY

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ABSTRACT OF THE DISSERTATION
THE ANTI-INFLAMMATORY ACTIVITY AND BIOAVAILABILITY OF MORINGA ISOTHIOCYANATES

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Moringa (*Moringa oleifera* Lam.) seed extract (MSE) and its main bioactive component, moringa isothiocyanate-1(MIC-1), mitigate inflammation, oxidative stress, diabetes, and cancer in both *in vitro* and *in vivo* models. Moringa is a member of the Moringaceae family of 13 known species. All, including *Moringa oleifera*, are traditionally used as food, medicine, or for industrial purposes. The pods, seeds leaves, bark, and roots are all used. Investigating the therapeutic benefits of MSE and MIC-1 for both oral and topical delivery will help to fill the gap of knowledge in better understanding their potential as botanicals. This thesis provides information necessary to address these benefits, through the following aims: (1) investigate the bioavailability and bioaccessibility of MSE and MIC-1 *in vitro* and *in vivo*; (2) evaluate the ability of MSE to attenuate inflammation orally in a collagen-induced arthritis model; (3) evaluate the ability of MSE to reduce inflammation topically in a carrageenan paw edema model; (4) examine the anti-inflammatory effects of MSE and MIC-1in a 12-O-tetradecanoylphorbol-13-acetate (TPA) induced ear edema model.

Chapter 1 provides an introduction to the background and goals of the dissertation including a review of moringa, MSE and MIC-1 in relation to the key

subjects discussed in this dissertation such as bioavailability, bioaccessibility and inflammation.

Chapter 2 investigated the bioaccessibility using a human intestinal model and bioavailability in rats. Bioaccessibility of MIC-1, using the TNO Intestinal Model (TIM-1), was determined to be 61% and 62% in the fasted and fed states respectively. Bioavailability and pharmacokinetic studies were conducted in Sprague-Dawley rats treated with 50 mg/kg of MIC-1, either intravenously with pure MIC-1, or orally gavaged with MSE or MIC-1. Serum levels of MIC-1 were 6 to 12 times higher in animals dosed intravenously than in animals dosed by gavage with a half-life of about 2 h. Serum levels of MIC-1 dropped to zero between 8 and 24 h for all three treatments. These results suggested that MIC-1 remains largely unmodified during uptake, unlike other isothiocyanates, and has favorable bioaccessibility and bioavailability characteristics for a potential therapeutic agent.

Chapter 3 evaluated the ability of MSE to mitigate inflammation, addressing the hypothesis that MSE would attenuate inflammation in a Collagen Induced Arthritis (CIA) rodent model. Inflammation was induced in rats using an emulsion of Complete Freund's Adjuvant (CFA) and type II collagen, injected into the tail followed by administration of MSE and MIC-1 with the onset of symptoms. Swelling in the paws of the animals was measured using a plethysmometer. There were no significant reductions in the amount of swelling observed in the animals after treatment with MSE, which provided useful feedback for future experiments, such as the appropriate timing of MSE treatment, from intervention to preventative.

Chapter 4 addressed the development of a topical formulation of MSE to evaluate the anti-inflammatory effects in an acute model of inflammation. Various topical formulations were developed and evaluated in a carrageenan paw edema (CPE) model of acute inflammation. MSE formulations at various doses of 1,2 and 5% were applied directly to the hind paws of rats, 30 min before administering a 1% carrageenan-saline solution. No significant difference was observed when MSE was applied to the paw before the injection of carrageenan, as measured using a plethysmometer. These results led to further investigation into the delivery of MSE and MIC-1, so that those changes can be implemented in future inflammation experiments.

Lastly, Chapter 5 elucidated the topical anti-inflammatory effects and mechanisms of action of MSE and MIC-1 using a mouse ear edema model treated with a pro-inflammatory agent, 12-O-tetradecanoylphorbol-13-acetate (TPA). A time-dependent and dose-dependent response was determined by pretreating CD-1 mice with various doses of MSE and MIC-1, dexamethasone, a glucocorticoid as the positive control, or the vehicle control, followed by TPA. The difference in thickness of the ears was measured using a pair of digital Vernier calipers. The most effective doses of MSE and MIC-1 at 2 mg/ear and 0.8 mg/ear respectively were then selected for evaluating the change in weight of the ears using 6 mm biopsy punches. MSE and MIC-1 were both shown to be effective in a dose dependent manner, as assessed by reduction in ear thickness and a 44 % and 48 % decrease in ear punch weight when treated with MSE and MIC-1 respectively. The MSE and MIC-1 treated ears also resulted in a reduction in the levels of cytokine and chemokines, IL-6, MCP-1, and KC. MSE and MIC-1 reduced IL-6 expression by 70 % and 74 %, MCP 1 by 74 % and 73 % and KC by 56 % and 43 %

respectively. The anti-inflammatory effect of MSE and MIC-1 was further confirmed by H&E staining, used to assess the thickness in swelling in the ears. MSE significantly reduced the thickness of the ears to 33 % compared to TPA at 47 %. These data validate the anti-inflammatory properties of MSE and MIC-1 involving the inhibition of the NF-κB and Nrf2 pathways.

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Rukia, Kirsten, Keyaara, Chisom, Jessica, Harna, and all those who have been instrumental in my journey, from the bottom of my heart, thank you.

"The heights by great men reached and kept were not attained by sudden flight, but they, while their companions slept, were toiling upward in the night."-

Henry Longfellow.

Dedication

To every little girl who has ever dreamt of achieving greatness.

May you always believe that you are capable.

If you dream it, and believe in it, I am proof that you can achieve it.

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CHAPTER 1: INTRODUCTION

The use of naturally derived plant products for medicine, while seemingly gaining in popularity now, has been around for thousands of years. Since ancient times, people have been turning to nature for the treatment of ailments- from barks, seeds, fruit bodies and many other plant parts [1]. The first documented use of plants for medicine goes as far back as 2600 BC. Written on clay tablets in Mesopotamia were descriptions on the use of materials including oils of *Cedrus species* (Cedar) and *Cupressus sempervirens* (Cypress), *Glycyrrhiza glabra* (Licorice), *Commiphora species* (Myrrh) and *Papaver somniferum* (Poppy juice) [2]. All of these plant oils are still used in some form of treatment for a variety of conditions today. It is of interest that many natural plant compounds, those safe for human consumption, can be used in place of, or in addition to, modern pharmaceuticals since most have little to no harmful side effects, are cheaper to source and safer to consume over longer periods of time [3]. The general terms for commercial plant extracts used to support the normal functioning of the human body are "dietary supplements" or "botanicals" [4]. As the familiarity with these botanicals increases, researchers are studying their effects in treating a variety of diseases, thereby narrowing the gap between natural / traditional medicines and modern pharmaceuticals.

***Moringa oleifera* Lam.**

In tropical and sub–tropical environments, among the abundant plant life, is a drought resistant tree that can be grown to about 7-10 meters tall with sparse foliage and distinctive stick-like pods, or drumsticks as they are commonly referred [5]. This

Moringa oleifera tree, commonly referred to as moringa, is a member of the Moringaceae family of 13 known species. All, including *Moringa oleifera*, are traditionally used for food, medicine, or industrial purposes, from the pods, seeds leaves, bark, and roots [6]. Moringa's traditional uses focus mainly on the leaves. Dried leaves have shown to have about 30.3% protein, 3.7% calcium, 0.3% phosphorus, 1.5% potassium, 0.2% sodium, 0.6% sulfur, 8.2% copper, 13.0mg/kg zinc, 86.8mg/kg manganese, 490mg/kg iron, and 363mg/kg selenium [7] . They are also rich in antioxidants such as beta carotene, vitamin C, quercetin and chlorogenic acid which is used to lower blood sugar [8]. Even before official reports of their phytochemical analysis, the leaves have been used as a poultice or paste and applied externally to sores and sprains, and consumed for the treatment of anemia, menstrual irregularities, stomach aches, constipation, and piles [9]. The leaves have also been used to combat malnutrition in infants and nursing mothers and serve as a short-term alternative to chemoprophylaxis (disease prevention) in livestock [7]. Studies have identified about 16 bioactives in a methanolic extract of the leaves. The phytochemicals present were found to be phenols, flavonoids, saponins, anthocyanins and alkaloids. With the exception of the phenols and alkaloids, all these phytochemicals were found in higher concentrations in the seeds of moringa [10].

Moringa seeds are the focal point of this thesis, and not unlike the leaves, have many traditional purposes. Long established uses include filtering the sediment for water purification and arsenic removal [8, 11]; adding defatted seed meal to sheep diet in order to improve rumen fermentation [12]; supplementing the poor diets of cows with moringa protein to increase milk production [13]; and extracting the oil for cooking and frying due to its optimal stability [14]. Similar to the leaves, the phytochemical composition of the

seeds includes both essential and nonessential amino acids including alanine, glutamate, glycine, lysine, threonine and methionine. The seeds are also rich in calcium, phosphorus, iron, potassium, magnesium, zinc, copper and manganese [15]. Due to their plethora of phytochemicals, the biological activities reported in both the leaves and seeds of the plant includes anti-inflammatory, antibacterial, antioxidant, radical scavenging, antidiabetic, wound healing, antiepileptic, anti-convulsant, cardiovascular, anti-hypertensive, anti-fertility, anti-urolithiatic, anti-asthmatic, hepatoprotective, and protection of the central nervous system [8][16].

While the leaves and seeds are the most utilized parts of the plant, it is worth noting that all parts of the plant contain some bioactives and have been used for both medicinal and traditional purposes. An extract of the roots of moringa was reported to contain an active and powerful antibiotic and fungicidal, pterygospermin [17]. An alcoholic extract of the bark contains a lesser amount of the main glucosinolates studied in the seeds and leaves, 4-(alpha-L-rhamnopyranosyloxy) benzylglucosinolate; A hydro-alcoholic extract of the flowers contains antioxidants such as quercetin, kaempferol, isoquercetin, ascorbic acid; An aqueous and hydroalcoholic extract of the stem has 4-hydroxyl mullein, vanillin, octacosonoic acid, beta-sitosterone and beta-sitosterol, all used for cosmetic or medicinal purposes [8]. For all its multipurpose uses and vast number of nutrients found in all parts of the plant, moringa is often called ‘the Miracle Tree.’

Moringa oleifera's Glucosinolates and Isothiocyanates

Similar to the Moringaceae family, the phytochemicals in the members of the Brassicaceae family range from folic acid, phenolics, carotenoids, selenium, ascorbic acids, and a group of compounds called glucosinolates [18] . Glucosinolates are β -thioglucoside N-hydroxysulfates, (Z)-(or cis)-N-hydroximinosulfate esters, or S-glucopyranosyl thiohydroximates, with a side chain (R) and a sulfur-linked β -D-glucopyranose moiety [19].

Glucosinolates are sulfur-containing secondary metabolites that are precursors to the bioactive isothiocyanates (ITCs), a product of the hydrolysis reaction that is mediated by myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1). The conversion also occurs by the microflora of the intestines [20]. The 120 known glucosinolates are biologically inactive molecules that could have adverse effects on animals that ingest them in high amounts [21]. They make up roughly 1% of dry weight in the tissue of most *Brassica* vegetables [19]. The glucosinolates co-occur with the enzymes that catalyze their conversion into their isothiocyanates, nitriles, thiocyanates, epithionitriles and oxazolidine counterparts. The products are dependent on conditions including pH, metal ions and other protein elements, however, isothiocyanates are the most commonly produced metabolites of glucosinolates [22].

Isothiocyanates are a class of secondary metabolites that have a characteristically bitter taste and pungent odor, mostly found in the Cruciferae family of plants [23]. They are thioglycoside conjugates of glucosinolates and are formed when the cells of the plant

are damaged, by chewing for example, and an enzyme called myrosinase is released. This enzyme aids in catalyzing the hydrolysis of glucosinolates via the Lossen rearrangement-cleaving the thio-linked glucose and leaving the aglycone [24, 25]. Research into the biological effects of isothiocyanates has ranged from metabolism regulation, carcinogenesis, cardiovascular protection, protection of the central nervous system, diabetic nephropathy and neuropathy antimicrobial and restoration of skin integrity [26, 27]. More specifically, studies have shown that a number of naturally derived ITCs, including the ITCs from broccoli, watercress, and synthetic analogs have been effective at inhibiting chemically induced tumors in organ sites of rodents including the pancreas, stomach, bladder, colon, and esophagus [38]. Moreover, approximately 20 natural and synthetic isothiocyanates have shown to inhibit chemically induced carcinogenesis *in vivo* and *in vitro* [28]. The consumption and bioavailability of sulforaphane has been thoroughly investigated and is well documented and serves as a gold standard for other isothiocyanates [29-33].

Many isothiocyanates are volatile compounds due to the isothiocyanate (-N=C=S) group. They are known for their biological functions such as being an herbivore deterrent, fungicidal, bactericidal, nematocidal and being allelopathic and in the treatment of many ailments [34]. In moringa, there are 4 isothiocyanate products, with MIC-1, 4-(α-L-rhamnopyranosyloxy) benzyl isothiocyanate, being the most abundant. The other three, MIC-2, MIC-3, MIC-4 are named based on the position of an acetyl group on the R2, R3 or R4 position. These MIC's are produced in lower amounts, but all four contain an additional sugar moiety in the aglycone/ITC part of the molecule, which differs from the ITCs of other cruciferous vegetables [25] .

The aromatic ring and rhamnose moiety on MIC-1 that sets it apart from other isothiocyanates makes it a high-yielding stable, white powder as opposed to a low-yielding, volatile oil [35] [33, 36]. The presence of this sugar moiety allows for the unique property of moringa's isothiocyanates compared to those from other cruciferous vegetables.

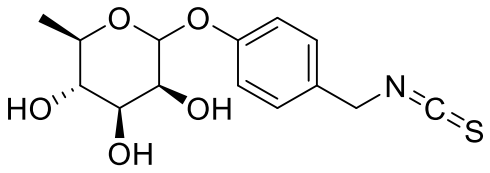

Figure 1.1: Structure of MIC-1 (4-Hydroxybenzyl isothiocyanate rhamnoside or 4-(α-L-rhamnopyranosyloxy) benzyl isothiocyanate). The distinctive sugar moiety attached is proposed to allow MIC-1 to be a stable, high-yielding white powder.

The consumption of fruits and vegetables with high nutritional value like moringa and other cruciferous vegetables like cabbage, kale and Brussel sprouts is associated with a reduced risk of diseases [37] . The most well studied isothiocyanate, sulforaphane, is an isothiocyanate found in high amounts in broccoli, another cruciferous plant. The precursor glucoraphanin is converted via myrosinase. Studies have shown that eating raw broccoli leads to higher amount of sulforaphane and its conjugates in the blood and urine of persons rather than cooked broccoli [37].

Bioavailability and Bioaccessiblity of ITCs

Understanding the bioavailability of any pharmacologically active compound is an important step for its clinical development. Bioaccessiblity is the first stage in defining the bioavailability of a compound and is performed *in vitro,* while bioavailability testing is done *in vivo*, as it requires the blood system [38]. It is important to understand how MIC-1, an isolated pure compound, and MSE perform within the human digestive system by defining the bioaccessiblity and later, its bioavailability. The investigation into whether isothiocyanates are both bioavailable and bioaccessible is important for their functional validation as future dietary supplements or as conventional pharmaceuticals [39].

Bioaccessiblity can be defined as "the amount of the compound that is released from the food matrix in the GI tract that is available for absorption" [40] .While the human digestive system is a very complex and dynamic environment, there exist *in vitro* models that at least partially mimic the stomach and small and large intestines. Models include those that mirror stomach-to-colon (SHIME) [41] or just the colon (HMI or Gut-on-a-chip) [42, 43] among others. While *in vivo* studies mainly sample the end point of the digestive system (urine or feces) to see the effect of a compound, intestinal *in vitro* models can rely on sampling different regions of the gut, making it possible to monitor changes at various locations [44]. The multi-compartment computer-controlled TNO gastrointestinal model (TIM-1 model) simulates the *in vivo* conditions and kinetic events of the stomach, duodenum, jejunum, and ileum of the small intestines [45]. The dynamic digestion of TIM-1 suggests that it can stimulate continuous changes of the physiochemical conditions of the digestive system, from mouth to stomach, and changing

pH and enzyme secretion concentrations [46]. This system has compartments representing the stomach, duodenal, jejunal and ileal parts of the gastrointestinal tract. Each of these four compartments has two glass jackets that are lined with flexible walls. It stimulates gastric emptying rate, peristaltic movements, and gradual pH changes in the different compartments [46]). This model was ideal to explore the workings of MIC-1 while consumed in both a fed and fasted state. It was found that MIC-1 was bioaccessible, with no statistical difference in the state. MIC-1 was also found to be bioavailable when used in an *in vivo* mouse model that investigated the amount of MIC-1 in their blood serum over time (Figure 2.3). A subset of animals was administered MIC-1 via IV, another subset received MIC-1 via oral gavage and another subset received MSE via oral gavage. The IV-administered MIC-1 would show that about 20% is bioavailable and even when orally administered, unlike other isothiocyanates that are conjugated with glutathione and metabolized into mercapturic acid or bithiocarbamate products, MIC-1 remained unchanged [47].

Bioavailability is the fraction of the consumed compound that gets into the circulatory system (blood) that can then be distributed to tissues and organs [48]. It incorporates the following four components: 1) availability for absorption or bioaccessibility; 2) absorption; 3) tissue distribution; 4) bioactivity [49]. Determining the bioavailability of a botanical such as moringa in an *in vivo* model is important because in order to be consumed as part of the diet, a significant therapeutic level must be sustained when ingested orally [50]. Evaluating the bioavailability of MIC-1 both isolated and in MSE was performed by orally gavaging a group of mice and collecting the blood serum at different time points, going from the mouth, stomach, small intestine then large

intestine and colon involves various temperature, pH conditions and enzyme secretions with the breaking down of the supplement along the way. The chemical structure and properties of the compound are important to consider as they are affected by the gut physiological and physiochemical factors [50].

Studies have shown that consumed isothiocyanates are conjugated to glutathione and then further metabolized into mercapturic acids, predominantly an N-acetyl cysteine conjugate, which is then excreted into the urine. Therefore, the presence of mercapturic acid in the urine indicates that isothiocyanates were ingested [37, 51]. The most well researched bioavailable isothiocyanate is sulforaphane, usually sourced from broccoli. In rats, sulforaphane is rapidly absorbed, achieving high bioavailability at low doses after being fed fresh broccoli, with peak intracellular concentration within 3h of doses. Oral absolute bioavailability was over 80% at the lowest dose of 0.5mg/kg but decreased as concentrations increased [52].

Inflammation

The World Health Organization (WHO) reported that in the year 2000, nearly 125 million Americans live with chronic conditions which includes inflammatory diseases [53]. Inflammation, the body's defense mechanism, is involved in diseases such as diabetes, cancer, obesity, cardiovascular disease, arthritis, allergies, multiple sclerosis, and chronic obstructive pulmonary disease [53][54].

Inflammation can be defined as the body's recognition and defense mechanism against harmful and foreign stimuli. It involves the activation of immune and non-

immune cells that protect against bacteria, viruses, toxins, and infections, and can be categorized as acute or chronic, depending on the severity and length of time of the inflammatory response [55]. This process involves the release of substances such as cytokines and chemokines that are responsible for the dilation and increase in permeability of capillaries, which results in tissue that is swollen and inflamed [55]. This acute response contributes to the resolution of inflammation and restoration of tissue homeostasis, but if it is uncontrolled, it may become chronic and lead to a range of chronic inflammatory conditions [56].

Many inflammatory diseases are the result of acute or chronic inflammation. Acute inflammation is typically reserved to the tissue level, for example, the response to a cut of the finger. It is characterized by redness, swelling, heat, pain, and loss of the function of the tissue, due to local immune, vascular, and inflammatory cell responses to the infection or injury. The microcirculatory events include changes to the vascular permeability, leukocyte recruitment and accumulation and release of inflammatory mediators [56]

Chronic inflammatory conditions include but are not limited to cardiovascular, arthritis, cancer, fatty liver, type 2 diabetes mellitus, type 1 diabetes mellitus, inflammatory bowel disease and asthma that are not limited to age, gender or socioeconomic background [57].

In general, the inflammatory response process can be summarized by the following processes: cell surface pattern receptors recognizing the stimuli, activation of inflammatory pathways, release of inflammatory markers, and recruitment of inflammatory cells [56]. Various transcription factors and pathways are activated during

this time, such as activator protein-1 (AP-1), STAT3, and TNF pathway [57]. AP-1 transcription factors are found to be the most important regulators in the epidermis of the skin in regulating epidermal homeostasis and diseases such as cancer [58]. Some key cytokines present during the inflammatory response include stimulators of acute phase proteins such as IL-6, IL-1β, tumor necrosis factor-β, interferon-γ, transforming growth factor-β and IL-8 [59]. Another important transcription factor to note is NF-κβ. This transcription family has five members which include p65 (ReIA), RelB, c-Rel, NF-κβ1 and NF-κβ2. They all share some structural features that are bound to Iκβ proteins, the inhibitor of NF-κβ, in quiescent cells [60]. It is known that NF-κB controls the expression of cytokines IL-1β and TNF, important markers in inflammation, and in turn induce their expression. Along with their expression, there are also elevated levels of cytokines, including IL-6 and IL-15, adhesion molecules and chemokines IL-8, MCP-1 and MIP-1α, all to promote the activation and recruitment of inflammatory cells [61].

The skin, the largest organ of the body, is composed of three main parts, from outermost to innermost, the epidermis, the dermis and the subcutis [62]. It provides a life-sustaining barrier between the body and the environment, restricting water loss and preventing entry of harmful microorganisms and substances [57]. While sometimes the cause of skin inflammation may be due to external factors, others are the results of autoimmune diseases. The development of such skin pathologies involves a possible combination of genetic susceptibility, environmental influence such as infections, smoking, drinking, or drugs and the presence of an autoantigen [63]. An example of an autoimmune disease would be rheumatoid arthritis (RA). It is common, affecting about 2-3% of the population or 125 million people according to the National Psoriasis

Foundation. It is characterized by inflammation and hyperplasia of the synovial fluid, cartilage and bone destruction, and even systemic features such as cardiovascular, pulmonary psychological and skeletal disorders [64]. The initiators of the inflammatory process are the keratinocytes in the epidermis, which receive signals and transit them to the immune cells. The receptors sense the presence of stimuli, microorganisms for example, and produce cytokines, chemokines, and their receptors. The CIA model in rats is used in this thesis to mimic the pathology of RA [65].

Two other *in vivo* models were used to evaluate the effectiveness of MIC-1 and MSE in an acute inflammatory response characterized by swelling, redness, heat and pain to a specific site. One model was a carrageenan paw edema model where in the inflammation site is the hind paws of the animals. Injection of a 1% carrageenan solution elicits an inflammatory granulomatous reaction that is characterized by a production of prostaglandin, leukotriene and polymorphonuclear leukocytes (PMNs) and macrophages [66]. The lambda form of this family of polysaccharides has historically been used as a stimulant for an *in vivo* model of a local inflammatory response. It causes local edema, infiltration of white blood cells, increased levels of local PGE2, increased interleukin-8 secretion in cell tissue culture via nuclear localization of NFκB [67]. The second model, TPA induced ear edema model, uses 12-O-tetradecanoylphorbol-13-acetate that elicits the inflammatory response by production of ROS and pro-inflammatory mediators that include cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6), interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), and is closely associated with the nuclear factor-kappaB (NF-kB) and the mitogen-activated protein kinase (MAPK) pathways [68].

While *in vivo* studies are performed on mice and rats, it is important to note that while they are similar, the skin of rodents does differ from the skin of humans. The anatomical and immunological differences between murine and human skin include the following: murine skin is covered with a thick layer of hair while humans have sparse hair coverage. Mouse skin is thinner than human's and has a superficial muscle layer that contracts allowing for healing without scars while humans use re-epithelization and granulation to heal that would leave behind scars [69]. Mouse skin also contains specific types of dendritic cells CD207(langerin)+CD103+ dermal DCs (DDCs) and Vγ5Vδ1 T cells also known as dendritic epidermal T cells (DETCs) while humans either lack or have not been identified [69].

While there are differences, the use of mouse skin for topical experiments is the gold standard when long and costly clinical trials are not an option. The use of CD-1 mice to investigate the topical anti-inflammatory effects of an MSE and its isothiocyanate is relevant to the further development of a topical treatment, perhaps in combination with others, for the symptoms of inflammatory conditions.

The inflammatory conditions of concern in this dissertation range from acute inflammation, such as eczema, to chronic inflammation like RA. With the comprehensive research presented in this thesis, these findings could provide the necessary steps to formulating MSE or MIC-1 as a preventative therapeutic agent to be used in combination with prescribed medicine. The use of a naturally derived plant product incorporated into a daily regimen could be the added therapeutic in delivering health and wellness.

ITCs and inflammation

The sharp taste of cruciferous vegetables, the result of an active defense system, is due to the presence of isothiocyanates. These compounds possess strong anti-inflammatory, anti-carcinogenic, antioxidant, anti-microbial, neuroprotective, and cardioprotective activities [70]. Vegetables such as broccoli, watercress, brussels sprouts, cabbage, Japanese radish and cauliflower are abundant in isothiocyanates. Some of these include sulforaphane, phenethyl isothiocyanate (PEITC), and benzyl isothiocyanate (BITC), all of which have been shown to be highly effective at reducing the risk of cancer in carcinogen animal models, and in vitro, limiting the growth of various cancer cells [28].

Studies have demonstrated the effects of isothiocyanates in alleviating inflammation in different inflammatory models. Water-extractable isothiocyanates from moringa leaves have decreased gene expression and decreased production of inflammatory markers iNOS, IL-1β, nitric oxide and TNFα *in vitro* [25]. Topically, a UVB radiation study showed that skin erythema was reduced by 40% at the sites that received a topical treatment with sulforaphane-containing broccoli extract compared to the sites that received the vehicle alone [26]. Again, as a topical treatment, 2% moringin cream was shown to relieve neuropathic pain due to the inhibition of inflammatory pathway and blockade of voltage-gated ion channels [71]. Another study showed that encapsulated liposomes of naringin with phenyl isothiocyanate and sulforaphane, two isothiocyanates, were able to decrease inflammation in both an acute carrageenan paw edema model and a chronic arthritis model in rats [72]

All these findings confirm that an investigation into the effects of a *Moringa oleifera*

seed extract and the main isothiocyanate, 4-(α-L-rhamnopyranosyloxy) benzyl isothiocyanate, MIC-1 in an acute skin inflammation model needs to be performed. The results may suggest a form of combination therapy to target the many mechanisms and pathways involved in a variety of inflammatory conditions.

Thesis Summary and Hypothesis

The work in this thesis focuses on the hypothesis that one specific botanical, *Moringa oleifera* Lam. (moringa), contains bioactive components that can be used as a therapeutic agent. My experiments focus on an isothiocyanate-enriched moringa seed extract (MSE) and its main isothiocyanate, moringa isothiocyanate-1 (MIC-1). This thesis adds to the existing knowledge by evaluating the bioavailability and bioaccessiblity of MSE and MIC-1 *in vitro* and *in vivo* using an intestinal model. The TNO Gastro-Intestinal Model (TIM)-1, along with the topical anti-inflammatory effects of it in a TPA induced ear edema model. These investigations may aid in the further development of MSE or MIC-1 for therapeutic treatments, such as metabolic syndrome or in alleviating the symptoms of conditions such as psoriasis. This thesis includes work that investigated the effects of MSE and MIC-1 in two inflammatory models and yielded unexpected but useful results that contributed to the success of the TPA induced ear edema manuscript (Chapter 4). The impact of this dissertation could lead to the development of a botanical to prevent the onset of both oral and topical inflammatory conditions, or to complement the pharmaceutical drugs for various disorders. My research also adds to the fundamental understanding of the biochemical and molecular processes involved in inflammation and suggests strategies for further studies of the modes of pharmacological action of plant natural products.

With the understanding that MSE and MIC-1 can be used in alleviating the symptoms of inflammatory conditions, there had been no prior research that focused on its pharmacokinetic effects. It was therefore important to understand how MIC-1, whether administered as a pure compound or in MSE, is metabolized within the system. If it's bioaccessiblity or bioavailability is not defined, the use of it as an orally administered therapeutic agent could not be developed. Chapter 2 concentrates on investigating the bioavailability and bioaccessiblity of MSE and MIC-1 *in vitro* and *in vivo*. First, an *in vitro* intestinal model TIM-1 was used to evaluate how it responds after passing through the stomach and lower intestines in both a fed or fasted state. This research was followed by an *in vivo* model where mice were administered the MSE and MIC-1 via IV or oral gavage, after which their blood serum was measured for levels of MIC-1 over time. It was shown that MIC-1 remains unchanged within the serum, unique for a botanical compound.

It is of growing interest that inflammation, acute or chronic, can be addressed with not only traditionally prescribed pharmaceutical drugs, but in combination with botanicals. Chapter 3 investigates the use of MSE in a chronic inflammatory model, The chronic model is a Collagen Induced Arthritis (CIA) model that simulates the pathogenesis of rheumatoid arthritis (RA) in Wistar rats. The rats were induced with a mixture of Complete Freund's Adjuvant (CFA) and type II bovine collagen with incidence of arthritis developing roughly two weeks after a booster of the same mixture. The subsequent swelling was monitored and measured using a plethysmometer while treatments were administered to the animals via oral gavage. MSE at 50, 100 and 200 mg/kg MIC-1 was not effective at reducing the inflammation in the paws of the rats.

Chapter 4 focuses on the use of MSE in an acute inflammatory model, a carrageenan paw edema model. As previously stated, inflammation, even of the skin, is a challenge that includes psoriasis, eczema, acne and even tumorigenesis [73]. Because the skin is the largest organ of the body, it defends against many environmental threats, which can lead up to a plethora of negative health conditions. However, there are not many naturally derived topical treatments that can be effectively used over time without negative effects. This chapter evaluates the topical application of MSE and MIC-1 in a Carrageenan Paw Edema (CPE) model, which represents an acute inflammatory model that induces edema in the paw, with effects only lasting up to a few days. Gel, cream and ointment topical treatments were prepared, each with a concentration of 5% MSE. Results from the final experiment showed that there was a trending decrease in swelling in the animals treated with MSE and MIC-1 compared to the vehicle and lidocaine treated positive control animals, however, it did not reach statistical significance of $p<0.05$. These results were promising, but the lack of statistical significance was disappointing. It is possible that the negative responses were the result of suboptimal formulation and delivery for MSE and not including purified MIC-1 in the study. Additionally, a switch from a paw edema to ear edema was made because while both were effective at inducing inflammation, the ear edema model was a simpler and quicker way to induce swelling, with no injecting of inflammatory inducing material required. The decision made was therefore to consider a new solvent system and use a TPA induced ear edema model, discussed in Chapter 5.

Chapter 5 investigated in the anti-inflammatory effects of MIC-1 in MSE and isolated MIC-1 in an ear edema model induced by TPA. A time dependent dose response was performed, at which point the most effective doses was chosen to evaluate different

endpoints. Those endpoints included: morphological changes in ear thickness using a pair of digital Vernier calipers, ear punch weights using biopsy punches, thickness as seen in hematoxylin and eosin (H&E)-stained slides, and at the molecular level, the effect of cytokines and chemokines during the response. MSE and MIC-1 were effective at reducing ear punch weights, ear thickness and limiting the expression of specific cytokines and chemokines involved in specific inflammatory response pathways. It is hopeful that these findings can begin the development of a topical therapeutic means of relieving the symptoms of inflammation.

The overarching goal of each of these chapters was to evaluate the anti-inflammatory benefits of moringa, its seed extract and isothiocyanate, the active component of the extract, in various models for the possible development of a therapeutic moringa-based product to improve human health, for either oral or topical application. The work of this thesis can be broken down into the following broad questions/objectives:

1. What happens when MSE and MIC-1 are consumed? Are they bioaccessible and bioavailable within the body?
2. Can MSE and MIC-1, when orally supplemented, be used to alleviate inflammation in a rodent model of rheumatoid arthritis?
3. Can MSE and MIC-1 be formulated as a topical application to alleviate the symptoms of acute inflammation in a mouse paw edema model?
4. As topical agents, can MSE and MIC-1 be used to attenuate acute inflammation in a mouse ear edema model?

Experiments were performed and successful at providing a better understanding of its delivery both orally and topically. Each of the following chapters are presented as standalone projects that includes an introduction, methodology, discussion, and conclusion, allowing for a better understanding of the goals in order to investigate the pharmacological activity of an isothiocyanate-rich moringa seed extract.

CHAPTER 2: Moringa isothiocyanate-1 is bioaccessible and bioavailable as a stable unmodified compound

Phytochemistry Letters 38 (2020) 10.1016

Abstract

Moringa oleifera Lam. is a widely cultivated subtropical tree with a variety of documented medicinal properties. An ethanolic moringa seed extract (MSE) was shown to contain high concentrations of the stable moringa isothiocyanate-1 (MIC-1). MIC-1 has anti-inflammatory and antidiabetic properties but has not been characterized metabolically. The objective of this study was to understand its bioaccessibility using a human intestinal model and bioavailability using serum from treated rats. Bioaccessibility of MIC-1, using the TNO Intestinal Model (TIM-1), was determined to be 61% and 62% in the fasted and fed states respectively. MIC-1 from the serum of treated animals was measured directly without prior chemical or enzymatic digestion. Bioavailability and pharmacokinetic studies were conducted in Sprague-Dawley rats treated with 50 mg/kg of MIC-1, either intravenously with pure MIC-1, or orally gavaged with MSE or MIC-1. Serum levels of MIC-1 were 6 to 12 times higher in animals dosed intravenously than in animals dosed by gavage with a half-life of about 2 h. Serum levels of MIC-1 dropped to zero between 8 and 24 h for all three treatments. These results suggest MIC-1 remains largely unmodified during uptake, unlike other isothiocyanates, and has favorable bioaccessibility and bioavailability characteristics for a potential therapeutic agent.

Introduction

Moringa oleifera Lam. (moringa), also known as the 'Drumstick tree,' is indigenous to the South India region and known for its wide range of uses as a nutritional food and traditional medicine [74]. It is cultivated and grown in many parts of the world including tropical parts of Africa, South and Central America and Asia. Moringa leaf and seed extracts were shown to have anti-inflammatory and antidiabetic properties *in vitro* and *in vivo* [25, 35, 75]. Moringa seeds have also been shown to possess antimicrobial and anti-hepatoxic properties [76]. Moringa seeds have been identified as a richer source of medicinal activity than the leaves, presumably because of their much higher concentrations of isothiocyanates [77]. Moringa isothiocyanates (MICs) have been reported to reduce weight gain, insulin resistance and hepatic gluconeogenesis and improve glucose tolerance in obese mice [75, 78]. MICs were also shown to mitigate ulcerative colitis pathologies in a mouse model through an anti-inflammatory mechanism likely mediated through the nuclear factor erythroid 2-related factor 2 (Nrf-2) signaling pathway [79].

Isothiocyanates are small molecules characterized by the presence of a -N=C=S group and are hydrolyzed from their glucosinolate precursors via a myrosinase (b-thioglucoside glucohydrolase) enzyme reaction [52, 80]. The conversion happens as a plant defense mechanism after plant cells have been physically damaged from chewing, but can also take place in the gut by microfloral digestion [81]. Isothiocyanates are common and widely distributed in cruciferous vegetables such as moringa, broccoli and cauliflower, however, the structure and stability of isothiocyanates vary greatly [25]. In humans and other mammals, dietary isothiocyanates are generally conjugated with

glutathione and metabolized through the mercapturic acid pathway which has been well characterized as a result of anticancer studies using isothiocyanates [47]. The most widely studied isothiocyanate is sulforaphane (1-isothiocyanato-4-methylsulfinyl butane) from broccoli which has many reported chemoprotective properties, including the prevention of carcinogen activation and enhancement of carcinogen detoxification [52]. Sulforaphane, which is derived from its glucosinolate precursor, glucoraphanin, is an unstable viscous, yellow liquid at room temperature [82]. Moringa isothiocyante-1 (MIC-1), however, is a much larger molecule with a more stable structure due to the attached sugar moiety (rhamnose) [78]. MIC-1 is a stable white crystalline powder at room temperature. We have previously optimized an extraction method of moringa seeds to maximize the yield of MIC-1 and reported as much as 38.9% MIC-1 in an ethanolic moringa seed extract (MSE). This MSE was shown to be safe with a no observed adverse effect level (NOAEL) of 257 mg/kg bw in rats [83]. We have also been successful at isolating and purifying MIC-1 from MSE for use as a chemical standard [77].

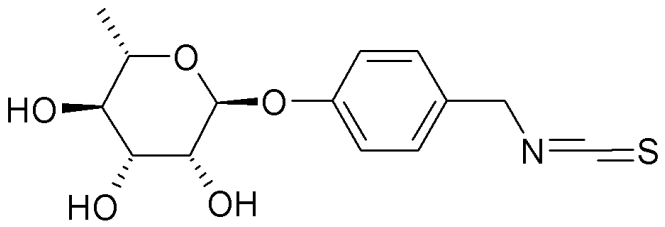

Fig 2.1 Structure of compound, MIC-1.

Very little is currently known about the metabolism of MIC-1, despite significant recent interest in the pharmacological benefits of this compound. During absorption, MIC-1 could be conjugated, bound to plasma/blood proteins and/or remain as an

unmodified compound in the blood when ingested, as observed for other aromatic isothiocyanates, such as phenethyl isothiocyanate (PEITC) [84]. Bioaccessibility, bioavailability and pharmacokinetics of MIC-1 have not been evaluated, although such studies would be useful for the development of moringa products as dietary supplements or foods and to standardize dosing in future clinical trials. In this study we investigated the bioaccessibility of MIC-1 from MSE using a model of the upper gastrointestinal tract of humans; developed methods for measuring MIC-1 in the serum of animals; and conducted a preliminary pharmacokinetic study of MIC-1 in treated rats, to gain biological insight into the metabolic fate and pharmacokinetics of this compound.

Materials and Methods

Reagents and materials

Moringa seeds used for extraction were supplied from the Jamaica Moringa Farmer's Association in Kingston, Jamaica (voucher accession number 146375 in the Rutgers University Chrysler Herbarium, CHRB). Rat serum was purchased from EMD Millipore (S24-100ml). All chemicals, including solvents such as hexane, ethyl acetate and ethanol were purchased from Sigma-Aldrich. Pepsin A from porcine stomach mucosa (2500-3500 units/mg, P-7012), trypsin from bovine pancreas (7500 N-α-benzoyl-L-arginine ethyl ester (BAEE) units/mg, T9201), and α-Amylase Type II-A: from *Bacillus* species (1333 units/mg A-6380) were obtained from Sigma-Aldrich (Stockholm, Sweden). Fresh-frozen pig bile was obtained from a local slaughterhouse collected from non-suckling pigs (Farm to Pharm, L.L.C., Warren NJ). *Rhizopus* lipase (150,000 units/mg F-AP-15) was obtained from Amano Enzyme Inc. (Nagoya, Japan).

Preparation of MSE and MIC-1

Seeds were ground and incubated in Millipore water at a ratio of 1g of seeds: 3ml of water at 35-37°C for 2 hours. To every 1ml of water, 4 ml of 95% ethanol was added to the slurry and filtered through a vacuum filtration system. Ethanol was removed from the mixture by rotary evaporation. Water was removed by lyophilization and the remaining dried extract was weighed, quantified for the amount of MIC-1 by LC-MS/MS and stored at -20° C. MIC-1 was isolated and purified for use as a chemical standard for quantification as previously described [77].

In vitro TIM-1 bioaccessibility

The TNO Intestinal Model (TIM-1) was used to determine the bioaccessibility of MIC-1 from MSE in both fed and fasted state conditions. Bioaccessibility is defined as the amount of compound released from a food matrix and absorbed by simulated jejunal and ileal membranes (with a cutoff of approximately 5 kDa) reflecting availability for intestinal absorption *in vivo*, i.e. an indicator of bioavailability [85]. TIM-1 is a dynamic, computer-controlled model of the upper gastrointestinal tract of humans and has been described at length [85]. Briefly, TIM-1 consists of stomach, duodenum, jejunum and ileum compartments, each composed of a glass capsule encasing a flexible inner silicone jacket. Water heated to 37 ± 1 °C is pumped through the space between the glass and silicone to maintain body temperature and mechanically compress and release the silicone jackets to imitate peristalsis and mixing of chyme. Secretions of amylase (saliva),

gastric juice, bile and pancreatin/ pancreatic juice [86] are introduced via pumps connected to GI compartments while pH is regulated by secretion of hydrochloric acid in the stomach and sodium bicarbonate in intestinal compartments. Simulated gastric (pepsin and lipase), biliary (fresh pig bile) and pancreatic secretions are introduced into compartments by computer-controlled pumps. Gastric emptying and intestinal transit time are controlled by three peristaltic valves that move specific volumes of chyme with each open-close cycle, which is altered to simulate either the fasted state, corresponding to intake of compounds with water, or the fed state, for intake of compounds with a meal/food matrix. Connected to jejunal and ileal compartments are hollow fiber filtration devices composed of semi-permeable membranes (0.05 μm pore size, Spectrum Milikros modules M80S-300-01P) that simulate absorption of released/digested water- or fat-soluble compounds less than 5 kDa in size. During TIM-1 digestion, samples were collected hourly from fluids absorbed through jejunal and ileal filtration devices as well as the ileal efflux, fluids that pass through the ileocecal valve of the model and contain compounds that would theoretically be delivered to the colon.

TIM-1 bioaccessibility experimental conditions

MSE (520 mg) containing 202 mg of MIC-1 was mixed with 100 g of food, artificial saliva consisting of 100 ml electrolyte solution (0.62 g/l sodium chloride, 0.22 g/l potassium chloride and 0.03 g/l calcium chloride di-hydrate) and 11.5 mg amylase, and 100 ml of water, together adjusted to pH 5.5 for the fed state. The food consisted of a standardized High Fat Meal that fulfills the requirements of the U.S. Food and Drug Administration (FDA) and Center for Drug Evaluation and Research (CDER) for

experiments with drugs during intake with a high fat meal are described in full (Guidance for Industry, December 2002) and previously used for similar studies [45]. The same MSE was mixed with 50 ml of the electrolyte solution, 2.5 mg of amylase and 250 ml of water, together adjusted to pH 2.2 for the fasted state. Both the fed and fasted state mixtures were adjusted to a final volume of 300 ml. The final mixture was introduced into the gastric compartment of TIM-1 and digestion was initiated. Fed state parameters utilized 500 ml of porcine bile, 17.5 g of pancreatin, 37.5 mg of lipase and 30 mg of pepsin. Fasted state parameters utilized 100 ml of porcine bile, 3.5 g of pancreatin, 7.5 mg lipase and 6 mg pepsin. Both conditions were provided with 2 mg of trypsin. Each TIM-1 digestion experiment was terminated at 240 min (4 h) when approximately 80% of the stomach contents had passed the ileocecal valve of the model and become the ileal efflux. Bioaccessibility was determined from quantifications of MIC-1 in jejunum, ileum and ileal efflux samples at 1, 2, 3, and 4 h after initiation of digestion relative to the starting amounts. Residues, which comprised fluids remaining in the stomach, duodenum, jejunum and ileum compartments of the model after the 4 h digestion period were also analyzed. For MIC-1 quantification, 2 ml from each sample were diluted with 1 ml of Millipore water, defatted with 2 X 2 ml hexane and partitioned into 3 X 2 ml of ethyl acetate. Ethyl acetate was removed by speed vac and samples were resuspended in 200 µl of 95% ethanol for LC-MS analysis. Each condition was repeated in triplicate.

Animal bioavailability study

Twenty male Sprague-Dawley rats (230-260 g) were purchased from Charles River Laboratories (Malvern, PA). Rats were acclimated for 2 weeks in the animal

facility at 22 ± 2 °C on a light/dark cycle of 12 h. They were housed in groups of 5 per cage and were allowed access to both food and water *ad libitum*. The MSE used in the experiment was standardized to 38.9% w/w MIC-1 in MSE. The animals were randomly assigned to four treatment groups. MIC-1 was dosed at 50 mg/kg body weight for intravenous and oral gavage treatments. MSE was dosed at 128 mg/kg to deliver 50 mg/kg body weight of MIC-1 in 0.1% DMSO vehicle. After dosing, animals were subsequently bled at 0.5, 1, 2, 4, 8 and 24 h through terminal trunk bleeds. The experiments were carried out using an approved protocol by the Rutgers University Institutional Animal Care and Use Committee (Protocol # 05-037). A small subset of rats was gavaged with 200 mg/kg of MSE then bled, to be used for validation and method development.

Biological sample collection and processing

Recovery of MIC-1 Developing a method to recover MIC-1 from serum spiked with MIC-1 was the first step in examining its potential metabolites. In general, isothiocyanates from most cruciferous vegetables are known to be unstable compounds with rapid metabolism. Since we were able to detect unmodified MIC-1 in the serum of animals treated with MSE, we decided to evaluate the presence and stability of MIC-1 in the blood and serum overtime from treated animals using 2 alternative approaches consisting of hydrolysis (chemical or enzyme) or simple partitioning.

Acid Precipitation/Hydrolysis Serum samples were spiked with known amounts of MIC-1 for a final concentration of 10 µg/ml and 50 µg/ml. At a ratio of 1:4 (sample:

solvent), 0.1 M HCl in acetonitrile was added to the spiked serum in 8 ml glass vials. Vials were incubated at 40 °C for 6 h and centrifuged at 20,000 X G for 10 min. The supernatant was collected and dried in a speed vac. The precipitate was resuspended in 500 µl of 80% ethanol, filtered and prepared for UPLC-MS/MS analysis.

Liquid Partitioning Trunk blood samples (1 ml) were collected from animals into Eppendorf tubes. Aliquots of whole blood were kept in Eppendorf tubes at room temperature for 30 min and then kept on ice for 20 min before being centrifuged at 1200 X G for fifteen min at 4 °C. Serum was collected and placed into 1.5 ml Eppendorf tubes and stored at -80 °C until processed. A 200 µl aliquot of serum was combined with 1 ml of Millipore water in 8 ml glass vials in triplicate. The samples were defatted with hexane (1 ml X 2), then partitioned with ethyl acetate (2 ml X 3) and dried for 2 h in a speed vac. Samples were re-suspended in 500 µl of 80% ethanol, vortexed for 1 min, sonicated for 15 min, filtered and placed in vials for analysis of MIC-1 by UPLC-MS/MS.

β-Glucuronidase hydrolysis This method was similar to the nonhydrolyzed partitioning method described above, with the addition of B-glucuronidase enzyme digestion. β-glucuronidase enzyme hydrolysis was performed on the serum samples from MSE treated animals to determine if any MIC-1 may have become glucuronidated during metabolism, which is common for many types of bioactive botanical compounds [87]. Serum samples (200 µL) were combined with 200 µL of sodium acetate buffer (pH 5.5), 500 µL of Millipore water and 25 µL of β-glucuronidase for digestion. Samples were vortexed and incubated at 37 °C for 15 h, cooled on ice and transferred to glass tubes. One mL of water was added to each sample, then defatted with hexane (1 ml x 2),

partitioned with ethyl acetate (2 ml x 3) and dried for 2 hours by speed vac. Samples were resuspended in 125 µL of 90% ethanol for UPLC-MS/MS analysis.

UPLC-MS/MS analysis of MIC-1 from serum samples

UPLC-MS/MS analysis was performed using a Dionex® UltiMate 3000 RSLC ultra-high-pressure liquid chromatography system, a photodiode array detector DAD-3000RS, and a Q Exactive Plus Orbitrap high-resolution high-mass-accuracy mass spectrometer (MS). Mass detection with an electrospray (ESI) interface was full MS scan with low energy collision induced dissociation (CID) from 100 to 1000 m/z in negative ionization mode. Analytes were separated on a Phenomenex^{TM} Kinetex C8 reverse phase column (100 x 2 mm, 2.6 um/ 100 Å particles). The mobile phase consisted of two components: solvent A (0.5% ACS grade acetic acid in LC-MS grade water, pH 3-3.5), and solvent B (100% LC-MS grade acetonitrile). The mobile phase gradient consisted of 95% A and 5% B to 5% A and 95% B over 30 min at 0.20 mL/min. Quantification of MIC-1 in serum samples was calculated relative to a calibration curve of pure MIC-1 standard. Identification of MIC-1 and possible metabolites utilized libraries of spectral database searches (reaxys.com, Elsevier RELX Intellectual Properties SA); SciFinder, American Chemical Society).

Pharmacokinetic analysis

Serial MIC-1 serum samples obtained at 0.5, 1, 2, 4, 8, and 24 h were used to calculate pharmacokinetic parameters (e.g., area-under-the- concentration time curve

[AUC0-(t-last), AUC0-∞], maximum serum concentration [Cmax], time to maximum concentration [Tmax], bioavailability (F), clearance (CL) and apparent clearance [CL/F]) via model independent methods. Elimination rate constants (ke) were calculated from slopes of the log-linear phase of MIC-1 concentration-time profiles. Terminal elimination half-lives (T1/2) were calculated as 0.693/ke. AUC0-t-last was determined via the linear trapezoidal method and AUC(t-last)-∞ was calculated from the quotient of the last measurable MIC-1 concentration (t-last) and ke. AUC0-∞ was calculated as the sum of AUC0-(t-last) and AUC(t-last)-∞. Clearance was calculated as Dose/AUC0-∞. As both intravenous and oral concentration-time profiles were available, both CL and CL/F could be determined. Absolute bioavailability (F) was calculated as [AUC0-∞ (oral)/AUC0-∞ (IV)]. Following IV administration, the concentration of MIC-1 at 0 h was determined by back extrapolating from the 0.5-h concentration using k_e. This value, in turn was used to calculate AUC.

Statistical analysis

Results are expressed as mean ± standard deviation unless otherwise stated. Differences in the MIC-1 and MSE oral gavage with MIC-1 IV were statistically analyzed using the Holm-Sidak method with alpha=0.05. All data analysis was performed using GraphPad Prism 7.03. A p value < 0.05 was considered statistically significant. Statistical specifics are listed in the figure legends of each study.

Results and Discussion

Bioaccessibility of MIC-1, delivered by MSE, was determined using the TIM-1, an *in vitro* digestion apparatus used to predict the behavior of a compound in the human gastrointestinal tract. Bioaccessibility represents the amount of compound potentially available for absorption. For MIC-1, bioaccessibility was determined to be 60.9 ± 3.7% and 62.4 ± 3.0% in the fasted and fed states, respectively (Figure 2.2 A & B). The highest total absorption was observed during the second hour for both conditions. The lower absorption of MIC-1 observed during the first hour in the fed state is likely due to the slower rate of gastric emptying in the fed state (as determined by setpoints of TIM-1). These *in vitro* findings suggest good bioavailability of MIC-1 irrespective of feeding status. However, if isothiocyanates are delivered through dietary sources, as they usually are, they would be delivered in a fed state, which showed a slight increase in bioaccessibility.

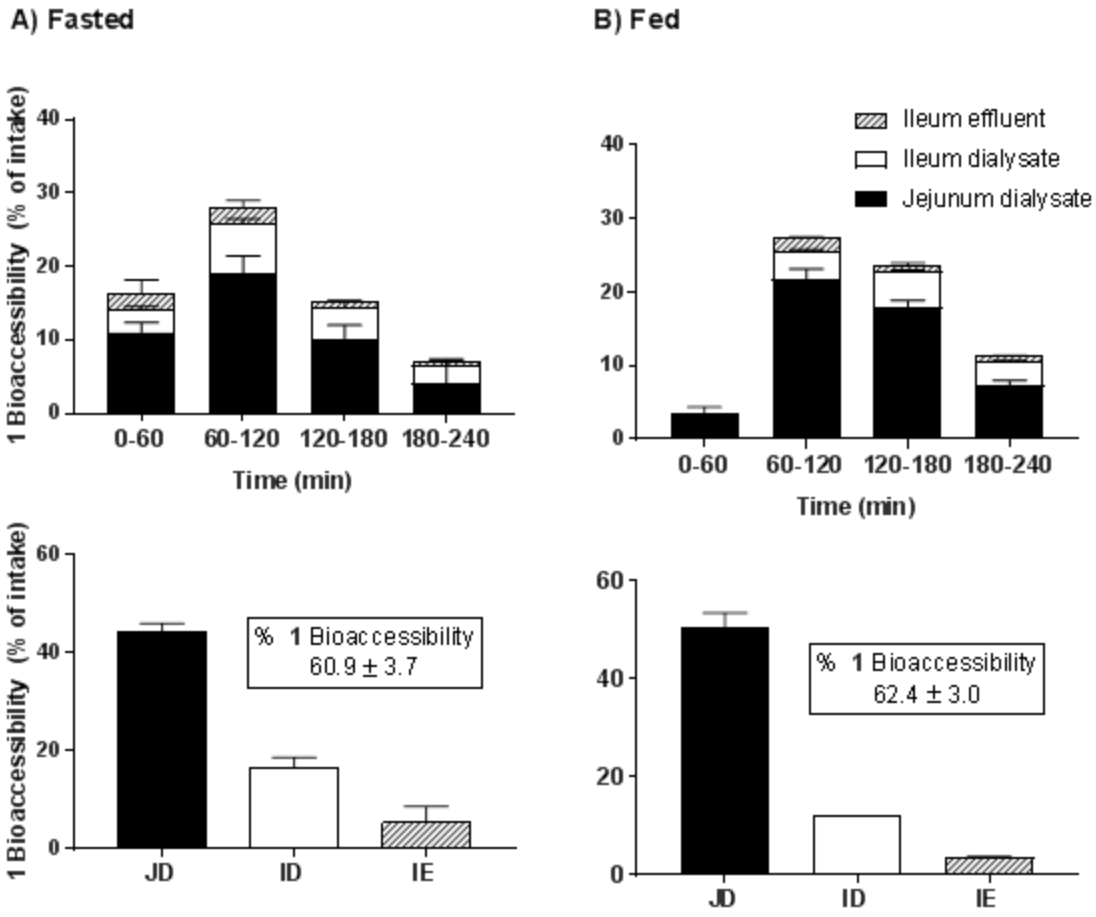

Figure 2.2. TIM-1 bioaccessibility measurements of MIC-1 delivered in MSE in A) fasted and B) fed states. Bioaccessibility is represented as the sum of the jejunum dialysate (JD) and the ileum dialysate (ID), while the ileum effluent (IE) indicates the portion entering the colon.

MSE is a unique isothiocyanate source because the extract is prepared in such a way that the MIC-1 precursor glucosinolate is converted efficiently to MIC-1 unlike dietary sources of isothiocyanates that may be incompletely converted. The efficient bioaccessibility of MIC-1 from MSE also confirms the stability of MIC-1 as a bioactive compound since it was not significantly degraded under the conditions of the upper gastrointestinal tract. For comparison, other plant compounds, such as anthocyanins from blueberry juice, were only 20% bioaccessible [45]. MIC-1 in MSE could therefore be effectively provided as a therapeutic through a variety of vehicles.

Isothiocyanates are often metabolized *in vivo* through well-documented pathways like the mercapturic acid pathway [47]. However, due to the unique structural motif of MIC-1, containing a conjugated rhamnose sugar, a variety of methods were evaluated to optimize serum sample preparation for MIC-1 quantification. Since we were able to detect unmodified MIC-1 from serum of animals treated with MSE, we needed to evaluate the persistence of MIC-1 in the serum from treated animals using alternative approaches consisting of hydrolysis (chemical or enzymatic) or direct partitioning. Acid hydrolysis has been used for the analysis of specific isothiocyanates [47] and should promote the release of MIC-1 from soluble proteins. The amount of MIC-1 recovered from acid hydrolyzed serum samples spiked with MIC-1 (10.8 +/- 1.5 µg/ml) was, however, the same as the MIC-1 recovered from spiked samples processed by simple partitioning (9.5 +/- 0.5 µg/ml). Moreover, serum samples from animals treated with MIC-1 from MSE and processed by acid hydrolysis released only 0.06 µg/ml MIC-1 while direct solvent partitioning of the same samples released 0.43 +/- 0.02 µg/mL, over 7-fold higher recovery. The recovery of MIC-1 from serum samples spiked with MIC-1

and processed using the solvent partitioning method was about 90%. Enzymatic hydrolysis was also investigated as a possible mechanism to release MIC-1 from a potential metabolically modified form using β glucuronidase/sulfatase digestion since many natural products, as well as drugs and toxins, are present in serum of animals or humans as glucuronide or sulfate conjugates [88]. Enzymatic digestion had a non-statistically significant negative impact on the amount of recoverable MIC-1 from serum of animals treated with MSE (3.81 +/- 0.78 µg/ml) relative to the same samples processed without enzyme treatment (5.65 +/- 3.92 µg/ml), suggesting that MIC-1 is not modified with glucuronide or sulfate. Based on the relative results from these methods, direct solvent partitioning was determined to be the most appropriate method to process samples for the bioavailability and pharmacokinetic studies with MIC-1 and MSE.

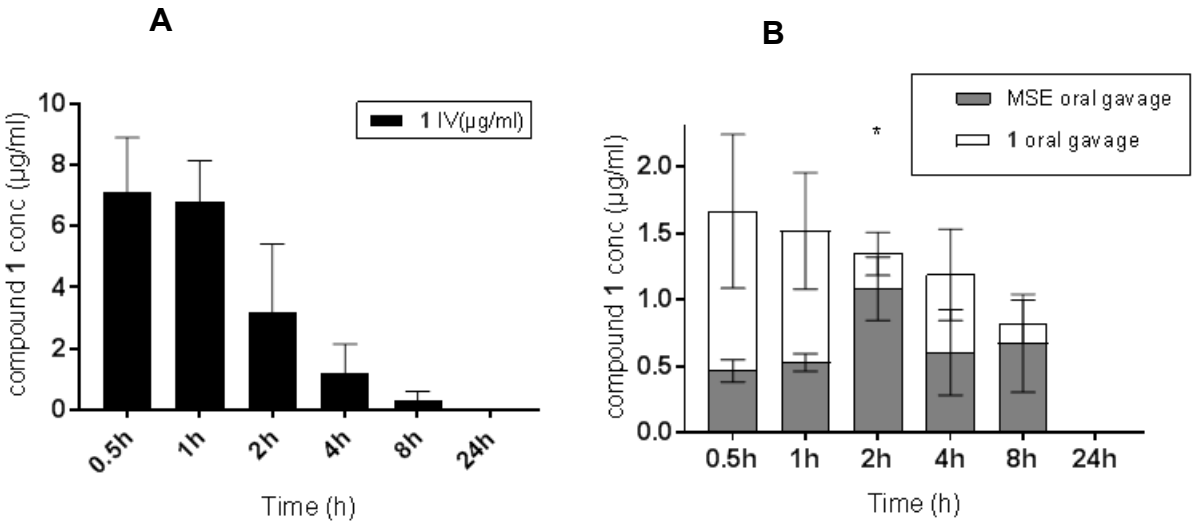

Figure 2.3. Serum concentration of compound 1 (MIC-1) recovered from rats after intravenous administration (IV) of pure MIC-1 at a dose of 50 mg/kg (A). Serum concentration of MIC-1 recovered in mice gavaged with either 50 mg/kg MIC-1 equivalent in a moringa seed extract (MSE) or 50 mg/kg of pure MIC-1 (B). Samples were analyzed for MIC-1 by UPLC–MS/MS. Data (n = 5) are represented as mean ± SD. Statistical significance determined using the Holm-Sidak method with $\alpha = 0.05$.

MIC-1 bioavailability and pharmacokinetics were evaluated from blood serum samples of rats dosed with MIC-1 or MSE, intravenously or via oral gavage. The half-life of MIC-1, administered intravenously, can be estimated to be close to 2 h (Figure 2.2A). Serum levels of MIC-1 were 6-12 times higher at 0.5 h in animals dosed intravenously compared to animals treated by gavage with MIC-1 or MSE at equivalent doses of MIC-1 (Figure 2.2 A & B). Serum concentrations of MIC-1, delivered by oral gavage, was initially much higher when purified MIC-1 was administered compared to levels from MSE administration. However, by 2 h, MIC-1 from MSE was at a maximum higher than

from the purified MIC-1. The 2 h maximum concentration of MIC-1 agrees with uptake pharmacokinetics of other isothiocyanates [89]. The differences in serum concentrations of MIC-1 between treatments with pure MIC-1 or MSE at the 2 h time point could be due to differences in bioavailability of MIC-1 from each of the preparations over time. Alternatively, an increase in the elimination of MIC-1 induced by the initially higher concentrations of MIC-1 in the serum could account for the difference, but elimination amounts were not measured. Between 8 and 24 h, serum concentrations of MIC-1 for all of the treatments fell below detection, suggesting that MIC-1 was either metabolized or eliminated entirely. MIC-1 concentrations in the feces or urine were not investigated in these experiments. UPLC-MS/MS analysis did not reveal any breakdown products of MIC-1, as determined by searching for mass fragments of MIC-1 in other peaks during the serum analysis. Interestingly, MIC-1 detection in serum did not require chemical or enzymatic hydrolysis. This is unusual, since most isothiocyanates are conjugated with glutathione and metabolized into mercapturic acid or dithiocarbamate products [47]. Other natural products such as polyphenols are typically glucuronidated or sulfated in the serum as a result of liver metabolism [90]. Perhaps MIC-1 is protected from typical isothiocyanate metabolism and other metabolic modifications because of its unique glycosidic motif.

Table 2.1.
Pharmacokinetic parameters of MIC-1**.**

	Treatment		
Parameter	MIC-1 IV	MIC-1 gavage	MSE gavage
Dose of MIC-1 (mg/kg B.W.)	50	50	50
$AUC_{0\text{-}8}$ (mg·h/L)	20.7	3.8	5.4
$AUC_{0\text{-}\infty}$ (mg·h/L)	21.5	4.2	n.d.
C_{max} (mg/L)	7.1	1.2	1.1
T_{max} (h)	0.5	0.5	2.0
CL (L/hr/kg)	2.3	--	--
CL/F (L/hr/kg)	--	2.3	n.d.
k_e (hr^{-1})	0.38	0.34	n.d.
T½ (hr)	1.8	2.0	n.d.
F*abs* (%)	100	19.5	n.d.

-- = not applicable
n.d. = not determined

The oral bioavailability of MIC-1 when administered to rats as a pure compound is approximately 20%, which is in line with our bioaccessibility determinations. The translatability of this value to humans, however, remains to be determined, but bioaccessibility infers that absorption would likely be significant. Unlike most isothiocyanates, we were able to determine the absolute oral bioavailability of MIC-1 when administered in a purified form. This is a rare and important accomplishment as the absolute oral bioavailability for most phytochemicals is unknown. Future studies utilizing a more rigorous blood sampling scheme between 8 and 24 h may allow for a more accurate assessment of MIC-1 bioavailability and its elimination when administered as MSE.

Calculations based on area under the curve indicate that 58% of MIC-1 delivered in MSE and 25% of MIC-1 delivered as a pure compound were recovered in blood. This is based on the assumption that the intravenous treated area under the curve was 100%.

The decreased MIC-1 serum concentration from orally gavaged MIC-1 may be due to incomplete absorption or metabolism prior to entering systemic circulation. Higher serum concentrations of MIC-1 from orally gavaged MSE relative to pure MIC-1 may be due to higher bioavailability of MIC-1 in the context of the seed extract versus the pure compound. Alternatively, the large error observed in serum levels of MIC-1 from gavaged animals, and the non-linear decrease observed at 2 h, may account for the lower actual differences for animals treated with MIC-1 than calculated by area under the curve. Differences in elimination may also have an effect as previously mentioned.

In conclusion, MIC-1 is a unique, bioactive isothiocyanate that is readily bioaccessible in both the fed and fasted state from MSE as measured using an *in vitro* model of the upper gastrointestinal system. The unique chemical structure of MIC-1 may protect it from typical isothiocyanate metabolism and other biochemical modification *in vivo*. Pharmacokinetic studies of MIC-1 follow similar trends to other isothiocyanates and show clearance of MIC-1 between 8 and 24 h. Although no metabolites of MIC-1 have yet been determined, these pharmacokinetic studies, together with previous *in vitro* and *in vivo* studies [75] suggest MIC-1 is bioactive in its unmodified form. Additional studies should explore elimination products of MIC-1 metabolism and include human pharmacokinetic studies with greater statistical power. Such work may provide the insight necessary to better understanding MIC-1 metabolism, and ultimately for the therapeutic utilization of this stable, bioactive isothiocyanate.

Acknowledgments

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CHAPTER 3: Anti-inflammatory effects of an isothiocyanate-rich moringa seed extract in a Collagen Induced Arthritis model

Abstract

Inflammation, the body's defense against pathogens and foreign matter, is characterized by redness, swelling, pain and heat. It is a major contributor to various diseases, including rheumatoid arthritis, cancer, chronic hepatitis, and atherosclerosis. This work evaluates the effectiveness of a moringa seed extract (MSE) that is rich in its main isothiocyanate moringa isothiocyanate-1 (MIC-1), against chronic inflammation. The Collagen Induced Arthritis model (CIA) mimics the symptoms and pathology of the autoimmune disease rheumatoid arthritis (RA) by injecting an emulsion of type II collagen and Complete Freund's Adjuvant (CFA) into the tail of Wistar rats. Upon onset of the disease, symptoms of a CIA model include edema, erythema, and pain at the hind and front paws of the rats. MSE was administered as an intervention, through oral gavage, at doses of 50, 100 and 200 mg/kg MIC-1 equivalent in MSE. Dexamethasone, a common glucocorticoid, was used as the positive control at 0.25 mg/kg. These treatments were administered at the inception of the disease's symptoms. The inflammatory response was monitored in two ways: arthritic scores and paw volume, measured with a plethysmometer. Both measurements were taken over time for a total of 15 days. The dexamethasone treated animals received its peak arthritic score of 4.3 on Day 1 and average change in paw volume of -0.29 ± 0.7 ml. The group that received only the CFA and type II collagen emulsion had its peak arthritic score of 4.9 on Day 5 and an average change in paw volume of 0.67 ±0.44 ml. MSE at 200 mg/kg MIC-1 had the highest score of all the treatments, with a peak score of 8.3 during Days 8-10, MSE at 100 mg/kg MIC-

1, peak score of 5.8 on Day 10, and MSE at 50 mg/kg MIC-1, a peak score of 6.4 on Days 5-12. Their respective changes in paw volume were 1.05 ± 0.69 ml, 0.56 ± 0.37 ml and 1.09 ± 0.74 ml. There was no effective dose of MSE at alleviating inflammation. However, previous research suggests that there is potential for MSE and MIC-1's use as therapeutic agents to be used on their own or in combination with prescribed treatments. It is therefore worth examining, in future research, the time of treatment at which MSE is administered during the development of this chronic inflammatory response.

Introduction

Inflammation, the body's defense against pathogens, is characterized by redness, swelling, pain and heat [91]. It can be acute or chronic, with the acute response defined as an increase in vascular permeability and cellular infiltration including fluid, proteins and leukocytes that are collected at the site of inflammation [92]. With chronic inflammation the symptoms occur over an extended period of time. It is a major contributor to various diseases that include rheumatoid arthritis, cancer, chronic hepatitis, and atherosclerosis [93] . In rheumatoid arthritis (RA), this autoimmune disease targets the smaller synovial joints that eventually lead to bone erosion and deformity. It affects 1-2% of the world's population, with an increased prevalence in the aging population [94].

Upon progression of the disease, the CIA model mimics several pathological features of RA. This includes synovial thickening, infiltration of mononuclear cells such us lymphocytes and monocytes, degradation of cartilage with susceptibility linked to the expression of MHC class II genes. The differences between the two include no

rheumatoid factor in CIA, CIA has no sex bias, and it is usually monophasic [65]. CIA is induced by injecting type II collagen, a major protein in cartilage and the target tissue of RA, and Complete Freund's Adjuvant into the tail of the rat, which leads to symptoms of edema, erythema, and pain at the hind and front paws of the animals. The development of CIA includes an infiltration of neutrophils and macrophages into the joint, along with T and C cell responses to type II collagen [95, 96]. We expect there to be an increase in expression of proinflammatory cytokines such as IL-1 and TNF-α at the area of inflammation as well as systemically, in the serum of blood [94]. The treatment of RA usually includes non-steroidal anti-inflammatory agents (NSAIDs), corticosteroids or disease-modifying anti-rheumatic drugs (DMARDs) [97]. There is no drug that can cure it completely. Since treatments are taken over prolonged periods of time, the introduction of complementary or alternative relief complementary or alternative relief from botanicals with little to no side effects is of increasing interest.

Moringa oleifera Lam., commonly referred to as moringa, is a member of the Moringaecea family and has been traditionally used for food and medicine for centuries. It is native to the sub-Himalayan tracts but can be found in tropical and subtropical climates [98]. The leaves, fruits, flowers, pods, and seeds of the plant are highly nutritious and contain many phytochemicals such as amino acids, alkaloids, antioxidants, glucosinolates, isothiocyanates [99]. The isothiocyanates have many beneficial biological effects including anti-inflammatory and anticarcinogenic actions and are therefore of special interest. These isothiocyanates are derived from their glucosinolates precursors, with the hydrolyzation of the glucosinolates to an aglycone R-C(-SH)=N-O-SO_3^- in the presence of the myrosinase enzyme (thioglucoside glycohydrolase) [28]. Moringa's main

isothiocyanate has been optimized in an ethanolic extraction method that involves a process of seed grinding and incubation in water and ethanol. This yields a seed extract that contains 35-45% of MIC-1 [100]. These MIC's, whether obtained from the seeds or in lower amounts from the leaves, have shown to be effective *in vitro* and *in vivo* in attenuating inflammation, hyperglycemia, hyperlipidemia while also linked to having antibacterial and antioxidant properties [25]. It is important to note that oral toxicology reports have shown that doses of up to 260 mg/kg bw/day MIC-1 are safe for rats [83].

This chapter focuses on the evaluation of a moringa seed extract (MSE) rich in its main isothiocyanate, moringa isothiocyanate-1 (MIC-1) as an effective anti-inflammatory agent in a chronic inflammatory model. The study evaluated MSE with 38.9% MIC-1 and its ability to attenuate the inflammatory response of Wistar rats challenged with RA in a CIA model. The animals were grouped into three treatment groups: 50, 100 and 200 mg/kg (all doses calculated by b/w) MIC-1 in MSE. A plethysmometer was used to measure the change in paw volume of the hind paws of the animals, along with a visual, subjective arthritic scores based on the intensity of swelling observed. MSE did not mitigate the swelling in the hind paws of the animals, and therefore no further investigations were performed.

Materials and Methods

Induction of RA using CIA model

The protocol of David Brand was adapted to induce CIA in Wistar rats [65]. Briefly, Complete Freund's Adjuvant (CFA) and type II collagen (from Chondrex) were

emulsified at a ratio of 1:1 using a tissue homogenizer until it was a thick, mayonnaise like consistency. The emulsion was carefully transferred to a syringe with a 26x ½ gauge needle and placed on ice until ready to be used. The animals were anesthetized with isoflurane with an injection site of 1.5cm-2cm distal from the base of the tail. Blood vessels were avoided. Limbs were observed 2-3 times per week with swelling of paws expected in 2-3 weeks, with a booster given at Day 14.

Experimental setup

Male and female albino Wistar rats were obtained from Charles River Laboratories (Malvern, PA). The animals were housed and acclimatized at Bartlett Hall vivarium in 12:12 h light dark schedule with access to food and water *ad libitum.* The MSE used in the experiment was standardized to 38.9 % w/w MIC-1 in MSE. The animals were randomly assigned to six treatment groups, five male, five female each: moringa seed extract (MSE) at 50 mg/kg, MSE at 100 mg/kg MIC-1, MSE at 150 mg/kg MIC-1, the positive control dexamethasone at 0.25 mg/kg, water only and the non-immunized control animals. Day 0 plethysmometer measurements were taken followed by induction of RA with the CFA collagen mixture. A booster was administered 14 days after, and incidence of arthritis was monitored for 2-3 weeks. Administration of the treatment commenced when swelling began. A plethysmometer was used to measure the paw volumes and an arthritic score was given by a single experimenter. The scores were assigned from a range of 0-4, with 0 being no evidence of swelling and erythema, 1, erythema and mild swelling confined to the tarsals or ankle joint, 2, erythema and mild swelling extending from the ankle to the tarsals, 3, erythema and moderate swelling

extending from the ankle to metatarsals joints, 4, erythema and severe swelling encompass the ankle, foot, and digits, or ankylosis of the limb. Each limb was scored and the average score for the individual animal was recorded. The animals were dosed once a day (10 ml/kg b/w) with the corresponding treatment at 10 ml/kg body weight. All dosing materials were prepared each day in deionized water. The animals were dosed, monitored, and measured for 15 days after incidence of inflammation. The experiments were carried out using an approved protocol by the Rutgers University Institutional Animal Care and Use Committee (Protocol # 15-064).

Arthritic scoring and measuring paw volume with plethysmometer

The paw volume was measured at the natural hairline using a plethysmometer (IITC Life Science Inc., Woodland Hills, CA). Scoring was done in accordance with the Brand protocol, where 0 was no inflammation, 1 is edema or erythema of one joint, 2 is edema or erythema one or two joints, 3 is edema or erythema of more than two joints on 1-3 digits, and 4 is severe arthritis with deformation or ankylosis of the paws. The arthritic index (AI) is the combined score of all the paws, with a maximum possible score of 16.

Results

Arthritic Score

Figure 3.1 reveals that the dexamethasone treated group had the lowest average arthritic score of all the treatments, with an average score of 4.3 on day 1, 1.4 on day 3, followed by a complete reduction in swelling at Day 5. The water treated animals maintained swollen paws for the duration of the experiment, with a peak score of 4.9 at Day 5. The scores decreased to 3.0 at Day 15. The 'not immunized' rats remained at a score of 0 throughout the entirety of the 15 days. MSE at 50 mg/kg MIC-1 peaked from Day 5-12 with a score range of 6.3- 6.4, decreasing to a score of 4.7 at Day 15. MSE at 100 mg/kg MIC-1 followed a similar trend of the water treatment group, with a peak of 5.8 at Day 10, decreasing to 4.0 at Day 15. MSE at 200 mg/kg MIC-1 had the highest incidence of swelling, peaking at 8.3 on Days 8-10 and maintaining a high score of 7.1 towards the end of the study, until Day 15.

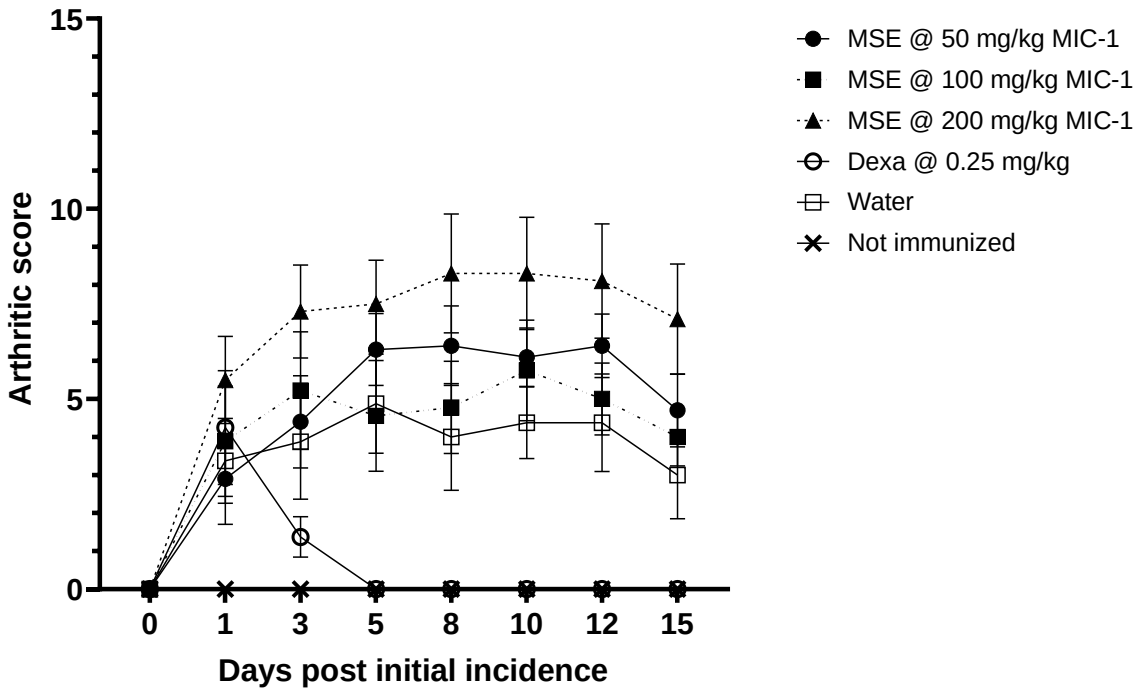

Figure 3.1: Dexamethasone reverses assigned score in an arthritic mouse model. Mice (n=10 per group) were assigned to treatment groups MSE at 50 mg/kg, 100 mg/kg, 200 mg/kg MIC-1, dexamethasone at 0.25mg/kg and some left untreated. The treated mice paws were scored based on a 0-4 scale for a total of 15 days. The scoring scale was based on the following features: 0: No evidence of swelling and erythema; 1: Erythema and mild swelling confined to the tarsals or ankle joint; 2: Erythema and mild swelling extending from the ankle to the tarsals; 3: Erythema and moderate swelling extending from the ankle to metatarsals joints; 4: Erythema and severe swelling encompass the ankle, foot, and digits, or ankylosis of the limb. Data is represented as Mean ± SEM. Data were analyzed using two-way ANOVA followed by Tukey's post-hoc test.

Change in paw volume

The paw volumes were measured simultaneously with the arthritic scores. Figure 3.2 displays the data obtained from the plethysmometer measuring the displacement of water in ml. The change in paw volume was calculated by subtracting each measurement from the initial Day 0 reading. There was an increase in paw volumes with the MSE treated animals when compared to the control groups. Dexamethasone was effective at reducing the paw volume with an average change of -0.29 ± 0.7 ml, going as low as -0.46 ml on Day 8. The water treated animals had an average change of 0.67 ± 0.44 ml and the 'not immunized' animals measured an average of 0.02 ± 0.16 ml. MSE at 100 mg/kg MIC-1 had the lowest change of all the MSE treated animals at 0.56 ± 0.37 ml. The highest MSE dose of 200mg/kg MIC-1 had an average of 1.05 ± 0.69 ml, more than twice the volume of the animals that received water. The lowest dose of 50mg/kg MIC-1 had the greatest change in the paw volume of 1.09 ± 0.74 ml. While MSE at 100 mg/kg MIC-1 seemed to have a lower change in paw volume than the water treated animals, there was no statistical significance observed.

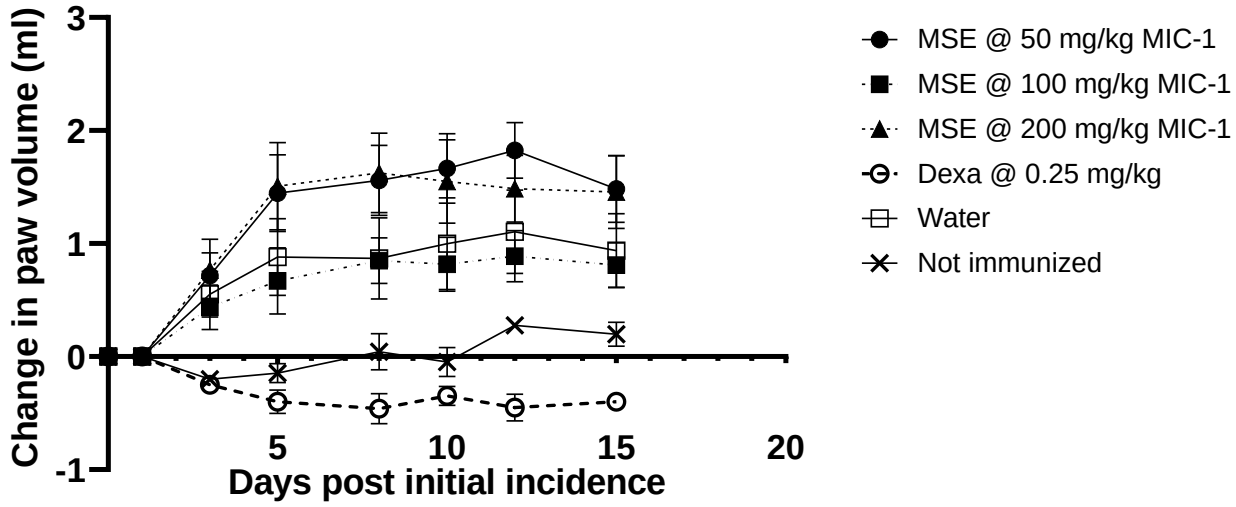

Figure 3.2: Dexamethasone reverses symptoms in an arthritic mouse model. Mice (n=10 per group) were assigned to treatment groups MSE at 50 mg/kg, 100 mg/kg, 200 mg/kg MIC-1, dexamethasone at 0.25mg/kg and some left untreated. The treated mice paws were measured using a plethysmometer. Data is represented as Mean ± SEM. Data were analyzed using two-way ANOVA followed by Tukey's post-hoc test.

Discussion

The CIA model was used to evaluate the effectiveness of a MIC-1-rich moringa seed extract as a potential anti-inflammatory treatment. Similar to human RA, the disease in rats is characterized by fibrin deposition, hyperplasia of synovial cells, mononuclear infiltrates, periosteal bone formation, and ankylosis (immobility of the joint) [101]. While there are many arthritic models used in research today, the CIA has been the most widely studied and shares several pathological features with RA. It is induced by the major protein in cartilage that is also the target tissue of RA, collagen type II (CII) [65]. The susceptibility to both CIA and RA are associated with the major histocompatibility complex (MHC) class II molecules, involving both T cells and B cells, that are mainly controlled by Th1and Th2 cytokines [101].

Many botanicals attenuate the effects of a CIA induced arthritis model. A sappanchalcone compound from the dried heartwood of *C. sappan* was administered via intraperitoneal injection and showed to mediate the effects of a CIA model in male DBA/1J mice. In doing so, it also regulated the level of pro-inflammatory cytokines including TNF-α, IL-1B and IL-6 [94]. Curcumin from *Curcuma longa*, a major active compound of turmeric and gingerol from *Zingiber officianali* (ginger) had a similar molecular response [103].

More recently, research showed that a combination of isothiocyanates sulforaphane and phenethyl isothiocyanate, along with naringin, decrease inflammation in a chromic rheumatoid arthritis model. Because of the poor bioavailability of these compounds on their own, they were encapsulated in liposomal formulations in order to improve delivery to the target site. Two biomarkers measured during the course of the

arthritis was C-reactive protein (CRP) released from the river in response to IL-6 and serum rheumatoid factor (RF) due to the activations of T cells and B cells via toll-like receptors [72].

Our laboratory has demonstrated the anti-inflammatory capabilities of MIC-1 and MIC-1 in MSE in many ways. RA in rats involve the expression of cytokines IL-1β, IL-6, TNF-α, INF-γ, granulocyte-macrophage colony stimulating factors and prostaglandins [72] Most recently, studies of MSE and MIC-1 have highlighted the effect on the Nf-κB and Nrf2 pathways by decreasing pro-inflammatory cytokines in an LPS induced sepsis model [100]. Many other studies also describe their efficacy [35, 36, 39, 75, 77, 79, 104] , so the success of MIC-1 in MSE in this specific inflammatory model was highly anticipated. Regrettably, the results did not support the hypothesis (Figure 3.1 and 3.2). Nevertheless, the data offers useful information to move forward with the next experiment. MSE administered at those specific doses did not reduce the arthritic scores or decrease the paw volumes, but rather seemed to have no effect and allowed for the uninterrupted continuation of disease progression. Further experimentation to investigate the molecular response or to visualize the effects with CT imaging could have been performed, but a decision was made to test another model (CPE: Chapter 4). Possible explanations for the ineffectiveness of MIC-1 in MSE could be the timing of administration. The oral dosing of moringa occurred post observable paw edema, at which point, the effects of RA may have been too severe for MSE to be effective. A positive result may have been observed if MSE was administered as a preventative, as is usually done with vitamins and supplements. The isolated MIC-1 could have also been tested alongside the extract to validate its bioactivity.

Acknowledgements

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CHAPTER 4: Anti-inflammatory effects of an isothiocyanate-rich moringa seed extract in a Carrageenan Paw Edema model

Abstract

Inflammation occurs frequently and is usually the result of defense against infection or other foreign material. The process is highly complex, but at the cellular level, it involves interactions among cytokines, along with leukocytes and endothelial cells that specifically play a role in the acute inflammatory response. Orally applied *Moringa oleifera* Lam., or moringa extracts, and its major bioactive, isothiocyanates, have been reported to attenuate major inflammatory processes. However, no studies have addressed the potential of moringa preparations for targeting inflammation when applied topically in an edema model. The goal of this work was to evaluate the effects of a 38.9% isothiocyanate-rich moringa seed extract (MSE) in an acute carrageenan-induced paw edema model. Carrageenan was made up into a 1% saline solution and injected into the subplantar surface of the hind paw of a Sprague Dawley rat. A formulation test was performed where the animals were treated 30 min prior to the injection of carrageenan with an ointment, gel or cream MSE enriched topical formulation. The ointment formula was selected and retested. At the 6 h time point, there was a 31.0 % increase in the vehicle control, diclofenac at 16 % increase, 1 % MIC-1 in MSE at 15.6 % and 5 % MIC-1 in MSE at 18.8 %. While there was a positive trend, there was no statistical significance observed $p>0.05$. Both lidocaine and diclofenac sodium gel, used in separate experiments, were shown to reduce paw volume. Further investigation into the ideal solvent, formulation, and delivery of MSE and MIC-1 could lead to their use as potential topical botanicals.

Introduction

Inflammation occurs frequently and is usually the result of defense against infection or other foreign material [105]. The body's initial response to trauma or infection is acute inflammation, a non-specific reaction and the first line of defense [106]. The acute inflammatory process is still highly complex with interactions involving cytokines along with leukocytes and endothelial cells [107]. The symptoms include edema, leukocyte infiltration and granuloma formation [96]. The concern in any inflammatory response is not necessarily how it starts, but how it resolves itself. What differentiates acute and chronic inflammation is acute inflammation resolves over a few hours or days, while chronic forms can persist indefinitely [105].

The acute inflammatory model evaluated in this study was a carrageenan paw edema (CPE) model, where MSE was evaluated as a topical anti-inflammatory treatment in Wistar rats. Carrageenan, a seaweed polysaccharide used to illicit inflammation, is made up into a 1% saline solution and injected into the subplantar surface of the hind paw of a Wistar rat [108]. This treatment induces a biphasic edema- an early inflammatory response that lasts 6 hours and a second late response that peaks at 72 hours, declining at 96 hours [91, 109].

The topical administration of therapeutics has benefits that include bypassing the body's gastrointestinal tract, reducing the systems drug levels, and optimizing the concentration while reducing drug interactions [110]. One example of treatment is a drug called Methotrexate, an orally administered disease-modifying antirheumatic drugs

(DMARDs) prescribed to RA and psoriasis patients. Its side effects include gastrointestinal disorders, pneumonitis, nephrotoxicity, hepatic dysregulations, hematological disorders, and infections [111]. The development of botanical therapeutics such as MIC-1 and MSE to be used alone or to complement the effects of topical anti-inflammatory drugs could be an alternative answer to the long-term side effects of oral drugs.

Moringa oleifera Lam. (moringa), a tropical tree native to Asia, is traditionally used as food and medicine for the alleviation of inflammatory-mediated conditions, antioxidant, antibacterial, hypotensive along with chemopreventative properties [77]. Cultivated in tropical and sub-tropical regions, it has an elevated nutrient content, rich in protein, vitamins and photoactives, which include a group of compounds referred to as isothiocyanates (ITCs) [25]. ITCs are formed from the enzymatic bioconversion of their glucosinolate precursors that are in the highest concentration in the seeds of the plant [75]). For our studies, we have used an optimized extraction method that, in brief, involves a process of seed grinding, water soaking and ethanol extraction (MSE) that yields 35-45% of MIC-1 w/w [100].

The goal of this work was to investigate the efficacy of a moringa seed extract with 38.9% MIC-1 at attenuating the inflammatory effects in a carrageenan-induced paw edema model. Male and female Sprague Dawley rats were used to assess the topical effects of MSE, prepared in three different formulations (ointment, cream, and gel) and applied directly and preventatively to the hind paws. Control formulations without MSE and typically prescribed medicines, anesthetic lidocaine and NSAID, diclofenac sodium were used. The most effective formula was selected and evaluated again to confirm

efficacy. Measurements were taken using a plethysmometer by measuring the change in volume through the displacement of water.

Materials and Methods

Animals

Sprague Dawley rats were obtained from Jackson Laboratories and acclimated at Bartlett Vivarium for one week in 12:12h light dark schedule. Access to food and water was ad libitum. The MSE used in the experiment was standardized to 38.9% w/w MIC-1 in MSE. In the first experiment, the animals were randomly assigned to six treatment groups with 4 animals each: gel control, 2 % MIC-1 in MSE gel, ointment control, 2 % MIC-1 in MSE ointment, cream control, 2 % MIC-1 in MSE cream, and lidocaine as the positive control. In the subsequent experiment, new animals were placed into four treatment groups, 10 animals per group. The treatments included diclofenac, 1% MIC-1 in MSE in ointment, 5% MIC-1 in MSE ointment, and an ointment control. The dose material was applied in even amounts by smearing over the paw with a sterile cotton swab.

Formulation development

Gel: The 2% MIC-1 in MSE (w/w) gel formula was created by mixing 15ml of water with 1g xanthan gum, 1g Brij and 0.87g of MSE. Half the water and xanthan gum were heated in a beaker on a hot plate. The remaining water was used to solubilize the MSE. It was vortexed, sonicated then added to the existing water and xanthan gum mixture. The

mixture was heated to 30-40°C and mixed using a stir bar while Brij was slowly added. The control gel formulation was the same, excluding the MSE.

Cream: The 2% MIC-1 in MSE (w/w) cream formulation was a mixture of water, beeswax, and oil. Grapeseed oil, 8.5 g, was used to as to limit any adverse allergic reactions from any nut-based oils. The oil was first heated slowly in a beaker on a hot plate. The beeswax, 1g, was cut into smaller pieces and added to the hot oil and allowed to slowly melt. An emulsifier was used to mix 4 ml water containing 0.7 g MSE into the oil and beeswax mixture to create a fluffy, smooth texture. The water was slowly added using a Pasteur pipette. The control cream formulation did not have MSE in the water.

Ointment: The 2% MIC-1 in MSE (w/w) ointment formulation was a combination of grapeseed oil and beeswax at a ratio of 8.5 g oil to 1g of beeswax. The two were melted in a beaker on a hot plate slowly over low heat. MSE, 0.5 g, was weighed out and added to 1 ml DMSO. DMSO was used to solubilize the MSE. The MSE infused DMSO was added to the melted oil and beeswax in small amounts and mixed to combine. The control ointment formulation did not have MSE added.

Preparation of the Carrageenan solution

To prepare the carrageenan solution, a beaker of 50 ml Millipore water was heated to about 90°C on a hot plate. It was important that it was not allowed to come to a boil. While heating, the top of the beaker was covered with parafilm first, then a glass dish to prevent evaporation of the water. When the temperature was reached, 0.5 g of carrageenan powder was added to the water so as to create a 1% solution. It was mixed

with a stir bar until completely dissolved (about 45 minutes). The solution was added to a bottle and autoclaved before use.

Carrageenan-induced inflammation

The model for inflammation in rat paws was induced by following the protocol as mentioned by Rahmawati [112]. In short, the animals were treated topically with the corresponding treatments 30 minutes before 100ul of a 1% w/v λ-carrageenan solution in saline was injected into the plantar region of the right hind paw of each animal after anesthesia. The volume of the paw was measured using a plethysmometer (IITC Life Science Inc., Woodland Hills, CA) before treatment, time 0, and then every hour up to 4 hours post carrageenan induction.

Results

At hour 2, there was a statistically significant difference in the paw edema between the gel control and 5 % MIC-1 in MSE in gel, and the gel control and the positive control lidocaine (Figure 4.1). The gel control had an increase of 15 % while the 5% MIC-1 in MSE gel had an increase of 0.8 %. Lidocaine decreased the paw volume, with a decrease of 5.4 %. At hour 4 and 6, the peak percent increase in paw edema was seen in the controls of the gel, cream, and ointment respectively. At hour 4, each of the control formulations had larger increases in paw volume with the gel control at 32.1 %,

cream control at 30.4 % and the ointment control at 26.0 %. Their MIC-1 in MSE equivalents did not have statistically significant differences, however, there was a statistically significant difference observed between each of the control formulations and lidocaine, which had a decrease of 1.3%. There was also a statistically significant difference at between the gel control and the 5 % MIC-1 in MSE ointment, and the cream control and 5 % MIC-1 in MSE ointment. The 5 % MIC-1 in MSE ointment had 5.7 % increase. At hour 6, there is a statistically significant difference between gel control and lidocaine, cream control and lidocaine and 5 % MIC-1 in MSE gel and the cream control. At this time point, lidocaine had 0.9 % increase with gel control at 33.8 %, 5% MIC-1 in MSE gel at 10.9 %, and cream control at 28.5 %. The ointment was selected as the most effective formulation based on the consistently lower results in the response over the 6-hour test period. The following experiment would include an ointment formulation at 1% MIC-1 in MSE, 5% MIC-1 in MSE, the vehicle control and diclofenac sodium gel formulated in the ointment vehicle for consistency.

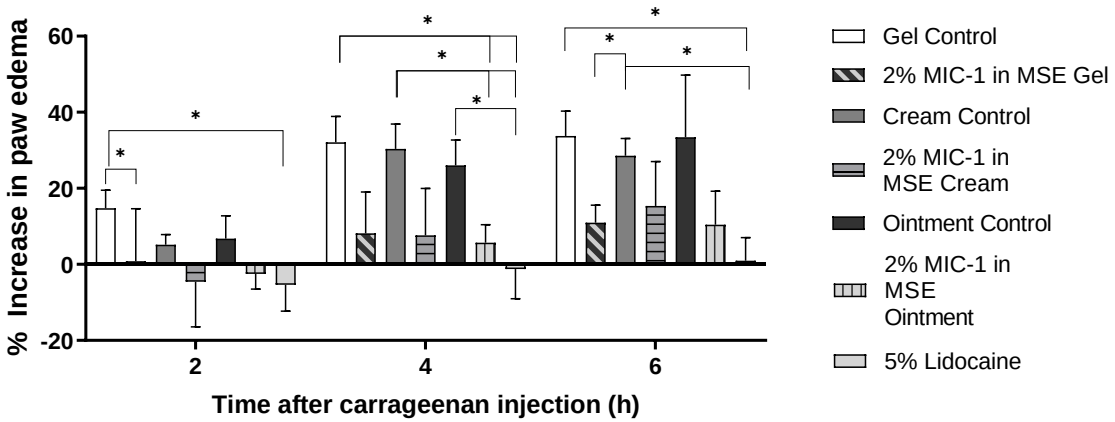

Figure 4.1 Effect of 2% MSE in a gel, cream and ointment formulation in a Carrageenan induced ear edema model. Graph shows the percentage of increase in paw edema as measured by a plethysmometer. The 2% gel, cream and ointment were able to significantly reduce the paw volume. Data is represented as Mean ± SD of 4 animals. Statistical significance determined by Two-way ANOVA with Tukey's post-hoc test; *$p<0.05$.

When the experiment was repeated with a new subset of animals, 10 animals per treatment group, there was no statistically significant difference observed, however, there was a trend of both 1% and 5 % MSE decreasing the paw volume at each of the time points, compared to the vehicle control, with 1 % MSE being consistently lower (Figure 4.2). At the 6 h time point, there was a 31.0 % increase in the vehicle control, diclofenac at 16 % increase, 1 % MIC-1 in MSE at 15.6 % and 5 % MIC-1 in MSE at 18.8 %. While there was a positive trend, there was no statistical significance observed.

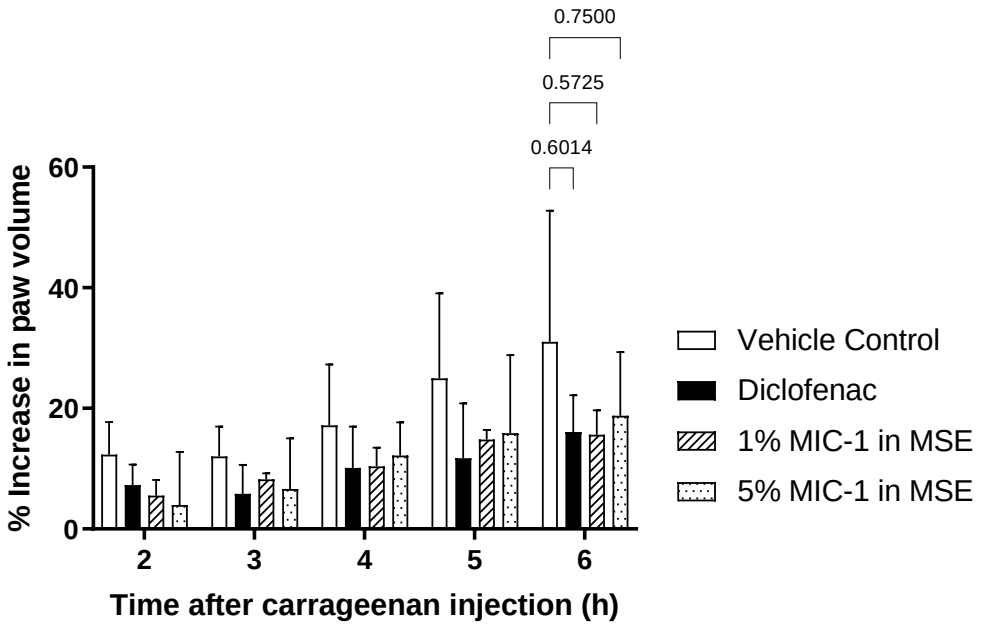

Figure 4.2 Comparison between 1% and 5% MSE in ointment formulation in a carrageenan induced paw edema model. Data is represented as Mean ± SD of 10 animal. Statistical significance determined by Two-way ANOVA with Tukey's post-hoc test. There was no statistical significance at $p>0.05$.

Discussion

The CPE model is an acute biphasic inflammatory model where the mediators released in the first phase are histamine, 5-hydroxytrptamine, bradykinin and platelet activating factor. After hour 3, there is an increase in phagocytic inflammation with an influx of neutrophils and tissue edema [113]. There are 4 stages in an acute inflammatory response: fluid buildup that delivers plasma proteins to the site, influx of neutrophils in the attempt to remove pathogens and cellular fragments, vasodilation of blood vessels

and an increase in temperature and lastly, pain or loss of function further causing tissue damage [114].

An earlier research study demonstrated that the use of a combination of isothiocyanates sulforaphane and phenethyl isothiocyanate, along with and another botanical naringin, could decrease inflammation in a chromic rheumatoid arthritis model. However, because of the poor bioavailability of these compounds on their own, they were encapsulated in liposomal formulations in order to improve delivery to the target site. In a carrageenan induced paw edema model, the release of mediators serotonin and histamine occur in the first phase, then kinins in the second and then prostaglandin in the third [72].

Moringa seed extract (MSE) has traditionally been used for medicinal purposes and more recently, there is reported evidence of its strong anti-inflammatory effects. A study showed that 30-300mg/kg p.o. doses of moringa leaf extracts were administered orally to rats to investigate their anti-inflammatory responses in both a carrageenan induced paw edema and collagen induced arthritis models. The moringa leaf ethanol extract showed a non-dose but significant effect against inflammation through inhibition of the prostaglandins-mediated potential of analgesic action of bradykinin [114]. The goal of this current study was to evaluate the topical efficacy of a moringa seed extract in an acute inflammatory model. While many previous studies have been performed on the oral anti-inflammatory efficacy of MSE and MIC-1 [7, 25, 35, 36, 39, 75, 77, 104, 116], there is no research evaluating the topical effects of MIC-1 derived from seeds in an edema model. It was expected that MSE would alleviate the swelling and subsequent infiltration of neutrophils, cytokines, or chemokines at the site and within the system of the animals. However, while the data did show a potentially meaningful outcome in the first

experiment (figure 3.1), and while there was a positive trend in the second (Figure 3.2), the results were not statistically significant. No further molecular work was done due to lack of significant treatment efficacy.

There were a few possibilities as to why the model was not effective. While the formulations included 1, 2 or 5 % MIC-1 in MSE, there was no way of measuring the diffusion of active compounds through the skin-barrier into the affected area of the paw. The formulations may need to be improved by testing the solubility of MIC-1 and selecting the ideal solvent that allows for ultimate topical or even transdermal delivery. Lastly, isolated MIC-1 would need to be included in the testing, to confirm its effectiveness as the main active in MSE. After all these considerations, formulation development could follow, and pharmacological effects of moringa botanicals could be assessed. Therefore, a new model, TPA induced ear edema, was chosen to further study the topical application of an isothiocyanate rich-MSE, with the inclusion of the purified MIC-1. It is also important to note that the switch from lidocaine to diclofenac sodium was made to include a more appropriate positive control. Diclofenac sodium is a an NSAID typically used to reduce inflammation as opposed lidocaine which is typically used as an anesthetic [117]. Diclofenac sodium was purchased as a salt and was formulated in the ointment similarly to the MSE for consistency.

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The author would like to thank Dr. Asha Jaja-Chimedza, Dr. Alexander Poulev, Dr. Charlene Van Buiten, Kristin Moskal, Irina Tmenova, Dr. Peter Kuhn and Dr. Sarah Moroianu for their help in performing the experiments for this study.

CHAPTER 5: Anti-inflammatory effects of an isothiocyanate rich moringa seed extract in a TPA induced ear edema mouse model

Abstract

Moringa (*Moringa oleifera* Lam.) seed extract (MSE) and its primary bioactive compound, moringa isothiocyanate-1(MIC-1), mitigate inflammation, oxidative stress, diabetes, and cancer in both *in vitro* and *in vivo* models. The present study elucidates the topical anti-inflammatory effects and mechanisms of action of MSE, containing 38% of MIC-1 and purified MIC-1 using a mouse ear edema model which utilizes 12-O-tetradecanoylphorbol-13-acetate (TPA), as the pro-inflammatory agent. A time-dependent and dose-dependent response was determined by pretreating CD-1 mice with various doses of MSE and MIC-1, dexamethasone, or vehicle control, followed by TPA, and the subsequent difference in ear thickness was measured using digital Vernier calipers. The effective doses of MSE and MIC-1, at 2 mg/ear and 0.8 mg/ear; respectively, were then selected to evaluate the change in weight of the ears using 6 mm biopsy punches. MSE and MIC-1 were effective in a dose-dependent manner in a TPA-induced ear edema model, causing a reduction in ear thickness and a 48% and 49 % decrease in ear punch weight, respectively. MSE and MIC-1 also caused a reduction in the levels of cytokine and chemokines, interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and keratinocyte chemoattractant (KC) in the ear tissue. MSE and MIC-1 reduced IL-6 expression by 84% and 78%, monocyte chemoattractant protein-1 (MCP1) by 74% and 73%, and KC by 56% and 43%, respectively. Additionally, the anti-inflammatory effect of MSE and MIC-1 was confirmed by hematoxylin and eosin (H&E)

staining, used to assess the thickness of the ear swelling. MSE significantly reduced the thickness of the ears by 20% compared to TPA. These results further support the general anti-inflammatory properties of MSE, and MIC-1 likely transmitted via the nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor-kappa B (NF-κB) pathways as mentioned in previous studies. This work may also suggest therapeutic uses of MSE and/or MIC-1 for skin inflammation.

Introduction

In addition to its barrier function, inflammation is the skin's natural defense mechanism against a foreign substance [118]. There is an extensive relationship among epithelial, stromal, and immune cells that regulate the skin's immune response in skin to ensure an effective defense and maintain homeostasis [57]. When an offending agent such as bacteria, chemicals, or injury is present, the early inflammatory response attracts neutrophils and macrophages directly to the site. This elicits two responses, innate or adaptive, both of which involve immune cells that target the site of inflammation [119]. The severity of topical inflammatory conditions can range from acute to chronic and include disorders such as atopic dermatitis, psoriasis, and hidradenitis suppurativa [120]. All of these conditions involve the recruitment and activation of specific immune cells that are regulated by transcription factors such as Activator Protein 1 (AP-1), Nuclear Factor-κB (NF-κB), Nuclear Factor of Activated T-cells (NFATs), Signal Transducer and Activator of Transcription (STATs) proteins, and production of numerous cytokines and chemokines [121, 122].

While oral non-steroidal anti-inflammatory drugs (NSAIDs) are typically prescribed for the treatment of inflammatory conditions, they are responsible for about

one-quarter of all adverse drug reactions reports that are made globally [123]. Given the burden that inflammatory skin conditions have on older adults and persons who cannot be prescribed oral drug products for extended periods, the use of topical NSAIDs or corticosteroids has been increasingly preferred by patients and prescribed by healthcare providers [124]. The introduction and use of topical corticosteroids is the most significant contribution to the treatment of dermatological disorders. However, patients may develop side effects as a result of prolonged use of topical treatments for inflammation. For example, cutaneous atrophy, steroid rosacea, perioral dermatitis, the development of telangiectasia (widened blood vessels), and skin infections are a few of the side effects that have been observed [125]. Therefore, the use of a natural, plant-derived anti-inflammatory agent may provide a safer, long-term alternative to traditional pharmaceutical treatment options.

Commonly known as moringa, *Moringa Oleifera* Lam., a plant which has been shown to be effective against inflammation. Moringa is traditionally grown and cultivated in tropical and sub-tropical climates and has been used for many years as food and medicine. The Moringacea and Brassicaceae families contain a group of compounds called glucosinolates which are converted to isothiocyanates by the enzyme myrosinase. Isothiocyanates have several health benefits including anti-inflammatory, chemoprotective, and antioxidant effects. Moringa isothiocyanate-1 (MIC-1), the isothiocyanate which is produced in the most significant amount in the seed extract of moringa, also has these same properties [100]. Moreover, it is uniquely stable, bioaccessible, and bioavailable, possibly due to the addition of a sugar moiety that other isothiocyanates lack [126]. Moringa seeds and their main bioactives, isothiocyanates, are

known to mitigate weight gain, insulin resistance and bowel inflammation by activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant pathway and inhibition of nuclear factor-kappaB (NF-κB) pathways [75, 79, 100]. Additionally, oral toxicological studies in rats suggest that moringa seed extract (MSE) is safe at 260 mg/kg bw/day [83].

This study evaluates the ability of an isothiocyanate-rich moringa seed extract and its main active, MIC-1, to reduce inflammation in a 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced mouse ear edema model. We found that both MSE and MIC-1 suppressed skin inflammation in a dose-dependent manner, reduced the thickness of the skin in the ear model, and decreased cytokine and chemokine, MCP-1, KC and IL-6 infiltration. These are important markers involved in the inflammatory process, specifically the NF-κB pathway. We suggest that MSE and MIC-1 may be further studied and possibly developed as natural, topical anti-inflammatory agents.

Materials and Methods

Preparation of MSE and MIC-1

The moringa seed extract (MSE) obtained from The Jamaica Moringa Farmer's Association (Kingston, Jamaica) was prepared as previously described [77]. Briefly, moringa seeds were weighed, ground in a blender, and incubated on a shaker kept at 37^{o}C for 2 h in water at a ratio of 1 g seed powder: 3 ml water. Thereafter, ethanol was added to the mixture in a ratio of 1 ml water: 4 ml ethanol, and the slurry filtered and dried using a rotary evaporator and a freeze drier. The MSE was then stored at -20^{o} C. MIC-1 content in MSE was quantified by LCMS as described [126]MIC-1 was isolated and

purified by resuspending 2 g of the freeze-dried MSE in a 10 mL solvent system comprising a mixture of hexane, ethyl acetate, methanol, and water in a 4:6:4:6 ratio [127]. The resuspension was vortexed, filtered, sonicated for 10 minutes, and subjected to Fast Centrifugal Partition Chromatography (liquid-liquid chromatography) in FCPC®1000 Kromaton v1.0. The wavelength on the UV detector was set to 229 nm. The peak at about 100 minutes was collected, and solvents the removed via rotary evaporation and freeze drying. Purified MIC-1 (at least 90% purity) is a white, crystalline powder and was stored at -20^{o}C for later use. For animal experiments, MSE and MIC-1 were prepared in 10% ethanol solution.

Mice

Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Rutgers University (protocol # ID999900474), following ethical guidelines. Male CD-1 mice, 6-8 weeks old, were purchased from Charles River Laboratories (Malvern, PA) and acclimated for one week under controlled temperature (22 ± 2°C), humidity (40-60%), and in a light/dark cycle for 12 h. The experimental and control mice were co-housed, 4 per cage, and allowed access to food and water *ad libitum*.

Optimization of MSE and MIC-1 in topical delivery

The delivery of isolated MIC-1 or MIC-1 in the MSE needed to be optimized to improve its permeation across human cadaver skin. Since the properties of both the compound and solvent affect its ability to cross the skin barrier, we investigated the

permeation of MIC-1 in solvents with a variety of properties including methanol, ethanol, propylene glycol, polyethylene glycol, 50% ethanol in water, 50% methanol in water, PBS, 50% ethanol in PBS. In vitro permeation studies were performed using the Franz Diffusion cell technique to determine the compound-solvent system which would provide the highest rate of release for MIC-1 and therefore the best topical/transdermal delivery. Briefly, human cadaver skin was prepared by thawing in PBS (pH 7.4), and cut into roughly 2-inch sections to cover the receptor compartment. The cells were maintained at 37°C with continuous stirring to allow for complete mixing in the receptor compartment and uniform sampling for the entirety of the test. The donor compartment was loaded with MSE or MIC-1 in solvents at various concentrations and samples were collected from the receptor compartment at 0, 2, 4, 8, 12 and 24 hours. Samples were analyzed using LCMS to quantify the amount of MIC-1 that was released over time. 10% ethanol provided the highest release rate of MIC-1 over time. This was the first step in optimizing a formulation for MIC-1. The melting point was found to be between 95-100°C when using a standard chemistry lab melting point apparatus. This work was performed in collaboration with the Center of Dermal Research, lab of Dr. Bozena Michniak-Kohn with graduate student Keyaara Robinson.

TPA-Induced Mouse Ear Edema Model

Mice were randomly assigned to five treatment groups and anesthetized under 2-5% isoflurane for 10 minutes for the ear edema experiments. TPA was prepared at a concentration of 0.1 µg/µl (2 µg/ear) and dexamethasone was prepared at a concentration of 2.5 µg/µl (0.05 mg/ear) in acetone. MSE and MIC-1 were both prepared in 10%

ethanol, with the MSE dosed at 6.4 (0.128 mg/ear), 12.8 (0.256 mg/ear), 50 (1 mg/ear), 100 (2 mg/ear) μg/μl in 20 μl. MIC-1 doses included 2.5, 5, 20, 40 μg/μl or 0.05, 0.1, 0.4, 0.8 mg/ear in 20 μl.

'Untreated control' animals received no treatment. 'TPA' animals received only TPA on the right ear, 2 μg/ear, and 20 μl of the vehicle control, acetone, on the left. The 'TPA + dexamethasone' group, or positive control animals, received 0.05 mg in 20 μl dexamethasone in acetone on the right ear followed by TPA, with the left control ear receiving 20 μl of acetone. The 'TPA + MSE' group received the dose of MSE (dependent on the experiment) and then TPA, with the left ear receiving 20 μl of both the vehicle controls, 10 % ethanol followed by acetone, the vehicles for MSE and TPA respectively. Similarly, for the 'TPA + MIC-1' group, receiving the specific dose of MIC-1 on the right ear, followed by TPA, with the left ear receiving 10 % ethanol and then acetone. Consistent for all treatments was the administration of TPA 20 min posttreatment application.

At each time point, 2, 4, and 8 h post-TPA treatment, the ear thickness was measured using digital Vernier caliper. A sterile 6 mm biopsy punch (VWR) was used to collect the biopsy samples which were then used for cytokine and chemokine evaluation with LUMINEX as reported [128].

Ear tissue and serum collection

The animals were euthanized via CO_2 inhalation and then cardiac puncture to prepare the samples for cytokine and chemokine analysis. The blood collected from the cardiac puncture was allowed to clot for 30 min at room temperature before

centrifugation to collect the serum. After cardiac puncture, 6 mm biopsy punches of the ear tissue were collected and immediately placed into tubes with homogenizing beads and extracted using 10 mL of Tissue Extraction Reagent, a Tris-based lysis buffer for total protein extraction, supplemented with proteinase inhibitor, for every 1 g of tissue. For serum collection, the blood was centrifuged at 1500 x *g* for 10 min and then placed in Eppendorf tubes on ice. The ear tissue samples were homogenized, centrifuged, and the supernatant collected. The amount of protein was quantified using a standard protein assay kit. The ear tissue samples were prepared at 1µg/µl of protein and along with the serum samples, analyzed at the Cancer Institute of New Jersey core facility.

LUMINEX Analysis

The Luminex assay was performed following the vendors' protocol at the Rutgers Cancer Institute of New Jersey Immune Monitoring Shared Resource. Briefly, cytokines and chemokines were measured using the 10-plex Millipore Milliplex Catalog ID. MCYTOMAG-70K-10 MouseCytokine MAGNETIC Kit which contains; interleukin-1α (IL-1α), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-1B (IL-1B), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1), keratinocyte chemoattractant (KC), interferon gamma (IFNγ), interleukin-12p40 (IL-12p40), interleukin-12p70 (IL-12p70). Ear protein samples and serum samples were analyzed on a 96-well plate. Using a Luminex 200 system with Luminex xPONENT software (Luminex Corp.; Austin, TX, USA) using a 5PL curve. For a given sample and analyte, concentration values were discarded if readings were <30 beads. Analytes falling above

or below these values were inputted to the lower or upper limit of quantification, respectively.

Hematoxylin and Eosin Staining

Two different subsets animals were used (n=14 each) to collect ear biopsy samples and whole ears for hematoxylin and eosin (H&E) staining. Staining and analysis was performed by the Research Pathology Services in the Office for Research (Rutgers, New Jersey, USA). Whole ear samples were collected 7 h posttreatment and fixed in 10% neutral-buffered formalin solution at room temperature. The H&E staining from 4-6 biological replicates per treatment group was performed blinded. During the preparation and evaluation of the slides, the pathologist was also blinded to the treatments performed for each sample. The edema scores were assigned as follows: 0= no edema, 1=mild, 2= moderate, 3=severe. Inflammation scores were also assigned, both based on the degree of increase in dermal interstitial fluid. The Aperio ImageScope software was used to analyze the thickness of the slides at three random locations in the images of each of the treatments.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism Version 9. Comparisons between treatment groups were performed using one-way and two-way ANOVA, followed by Tukey's multiple comparison test. A p value < 0.05 was considered statistically significant.

Results

MSE and MIC-1 reduce ear edema in a dose dependent manner

To investigate the dose and time effects of MSE and MIC-1 on the TPA-induced ear inflammation, the ear thickness was measured at 0 h for the baseline, then at 2 and 4 h post-TPA application as per experimental diagram (Fig. 1). The animals were pre-treated on their right ears with either the vehicle, 0.5 mg/ear dexamethasone, varying concentrations of MSE, 0.128 mg, 0.256 mg, 1 mg, and 2 mg/ear in 10% ethanol or the equivalent dose of MIC-1, 0.05 mg, 0.1 mg, 0.4 mg, and 0.8 mg/ear also in 10% ethanol. The left ears received 10% ethanol and/or acetone as the vehicle controls. To quantify the treatment's effect, the difference between the thickness of the right ear and the left control ear was calculated. Data indicated that 4 h is the least amount of time, post-TPA administration, to observe a statistically significant response in edema (Figs. 2B-C). Fig. 2A shows images of the changes in the ear morphology at the 4 h time point for each treatment. Untreated ears (i) are thin and white, thinner towards the tips with visible capillaries. The right ear of the TPA treatment (ii) is thicker and redder in appearance than the left control ear of the animal and compared to the right ears of the other treatment groups. The animals that received the TPA + dexamethasone (iii), TPA + MSE (iv) and TPA + MIC-1(v) showed a reduction in the TPA-induced swelling and redness of the right (treated) ear.

Four hours after treatment, there was a statistically significant decrease in the ear thickness in the TPA + MSE group at all doses as compared to the TPA group (Fig. 2B). TPA alone increased the thickness of the right ear by 0.171 ± 0.01 mm. The lowest concentration of MSE, 0.128 mg/ear, reduced thickness deferential to 0.102 ± 0.02 mm, or a 40% decrease compared to TPA alone. MSE at 0.256 mg/ear reduced thickness deferential to 0.099 ± 0.01 mm, or a 42% decrease, MSE at 1mg/ear resulted in 0.091 ± 0.01 mm thickness differential or a 47% decrease and MSE at 2 mg/ear produced 0.032 ± 0.01 mm differential, an 81% decrease compared to TPA treatment. The positive control, dexamethasone, reduced the swelling thickness by 0.075 ± 0.012 mm or 58%. There was no statistical difference observed at 2 h for any of the treatments. A similar trend 4 h posttreatment was observed in animals treated with TPA and MIC-1 (normalized for a dose provided by the MSE), with a decrease in ear thickness of 0.141 ± 0.03 mm or 18% for MIC-1 at 0.05 mg/ear, 0.110 ± 0.03 mm or 36% for MIC-1 at 0.1 mg/ear, 0.030 ± 0.01 mm or 82% for MIC-1 at 0.4 mg/ear and 0.014 ± 0.01 mm or 92% for MIC-1 at 0.8 mg/ear (Fig. 2C). Again, there was no statistically significance reduction in thickness at 2 h. MSE at 2 mg/ear and MIC-1 at 0.4 mg/ear and 0.8 mg/ear outperformed the positive control dexamethasone. Therefore, MSE at 2 mg/ear and MIC-1 at 0.8 mg/ear were selected for further experiments.

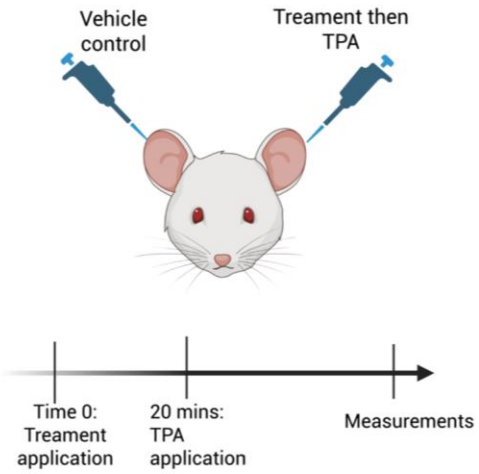

Figure 5.1. Diagram of the experimental design for the TPA-induced ear edema model. The right ear of the animals received the experimental treatment (dexamethasone at 0.05 mg/ear, MSE at 0.128, 0.256, 1, 0r 2 mg/ear, MIC-1 at 0.05 mg, 0.1 mg, 0.4 mg and 0.8 mg/ear) applied 20 min before TPA (2ug/ear), while the left ear received the vehicle control (20 µl of 10% ethanol and/or acetone) at the time of the TPA treatment. Untreated control animals received nothing, while TPA animals received TPA on the right ear and acetone on the left. For the ear thickness, the measurements were taken at 2- and 4-h post TPA application. For the ear biopsy experiments, measurements were taken 4 h post TPA application. For the H&E staining, ears were collected at 7 h post TPA treatment.

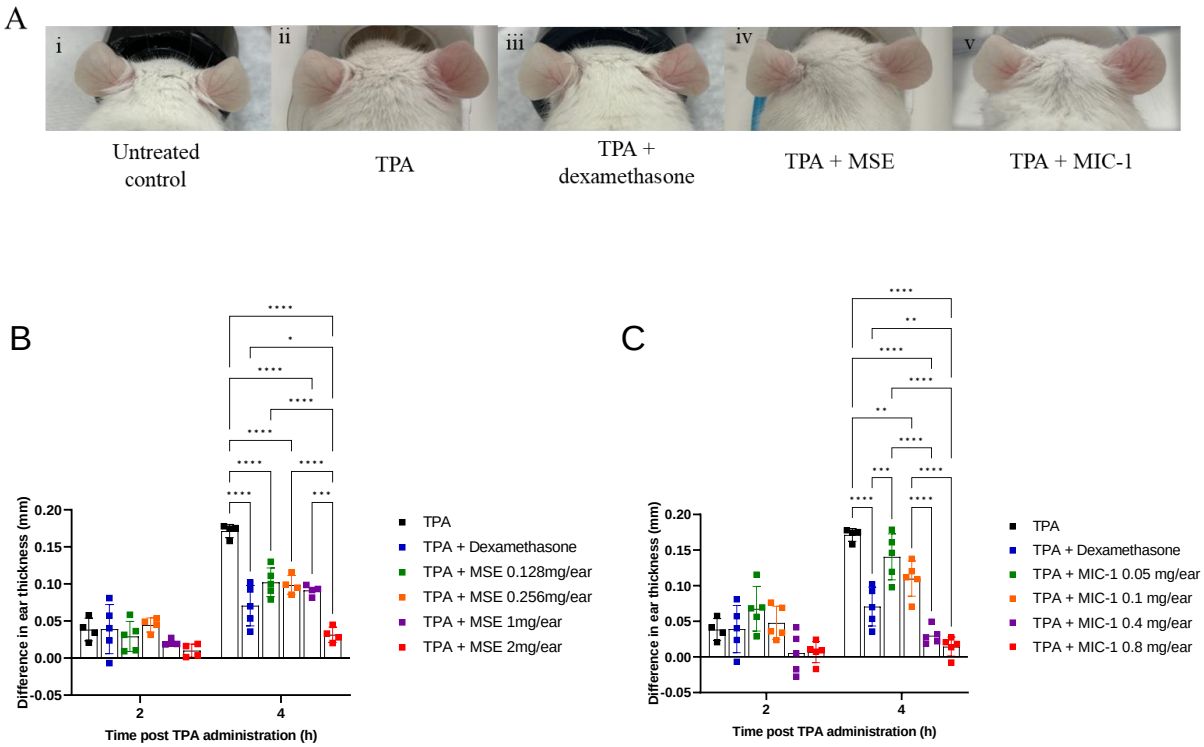

Figure 5.2. Images of mouse ears subjected to experimental treatments (A). Effects of MSE-1 (B) and MIC-1 (C) on the TPA-induced ear edema of the right ear, measured as reduction of the ear thickness compared to the untreated 20 µl of 10% ethanol and/or acetone left ear. Photos represent each of the treatment groups, TPA (2 µg/ear), TPA + dexamethasone (0.5 mg/ear), TPA + MSE (2 mg/ear) and TPA + MIC-1 (0.8 mg/ear). TPA was administered 20 min post treatment. The photos were taken 4 h post TPA administration. Data (mm) were expressed as difference in thickness between treated (right) and untreated (left) ears. Data are represented as mean ± SD of 4-5 animals. Statistical significance was determined by two-way ANOVA followed by Tukey's post-hoc test; *p<0.05, **p< 0.005, ***p< 0.0005, ****p<0.0001. No significance was observed at 2 h.

MSE and MIC-1 reduce ear biopsy punch weights

Six mm ear biopsy samples were collected using sterile biopsy punches from the control (left) and treated (right) ears. The biopsy punches were weighed, and the results calculated as the difference in ear punch weight in mg compared to the controlled left ear (Fig. 3). Untreated control ears showed a change of 3.2 ± 1.2 mg in the punch weight deferential, suggesting that the left and right ear of the mice are not identical in size or thickness. The weight differential increased to 17.4 ± 4.3 mg, compared to the control (left ear) punches, for the animals receiving TPA alone, a 443% or 4-fold increase. Dexamethasone limited the TPA-induced weight gain to a 25% increase with a 4.0 ± 3.1 mg change, MSE at 2 mg/ear caused a 184% increase or 2-fold increase in TPA-induced weight gain of the biopsy punches or 9.1 ± 3.9 mg and MIC-1 at 0.8 mg/ear, resulted in 175% increase with a change of 8.8. ± 1.4 mg.

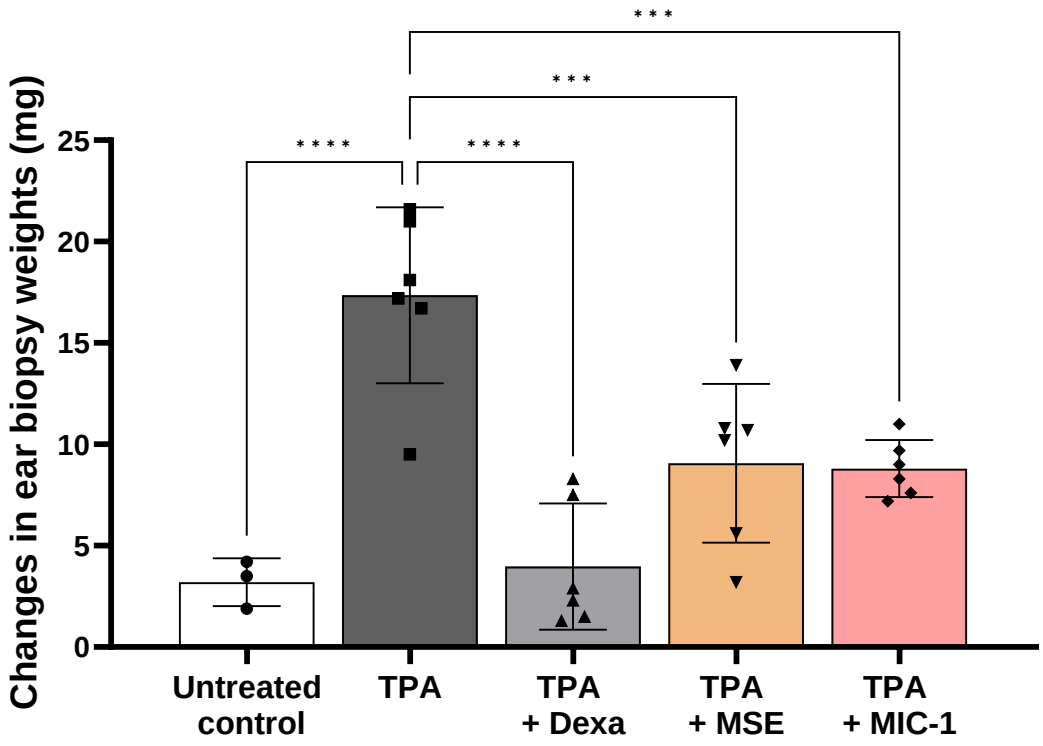

Figure 5.3. Effects of MSE and MIC-1 on the attenuation of the TPA-induced ear edema measured as differences in weights (mg) of 6 mm ear punch biopsies. Treatment groups included untreated control, TPA (2 μg/ear), TPA + dexamethasone (0.5 mg/ear), TPA + MSE (2 mg/ear) and TPA + MIC-1 (0.8 mg/ear). TPA was administered 20 min post treatment. Biopsy punches were collected 4 h post TPA administration. Data are expressed as a difference between the weights (mg) of the biopsy punches from the treated (right) and untreated (left) ears. Data are represented as mean ± SD of 4-5 animals. Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test; **p< 0.005, ****p<0.0001.

MSE and MIC-1 selectively decreases levels of MCP-1, KC, and IL-6 in mouse ear tissue

To evaluate the effects of MSE and MIC-1 on the production of the inflammatory markers in the ear tissue, LUMINEX assay was performed on 6 mm ear biopsy punch samples, 4 h post-TPA application. Similarly, to the effect of positive control, dexamethasone, MIC-1 at 0.8 mg/ear and MSE at 2 mg/ear significantly reduced the levels of chemokines MCP-1 and KC along with cytokine IL-6 in the lysate of the ear tissue (Fig. 4). Control left ears expressed IL-6 at 69 pg/ml. In contrast, the TPA-treated right ear boosted IL-6 production to 2085 pg/ml, a 2922%- or 29-fold increase (Fig 4A). MSE and MIC-1 lowered the TPA-induced levels of IL-6 by 84% and 78% (330 pg/ml and 467 pg/ml, respectively). Dexamethasone brought the TPA-induced IL-6 levels down by 82% or to 369 pg/ml.

Chemokine MCP-1 showed a similar trend. The control left ear expressed MCP-1 at 219 pg/ml, while the TPA-treated right ear boosted MCP-1 production to 2669 pg/ml, a 1119% or 11-fold increase (Fig. 4B). MSE and MIC-1 lowered the TPA-induced levels of MCP-1 in the right ear by 74% and 73%, to 698 pg/ml and 713 pg/ml, respectively (Fig 4B). Dexamethasone reduced MCP-1 expression by 86% to 362 pg/ml.

A chemokine KC in (Fig 4C) was affected similarly to the previous two markers. Control, left ear expressed 126 pg/ml of KC. MSE and MIC-1 significantly reduced TPA-induced KC expression in the right ear by 56% and 43%, respectively to 662 pg/ml and 874 pg/ml when compared to TPA-induced expression at 1522 pg/ml. Dexamethasone performed similarly, reducing the expression by 59% at 626 pg/ml. Effects of MSE and MIC-1 on IFN-γ, IL-1α, IL-1B, IL-10, IL-12p40, IL-12p70 and TNF-α were also

measured. These inflammatory markers did not show statistically significant changes in response to the experimental treatments (data not shown).

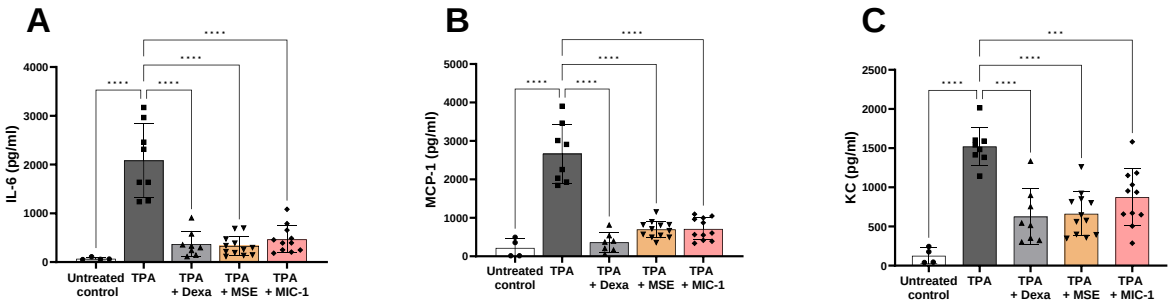

Figure 5.4. MSE and MIC-1 suppressed the expression of (**A**) IL-6 and (**B**) MCP-1 and (**C**) KC in TPA inflamed ear tissue. Ear biopsy samples, 6 mm, were collected 4 h post TPA administration. Data shown as mean ± SD and are representative of two independent experiments of 4-8 mouse ear samples. Statistical significance was determined by two-way ANOVA followed by Tukey's post-hoc test; ***p< 0.0005, ****p<0.0001.

MSE and MIC-1 selectively reduce IL-6 and KC level in serum

To determine whether topical treatment with MSE and MIC-1 exhibited a systemic response beyond the ear tissue, blood serum from the experimental animals was collected and analyzed. The trend of IL-6 in serum was similar to IL-6 in the tissue lysate with the difference being the amount of the cytokine expressed. There was a 90% decrease in the amount of IL-6 in the serum as compared to the ear tissue lysate with the TPA-induced levels in the lysate at 2084 pg/ml and the TPA-induced levels in the serum at 198 pg/ml. MSE and MIC-1 reduced IL-6 serum levels by 99% and 97% to 29 pg/ml

and 67 pg/ml respectively with dexamethasone reducing the content by 98% at 46 pg/ml (Fig. 5A)

MCP-1 showed no statistically significant differences among the treatment groups (Fig 5. B), while TPA-induced KC serum levels were significantly reduced by MSE and MIC-1 by 63% and 59%, from 289 pg/ml to 107 pg/ml and 119 pg/ml, respectively (Fig. 5C). Dexamethasone did not significantly reduce TPA-induced KC levels.

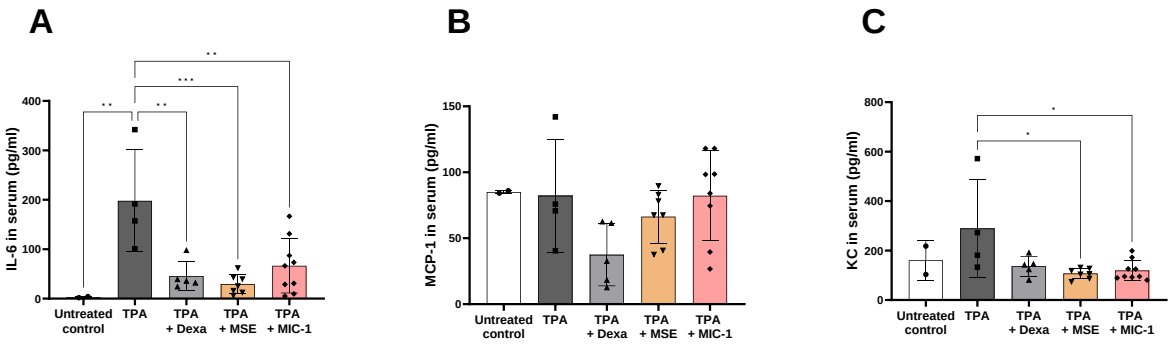

Figure 5.5. Effects of MSE and MIC-1 on (**A**) IL-6 and (**B**) MCP-1 and (**C**) KC in serum. Serum was collected 4 h post TPA administration. Data shown as mean ± SD and are representative of two independent experiments of 4-24 ear samples. Statistical significance was determined by two-way ANOVA followed by Tukey's post-hoc test; *p<0.05, **p< 0.005, ***p< 0.0005. No significance was observed for chemokine MCP-1.

MSE reduces the thickness in the ear tissue

Treatment-induced microscopic changes in ear morphology were evaluated using H&E staining on the ear biopsy samples. The Aperio ImageScope software was used to analyze the thickness of the H&E-stained slides at similar locations in the representative images of each of the treatments (Fig. 6). When comparing the average thickness of the cross section of the vehicle control ear samples, 412 μm, there was a 47% thickness

increase in the TPA-treated samples of 778 µm, indicative of inflammation. MSE and MIC-1, increased the thickness of the TPA-treated ear samples increased by 33% and 38% to 618 µm and 669 µm, respectively. The dexamethasone-treated samples had at 21% increase to 526 µm. MSE significantly ($p<0.05$) attenuated the TPA-induced ear thickening. MIC-1 responded similarly to MSE, but the results were not statistically significant at $p<0.05$.

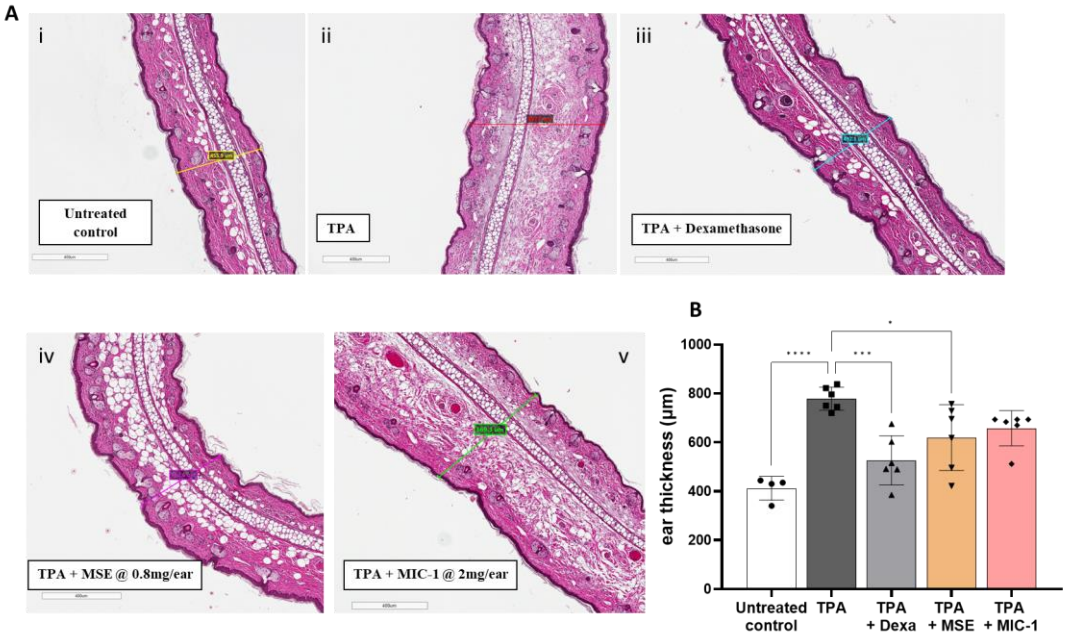

Figure 5.6. Thickness of transversal cuts of H&E-stained ears. Ear samples were collected at 4 h post TPA administration. Treatment groups included ears that were left untreated (Untreated control) (i), ears treated with TPA (ii), TPA 20 min post dexamethasone at 0.5 mg/ear (Dexa) (iii), MSE (2 mg/ear) (iv) or MIC-1 (0.8 mg/ear) (v). Images of H&E slides (**A**) were analyzed using Aperio ImageScope software. Data (**B**) is represented as mean ± SD of 4-6 ear samples. Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test; *$p<0.05$, **$p< 0.005$, ***$p< 0.0005$, ****$p<0.0001$.

Discussion

This study demonstrates the anti-inflammatory effect of an isothiocyanate-rich MSE and its main isothiocyanate, MIC-1, in a TPA-induced mouse ear edema model. Oral anti-inflammatory effects of MSE and MIC-1 were previously investigated in the *in vivo* sepsis model [77] , and ulcerative colitis model [79] which demonstrated that the specific anti-inflammatory benefits of MSE can be attributed to its high MIC-1 content. In addition, MSE was effective in the mouse metabolic syndrome model, where it improved insulin resistance and reduced body mass in mice fed high fat diet [75]. Both metabolic syndrome and diabetes are also associated with a low grade, systemic inflammation [129].

This study demonstrates, for the first time, that topical application of MSE and MIC-1 inhibits inflammation in the TPA-induced mouse ear edema model (Figs. 2,3,6). The magnitude of the anti-inflammatory effects of MIC-1 and MSE containing 38% of MIC-1 were comparable to the positive control, dexamethasone, a glucocorticoid often used to treat inflammation-related skin diseases. The inhibition of skin inflammation after treatment with MSE or MIC-1 was associated with decreases in the pro-inflammatory cytokine and chemokines IL-6, MCP-1, and KC (Figs. 4,5). These mediator molecules play an essential role in the inflammation process. Lipidated peptidomimetics were shown earlier to decrease cytokine and chemokine expression in the TPA-induced mouse ear inflammation model [128]. Our study indicates that IL-6, MCP-1, and KC are critical players in the topical anti-inflammatory effect of MSE and MIC-1, likely mediated by the Nrf2 and NF-κB pathways [100].

In general, isothiocyanates, such as sulforaphane from Brassicaceae, are low yielding in quantity, unstable and liquid, while MIC-1 from MSE is a high yielding, stable white powder [130]. Isothiocyanates from Brassicaceae have many documented health benefits such as anti-inflammatory, anticarcinogenic, antidiabetic, cardioprotective, antioxidant and antimicrobial [131]. MSE contains 38% MIC-1, because its precursor glucosinolate, glucomoringinin, is fully converted to MIC-1 under the MSE extraction method we developed [75, 77] . The primary mode of action of Brassicaceae isothiocyanates, i.e., sulforaphane, is the activation of Nrf2 signaling cascade [70, 119, 132-134]. The results of this investigation further support our earlier observations that glycosylated moringa isothiocyanates, such as MIC-1, enhance Nrf2 signaling, at least as much as sulforaphane, but may also be active in the inhibition of the NF-κB signaling cascade [100]. This work also supports the conclusion that MIC-1 is the primary active component of MSE, since both produce similar pharmacological results at the same levels of MIC-1.

Skin inflammation is part of the defense against various environmental factors, pathogens and chemical agents [73]. Keratinocytes, stimulated by these external causes, secrete pro-inflammatory cytokines that stimulate innate and adaptive immune responses [135]. Secreted factors include molecules such as cytokines and growth factors, which affect multiple inflammatory processes [92, 100, 136-140]. These include TNFα, the migration of T-helper (Th)1-polarized T lymphocytes, interleukins- 6,7,12,15,18, IFN-γ, or granulocyte-macrophage colony-stimulating factor (GM-CSF) as well as mediators that may reduce inflammation, such as IL-1Ra, IL-10, CXCL10 and prostaglandin E2 (PGE2) [73]. TPA, a phorbol ester, induces topical inflammation by activating protein

kinase C, and a downstream transcriptional regulator of inflammation NF-κB [73, 141, 142]. The TPA-induced ear edema is a robust and often used model to evaluate the anti-inflammatory effects of compounds [92, 100, 136-140]. A single dose of TPA to the ears of mice could elicit ear swelling, thickness, redness, and infiltration of neutrophils and local and systemic secretion of cytokines and chemokines; all these effects are associated with an acute inflammatory response [128, 138, 139, 143] .

This study demonstrated that both MSE and MIC-1 limit the expression of IL-6, MCP-1, and KC in the ear tissue (Fig. 4) and in the serum (Fig. 5) although the decrease in MCP-1in serum was not statistically significant at $p<0.05$. Since very little to no IFN-γ, IL-1α, IL-1B, IL-10, IL-12p40, IL-12p70 and TNF-α were detected in the ear samples at the 4 h time point, 4 h may be too soon to detect the changes, or the changes were not detectable because of a low basal level of these inflammation mediators.

Modes of action of the dexamethasone and other glucocorticoids include suppressing the migration of neutrophils and decreasing lymphocyte colony proliferation and the permeability of the capillary membranes. They also inhibit a variety of cytokines including IL-1, IL-12, IL-18, TNFα, IFN-γ and granulocyte-macrophage colony-stimulating factor [144]. It is tempting to speculate that combining MIC-1 with glucocorticoid drugs may have an additive, or even synergistic effect on reducing topical and systemic inflammation.

In conclusion, MIC-1 and MSE normalized for MIC-1 content effectively reduced skin inflammation in a TPA-induced ear edema model in a dose-dependent manner. Their effectiveness approached that of a widely used anti-inflammatory agent, dexamethasone. Upon treatment with both moringa seed-derived agents, there was an observable decrease

in the TPA-induced ear edema, a decrease in the ear biopsy punch weights, a decrease in the dermal thickness, and a decrease in pro-inflammatory cytokines and chemokines MCP-1, KC, IL-6 and IL-1α in the ear tissue lysate. These results indicate that MSE and MIC-1 have the potential to mitigate skin inflammation and possibly treat diseases associated with skin inflammation. MIC-1-based remedies may be used alone or in combination with other anti-inflammatory agents.

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CHAPTER 6: SUMMARY

This dissertation focused on the pharmacological activity of isothiocyanate rich *Moringa oleifera* Lam. (moringa) and its main bioactive MIC-1. Our goal was to further understand the mechanisms of their anti-inflammatory action and to assess their potential

as candidates for further development as topical therapeutics. Moringa is rich in protein, calcium, potassium, among many other compounds, but the most notable bioactive, moringa isothiocyanate-1 (MIC-1) is similar to other isothiocyanates from cruciferous vegetables. It is enzymatically formed from a glucosinolate precursor that has been researched for the treatment of the conditions ranging from metabolic regulation, gut inflammation, and central nervous system protection. My research was the first to focus on the bioavailability and bioaccessibility of MIC-1, along with its effectiveness as a topical anti-inflammatory mediator.

The most effective anti-inflammatory effects were achieved when MSE and MIC-1 (in a 10% ethanol) were used in the ear edema model. Topical application of both MSE and MIC-1 reduced ear thickness, ear weights and also attenuated the expression of inflammatory cytokines IL6, MCP-1 and KC. A TPA-induced ear edema model allowed me to propose that MSE and MIC-1 are candidates for the topical treatment of skin inflammation. Prior to this, two other inflammation models were tested. First, a Collagen Induced Arthritis (CIA) model, a chronic inflammation model that mimics Rheumatoid Arthritis (RA) in the paws of rats and a Carrageenan Paw Edema (CPE) model, an acute inflammatory model that produces edema in the paws of rats. With the CIA experiment, MSE was administered orally gavaged during the onset of inflammation but was unable to significantly attenuate the paw swelling in the animals as measured by a plethysmometer. While the acute CPE model showed some promising results, the effect was not statistically significant.

This thesis also measured the bioaccessibility and bioavailability of MIC-1 and MSE using an *in vitro* TNO-Intestinal Model (TIM)-1. MIC-1 in MSE was about 60%

bioaccessible. Interestingly, it was observed in the blood as an intact, stable compound that remained present in the blood stream of rats up to 8 hours. Optimized extraction methods to isolate and quantitatively assess MIC-1 from serum were developed and published. While acid precipitation/hydrolysis and β-glucuronidase hydrolysis methods were tested, a simple liquid partitioning with hexane and ethyl acetate was the most effective method used for measuring MIC-1 in blood.

My research allows for better understanding of pharmacological endpoints and properties MSE and MIC-1 and their potential use as natural therapeutics against various inflammation-related conditions, through either oral or topical administration. Further studies should emphasize optimizing delivery systems and formulations that increase the efficacy of both MSE and MIC-1. My results on MIC-1's bioavailability and bioaccessibility provide a foundation for future development of anti-inflammatory botanical therapeutic containing moringa isothiocyanates.

Growing up in the West Indies where ethnobotany is simply our way of life, being able to contribute to the validation of this science has been a highly rewarding experience. My dissertation research has fortified my passion for the use of natural plant products as a means of disease treatment or promotion of wellness. I hope that my work will make a meaningful contribution to further use of plant natural products in medicine.

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