PROTEOMIC ANALYSIS OF *MORINGA OLEIFERA'S* ANTI-ARTHRITIC EFFECTS ON HUMAN FIBROBLAST-LIKE SYNOVIOCYTES

A Thesis
by
BRIANNA NICOLE TATE

Submitted to the Graduate School
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

December 2018
Department of Biology

PROTEOMIC ANALYSIS OF *MORINGA OLEIFERA'S* ANTI-ARTHRITIC EFFECTS ON HUMAN FIBROBLAST-LIKE SYNOVIOCYTES

A Thesis
by
BRIANNA NICOLE TATE
December 2018

APPROVED BY:

Chishimba Nathan Mowa, Ph.D.
Chairperson, Thesis Committee

Maryam Ahmed, Ph.D.
Member, Thesis Committee

Michael Opata, Ph.D.
Member, Thesis Committee

Zack E. Murrell, Ph.D.
Chairperson, Department of Biology

Mike, J. McKenzie, Ph.D.
Dean, Cratis D. Williams, School of Graduate Studies

Abstract

PROTEOMIC ANALYSIS OF *MORINGA OLEIFERA'S* ANTI-ARTHRITIC EFFECTS ON HUMAN FIBROBLAST-LIKE SYNOVIOCYTES

Brianna Nicole Tate

B.A., Appalachian State University

Chairperson: Chishimba Nathan Mowa, Ph.D.

Rheumatoid arthritis (RA) affects more than 1.3 million Americans, making it the most common auto-immune arthritic disorder in the U.S. Current treatments are largely based on non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids. However, these drugs are either ineffective, costly or have a plethora of side effects, notably immune suppression and increased risks of heart attacks. Therefore, here, we sought to examine the anti-arthritic effects of a tropical medicinal plant, *Moringa oleifera* (MO), and its underlying mechanism using proteomics analysis and primary human fibroblast-like synoviocytes (HFLS) harvested from healthy people and patients with arthritis (HFLS-RA). Initial experiments optimized MO's optimal dosage by examining MO's effect on cell viability using MTT assay. Cells were divided into the following groups: **1**) *Negative control groups* [**a**) HFLS and **b**) HFLS-RA, 0.1M phosphate-buffered saline (PBS) only] and **2**) *MO treatment group* (HFLS and HFLS-RA), dose-dependent treatment (500 µg/mL, 750 µg/mL, 1000µg/mL, 50 mg/mL, 75 mg/mL, 100 mg/mL, 150 mg/mL and 200 mg/mL). For proteomics studies, cells were divided into the following groups: **1**) *Negative control group* (HFLS-RA), vehicle only (as above) and **2**) *MO Treatment group* (HFLS-RA), 75 mg/mL (optimal concentration). After

24 hours of treatment, cells were harvested and analyzed for cell viability (MTT assay) or proteome-wide expression (Proteomics). Data generated from proteomics were verified by confocal immunofluorescence. The MTT assay data shows that below 75 mg/mL, MO is not cytotoxic and therefore does not affect cell viability. Proteomics data revealed that MO alters expression of all the 40 proteins that are aberrantly expressed in HFLS-RA by either mitigating their expression (35 proteins) or enhancing (5 proteins) them. Specifically, the 35 proteins were mostly associated with pathological processes, such as inflammation, aberrant proliferation and cell adhesion, whereas the 5 proteins (Heat shock 70 kDa protein 1A/1B, Kallistatin, programmed cell death 6-interacting protein, Hemoglobin subunit alpha, and Aldo-keto reductase family 1 member C1) were associated overall with normal protective processes, such as anti-inflammatory and apoptotic activities, were down regulated in HFLS-RA. We conclude that MO is potentially a good candidate for developing alternative therapy for managing arthritis.

Acknowledgments

Firstly, I would like to express my sincere gratitude to my advisor Dr. Chishimba Nathan Mowa for the continuous support he has provided during my M.S. studies and related research, for his being a constant source of motivation, and his endowment in wisdom and knowledge. His guidance was a source of great help to me immensely during my entire time of conducting research and writing of this thesis. I could not have imagined having a better advisor and mentor for my M.S. study.

In addition to my advisor, I would like to thank the rest of my thesis committee members, Dr. Maryam Ahmed and Dr. Michael Opata, for challenging me and providing insights and encouragement during my research. Without them this thesis would not have been possible.

I thank my fellow lab mates and those of other labs that have aided me in learning a great many techniques and providing me with the assistance to grow as a scientist. I also thank the friends I have made at Appalachian State University.

Finally, I would like to thank my mother and father for supporting me financially and emotionally through the tumultuous times I have experienced during my time of my M.S. study, as well as in life in general.

Dedication

I would like to dedicate this thesis to my parents who have motivated and supported me throughout my entire life. This is just as much their achievement as it is mine. I would also like to dedicate this thesis to my close friends and confidants that have helped me persevere and make this accomplishment possible.

Table of Contents

List of Figures

Introduction

Study overview

The present study investigates the suitability of a tropical medicinal plant, *Moringa oleifera* (MO), as an alternative therapy to Rheumatoid arthritis (RA), one of the prevalent diseases in the US that disproportionately affect women. Current treatments are not only costly but are associated with multiple side effects. MO was considered for investigation because it is a potent modulator of inflammation and proliferation, two prominent pathogenic processes associated with RA.

Rheumatoid arthritis

Overview of RA

RA is a chronic inflammatory auto-immune disease characterized by persistent synovitis, systemic inflammation, production of auto-antibodies and destruction of affected bone and cartilage, which leads to progressive damage and loss of function of the joint (Scott et al, 2010). While RA primarily targets the joints, the disease can result in systemic complications, such as rheumatoid nodules, pulmonary involvement or vasculitis, and systemic comorbidities (Smolen, 2016). Although RA is the most prevalent autoimmune disease, affecting 1% of the American adult population, its cause has remained elusive (Parker, 2017). In addition, RA's treatment is costly and its' effectiveness is wanting.

Epidemiology and cost of RA

RA is most prevalent in women and the elderly, i.e., seventy-five percent of RA patients are women, making them three times more susceptible than their male counterparts (Scott, 2010). Women also have an earlier peak onset age of arthritis, occurring in the fourth to fifth decades, whereas in men, the prevalence increases in the sixth to eighth decades (Parker, 2017). The exact reason for the heightened risk in females has not be elucidated but it has been hypothesized that it could be due to hormonal changes that take place in menopausal women as well as genetic factors pertaining to genes associated with RA that are located on the X chromosome (Scott, 2010).

The costs of medications to manage the disease, such as methotrexate, are prohibitive; averaging approximately $5825 per annum in pharmacy-related expenditures, compared to $1264 for patients without arthritis (Mukherjee, 2017). Given these conditions, namely prohibitive costs and associated side effects, it is important that we develop alternative therapies that are more effective as well as affordable and safer.

Pathogenesis and signaling pathway of RA

The exact onset of RA pathogenesis is not clear but it (RA) is initiated when antigen-presenting cells, such as dendritic cells and macrophages (and others) present auto-antigens, which subsequently activates auto-reactive T-cells. Auto-reactive T-cells are immune cells that recognize endogenous antigens within the host and, thus, attack the body's own tissues. This anomaly is one of the fundamental characteristics of the early events of the disease (Weisman, 2011). Following these changes, the synovium, or synovial membrane, is then infiltrated by an influx of immune cells, including T cells, B cells and macrophages via

activated endothelial cells that secrete adhesion molecules (Smolen et al., 2007; Weisman, 2011). The previously activated T cells accumulate in the joint and begin to secrete interleukin-2 and interferon-γ which, in turn, activates and differentiates other immune cells, such as macrophages and B cells, as well as cells found within the joint, such as fibroblasts and osteoclasts (Smolen et al., 2007; Weisman, 2011). The B cells, once activated, can either serve as antigen-presenting cells to the other T-cells, subsequently activating these cells (T-cells), thus perpetuating a cycle of positive feedback among the two cell types. Alternatively, these cells can differentiate into plasma cells, which then produce auto-antibodies to a variety of target molecules, including rheumatoid factor, vimentin or fibrinogen (Smolen et al., 2007). These auto-antibodies can then go on to form immune complexes that result in the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF) via complement and Fc-receptor activation (Smolen et al., 2007; Weisman, 2011). The stimulated macrophages also produce pro-inflammatory cytokines, as well as activate synoviocytes in the joint, which then secrete inflammatory cytokines of their own that ultimately results in the breakdown of the proximate cartilage and bone (Weisman, 2011).

The synovium

The synovial membrane, or synovium, is a layer of tissue that encases diarthrodial joints (Bottini & Firestein, 2013). This layer of tissue is populated by two key cell types, namely macrophage-like type A synoviocytes and fibroblast-like type B synoviocytes (Firestein, 1996). In a patient affected by RA, the macrophage-like cells display an "activated" phenotype and produce pro-inflammatory cytokines, chemokines and growth factors. These inflammatory signals activate local fibroblast-like synoviocytes in the

synovium and they, in turn, produce more inflammatory mediators, namely IL-6, prostanoids, and matrix metalloproteinases (MMPs) (Bartok & Firestein, 2010).

The fibroblast-like synoviocytes are the key cells in joint erosion and their activation and subsequent secretion of matrix-degrading enzymes and inflammatory factors that further enhance the inflammatory cycle in RA and systematically break down the extracellular matrix, ultimately resulting in damage to the bone and cartilage (Bartok & Firestein, 2010; Neumann et al., 2010). Further, these cells have been implicated in the hyperplasia of the synovium. Once activated, these cells begin to exhibit characteristics analogous to that of tumor cells. They form an expansive synovial tissue called 'pannus' at the cartilage-bone interface (Bartok & Firestein, 2010). This is likely due to their upregulation of oncogenes involved in cell cycle regulation or that act as transcription factors, such as *c-fos*, *ras*, *raf*, *myc* and *myb*. Their lack of apoptosis and their uptick in proliferation result in the synovium, which under physiological conditions is usually only 1-3 cells thick, to instead become 10-15 cells thick (Bartok & Firestein, 2010; Neumann et al., 2010). The pannus is said to behave like a locally invasive tumor (Bartok & Firestein, 2010). This tissue cloaks the cartilage and erodes into bone.

Bone Erosion

Bone erosion is a key hallmark of RA and usually occurs in the latter stages of the disease (Schett & Gravallese, 2012). The cells responsible for this phenomenon are called osteoclasts. Osteoclasts are giant multinucleated cells of a monocyte/macrophage origin which populate bone surfaces throughout the body (Takayanagi et al., 2000). Their normal function in the body is bone reabsorption. They do so by adhering tightly to the bone surface via interactions with both integrins and extracellular matrix proteins, as well as by

assembling tight junctions. This process seals the bone surface and the osteoclast together and separates the bone from the surrounding extracellular space. Proton pumps located along the peripheral of the osteoclasts create an acidic milieu, enabling solubilization of calcium from bone (Schett & Gravallese, 2012).

However, upon onset of disease, this regulated process becomes destructive. Synovitis which, as mentioned before, takes place due to the dysregulation of the apoptotic and proliferative mechanisms in the fibroblast-like synoviocytes of the joint, provides a source of pro-inflammatory cytokines, which, in turn, drives the process of osteoclast differentiation (Schett & Gravallese, 2012). The TNF, IL-1, and IL-6 secreted by the synoviocytes augments the osteoclastogenesis process. They do so through upregulation of RANK-L expression in mesenchymal cells and through direct effects on osteoclast precursor cells. TNFα stimulates differentiation of osteoclast precursor cells into mature osteoclasts and IL-1 acts on osteoclasts to increase their bone-resorbing capacity (Goldring, 2003). The expression of receptors for osteoclast differentiation, such as RANK-L, is also stimulated by cytokines derived from the synovium (Schett & Gravallese, 2012). T cells can have a similar effect on the differentiation of osteoclasts through their secretion of RANK-L (Firestein, 2003). In fact, in one murine *in vivo* study, it was found that with adjuvant-induced arthritis T-cell activation lead to a RANK-L-mediated increase in osteoclasts and subsequent bone loss (Kong et al., 2009). Additionally, in RANK-L knockout mice there is significant reduction in bone erosion, even in arthritic models (Pettit et al., 2001). Once differentiated, osteoclasts then begin to efflux calcium from the surrounding bone, resulting in irreversible damage to the joint.

Cartilage Damage

The cartilage remodeling process is entirely mediated by a single type of cell: the chondrocyte (Goldring et al., 2003). These cells are responsible for the synthesis of the complex extracellular matrix of the articular cartilage. They also produce proteinases and other mediators that degrade the damaged matrices to permit repair. Unlike bone, which can be, and is, continuously remodeled throughout life, cartilage turnover is limited and, once damaged, this tissue is restricted in its ability to repair itself (Goldring et al., 2003).

There are many similarities between the mechanisms that result in bone erosion and those that play a role in cartilage degradation in RA. Both are mediated by the activity of synoviocytes in the synovium and the cytokines they secrete (Goldring et al., 2003). There are two key mechanisms by which the arthritic synovial tissues contribute to cartilage loss. The first is the effect that their cytokines and other mediators that they release have on the dysregulation of chondrocyte function. IL-1 and TNFα are two key products of synovial fibroblasts that function in this manner. IL-1 is said to be more potent than TNFα in respect to their effects. Studies have shown that IL-1 stimulates chondrocytes to produce matrix metalloproteinases, also known as MMPs, and other degenerative products, such as nitric oxide (Neumann et al, 2010). In addition to chondrocyte dysfunction and destruction of the extracellular matrix of the cartilage, the second mechanism by which synovial tissues cause cartilage degradation is through decreasing the synthesis of cartilage-specific collagens and proteoglycans, which would otherwise aid in the remodeling of the cartilage tissue (Saklatvala et al., 1986). By mediating these two pathways, the synovial tissue is directly responsible for the characteristic cartilage damage observed in RA patients.

Current treatments of RA and associated shortfall

As prevalent and debilitating as RA is, it should come as no surprise that there is a wide variety of pharmaceuticals currently on the market that seek to attenuate the symptoms of the disease, and more are still currently being developed. The primary goal of arthritic treatments is to slow the disease progression, reduce pain, aid in mobility, and improve the overall quality of life for the patient. There are three major classes of drugs that are typically used to treat RA: Disease Modifying Anti-Rheumatic Drugs (DMARDs), Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), and corticosteroids (Scott et al, 2010).

Non-Steroidal Anti-Inflammatory Drugs

The NSAIDs class of drugs are used for managing the pain experienced by patients with RA (Scott et al, 2010). Drugs included in this category are aspirin and ibuprofen. This class of drug primarily works, in low doses, by inhibiting the activity of cyclooxygenases and, consequently, inhibits the synthesis of prostaglandins (Brooks & Day, 1991). It should be noted, however, that this is not the sole way in which NSAIDs attenuate inflammation. Certain NSAIDs have been found to inhibit various lipoxygenase enzymes *in vitro* and *in vivo*. The NSAIDs responsible for this do so by reducing the production of leukotrienes and prostaglandins from leukocytes and synovial cells by allowing for the reincorporation of free arachidonic acid into triglycerides, instead of allowing it to be metabolized into lipoxygenase enzymes, which play a role in the inflammatory process (Brooks & Day, 1991). NSAIDs, have also demonstrated inhibiting effects on neutrophil function, including cell-cell aggregation. At high doses, NSAIDs have been found to interfere with processes not dependent on prostaglandins, such as the activity of enzymes (i.e., phospholipase C), the

synthesis of proteoglycan by chondrocytes, transmembrane ion fluxes and cell-cell binding. These actions of NSAIDs, however, have rarely been demonstrated in humans (Brooks & Day, 1991).

Disease Modifying Anti-Rheumatic Drugs

This class of drugs, also known as DMARDs, is the most widely utilized drug to manage RA. There are two classes of DMARDs: biologics and non-biologics (Scott et al 2010). Methotrexate, a non-biologic, is usually the first drug-of-choice in the management of arthritic symptoms, regardless of duration or severity of the disease (Scott et al, 2010). It is believed that methotrexate works by "starving" malignant cells for the purine and pyrimidine precursors of DNA and RNA. These molecules (purine and pyrimidine) are required for proliferation as they inhibit dihydrofolate reductase, the enzyme responsible for producing reduced folate, which is necessary for the synthesis of many molecules, including purines and pyrimidines (Cronstein, 1997). Nonetheless, the mechanisms of methotrexate, and DMARDs as a whole, are poorly understood. Due to its (Methotrexate) ability to reduce synovitis, methotrexate and leflunomide (an alternative to methotrexate) appear to have bone-sparing effects (Schett & Gravallese, 2012).

The biologic DMARDs are usually used to treat patients with moderate to severe RA and are typically only used if the patient does not respond well to the non-biologics DMARDs. They work in a multitude of ways, depending on the drug. Some are TNF inhibitors, others inhibit T-cell activation, and still others serve as agonists for targets such as IL-1 and IL-6 (Scott et al, 2010). However, as stated before, the mechanisms of action for DMARDs, as a whole, is still not fully understood.

Corticosteroids

This class of drugs is used by about 25%-75% of arthritic patients, though its use is highly controversial due to its harsh side effects, including hypertension, adrenal suppression, increased risk of infection and GI bleeding (Scott et al, 2010). Corticosteroids work by preventing the formation of both prostaglandins and leukotrienes, two key players in the inflammatory response, by causing the release of lipocortin. This molecule inhibits the production of phospholipase A2 and, thus, reduces the production of arachidonic acid, implying that the arachidonic acid cannot be metabolized into lipoxygenase enzymes, which would further exacerbate the inflammatory response (Vane & Botting, 1987).

Moringa Oleifera **(MO):**

Overview of MO

Historically, MO has been renowned for its many medicinal properties for hundreds of years (Sujatha & Patel, 2017). Aside from being consumed as a daily vegetable, MO has been utilized in folk medicine in order to treat a wide array of different maladies (Abdull Razis et al., 2014). All parts of the plant are edible and contain constituents that lend themselves to the versatility of the MO plant in its ability to treat multiple ailments, including, but not limited to, microbial infections, ulcers, diabetes and cancers (Sujatha & Patel, 2017; Abdull Razis et al., 2014; Arulselvan et al, 2016). As MO becomes more widely studied, more of its properties and mechanisms of action are being elucidated, especially those associated with its anti-inflammatory characteristics (Abdull Razis et al., 2014; Mittal et al., 2017). Through extensive research, it has been found that the MO contains compounds that exhibit potent anti-inflammatory effects, notably quercetin and kaempferol, which are

produced in high levels by the plant (Coppin et al, 2013). Due to the anti-inflammatory nature of the MO plant, in addition to its accessibility, low costs, and lack of known significant side effects, it is possible that it could be effectively utilized to treat many inflammation-based diseases, such as diabetes, cancer and RA, as an alternative to the current therapeutics on the market. However, in order to develop and commercialize phytomedicines for use in a clinical setting, it is important to understand the mechanisms by which MO is able to attenuate inflammatory and related pathways.

Use of MO in inflammation-related disorders

It should come as no surprise that due to the abundance of anti-inflammatory compounds, researchers have turned to MO to develop alternative therapies to current pharmaceuticals. The over-expression of pro-inflammatory cytokines has been implicated in a number of autoimmune disorders, such as RA, inflammatory bowel disease (IBD), psoriasis, systemic lupus erythematosus (SLE), and organ graft rejection (Koneni et al, 2009). Since MO bioactive compounds have been shown to effectively downregulate the NF-κB pathway (REF), it shows great promise in the treatment and management of certain autoimmune diseases, including RA.

Indeed, studies are already utilizing MO to treat autoimmune diseases, including RA, and IBD. These studies show that MO is effective in attenuating the symptoms of these conditions (Das & Kanodia, 2012; Mahajan et al, 2007). In one study, rats induced with IBD were treated with the ethanolic extract of MO leaves. It was found that the MO was effective in protecting the gut of the rats from oxidative stress, a process thought to play a significant role in the pathogenesis of IBD. There was a significant increase in antioxidant parameters and a reduction in the markers of oxidative stress (Das & Kanodia, 2012). In another study

where rats were induced with experimental arthritis and treated with the ethanolic extract of MO seeds, a similar pattern was observed. There was a significant increase in the antioxidant markers, although there were no significant changes in the markers of oxidative stress. The extract did, however, cause a significant reduction in the swelling of the joints of the rats, as well as in the expression of rheumatoid factor (Mahajan et al, 2007). It has also been shown that the ethanolic extract of MO also has immunomodulatory effects as well. One study found that MO is able to suppress macrophage phagocytic activity, which in turn affects the role of the B-cells in a humoral immune response (Mahajan & Mehta, 2010). Humoral immunity is an important target in the management of certain autoimmune diseases, especially rheumatoid arthritis, which is caused by dysregulation in the activity of key immune cells (Drexler et al 2008). These *in vivo* studies show that MO extracts have the medicinal potential to mediate and manage the progression of certain autoimmune diseases.

Objectives of current study

Recently, an increasing number of studies have demonstrated the multiple medicinal properties of MO (Gilani, 2006), notably anti-inflammatory using ethanolic extract (Mahdi et al., 2017). In fact, the ethanolic extract was found to attenuate arthritic symptoms both in an *in vitro* and *in vivo* model of RA (Coppin et al, 2013, Mahdi et al., 2017). Therefore, in the present study, we sought to elucidate the effect and underlying mechanism of the ethanolic extract of MO on human fibroblast-like synoviocytes *in vitro* from arthritic patients using proteomics. The leaves of MO contain polyphenols and flavonoids which have potent anti-inflammatory properties (Coppin et al, 2013; Sherma et al., 2014).

Materials and Methods

Cell culture and Moringa oleifera treatment

Human Fibroblast-like synoviocytes harvested from a 47-year old Caucasian male with rheumatoid arthritis (HFLS-RA) were purchased from Cell Applications (San Diego, CA, USA). These cells were cultured in Synoviocyte growth media (Cell Applications, San Diego, CA, USA). Cells were seeded in 10-cm culture plates (VWR, Radnor, PA) at a density of 7, 000 cells/cm^2, based on the manufacturer's recommendations and previous studies (Sable et al., 2016). All cells were cultured for 24 hours at 37℃ and 5% CO_2. After treatments (see below), cells were harvested and analyzed for cell viability using MTT assay and proteome-wide protein expression, which was verified by confocal immunofluorescence. After cells reached a high confluency (>90%), they were treated based on the analysis (described earlier), i.e., **A**) MTT assay treatment groups: divided into the following groups, based on their treatment: **1**) Vehicle groups [**a**) HFLS and **b**) HFLS-RA, 0.1M phosphate-buffered saline (PBS) only], which served as negative control groups and **2**) dose-dependent treatment of MO (500 µg/mL, 750 µg/mL, 1000µg/mL, 50 mg/mL, 75 mg/mL, 100 mg/mL). **B**) Proteomics/confocal immunofluorescence treatment groups: **1**) Negative control groups (HFLS-RA), vehicle only (0.1M PBS), **2**) MO Treatment group (HFLS-RA), 75 mg/mL MO. Based on initial optimization experiments, passages 3-6 and MO dosage of 75 mg/dL were determined to be optimal and were, thereafter, used in all subsequent experiments. MO was grown, harvested, processed and obtained from North Carolina A & T University (Greensboro, NC).

MTT Assay

In order to determine the optimal dosage of MO that is not toxic to cells, initial studies were conducted using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Sigma Aldrich, St. Louis, MO). Briefly, HFLS-RA cells and non-arthritic cells (HFLS) were seeded in 96-well plates at a density of 3×10^4 cells/well, and were treated with 500, 750, 1000µg/mL and 50, 75, and 100 mg/mL of ethanolic MO whole leaf extract or 0.1M PBS only, as described earlier. After 24 and 48 h of incubation, 2.5 mg/mL of MTT solution was added to the wells, and the cells were then incubated for 2 h. The absorbance of each well was measured using a Bio-Rad 680 microplate reader (Bio-Rad laboratories, Hercules, CA, USA) at 570 nm. Based on MTT assay results, 75 mg/mL was determined as the optimal concentration and was, therefore, used in all subsequent experiments.

Proteomics

Proteomics analysis was used to delineate the proteome-wide effect of MO on arthritic cells, identify the signature proteins of MO's biological effects, as well as determine its likely underlying mechanism on arthritic cells.

Briefly, harvested cells were snap-frozen and stored at –80ºC until processing. The total protein was extracted and quantified using a BCA assay (Thermo Scientific, Waltham, MA). To extract protein, cells were rinsed with ice-cold PBS then lysed within the vessel with RIPA buffer. Then, using a cell scraper, cells were harvested and transferred to a pre-chilled 1.5mL Eppendorf tube. The cell suspension was then incubated on ice for 10 minutes before being centrifuged for 10 minutes at 10,000 x g at 4℃. The supernatant was then collected and stored in a clean Eppendorf tube at -80℃.

Study Samples:

Eight (4) whole protein samples were extracted from human Fibroblast-like-synoviocyte cell culture lysates (normal and diseased) (n=2) using RIPA buffer (Thermo Scientific, Waltham, MA) based on the following treatment groups: **a**) Control group: UNT-RA and **b**) Treatment group: (RA + Moringa [M]). The supernatant was then immediately stored at -80°C and later transported to the core facility Laboratory of the David Murdock Research Institute under dry ice for proteomics analysis.

Sample Preparation:

The samples were assayed using the Thermo Scientific Micro BCA Protein Assay kit to determine protein concentration. The samples were diluted to fall within the linear working range of the kit (5-200µg/mL) and the concentrations were calculated based on absorbance values compared to a BSA standard curve. The samples were then filtered using a 3kD ultra centrifugal filter to exchange the dissolution solvent with a mass spectrometry friendly buffer. Hundred (100) µL of sample was diluted to 500µL with 50mM ammonium bicarbonate and filtered. The samples were rinsed with 200µL ammonium bicarbonate and filtered. The tubes were inverted and centrifuged to collect the sample. The sample identification, the stated concentration, and 10 µg volumes are listed below in Table 1:

Table 1: Identification and protein concentration of each sample analyzed.

Sample ID	Protein Concentration ng/mL	10 µg of protein in µL
UNT-RA - A	429.57	23.3
UNT-RA - B	302.25	33.1
RA+MO - A	362.57	27.6
RA+MO - B	344.43	29.1

A volume of each sample corresponding to 35µg of protein (based on the protein quantization results) was used. The sample volumes were made equal by adding 50mM ammonium bicarbonate (AmBic) to a volume of 29.8µL. A 1% solution of Rapigest was added to each sample to denature the proteins and the mixture was placed in a shaking heated mixer at 40°C for 10 minutes. Disulfide bonds were reduced by adding 200mM dithiothreitol (DTT) to each sample and heating the tubes to 80ºC for 15 minutes. Free sulfur atoms were alkylated with 400mM Iodoacetamide (IA) by placing the tubes in the dark for 30 minutes at room temperature. A tryptic digest was performed by adding 0.7µg Gold-Mass Spectrometry grade Trypsin to each tube and incubating overnight at 37ºC. Alcohol dehydrogenase (ADH) digest from yeast was added to a final concentration of 50fmol/µg protein. The trypsin reaction was stopped and the Rapigest was degraded with the addition of 10% TFA/20% acetonitrile/70% water that was then heated to 60ºC for 2 hours. The samples were centrifuged and the supernatant pipetted into auto sampler vials.

A protein standard (BSA) was performed through the reduction/alkylation and digestion steps and used as a QC of the sample preparation steps and instrument performance. LC solvents for peptide separation included **a**) water containing 0.1% formic acid; and **b**) acetonitrile containing 0.1% formic acid. The study sequence consisted of the study sample

injections bracketed by a pair of QC injections. Data from all study samples were acquired using Data Dependent™ scans (Nth order double play) on the LTQ Orbit rap XL. Database searches were performed in Elucidator (Rosetta Bio software) using MASCOT (Matrix Sciences, London, UK). Analytical results were also viewed in Scaffold (Proteome Software, Portland, OR). QC and study samples were evaluated to confirm data quality. Liquid chromatography total ion current (TIC) outputs were assessed for signal quality and changes in signal intensity. Results were also monitored for signal trends, such as a consistent increase or decrease in TIC maximum values, and MASCOT search results were used to monitor the quality of the MS data.

Confocal immunofluorescence

Confocal immunofluorescence was employed to verify and complement proteomics data, as well as for cellular localization. Briefly, cells were cultivated in 35 mm glass bottom cell culture dishes (Cellvis, Mountain View, CA) and then fixed in the culture using a 3% formaldehyde/PBS solution for 15 minutes at room temperature, followed immediately by three washes in 0.1M PBS. The cells were then permeabilized in 0.4% Triton X / PBS solution for 5 minutes, washed again as previously described in 0.1M PBS. Immediately following the washes, 50 µL of a primary staining solution containing 5% donkey serum (Sigma Aldrich, St. Louis, MO) and primary antibody (Cell Signaling, Beverly, MA) in PBS at a dilution of 1:100 was added to each coverslip. The primary antibody used was an anti-NF-ƙB p65. The cover slips were incubated at room temperature overnight in the dark then washed with PBS as described previously. The secondary staining solution containing 5% donkey serum and diluted at 1:600 with anti-rabbit IgG secondary antibody (Cell Signaling,

Beverly, MA) was added (50 µL) to the cells and incubated for 1 hour at room temperature in the dark followed by three washes in PBS. Ten microliters of mounting media containing DAPI (Thermo Scientific, Waltham, MA) was then added to the coverslip and allowed to mount for 24 hours. Cells were imaged within the cell dishes using the Ziess LSM 510 microscope. Default files in the software for TRITC and DAPI were used in order to visualize the fluorescent probes. Images were taken using the Ziess LD Plan-Neofluar 40x/0.6 Kor objective lens.

Statistical Analysis

Proteomics:

Raw mass spectrometry (MS) data files for the study samples, collected on the Thermo Orbit rap XL system, were processed in Elucidator. MS data were grouped in Elucidator based on sample group and aligned. Sample groups were used to assist in data alignment, feature identification, and were utilized for QC assessment and group comparisons. Data were processed from retention time, 8-90 min. Thermo Orbit rap data files were searched using the Mascot search engine against the SwissProt mouse database (appended with yeast ADH, March 2, 2010). The aligned mass features were annotated with these database search results using the results from the system Peptide Tellers and a predicted error rate of 1%. MS data were summarized to the feature level, normalized, and an error-weighted ANOVA test was performed to compare the expression results between sample groups. Candidate differentially expressed markers were determined based on a $p < 0.01$. Features were summarized by protein based on the results of the database search.

Results

MTT Assay

MO has an inhibitory effect on the cell viability of healthy and arthritic synoviocytes. Initial preliminary MTT assay screening, which sought to determine the optimal non-toxic concentration of MO treatment for the synoviocytes, revealed that concentrations above 100mg/mL exhibited cytotoxic effects on the cells [Data not shown]. Follow up experiments showed that concentrations below 75 mg/mL preserved cell viability of both synoviocytes (Figure 1).

Proteomics

Forty proteins were identified in the untreated arthritic cells and the Moringa-treated arthritic cells as having their expression changed as a result of treatment. A total of 40 distinct proteins were identified in arthritic cells of both the untreated and MO-treated groups (Figure 2). Out of these 40, the expression of five proteins were down regulated by arthritis (untreated arthritic cells) but MO reversed their expression, i.e., MO up-regulated the expression of these five proteins that were down regulated in untreated arthritic cells (Figure 2). In contrast, while the remaining 35 proteins were upregulated by arthritis in the untreated cellular disease model (Figure 3.), MO treatment mitigated this expression pattern by down regulating their expression (Figures 2, 3).

***Moringa oleifera* mitigates enhanced expression of diverse pathological pathways in arthritic cells.** When the 27 proteins (Figure 2), with consistent enhanced expression in untreated arthritic cells, discussed earlier, were categorized in biological themes using the UniProt tools, the identified themes included: **a**) translation (ribosome-,

transport-, protein quality- and endoplasmic reticulum-associated proteins), **b**) cell adhesion-associated proteins, **c**) inflammation-associated proteins, **d**) oxidative stress resistance-associated proteins, **e**) Cell signaling, **f)** cytoskeleton-associated and **g**) miscellaneous (Figure 3A). Interestingly, when these arthritic cells were treated with MO, the enhanced expression of all the proteins in all the biological themes were mitigated by MO (Figures 4-9).

***Moringa oleifera* reversed the diminished protein expression of anti-inflammatory and pro-apoptotic pathways.** The 5 proteins (see Figures 3B, 10) whose expression were diminished in arthritic cells were categorized in 3 major biological themes, notably apoptosis, anti-inflammation and stress resistance (Figure 3B). Treatment of these diseased cells with MO reversed the protein expression trend by increasing their expression (Figure 3B, 10).

Confocal Immunofluorescence

Moringa down regulates expression of NF-ƙB p65. Expression of NF-ƙB p65 appeared to decrease in arthritic cells treated with MO compared with the control group (Figure 11). This data verified proteomics data.

Discussion

Previous *in vivo* studies have shown MO's anti-arthritic effects but not its underlying mechanisms. Therefore, the aim of the present study was to elucidate the anti-arthritic effects of the ethanolic extract of MO using an *in vitro* arthritic model and proteomics. Cell viability assays determined that the optimal concentration of MO in the arthritic cells was 75 mg/mL. We show here that MO mitigates the effects of arthritis by reversing the enhanced expression of proteins associated with a number of aberrant pathways, notably those associated with inflammation and proliferation. Also, we show that MO enhances expression of another set of biological themes, which were weakly expressed in arthritic cells, notably apoptotic and anti-inflammatory proteins.

Arthritis is characterized by various aberrant pathways, notably pronounced inflammation (Weisman, 2011). The present data is consistent with earlier findings and specifically revealed increased expression of two inflammatory-related proteins, namely NEDD8 and Leucine-rich pentatricopeptide repeat cassette (LPPRC), whose expression were mitigated by MO. NEDD8 is a mammalian member of the ubiquitin-like (UBL) family of proteins and it covalently ligates to target proteins through "neddylation" (Takashima et al, 2012; Amir et al, 2004), which triggers changes within the target protein, subsequently initiating other pathways. For example, NEDD8 is known to target cullin/Cdc53 family proteins leading to formation of an E2-E3 complex and subsequently increased activity of Skp, Cullin, F-box containing (SCF) complexes (Amir et al., 2004), which ubiquitylates phosphorylated IκBα ultimately transporting NF-κB, the master transcription of inflammation, to the nucleus for initiation of inflammation (Kawakami et al., 2001). NEDD8 is also known to block the function of tumor suppressor p53, which is involved in the

regulation of proliferation and apoptosis, via proteasomal degradation. This inactivation (via NEDD8) causes cells, such as the fibroblast-like synoviocytes, to lose their proliferative activity (Keisuke et al, 2001; Yang et al, 2005).

Leucine-rich PPR motif-containing protein (LPPRC) is known to exert multiple roles, including those involving mitochondrial activity and the expression and stability of the COX subunits, namely CO1 and CO3, which are essential for the formation of cytochrome c oxidase (Xu et al, 2004). Further, LPPRC expression is also significantly upregulated in hepatocellular carcinoma cell lines and when LPPRC was knocked down *in vitro*, their tumorigenic activity, such as colony formation and invasive abilities, was significantly diminished (Michaud et al, 2011). Lastly, LRPPRC prevents apoptosis via enhancing expression of pro-survival Bcl-2 family members, such as Bcl-2, BclxL and Mcl-1. Collectively, these data highlight the role of LPPRC in proliferation and MO likely inhibits the aberrant cellular proliferation and inflammation in arthritic cells, in part, by down regulating the expression of LPPRC and NEDD8.

D-dopachrome decarboxylase, or DOPD, is also implicated to play a role in positive regulation of inflammatory activity. In one study, it was found that recombinant DOPD binds CD74 with high affinity, leading to activation of ERK1/2 MAP kinase and downstream pro-inflammatory pathways. DOPD also exhibits positive regulation of TNFα, IFNγ, and IL-1β production as well as immunoneutralization of DOPD was found to significantly reduce levels of these inflammatory chemicals (Merk et al, 2011).

Pathogenesis of rheumatoid arthritis involves multiple cells, including macrophage, T- and B cells, angiogenic, osteoclasts and synoviocytes. The multiplicity of cell types requires complex signaling activity and molecules, possibly including S10A6, IPRI and

MCTPI, as revealed by the present study. For instance, IPRI and MCTP1 are involved in the release and modulation of calcium, which modulates multiple cellular responses, such as exocytosis, motility, proliferation and gene expression (Van Rossum et al, 2006; Shin et al, 2005). S10A6 also plays a role in normal cell proliferation and is also localized in a variety of cancer phenotypes (Breen et al, 2003) and enhances pro-survival pathways in cancer. If these molecules induce similar proliferative effects in arthritic synoviocytes, they likely participate in promoting one of the hallmarks of RA progression (Emberley et al, 2004).

This aberrant proliferation observed in the current study in arthritic cells could be closely linked to an increase in translation, which likely "fuels" or supports proliferation. Indeed, this may also likely explain the increase in the expression of proteins, such as LPPRC, discussed earlier. Interestingly, MO was able to not only block the expression of proliferation-promoting molecules, such as LPPRC and the aberrant cellular proliferation, but also inhibited expression of translation-associated proteins, implying that MO may repair dysregulation of cell proliferation in arthritic synoviocytes. Specifically, the proteins associated with translation that were downregulated by MO include IF4G1, involved in mRNA recruitment; WBP11, involved in mRNA processing, and RS4X, which is involved in ribosomal function (Gladyshev et al, 2010; Holcik & Sonenberg, 2005; Sudol et al, 2001; Watanabe et al, 1993). Decreased expression of IF4G1 may also have implications in the proliferative and apoptotic nature of the arthritic cells. Increased expression of IF4G1, which is common in breast cancers, was found to increase translation of mRNAs involved in cell survival, preventing autophagy and apoptosis. Reduced expression of the gene had the inverse effect (Badura et al, 2012). Therefore, the reduction in the expression of this gene in

the synoviocytes as a result of treatment with MO may reduce the overall proliferative activity of the cells.

Proteins that are involved in protection and resistance to oxidative stress were found to be downregulated as a result of MO treatment of the arthritic cells. We deduce from these findings that the HFLS-RA cells may be upregulating these proteins in order to protect themselves from an environment high in ROSs to which they are exposed as exposure to these molecules can induce apoptosis (Hitchon & El-Gabalawy, 2004). While MO is known to be an antioxidant, we believe that MO likely acts upon the arthritic cells and makes them less resistant to oxidative stress by inhibiting the expression of the antioxidant proteins. This, in turn, would make them more susceptible to their hypoxic environment and thus may initiate apoptosis in the cells (Aruselvan et al., 2016; Hitchon & El-Gabalawy, 2004).

Other than proliferation, arthritic cells also undergo changes in their shape and mobility (Kollias & Gkretsi, 2007). These two changes require cytoskeletal elements and possibly adhesion molecules (Janmey, 1998). For example, expression of MICA2, which has been implicated in cytoskeleton remodeling in metastatic cancer progression, cell-cell and cell-substrate adhesion, motility and invasion, was up regulated in arthritic cells, and has been previously reported to be down regulated by MO, consistent with the present data (Mariotti et al, 2016; Fremont et al, 2017). It has also been demonstrated in previous studies that depletion of MICAL1 (the family of molecules to which MICA2 belongs) reduced cell migration and invasion as well as ROS generation in breast cancer cells (Deng et al, 2016). Similarly to MICA2, CO6A3 is upregulated in cancer cells, specifically ovarian cancer cells. This protein is involved in both cell adhesion and cytoskeleton remodeling (Sherman-Baust et al, 2003). Staining of ovarian tumors with collagen VI antibodies confirmed its expression

in vivo and suggested reorganization of the extracellular matrix around the tumor. Also, the presence of CO6A3 was found to correlate with tumor grade, an ovarian cancer prognostic factor, suggesting that tumor cells may directly remodel their microenvironment to increase their survival in the presence of chemotherapeutic drugs, as higher expression of CO6A3 was found in ovarian cells resistant to chemotherapeutic treatment. (Sherman-Baust et al, 2003).

In addition to reducing the expression of certain potentially clinically relevant proteins in the pathogenesis of RA, MO was also found to upregulate certain proteins. The functions of these proteins fell into one of three categories: apoptosis, stress resistance, and protection from inflammation. The pro-apoptotic gene of PDC61, encodes for a protein that binds to the products of the PDCD6 gene, which is involved in the apoptosis pathway (Hashemi et al 2015). Increase in its expression would correlate to an increase in apoptotic pathways. Kallistatin is responsible for conferring an anti-inflammatory response, as well as possessing anti-angiogenic properties and inhibiting tumor growth (Miao et al, 2002). HSP71 is also a protective protein in that it protects the proteasome from environmental stressors, such as inflammation. All the three proteins upregulated here have therapeutic implications in arthritis, i.e., protecting the cell from inflammatory and oxidative stressors is important as RA is a highly inflammatory condition with notable increases in the production of ROSs. If MO is able to restore cellular regulation of apoptosis while also protecting the cells of the joint from the unfavorable environment in the joint created by arthritis, then it can be deduced that MO would be a potential therapy in targeting the symptoms of arthritis and hampering disease progression.

The limitations of the present study are many and include the following: 1). Since the development and progression of RA involves an intricate interplay of many cell types,

including immune cells, synoviocytes, blood vessel cells and their associated cytokines and chemokines, an *in vivo* model is more ideal; 2) Secondly, this study utilized the whole leaf ethanolic extract of MO. It would have been more beneficial if the subfractions and active compounds within the extract could have been identified so that the subfractions responsible for the observed desired effects could be isolated for further experimentation. 3) Lastly, it would have been more useful if a reference drug was used to which MO could have been compared in terms of efficacy. Future studies could utilize a widely used drug, such as methotrexate in order to determine how MO measures up to pharmaceuticals and whether it would be a suitable substitute in a clinical setting.

We conclude that MO mitigates the expression of proteins implicated in the pathogenesis and progression of arthritis, notably inflammation, proliferation and oxidative stress, whereas on the other hand it (MO) enhances expression of proteins with potentially protective activities, such as apoptosis and anti-inflammation. These findings show that MO is a strong or potential candidate as an alternative therapy for RA.

References

Abdull Razis AF, Ibrahim MD, and Kntayya SB (2014). Health benefits of Moringa oleifera. *Asian Pacific Journal of Cancer Prevention* 15: 8571–8576.

Amir RE, Haecker H, Karin M, and Ciechanover, A (2004). Mechanism of processing of the NF-kB2 p100 precursor: Identification of the specific polyubiquitin chain-anchoring lysine residue and analysis of the role of NEDD8-modification on the SCF(-TrCP) ubiquitin ligase. *Oncogene* 23: 2540–2547.

Arulselvan P, Tan W, Gothai S, Muniandy K, Fakurazi S, Esa N, Alarfaj A, and Kumar S (2016). Anti-Inflammatory potential of ethyl acetate fraction of Moringa oleifera in downregulating the NF-κB signaling pathway in lipopolysaccharide-stimulated macrophages. *Molecules* 21: 1452.

Badura M, Braunstein S, Zavadil J, Schneider RJ. (2012). DNA damage and eIF4G1 in breast cancer cells reprogram translation for survival and DNA repair mRNAs. *Proceedings of the National Academy of Science USA* 109: 18767–18772.

Bartok B and Firestein GS (2010). Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. *Journal of Immunology Review* 233: 233–255.

Bottini N and Firestein GS (2013). Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors. *Nature Reviews Rheumatology*. 9: 24–33.

Breen EC, and Tang K (2003). Calcyclin (S100A6) regulates pulmonary fibroblast proliferation, morphology, and cytoskeletal organization in vitro. *Journal of Cell Biochemistry* 88: 848–854.

Brooks P and Day R (1991). Nonsteroidal anti-inflammatory drugs--differences and similarities. *New England Journal of Medicine*. 324(24): 1716.

Coppin JP, Xu Y, Chen H, Pan MH, Ho CT, Juliani R, Simon JE, Wu Q (2013). Determination of flavonoids by LC/MS and anti-inflammatory activity in Moringa oleifera. *Journal of Functional Foods* 5: 1892–1899.

Cronstein BN (1997). The mechanism of action of methotrexate. *Rheumatic Disease Clinics* 23(4): 739 – 755.

Das S and Kanodia L (2012) Effect of ethanolic extract of leaves of Moringa oleifera lam on acetic acid induced colitis in albino rats. *Asian Journal of Pharmaceutical and Clinical Research* 5: 110–114.

Deng, W et al., (2016). MICAL1 controls cell invasive phenotype via regulating oxidative stress in breast cancer cells. *BMC Cancer* 16: 4869.

Drexler SK, Kong PL, Wales J, Foxwell, BM (2008). Cell signaling in macrophages, the principal innate immune effector cells of rheumatoid arthritis. *Arthritis Research & Therapy* 10(5): 216

Emberley ED, Murphy LC, Watson PH (2004). S100 proteins and their influence on pro-survival pathways in cancer. *Biochemistry and Cell Biology* 82(4): 508–15

Firestein GS (1996). Invasive fibroblast-like synoviocytes in rheumatoid arthritis: passive responders or transformed aggressors? *Arthritis and Rheumatology* 39: 1781–1790.

Firestein, GS (2003). Evolving concepts of rheumatoid arthritis. *Nature* 423(6937): 356.

Fremont S, Hammich H, Bai J, et al. (2017). Oxidation of F-actin controls the terminal steps of cytokinesis. *Nature Communications* 8:14528.

Gilani A, et al. (2006). Moringa oleifera: A food plant with multiple medicinal uses. *Phytotherapy Research* 21(1): 17-25.

Gladyshev V, Shchedrina VA, Zhang Y, Labunskyy VM, Gladyshev V N, Hatfield DL (2010). Structure-Function Relations, Physiological Roles, and Evolution of Mammalian ER-Resident Selenoproteins. *Antioxidants & Redox Signaling*, 12(7): 839-849.

Goldring, SR (2003). Pathogenesis of bone and cartilage destruction in rheumatoid arthritis. *Rheumatology* 42(Suppl. 2): ii1–ii6.

Hashemi M, Yousefi J, Hashemi SM, Amininia S, Ebrahimi M, Taheri M, Ghavami S (2015). Association between programmed cell death 6 interacting protein insertion/deletion polymorphism and the risk of breast cancer in a sample of Iranian population. *Dis Markers* 854621.

Hitchon CA and El-Gabalawy HS (2004). Oxidation in rheumatoid arthritis. *Arthritis Research and Therapy* 6: 265–78.

Holcik M & Sonenberg N (2005). Translational control in stress and apoptosis. *Nature Reviews of Molecular Cell Biology* 6: 318–327.

Janmey, PA (1998). The cytoskeleton and cell signaling: component localization and mechanical coupling. *Physiological Reviews* 78: 763-781.

Kawakami T, Chiba T, Suzuki T, Iwai K, Yamanaka K, Minato N, Suzuki H, Shimbara N, Hidaka Y, Osaka F, Omata M, and Tanaka K (2001) NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *EMBO Journal* 20: 4003–4012.

Keisuke T, Masao O, Keiji T, and Tomoki C (2001). The NEDD8 System Is Essential for cell cycle progression and morphogenetic pathway in mice. *The Journal of Cell Biology* (4), 571.

Kollias G and Gkretsi, V (2007). Actin cytoskeleton dynamics linked to synovial fibroblast activation as a novel pathogenic principle in TNF-driven arthritis. *Annals of The Rheumatic Diseases* 6623–28.

Koneni S, Siron Mani R, Sashidhara KV, Rosaiah, JN, Tyagi E, Shukla R, Rajendran, SM (2009). Rare dipeptide and urea derivatives from roots of Moringa oleifera as potential anti-inflammatory and antinociceptive agents. *European Journal of Medicinal Chemistry* 44(1): 432–436.

Kong YY et al. (1999). Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 402: 304–309.

Mahajan SG and Mehta AA (2010). Immunosuppressive activity of ethanolic extract of seeds of Moringa oleifera Lam. in experimental immune inflammation. *Journal of Ethnopharmacology* 130(1): 183 –186.

Mahajan SG, Mali RG, Mehta AA. (2007). Protective effect of ethanolic extract of seeds of Moringa oleifera Lam. against inflammation associated with development of arthritis in rats. *Journal of Immunotoxicology* 4(1): 39–47.

Mahdi, HJ, et al. (2017). Original article: In vivo anti-arthritic and anti-nociceptive effects of ethanol extract of Moringa oleifera leaves on complete Freund's adjuvant (CFA)-induced arthritis in rats. *Integrative Medicine Research* 7(1): 85–94.

Mariotti S, Barravecchia I, Vindigni C, Pucci A, Balsamo M, Libro R, Senchenko V, Dmitriev A, Jacchetti E, Cecchini M, et al. (2016). MICAL2 is a novel human cancer gene controlling mesenchymal to epithelial transition involved in cancer growth and invasion. *Oncotarget* 7(2): 1808–25.

Merk M, Zierow S, Leng L et al. (2011). The D-dopachrome tautomerase (DDT) gene product is a cytokine and functional homolog of macrophage migration inhibitory factor (MIF). *Proceedings of the National Academy of Sciences of the United States of America* 108(34): E577–E585.

Miao RQ, Agata J, Chao L, Chao J (2002) Kallistatin is a new inhibitor of angiogenesis and tumor growth. *Blood* 100: 3245–3252.

Michaud M, Barakat S, Magnard S, Rigal D, Baggetto LG (2011) Leucine-rich protein 130 contributes to apoptosis resistance of human hepatocarcinoma cells. *International Journal of Oncology* 38: 169–178.

Mittal A, Sharma M, David A, Vishwakarma P, Saini M, Goel M, and Saxena KK (2017). An experimental study to evaluate the anti-inflammatory effect of moringa oleifera leaves in animal models. *International Journal of Basic & Clinical Pharmacology* 6: 452.

Mukherjee K, Khalid MK (2017). "Sociodemographic Determinants of Out-of-Pocket Expenditures for Patients Using Prescription Drugs for Rheumatoid Arthritis." *American Health & Drug Benefits* 10(1): 7–14.

Neumann E, Lefèvre S, Zimmermann B, Gay S, and Müller-Ladner, U. (2010). Review: Rheumatoid arthritis progression mediated by activated synovial fibroblasts. *Trends in Molecular Medicine* 16458–468.

Parker E, Elam A (2017). Rheumatoid arthritis: diagnosis and treatment. Clinical Advisor 20(5): 19–25.

Pettit, AR et al. (2001). TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. *American Journal of Pathology* 159: 1689–1699.

Sable R, Durek T, Taneja V, Craik DJ, Pallerla S, Gauthier T, and Jois S (2016). Constrained cyclic peptides as immunomodulatory inhibitors of the CD2:CD58 protein-protein interaction. *ACS Chemical Biology* 11: 2366–2374.

Saklatvala J (1986). Tumor necrosis factor α stimulates resorption and inhibits synthesis of proteoglycan in cartilage. *Nature* 322: 547–9.

Schett G and Gravallese E (2012). Bone erosion in rheumatoid arthritis: mechanisms, diagnosis and treatment. *Nature Reviews Rheumatology*, 8(11), 656–664.

Scott DL, Wolfe F, Huizinga TW (2010). Rheumatoid arthritis. *The Lancet* 376: 1094–1108.

Sherma Z, Preedy VR, Watson RR (2014). Polyphenols in Human Health and Disease. London: Academic Press.

Sherman-Baust, CA, et al. (2003). Remodeling of the extracellular matrix through overexpression of collagen VI contributes to cisplatin resistance in ovarian cancer cells. *Cancer Cell* 4:377-386.

Shin OH, Han W, Wang Y, Sudhof TC (2005). Evolutionarily conserved multiple C2 domain proteins with two transmembrane regions (MCTPs) and unusual Ca2+ binding properties. *Journal of Biological Chemistry* 280:1641-1651.

Smolen JS, Aletaha D, Koeller M, et al. (2007). New therapies for treatment of rheumatoid arthritis. *The Lancet*, 370(9602): 1861-1874.

Smolen JS, Aletaha D, McInnes IB (2016). "Seminar: Rheumatoid arthritis." *The Lancet* 388: 2023-2038.

Sudol M, Sliwa K, Russo T (2001). Functions of WW domains in the nucleus. *FEBS Letters* 490: 190–195.

Sujatha BK, Patel P (2017). Moringa Oleifera – Nature's Gold. Imperial Journal of Interdisciplinary Research 3(5): 2454-1362.

Takashima, O., Tsuruta, F., Ebina, M., Kigoshi, Y., Nakamura, S., & Chiba, T. (2012). A novel NEDD8-binding protein modulates NF-κB signaling pathway. Arthritis Research & Therapy (Formerly Arthritis Research), 14(Supplement 1), 1.

Takayanagi H, Iizuka, H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, Koshihara Y, Oda H, Nakamura K, Tanaka S (2000). Involvement of receptor activator of nuclear factor κB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. *Arthritis Rheumatology* 43, 259–269.

Van Rossum DB., Patterson RL., Cheung KH et al (2006) DANGER: A novel regulatory protein of IP3-receptor activity. J Biol Chem. 281: 37111–37116.

Vane JR, Botting R (1987). Inflammation and the mechanism of action of anti-inflammatory drugs. *FASEB Journal* 7: 89-96.

Watanabe M, Zinn AR, Page DC, Nishimoto T (1993). Functional equivalence of human X- and Y-encoded isoforms of ribosomal protein S4 consistent with a role in Turner syndrome. *Nature Genetics*. 4: 268–271.

Weisman, MH (2011). Rheumatoid arthritis. New York: Oxford University Press, 2011.

Xu F, Morin C, Mitchell G, Ackerley C, Robinson BH (2004). The role of the LRPPRC gene in cytochrome oxidase assembly: mutation causes lowered levels of COX (cytochrome c oxidase) I and COX III mRNA. *Biochemical Journal* 382: 331–6.

Yang X, Wang J, Liu C, Grizzle WE, Yu S, Zhang S, Barnes S, Koopman WJ, Mountz JD, Kimberly RP, Zhang HG (2005). Cleavage of p53-vimentin complex enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis of rheumatoid arthritis synovial fibroblasts. *American Journal of Pathology* 167: 705–719.

Figures

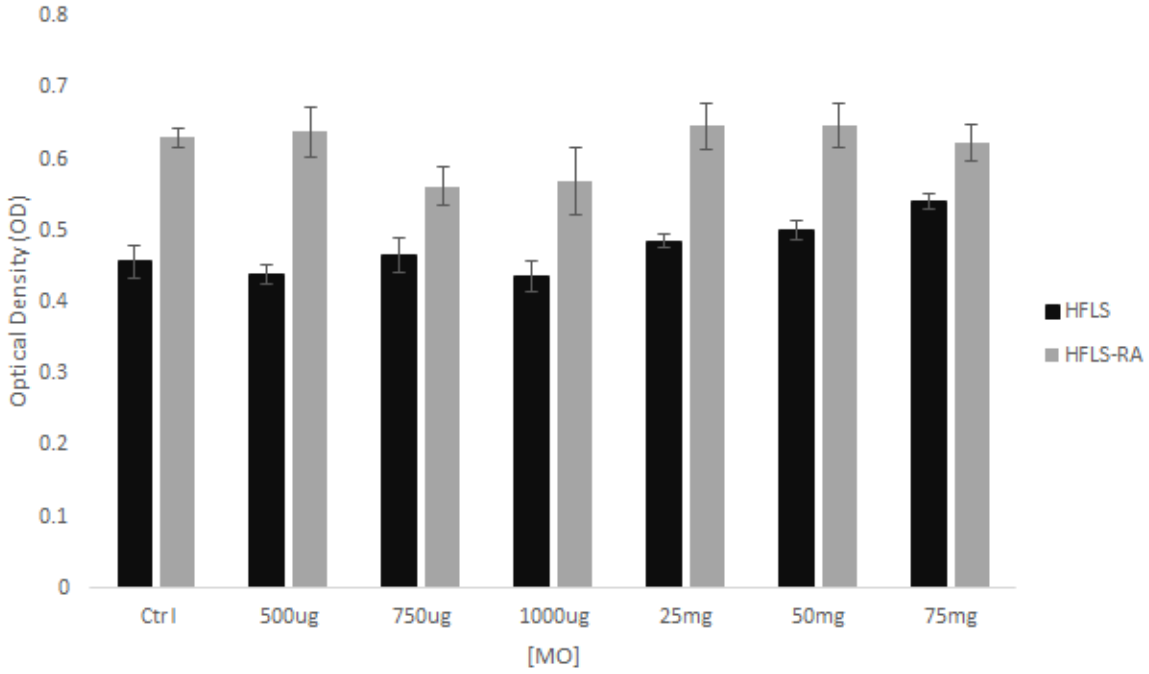


Figure 1. Data from MTT Assay shows that *Moringa oleifera* is not toxic to both healthy and arthritic cells. Healthy and arthritic cells were treated with varying concentrations (up to 75 mg/mL) of MO for 24 hours. Cell viability was not affected in both arthritic and healthy cells below the concentration of 75 mg/mL.

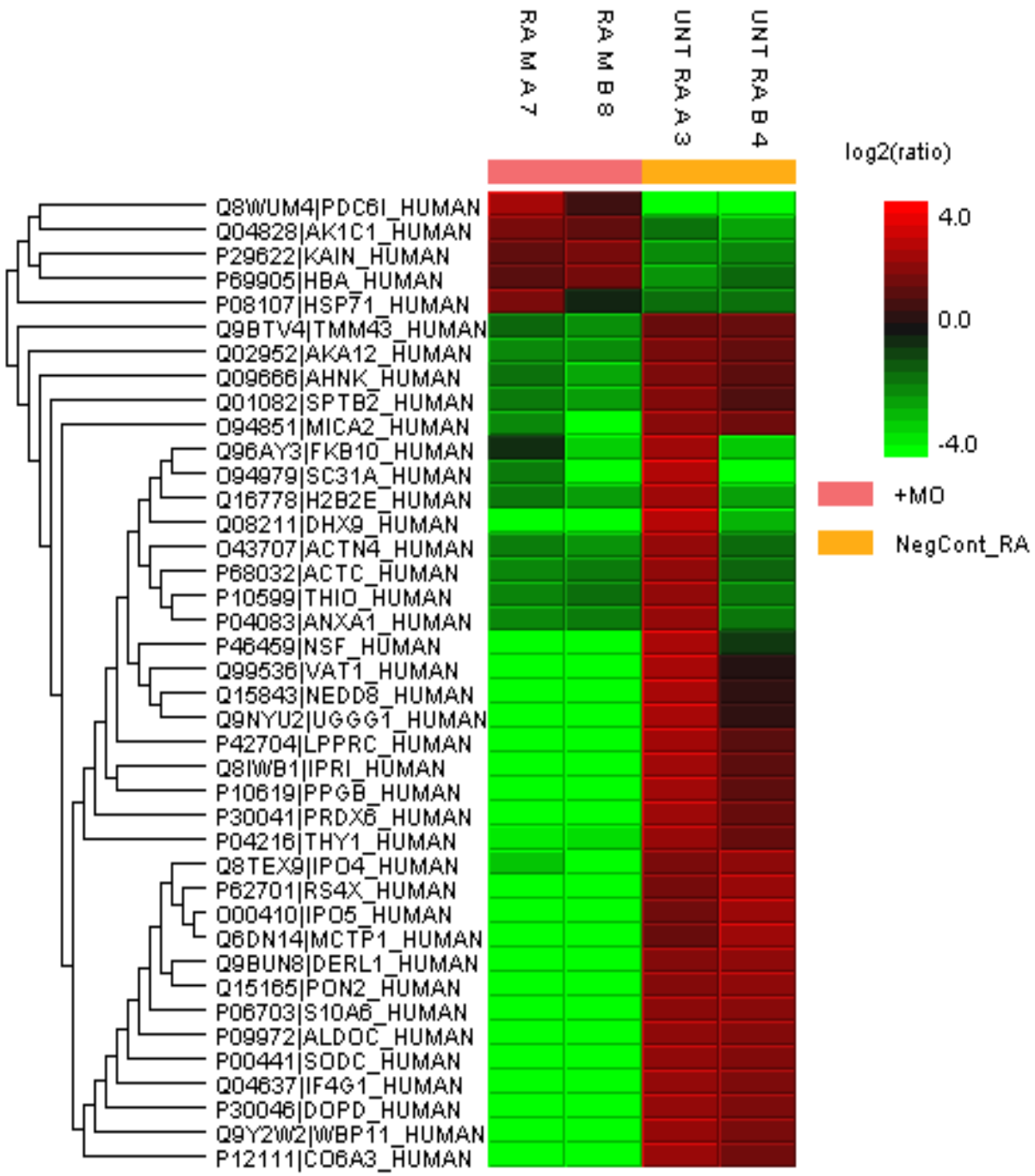
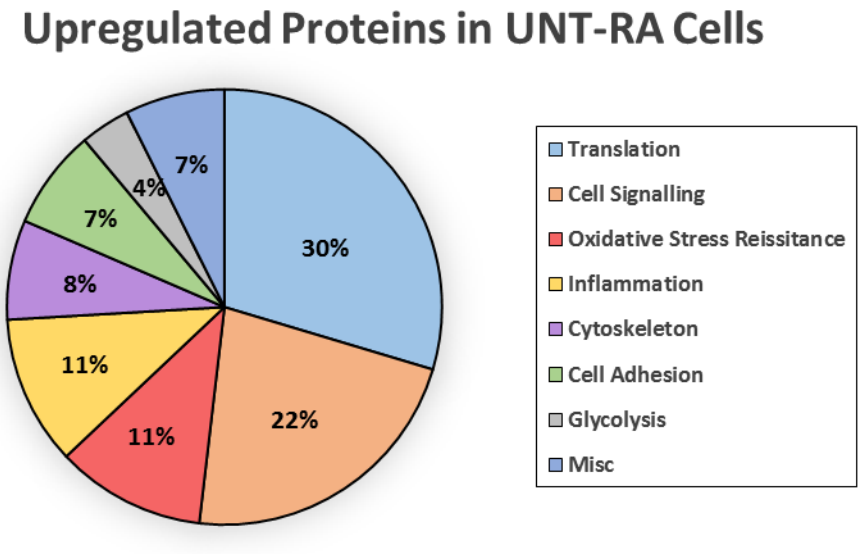


Figure 2. Heat map of the proteomic analysis shows *Moringa oleifera's* effects on protein expression in human arthritic synoviocytes (HFLS-RA). *Moringa oleifera* reverses the aberrant expression of almost all the proteins in human fibroblast-like synoviocytes (HFLS-RA), by down regulated most of their expression but up regulated a few of them.

A.



B.

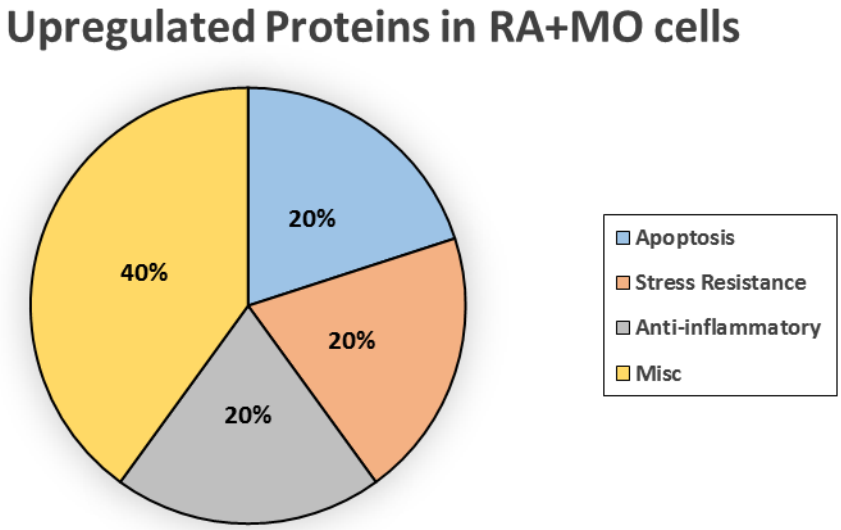
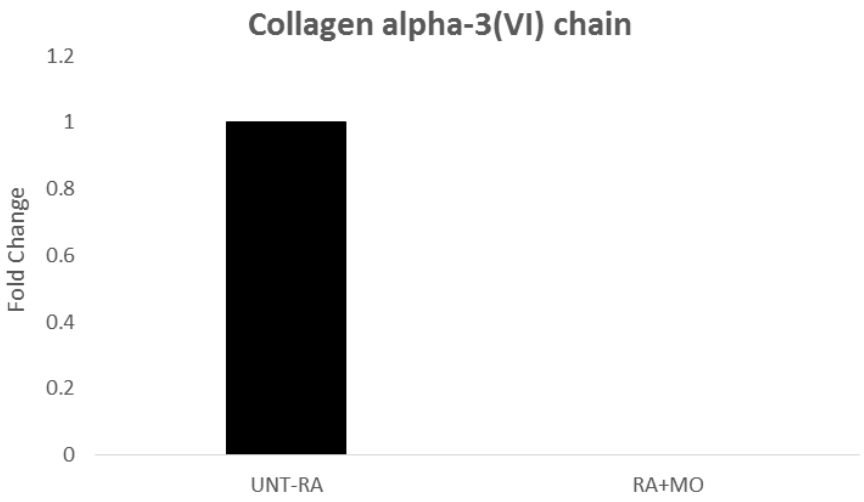


Figure 3. Pie charts show that *Moringa oleifera* modulates protein expression in a cell model of arthritis. *Moringa oleifera* alters protein expression in biological processes such as: (**A**) translation, cell adhesion, cell signaling, inflammation and glycolysis by down regulation; (B) stress resistance, apoptosis, and anti-inflammation by enhancement.

A



B

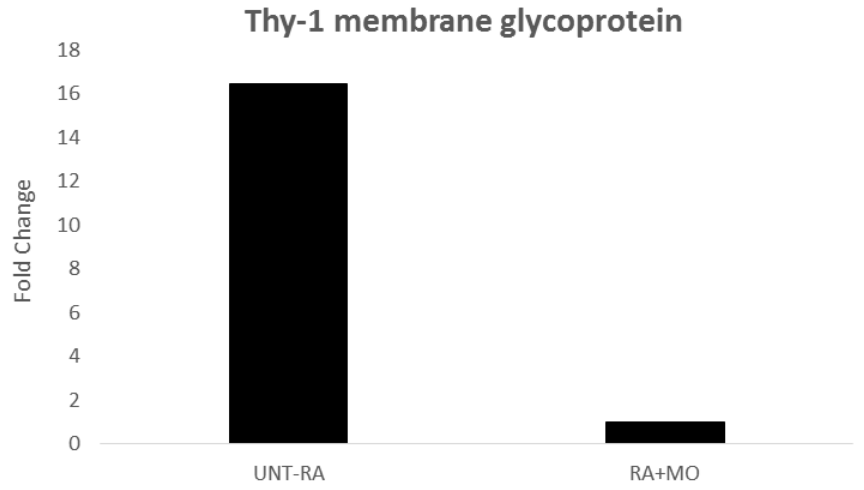


Figure 4. Proteomics analysis shows that *Moringa oleifera* down regulates expression of cell adhesion-associated proteins in human arthritic synoviocytes (HFLS-RA). The two proteins are: (**A**). Collagen alpha-3(VI) chain (CO6A3) and (**B**) Thy-1 membrane glycoprotein (THY1).

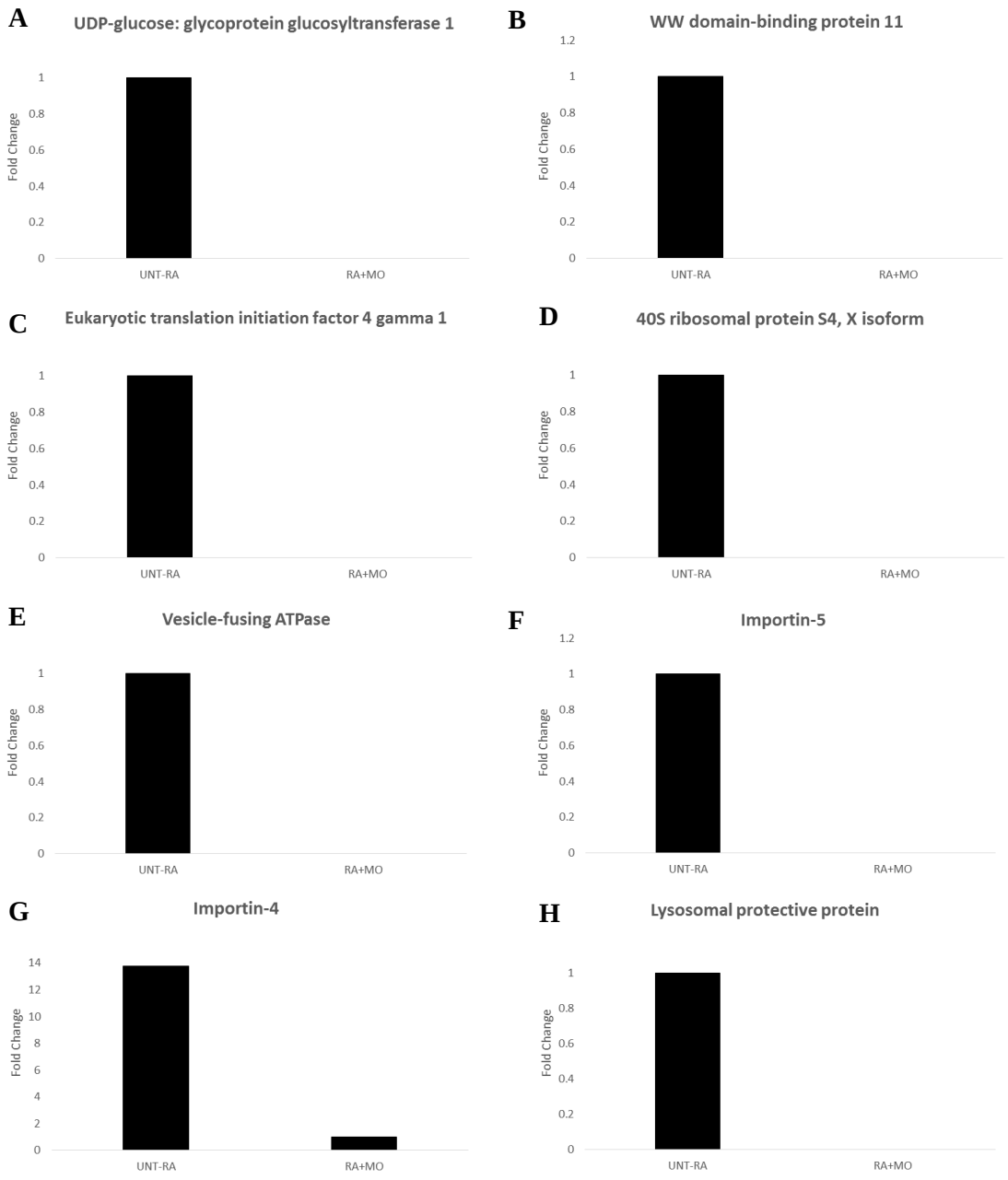


Figure 5. Proteomics analysis shows that *Moringa oleifera* down regulates expression of translation-associated proteins in human arthritic synoviocytes (HFLS-RA). These proteins include: (**A**) UDP-glucose: glycoprotein glucosyltransferase 1 (UGG1), (**B**) WW domain-binding protein 11 (WBP11), (**C**) Eukaryotic translation initiation factor 4 gamma 1 (IF4G1), (**D**) 40S ribosomal protein S4, X isoform (RS4X), (**E**) Vesicle-fusing ATPase (NSF), (**F**) Importin-5 (IPO5), (**G**) Importin-4 (IPO4), (**H**) Lysosomal protective protein (PPGB).

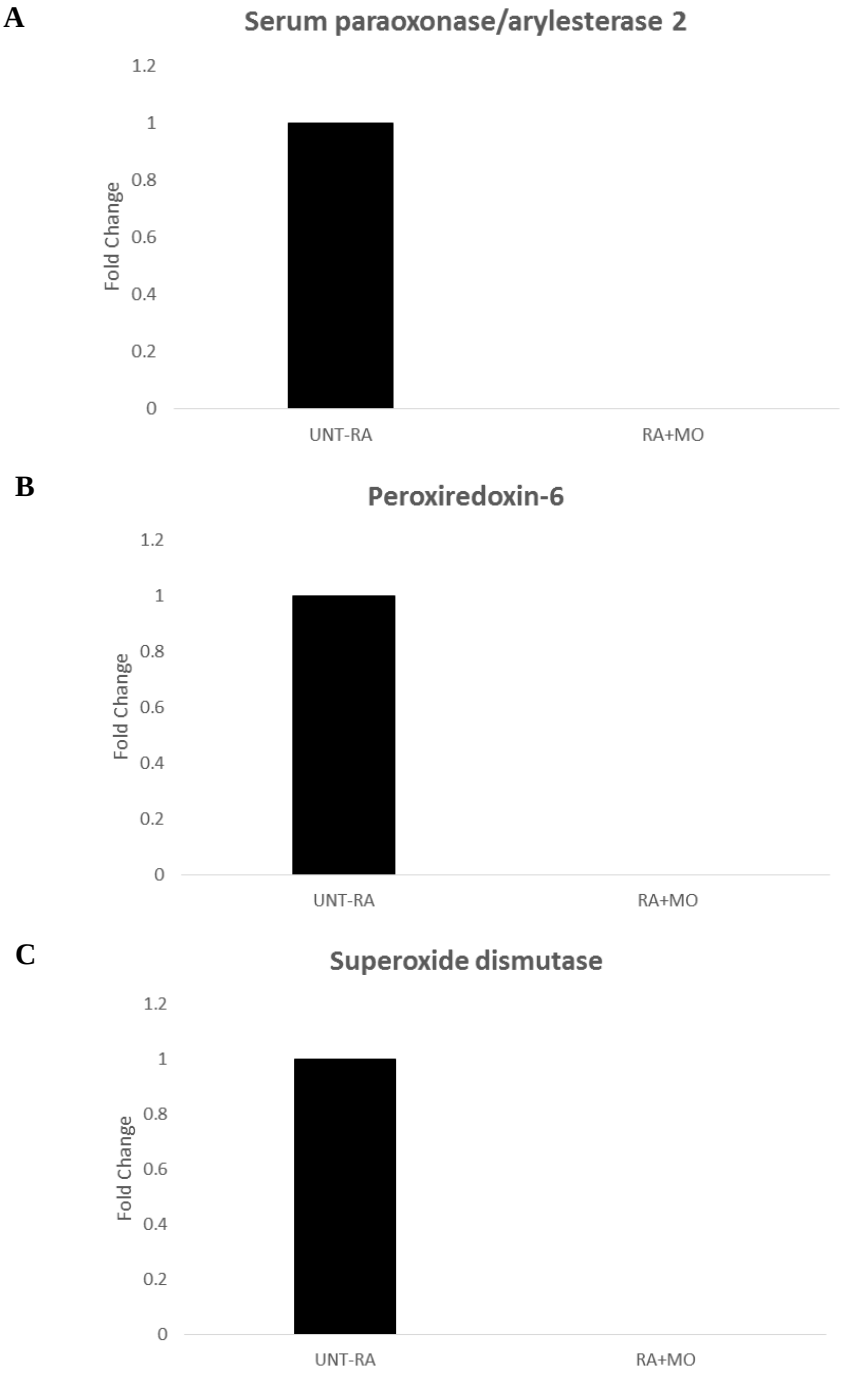


Figure 6. Proteomics analysis shows that *Moringa oleifera* down regulates expression of oxidative stress resistance-associated proteins in human arthritic synoviocytes (HFLS-RA). The three proteins include: (**A**) Serum para-oxonase/arylesterase 2 (PON2), (**B**) Peroxiredoxin-6 (PRDX6), (**C**) Superoxide dismutase (SODC).

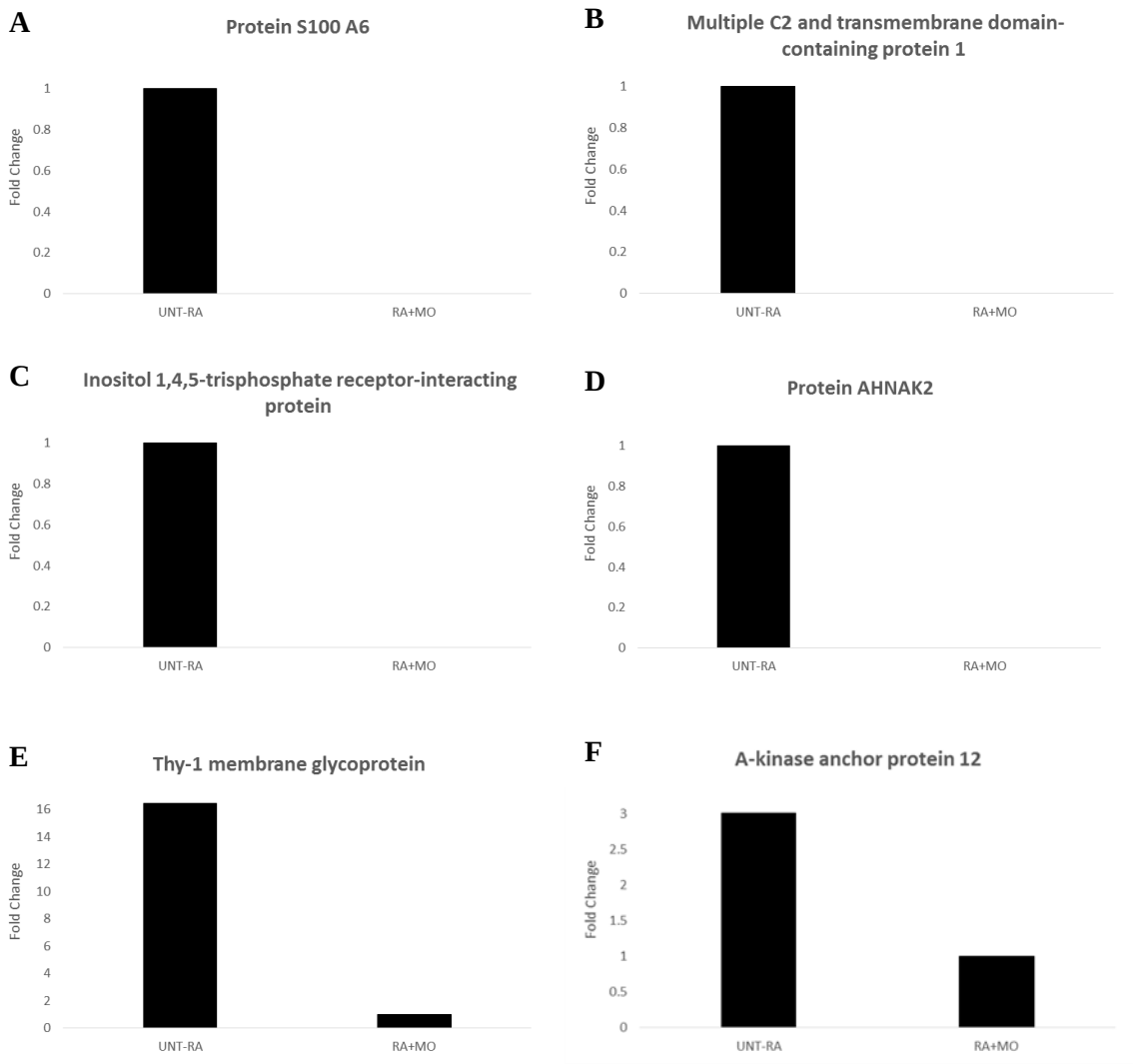


Figure 7. Proteomics analysis shows that *Moringa oleifera* down regulates expression of cell signaling-associated proteins in human arthritic synoviocytes (HFLS-RA). The six proteins include: (A) Protein S100 A6 (S10A6), (**B**) Multiple C2 and transmembrane domain-containing protein 1 (MCTP1), (**C**) Inositol 1,4,5-trisphosphate receptor-interacting protein (IPRI), (**D**) Protein AHNAK2 (AHNK), (**E**) Thy-1 membrane glycoprotein (THY1), (**F**) A-kinase anchor protein 12 (AKA12).

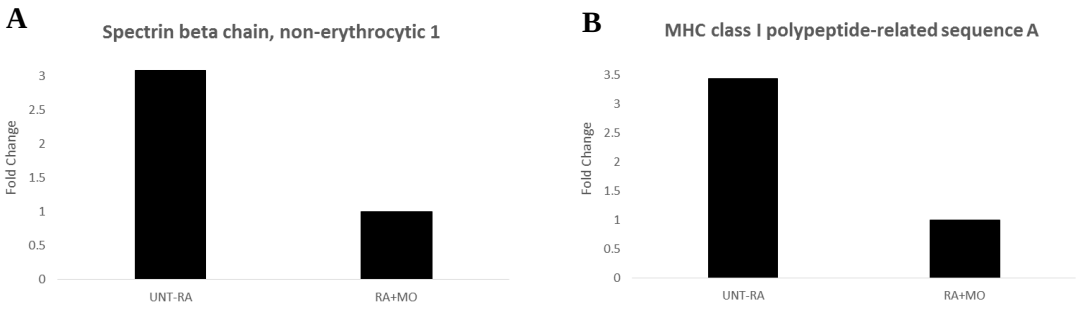


Figure 8. Proteomics analysis shows that *Moringa oleifera* down regulates expression of cytoskeleton-associated proteins in human arthritic synoviocytes (HFLS-RA). Two proteins – Spectrin beta chain, non-erythrocytic 1 (SPTB2), Protein-methionine sulfoxide oxidase MICAL2 (MICA2) – were found to be elevated in the proteome of the untreated arthritic cells.

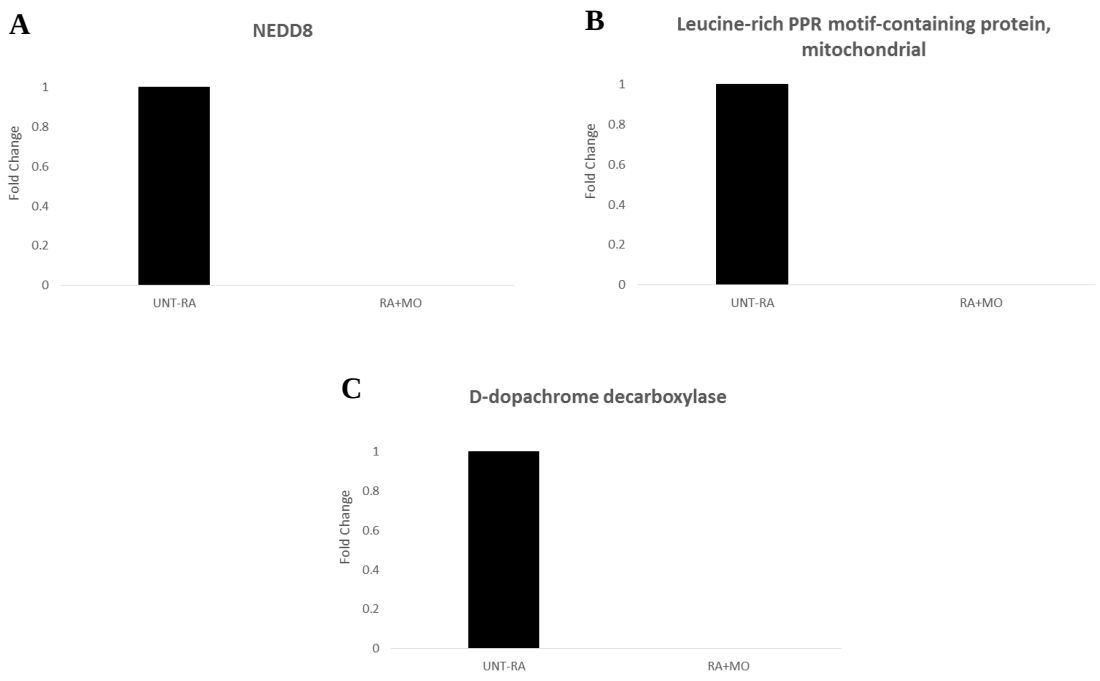


Figure 9. Proteomics analysis shows that *Moringa oleifera* down regulates expression of inflammation-associated proteins in human arthritic synoviocytes (HFLS-RA). The three proteins include: (**A**) NEDD8, (**B**) Leucine-rich PPR motif-containing protein, mitochondrial (LPPRC) and (**C**) D-dopachrome decarboxylase.

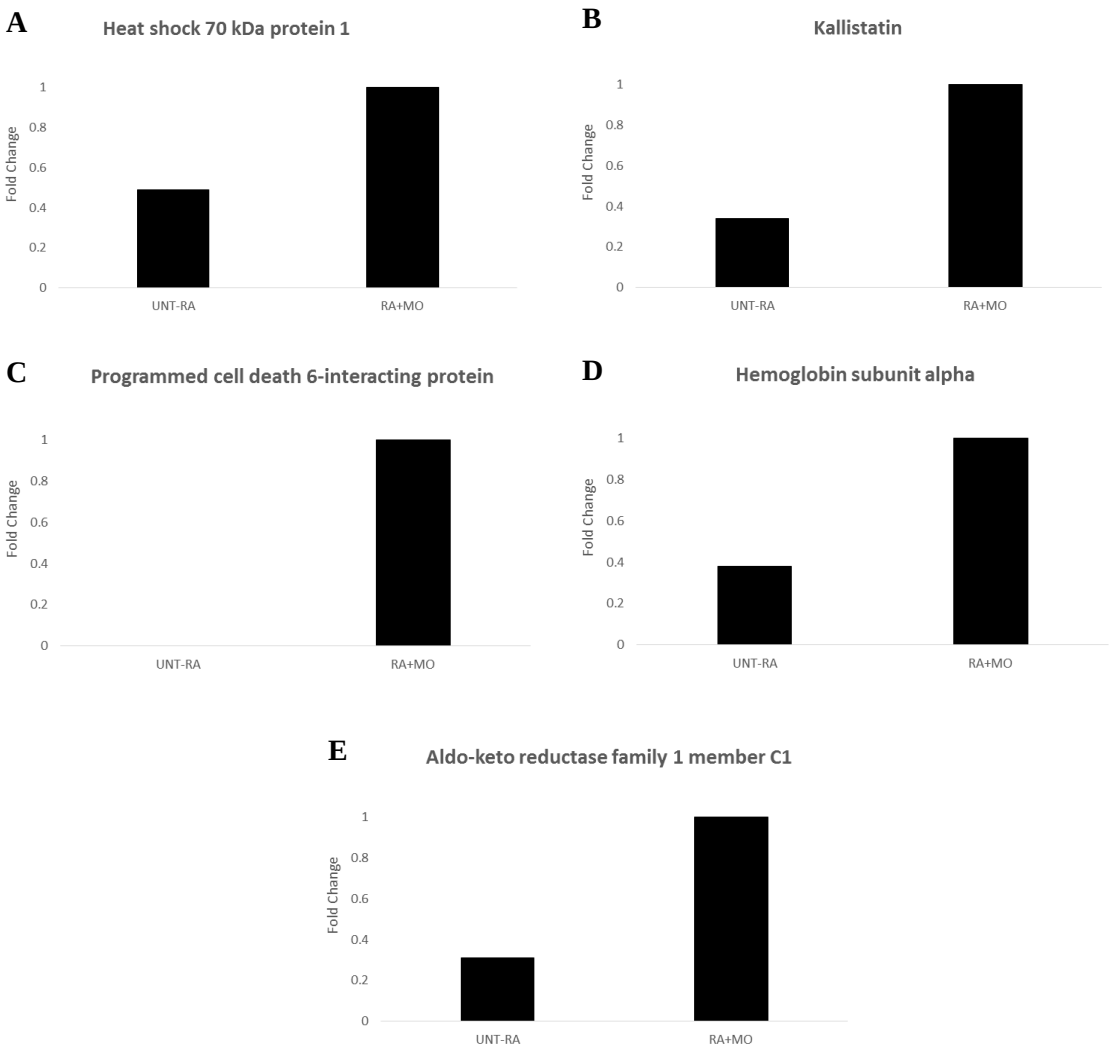


Figure 10. Proteomics analysis shows that *Moringa oleifera* overall down regulates expression of proteins with cyto-protective roles in human arthritic synoviocytes (HFLS-RA). These proteins include: (**A**) Heat shock 70 kDa protein 1A/1B (HSP71), (**B**) Kallistatin (KAIN), (**C**) Programmed cell death 6-interacting protein (PDC61), (**D**) Hemoglobin subunit alpha (HBA) and (**E**) Aldo-keto reductase family 1 member C1 (AK1C1).

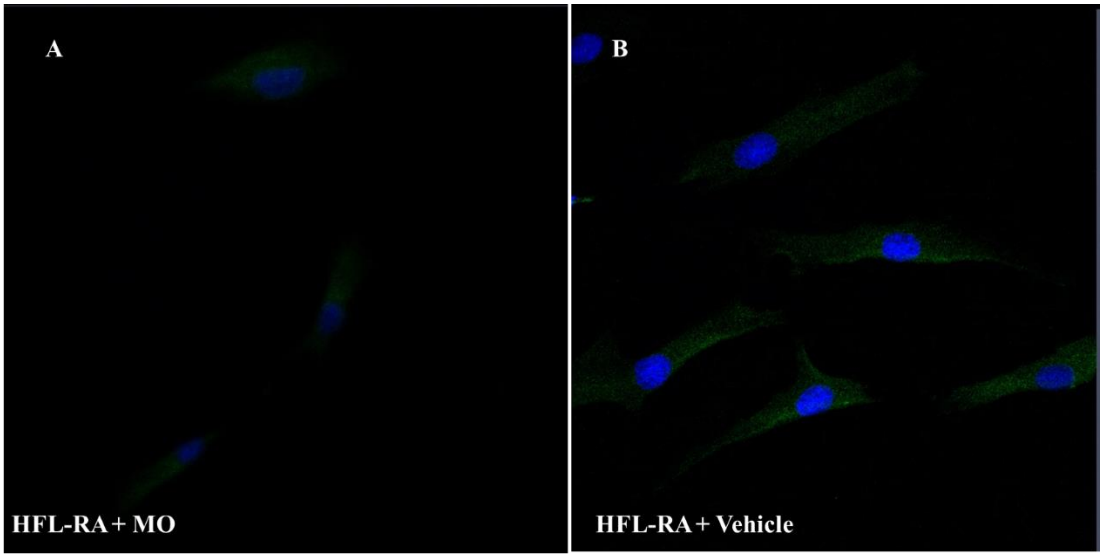


Figure 11. Confocal images show that *Moringa oleifera* down regulates the expression of NF-ƙB in human fibroblast-like synoviocytes (HFLS-RA) (**A**) compared to (**B**) vehicle.

Vita

Brianna Nicole Tate was born in Greensboro, North Carolina, to Caroline Tomlinson Pemberton and William Briceson Tate III. She graduated from Grimsley High School in North Carolina with an International Baccalaureate diploma in June 2012. The following autumn, she entered Appalachian State University to study Biology and, in May, 2016, she was awarded a Bachelor of Arts degree. In the fall of 2016, she began her study toward a Master of Science degree in Cell and Molecular Biology at Appalachian State University. The M.S. was awarded in December, 2018. In August, 2018, Ms. Tate commenced work toward her Ph.D. in Animal Science at Cornell University.