LEISHMANICIDAL, ANTI-INFLAMMATORY AND ANTI-OBESITY PROPERTIES OF NATURAL PRODUCTS FROM COMMON MEDICINAL AND EDIBLE PLANTS

by

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ABSTRACT OF THE DISSERTATION

Leishmanicidal, Anti-Inflammatory and Anti-Obesity Properties of

Natural Products from Common Medicinal and Edible Plants

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The dissertation aim was to present natural products derived from four common edible medicinal plants that could be applied for solving leishmaniasis, obesity and type 2 diabetes. This research showed leishmanicidal natural compounds isolated from *Cichorium intybus* L. (Asteraceae), *Cornus florida* L. (Cornaceae), *Eryngium foetidum* L. (Apiaceae), which have been used traditionally as antiparasitic remedies. The roots of *C. intybus* (chicory) yielded four sesquiterpene lactones: (1) 11(S),13-dihydrolactucopicrin, (2) lactucopicrin, (3) 11(S),13-dihydrolactucin and (4) lactucin. Only compound 2 presented leishmanicidal activity (IC_{50} 24.8 μM). The bark of *C. florida* (flowering dogwood) afforded eight compounds: (1) betulinic acid, (2) ursolic acid, (3) *β*-sitosterol, (4) ergosta-4,6,8,22-tetraene-3-one, (5) 3*β*-*O*-acetyl betulinic acid, (6) 3-epideoxyflindissol, (7) 3*β*-*O*-*cis*-coumaroyl betulinic acid, (8) 3*β*-*O*-*trans*-coumaroyl betulinic acid. The most active leishmanicidal compounds were (4) 11.5 μM, (6) 1.8 μM, (7) 8.3 μM and (8) 2.2 μM. The aerial parts of *E. foetidum* (culantro) generated two natural products: (1) lasidiol *p*-methoxybenzoate and (2) a terpene aldehyde ester

derivative. Only compound 1 inhibited *L. tarentolae* and *L. donovani* with IC_{50} values of 14.33 and 7.84 μM, respectively.

Obesity and type 2 diabetes are reaching alarming levels worldwide. This work presented the anti-inflammatory, anti-obesity and anti-diabetic effect in vitro and in vivo of *Moringa oleifera* Lam. (Moringaceae), which contains four bioactive isothiocyanates (MICs). Fresh leaves of *M. oleifera* were extracted with water to obtain a moringa concentrate (MC) containing 1.66% of total MICs. Also, MIC-1 and 4 were isolated from leaves. MC, MIC-1, and MIC-4 significantly decreased gene expression and production of inflammatory markers (NO, TNFα, IL-1β and IL-8) in LPS-stimulated RAW macrophages and Caco2 cells. The MC-treated animals, fed high-fat diet did not gain weight and did not develop fat liver disease compared to control animals. Also, when compared to control animals, the blood metabolic and inflammatory biomarkers from MC-treated mice were in the normal range. In addition MC-treated animals had normal levels of insulin signaling and inflammatory markers in liver, skeletal muscle, white adipose tissue and ileum. MC and MIC inhibited liver gluconeogenesis in vivo as well as in vitro. Finally, an indirect calorimetry acute study indicated that MC-treated mice had a higher fat oxidation rate compared to control mice.

DEDICATION

I dedicate this work to my parents, María de Lourdes Silva and Patricio Rojas-Sánchez, and to my wife, Rosita J. Mateus-Herrera.

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CHAPTER 1

Introduction

"Let food be thy medicine and medicine be thy food"

— Hippocrates

1.1 Aims of the thesis

The main aim of this work was to study the natural products isolated from common medicinal and edible plants that can be applied in the treatment or prevention of human health problems. The studied human health issues were leishmaniasis, and obesity and type 2 diabetes. For this reason, the proposal had two specific aims: the first one was dedicated to the research on leishmanicidal bioactive natural products isolated from the following common medicinal plants *Cichorium intybus* L. (Asteraceae), *Cornus florida* L. (Cornaceae), and *Eryngium foetidum* L. (Apiaceae). The second aim was devoted to the investigation of the anti-inflammatory and anti-obesity properties of a botanical enriched extract and the isothiocyanates isolated from *Moringa oleifera* Lam. (Moringaceae) on in vitro as well as in vivo models.

The thesis outline is as follows**:** The first chapter gives a general introduction, justification of the research and an overview of the used methods (Ch. 1). Chapters 2 to 4 are dedicated to the leishmanicidal natural products isolated from *Cichorium intybus* (Ch. 2), *Cornus florida* (Ch. 3), and *Eryngium foetidum* (Ch. 4).

In chapter 3, Dr. Rocky Graziose, from Dr. Ilya Raskin laboratory, was in charge of extraction, isolation and identification of the natural products while I performed the in vitro leishmanicidal and cytotoxicity assays. In chapter 4, Mr. Brian Vesely, from Dr.

Dennis Kyle Laboratory (University of South Florida), performed the leishmanicidal assay using amastigotes of *Leishmania donovani*. Chapter 5 deals with the biological activity of *Moringa oleifera* related to inflammation and obesity-type 2 diabetes in vitro and in vivo. Dr. Carrie Waterman was in charge of preparing the extract and isolating the *Moringa* isothiocyanates while I was responsible for the biological assays. For the animal study, in Chapter 5, Dr. Waterman and I were co-responsible for the study. I was in charge for most of the biological assays with the exception of the following experiments: gene expression in adipose tissue, hepatic protein expression, glycerol production and the indirect calorimetry study that were performed at Dr. William Cefalu Laboratory at Pennington Biomedical Research Center, LSU; and histology analysis of liver samples that was performed at Dr. Kenneth Reuhl Laboratory at Rutgers University. However, Dr. Waterman and I were in charge of analyzing and interpreting the data provided from our collaborators. The last chapter (Ch. 6) presents the conclusions and discusses the proposed experiments for a follow up based on the data provided on this research. Finally, the appendix section contains three abstracts of published research in peer-reviewed journals and one manuscript that have submitted for publication. These publications are related to leishmanicidal or antidiabetic natural products.

1.2 Leishmanicidal natural products from common medicinal plants

The rationale behind my research was that common medicinal plants that have been used traditionally as anti-parasitic remedies can harbor not yet discovered leishmanicidal natural products. The objectives were isolate, characterize and test leishmanicidal natural products from the following common medicinal plants *Cichorium intybus* L. (Asteraceae), *Cornus florida* L. (Cornaceae) and *Eryngium foetidum* L. (Apiaceae). The methodology employed was bioassay-guided fractionation, using the *Leishmania tarentolae* as testing in vitro organism for leishmanicidal activity, and mammalian cells to check the cytotoxicity of isolated compounds.

1.2.1 The biology, global burden and current therapy of leishmaniasis

Leishmaniasis is the most devastating human protozoan-parasitic infection in the world after malaria [1]. Leishmaniasis urgently requires new chemotherapeutic agents or at least new lead compounds to be evaluated as potential drug candidates [1]. Despite the importance of this parasitic infection, the disease has not received proper attention from the policy makers and scientific community, especially in developed countries. There[1]fore, leishmaniasis is considered a neglected tropical disease [1].

The biological agents of the infection are protozoan parasites from the genus *Leishmania* (Kinetoplastida, Trypanosomatidae), with 21 different human pathogenic species distributed in the Old and New Worlds (Table 1.1) [2]. The species differ in terms of the species vector, the type of syndrome produced (see below) and if they prefer to infect mainly humans (anthroponotic infections) or animals and humans (anthropozoonotic) [3]. The infection is vector-borne, transmitted through the bite of

female phlebotomine sand flies from the genera *Lutzomyia* in the New World and *Phlebotomus* in the Old World (ca. 30 species in total) [4]. The biological cycles starts when the parasites are injected at the same time during the blood meal [5]. This infectious form is known as metacyclic promastigote (which displays a flagellum), and are mobilized from the midgut to the pharynx of the vector [5]. Then, after the parasites have been injected into the skin, they immediately seek to infect macrophages [5]. Once the promastigotes are engulfed, they transform into amastigotes: the flagellum is reabsorbed, the parasite express a different set of cellular membrane proteins, the phagocytosis process and the immune response are modified, and thus, the parasites can live and thrive inside the infected macrophages [6]. Finally, the biological cycle is completed when female sand flies ingest infected macrophages, and then in the midgut of the vector the parasites become infectious again [5].

In terms of the disease epidemiology, around 12 million people are currently infected, with 2 million new cases per year, and approximately 350 to 600 million people are exposed worldwide [7]. However, it is presumed there is a high number of cases that are not reported to the national medical systems since most of the affected population live in rural areas without access to medical attention [8]. The disease mainly affects poor people in developing countries in the following regions: Indian subcontinent, Central Asia, Middle East, Mediterranean Basin, North Africa [9], and in America from southern USA to northern Argentina, with the exception of Chile, Uruguay and some Caribbean Islands [10].

Leishmaniasis includes three major clinical syndromes: 1) cutaneous, which is the most common form, involving skin chronic sores and ulcers as the main symptom; 2)

muco-cutaneous that includes skin sores, scars with facial disfiguration, especially in the mouth, palate and nose (this form is only seen in the New World); and 3) the most lethal version of the infection known as visceral or kala-azar that produces unspecific symptoms like weakness, fever, cough, loss of appetite and weight, while the parasites are invading the macrophages in liver, lymph nodes and spleen producing enlargement of these organs. The death is usually caused by hemorrhage (liver failure) or co-infection (the innate immune system is unable to stop other pathogens); this fatal outcome can be produced even if the infection is detected and treated on time [11]. After treatment, the disease can relapse with skin lesions (post kala-azar dermal leishmaniasis) [2,4]. More than mortality, which reaches a tentative estimation of 20,000 to 40,000 deaths per year [8], the problems created by this parasitic disease are the great disability and morbidity [12] that is generated by the chronic infection, and the co-infection with other virulent infectious diseases like HIV and tuberculosis [13]. These are the reasons why leishmaniasis is the second most serious parasitic infection in the world, after malaria [14].

The current treatments have many drawbacks (Table 1.2): moderate to high toxicity, difficult to get in rural areas, most have to be administered via parenteral routes (intramuscular, intravenous or intralesional), the length of the treatment (usually from one to six months), and all treatments require close medical control [15,16]. The most common drugs currently in use contain a pentavalent antimony atom (sodium stibogluconate and meglumine antimonite), a metalloid element that is poorly metabolized and eliminated causing blood, cardiac and renal toxicity plus a really painful intramuscular or intralesional administration [17]. The other options, liposomal

amphotericin B (AmBisome®), paromomycin (Aminosidine®), pentamidine (Pentam 300®), can be toxic for the kidneys, liver and internal ear and produce other uncomfortable secondary effects as well [17-19]. None of the current treatments were developed specifically for leishmaniasis, with the exception of the pentavalent antimonials; and only amphotericin B and paromomycin have a natural origin (fermentation products of *Streptomyces* species) [17-19].

Despite all the ongoing efforts of some research groups in order to discover a vaccine or a way to prevent the transmission of the infection [20], still does not exist a reliable method available to be used immediately [21]. In addition, there are three new complications with the infection: first, some strains have developed resistance to the current drugs [15]; second, there is a growing number of patients co-infected with HIV [13,22]; and third, there is a high risk for travelers and military personnel that visit endemic regions to acquire the infection [7]. Finally, few new drugs or treatments have been developed or discovered for all parasitic diseases in recent years (except for malaria) [23]. In the case of leishmaniasis, the last drug approved for use in humans was miltefosine which was originally developed as a chemotherapeutic agent to treat breast cancer, skin metastasis and other type of malignant tumors [22,24].

1.2.2 Natural products with leishmanicidal activity

Bioactive natural products from medicinal plants represent an excellent source for novel compounds to be considered as drug candidates to treat protozoan-parasitic infections [25]. For instance, there is a successful story in the case of malaria: Artemisinins are a group of natural sesquiterpene lactones isolated originally from

Artemisia annua L. (Asteraceae), a medicinal plant used to treat malaria in China. The semi-synthetic derivatives have been used with great success on multidrug resistant malaria caused by *Plasmodium falciparum* [26]. Interestingly, the artemisinin derivatives have been also tested against leishmania parasites in vitro [27] as well as in vivo [28] models, displaying some leishmanicidal activity.

Medicinal plants and other biological sources have provided several leishmanicidal natural products, including: alkaloids, tritperpenes, sesquiterpenoids, quinines, lactones, coumarins, chalcones, lignans and saponins [29]. For instance, a survey of traditional medicinal plants from Ecuador, tested against *Leishmania donovani* amastigotes, found seven active plant extracts with IC_{50} values < 10 µg/mL [30]. Two of the plant extracts prepared from *Gouania lupuloides* Urb. (Rhamnaceae) and *Minquartia guianensis* Aubl. (Olacaceae), showed also a high selectivity index (> 10) indicating the activity is not due to general cytotoxicity [30]. Another example constitutes the sesquiterpene lactones psilostachyin and peruvin that were isolated from the Argentinean medicinal plant *Ambrosia tenuifolia* Sprengel (Asteraceae) and showed potent IC_{50} values (0.12 µg/mL and 0.39 µg/mL, respectively) in promastigotes of *Leishmania spp.* [31]. These cases clearly evidence the potential of plant natural products to be applied in leishmaniasis. Therefore, there is a high probability that common medicinal plants could yield new leishmanicidal natural compounds. The reasons to research on *Cichorium intybus*, *Cornus florida*, and *Eryngium foetidum* were: 1) important ethnobotanic tradition of use, 2) antiparasitic properties, and 3) easy to grow in greenhouse or field, or easy to find in nature. The bio-guided fractionation approach was employed to evaluate and identify the

active extracts, fractions and compounds [32]. The non-pathogenic strain *Leishmania tarentolae* was the model organism used to perform the leishmanicidal assays.

1.2.3 Leishmanicidal assay using Leishmania tarentolae

The species *Leishmania tarentolae* is a non-pathogenic species for mammals and it was isolated for the first time from a gecko lizard named *Tarentola mauritanica* [33]. *L. tarentolae* belongs to the *Sauroleishmania* subgenus, which is the sister taxa of the subgenus *Leishmania (Leishmania*), a human pathogenic clade [34]. The promastigote and amastigote stages have been observed in *L. tarentolae* and it is also capable to infect mammalian macrophages, mimicking the pathogenic species [35]. The lack of human pathogenicity makes this species very suitable to be used as a model organism while screening botanical crude extracts, fractions and even pure compounds. Therefore, the rationale for use this strain it is explained because it is easy and cheap to grow, do not require a high biohazard level of precautions, and it can be used in the field if needed [36]. I was in charge of researching, implementing and testing this bioassay. A simplified version of the assay is available for the GIBEX initiative in order to be used in the field.

1.2.4 Methodology

1.2.4.1 Procedure and chemicals for extraction, isolation and identification of natural products

Plant extracts were subjected to bioactivity-guided fractionation for the isolation of leishmanicidal compounds [37]. This procedure requires testing the extracts, fractions and subfractions in the leishmanicidal assay in order to select the bioactive ones. The cut-

off concentration value to consider a crude extract or fraction active was ≥ 30% growth inhibition at 20 μg/mL [37].

The plant material was dried and pulverized before the extraction process. Organic solvents (HPLC grade) were employed for extraction, fractionation and isolation. The plant material was first defatted with *n*-hexane in a ratio 4:1 (v/w). The objective was to get rid of most of lipids and pigments (e.g. chlorophyll and carotenes) that can interfere with the extraction and isolation process of potential active compounds. Then, the extracted material was dried and partitioned using mixtures of organic solvents with different polarities, from highly non-polar to hydrophilic. This procedure allowed the separation and initial purification of compounds based on their polarity. The purified extracts were subjected to the fractionation process which involves different chromatography techniques like column chromatography, thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC). The conditions and specifications varied for each plant extract and their fractions, and details will be described for each plant extract on each dissertation chapter. The identification of isolated compounds was made by ultra-high performance liquid chromatography coupled with mass spectrometry (LCMS), and nuclear magnetic resonance (NMR) experiments. LCMS can detect the molecular mass of a particular compound based on molecular fragmentation patterns and NMR (^{1}H and ^{13}C) gives the possible structure of the compound. This whole approach has proven to be very successful when working with several natural products isolated from different plant extracts [37].

HPLC grade organic solvents were purchased from Sigma-Aldrich (St. Louis, MO) or VWR. Precoated silica gel (Si25, F254) plates from Fluka Analytical (Germany) were

used for analytical TLC and detection was performed under UV at 254 and 366 nm, *p*-anisaldehyde/H_2SO_4 staining plus heating. Column chromatography was performed using silica gel 0.035–0.070 mm, 60 Å (Acros Organics, USA) with positive air flow pressure. Prep-HPLC was performed with a Waters system: autosampler, pump with semi-preparative heads, pump controller and a 490E multi-wavelength detector. High resolution mass spectral data were acquired by Ion-Trap Time-of-Flight Mass Spectrometry (IT-TOF-MS), on a Shimadzu LC-MS-IT-TOF (Scientific Instruments, Columbia, MD) instrument equipped with a Prominence HPLC system, designed to perform high-precision $LCMS^n$ analyses. Ionization was performed using APCI or ESI source in the positive ionization mode. Shimadzu's LCMS Solution software was used for data analysis. The molecular formulae were generated by the formula predictor function of LCMS Solution. 1D and 2D NMR spectra (^1H, ^{13}C, COSY, DEPT, HMQC and HMBC) were recorded at 298 K on Varian VNMRS 400 and 500 MHz spectrometers using $CDCl_3$ and deuterated acetone as solvents, and TMS as internal standard (Sigma, St. Louis, MO).

1.2.4.2 Leishmanicidal in vitro assay

Promastigotes of *Leishmania tarentolae* strain UC were donated by Dr. Larry Simpson (UCLA). The parasites were maintained in brain heart infusion (BHI) supplemented with hemin (10 μg/mL) and subcultured every third day. One hundred microliters of culture with 1×10^6 cells per mL were seeded in 96-well plates. Serial dilutions of the extract or compounds were prepared covering a range of 8 to 12 points from 200 to 0.01 μg/mL, and the different concentrations tested by triplicate. The plates were incubated at 27 ºC for 48 hours in darkness. After this period, the plates were

inspected under an inverted microscope to assure sterile conditions and growth of controls. Then, 10 μL of 5 mg/mL of the tetrazolium salt MTT were added to each well to evaluate cell viability. The incubation continued for another 4 hours. The blue dye formazan were formed by the reduction of MTT inside the mitochondria, lower the amount of formazan higher the leishmanicidal activity [38]. Next, formazan was solubilized with 100 % DMSO or acidic i-PrOH (0.1 N HCl). Finally, absorbance was read using a Synergy^{TM} HT multidetection microplate reader (BioTek® Instruments, Inc. Winooski, VT) at 570 nm and correction at 630 nm. Pentamidine (Sigma) was the reference drug and the IC_{50} value was calculated based on the dose response curve covering a range of 13 points from 3.75 to 0.125 μg/mL. In Chapter 4, amastigotes of *Leishmania donovani* were also employed. The axenic amastigote leishmanicidal bioassay were performed for 72 h assay using ca. 66000 amastigotes per well and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] was used to evaluate viability of the parasites. The IC_{50} value was calculated as mentioned here before.

1.2.4.3 Cytotoxicity in vitro assay

Rat skeletal myoblast L6 cells were used as a mammalian model cell to assess cytotoxicity of the isolated compounds. The well-known cytotoxic compound emetine (Sigma, USA) was employed as reference drug. The cells grew DMEM supplemented with 10% FBS at 37°C in 95% air-5% CO_2 humidified environment. The assay was conducted in 96-well plates and each well was filled with 100 μL of culture medium with 1×10^4 cells per mL. After 2 hours, the tested compounds were added by triplicate in different 12 concentrations (serial dilution from 200 to 0.01 μg/mL). After 72 hours of

incubation, the plates were checked under an inverted microscope to assure growth in sterile conditions. Then, 10 μL of MTT (5 mg/mL) sterile solution was added to each well, and incubation continued for another 4 hours [39]. Finally, absorbance was measured as mentioned in section 1.2.4.2.

1.2.4.4 Statistical Analysis.

Data were analyzed using GraphPad Prism 6.02 (GraphPad Software Inc., La Jolla, CA). The decrease of absorbance was expressed as percentage of the absorbance of the growth control and plotted against the drug concentrations. The IC_{50} values were calculated using a non-linear dose-response curve fitting analysis [40]. The IC_{50} values reported were the mean of three independent experiments.

1.3 Anti-inflammatory and anti-obesity properties of *Moringa oleifera* Lam.

The first objective was to test the anti-inflammatory in vitro activity of *M. oleifera* food-grade botanical extract (MC) and the isolated *M. oleifera* isothiocyanates (MICs) from leaves. For this purpose, in vitro experiments were performed with RAW 264.1 murine macrophages and Caco-2 human intestinal cells that involved the quantification of inflammatory biomarkers by ELISA and gene expression.

The second objective was to evaluate the same ME incorporated in the food a diet-induced obese model and determine whether this intervention could ameliorate the pathological changes originated by a high fat diet. The methodology applied was a preventive design with the diet-induced obese C57BL/6J murine model that involved evaluation of biometric indicators and the quantification of metabolic and inflammatory biomarkers by ELISA, gene expression, immunoblot and histology.

1.3.1 The epidemiology and physiopathology of obesity and type 2 diabetes

The pandemic of overweight and obesity has reached alarming levels: one third of the population (ca. 2.1 billion) is affected, including children and teenagers, and has generated 3.4 million deaths just in 2010 [41]. The trend of the epidemic is to increase in the years to come and developing countries will be the most affected [41]. The same is true for type 2 diabetes (T2D) around the world [42], and in USA the prevalence has increased between 2001 and 2009, at this rate by 2050 one in every three people in USA will have T2D [43] [44]. Obesity is conventionally defined as body-mass index (BMI) $\geq$30 kg/m^2, while overweight is $\geq$25 to <30 kg/m^2 in adults (>18 years old). For children (2 to 18 years), the definition is based on International Obesity Task Force [IOTF]. Both, overweight and obesity, increase the risk of insulin resistance in men [45], women [46],

and children [47]; and thus, the development of T2D, metabolic syndrome (MS), some types of cancer and cardio-vascular problems (the chronic non-communicable diseases) [45-48]. For example, adults from both sexes who have a BMI >35 kg/m^2 have almost 20-fold higher chance of developing T2D compared to people with a BMI of 18.5–24.9 kg/m^2 [49].

The two main environmental contributors for the obesity pandemic is a sedentary lifestyle and diets with high caloric content, but there are also intrinsic factors that predispose the propensity for weight gain (e.g. mitochondrial function) [50]. An interesting observation about human obesity is related with the successful evolutionary mechanism that allows the white adipose tissue to store calories in form of fat that can be utilized for periods with little or no food, a well-known strategy in mammals. However, this physiological mechanism seems to be disturbed due to changes in cellular processes (high lipogenesis with low lipolysis rates) and environmental pressures (low energy expenditure, high caloric diets, availability of energy-dense food like products, and exposition to hormonal disruptors like some synthetic chemicals) [51].

Behind chronic metabolic diseases there are two pathophysiologic conditions: chronic inflammation and insulin resistance. These two conditions are interconnected and generate a profound disturbance in cell homeostasis. The insulin resistance state, which is defined as the inability of insulin to increase glucose uptake and its utilization by cells [52], can be triggered by chronic inflammation [53]. In a normal state, cells are able to uptake and use glucose. The glucose uptake is a fine-tuned process in order to maintain cellular homeostasis and avoid low or high glucose levels in the blood (hypo or hyperglycemic states). Insulin is the key hormone that regulates this whole process.

Insulin is secreted by pancreatic β cells after a meal is consumed and allows the hepatic, adipose and skeletal muscle tissues to uptake glucose. Insulin is sensed by the membrane receptor IR and immediately starts a chain of intracellular signaling that involves the phosphorylation of different proteins via IRS 1-2, Akt 1-2 and mTOR. The outcome of this signaling process is the membrane expression of the glucose receptor GLUT-4, which allows the incorporation of glucose, the activation of glycolysis, glycogen synthesis (in liver and muscle), and inhibiting gluconeogenesis (liver) and lipolysis (adipose tissue). In a insulin resistance state, the IR is not working properly, thus the signaling process is altered and cells cannot incorporate glucose [44] [54]. Without the proper regulation of glucose levels, the body starts to overcome the apparently lack of glucose by increasing gluconeogenesis and *de novo* lipogenesis (liver), and lipolysis and pathological lipogenesis (adipose tissue). One of the reasons to disturb the IR signaling is chronic inflammation. The low grade chronic inflammation is now recognized as a clear signature in metabolic diseases such as obesity, T2D, MS, atherosclerosis [53], nonalcoholic fatty liver disease [55], neurological degenerative diseases, like Alzheimer, and several types of cancer [56]. The upregulation of several pro-inflammatory biomarkers have been identified as hallmark signs of the low grade chronic inflammatory response found in metabolic diseases. These biomarkers include pro-inflammatory cytokines (TNF-α, IL-1β, IL-8 and IL-6 mainly), C-reactive protein, the transcription nuclear factor NF-κB, the family of the peroxisome proliferator-activated receptors (PPAR), and the genes of the enzymes inducible nitric-oxide synthase (*iNOS*) and cyclo-oxygenase (*COX-2*) with their final products nitric oxide (NO), and prostaglandine E_2 (PGE_2), respectively [53,57]. The overexpression of these biomarkers can be pleiotropic

and deleterious on cell homeostasis. For example, TNF-α has been shown to directly interfere with insulin signaling reducing the kinase activity of the insulin receptor [58], thus generating insulin resistance on tissues affected by this cytokine [59]; and NO overproduction have been involved in the pathogenesis of various chronic disease states [60]. Interestingly, the daily consumption of high amounts of refined dietary sugars and saturated fatty acids can trigger chronic inflammation and the development of insulin resistance state in key metabolic tissues like liver, visceral white adipose tissue, skeletal muscle and intestines [61].

The negative impact of low grade chronic inflammation and the hyperglycemic state developed by the insulin resistance originates the well-known complications in retina, kidneys, heart, aorta, brain, peripheral nervous system, immune system (which leads to high susceptibility for bacterial and fungal infections) and feet [62]. The injury in these organs are due a rate increase in the non-enzymatic glycosylation process of proteins, lipids and nucleic acids, known as advanced glycation end products, that results in the irreversible damage of the myelin sheath of nerves and the endothelium (the inner cellular layer of blood vessels) [63].

The healthcare for metabolic diseases is a sensitive issue. For instance, T2D and its complications represent a great challenge for the patient and the medical team, but it is also expensive: the total medical cost was $ 306 billion in USA by 2012 [64] . Therefore, the public health measures to counteract the metabolic diseases should be prioritized towards prevention rather than treatment since diet and exercise are important factors in these type of health problems [65].

Finally, nowadays there is a great necessity for novel, comprehensive and innovative approaches to prevent the onset of obesity and T2D, rather than just the development of new drugs [66]. Since diet plays a crucial role in the development of non-communicable metabolic diseases, the use of edible and medicinal plants is an interesting and valid option for prevention.

1.3.2 The use of plants to prevent and treat obesity and diabetes

Diet and exercise are the pivotal variables of obesity, T2D, and MS, despite of the genetic, social and economic aspects that can determine the epidemic of these metabolic alterations [67]. A diet rich in refined carbohydrates, saturated fatty acids and sodium, like the Western diet, is accused to be one of the reasons for the pandemic of obesity. On the other hand, a plant based diet rich in vegetables, fruits, nuts, and fiber seems to prevent the development of obesity and T2D [61].

It was recently reported that a Mediterranean-like diet supplemented with extra nuts and olive oil could reduce the incidence of major cardiovascular events (heart attack or stroke) in people with cardiovascular risk [68]. Similarly, another study found that eating whole fruits, especially blueberries, grapes and raisins, can reduce the chance to develop diabetes [69]. In a similar way, the consumption of the seeds from *Lupinus mutabilis* Sweet, the lupinus beans traditionally use in the Andes region, demonstrated an improvement on the insulin resistant indicators (fasting glycemia and insulinemia) on dysglycemic [70] and diabetic patients [71]. In another clinical study, the oral administration of the brown seaweed *Undaria pinnatifida* (known as wakame and largely consumed in Japan) revealed an improvement of biometric indicators on women patients

diagnosed with MS [72]. In addition, the oral administration of a standardized anthocyanin rich extract from maqui berries (*Aristotelia chilensis* Stuntz) improved fasting blood glucose levels and glucose tolerance in a diabetic mouse model, the hyperglycemic obese C57BL/6J mice fed with a high fat diet [73]. These few examples confirm the potential of a plant-rich diet to prevent the development of metabolic diseases like obesity and T2D.

In terms of medicinal plants, there are reports of hundreds of traditional plants that have the potential to treat obesity, T2D and their complications. Medicinal plants for the treatment and prevention of obesity [74] and T2D have been used in Europe [75], China [76], India [77], and Mexico[78]. Some plants or botanicals have been tested in small clinical studies and have demonstrated efficacy, although larger studies will be needed to confirm their activity, rule out toxicity or severe side effects, and therefore justify their increased use [74].

One remarkable case of a antidiabetic medicinal plant is the European medicinal herb *Galega officinalis* L. (Fabaceae), commonly known as French lilac, galega or goat's rue, and the antidiabetic drug metformin (Glucophage®). The leaves of this plant have been used since medieval times to treat several diseases including diabetes. In the 1920s the natural products guanidine and galegin were isolated from *G. officinalis* and demonstrated hypoglycemic effects, although with toxic effects. By 1929, German scientists had developed different semisynthetic less toxic versions known as biguanides, metformin been one of them. Finally, the French doctor Jean Stern tested metformin on animals and, for the first time, in T2D patients by 1956 [79]. Today, metformin is considered a safe and effective drug, and one of the most prescribed chemotherapeutic

agents in the world. It is important to mention that obese patients that suffer T2D diabetes receive the higher benefit from metformin since they had fewer hypoglycemic attacks and gained less weight when compared to insulin or sulfonylureas treatments [80]. Moreover, an ethanolic extract of *G. officinalis* demonstrated a weight loss effect due to decrease of white adipose tissue content in mice [81].

In conclusion, the research performed so far on medicinal and edible plants provide evidence of their use in order to prevent or ameliorate obesity and T2D. Based on this premise, the research of *Moringa oleifera* Lam. in the prevention of obesity is justified.

1.3.3. Methodology

1.3.3.1 In vitro anti-inflammatory experiments

RAW 264.7 murine macrophages (ATCC® TIB-71™), Caco-2 (ATCC® HTB-37™) and HT-29 (ATCC® HTB-38™) human intestinal cell lines were used as mammalian model cells to assess cytotoxicity (cell viability) and anti-inflammatory bioactivity of the food-grade *Moringa oleifera* extract (MC) and the isolated compounds (MICs). The cell lines were obtained from American Type Culture Collection (ATCC). In order to evaluate the anti-inflammatory activity in vitro, cells were first treated and then inflammation was induced (preventive approach). The assays to measure changes in inflammatory markers involved ELISA, nitric oxide production and gene expression experiments.

1.3.3.2 In vivo and in vitro anti-obesity experiments

The diet-induced obesity C57BL/6J mouse model was employed [82]. This mouse model develops obesity and insulin resistance/glucose intolerance state when it receives a

high fat diet (40 to 60% kcal from fat) [83]. The study was designed to prevent the onset of obesity and insulin resistance. In addition, in vitro studies were performed to determine the mechanism of action of MC and MICs.

1.4. Tables

Table 1.1 World distribution of human *Leishmania* parasites and the syndrome produced [2,7,17,22].

Region	Countries	Parasite (Subgenus)	Syndrome
New World	From Mexico to Argentina and the Caribbean islands of Guadeloupe and Martinique	*L. (Leishmania) infantum* previously known as *L. chagasi*	Visceral
	From Costa Rica to Bolivia, including Brazil	*L.(Leishmania) amazonensis*	Cutaneous
	From Mexico to Ecuador and Hispaniola island	*L. (Leishmania) mexicana*	Muco-cutaneous
	Venezuela	*L. (Leishmania) garnhami*	Cutaneous
	Peru	*L. (Viannia) peruviana*	Cutaneous
	From Guatemala to Argentina	*L. (Viannia) braziliensis*	Muco-Cutaneous
	From Venezuela to Peru, including Brazil, and the Guianas.	*L. (Viannia) guyanensis*	Muco-Cutaneous
	From Belize to Ecuador	*L. (Viannia) panamensis*	Muco-Cutaneous
	Brazil, French Guiana, Ecuador and Peru	*L. (Viannia) naiffi*	Cutaneous
	Brazil, Bolivia and Peru	*L. (Viannia) lainsoni*	Cutaneous
Old World	China, Indian subcontinent, Iran, Northeastern Africa and the Arabic Peninsula	*L.(Leihsmania) donovani*	Visceral and PKDL*
	All the Mediterranean region including Portugal, and Yemen	*L. (Leishmania) infantum*	Visceral
	India, Northeastern Africa, Lebanon and Israel	*L. (Leishmania) archibaldi*	Cutaneous
	Middle East, including Arabic Peninsula, Caspian Sea and Mediterranean regions	*L. (Leishmania) tropica*	Cutaneous
	Ethiopia and Kenya	*L. (Leishmania) aetiopica*	Cutaneous
	Middle East from Afghanistan to Israel, North Africa including Chad and Sudan	*L. (Leishmania) major*	Cutaneous

*PKDL: post kala-azar dermal leishmaniasis

Table 1.2 Current treatments for leishmaniasis [17,18,24].

Drug	Type	Source/Year	Main concerns	Route of administration
sodium stibogluconate (Pentostam®) meglumine antimoniate (Glucantime ®)	organo-metal complexes of pentavalent antimonial * (Sb^v)	synthetic 1940s	bone marrow, cardiac and renal toxicity, teratogenic, painful administration, length of treatment, resistant strains	parenteral: intramuscular and intralesional
pentamidine isethionate (Pentam®)	aromatic diamidines	synthetic 1950s	only for cutaneous cases in South America, cardiac toxicity, low to medium cure rate	parenteral: intramuscular
amphotericin B (liposomal) (AmBisome®)	polyene antibiotic	semisynthetic *Streptomyces nodus* 1997	renal toxicity, expensive, hospitalization for administration	parenteral: intravenous only
paromomycin (Aminosidine ®)	aminoglycoside antibiotic	semisynthetic *Streptomyces rimosus* 2006	nephro and ototoxicity, low to medium cure rate	parenteral and topical
miltefosine (Impavido ™)	alkylphospho-choline	synthetic 2002	expensive, teratogenic, pending approval for cutaneous use	Oral

*Pentavalent antimonials are the only drugs that were specifically developed to treat leishmaniasis.

CHAPTER 2

Leishmanicidal sesquiterpene lactones isolated from roots of *Cichorium intybus* L.

Rathinasabapathy T, **Rojas-Silva P**, Poulev A, Komarnytsky S, Raskin I. Isolation of sesquiterpene lactones from roots of *Cichorium intybus* L. with leishmanicidal activity. *Pharmaceutical Biology* 2012, 50(2):573.

2.1 Abstract

Context: *Cichorium intybus* L. is a traditional medicinal herb that has been used to treat different type of illnesses including parasitic infections.

Objective: This study evaluates the in vitro leishmanicidal activity of sesquiterpene lactones isolated from chicory roots.

Materials and methods: Root powder was extracted with methanol followed by partitioning with *n*-hexane and EtOAc**,** then fractionated by FCPC and fractions V, VIII and IX were purified by HPLC. Compound identity was confirmed by LCMS and NMR. All extracts and fractions were tested at 20 μg/mL.

Results: The FCPC fractionation of EtOAc extract generated 13 fractions. Fractions V, VIII and IX were active in the leishmanicidal assay. Fraction V yielded (1) 11(S),13-dihydrolactucopicrin and (2) lactucopicrin, and fractions VIII+IX yielded (3) 11(S),13-dihydrolactucin and (4) lactucin. Compounds 1, 3 and 4 did not show activity (IC_{50} >50 μM), but compound 2 presented moderate activity (IC_{50} 24.8 μM).

Discussion and conclusion: This is study presented the first report of leishmanicidal bioactivity of bitter sesquiterpene lactones isolated from chicory roots.

Keywords: sesquiterpene lactones, leishmaniasis, chicory, FCPC, HPLC, Asteraceae.

2.2 Introduction

Cichorium intybus L. (Asteraceae) is a cosmopolitan medicinal and edible plant, known as chicory, chicorée, French endive, witloof, and succory [84]. The genus *Cichorium* includes another four species with a major geographical distribution in Asia and Europe [84]. *Cichorium intybus* is an erect perennial herb that can reach up to 2 m in height; with a strong deep fleshy taproot, and very distinctive bright blue florets (known as "blue sailors" in USA), but the florets can be bluish white or pink [85]. The place of origin is uncertain, but it could be somewhere in the Middle East or Mediterranean regions [86].

Chicory was well known and cultivated by the ancient cultures of Egypt, Greece and Rome [87]. The plant was employed as a vegetable crop for human and animal consumption, but also as a medicinal plant [87]. Nowadays, people around the world are still using this herb as food, medicine and ornamental. For example, dried and toasted roots are employed as the most common coffee substitute [88], young roots are boiled and eaten as well as leaves are used in salads [86]. Chicory is commonly used as medicine too, specially the roots. Different type of preparations, water and alcoholic extracts, are used worldwide as laxative, diuretic, mild sedative, fever and jaundice treatments, antiparasitic and antidiabetic [86]. Chicory is considered a medicinal plant safety class 1 (an herb that can be safely consumed when used appropriately) by the American Botanical Safety Handbook 2013 [89]. A concentrated ethanolic extract of chicory roots did not show in vitro mutagenic activity neither toxicity effects in rats even at 1000 mg/kg/day [90]. In addition, extracts are cataloged as safe by the FDA and

appears in the official list of “Everything Added to Food” in the United States (EAFUS) [90].

Chicory roots are recognized for their high fiber content, mainly inulin (a β-2,1 linked fructose polymer with a terminal glucose residue) and oligofructo-saccharides, which avoid constipation in humans after oral consumption [91]. The root extracts and isolated compounds have also demonstrated remarkable biological properties like in vitro anti-inflammatory effect on human colonic intestinal cells [92], analgesic and sedative effects on mice [93], decreasing cholesterol uptake in rats [94], hypoglycemic and hypolipidemic effects on rats [95], antitumoral activity [96] and amelioration of ethanolic immunotoxicity [97] both in mice. Finally, chicory roots extract has an important application as antiparasitic remedy: an overnight root infusion have been used as an effective antimalarial treatment in Afghanistan according to the local folklore [98], a country where the prevalence of leishmaniasis is also very high [99]. In another study, a condensed tannins extract from leaves and a crude sesquiterpene lactone extract from roots showed activity against pulmonary and gastrointestinal nematodes in farmed red deers [100]. This evidence suggests the chicory potential as an antiparasitic plant.

In terms of phytochemistry, *C. intybus* has been extensively studied and its secondary metabolites are well known. The most remarkable chemical constituents in roots and aerial parts are the bitter guaianolides type sesquiterpene lactones and their glycosides derivatives [101],which have been responsible for most of the reported biological activity [84]. Phenolics like chlorogenic acid, kaempeferol and quercetin derivatives are the most common phytochemicals in leaves [102]. The main role of these

compounds is to protect the plant from nematodes and fungal infections; and also to deter insect feeding [103].

2.3 Materials and methods

2.3.1 Plant material

Chicory cv. Sacson root powder was provided by Leroux (Lille-Valenciennes, France). The authenticity of the material was confirmed with the company.

2.3.2. Instrumentation and chemicals

Please see section *1.2.4.1.*

2.3.3. Extraction, isolation and identification

One hundred and fifty grams of root powder were extracted twice by constant agitation with MeOH (1L) for 24 hours at room temperature, and then filtered, concentrated and dried under vacuum. All flasks and containers were protected from light and air during the extraction procedure to avoid the degradation of compounds.

The MeOH crude extract was dissolved in H_2O (200 mL) and partitioned first with *n*-hexane (3×150 mL) and then with EtOAc (3×150 mL). The EtOAc extract was concentrated and dried under vacuum, the yield was 2.215 grams. Then, two grams of the EtOAc extract were dissolved in 10 mL of the solvent system (see below), sonicated briefly, filtered through a 0.45 µm pore, and injected through the Rheodyne valve in the FCPC equipment. The fractionation was done using FCPC in a Kromaton-FCPC® 1000, v1.0 equipment (Annonay, France) with a rotor volume of 1000 ml and maximum pressure of 860 psi. A three phase solvent system was employed consisted of *n*-hexane, MeOAc, ACN and H_2O in the following proportion 1:1:0.75:1, respectively. The

stationary phase was the middle phase (MP) plus lower phase (LP) in a proportion of 7:3 and injected at a flow rate of 80 ml/min while rotating at 300 rpm, the system was then equilibrated with the upper phase (UP) at a flow rate of 10 ml/min and 750 rpm. The mobile phase started with the UP, followed by the MP and ending with the LP. The elution volume for UP and MP was 1.5 L and 1.0 L for LP. The rotor speed was 750 rpm with a flow rate of 10 ml/min and detection was set up at UV 258 nm. The fractionation process yielded 13 different fractions that were collected every 2 min with a CHF122 SC Advantec fraction collector (Dublin, CA).

Further purification was achieved by preparative HPLC following a gradient of MeOH and 0.1% TFA in H_2O. The gradient started with a MeOH-TFA ratio of 20:80 and switched to 95:5 by 60 min. Masses were acquired from LCMS (Ultimate 3000 RSLC, Dionex®) ultra-high pressure liquid chromatography system, consisting of a workstation with Dionex Chromeleon v. 6.8 software package, solvent rack/degasser SRD-3400, pulseless chromatography pump HPG-3400RS, autosampler WPS-3000RS, column compartment TCC-3000RS, and photodiode array detector DAD-3000RS. After the photodiode array detector, the eluent flow was guided to a Varian 1200L (Varian Inc., Palo Alto, CA) triple quadrupole mass detector with electrospray ionization interface, operating in the negative ionization mode. The voltage was adjusted to -4.5 kV, heated capillary temperature was 280 °C, and sheath gas (zero grade compressed air) was used for the negative ionization mode. The mass detector was used in scanning mode from 65 to 1500 atomic mass units. Data from the Varian 1200L mass detector was collected, compiled and analyzed using Varian's MS Workstation, v. 6.9, SP2. The structures were

confirmed by ^{1}H-NMR at 298 K on Varian VNMRS 400 MHz spectrometers using $CDCl_3$ as solvent and TMS as internal standard.

2.3.4. Leishmanicidal, cytotoxicity assays and statistical analysis

Promastigotes of *Leishmania tarentolae* strain UC were donated by Dr. Larry Simpson (UCLA). The parasites were maintained in brain heart infusion (BHI) supplemented with hemin (10 μg/mL) and subcultured every third day. One hundred microliters of culture with 1×10^6 cells per mL were seeded in 96-well plates. Serial dilutions of the extract or compounds were prepared covering a range of 8 to 12 points from 200 to 0.01 μg/mL, and the different concentrations tested by triplicate. The plates were incubated at 27 ºC for 48 hours in darkness. After this period, the plates were inspected under an inverted microscope to assure sterile conditions and growth of controls. Then, 10 μL of 5 mg/mL of the tetrazolium salt MTT were added to each well to evaluate cell viability. The incubation continued for another 4 hours. The blue dye formazan were formed by the reduction of MTT inside the mitochondria, lower the amount of formazan higher the leishmanicidal activity [38]. Next, formazan was solubilized with 100 % DMSO or acidic i-PrOH (0.1 N HCl). Finally, absorbance was read using a Synergy™ HT multidetection microplate reader (BioTek® Instruments, Inc. Winooski, VT) at 570 nm and correction at 630 nm. Pentamidine (Sigma) was the reference drug and the IC_{50} value was calculated based on the dose response curve covering a range of 13 points from 3.75 to 0.125 μg/mL. In Chapter 4, amastigotes of *Leishmania donovani* were also employed. The axenic amastigote leishmanicidal bioassay were performed for 72 h assay using ca. 66000 amastigotes per well and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-

tetrazolium] was used to evaluate viability of the parasites. The IC_{50} value was calculated as mentioned here before.

Rat skeletal myoblast L6 cells were used as a mammalian model cell to assess cytotoxicity of the isolated compounds. The well-known cytotoxic compound emetine (Sigma, USA) was employed as reference drug. The cells grew DMEM supplemented with 10% FBS at 37°C in 95% air-5% CO_2 humidified environment. The assay was conducted in 96-well plates and each well was filled with 100 µL of culture medium with 1×10^4 cells per mL. After 2 hours, the tested compounds were added by triplicate in different 12 concentrations (serial dilution from 200 to 0.01 µg/mL). After 72 hours of incubation, the plates were checked under an inverted microscope to assure growth in sterile conditions. Then, 10 µL of MTT (5 mg/mL) sterile solution was added to each well, and incubation continued for another 4 hours [39]. Finally, absorbance was measured as mentioned in section 1.2.4.2.

Data were analyzed using GraphPad Prism 6.02 (GraphPad Software Inc., La Jolla, CA). The decrease of absorbance was expressed as percentage of the absorbance of the growth control and plotted against the drug concentrations. The IC_{50} values were calculated using a non-linear dose-response curve fitting analysis [40]. The IC_{50} values reported were the mean of three independent experiments.

2.4 Results and discussion

The FCPC experiment generated 12 fractions. Fractions V and VIII+IX were the only ones to demonstrate relevant growth inhibition (≥ 30% at 20 µg/mL) and were subjected to further purification. Fraction V yielded 2 compounds identified by LCMS as

(1) 11(S),13-dihydrolactucopicrin and (2) lactucopicrin. Fractions VIII and IX were combined and purified together and yielded 2 compounds identified as (3) 11(S),13-dihydrolactucin and (4) lactucin. These compounds belong to the guaianolide type of sesquiterpene lactones. The isolated compounds are represented in Fig. 2.2 and were previously reported from chicory roots [101,104] and *Lactuca virosa* L. (Asteraceae) [105]. One major issue with the isolated compounds was stability because the compounds were light sensitive, degraded quickly in aerobic conditions and were unstable in aqueous solution which could affect their biological activity. The four sesquiterpene lactones were then tested in the leishmanicidal bioassay and results are presented in Table 2.1. Compounds 1, 3 and 4 did not show activity (IC_{50} >50 μM), but compound 2 presented moderate activity with an IC_{50} of 24.8 μM (10.17 μg/mL). This activity contrast with the reported activity for other natural sesquiterpene lactones isolated from plants. For example, psilostachyin and peruvin that were isolated from the Argentinean medicinal plant *Ambrosia tenuifolia* Sprengel (Asteraceae) showed potent IC_{50} values (0.12 μg/mL and 0.39 μg/mL, respectively) in promastigotes of *Leishmania spp.* [31]. Parthenolide was isolated from the aerial parts of *Tanacetum parthenium* Sch. Bip. (Asteraceae) and showed a promising activity against promastigotes and amastigotes of *L. amazonensis*, IC_{50} values of 0.37 and 0.81 μg/ml respectively [106]. Similarly, another three natural sesquiterpene lactones, helenalin, mexicanine and dehydroleucodine isolated from the aerial parts of *Gaillardia megapotamica* Baker (Asteraceae) and *Artemisia douglasiana* Besser (Asteraceae) were evaluated using *L. mexicana* promastigotes and demonstrated a rapid and irreversible effect with IC_{50} values between 2-4 μM [107]. All the sesquiterpene mentioned above do not belong to the guaianolide group, and that could

explain the difference in biological activity. Psilostachyin, peruvin, mexicanin and helenalin are pseudoguaianolides [108,109] and parthenolide is a germacrane sesquiterpene [110]. These results could be beneficial for two reasons. First, the active molecule (2) may be used as a blue print for new semi-synthetic molecules using medicinal chemistry approaches. Thus, the new developed molecules could have improved activity and higher stability. The second application implies that the non-active sesquiterpene lactones (1, 3 and 4) can be incorporated in public databases, like PubChem, ChemBL or Collaborative Drug Discovery, avoiding repetitive screening and tests [111].

In conclusion, in this study we reported the isolation of four sesquiterpene lactones from chicory roots and for the first time their leishmanicidal activity in vitro. Only (2) lactucopicrin showed moderate activity, although other types of sesquiterpene lactones proved to be leishmanicidal in submicromolar ranges. One possible explanation for the low activity could be the typical high chemical instability of guaianolide-like sesquiterpene lactones.

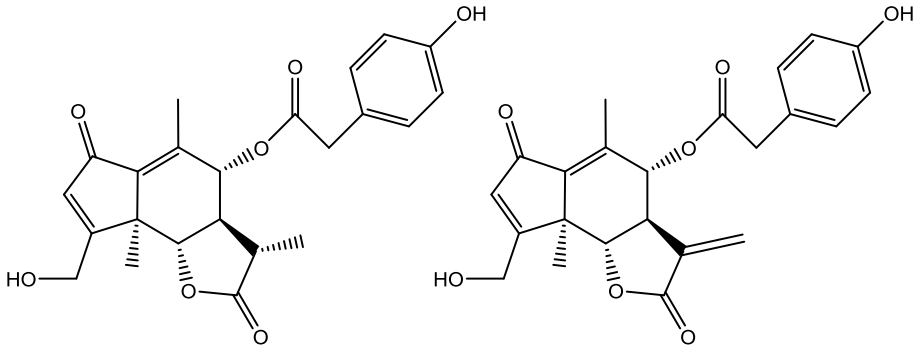
2.5 Tables and figures

Table 2.1 Leishmanicidal activity of sesquiterpene lactones isolated from the roots of *Cichorium intybus* L. Data are IC_{50} (μM) ± SD, $n = 3$.

Compound	Leishmanicidal activity
(1) 11(S),13-dihydrolactucopicrin	> 50
(2) lactucopicrin	24.8 (± 2.1)
(3) 11(S),13-dihydrolactucin	> 50
(4) lactucin	> 50
†pentamidine	1.6 (± 0.1)

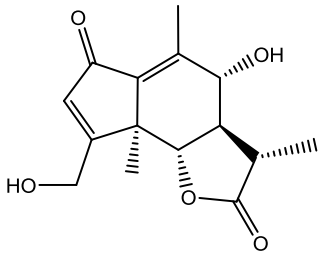
†Positive control (leishmanicidal drug).

Figure 2.1 Chemical structure of isolated sesquiterpene lactones from *C. intybus* roots.

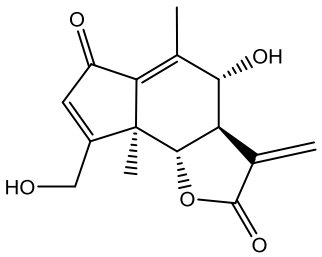


(**1**) 11(S),13,-dihydrolactucopicrin

(**2**) lactucopicrin



(**3**) 11(S),13,-dihydrolactucin



(**4**) lactucin

CHAPTER 3

Leishmanicidal activity of natural products isolated from *Cornus florida* L.

Graziose R, **Rojas-Silva P**, Rathinasabapathy T, Dekock C, Grace MH, Poulev A, Lila MA, Smith P, Raskin I. 2012. Antiparasitic compounds from *Cornus florida* L. with activities against *Plasmodium falciparum* and *Leishmania tarentolae*. Journal of Ethnopharmacology 2012, 142(2):456-61.

3.1 Abstract

Context: *Cornus florida* L. is a traditional horticultural and medicinal tree from North Eastern North America that was used in parasitic infections by North American natives.

Objective: To identify leishmanicidal constituents from the bark of *Cornus florida* L.

Materials and methods: Dried and powdered bark was extracted with 95% ethanol. The resultant extract was subjected to in vitro leishmanicidal-guided fractionation against *Leishmania tarentolae* and rat skeletal myoblast L6 cells to assess cytotoxicity.

Results: Guided fractionation afforded 8 compounds: (1) betulinic acid, (2) ursolic acid, (3) *β*-sitosterol, (4) ergosta-4,6,8,22-tetraene-3-one, (5) 3*β*-*O*-acetyl betulinic acid, (6) 3-epideoxyflindissol, (7) 3*β*-*O*-*cis*-coumaroyl betulinic acid, (8) 3*β*-*O*-*trans*-coumaroyl betulinic acid, of which, (6) is for the first time here isolated from a natural source and (4), (7) and (8) are reported for the first time from this genus. Leishmanicidal IC_{50} values are reported here for the first time for (4) 11.5 µM, (6) 1.8 µM, (7) 8.3 µM and (8) 2.2 µM. Cytotoxicity against L6 cells is reported for all compounds.

Conclusion: This work showed that *C. florida* natural products possess promising in vitro leishmanicidal activity.

Keywords: *Cornus*, Cornaceae, leishmaniasis, cytotoxicity, betulinic acid.

3.2 Introduction

Cornus florida L. (Cornaceae) is a perennial tree native to the East and Central regions of North America. It is known as flowering dogwood, American dogwood or just dogwood [112,113]. The species of the *Cornus* genus are mainly distributed in the Northern hemisphere, eastern Asia and North America, including 55 species; although two are endemic in South America and one in Africa [114]. *Cornus florida* is one of the most common ornamental trees in the eastern United States with more than 100 cultivars [115]. The tree is highly recognized for the showy snow white (rarely pink or red) petal-like bracts (late spring) and shiny red berries (early fall); as well as vivid red leaves during fall season [116].

Different type of natural products have been identified in *Cornus* spp. mainly: iridoids, sterols, saponins, terpenoids, tannins, flavonoids and anthocyanins [117]. However, there is no much information about phytochemicals isolated from *C. florida.* The presence of anthocyanins in the fruits and saponins from the bark has been reported [118]. Betulinic and ursolic acids (pentacyclic triterpenoids), verbenalin (alkaloid), quercetin, kaempeferol (flavonols) and gallic acid (phenolic acid) have also been identified [113].

The bark of this species has been historically used to treat malaria by Native Americans [119]. During the World War II, Spencer et al. [120] demonstrated the bioactivity of the bark against avian malaria parasites. Despite the traditional use against malaria, *C. florida* could be good sources for new leishmanicidal natural products.

3.3 Materials and methods

3.3.1 Plant material

Bark was collected from *Cornus florida* in September of 2009 at West Hills Park, Huntington Station, NY (40°48'36.38"N, 73°26'14.90"W). The plant material was collected by Rocky Graziose and species identity was confirmed by Lena Struwe; a voucher (R.Graziose #45) is stored at the Chrysler Herbarium (CHRB).

3.3.2 Instrumentation and chemicals

Please see section *1.2.4.1*.

3.3.3 Extraction, isolation and identification

Dr. Rocky Graziose was in charge of this section. Details can be found in the publication [121].

3.3.4 Leishmanicidal, cytotoxicity assays, and statistical analysis

Promastigotes of *Leishmania tarentolae* strain UC were donated by Dr. Larry Simpson (UCLA). The parasites were maintained in brain heart infusion (BHI) supplemented with hemin (10 μg/mL) and subcultured every third day. One hundred microliters of culture with 1×10^6 cells per mL were seeded in 96-well plates. Serial dilutions of the extract or compounds were prepared covering a range of 8 to 12 points from 200 to 0.01 μg/mL, and the different concentrations tested by triplicate. The plates were incubated at 27 ºC for 48 hours in darkness. After this period, the plates were inspected under an inverted microscope to assure sterile conditions and growth of controls. Then, 10 μL of 5 mg/mL of the tetrazolium salt MTT were added to each well to evaluate cell viability. The incubation continued for another 4 hours. The blue dye formazan were formed by the reduction of MTT inside the mitochondria, lower the

amount of formazan higher the leishmanicidal activity [38]. Next, formazan was solubilized with 100 % DMSO or acidic i-PrOH (0.1 N HCl). Finally, absorbance was read using a Synergy^{TM} HT multidetection microplate reader (BioTek® Instruments, Inc. Winooski, VT) at 570 nm and correction at 630 nm. Pentamidine (Sigma) was the reference drug and the IC_{50} value was calculated based on the dose response curve covering a range of 13 points from 3.75 to 0.125 μg/mL. In Chapter 4, amastigotes of *Leishmania donovani* were also employed. The axenic amastigote leishmanicidal bioassay were performed for 72 h assay using ca. 66000 amastigotes per well and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] was used to evaluate viability of the parasites. The IC_{50} value was calculated as mentioned here before.

Rat skeletal myoblast L6 cells were used as a mammalian model cell to assess cytotoxicity of the isolated compounds. The well-known cytotoxic compound emetine (Sigma, USA) was employed as reference drug. The cells grew DMEM supplemented with 10% FBS at 37°C in 95% air-5% CO_2 humidified environment. The assay was conducted in 96-well plates and each well was filled with 100 μL of culture medium with 1×10^4 cells per mL. After 2 hours, the tested compounds were added by triplicate in different 12 concentrations (serial dilution from 200 to 0.01 μg/mL). After 72 hours of incubation, the plates were checked under an inverted microscope to assure growth in sterile conditions. Then, 10 μL of MTT (5 mg/mL) sterile solution was added to each well, and incubation continued for another 4 hours [39]. Finally, absorbance was measured as mentioned in section 1.2.4.2.

Data were analyzed using GraphPad Prism 6.02 (GraphPad Software Inc., La Jolla, CA). The decrease of absorbance was expressed as percentage of the absorbance of the growth control and plotted against the drug concentrations. The IC_{50} values were calculated using a non-linear dose-response curve fitting analysis [40]. The IC_{50} values reported were the mean of three independent experiments.

3.4 Results and discussion

A summary of isolated compounds and their bioactivity are presented in Table 3.1 and their chemical structures in Fig. 3.1. Compounds (1) betulinic acid, a very common pentacyclic triterpenoid found in the bark of many trees [122], and (3) *β*-sitosterol, one of the most ubiquitous plant sterols [123], were inactive against *L. tarentolae* like in [108]previous reports [124]. Compounds (2) ursolic acid and (5) 3*β*-*O*-acetyl betulinic acids, which are chemically related to (1), showed significant activity against *L. tarentolae* promastigotes with IC_{50} values of 9.9 μM and 0.9 μM, respectively. Previous studies showed different results for (2) and (5). For example, compound (2) had moderate activity against *L. amazonensis* but was inactive against *L. infantum* [125], and 3*β*-*O*-acetyl betulinic acid demonstrated a high IC_{50} value (44.9 μM) against *L. amazonensis*, but was completely inactive (>200 μM) against *L. braziliensis* [126]. Compounds (7) 3*β*-*O*-cis-coumaroyl betulinic acid and (8) 3*β*-*O*-trans-coumaroyl betulinic were isolated for the first time from a *Cornus* species. Both compounds are betulinic acid derivaties containing phenolic esters which displayed moderate leishmanicidal activity (IC_{50} 10.4 μM and 15.3 μM, respectively). However, it is remarkable that compounds (7) and (8) have a higher activity compared to (1), suggesting that the phenolic moiety plays an important role in the leishmanicidal bioactivity. Compound (4), ergosta-4,6,8,22-

tetraene-3-one, showed moderate activity against *L. tarentolae* (IC_{50} 11.5 μM). This natural product belongs to ergosterol-like metabolites, which are synthetized by fungi [127]. The presence of this fungal natural product could be an indication of a bark epi- or endophyte. It is worth to mention that the collected bark did not have any visible mold and was dried shortly after collection to prevent microbial contamination. Compound (6) 3- epideoxyflindissol is a tirucallane triterpenoid that possessed a strong leishmanicidal activity, which was comparable to the positive control pentamidine (IC_{50}= 1.8 vs. 1.6 μM, respectively), and the highest selectivity index (8.1). This compound has never been isolated before from a *Cornus* species, although similar compounds were isolated from *C. walteri* [128].

In conclusion, *C. florida* bark demonstrated to possess several leishmanicidal compounds, although has never been used traditionally to treat leishmaniasis. Due to a low selectivity index (< 10) for all of the active compounds a following research it is necessary to test the leishmanicidal activity in amastigotes and cytotoxicity in macro-phages, before proceeding with an in vivo study in animals.

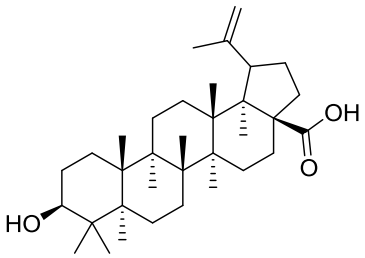
3.5 Tables and figures

Table 3.1 Leishmanicidal bioactivity of isolated compounds from bark of *Cornus florida* L. Results expressed are IC_{50} (µM) ± SD, $n = 3$.

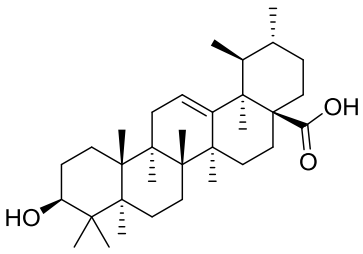
Compound	Leishmanicidal activity	Cytotoxicity	Selectivity Index
(1) betulinic acid	> 40	10.6 ± 2.6	nd
(2) ursolic acid	9.9 ± 3.2	12.7 ± 0.7	1.3
(3) *β*-sitosterol	> 40	6.2 ± 1.0	nd
(4) ergosta-4,6,8,22-tetraene-3-one	11.5 ± 0.4	27 ± 5.4	2.4
(5) 3*β*-*O*-acetyl betulinic acid	0.9 ± 0.3	5.2 ± 0.1	5.7
(6) 3-epideoxyflindissol	1.8 ± 1.1	14.7 ± 1.1	8.2
(7) 3*β*-*O*-*cis*-coumaroyl betulinic acid	8.3 ± 4.0	1.6 ± 1.4	0.2
(8) 3*β*-*O*-*trans*-coumaroyl betulinic acid	2.2 ± 0.1	9.3 ± 0.7	4.2
†pentamidine	1.6 ± 0.1	nd	nd
†emetine	nd	0.04 ± 0.001	nd

†Positive controls, nd = not determined.

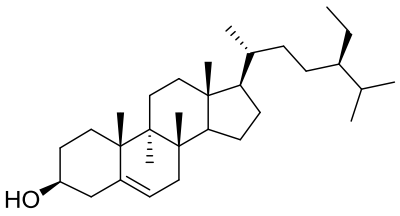
Figure 3.1 Chemical structures of isolated compounds from *C. florida* bark**.**



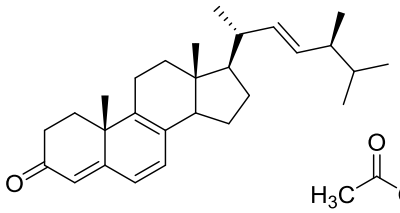
(**1**) betulinic acid



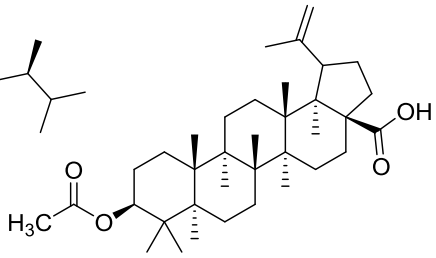
(**2**) ursolic acid



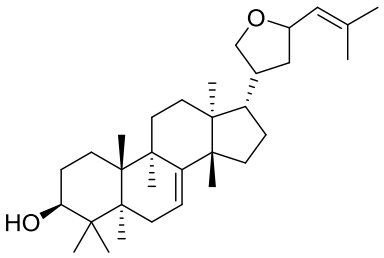
(**3**) sitosterol



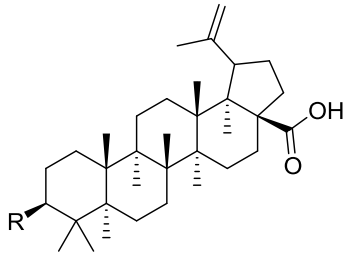
(**4**) ergosta-4,6,8,22-tetraene-3-one



(**5**) 3beta-*O*-acetyl betulinic acid



(**6**) 3-epideoxyflindissol



7: 3beta-*O*-*cis*-coumaroyl betulinic acid
8: 3beta-*O*-*trans*-coumaroyl betulinic acid

CHAPTER 4

Leishmanicidal activity of natural products isolated from *Eryngium foetidum* L.

Rojas-Silva P, Graziose R, Vesely B, Poulev A, Mbeunkui F, Grace MH, Kyle DE, Lila MA, Raskin. Leishmanicidal activity of a daucane sesquiterpene isolated from *Eryngium foetidum*. Pharmaceutical Biology 2014, 52(3):398-401.

4.1 Abstract

Context: *Eryngium foetidum* L. (Apiaceae) is a traditional herb that has been used to treat different parasitic infections in South America and Caribbean islands.

Objective: To evaluate in vitro leishmanicidal and cytotoxicity activities of isolated compounds based on a bioassay guided fractionation approach.

Materials and methods: Defatted aerial parts of *E. foetidum* were extracted with MeOH followed by partitioning with *n*-hexane, EtOAc and 50% MeOH. Then, the first two fractions were subsequently fractionated by column chromatography and HPLC. Compound identity was confirmed by LCMS and NMR. *Leishmania tarentolae* (promastigotes) and *L. donovani* (amastigotes) were used as testing parasites. L6 rat myoblasts were used for cytotoxicity.

Results: The *n*-hexane and EtOAc fractions showed ca. 40% growth inhibition (*L. tarentolae* promastigotes). The following compounds were isolated from these two fractions: lasidiol *p*-methoxybenzoate (**1**), and 4-hydroxy-1,1,5-trimethyl-2-formyl-cyclohexadien-(2,5)-[α-acetoxymethyl-cis-crotonate] (**2**). Compound **1** inhibited the growth of both *L. tarentolae* and *L. donovani* with IC_{50} values of 14.33 and 7.84 μM, respectively; and showed no cytotoxicity (IC_{50} >50 μM). Compound **2** was inactive in the *L. tarentolae* assay.

Discussion and conclusion: This is study presented the bioassay guided fractionation with the leishmanicidal and cytotoxicity activities of two compounds isolated for the first time from an *Eryngium* species.

Keywords: Apiaceae, sesquiterpenoids, leishmaniasis, cytotoxicity, bioassay guided fractionation, LCMS, NMR.

4.2 Introduction

Eryngium foetidum L. (Apiaceae) is a native plant from tropical America [129]. The genus *Eryngium* includes 250 species being the largest and complex group within Apiaceae [130]. *Eryngium* members are distributed along the world, and the place of origin is considered in Southwest Asia [130] . However, the majority (ca. two-thirds) of *Eryngium* species can be found in the Americas [130]. Some *Eryngium* species have been used as ornamentals, vegetable or medicinal plants [131].

Eryngium foetidum is described as a biennial herbaceous plant that can grow from 8 to 40 cm long with a fibrous taproot. It presents a characteristic basal rosette with oblanceolate leaves that have very short petioles and fine spiny blade margins. The inflorescences are terminal with cylindrical flower heads that are subtended by 4 to 7 bracts [85]. Since the plant is well known around the world it has adopted several common names like culantro, recao [129] or cilantro de monte [132], Mexican coriander, long coriander, fit weed, spirit weed, shado beni, or recao [133].

The leaves of *E. foetidum* are highly aromatic and used as condiment like its relative *Coriander sativum* L. (Apiaceae) which is known as cilantro and share a similar penetrating scent [133]. *Eryngium foetidum* has a long tradition as a popular culinary and

medicinal plant in Latin America, including the Caribbean Islands, but also in China, South-East Asia and the Pacific Islands where it was introduced [129].

Various medicinal properties have been attributed to *E. foetidum* leaves and roots. These properties include the following: treatment against fevers, chills, and malaria [134,135]; anti-inflammatory activity demonstrated in vitro and in vivo [136,137]; antihelmintic, anticonvulsant and antidiabetic [129]; to relief menstrual and abdominal pain, and to treat constipation as well as vomit and diarrhea [135,138].

Several chemical constituents have been identified from *E. foetidum* including essential oils like (*E*)-2-dodecenal or eryngial, triterpenoid glycosides, steroids (e.g. campesterol, stigmasterol, *β*-sitosterol and their derivatives), terpenes (e.g. limonene) and saponins [131,133,137]. Several anti-oxidants have also been isolated: phenolic acids (gallic acid, protocatechuic acid, syringic acid, *p*-coumaric acid, ferulic acid and sinapic acid); carotenoids (lutein, zeaxanthin, *β*-cryptoxanthin, and *β*-carotene); and anthroquinones (norlichexanthone, telochistin, secalonic acid D, citreorosein, emodin and parietin) [135].

Following the previously reported antiparasitic properties of *E. foetidum* [133], we subjected this plant to a bioassay guided fractionation procedure using a leishmanicidal assay. As a result, two compounds newly described for the *Eryngium* genus were isolated from the active fractions. This paper describes the extraction, isolation, identification and leishmanicidal activity of the extracts, fractions, and two compounds from the aerial parts of *E. foetidum.*

4.3 Materials and methods

4.3.1 Plant material

Seeds of *E. foetidum* were purchased from Johnny's Selected Seeds (http://www.johnnyseeds.com). The plants were grown in the Rutgers Experimental Greenhouse and were harvested after four months. The species was identified by P. Rojas-Silva and confirmed by Dr. Lena Struwe. Two voucher specimens were deposited in the Chrysler Herbarium (CHRB) at Rutgers University, collection numbers: P.Rojas 14 and P.Rojas 15. The fresh aerial parts were frozen overnight and then lyophilized until dry.

4.3.2 Instrumentation and chemicals

Please see section *1.2.4.1.*

4.3.3 Extraction and isolation

Dried and powdered aerial parts (800 g) were defatted with *n*-hexane (1×4.5 L). The plant material was then dried and extracted with MeOH (4×1 L). The MeOH crude extract was dried, and resuspended in 1000 mL 50% MeOH. The solution was partitioned first with *n*-hexane, followed by EtOAc (1 L each): the yield was 10, 9 and 18 g for the *n*-hexane, EtOAc and 50% MeOH fractions, respectively. A portion of the *n*-hexane fraction (2.7 g) was applied to a silica gel column (200 g) with a stepwise elution gradient (500 mL each 0, 10, 20, 30, 40, 50, 60, and 80% EtOAc in *n*-hexane). The eluent was collected in 125 mL flasks, which were pooled according to their TLC profile to generate ten fractions (1-10). Fraction 4 (215 mg) was purified by column chromatography over silica gel (30 g) using *n*-hexane-EtOAc-MeOH (100 mL each 90:10:0, 80:20:0, 70:30:0, 60:40:0, 0:50:50). The eluent was collected in 30 mL vials, and then vials were pooled

based on their TLC profile to generate 3 subfractions. Subfraction 2 yield was 197 mg and a portion of it (50.9 mg) was purified by prep-HPLC (column CuroSil-PFP 5 μm, 21.20×250 mm; ACN 0.5% AcOH 75:25, isocratic, 10 ml/min) yielding compound **1** (*R*t 9.7 min, 5.4 mg).

A portion of the EtOAc fraction (2 g) was fractionated using a silica gel column (100 g) with *n*-hexane-EtOAc in a stepwise elution gradient (500 mL each of 0, 10, 20, 30, 40, 50, 60, and 80% EtOAc in *n*-hexane). Four fractions (1-4) were recovered and fraction 3 (640 mg) was subjected to column chromatography (silica gel 100 g) using *n*-hexane-EtOAc (300 ml, 70:30 isocratic). Eight subfractions were generated. Subfraction 2 (100 mg) was passed through a silica gel column (25 g) using *n*-hexane-EtOAc (300 ml, 70:30 isocratic) and yielded another 3 subfractions. Finally, subfraction 2 was subjected to prep-HPLC (column SymetryPrep™ C8 7 μm, 19×300 mm; ACN: 0.5% AcOH 90:10, isocratic, 10 ml/min) and compound **2** was isolated (*R*t 8.1 min,14 mg).

4.3.4 Identification

Lasidiol *p*-methoxybenzoate (1): clear oil. ESI-MS *m/z* 355.1 $[M + H - H_2O]^+$, 373.6 $[M + H]^+$, 395.7 $[M + Na]^+$; HR-ESI-IT-TOF-MS *m/z* 355.229 $[M + H - H_2O]^+$, 221.175 $[M - ArCO_2H]^+$, 203.168 $[M - ArCO_2H - H_2O]^+$, molecular formula $C_{23}H_{32}O_4$. ^{1}H NMR ($CDCl_3$-d, 500 MHz) δ 8.00 (2H, d, *J* = 8.8 Hz), 6.93 (1H, d, *J* = 8.8 Hz), 5.51 (1H, d, *J* = 6.6 Hz), 5.30 (1H, d, *J* = 6.8 Hz), 3.86 (3H, s), 2.46 – 2.24 (2H, m), 2.13 – 2.01 (2H, m), 1.88 (1H, dsept, *J* = 6.5, 5.0 Hz), 1.80 – 1.70 (1H, m), 1.70 (3H, s), 1.73 – 1.39 (4H, m), 1.07 (3H, s), 1.05 (3H, d, *J* = 6.8 Hz), 0.96 (3H, d, *J* = 6.7 Hz). ^{13}C NMR (125 MHz) δ_c 165.90, 163.30, 142.67, 131.53, 123.13, 121.64, 113.64, 83.29, 77.91, 56.78, 55.46, 53.58,

36.15, 35.59, 30.26, 26.66, 25.78, 24.83, 24.58, 22.86, 21.28. Compared to Cumanda et al.,1991[139] .

4-Hydroxy-1,1,5-trimethyl-2-formyl-cyclohexadien-(2,5)-[α-acetoxymethyl-cis-crotonate] (2): clear oil. ESI-MS *m/z* 306.35 $[M+H]^+$, molecular formula $C_{17}H_{22}O_5$. ^{1}H NMR (acetone d_6, 400 MHz) δ: 9.49 (1H, s), 6.65 (1H, d, *J* = 3.6 Hz), 6.50 (1H, q, *J* = 7.3 Hz), 6.01 (1H, d, *J* = 3.3 Hz), 5.51 (1H, s), 4.74 (2H, m), 2.13 (3H, d, *J* = 7.3 Hz), 2.03 (3H, s), 1.77 (3H s), 1.30 (3H, s), 1.25 (3H, s). ^{13}C NMR (125 MHz) δ_c 193.55, 170.49, 165.61, 148.65, 144.87, 143.34, 138.57, 127.38, 124.72, 68.26, 65.54, 35.19, 27.08, 26.43, 20.91, 19.18, 15.94. Compared to Bohlmann and Zdero, 1969 [140].

4.3.5 Leishmanicidal and cytotoxicity assays, and statistical analysis

Promastigotes of *Leishmania tarentolae* strain UC were donated by Dr. Larry Simpson (UCLA). The parasites were maintained in brain heart infusion (BHI) supplemented with hemin (10 μg/mL) and subcultured every third day. One hundred microliters of culture with 1×10^6 cells per mL were seeded in 96-well plates. Serial dilutions of the extract or compounds were prepared covering a range of 8 to 12 points from 200 to 0.01 μg/mL, and the different concentrations tested by triplicate. The plates were incubated at 27 ºC for 48 hours in darkness. After this period, the plates were inspected under an inverted microscope to assure sterile conditions and growth of controls. Then, 10 μL of 5 mg/mL of the tetrazolium salt MTT were added to each well to evaluate cell viability. The incubation continued for another 4 hours. The blue dye formazan were formed by the reduction of MTT inside the mitochondria, lower the amount of formazan higher the leishmanicidal activity [38]. Next, formazan was solubilized with 100 % DMSO or acidic i-PrOH (0.1 N HCl). Finally, absorbance was

read using a Synergy™ HT multidetection microplate reader (BioTek® Instruments, Inc. Winooski, VT) at 570 nm and correction at 630 nm. Pentamidine (Sigma) was the reference drug and the IC_{50} value was calculated based on the dose response curve covering a range of 13 points from 3.75 to 0.125 μg/mL. In Chapter 4, amastigotes of *Leishmania donovani* were also employed. The axenic amastigote leishmanicidal bioassay were performed for 72 h assay using ca. 66000 amastigotes per well and MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] was used to evaluate viability of the parasites. The IC_{50} value was calculated as mentioned here before.

Rat skeletal myoblast L6 cells were used as a mammalian model cell to assess cytotoxicity of the isolated compounds. The well-known cytotoxic compound emetine (Sigma, USA) was employed as reference drug. The cells grew DMEM supplemented with 10% FBS at 37°C in 95% air-5% CO_2 humidified environment. The assay was conducted in 96-well plates and each well was filled with 100 μL of culture medium with 1×10^4 cells per mL. After 2 hours, the tested compounds were added by triplicate in different 12 concentrations (serial dilution from 200 to 0.01 μg/mL). After 72 hours of incubation, the plates were checked under an inverted microscope to assure growth in sterile conditions. Then, 10 μL of MTT (5 mg/mL) sterile solution was added to each well, and incubation continued for another 4 hours [39]. Finally, absorbance was measured as mentioned in section 1.2.4.2.

Data were analyzed using GraphPad Prism 6.02 (GraphPad Software Inc., La Jolla, CA). The decrease of absorbance was expressed as percentage of the absorbance of the growth control and plotted against the drug concentrations. The IC_{50} values were

calculated using a non-linear dose-response curve fitting analysis [40]. The IC_{50} values reported were the mean of three independent experiments.

4.4 Results and discussion

The leishmanicidal activity of the crude MeOH extract from the aerial parts of *E. foetidum* and their initial fractions were tested on an in vitro culture of *L. tarentolae* promastigotes using 96-well microplates by evaluating the growth inhibition with a tetrazolium salt (MTT) staining. The crude extract showed 20% growth inhibition at 20 μg/mL, but the *n*-hexane and EtOAc fractions showed a promising bioactivity (see below). The 50% MeOH fraction showed just 4% growth inhibition and was not pursued any further. Therefore, in order to identify the active compounds, the active fractions were subjected to bioactivity guided fractionation. The *n*-hexane fraction demonstrated leishmanicidal activity of 41.3 %. From this one, ten fractions were isolated and subjected to bioassay. Fraction 4 showed the highest growth inhibition (59.1%) and was further fractionated. The subfraction 2 exhibited a 98.1% growth inhibition and contained one major compound as judged by TLC. Compound 1 was isolated from this subfraction using prep-HPLC. The EtOAc fraction exhibited a 41.4% leishmanicidal activity. Four fractions were generated, and fraction 3 showed the strongest activity (50.2%). From this fraction, eight subfractions were obtained. Only subfraction 2 showed activity (21.1%) which contained a major compound based on TLC. This subfraction was subjected to prep-HPLC, resulting in the isolation of compound 2.

Leishmanicidal and cytotoxic activities of the isolated compounds are reported in Table 4.1. The structures of compounds are depicted in Fig. 4.1. Compound 1 was identified as lasidiol *p*-methoxibenzoate [139] based on the data from high resolution

mass spectrometry and NMR experiments. This compound showed a good dose response in the growth inhibition assays with an IC_{50} value of 14.33 μM for *L. tarentolae* promastigotes and 7.84 μM for *L. donovani* amastigotes. Interestingly, compound 1 did not show cytotoxicity on L6 myoblast cells (IC_{50} >50 μM). These results are promising based on the leishmanicidal activity, especially against amastigotes, and the low *in vitro* cytotoxicity. Further studies will be necessary to investigate the medicinal applicability and mode of action. Although other types of sesquiterpenes have been identified in *Eryngium maritimum* L. [141], there are no reports of the isolation of daucane sesquiterpenes from the *Eryngium* genus. Lasidiol *p*-methoxibenzoate was previously isolated from the roots of the Ecuadorian medicinal plant *Xanthium catharticum* Kunth (Asteraceae), but the authors did not report any biological activity for this compound [139]. The chemical structure of compound 1 is closely related to 8-ketolasidiol *p*-anisate (fercomin), lasidiol, lasidiol angelate and ferutidin [139,142]. Most of these compounds have been isolated from medicinal plants in the genera *Ferulis* and *Ferulago* (Apiaceae), and they have shown antiproliferative activity against human tumor cells *in vitro* [143].

Compound 2 was identified as a terpene aldehyde ester (4-hydroxy-1,1,5-trimethyl-2-formyl-cyclohexadien-(2,5)-[α-acetoxymethyl-cis-crotonate]) based on LCMS and NMR experiments. This compound has never been isolated from any *Eryngium* species, but was previously found in *Ferula hispanica* Rouy (Apiaceae) [140]. However, the compound did not show any activity on the axenic *L. tarentolae* growth inhibition assay (IC_{50} >50 μM) and thus was not tested against *L. donovani*.

In conclusion, a leishmanicidal bioassay guided fractionation approach generated two compounds from the aerial parts of *E. foetidum*. These compounds have never been

isolated before from this genus, and here we demonstrate their leishmanicidal activity for the first time. Based on the absence of cytotoxicity for compound 1, our research provides additional information that may be useful for the development of new natural product-based pharmaceutical agents for treatment and prevention of leishmaniasis.

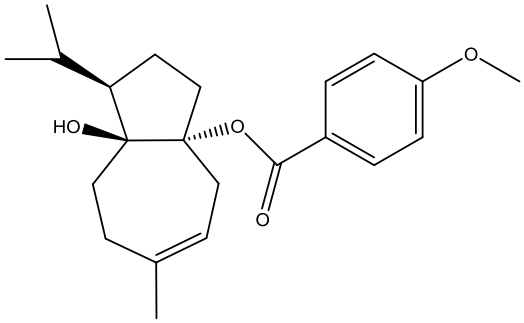
4.5. Tables and figures

Table 4.1 Leishmanicidal and cytotoxicity activities of isolated compounds from *Eryngium foetidum* L. aerial parts. IC_{50} values are expressed in μM ± SD, $n = 3$.

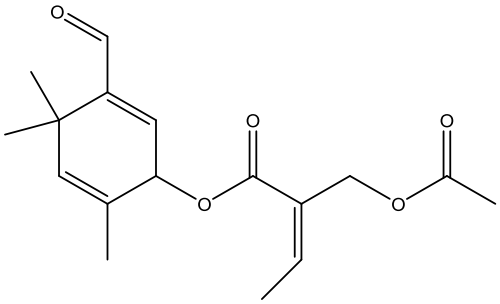
Compound	***L. tarentolae***	***L. donovani***	**L6 cells**
(1) lasidiol *p*-methoxibenzoate	14.33 (± 0.98)	7.84 (±1.3)	>50
(2) terpene aldehyde ester	> 50	nd	nd
†pentamidine	1.6 (± 0.04)	nd	nd
†miltefosine	nd	5.64 (±0.4)	nd
†emetine	nd	nd	0.03 (± 0.003)

†Positive controls. nd: non-determined.

Figure 4.1. Chemical structures of two isolated compounds, from aerial parts of *Eryngium foetidum* L.



(**1**) lasidiol *p*-methoxibenzoate



(**2**) terpene aldehyde ester derivative

CHAPTER 5

Anti-inflammatory and anti-obesity properties of *Moringa oleifera* Lam.

Waterman C, Cheng DM, **Rojas-Silva P**, Poulev A, Dreifus J, Lila MA, Raskin I. 2014. Stable, water extractable isothiocyanates from *Moringa oleifera* leaves mediate inflammation in vitro. Phytochemistry 2014, 103:114-122.

Waterman C, **Rojas-Silva P**, Tumer TB, Kuhn P, Richard AJ, Wicks S, Stephens JM, Wang Z, Mynatt R, Raskin I. Isothiocyanates from *Moringa oleifera* reduce weight gain, insulin resistance and hepatic gluconeogenesis in mice. Submitted to Diabetes.

5.1. Abstract

Context: *Moringa oleifera* Lam. (Brasicales, Moringaceae) is an edible and medicinal plant that has been used as natural remedy for chronic inflammatory conditions as well against obesity and diabetes mellitus type 2 in tropical areas around the world.

Objectives: To evaluate the anti-inflammatory and the anti-obesity activities in vitro and in vivo of a *M. oleifera* food-grade extract (MC) and isolated isothiocyanates (MICs).

Materials and methods: Fresh leaves of *M. oleifera* were extracted with water in order to obtain a MC containing 1.66% of total MICs. Also, MIC-1 and 4 were isolated from leaves. The MC and MIC-1 and 4 were tested in anti-inflammatory assays in vitro using RAW macrophages and Caco2 intestinal cells. For the in vivo study, MC was incorporated into a high fat diet and fed C57BJ6 mice for 3 months. During this time, body weight, OGTT and body composition was recorded. At the end of the experiment, blood, white adipose tissue, liver, small and large intestines were collected for analysis.

Results: MC, MIC-1, and MIC-4 significantly decreased gene expression and production of inflammatory markers in RAW macrophages and Caco2 cells. MIC-1 and 4 attenuated expression of *iNOS* and *IL-1β* and production of NO, TNFα and IL-8 at micromolar doses.

The MC treated animals did not gain weight neither develop fat liver disease compared to control animals. The serum metabolic and inflammatory biomarkers and OGTT results from MC treated mice showed normal levels. The analysis of insulin signaling and inflammatory markers in tissues (liver, skeletal muscle, white adipose tissue and ileum) showed almost complete normality. In addition, MC and MIC proved to shut down gluconeogenesis in vivo as well as in vitro. Finally, the indirect calorimetry acute study indicated that mice consuming MC had a higher rate of fat oxidation.

Conclusion: These results suggested the ability of MC, MIC-1 and 4 to suppress inflammation and the development of insulin resistance in vitro and in vivo. The use of *M. oleifera* could potentially alleviate and prevent chronic low-grade inflammation and the metabolic changes associated with diseases like obesity and type 2 diabetes.

Keywords: Moringa, Moringaceae, glucosinolates, isothiocyanates, chronic inflammation, obesity, diabetes.

5.2 Introduction

Moringa oleifera Lam. is a member of the monogenic family Moringaceae, within the order Brassicales to which cruciferous vegetables (e.g. mustard, cabbage or broccoli) also belong [144]. The plant is a small fast growing tropical tree commonly known as moringa, moonga, drumstick, horseradish tree, Ben oil tree, or benzolive tree [145,146]. The place of origin is located in sub-Himalayan areas of India, Pakistan, Bangladesh and Afghanistan [146].

The leaves and fruits of *M. oleifera* have been historically used as nutritious food in some tropical areas of Asia and Africa. They contain ca. 30 to 25% protein by dry weight, with a unique profile of essential amino acids, high levels of vitamins, and beneficial phytochemicals known as isothiocyanates (ITCs) which are remarkable bioactive plant natural products [145,147]. Other important type of phytochemical found in *M. oleifera* are flavonoids derivatives of kaempferol, quercetin and isorhamnetin like glucosides, rutinosides, malonylglucosides and acetylglucosides [148].

Glucosinolates or mustard oil glucosides are sulfur-rich phytochemicals present almost exclusively in members of Brassicales (the exception is the unrelated genus *Drypetes*, Euphorbiaceae) [149]. Glucosinolates are the chemical precursors of the distinctive ITCs which the main function for plants is to avoid parasitism and herbivory [144]. Glucosinolates are converted enzimatically by myrosinase, a specific β thioglucoside glucohydrolase, which is activaed after tissue damage such as cutting, chewing, digestion or chopping of the raw plant material [144]. Myrosinase cleaves the thio-linked glucose in any glucosionolate, leaving the aglycone which rearranges quickly to form active ITCs, the most common products, but also thiocyanates or nitriles as by-

products [149]. The strong and typical flavor that are produced by cruciferous vegetables like cabagge (*Brassica oleracea* L.), white mustard (*Sinapis alba* L.), oriental mustard (*Brassica juncea* (L.) Czern.), radish (*Raphanus sativus* L.) , horseradish (*Armoracia lapathifolia* Gilib.) is due to the formation of ITCs [150].

Moringa oleifera contains four specific carbohydrate-modified aromatic glucosinolates (MGLs) which then are converted to four unique bioactive ITCs, referred here as *Moringa* isothiocyanates (MICs) (Fig. 5.1). The MGLs are prensent in leaves, fruits, seeds and roots of *M. oleifera* [151]. The most abundant ITCs formed in *M. oleifera* are MIC-1 (4-[(*α*-L-rhamnosyloxy)benzyl] isothiocyanate) and MIC-4 (4-[(4'-*O*-acetyl-*α*-L-rhamnosyloxy)benzyl] isothiocyanate) (Fig. 5.1) representing ca. 95%, the other two are MIC-2 (4-[(2'-*O*-acetyl-*α*-L-rhamnosyloxy) benzyl]isothiocyanate) and MIC-3 (4-[(3'-*O*-acetyl-*α*-L-rhamnosyloxy)benzyl] isothiocyanate), which are syntethized in small amounts [148].

The ITCs from edible plants in Brassicaceae, like sulforaphane (SF) from broccoli (*Brassica oleracea* L.) and phenethyl isothiocyanate (PEITC) from winter cress (*Barbarea vulgaris* W.T.Aiton), have been studied profoundly as anti-inflammatory [152] and anticancer natural products [150]. However, the main problem for a practical application is due to chemical instability. For example SF, formed from glucoraphanin (Fig. 5.1), is a volatile compound and degrades quickly [153]. An important difference is that MICs contain a benzyl plus and additional rhamnose moieties in the molecule which gives them chemical stability; in fact MICs are solid powders when they are isolated [154]. Nevertheless, there has not been extensive research on the biological activity of MICs, even though cruciferous ITCs have demonstrated a great potential as phytoactive

therapeutics in human health. Previous studies of MICs showed similar bioactivity to the well-studied crucifer ITCs. For example, MIC-2 did reduce NO synthesis at a lower level compared to SF [155]. In the same way, MIC-1 inhibited the expression of NF-κB [154], and MIC-1 and 4 lowered NO formation when tested at micromolar concentrations in macrophages [156].

Traditional uses of leaves, fruits, bark, seeds and roots of *M. oleifera* as a natural remedy include the treatment of diabetes mellitus, rheumatism, liver damage, ulcers, venomous bites, bacterial and fungal infections, parasitic worms, and also as diuretic, antihypertensive, antipyretic, among other uses [145,157]. Crude extracts of the plant obtained with organic solvents have been tested in animal models as treatment for inflammatory conditions [157], hyperglycemia and diabetes type 1 [158,159], liver fibrosis [160], hyperlipidemia [161], and also as immunosuppressant [162]. This particular bioactivity is linked to the anti-inflammatory and antioxidant properties of the plant's phytochemicals [161]. However, most of these activities have been attributed to the presence of phenolic compounds (e.g. flavonols and phenolic acids), not to MICs. Therefore, based on the ethnobotanical use and the ITC content in *M. oleifera*, the present study evaluates at the same time the anti-inflammatory and anti-obesity effects of a food grade extract from *M. oleifera* leaves (MC) and isolated MICs (1 and 4) in vitro and in vivo models. The well characterized RAW macrophages cell line and the human intestinal cell lines Caco-2 and HT-29 were used to evaluate the anti-inflammatory bioactivity. The obese diet-induced C57BL/6J mice were employed as an in vivo model to test the anti-obesity bioactivity in a preventive study design.

5.3 Materials and methods

5.3.1 Anti-inflammatory activity in vitro

5.3.1.1 Plant material

Fresh leaves from *M. oleifera* cultivar Indian PKM-1 were shipped overnight from Moringa Farms (Sherman Oaks, CA). The plant material was confirmed by Dr. Carrie Waterman and a voucher specimen (C.Waterman 1) was deposited at the Chrysler Herbarium (CHRB), Rutgers University.

5.3.1.2 Extraction and isolation

Fresh *M. oleifera* leaves were extracted the day of arrival with Millipore H_2O in a ratio 1:5 (w/v) to produce MC. MICs 1 and 4 were extracted also from the fresh leaves using a modified approach to previously published methods (Cheenpracha et al., 2010). The MC contained 1.15% of MIC-1, 0.51% of MIC-4 and approximately 0.06% of MIC-2 and MIC-3 combined. These procedures were performed by Dr. Carrie Waterman. The details are reported in Waterman et al., 2014 [163].

5.3.1.3 Cell culture conditions

RAW 264.7 murine macrophages (ATCC® TIB-71™), were maintained in Dulbecco's modified Eagle's medium (DMEM) (Caisson Labs, North Logan, UT) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum. Cells were incubated at 37 °C with 5% CO_2 humidified atmosphere and subcultured every three days. For the anti-inflammatory assay, RAW cells were plated at a density of 4×10^5 cells/mL in 24-well plates. Cells were incubated overnight (18 h),

washed with warm PBS, and replaced with fresh DMEM media. Cells were pretreated with designated doses of vehicle (EtOH-H_2O, 1:1, v/v), MC, MIC-1 or 4. LPS (1 μg/mL) was added after 2 h incubation with treatments to elicit inflammatory responses. Cells were treated in triplicate. After an additional 6 h incubation period, media were collected and then cells were washed with PBS prior to collection in TRIzol® Reagent (Life Technologies, Carlsbad, CA). Samples were stored at -80 °C prior to processing.

Caco2 grew in DMEM supplemented with 10% FBS, 100 μg/mL streptomycin and 100 U/mL penicillin, 1% NEAA, and 15 mM HEPES. Cells were plated either in 24-well plates or 6-well plates, the media is changed every three days and differentiation is followed by 21 days. At day 20, cells are washed twice with warm 1× PBS and media is replaced by DMEM without phenol red, 0.1% NEAA and 25 mM HEPES (serum starvation). Next day, media is replaced with DMEM without phenol red, 0.5% FBS, 0.1% NEAA and 25 mM HEPES The cells were treated by two hours and then inflammation was induced with 25 ng/mL IL-1β by 6 hours. Finally, media were collected for analysis of IL-8.

HT-29 cells were used just for cytotoxicity assay. Cells grew in McCoy 5A medium supplemented with 10% FBS, 100 μg/mL streptomycin and 100 UI/mL penicillin, 1% NEAA, and 15 mM HEPES. The assays were conducted in 24-well plates. HT-29 ca. 2×10^5 cells per mL were seeded and growth continued until it reaches 80% confluence (ca. 48 hours). Finally, cells were exposed to MC and MIC-1 and 4 to evaluate cytotoxicity.

5.3.1.4. Gene expression analyses

Total RNA was extracted from RAW macrophage cells according to manufacturer's specifications. Briefly, $CHCl_3$ (200 µL) was added to TRIzol® harvested samples (600 µL). Samples were vigorously mixed for 30 s, incubated at room temperature for 5 min, centrifuged at 12,400 *g* and i-PrOH was added to the aqueous phase to obtain a ratio of 0.7 supernatant to i-PrOH. Samples were mixed by inverting, vortexed briefly and incubated for 10 min at -20 °C. Samples were centrifuged at 12,400 *g* for 15 min at 4 °C. Next, supernatant was removed and the sample was washed twice with EtOH-H_2O (75:25, v/v) and centrifuged at 6000 *g* for 10 min. Samples were allowed to dry and resuspended in diethylpyrocarbonate (DEPC) treated-H_2O. RNA integrity was evaluated by running ca.1 µg of RNA on a 1% agarose gel. RNA was then treated with Deoxyribonuclease I Amplification grade (Life Technologies), following the manufacturer's guidelines. RNA quality was checked on the NanoDrop 1000 system (NanoDrop Technologies, Wilmington, DE). A ratio of OD 260/280 $\geq$ 2.0 and OD 260/230 $\geq$ 1.8 was considered to be good quality RNA. First strand cDNA synthesis was performed using ABI High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) with RNAse I inhibitor, according to the manufacturer's instructions using RNA (1 µg). The thermal cycle program was set as follows: 10 min, 25 °C; 60 min, 37 °C; 60 min, 37 °C; 5 s, 85 °C, and final hold at 4 °C.

Synthesized cDNAs were diluted 25 fold and the diluted sample (5 µL) was used for qPCR with Power SYBR Green PCR master mix (12.5 µL, Applied Biosystems), primers (0.5 µL, 6 µM) and Biotechnology Performance Certified (BPC) grade H_2O

(Sigma) to a final reaction volume (25 µL). Exon-spanning primer sequences were previously designed [152] on Primer Express® (Life Technologies) and are as follows: β-actin forward 5'- AAC CGT GAA AAG ATG ACC CAG AT - 3', reverse: 5'- CAC AGC CTG GAT GGC TAC GT-3', IL-1β forward 5'- CAA CCA ACA AGT GAT ATT CTC CAT - 3', reverse 5'- GAT CCA CAC TCT CCA GCT GCA - 3', iNOS forward 5'- CCC TCC TGA TCT TGT GTT GGA - 3', reverse 5'- TCA ACC CGA GCT CCT GGA A-3', COX-2 forward 5' – TGG TGC CTG GTC TGA TGA TG -3', reverse 5'- GTG GTA ACC GCT CAG GTG TTG-3', TNFα forward 5' – TGG GAG TAG ACA AGG TAC AAC CC – 3', reverse 5'- CAT CTT CTC AAA ATT CGA GTG AGA A - 3', IL-6 forward 5' - TCG GAG GCT TAA TTA CAC ATG TTC – 3', reverse 5' TGC CAT TGC ACA ACT CTT TTC T – 3'. All primers were validated by analyzing amplification efficiencies and melt curve profiles.

Quantitative PCR amplifications were performed on an ABI 7300 Real-Time PCR System (Applied Biosystems) with the following thermal cycler profile: 2 min, 50 °C; 10 min, 95 °C; 15 s, 95 °C; 1 min, 60 °C for the dissociation stage; 15 s, 95 °C; 1 min, 60 °C; 15 s, 95 °C. Inflammatory marker mRNA expressions were analyzed by the comparative $\Delta\Delta C_t$ method and normalized with respect to the average C_t value of β-actin. Vehicle with LPS treatment served as the calibrator for $\Delta\Delta C_t$ analysis and was assigned a value of 1.0. Lower values indicate inhibition of gene expression relative to vehicle treated with LPS control. All experimental samples were run in triplicate and each reaction plate included no template controls.

5.3.1.5 TNFα and IL-8 ELISA assays

The levels of TNF-a and IL-8 present in the media were measured by the solid phase sandwich ELISA kits provided by BD OptEIA™ (BD Bioscience, San Jose, CA). RAW 264.7 macrophages and Caco2 cells were cultured, treated with MC or MICs, and subjected to induced inflammation as stated above. After treatments, media (1 mL) was collected and immediately centrifuged at 16,000 RCF and 4 °C by 10 min. The supernatant was preserved at -20 °C until further processing. The samples were assayed following the manufacturer's protocol. One-hundred micro-liters of standards and samples were added to specific and previously treated ELISA 96-well plate by duplicate and incubated at room temperature for two hours. The specific interleukin present in the media were bound to the detection antibody attached to bottom of the wells. Then, the wells were washed five times and streptavidin-horseradish peroxidase conjugate mixed with biotinylated anti-human or anti-mouse specific interleukin antibody was added and followed by one hour incubation at room temperature. The captured interleukin plus the detection antibody marked with biotin created an antibody-antigen-antibody sandwich structure. Then, the wells were washed 7 times and TMB substrate solution is added followed by 30 min incubation at room temperature. A blue color is produced in direct proportion to the amount of the measured interleukin present in the initial sample. Finally, the stop solution was added and the color changes from blue to yellow. TNFα and IL-8 levels were quantified using a reference standard curve provided with the kit. The absorbance was read at 450 nm and 570 nm in the Synergy™ HT multidetection microplate reader (BioTek® Instruments, Inc. Winooski, VT). In order to know the concentration of the interleukine, the sample OD values were transformed by using the

standard curve generated in each experiment as follows: Concentration = (Sample Abs OD-intercept)/slope. The results were expressed as fold-change compared to control after normalization with total protein content.

5.3.1.6. Nitric oxide production analysis

Cells were cultured and treated as stated above. After treatments, media was collected and assayed immediately by duplicate following the Griess Reagent System provided by Promega (Promega Corporation; Madison, WI). The Griess method is designed to measure nitrates, since the half-life of nitric oxide (NO) is 2-3 seconds. The nitrite standard (0.1 M sodium nitrite) reference curve was built performing a serial dilution (0 to 100 µM). Absorbance is read at 540 nm in the Synergy™ HT multide-tection microplate reader (BioTek® Instruments, Inc. Winooski, VT). In order to know the concentration of nitrates, the sample OD values were transformed by using the standard curve generated in each experiment as follows: Concentration = (Sample Abs OD-intercept)/slope. The results were expressed as fold-change compared to control after normalization with total protein content.

5.3.1.7 Protein measurement

The total content of cell proteins were used to normalize the data obtained from the ELISA and NO production assays. After the collection of the media, cells were washed twice with cold 1X PBS and harvested with cold NET lysis buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris, and 0.5% Triton X 100). The harvested cells were sonicated for 5 min, and then spined-down at 14000 x g (RCF), 4°C by 10 min. The supernatant was used to measure the protein concentration following the BCA protocol

(Bicinchoninic Acid, Pierce™ Thermo Scientific Inc.) and each sample was assayed by duplicate.

5.3.1.8 Cell viability (cytotoxicity)

The cell viability assay was conducted in 96-well plates and each well filled with ca. 1×10^4 cells per mL in 200 μL of culture medium. After 2 hours, the extracts and tested compounds were added by triplicate in 8 different concentrations (serial dilution from 200 to 0.01 μg/mL). After 24 or 48 hours of incubation, the plates were checked under an inverted microscope to assure growth. Effect of treatments on cell viability was measured using MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide] (TCI, Portland, OR) [39]. MTT (5 mg/mL) was dissolved in PBS (Cayman Chemical, Ann Arbor, MI), filtered through a 0.22 μm membrane and 10 μL added to treated cells during the last 4 h of treatment. Media were carefully aspirated and discarded, and the cells were dissolved in DMSO. The absorbance was read at 570 nm and 630 nm as a background. using a Synergy™ HT multidetection microplate reader (BioTek® Instruments, Inc. Winooski, VT). The IC_{50} values were calculated as in section 1.2.4.3.

5.3.1.9 Statistical analysis

Statistical comparisons for anti-inflammatory experiments were made by ANOVA followed by Dunnett's or Wilcoxon's multiple comparisons post-test as indicated and $p < 0.05$ were considered significant. For statistical analysis GraphPad Prism version 6.02 for Windows (GraphPad Software, Inc.) was employed.

5.3.2 Anti-obesity and anti-diabetic study in vivo

5.3.2.1 Animals

Three month study. Twenty-four male C57BL/6J mice at 5 weeks of age were ordered from Jackson Laboratories (Bar Harbor, ME, USA). Mice were acclimated by 9 days and housed 4 per cage under a 12-h light/dark cycle, with *ad libitum* acces to water and a very high fat diet (VHFD) or VHFD + 5% MC for twelve weeks. Body weight and food intake of each mouse was recorded weekly. Food intake was estimated as follows: [total food consumed per cage]/[mice per cage]×[days of food consumption]. Body composition was determined at 4, 8, and 12 weeks by magnetic resonance imaging using an EchoMRI-100 instrument (Echo Medical Systems, Houston, TX, USA). Feces collection was performed over 3 days during the 12^{th} week of the experiment. Mice were removed from group cages and placed in clean individual cages. Feces were collected every hour, weighted and freeze-dried. At the end of the study mice were euthanized with CO_2. Blood and tissues (liver, epididymal white adipose tissue, gastrocnemius muscle and ileum) were collected immediately and preserved at -80 °C until processing. The animal protocols were approved by the Comparative Medicine Resources and the Office of Research and Sponsored Programs both from Rutgers University, NJ, USA.

Two week indirect calorimetry (metabolic chamber) study. Twenty-four male C57BL/6J mice at 4 weeks of age were purchased to Jackson laboratories (Bar Harbor, ME, USA). After exit from quarantine, mice were placed on the VHFD and placed in TSE training cages for one week. Then they were placed in the TSE for one week to establish baseline values. The mice were weighed and body composition measured and

then randomized into treatment groups. 12 mice per group were fed the VHFD or VHFD + 3.3% MC for an additional week before returning to their home cages.

5.3.2.2 Diet

Diets were formulated by Research Diets (New Brunswick, NJ, USA) to be isocaloric for fat, protein and carbohydrate content (Table 5.2). The VHFD contained ca. 60% kcal from fat (lard mainly). MC was incorporated into food and the formulation for both studies was standardized to deliver 800 mg of MICs/kg of food. In the initial 3 month study, the VHFD contained 5% MC (1.66% MIC by DW), and in the follow up 2 week indirect calorimetry study the VHFD contained 3.3% MC (2.40% MICs by DW) (Table 5.3).

5.3.2.3 Oral glucose tolerance test (OGTT)

Three month study. Mice in the three month study were first fasted overnight before fasting glycemic levels were recorded using a glucometer (AlphaTRAK® 32004-02, Abbott Animal Health) and finally gavaged with 2 g/kg of glucose. An additional six mice on the VHFD at the same age were gavage with 300 mg/kg of metformin 3 hours prior to glucose and used as a positive control treatment The glycemic levels were measured up to 120 min. The OGTT was performed at weeks 4, 8 and 12.

Acute OGTT. Nineteen C57BL/6J male mice were ordered, acclimated and housed as described in the 3 month study. Mice were fed *ad libitum* a VHFD for 12 weeks. The OGTT was performed as described about except for gavage treatements of 2 g/kg of MC (n = 5), 300 mg/kg of metformin (n = 3), or vehicle (H_2O, n = 3) 3 hours prior to glucose gavage.

5.3.2.4 Serum chemistry analysis

Animals from the three month study were fasted overnight and trunk blood was collected immediately after euthanization. Samples were allowed to clot and were then centrifuged for 10 min at 5000 rpm. Serum was aliquotted into cryovials and was stored at -80^{o}C for biochemical analysis. Insulin, leptin, resistin, interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) were measured using a multiplex assay (Millipore Temecula, CA, USA) measured on a Luminex 200 (Luminex, Austin, TX, USA). Total cholesterol and triglycerides were assayed on a DxC 600 Pro (Beckman Coulter, Inc., Indianapolis IN, USA).

5.3.2.5 Liver histology and total lipid extractions

Liver sections were fixed in 10% neutral buffered formalin for 48 h, then processed and embedded in paraplast. Six-micrometer sections were cut and stained in hematoxylin and eosin. A diagnosis of fatty liver was made based on the presence of macro or microvesicular fat >5% of the hepatocytes in a given slide. Total lipid content of liver and feces was determined by the Folch method [164]. Briefly, liver samples (~300 mg) and feces (~200 mg) were extracted 20:1 (v/w) with $CHCl_2/CH_3OH$ (2:1), followed by solvent evaporation before recording dry weights.

5.3.2.6 Gene expression analysis by quantitative PCR

Total RNA was isolated from liver and ileum for TNFα, IL-1β, IL-6 expression; and additionally for liver glucose-6 phosphatase (G6P), phosphoenol pyruvate kinase (PEPCK) and glucokinase (GcK). For extraction the PureLink® RNA mini kit plus on-column DNase treatment (Applied Biosystems, Foster City, CA, USA) were used. For

white adipose tissue: adiponectin, TNFα, monocyte chemoattractant protein-1 (MCP1), plasminogen activator inhibitor (PAI1), TNF binding protein (TBP), lipocalin-2 (LCN), uncoupling protein 1 (UCP1), PR domain containing 16 (PRDM16), beta-3 adrenergic receptor (ADRB3), carnitine palmitoyltransferase I (CPT1), Cell death-inducing DFFA-like effector a (CIDEA), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α).

Around 100 mg of tissue was homogenized either with lysis buffer or TRIzol® using zirconium beads triple pure with the BeadBug homogenizer (Benchmark Scientific, Inc. Edison, NJ, USA). First-strand cDNA was synthetized from 2 μg total RNA using the high capacity cDNA reverse transcription kit plus RNase inhibitor (Applied Biosystems) with oligo-d(T)s as primers. Quantitative PCR analyses were performed in 7300 Real-Time PCR system using the TaqMan Gene Expression Assays (Applied Biosystems). The housekeeping gene hydroxymethylbilane synthase (Hmbs) was used to normalize relative expression of target genes. The effect of treatment on relative gene expression levels was evaluated by the $\Delta\Delta C_t$ method ($2^{-[Ct \text{ target gene} - Ct \text{ housekeeping gene}]}$). Primers of genes were TaqMan assays. All selected assays were inventoried and labeled as best coverage.

5.3.2.7 Immunoblot analysis

Liver and muscle tissues were prepared for western blot analysis as previously described [165]. Briefly, tissue samples were homogenized and protein concentration was measured by the Bio-Rad protein assay kit (Bio-Rad laboratories, Hercules, CA, USA). Supernatants (50 μg) were resolved by SDS-PAGE and subjected to western blotting.

The protein abundance was detected with antibodies against p-tyr (PY20), insulin receptor substrate 1 (IRS-1), IRS-2, anti-phospho-IRS-1$^{(Tyr612)}$ (IRS-1 p), p85 of PI 3K (PI 3K), RAC-alpha serine/threonine-protein kinase (Akt1), RAC-beta serine/threonine-protein kinase (Akt2), phosph-Akt1$^{(Ser473)}$ (Akt1 p) (Lake Placid, NY, USA), phosph-Akt2$^{(Ser474)}$ (Akt2 p) (GenScript, Piscataway, NJ, USA), sterol regulatory element binding protein-1c (SREBP-1c), β-Klotho, cell death-inducing DFFA-like effector c (FSP27/CIDEA in humans), lipoprotein lipase (LPL), adipose triglyceride lipase (ATGL), Insulin Receptor beta (IR β) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GLUT4 (R&D Systems, Minneapolis, MN, USA), peroxisome proliferator-activated receptor alpha (PPARα), PPAR γ, PGC-1α, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) (Millipore), fatty acidy synthase (FAS) (Abcam, Cambridge, MA, USA), and β-actin using chemiluminescence Reagent Plus (PerkinElmer Life Science, Boston, MA, USA), and quantified via a densitometer. All proteins were normalized by β-actin, and specific protein phosphorylation was normalized by the corresponding protein.

5.3.2.8 In vitro gluconeogenesis studies

H4IIE rat hepatoma cells (CRL-1548, American Type Culture Collection, Manassas, VA, USA) were assayed for glucose production as previously described [166]. Cell viability was measured by the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT; TCI, Portland, OR, USA) assay [39]. Gene expression of PEPCK and G6P were also evaluated. RNA extraction, cDNA synthesis and qPCR were performed as described above, and β-actin was used to normalize the relative expression.

5.3.2.9 In vitro lipolysis assay

Mature adipocytes were treated with MC, MIC-1 or MIC-4 in 5% calf media to evaluate glycerol release into the media (lipolysis). The assay was initiated by replacing the calf media with the lipolysis assay media containing vehicle, isoproterenol (10µM, positive control) MC (50, 100 µg/mL) or MICs (5, 10 µM). After 3.2 hrs, the conditioned media was removed and assayed for glycerol using the free glycerol reagent (Sigma–Aldrich. St. Louis, MO, USA).

5.3.2.10 Statistical analysis

GraphPad Prism v.6.04 (GraphPad Software Inc., San Diego, CA) was used for statistical analysis. Statistical analyses are indicated in each figure legend.

5.4. Results and discussion

5.4.1 Anti-inflammatory activity in vitro

The cytotoxicity activity of MC, MIC-1 and 4 is presented on Table 5.1. The results showed that the applied doses for the other experiments were under the cytotoxicity level. Tested concentrations of MC ranged from 5 to 100 µg/mL containing roughly 0.08% to 1.66% MIC total content which expressed at molar level ranged from approximately 0.28 µM to 5.1 µM. MIC-1 and 4 were tested at 1 and 5 µM in RAW macrophages and 10 µM in Caco2 cells.

MC demonstrated a dose dependent inhibitory effect on *iNOS* and *IL-1β* gene expression (Fig. 5.1 A). The gene expression of *iNOS* and *IL-1β* was almost suppressed at 100 µg/mL of MC. When MIC-1 and 4 were tested at 1 and 5 µM, they also showed significant gene expression reduction of *iNOS* and *IL-1β* (Fig. 5.1 B). Additionally, MC tested at 100 µg/mL (Fig. 5.1 C) and MIC-4 at 5 µM (Fig. 5.1 D) decreased *IL-6* gene expression, although *TNFα* gene expression was not reduced at any of the concentrations of MC and MICs tested (Figs. 5.1 C & D).

MC and MICs did also reduce NO and TNFα production (Fig. 5.2 A & B). MC, MIC-1 and 4 inhibited the production of NO significantly. This result is similar with previously reported NO inhibition by MIC-1 and 4 that had IC_{50} values of 14.43 and 2.71 µM, respectively [156]. MC assayed at 100 µg/mL was able to inhibit the production of NO by 90%. MIC-1 and 4 are partially responsible for this effect, since they inhibited NO formation at 5 µM by 69% and 39%, respectively. Reduced *iNOS* expression and NO production by SF has been correlated to suppression of inflammation [167].The highest

reduction of MC over individual MICs was also evident in NO production, but to a lesser degree than observed in the TNFα production experiments. MC tested at 100 µg/mL inhibited TNFα production by 70% compared to the control. MIC-1 and 4 at 5 µM reduced TNFα production by 20% and 27%, respectively. Since *TNFα* gene expression was not significantly inhibited by MC or MICs, it is probable that that moringa phytochemicals may inhibit TNFα production at the translational level or at the level of TNFα turnover.

The enhanced anti-inflammatory activity of MC compared with MICs alone may be explained by the presence of polyphenols in combination with all the MICs, including MIC-2 and 3. These two last compounds have been reported to inhibit NO formation at low micromolar concentrations (IC_{50} of 1.67 µM and 2.66 µM, respectively [156]).

In addition, MIC-1 and 4 tested at 10 µM showed a significant reduction of IL-8 production on Caco2 cells (Fig. 5.4). However, MC did not demonstrate any activity at 100 µg/mL. This result is the first report of MICs reducing IL-8 production on Caco-2 cells.

In conclusion MC, MIC-1 and 4 demonstrated anti-inflammatory activity in vitro on murine macrophages and human intestinal cell models. This bioactivity provides preliminary support for the use *M. oleifera* in the prevention and treatment of conditions associated with chronic inflammation. Also, MIC-1 and 4 demonstrated to have equal pharmacological properties as well-known ITCs like SF or PEITC.

5.4.2 Anti-obesity and anti-diabetic study in vivo

5.4.2.1 Effect of VHFD + 5% MC diet on body weight, body composition, OGTT, liver composition and lipid content in liver and feces

The VHFD + 5% MC diet had ca. 800 mg of MICs/kg, thus this group was consuming ca. 66 mg total MICs/kg/day. The VHFD + 5% MC-fed mice did not gain weight as much as the VFHD control group during the 12 weeks (Fig 5.5 A). The final average weight for VHFD + 5% MC-fed mice was 38.42 g ± 1.04 (SEM) and for VHFD was 46.94 g ± 1.00 (SEM), almost 19% difference. In comparison, C57BL/6J mice fed a low fat diet (10% kcal from fat) typically gain 25-32% less weight than mice on a VHFD [168]. This result indicates that a 5% MC supplementation did prevent body weight gain.

Food consumption was stable along the 12 weeks study, the VHFD + 5% MC group consumed an average of 2.22 g/day ± 0.02 (SEM) while the VHFD control group ate 2.42 g/day ± 0.05 (SEM) in average. The food intake only became significantly different at the end of the study. Interestingly, the ratio of accumulated food intake to body weight was significantly higher in the VHFD + 5% MC-fed mice compared to the VHFD control group throughout the entire study (Fig. 5.5 B). These results in food consumption cannot explain the difference in boy weight. It is important to mention that the VHFD + 5% MC group had a normal behavior, look healthy and did not have food aversion or present any sign of gastrointestinal problem like diarrhea during the whole study (observational data). In addition, there was no difference in the lipid content as percent of dry fecal weight from the two experimental groups: for was VHFD 0.47% ±

0.14 (SD) vs. VHFD + 5% MC 0.46% ± 0.04 (SD). This result indicates that VHFD + 5% MC-fed mice were not losing lipids through the feces.

Body composition was evaluated at 4, 8 and 12 weeks by echo-MRI. The VHFD + 5% MC-fed mice showed less fat mass and high free fat mass (lean mass) as percentage of total body weight than VHFD control group (Fig. 5.5 C & D). The OGTT at 4, 8 and 12 weeks showed lower glycemic levels and faster return to fasting levels in VHFD + 5% MC group compared to VHFD animals, but not as effective as metformin (300 mg/kg) gavage treatment (Fig. 5.6). These results are in accordance with previously reports that observed a similar effect in diabetic rats [158,169]; although in those studies the animals were already hyperglycemic.

Macroscopic and histological examinations of the liver samples from the VHFD + 5% MC-fed mice revealed a healthy appearance compared with the fatty livers of VHFD control group (Fig. 5.7 A & B, D & E). The livers of VHFD + 5% MC-fed mice were lighter and contained lower levels of total lipids in relation to the VHFD-fed mice (Fig. 5.7 C & F). Therefore, the 5% MC supplementation did prevent the development of fatty liver disease, a common pathology found in obesity and T2D.

Finally, in order to explain the significant weight difference between treated and control mice, it is evident that 5% MC supplementation affected positively any of the pathways that regulate body energy like glucose homeostasis, inflammation, lipolysis, or thermogenesis. It is also probable that the intestinal microbiota was affected, although the study did not seek this possibility.

5.4.2.2 Effect of VHFD + 5% MC on serum markers, insulin signaling, and inflammation

VHFD + 5% MC-fed mice group had lower serum levels of the glucose and lipid regulator hormones insulin, leptin and resistin when compared to VHFD group (Fig. 5.8 A). They also had lower levels of the pro-inflammatory cytokines IL-1β and TNFα (Fig. 5.8 B), as well as cholesterol and triacylglicerides (Fig. 5.8 C) compared to the VHFD control group. The typical increment in serum levels of pro-inflammatory cytokines, insulin, leptin [170], resistin [171], triacylglycerides, and cholesterol [172] that is seen during insulin resistance [165,170-173] were reversed by MC treatment, indicating that the treated mice did not developed an insulin resistance state as their counterparts.

The VHFD + 5% MC-fed mice had different levels of hepatic and muscle insulin signaling phosphorylated proteins in relation to the VHFD-fed mice. In liver the levels of IRS-1, IRS-1p, PI-3K, Akt1p and Akt2p (Fig. 5.9 A) and in the skeletal muscle tissue the same proteins plus the glucose transporter GLUT-4 (Fig. 5.9 B) showed higher levels. This indicates a preservation of the insulin signaling pathway in the treated mice, which also is correlated with the serum levels of insulin corroborating the evidence that VHFD + 5% MC-fed mice did not develop an insulin resistance state.

The gene expression of pro-inflammatory markers TNFα, IL-6, IL-1β, was reduced in the liver (Fig. 5.10 A) and ileum (Fig. 5.10 B) from the VHFD + 5% MC-fed mice compared to the VHFD control group. In white adipose tissue, the gene expression of the inflammatory markers MCP-1, PAI-1, TBP and LCN did not show any change. However, the gene expression of two important adipokines was different between the two

groups: TNFα was reduced and adiponectin (ADPN) was induced in treated mice relative to the VHFD controls (Fig 5.10 C). These data reinforce the evidence of the anti-inflammatory activity that was previously mentioned in the in vitro section.

All this evidence clearly suggests that the treatment of 5% MC incorporated in the food did prevent the onset and development of insulin resistance state and chronic inflammation, despite of the very high fat diet that this group was consuming.

5.4.2.3. Effect of MC, MIC-4 and 4 on glucose production, gene expression, and acute OGTT study

Glucose production in vitro was reduced by 60% in HII4E liver cells by MC at 10 µg/mL and MIC-1 and 4 at 1µM (Fig. 5.11 A). MIC-1 and 4 showed higher activity than SF at the same concentrations (Fig. 5.11 A). The activity of MIC-4 was compared to metformin over a range of 5 different concentrations, showing an ED_{50} value of glucose production lower for MIC-4 (7 µM) compared to metformin (800 µM) (Fig. 5.11 B). Concomitantly, PEPCK and G6P gene expression was suppressed by MC and MICs in HII4E hepatic cells (Fig. 5.11 C), and again MIC-1 and 4 had a higher activity than SF at 10 µM. Liver PEPCK and GPG gene expression was also diminished in VHFD + 5% MC-fed mice compared to the VHFD controls (Fig. 5.11 D). In the acute OGTT, the MC-gavaged mice (2 g/kg) showed lower glycemic levels at 15 and 30 minutes compared to the vehicle (Fig. 5.11 E). Inhibition of pathological gluconeogenesis by metformin [174] and thiazolidi-nediones [175] have been successfully used in preventing and treating T2D patients [176]. In a similar way, MC could avoid the onset of this mechanism in treated mice, and thus, maintaining a normal glycemic level.

5.4.2.5 Effect of MC and MIC-1 and 4 on lipolysis and thermogenesis

The lipolysis and thermogenesis mechanisms were explored in vitro and in vivo in order to understand the reduced weight gain in VHFD + 5% MC-fed mice. There was no lipolytic activity in vitro, although MC had slight increase in glycerol production. (Fig. 5.12 A). Epididymal white adipose tissues from the VHFD + 5% MC-fed mice were tested for changes in gene expression of thermogenic and lipolytic markers. There was an increased expression on lipolytic genes like of PR domain containing 16 (PRDM16), β-3 adrenergic receptor (ADRβ3), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), although the expression of the thermogenic marker uncoupling protein 1 (UCP1) was decreased (Fig. 5.12 B). This difference in expression among the lipolytic and thermogenic genes could be explained since the white adipose assayed was from the epididymal region. Lipolysis and thermogenesis are interconnected processes, but are different [177]. Lipolysis is a physiological mechanism that occurs in insulin sensitive adipose tissue and it is coupled with lipogenesis [178]. In adult animals, thermogenesis is seen in special pockets distributed inside the white adipose tissue called "beige adipose tissue" (true brown adipose tissue is only found in young animals and have a different embryonic origin) [179]. Therefore, samples from other anatomical regions would have been relevant in order to determine if thermogenesis was active or not.

In liver tissue from the three month study the gene expression of glucokinase (GcK) was reduced in VHFD + 5% MC compared to the control mice (Fig. 5.12 C). In a similar way the lipogenic genes (FAS, SREBP1c and FSP27) were downregulated, but the lipolytic gene ATGL showed a clear induction (Fig. 5.12 D). High-fat feeding mice

has been shown to up regulate GcK [180], and through neural signaling subsequently down regulate thermogenesis-related genes in brown adipose tissue (BAT) and increase overall adiposity [180,181]. Moreover, the high activity of ATGL (an important hepatic lipase) in 5% MC treated mice indicates that are not accumulating fat in the liver and lipolysis is turn it on [182].

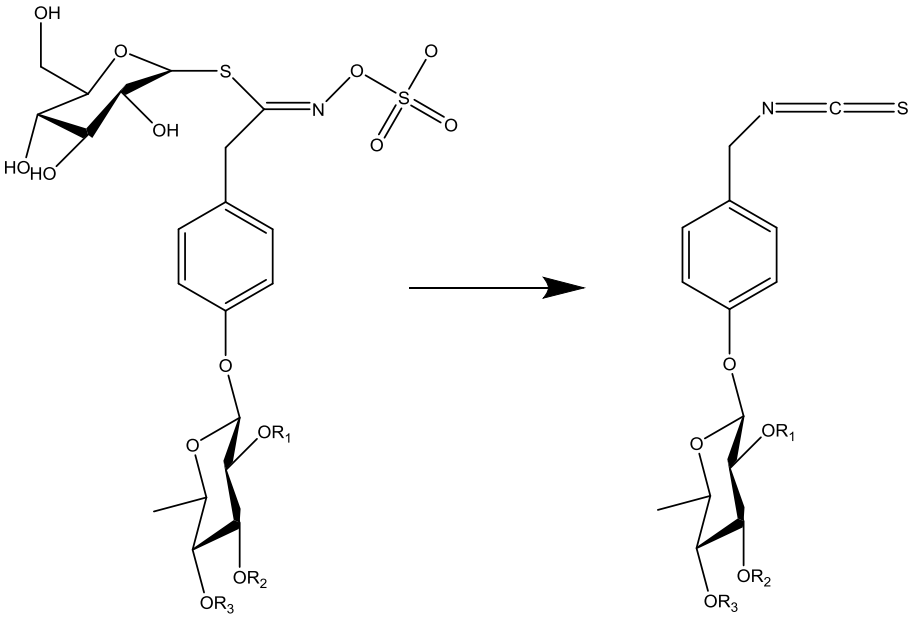
Finally, an additional short term study (2 weeks) was conducted in TSE metabolic chambers (indirect calorimetry study) with the objective to determine if MC induces thermogenesis by fat oxidation in vivo. For this purpose, mice were fed a VHFD or a VHFD + 3.3% MC (containing the same concentration of MICs as the 5% diet in the 3 month study). The study did not find differences in O_2 consumption or rearing activity between the two groups. Nevertheless, the VHFD + 3.3% MC-fed mice did show a lower respiratory exchange rate (RER). The RER was calculated as VCO_2/VO_2 and analyzed by covariate analysis accounting for the influence of light/dark, activity and weight (Fig. 5.12 E). This RER difference means that treated mice with 5% MC were oxidizing slightly more fat than control mice which could explain in part the difference in body weight [183].

In conclusion, the data from the in vivo and in vitro experiments presented in this section provide preliminary evidence for the hypothesis that MC and MICs could avoid the development of obesity, fatty-liver disease and insulin resistant state. The inhibition of gluconeogenesis and increased lipolysis coupled to a higher ratio of fat oxidation are among the possible mechanisms that could explain these results. However, it is not completely clear why MC prevented the weight gain in the treated mice. This is the first report of gluconeogenesis inhibition and its regulated gene expression by any ITC and *M.*

oleifera. Finally, MC and MICs have demonstrated to possess anti-inflammatory and anti-diabetic effects, and could be applied in the prevention of chronic metabolic diseases like obesity, T2D and metabolic syndrome. Therefore, *M. oleifera* could be included in the list of healthy and beneficial vegetables that are recommended to eat regularly.

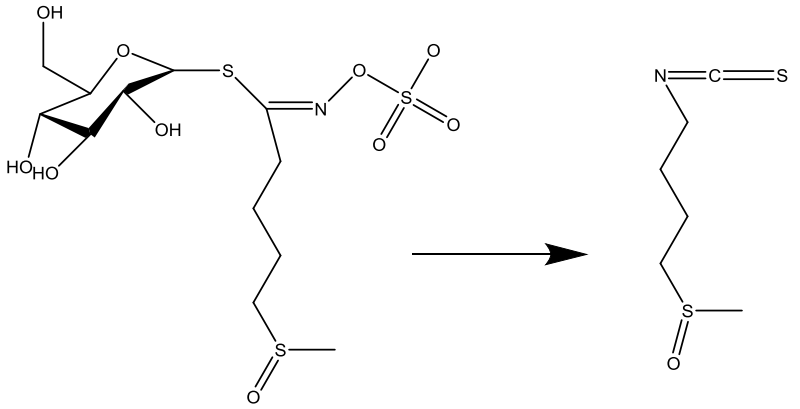
5.5 Tables and figures

Figure 5.1 Chemical structures of **A**) *M. oleifera* glucosinolates (MGLs) and **B**) *M. oleifera isothiocyanates* (MICs). For comparison is showed **C**) glucoraphanin, and **D**) sulforaphane (SF) from brocoli. The arrow shows the conversion catalyzed by myrosinase.



A) **GMG-1** R_1, R_2, R_3 = H
GMG-4 R_1,R_2 = H; R_3 = Acetyl

B) **MIC-1** R_1, R_2, R_3 = H
MIC-4 R_1,R_2 = H; R_3 = Acetyl



C) Glucoraphanin

D) Sulforaphane

Table 5.1 Cytotoxicity of *Moringa* extract (MC) and the isolated isothiocyanates (MICs). Cytotoxicity was evaluated using the MTT assay employing eight different concentrations of the *Moringa* extract and the compounds. The results are expressed as the IC_{50} values (mean ± SD, $n = 3$).

	RAW 264.7 macrophages	**Caco-2** Small intestinal epithelia	**HT-29** Large intestinal epithelia
Moringa extract (µg/mL)	536.5 (± 35.7)	370.9 (± 42.3)	321.8 (± 35.8)
MIC-1 (µM)	32.5 (± 2.8)	45.6 (± 9.0)	52.3 (± 1.5)
MIC-4 (µM)	29.5 (± 5.3)	30.3 (± 3.4)	33.0 (± 4.8)
†emetine (µM)	0.40 (± 0.02)	24.7 (± 4.8)	0.56 (± 0.02)

†Positive control.

Figure 5.2. Effect of MC, MIC-1 and 4 on gene expression of inflammatory markers. **A**) Effect of MC on *iNOS* and *IL-1β*. **B**) Effect of MIC-1 and 4 on *iNOS* and *IL-1β*. **C**) Effect of MC on *IL-6* and *TNFα*. **D**) Effect of MIC-1 and 4 on *IL-6* and *TNFα*. Each bar represents the mean ± SEM (n = 4), except for *TNFα* in **D** where n = 2. Comparison to control were made by ANOVA and Dunnett's post-test for *iNOS* measurements or Wilcoxon's post-test in all other experiments. *$p < 0.05$. **$p < 0.01$, *** $p < 0.001$.

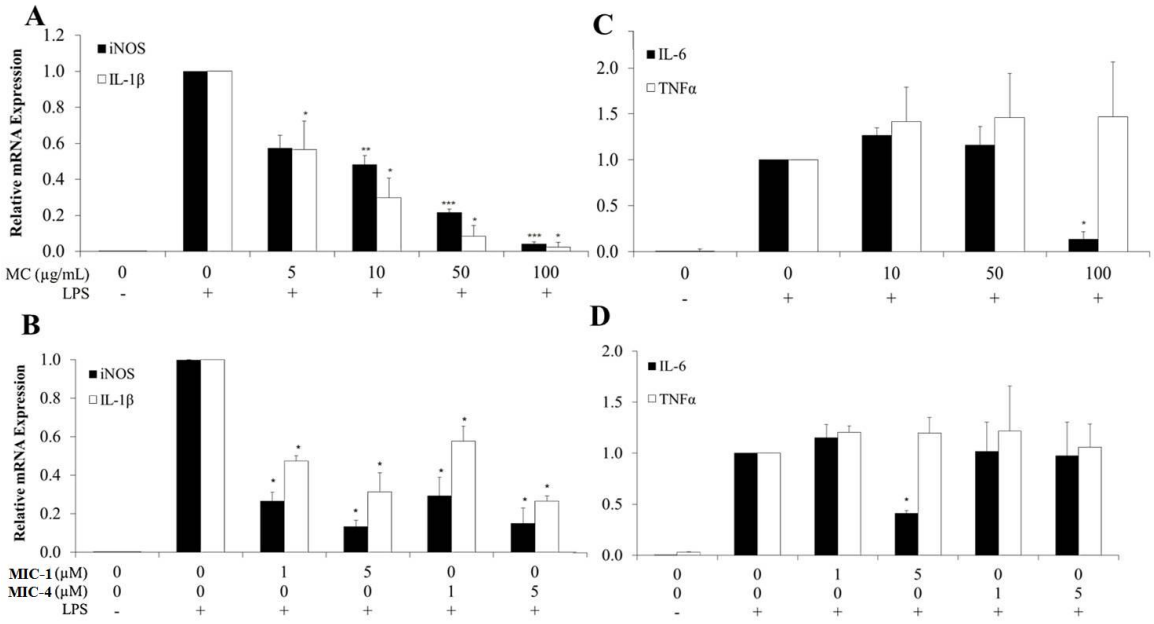


Figure 5.3 Effect of MC and MIC-1 and 4 on TNFα and NO production. **A**) Effect of MC on NO and TNFα production. **B**) Effect of MICs on NO and TNFα production. Each bar represents the mean ± SEM (n = 3). Comparison to control were made by ANOVA and Dunnett's post-test. *p < 0.05. **p < 0.01, *** p < 0.001.

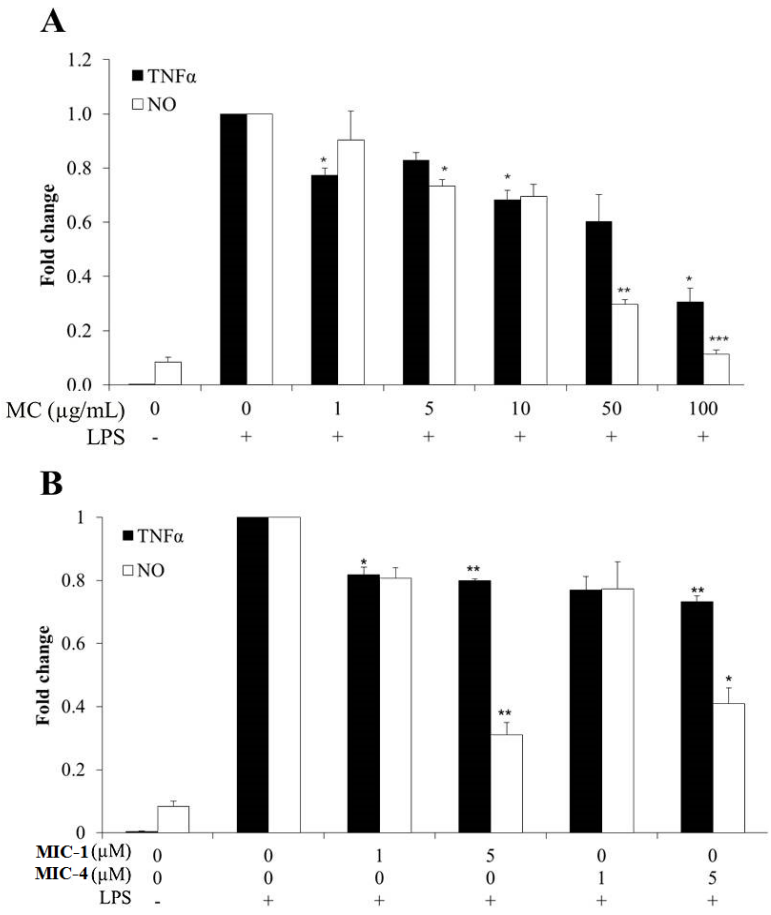


Figure 5.4 Effect of MC and MIC-1 and 8 on IL-8 production in Caco-2 intestinal cells. DX = dexamethasone, ME = *M. oleifera* extract, MIC = *M. oleifera* isothiocyanates. *$p <$ 0.001, bars represent standard deviation. (n = 3 independent experiments).

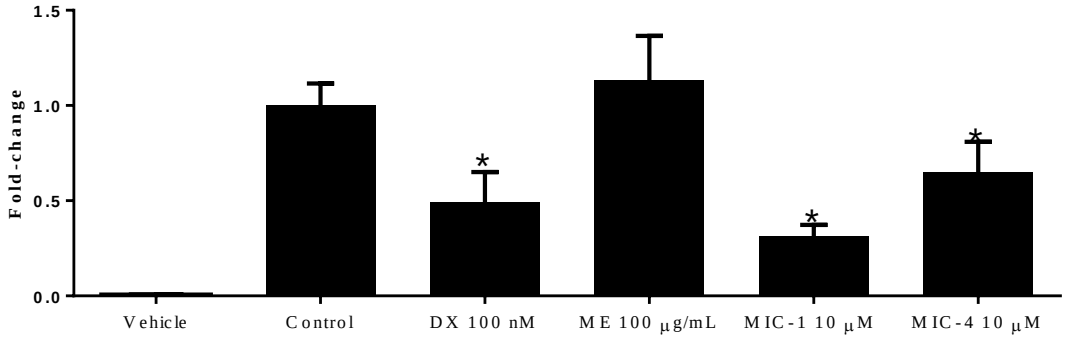


Table 5.2 Composition of experimental diets for the 3 month long term feeding study. Diet formulation was performed by Research Diets (New Brunswick, NJ, USA). Analysis of macronutrient, moisture, and ash was performed by New Jersey Feed Labs (Trenton, NJ, USA). VHFD: very high fat diet (ca. 60% energy from fat). 5% MC: moringa concentrate.

	VHFD	**VHFD + 5% MC**
Macronutrient	*grams*	*grams*
Protein	179	179
Carbohydrates	204	204
Fat	270	270
	kcal/kcal%	*kcal/kcal%*
Protein	716/18.1	716/18.1
Carbohydrates	815/20.6	815/20.6
Fat	2430/61.3	2430/61.3
Ingredient	*grams*	*grams*
Casein	200	196
L-cystine	3	3
Corn starch	0	0
Maltodextrin	125	102
Sucrose	68.8	68.8
Cellulose	50	50
Soybean oil	25	25
Lard	245	245
Mineral mix	10	10
Di-calcium phosphate	13	13
Calcium carbonate	5.5	5.5
Potassium citrate, 1 H_2O	16.5	16.5
Vitamin mix	10	10
Choline bitartrate	2	2
FD&C red dye #40	0	0.05
FD&C blue dye #1	0.05	0
Moringa concentrate	0	40

* kcal%: percentage of energy.

Table 5.3 Composition of experimental diets for 2 weeks indirect calorimetry study. Diet formulation was performed by Research Diets (New Brunswick, NJ, USA). Analysis of macronutrient, moisture, and ash was performed by New Jersey Feed Labs (Trenton, NJ, USA). VHFD: very high fat diet (ca. 60% energy from fat). 3.3% MC: moringa concentrate.

	VHFD	**VHFD + 3.3% MC**
Macronutrient	*grams*	*grams*
Protein	179	179
Carbohydrates	204	204
Fat	270	270
	kcal/kcal%	*kcal/kcal%*
Protein	716/18.1	716/18.1
Carbohydrates	815/20.6	815/20.6
Fat	2430/61.3	2430/61.3
Ingredient	*grams*	*grams*
Casein	200	189
L-cystine	3	3
Corn starch	0	0
Maltodextrin	125	115
Sucrose	68.8	68.8
Cellulose	50	50
Soybean oil	25	25
Lard	245	245
Mineral mix	10	10
Di-calcium phosphate	13	13
Calcium carbonate	5.5	5.5
Potassium citrate, 1 H_2O	16.5	16.5
Vitamin mix	10	10
Choline bitartrate	2	2
FD&C red dye #40	0	0.05
FD&C blue dye #1	0.05	0
Moringa concentrate	0	25.5

*kcal%: percentage of energy

Figure 5.5 Biometric data from the three month study in VHFD and VHFD + 5% MC-fed mice. (**A**) Body weight gain, (**B**) ratio of accumulated food intake to body weight, (**C**) fat mass, and (**D**) free fat mass. Data are means ± SEM, *n*=12 mice per group. Comparisons to control were made with t-test and Holm-Sidak's correction in **A** & **B**, and t-test with Welch's correction in **C** & **D**. **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

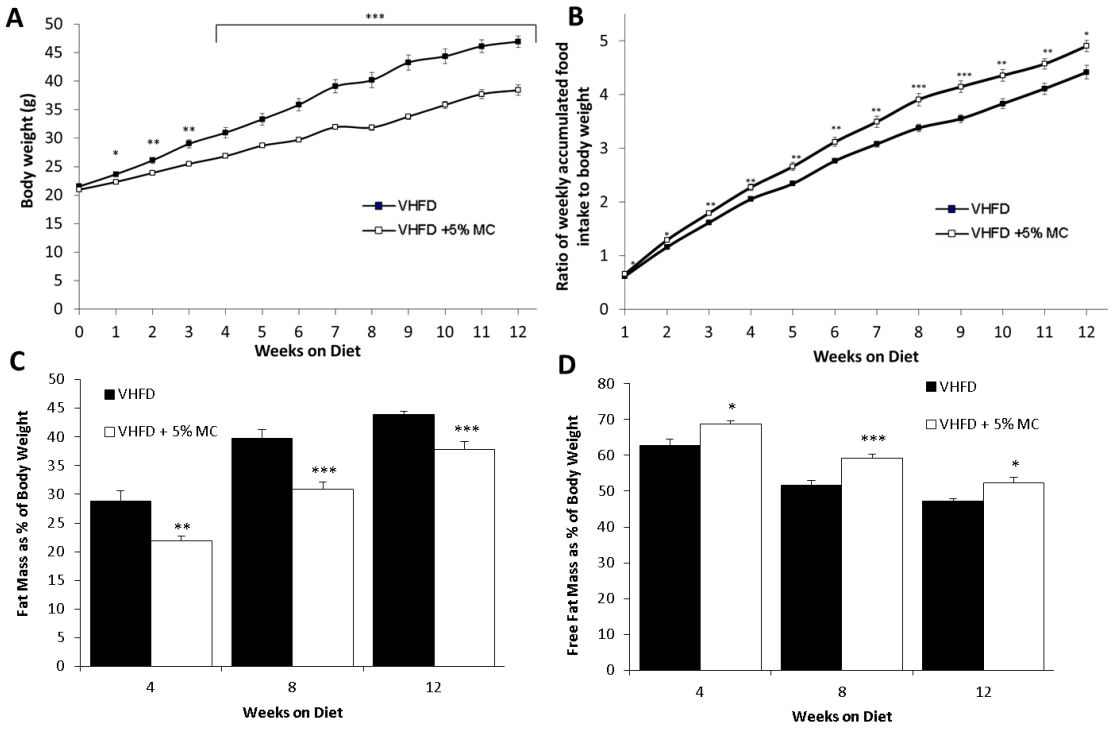


Figure 5.6 Oral glucose tolerance test (OGTT) performed at (**A**) 4, (**B**) 8 and (**C**) 12 weeks on VHDF and VHFD + 5% MC, and extra VHDF group gavaged with 300 mg/kg metformin on the day of the OGTT. (**D**) Area under the curve (AUC) of OGTT at 4, 8, and 12 weeks. Data are means ± SEM, n = 12 mice per group, except for metformin group where n = 6 and only shown as a reference group. Comparisons to control were made with t-test and Welch's correction. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

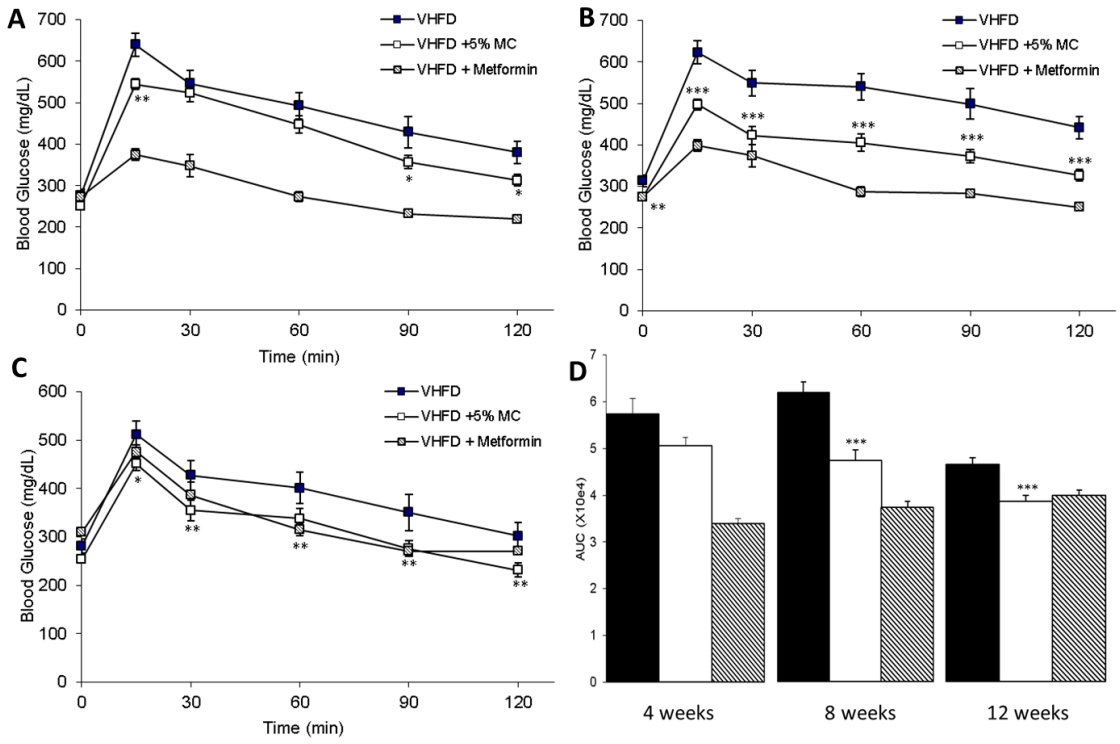


Figure 5.7 Liver histology, weight and total lipid content. Macroscopic examination of liver samples from (**A**) VHFD and (**B**) VHFD + 5% MC. (**C**) Liver weight in VHFD and VHFD + 5%MC. Histological examination of liver samples from (**D**) VHFD and (**E**) VHFD + 5% MC. (**F**) Total fat content in liver from VHFD and VHFD + 5% MC. Data are means ± SEM, $n = 12$. Comparisons to control were made with t-test and Welch's correction. $^{**}p < 0.01$, $^{***}p < 0.001$.

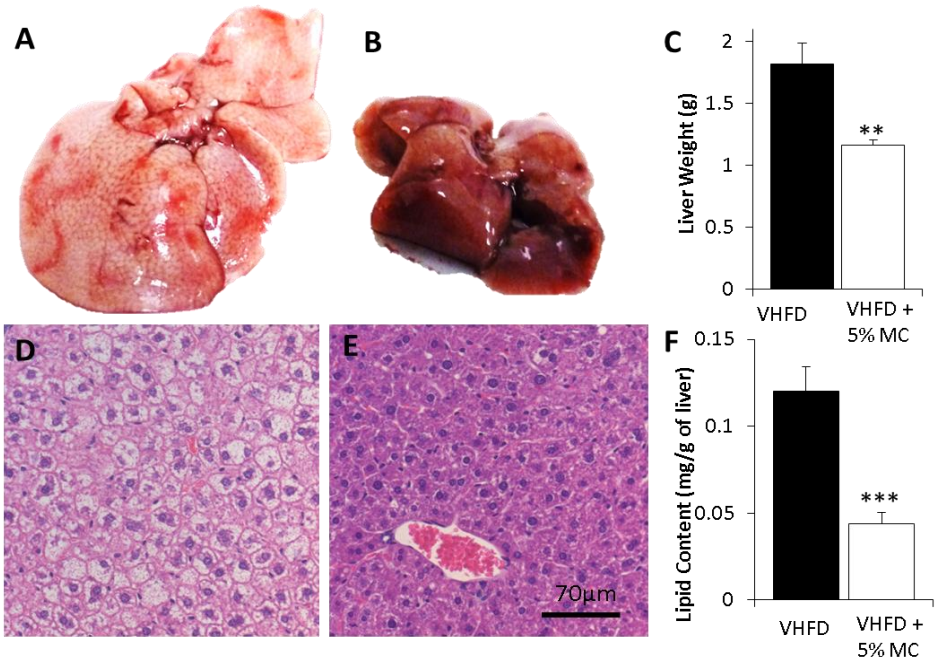


Figure 5.8 Serum levels of metabolic and inflammatory markers. Levels of (**A**) insulin, leptin, and resistin; (**B**) IL-1β and TNFα; and (**C**) total cholesterol and triacylglycerides in VHFD and VHFD + 5% MC. Data are means ± SEM, $n = 12$ mice per group except for IL-1β and TNF-α where $n = 5$. Comparisons to control were made with t-test and Welch's correction. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

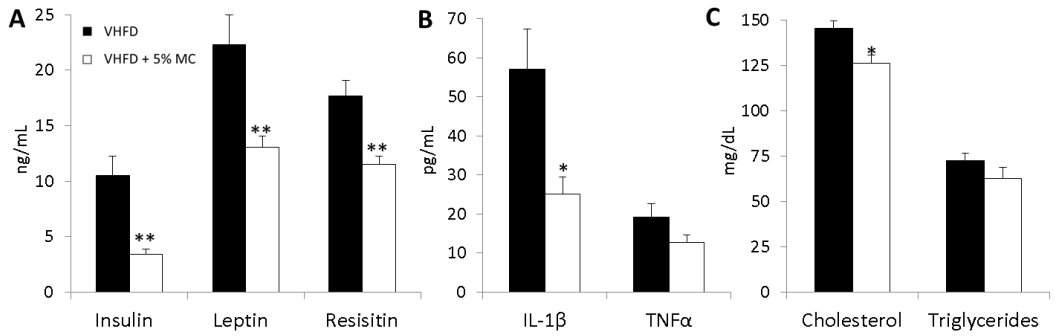


Figure 5.9 Insulin signaling protein levels in (**A**) liver and (**B**) skeletal muscle from VHFD and VHFD + 5% MC-fed mice. Data are means ± SEM, *n* =12 mice per group. Comparisons to control were made with t-test and Welch's correction **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

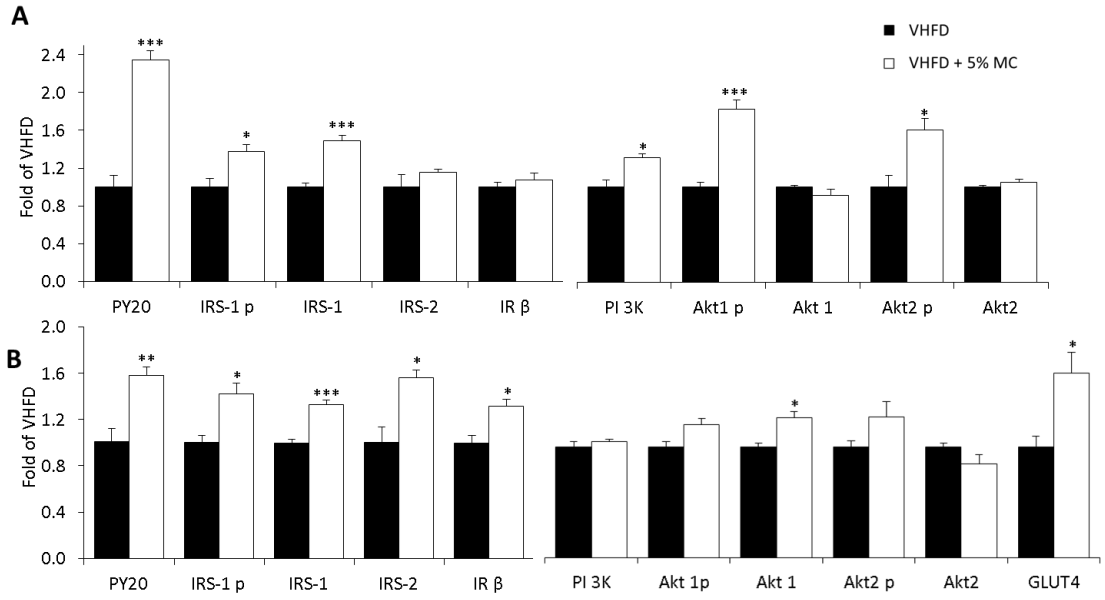


Figure 5.10 Gene expression of inflammatory markers in (**A**) liver, (**B**) ileum, and (**C**) white adipose tissue of VHFD and VHFD + 5% MC. Data are means ± SEM, n = 8-12 mice per group. Comparisons to control were made with t-test and Welch's correction. *p<0.05.

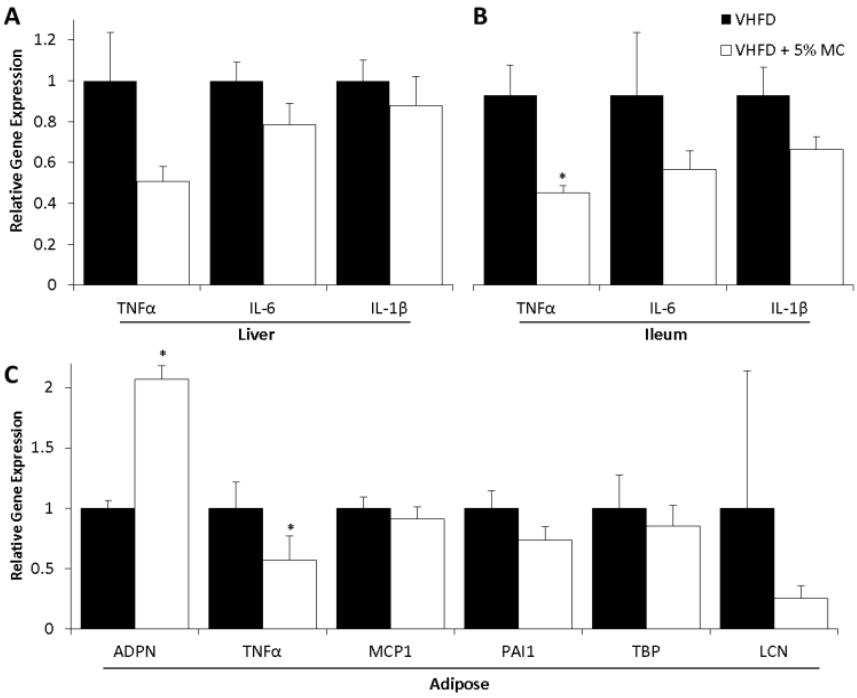


Figure 5.11 Effects of MICs, SF and MC on glucose metabolism in vitro (**A, B, C**) and in vivo (**D, E**). (**A & B**) Effects of MC, MIC-1, MIC-4 and SF on glucose production, and (**C**) gene expression of G6P and PEPCK in HII4E liver cells; $n = 3$. (**D**) Expression of G6P and PEPCK in hepatic tisse of VHFD and VHFD + 5% MC-fed mice, $n = 12$ mice per group. (**E**) Acute OGTT test in VHFD-fed mice gavaged with 2 g/kg of MC, $n = 6$ mice per group. Comparisons to controls were made by ANOVA with Dunnett's correction for **A** & **C**, and t-test with Welch's correction for **D** & **E**. Data are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

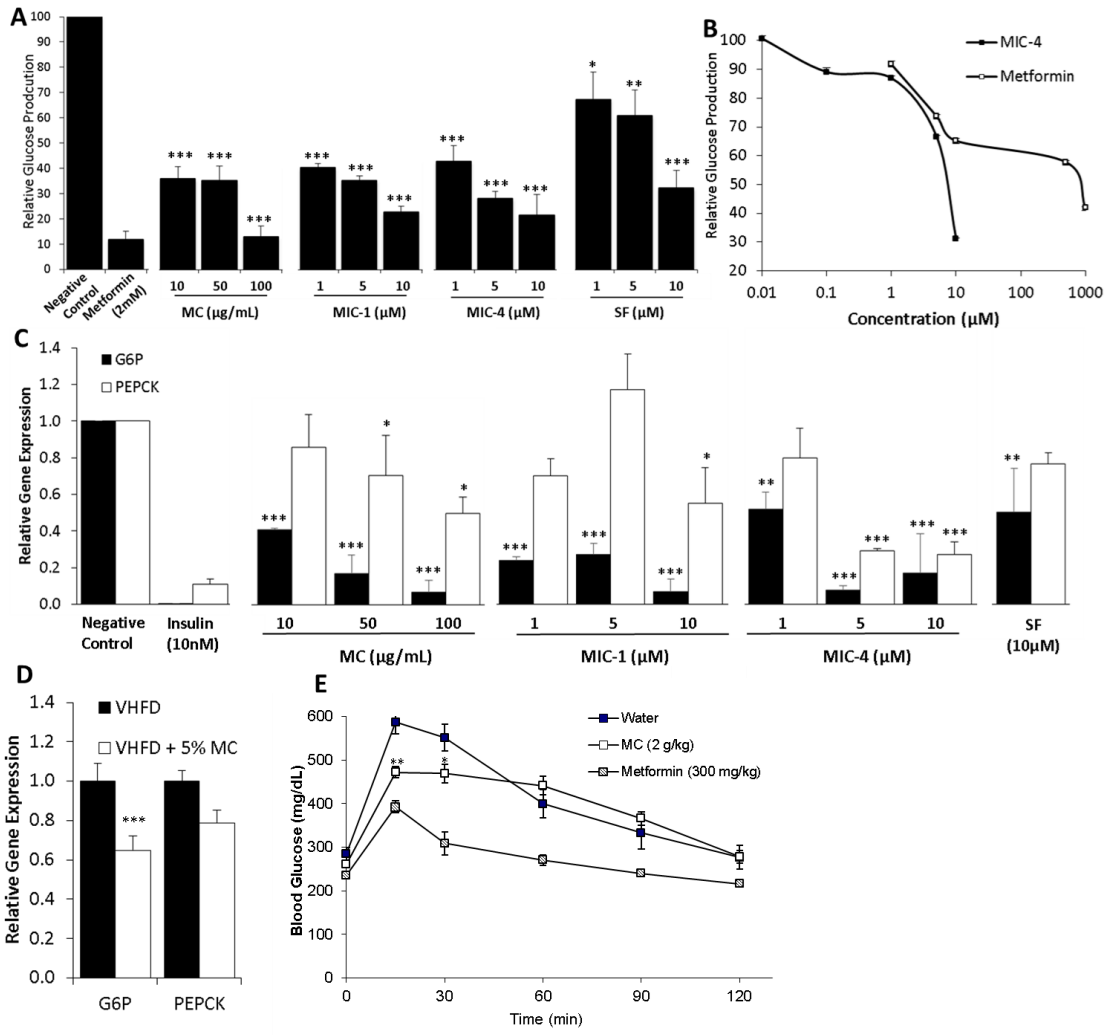
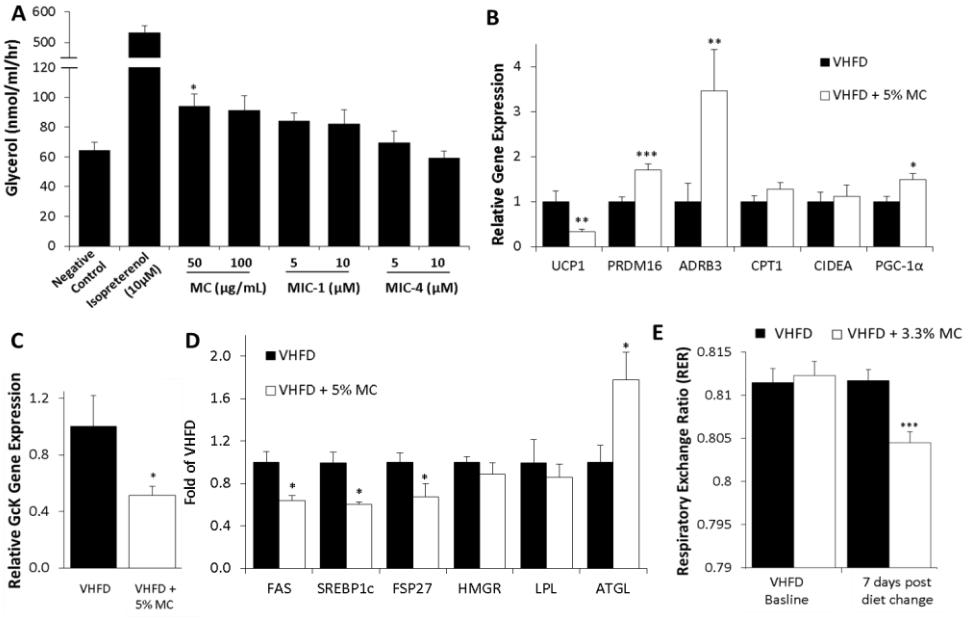


Figure 5.12 Effects of MC and MIC on lipolysis and thermogenesis. (**A**) Production in vitro of glycerol in adipocytes treated with MC, MIC-1 and MIC-4 (n = 3). (**B**) Expression of theromogenic and lipolytic genes in whithe adipose tissue, and (**C & D**) hepatic tissue from VHFD and VHFD + 5% MC-fed mice for 3 months (n = 12). (**E**) RER (indirect calorimetric study) in mice switched to a VHFD + 3.3% MC after 7 days compared to mice that remained on a VHFD (n = 12). Data are means ± SEM. Comparisons to controls were made by ANOVA with Dunnett's correction for **A**, t-test with Welch's correction for **B, C & D,** and ANCOVA for **E**. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$**.**



CHAPTER 6

Conclusions

"The right dose differentiates a poison from a useful medicine"

— Paracelsus

Plants and humans have had a long and close relationship. Human beings, as all terrestrial animals, depend largely on plants. But, we have maximized the benefits from plants like no other creature. Plants have been used mainly as a source of food, drinks, condiments, medicines and cosmetics, as ornamentals to decorate our bodies, homes, cities and burial sites, as well as tools for clothing, housing, fertilizing and even hunting. This relationship became more intimate when agriculture was invented and therefore we could domesticate and help plants to propagate. Therefore, plants have had an important, decisive and direct role in our survival as a species – maybe more crucial than any other life form on the planet. However, after thousands of years interacting with and using plants we still have major gaps in the understanding of their biology and phytochemistry.

Plants live in a chemical world that is almost completely invisible to us, with the exception of the volatiles and a few other chemicals that we can smell or taste. Plants have the ability to sense organic and inorganic compounds. This skill is the key for survival since the great majority of plants are autotrophs. But plants have also evolved the capacity to synthesize a great repertoire of different biochemicals collectively known as secondary metabolites or phytochemicals. These phytochemicals can be synthesized in all plant organs that grow under or above the surface. Plants are truly chemical factories

with more than 100,000 identified secondary metabolites so far. The most interesting characteristic of phytochemicals is their vast diversity in terms of chemical nature, from small simple molecules like phenolic acids or monoterpenes to really large and complex structures like tannins and lignins. Phytochemicals allow plants to survive and interact amongst themselves but also interact with other type of organisms like bacteria, fungi, insects and other arthropods, and vertebrates (mainly birds and mammals). These interactions help plants to capture nutrients, grow, reproduce, colonize new habitats, and defend from pathogens and predators .

Phytochemicals have been beneficial for humans in two ways. First, many natural products in vegetables, fruits, seeds and herbs are relevant since they can have multiple practical applications – one of them is to protect us from becoming sick or to ameliorate pathological conditions. Essential oils, phenolic acids, flavonoids, anthocyanins, tannins, carotenes, betalains, isothiocyanates, dietary fiber and other natural products isolated in edible plants and regularly consumed have demonstrated the ability to act as antioxidants, anti-inflammatory or anti-cancer [184]. Second, some of the phytochemicals isolated from medicinal plants have been used directly as drugs or have served as blueprint molecules to synthesize semi-synthetic pharmaceuticals, some of which are very useful nowadays like aspirin, metformin, and paclitaxel, among hundreds. Actually, around one quarter of all current pharmaceuticals comes from natural products, and most of them are isolated in plants. Most importantly, there is still a high probability of finding new types of phytochemicals that can be used as chemotherapeutic agents for different illnesses, but especially against infectious and parasitic diseases and cancer [185].

The systematic use of certain plants as medicine has occurred throughout human history, but certainly we are not the only species that have been using plants as medicine. For example, it is well documented that chimpanzees occasionally eat certain plants that are low in nutrients combined with dirt when they have high levels of malaria parasites in their blood [186]. These plants are not regularly in their diet, but are eaten in these cases to help the chimpanzees to fight the disease with phytochemicals found in the plants that can kill the parasites [186,187].

In any case, the empiric use of plants as natural remedies and the documentation of medicinal plants have been a constant in all cultures. Such traditional knowledge contains valuable information about the use of medicinal plants from simple preparations to complex herbal formulations. In some cases, the documentation process has been well preserved like the traditional herbal records, *materia medica* texts and pharmacopeias from Europe, China or India. From these catalogs many useful pharmaceuticals have been found in these medicinal plants [188]. In addition, medicinal plants from native Americans, Africans and Australians are being rediscovered that could help in the arduous task to provide new drugs, drug leads or new chemical entities [189].

For the reasons mentioned above, the investigation of new natural products or the new applications of already discovered molecules found in medicinal and edible plants should be a continuous process. The data presented in this dissertation was based on the research on this type of plants and could be considered a follow up on previous investigation. In *Cichorium intybus* L. (Asteraceae), *Cornus florida* L. (Cornaceae), *Eryngium foetidum* L. (Apiaceae), which have been used traditionally as antiparasitic

remedies, we found evidence that these common medicinal plants harbor leishmanicidal compounds, even though those plants were never used for this purpose before.

Fourteen natural products were isolated in total from these three medicinal and edible plants following the bioguided fractionation approach. The roots of *Cichorium intybus* L. (chicory) yielded four sesquiterpene lactones: (1) 11(S),13-dihydrolactucopicrin, (2) lactucopicrin, (3) 11(S),13-dihydrolactucin and (4) lactucin, although compound 2 presented leishmanicidal activity (IC_{50} 24.8 μM). The bark of *Cornus florida* L. (flowering dogwood) generated eight different natural products: (1) betulinic acid, (2) ursolic acid, (3) *β*-sitosterol, (4) ergosta-4,6,8,22-tetraene-3-one, (5) 3*β*-*O*-acetyl betulinic acid, (6) 3-epideoxyflindissol, (7) 3*β*-*O*-*cis*-coumaroyl betulinic acid, (8) 3*β*-*O*-*trans*-coumaroyl betulinic acid. The most active leishmanicidal compounds were (4) 11.5 μM, (6) 1.8 μM, (7) 8.3 μM and (8) 2.2 μM. The aerial parts of *Eryngium foetidum* L. (culantro) generated two natural products: (1) lasidiol *p*-methoxybenzoate and (2) a terpene aldehyde ester derivative. Only compound 1 inhibited the growth of *L. tarentolae* and *L. donovani* with IC_{50} values of 14.33 and 7.84 μM, respectively.

This evidence could promote the research in other medicinal plants or natural products previously isolated with a different goal. However, in order to truly claim leishmanicidal activity it is necessary to perform in vivo experiments to demonstrate the activity in a real scenario. In addition, studies about absorption, distribution, metabolism, toxicicity (AMET), and mode of action will be required. For the follow-up experiments, it is necessary to have large amounts (several grams) of the natural products – this is the first issue to be addressed since most of the phytochemicals do not occur in great

quantities and regular isolation processes only produce amounts measureable in milligrams. Finally, an idea that could help leverage the discovery of new antiparasitic compounds in general is to couple it with the discovery of natural anticancer products [190].

Since obesity and type 2 diabetes have a negative health impact in millions of people worldwide, we focused our attention in the anti-inflammatory, anti-obesity and anti-diabetic effects of *Moringa oleifera* Lam. (Moringaceae). The most interesting characteristic of this traditional Indian edible and medicinal plant is that contains four particular bioactive isothiocyanates, known as MICs. A food-grade (water extracted) moringa concentrate (MC) was prepared from leaves of *M. oleifera.* The MC contained 1.66% of total MICs. Also, MIC-1 and 4 were isolated from leaves. The anti-inflammatory and anti-obesity/diabetic evidence was truly remarkable.

MC, MIC-1, and MIC-4 could significantly decrease the production and gene expression of inflammatory markers in vitro (LPS-stimulated RAW macrophages and Caco2 cells). The in vivo experiments with MC-treated animals and fed a high-fat diet did showed promising results. The treated mice did not gain weight and did not develop fat liver disease compared to control animals. Also, when compared to control animals, the blood metabolic and inflammatory biomarkers from MC-treated mice were in the normal range. In addition MC-treated animals had normal levels of insulin signaling and inflammatory markers in liver, skeletal muscle, white adipose tissue and ileum. MC and MIC inhibited liver gluconeogenesis in vivo as well as in vitro. Finally, an indirect calorimetry acute study indicated that MC-treated mice had a higher fat oxidation rate compared to control mice.

The evidence demonstrating that the food-grade extract and the isolated MICs could prevent the onset of pathological alterations in vitro and in a diet-induced obesity model is compelling, though more data is needed to confirm these findings. For example, it is still not completely clear if MC could induce thermogenesis, or changes in the intestinal microbiota. Finally, pharmacokinetic and pharmacodynamic studies will be required if *M. oleifera* is going to be tested in clinical trials.

The main purpose of this research work was to provide new evidence about leishmanicidal, anti-inflammatory and anti-obesity activities found in common medicinal and edible plants, and therefore reinforce the idea that plants are still a valid source of novel and interesting phytochemicals that can be applied in human health for prevention or treatment.

"All nature is at the disposal of humankind. We are to

work with it. For without it, we cannot survive".

(Saint Hildegarde of Bingen, 12th Century,

quoted in Matthew Fox, Original Blessing)

APPENDIX A

In vitro and in vivo anti-diabetic effects of anthocyanins from Maqui-berry (*Aristotelia chilensis*)

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Abstract

We used a murine model of type II diabetes, which reproduces the major features of the human disease, and a number of cellular models to study the antidiabetic effect of ANC, a standardized anthocyanin-rich formulation from Maqui Berry (*Aristotelia chilensis*). We also isolated delphinidin 3-sambubioside-5-glucoside (D3S5G), a characteristic anthocyanin from Maqui Berry, and studied its antidiabetic properties. We observed that oral administration of ANC improved fasting blood glucose levels and glucose tolerance in hyperglycaemic obese C57BL/6J mice fed a high fat diet. In H4IIE rat liver cells, ANC decreased glucose production and enhanced the insulin-stimulated down regulation of the gluconeogenic enzyme, glucose-6-phosphatase. In L6 myotubes ANC treatment increased both insulin and non-insulin mediated glucose uptake. As with the ACN, oral administration of pure D3S5G dose-dependently decreased fasting blood glucose levels in obese C57BL/6J mice, and decreased glucose production in rat liver cells. D3S5G also increased glucose uptake in L6 myotubes and is at least partially responsible for ANC's anti-diabetic properties.

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APPENDIX B

Antiplasmodial activity of cucurbitacin glycosides from *Datisca glomerata* (C. Presl) Baill.

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Abstract

The traditionally used antimalarial plant, *Datisca glomerata* (C. Presl) Baill, was subjected to antiplasmodial assay guided fractionation. This led to the isolation of seven cucurbitacin glycosides, datiscosides I–O, along with two known compounds, datiscoside and datiscoside B, from the aerial parts of *D. glomerata*. Their structures and relative stereochemistry were determined on the basis of mass spectrometry, 1D and 2D NMR spectroscopic data. Antiplasmodial IC_{50} values were determined for all isolated compounds against a chloroquine sensitive strain of *Plasmodium falciparum* (D10), which were also evaluated in vitro for their antileishmanial activity against *Leishmania tarentolae*. Cytotoxicity was evaluated against rat skeletal muscle cells (L6) and Chinese ovarian hamster cells (CHO). The antiplasmodial activity of the compounds was moderate and ranged from 7.7 to 33.3 μM. None of the compounds showed appreciable antileishmanial activity. The compounds displayed cytotoxicity against L6 but not CHO mammalian cells.

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APPENDIX C

Polyphenol-rich Rutgers Scarlet Lettuce improves glucose metabolism and liver lipid accumulation in diet-induced obese C57BL/6 mice

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Abstract

Objective: The aims of the following experiments were to characterize anti-diabetic in vitro and in vivo activity of the polyphenol-rich aqueous extract of Rutgers Scarlet Lettuce. ***Materials / Methods:*** Rutgers Scarlet Lettuce (RSL) extract (RSLE) and isolated compounds were evaluated for inhibitory effects on glucose production as well as tumor necrosis factor alpha (TNFα)-dependent inhibition of insulin activity in H4IIE rat hepatoma cells. Additionally, high fat diet-induced obese mice were treated with RSLE (100 or 300 mg/kg), Metformin (250 mg/kg) or vehicle (water) for 28 days by oral administration and insulin and oral glucose tolerance tests were conducted. Tissues were harvested at the end of the study and evaluated for biochemical and physiological improvements in metabolic syndrome conditions. ***Results:*** A polyphenol-rich RSLE, containing chlorogenic acid, cyanidin malonyl-glucoside and quercetin malonyl-glucoside, was produced by simple boiling water extraction at pH 2. In vitro, RSLE and chlorogenic acid demonstrated dose-dependent inhibition of glucose production. In vivo, RSLE treatment improved glucose metabolism measured by oral glucose tolerance tests, but not insulin tolerance tests. RSLE treated groups had a lower ratio of liver weight to body weight as well as decreased total liver lipids compared to control group after 28 days of treatment. No significant differences in plasma glucose, insulin, cholesterol, and triglycerides were observed with RSLE treated groups compared to vehicle control. ***Conclusion:*** RSLE demonstrated anti-diabetic effects in vitro and in vivo and may improve metabolic syndrome conditions of fatty liver and glucose metabolism.

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APPENDIX D

Direct and indirect antioxidant activity of polyphenol and isothiocyanate-enriched fractions from *Moringa oleifera*

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Abstract

The present study describes an efficient preparatory strategy to fractionate *Moringa oleifera* leaves by fast partition counter current chromatography (FCPC) to lead polyphenol and isothiocyanate (ITC) rich fractions; the latter were further enriched by solid phase extraction 2.1 to 15 times to yield 12-30% ITC content. Moringa phenolics are highly potent as direct antioxidants assayed by oxygen radical absorbance capacity (ORAC), while moringa ITCs are unique components which effectively induce NAD(P)H quinone oxidoreductase 1 (NQO1) activity in Hepa1c1c7 cells and act as indirect antioxidants. In addition, 4-[(α-L-rhamnosyloxy) benzyl] isothiocyanate, and 4-[(4'-O-acetyl-α-L-rhamnosyloxy)benzyl]-isothiocyanate were further evaluated for their ORAC and NQO1 inducer potency in comparison with sulforaphane (SF), providing supportive data for results obtained from fractions and crude extract. Both ITCs were found to be as potent as SF to induce NQO1 activity. These findings suggest a general protective mechanism for numerous therapeutic benefits of moringa over a broad range of pathological conditions.

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LITERATURE CITED

1. Hotez, P.J., et al., *"Manifesto" for advancing the control and elimination of neglected tropical diseases*. PLoS Negl Trop Dis, 2010. **4**(5): e718.

2. Antinori, S., et al., *Leishmaniasis: new insights from an old and neglected disease*. Eur J Clin Microbiol Infect Dis, 2012. **31**(2): 109-118.

3. Bates, P.A., *Transmission of Leishmania metacyclic promastigotes by phlebotomine sand flies*. Int J Parasitol, 2007. **37**(10): 1097-1106.

4. Chappuis, F., et al., *Visceral leishmaniasis: what are the needs for diagnosis, treatment and control?* Nat Rev Microbiol, 2007. **5**(11): 873-882.

5. Magill, A.J., *Leishmania Species: Visceral (Kala-Azar), Cutaneous, and Mucosal Leishmaniasis.*, in *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*, G.L. Mandell, J.E. Bennett, and R. Dolin, Editors. 2010, Churchill-Livingstone: Philadelphia, PA. p. 3463-3480.

6. Kima, P.E., *The amastigote forms of Leishmania are experts at exploiting host cell processes to establish infection and persist*. Int J Parasitol, 2007. **37**(10): 1087-1096.

7. Pavli, A., et al., *Leishmaniasis, an emerging infection in travelers*. Int J Infect Dis, 2010. **14**(12): e1032-1039.

8. Alvar, J., et al., *Leishmaniasis worldwide and global estimates of its incidence*. PLoS One, 2012. **7**(5): e35671.

9. Hotez, P.J., et al., *Neglected tropical diseases of the Middle East and North Africa: review of their prevalence, distribution, and opportunities for control*. PLoS Negl Trop Dis, 2012. **6**(2): e1475.

10. Hotez, P.J., et al., *The neglected tropical diseases of Latin America and the Caribbean: a review of disease burden and distribution and a roadmap for control and elimination*. PLoS Negl Trop Dis, 2008. **2**(9): e300.

11. Belo, V.S., et al., *Risk factors for adverse prognosis and death in American visceral leishmaniasis: a meta-analysis*. PLoS Negl Trop Dis, 2014. **8**(7): e2982.

12. Hotez, P.J., et al., *The Global Burden of Disease Study 2010: Interpretation and Implications for the Neglected Tropical Diseases*. PLoS Negl Trop Dis, 2014. **8**(7): e2865.

13. Diro, E., et al., *Visceral Leishmaniasis and HIV coinfection in East Africa*. PLoS Negl Trop Dis, 2014. **8**(6): e2869.

14. Mathers, C.D., et al., *Measuring the burden of neglected tropical diseases: the global burden of disease framework*. PLoS Negl Trop Dis, 2007. **1**(2): e114.

15. Croft, S.L., et al., *Drug resistance in leishmaniasis*. Clin Microbiol Rev, 2006. **19**(1): 111-126.

16. Sundar, S., et al., *Leishmaniasis: an update of current pharmacotherapy*. Expert Opin Pharmacother, 2013. **14**(1): 53-63.

17. Murray, H.W., et al., *Advances in leishmaniasis*. Lancet, 2005. **366**(9496): 1561-1577.

18. Balasegaram, M., et al., *Liposomal amphotericin B as a treatment for human leishmaniasis*. Expert Opin Emerg Drugs, 2012. **17**(4): 493-510.

19. Bray, P.G., et al., *Pentamidine uptake and resistance in pathogenic protozoa: past, present and future*. Trends Parasitol, 2003. **19**(5): 232-239.

20. Kappagoda, S., et al., *Prevention and control of neglected tropical diseases: overview of randomized trials, systematic reviews and meta-analyses*. Bull World Health Organ, 2014. **92**(5): 356-366C.

21. Kobets, T., et al., *Leishmaniasis: prevention, parasite detection and treatment*. Curr Med Chem, 2012. **19**(10): 1443-1474.

22. Croft, S.L., et al., *Leishmaniasis chemotherapy--challenges and opportunities*. Clin Microbiol Infect, 2011. **17**(10): 1478-1483.

23. Richard, J.V., et al., *New antileishmanial candidates and lead compounds*. Curr Opin Chem Biol, 2010. **14**(4): 447-455.

24. Dorlo, T.P., et al., *Miltefosine: a review of its pharmacology and therapeutic efficacy in the treatment of leishmaniasis*. J Antimicrob Chemother, 2012. **67**(11): 2576-2597.

25. Schmidt, T.J., et al., *The potential of secondary metabolites from plants as drugs or leads against protozoan neglected diseases - part II*. Curr Med Chem, 2012. **19**(14): 2176-2228.

26. Krishna, S., et al., *Artemisinins: their growing importance in medicine*. Trends Pharmacol Sci, 2008. **29**(10): 520-527.

27. Chollet, C., et al., *In vitro antileishmanial activity of fluoro-artemisinin derivatives against Leishmania donovani*. Biomed Pharmacother, 2008. **62**(7): 462-465.

28. Yang, D.M., et al., *Effects of qinghaosu (artemisinin) and its derivatives on experimental cutaneous leishmaniasis*. Parasitology, 1993. **106 (Pt 1)**: 7-11.

29. Rocha, L.G., et al., *A review of natural products with antileishmanial activity*. Phytomedicine, 2005. **12**(6-7): 514-535.

30. Gachet, M.S., et al., *Assessment of anti-protozoal activity of plants traditionally used in Ecuador in the treatment of leishmaniasis*. J Ethnopharmacol, 2010. **128**(1): 184-197.

31. Sulsen, V.P., et al., *Trypanocidal and leishmanicidal activities of sesquiterpene lactones from Ambrosia tenuifolia Sprengel (Asteraceae)*. Antimicrob Agents Chemother, 2008. **52**(7): 2415-2419.

32. Pieters, L., et al., *Bioguided isolation of pharmacologically active plant components, still a valuable strategy for the finding of new lead compounds?* J Ethnopharmacol, 2005. **100**(1-2): 57-60.

33. Elwasila, M., *Leishmania tarentolae Wenyon, 1921 from the gecko Tarentola annularis in the Sudan*. Parasitol Res, 1988. **74**(6): 591-592.

34. Fraga, J., et al., *Phylogeny of Leishmania species based on the heat-shock protein 70 gene*. Infect Genet Evol, 2010. **10**(2): 238-245.

35. Raymond, F., et al., *Genome sequencing of the lizard parasite Leishmania tarentolae reveals loss of genes associated to the intracellular stage of human pathogenic species*. Nucleic Acids Res, 2012. **40**(3): 1131-1147.

36. Taylor, V.M., et al., *Leishmania tarentolae: utility as an in vitro model for screening of antileishmanial agents*. Exp Parasitol, 2010. **126**(4): 471-475.

37. Cos, P., et al., *Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept'*. J Ethnopharmacol, 2006. **106**(3): 290-302.

38. Berg, K., et al., *The use of a water-soluble formazan complex to quantitate the cell number and mitochondrial function of Leishmania major promastigotes*. Parasitol Res, 1994. **80**(3): 235-239.

39. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays*. J Immunol Methods, 1983. **65**(1-2): 55-63.

40. Motulsky, H., *Intuitive Biostatistics*. Second ed. 2012, New York: Oxford University Press.

41. Ng, M., et al., *Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013*. Lancet, 2014. **384**(9945): 766-781.

42. Wild, S., et al., *Global prevalence of diabetes: estimates for the year 2000 and projections for 2030*. Diabetes Care, 2004. **27**(5): 1047-1053.

43. Dabelea, D., et al., *Prevalence of type 1 and type 2 diabetes among children and adolescents from 2001 to 2009*. JAMA, 2014. **311**(17): 1778-1786.

44. Samuel, V.T., et al., *Mechanisms for insulin resistance: common threads and missing links*. Cell, 2012. **148**(5): 852-871.

45. Chan, J.M., et al., *Obesity, Fat Distribution, and Weight Gain as Risk Factors for Clinical Diabetes in Men*. Diabetes Care, 1994. **17**(9): 961-969.

46. Colditz, G.A., et al., *Weight as a risk factor for clinical diabetes in women*. Am J Epidemiol, 1990. **132**(3): 501-513.

47. Steinberger, J., et al., *Obesity, insulin resistance, diabetes, and cardiovascular risk in children: an American Heart Association scientific statement from the Atherosclerosis, Hypertension, and Obesity in the Young Committee (Council on Cardiovascular Disease in the Young) and the Diabetes Committee (Council on Nutrition, Physical Activity, and Metabolism)*. Circulation, 2003. **107**(10): 1448-1453.

48. Bianchini, F., et al., *Overweight, obesity, and cancer risk*. Lancet Oncol, 2002. **3**(9): 565-574.

49. Field, A.E., et al., *Impact of overweight on the risk of developing common chronic diseases during a 10-year period*. Arch Intern Med, 2001. **161**(13): 1581-1586.

50. Dunham-Snary, K.J., et al., *Mitochondrial genetics and obesity: evolutionary adaptation and contemporary disease susceptibility*. Free Radic Biol Med, 2013. **65**(0): 1229-1237.

51. Wells, J.C., *The evolution of human adiposity and obesity: where did it all go wrong?* Dis Model Mech, 2012. **5**(5): 595-607.

52. Lebovitz, H.E., *Insulin resistance: definition and consequences*. Exp Clin Endocrinol Diabetes, 2001. **109 Suppl 2**: S135-148.

53. Odegaard, J.I., et al., *Pleiotropic Actions of Insulin Resistance and Inflammation in Metabolic Homeostasis*. Science, 2013. **339**(6116): 172-177.

54. Johnson, A.M., et al., *The origins and drivers of insulin resistance*. Cell, 2013. **152**(4): 673-684.

55. Asrih, M., et al., *Inflammation as a potential link between nonalcoholic fatty liver disease and insulin resistance*. J Endocrinol, 2013. **218**(3): R25-36.

56. Tabas, I., et al., *Anti-inflammatory therapy in chronic disease: challenges and opportunities*. Science, 2013. **339**(6116): 166-172.

57. Ferrante, A.W., Jr., *Obesity-induced inflammation: a metabolic dialogue in the language of inflammation*. J Intern Med, 2007. **262**(4): 408-414.

58. Hotamisligil, G.S., et al., *Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes. Central role of tumor necrosis factor-alpha*. J Clin Invest, 1994. **94**(4): 1543-1549.

59. Hotamisligil, G.S., et al., *Tumor necrosis factor alpha inhibits signaling from the insulin receptor*. Proc Natl Acad Sci U S A, 1994. **91**(11): 4854-4858.

60. Hobbs, A.J., et al., *Inhibition of nitric oxide synthase as a potential therapeutic target*. Annu Rev Pharmacol Toxicol, 1999. **39**(1): 191-220.

61. Bosma-den Boer, M.M., et al., *Chronic inflammatory diseases are stimulated by current lifestyle: how diet, stress levels and medication prevent our body from recovering*. Nutr Metab (Lond), 2012. **9**(1): 32.

62. Fowler, M.J., *Microvascular and Macrovascular Complications of Diabetes*. Clin Diabetes, 2008. **26**(2): 77-82.

63. Peppa, M., et al., *Glucose, Advanced Glycation End Products, and Diabetes Complications: What Is New and What Works*. Clin Diabetes, 2003. **21**(4): 186-187.

64. Herman, W.H., *The Economic Costs of Diabetes: Is It Time for a New Treatment Paradigm?* Diabetes Care, 2013. **36**(4): 775-776.

65. Fowler, M.J., *Diabetes Treatment, Part 1: Diet and Exercise*. Clin Diabetes, 2007. **25**(3): 105-109.

66. Daar, A.S., et al., *Grand challenges in chronic non-communicable diseases*. Nature, 2007. **450**(7169): 494-496.

67. Wang, Y., et al., *The obesity epidemic in the United States--gender, age, socioeconomic, racial/ethnic, and geographic characteristics: a systematic review and meta-regression analysis*. Epidemiol Rev, 2007. **29**(1): 6-28.

68. Estruch, R., et al., *Primary prevention of cardiovascular disease with a Mediterranean diet*. N Engl J Med, 2013. **368**(14): 1279-1290.

69. Muraki, I., et al., *Fruit consumption and risk of type 2 diabetes: results from three prospective longitudinal cohort studies*. BMJ, 2013. **347**: f5001.

70. Fornasini, M., et al., *Hypoglycemic effect of Lupinus mutabilis in healthy volunteers and subjects with dysglycemia*. Nutr Hosp, 2012. **27**(2): 425-433.

71. Baldeon, M.E., et al., *Hypoglycemic effect of cooked Lupinus mutabilis and its purified alkaloids in subjects with type-2 diabetes*. Nutr Hosp, 2012. **27**(4): 1261-1266.

72. Teas, J., et al., *Could dietary seaweed reverse the metabolic syndrome?* Asia Pac J Clin Nutr, 2009. **18**(2): 145-154.

73. Rojo, L.E., et al., *In vitro and in vivo anti-diabetic effects of anthocyanins from Maqui Berry (Aristotelia chilensis)*. Food Chem, 2012. **131**(2): 387-396.

74. Hasani-Ranjbar, S., et al., *A systematic review of the efficacy and safety of herbal medicines used in the treatment of obesity*. World J Gastroenterol, 2009. **15**(25): 3073-3085.

75. Bailey, C.J., et al., *Traditional Plant Medicines as Treatments for Diabetes*. Diabetes Care, 1989. **12**(8): 553-564.

76. Li, W.L., et al., *Natural medicines used in the traditional Chinese medical system for therapy of diabetes mellitus*. J Ethnopharmacol, 2004. **92**(1): 1-21.

77. Grover, J.K., et al., *Medicinal plants of India with anti-diabetic potential*. J Ethnopharmacol, 2002. **81**(1): 81-100.

78. Andrade-Cetto, A., et al., *Mexican plants with hypoglycaemic effect used in the treatment of diabetes*. J Ethnopharmacol, 2005. **99**(3): 325-348.

79. Bailey, C.J., et al., *Metformin: its botanical background*. Practical Diabetes International, 2004. **21**(3): 115-117.

80. *United Kingdom Prospective Diabetes Study 24: a 6-year, randomized, controlled trial comparing sulfonylurea, insulin, and metformin therapy in patients with newly diagnosed type 2 diabetes that could not be controlled with diet therapy. United Kingdom Prospective Diabetes Study Group*. Ann Intern Med, 1998. **128**(3): 165-175.

81. Palit, P., et al., *Novel weight-reducing activity of Galega officinalis in mice*. J Pharm Pharmacol, 1999. **51**(11): 1313-1319.

82. Wang, C.Y., et al., *A mouse model of diet-induced obesity and insulin resistance*. Methods Mol Biol, 2012. **821**: 421-433.

83. Lin, S., et al., *Development of high fat diet-induced obesity and leptin resistance in C57Bl/6J mice*. Int J Obes Relat Metab Disord, 2000. **24**(5): 639-646.

84. Street, R.A., et al., *Cichorium intybus: Traditional Uses, Phytochemistry, Pharmacology, and Toxicology*. Evid Based Complement Alternat Med, 2013. **2013**: 579319.

85. eFloras. *Flora of China*. 2008 [cited 2014 15 January 2014].

86. Bais, H.P., et al., *Cichorium intybus L - cultivation, processing, utility, value addition and biotechnology, with an emphasis on current status and future prospects*. J Sci Food Agric, 2001. **81**(5): 467-484.

87. Grieve, M., *A Modern Herbal*. 1971, Mineola, NY.: Dover Publications.

88. Pazola, Z., *The chemistry of chicory and chicory-product beverages.*, in *Coffee*, R.I. Clarke and R. Macrae, Editors. 1987, Elsevier Applied Sci. Publishers Ltd.: New York, NY. p. 19-57.

89. Gardner, Z., et al., *American Herbal Products Association's Botanical Safety Handbook*. 2013, Boca Raton, FL: Taylor & Francis Group.

90. Schmidt, B.M., et al., *Toxicological evaluation of a chicory root extract*. Food Chem Toxicol, 2007. **45**(7): 1131-1139.

91. Den Hond, E., et al., *Effect of high performance chicory inulin on constipation*. Nutr Res, 2000. **20**(5): 731-736.

92. Cavin, C., et al., *Inhibition of the expression and activity of cyclooxygenase-2 by chicory extract*. Biochem Biophys Res Commun, 2005. **327**(3): 742-749.

93. Wesolowska, A., et al., *Analgesic and sedative activities of lactucin and some lactucin-like guaianolides in mice*. J Ethnopharmacol, 2006. **107**(2): 254-258.

94. Kim, M., *The water-soluble extract of chicory reduces cholesterol uptake in gut-perfused rats*. Nutr Res, 2000. **20**(7): 1017-1026.

95. Pushparaj, P.N., et al., *Anti-diabetic effects of Cichorium intybus in streptozotocin-induced diabetic rats*. J Ethnopharmacol, 2007. **111**(2): 430-434.

96. Hazra, B., et al., *Tumour inhibitory activity of chicory root extract against Ehrlich ascites carcinoma in mice*. Fitoterapia, 2002. **73**(7–8): 730-733.

97. Kim, J.H., et al., *Effects of the ethanol extract of Cichorium intybus on the immunotoxicity by ethanol in mice*. Int Immunopharmacol, 2002. **2**(6): 733-744.

98. Bischoff, T.A., et al., *Antimalarial activity of lactucin and lactucopicrin: sesquiterpene lactones isolated from Cichorium intybus L*. J Ethnopharmacol, 2004. **95**(2-3): 455-457.

99. Kassi, M., et al., *Marring leishmaniasis: the stigmatization and the impact of cutaneous leishmaniasis in Pakistan and Afghanistan*. PLoS Negl Trop Dis, 2008. **2**(10): e259.

100. Molan, A.L., et al., *Effects of condensed tannins and crude sesquiterpene lactones extracted from chicory on the motility of larvae of deer lungworm and gastrointestinal nematodes*. Parasitol Int, 2003. **52**(3): 209-218.

101. Van Beek, T.A., et al., *Bitter sesquiterpene lactones from chicory roots*. J Agric Food Chem, 1990. **38**(4): 1035-1038.

102. Heimler, D., et al., *Polyphenol content and antiradical activity of Cichorium intybus L. from biodynamic and conventional farming*. Food Chem, 2009. **114**(3): 765-770.

103. Nishimura, H., et al., *Allelochemicals in chicory and utilization in processed foods*. J Chem Ecol, 2000. **26**(9): 2233-2241.

104. Leclercq, E., *Determination of lactucin in roots of chicory (Cichorium intybus L.) by high-performance liquid chromatography*. J Chromatogr A, 1984. **283**: 441-444.

105. Kisiel, W., et al., *Minor sesquiterpene lactones from Lactuca virosa*. Phytochemistry, 1997. **46**(7): 1241-1243.

106. Tiuman, T.S., et al., *Antileishmanial activity of parthenolide, a sesquiterpene lactone isolated from Tanacetum parthenium*. Antimicrob Agents Chemother, 2005. **49**(1): 176-182.

107. Barrera, P.A., et al., *Natural sesquiterpene lactones are active against Leishmania mexicana*. J Parasitol, 2008. **94**(5): 1143-1149.

108. Schmidt, T.J., et al., *Anti-trypanosomal activity of helenalin and some structurally related sesquiterpene lactones*. Planta Med, 2002. **68**(8): 750-751.

109. Das, B., et al., *Acetylated pseudoguaianolides from Parthenium hysterophorus and their cytotoxic activity*. Phytochemistry, 2007. **68**(15): 2029-2034.

110. Long, J., et al., *Protection-group-free semisyntheses of parthenolide and its cyclopropyl analogue*. J Org Chem, 2013. **78**(20): 10512-10518.

111. Pollastri, M.P., *Finding new collaboration models for enabling neglected tropical disease drug discovery*. PLoS Negl Trop Dis, 2014. **8**(7): e2866.

112. McLemore, B.F., *Flowering Dogwood*, in *Silvics of North America*, R.M. Burns and B.H. Honkala, Editors. 1990, Forest Service, Department od Agriculture: Washington, DC.

113. Duke, J.A., *Handbook of Phytochemical Constituents of GRAS Herbs and Other Economic Plants*. 2nd ed. Vol. 1. 2000, Boca Raton, FL: CRC Press. 654.

114. Fan, C., et al., *Phylogenetic relationships within Cornus (Cornaceae) based on 26S rDNA sequences*. Am J Bot, 2001. **88**(6): 1131-1138.

115. Wadl, P.A., et al., *Molecular Identification Keys for Cultivars and Lines of Cornus florida and C. kousa Based on Simple Sequence Repeat Loci*. J Am Soc Hortic Sci, 2008. **133**(6): 783-793.

116. Cappiello, P., et al., *Dogwoods*. 2005, Portland, OR.: Timber Press.

117. Bate-Smith, E.C., et al., *Phytochemical interrelationships in the Cornaceae*. Biochem Syst Ecol, 1975. **3**(2): 79-89.

118. Vareed, S.K., et al., *Anthocyanins in Cornus alternifolia, Cornus controversa, Cornus kousa and Cornus florida fruits with health benefits*. Life Sci, 2006. **78**(7): 777-784; Hostettmann, K., et al., *Molluscicidal saponins from cornus florida L*. Helv Chim Acta, 1978. **61**(6): 1990-1995.

119. Moerman, D., *Native American Medicinal Plants: An Ethnobotanical Dictionary*. 2009, Portland, OR-USA: Timber Press.

120. Spencer, C.F., et al., *Survey of plants for antimalarial activity*. Lloydia, 1947. **10**: 145-174.

121. Graziose, R., et al., *Antiparasitic compounds from Cornus florida L. with activities against Plasmodium falciparum and Leishmania tarentolae*. J Ethnopharmacol, 2012. **142**(2): 456-461.

122. Yogeeswari, P., et al., *Betulinic acid and its derivatives: a review on their biological properties*. Curr Med Chem, 2005. **12**(6): 657-666.

123. Piironen, V., et al., *Plant sterols: biosynthesis, biological function and their importance to human nutrition*. J Sci Food Agric, 2000. **80**(7): 939-966.

124. Alakurtti, S., et al., *Anti-leishmanial activity of betulin derivatives*. J Antibiot, 2010. **63**(3): 123-126.

125. Gnoatto, S.C., et al., *Synthesis and preliminary evaluation of new ursolic and oleanolic acids derivatives as antileishmanial agents*. J Enzyme Inhib Med Chem, 2008. **23**(5): 604-610.

126. Chan-Bacab, M.J., et al., *Antiprotozoal activity of betulinic acid derivatives*. Phytomedicine, 2010. **17**: 379+.

127. Barrett-Bee, K., et al., *Ergosterol biosynthesis inhibition: a target for antifungal agents*. Acta Biochim Pol, 1995. **42**(4): 465-479.

128. Kim, K.H., et al., *Tirucallane triterpenoids from Cornus walteri*. J Nat Prod, 2011. **74**(1): 54-59.

129. Paul, J.H.A., et al., *Eryngium foetidum L.: A review*. Fitoterapia, 2011. **82**(3): 302-308.

130. Calvino, C.I., et al., *The evolutionary history of Eryngium (Apiaceae, Saniculoideae): rapid radiations, long distance dispersals, and hybridizations*. Mol Phylogenet Evol, 2008. **46**(3): 1129-1150.

131. Wang, P., et al., *Phytochemical Constituents and Pharmacological Activities of Eryngium L. (Apiaceae)*. Pharm Crop, 2012. **3**: 99-120.

132. Rios, M., et al., *Useful Plants of Ecuador: Applications, Challenges, and Perspectives*. 2007, Quito, Ecuador: Abya-Yala.

133. Paul, J.H., et al., *Eryngium foetidum L.: a review*. Fitoterapia, 2011. **82**(3): 302-308.

134. Roumy, V., et al., *Amazonian plants from Peru used by Quechua and Mestizo to treat malaria with evaluation of their activity*. J Ethnopharmacol, 2007. **112**(3): 482-489.

135. Singh, S., et al., *Determination of Bioactives and Antioxidant Activity in Eryngium foetidum L.: A Traditional Culinary and Medicinal Herb*. Proc Natl Acad Sci India Sect B Biol Sci, 2013. **83**(3): 453-460.

136. Mekhora, C., et al., *Eryngium foetidum suppresses inflammatory mediators produced by macrophages*. Asian Pac J Cancer Prev, 2012. **13**(2): 653-664.

137. Garcia, M., et al., *Topical antiinflammatory activity of phytosterols isolated from Eryngium foetidum on chronic and acute inflammation models*. Phytother Res, 1999. **13**(1): 78-80.

138. Lans, C., *Ethnomedicines used in Trinidad and Tobago for reproductive problems*. J Ethnobiol Ethnomed, 2007. **3**: 13.

139. Cumanda, J., et al., *New Sesquiterpenes from Xanthium-Catharticum*. J Nat Prod, 1991. **54**(2): 460-465.

140. Bohlmann, F., et al., *Über Terpenderivate ausFerula hispanica*. Chemische Berichte, 1969. **102**(7): 2211-2215.

141. Darriet, F., et al., *Bicyclo[4.4.0]decane oxygenated sesquiterpenes from Eryngium maritimum essential oil*. Planta Med, 2012. **78**(4): 386-389.

142. Miski, M., et al., *Daucane Esters from Ferula-Communis Subsp Communis*. Phytochemistry, 1985. **24**(8): 1735-1741.

143. Dall'Acqua, S., et al., *Natural daucane sesquiterpenes with antiproliferatice and proapoptotic activity against human tumor cells.* Bioorg Med Chem, 2011. **19**: 5876-5885.

144. Simpson, M.G., *Plant Systematics*. 2006, San Diego, CA: Elsevier Inc.

145. Anwar, F., et al., *Moringa oleifera: a food plant with multiple medicinal uses*. Phytother Res, 2007. **21**(1): 17-25.

146. Fahey, J.W., *Moringa oleifera: A Review of the Medical Evidence for Its Nutritional, Therapeutic, and Prophylactic Properties. Part 1*. Trees for Life Journal, 2005. **5**: 1.

147. Pandey, A., et al., *Moringa Oleifera Lam. (Sahijan) - A Plant with a Plethora of Diverse Therapeutic Benefits: An Updated Retrospection.* Medicinal Aromatic Plants, 2012. **1**(1).

148. Amaglo, N.K., et al., *Profiling selected phytochemicals and nutrients in different tissues of the multipurpose tree Moringa oleifera L., grown in Ghana*. Food Chem, 2010. **122**(4): 1047-1054.

149. Halkier, B.A., et al., *Biology and biochemistry of glucosinolates*. Annu Rev Plant Biol, 2006. **57**(1): 303-333.

150. Wu, X., et al., *Are isothiocyanates potential anti-cancer drugs?* Acta Pharmacol Sin, 2009. **30**(5): 501-512.

151. Bennett, R.N., et al., *Profiling glucosinolates and phenolics in vegetative and reproductive tissues of the multi-purpose trees Moringa oleifera L. (horseradish tree) and Moringa stenopetala L*. J Agric Food Chem, 2003. **51**(12): 3546-3553.

152. Dey, M., et al., *In vitro and in vivo anti-inflammatory activity of a seed preparation containing phenethylisothiocyanate*. J Pharmacol Exp Ther, 2006. **317**(1): 326-333.

153. Higdon, J.V., et al., *Cruciferous vegetables and human cancer risk: epidemiologic evidence and mechanistic basis*. Pharmacol Res, 2007. **55**(3): 224-236.

154. Brunelli, D., et al., *The isothiocyanate produced from glucomoringin inhibits NF-kB and reduces myeloma growth in nude mice in vivo*. Biochem Pharmacol, 2010. **79**(8): 1141-1148.

155. Park, E.J., et al., *Inhibition of lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression by 4-[(2'-O-acetyl-alpha-L-rhamnosyloxy)benzyl]isothiocyanate from Moringa oleifera*. Nutr Cancer, 2011. **63**(6): 971-982.

156. Cheenpracha, S., et al., *Potential anti-inflammatory phenolic glycosides from the medicinal plant Moringa oleifera fruits*. Bioorg Med Chem, 2010. **18**(17): 6598-6602.

157. Farooq, F., et al., *Medicinal properties of Moringa oleifera: An overview of promising healer*. J Med Plant Res, 2012. **6**(27): 4368-4374.

158. Jaiswal, D., et al., *Effect of Moringa oleifera Lam. leaves aqueous extract therapy on hyperglycemic rats*. J Ethnopharmacol, 2009. **123**(3): 392-396.

159. Gupta, R., et al., *Evaluation of antidiabetic and antioxidant activity of Moringa oleifera in experimental diabetes*. J Diabetes, 2012. **4**(2): 164-171.

160. Hamza, A.A., *Ameliorative effects of Moringa oleifera Lam seed extract on liver fibrosis in rats*. Food Chem Toxicol, 2010. **48**(1): 345-355.

161. Mbikay, M., *Therapeutic Potential of Moringa oleifera Leaves in Chronic Hyperglycemia and Dyslipidemia: A Review*. Front Pharmacol, 2012. **3**: 24.

162. Mahajan, S.G., et al., *Immunosuppressive activity of ethanolic extract of seeds of Moringa oleifera Lam. in experimental immune inflammation*. J Ethnopharmacol, 2010. **130**(1): 183-186.

163. Waterman, C., et al., *Stable, water extractable isothiocyanates from Moringa oleifera leaves attenuate inflammation in vitro*. Phytochemistry, 2014. **103**: 114-122.

164. Folch, J., et al., *A simple method for the isolation and purification of total lipids from animal tissues*. J Biol Chem, 1957. **226**(1): 497-509.

165. Wang, Z.Q., et al., *Bioactives from bitter melon enhance insulin signaling and modulate acyl carnitine content in skeletal muscle in high-fat diet-fed mice*. J Nutr Biochem, 2011. **22**(11): 1064-1073.

166. Cheng, D.M., et al., *In vivo and in vitro antidiabetic effects of aqueous cinnamon extract and cinnamon polyphenol-enhanced food matrix*. Food Chem, 2012. **135**(4): 2994-3002.

167. Liu, H., et al., *Coordinate regulation of enzyme markers for inflammation and for protection against oxidants and electrophiles*. Proc Natl Acad Sci U S A, 2008. **105**(41): 15926-15931.

168. Miller, R.S., et al., *Adipocyte gene expression is altered in formerly obese mice and as a function of diet composition*. J Nutr, 2008. **138**(6): 1033-1038.

169. Ndong, M., et al., *Effects of Oral Administration of Moringa oleifera Lam on Glucose Tolerance in Goto-Kakizaki and Wistar Rats*. J Clin Biochem Nutr, 2007. **40**(3): 229-233.

170. Widjaja, A., et al., *UKPDS 20: plasma leptin, obesity, and plasma insulin in type 2 diabetic subjects*. J Clin Endocrinol Metab, 1997. **82**(2): 654-657.

171. Steppan, C.M., et al., *The hormone resistin links obesity to diabetes*. Nature, 2001. **409**(6818): 307-312.

172. Srinivasan, K., et al., *Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening*. Pharmacol Res, 2005. **52**(4): 313-320.

173. El Messaoudi, S., et al., *The cardioprotective effects of metformin*. Curr Opin Lipidol, 2011. **22**(6): 445-453.

174. Hundal, R.S., et al., *Mechanism by which metformin reduces glucose production in type 2 diabetes*. Diabetes, 2000. **49**(12): 2063-2069.

175. Gastaldelli, A., et al., *The effect of rosiglitazone on the liver: decreased gluconeogenesis in patients with type 2 diabetes*. J Clin Endocrinol Metab, 2006. **91**(3): 806-812.

176. Knowler, W.C., et al., *Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin*. N Engl J Med, 2002. **346**(6): 393-403.

177. Kozak, L.P., et al., *Brown fat thermogenesis and body weight regulation in mice: relevance to humans*. Int J Obes, 2010. **34**(S1): S23-S27.

178. Duncan, R.E., et al., *Regulation of lipolysis in adipocytes*. Annu Rev Nutr, 2007. **27**(1): 79-101.

179. Giralt, M., et al., *White, brown, beige/brite: different adipose cells for different functions?* Endocrinology, 2013. **154**(9): 2992-3000.

180. Tsukita, S., et al., *Hepatic glucokinase modulates obesity predisposition by regulating BAT thermogenesis via neural signals*. Cell Metab, 2012. **16**(6): 825-832.

181. Ferre, T., et al., *Long-term overexpression of glucokinase in the liver of transgenic mice leads to insulin resistance*. Diabetologia, 2003. **46**(12): 1662-1668.

182. Zechner, R., et al., *FAT SIGNALS--lipases and lipolysis in lipid metabolism and signaling*. Cell Metab, 2012. **15**(3): 279-291.

183. Speakman, J.R., *Measuring energy metabolism in the mouse - theoretical, practical, and analytical considerations*. Front Physiol, 2013. **4**: 34.

184. Raskin, I., et al., *Plants and human health in the twenty-first century*. Trends Biotechnol, 2002. **20**(12): 522-531.

185. Fabricant, D.S., et al., *The value of plants used in traditional medicine for drug discovery*. Environ Health Perspect, 2001. **109 Suppl 1**: 69-75.

186. Krief, S., et al., *Ethnomedicinal and bioactive properties of plants ingested by wild chimpanzees in Uganda*. J Ethnopharmacol, 2005. **101**(1-3): 1-15.

187. Krief, S., et al., *Novel antimalarial compounds isolated in a survey of self-medicative behavior of wild chimpanzees in Uganda*. Antimicrob Agents Chemother, 2004. **48**(8): 3196-3199.

188. Patwardhan, B., et al., *Traditional medicine-inspired approaches to drug discovery: can Ayurveda show the way forward?* Drug Discov Today, 2009. **14**(15-16): 804-811.

189. Houghton, P.J., *The role of plants in traditional medicine and current therapy*. J Altern Complement Med, 1995. **1**(2): 131-143.

190. Wink, M., *Medicinal plants: a source of anti-parasitic secondary metabolites*. Molecules, 2012. **17**(11): 12771-12791.