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EVALUATION OF IN VITRO ANTI-INFLAMMATORY, ANTI-DIABETIC AND ANTI-LIPOGENIC ACTIVITY OF NATURAL POLYPHENOLIC EXTRACTS AND THEIR PURE CONSTITUENTS

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**EVALUATION OF IN VITRO ANTI-INFLAMMATORY, ANTI-DIABETIC AND
ANTI-LIPOGENIC ACTIVITY OF NATURAL POLYPHENOLIC EXTRACTS
AND THEIR PURE CONSTITUENTS**

BY

PRAGATI PANKAJ NAHAR

**A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
PHARMACEUTICAL SCIENCE**

UNIVERSITY OF RHODE ISLAND

2015

DOCTOR OF PHILOSOPHY DISSERTATION

OF

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ABSTRACT

The long-term goals and objectives of our group are to identify bioactive natural products relevant to human disease prevention and health promotion. To attain this goal, the objective of this thesis was to demonstrate that bioactive polyphenol-rich extracts exert positive health benefits beyond basic nutrition, thus impacting overall health and wellness. We believe that this project has immense scientific merit from a human health perspective and will be of great public impact given that consumers are seeking natural and organic choices for improving health. In this investigation, we have focused our attention on investigating the biological activities of two novel phenolic-rich preparations derived from curcumin and maple syrup for future nutraceutical applications.

The work herein assessed the anti-inflammatory properties of novel standardized curcumin formulation (Longvida®) and phenolic-enriched maple syrup extract using a well-known *in vitro* model of inflammation, lipopolysaccharide (LPS)-induced RAW 264.7 murine macrophages. RAW 264.7 cells were co-treated with 50 ng/mL LPS and test extracts for 24 hours and then inflammatory markers were measured using gene and protein expression. The activation of nuclear factor-kappa B (NFκB) was measured using luciferase activity. Both polyphenolic extracts were able to inhibit the LPS stimulated inflammation by down-regulating the inflammatory markers via targeting nuclear factor-kappa B (NFκB) (a major pathway modulated in inflammation) transcriptional activity in murine macrophages.

Next, the work herein studied the anti-hyperglycemic and anti-lipogenic properties of novel standardized nutraceutical grade phenolic rich extract, (MSX). Human HepG2 hepatoma cells were treated with MSX for 24 h. Glucose consumption, AMP activated protein kinase (AMPK) activation and its target gluconeogenic gene expression were measured. The glucose levels were reduced by MSX in the hepatocytes through AMPK activation. Subsequently, the anti-lipogenic effect of MSX treatment in mature differentiated 3T3-L1 murine adipocytes and human visceral adipocytes was evaluated. MSX treatment to mature adipocytes decreased lipid accumulation, compared to control, in both murine and human adipocytes. In 3T3-L1 adipocytes, this effect was associated with downregulation of adipo/lipogenic protein expression (e.g. PPAR γ , Srebp1c). We also observed reduced mRNA expression of the pro-inflammatory mediators, namely IL-6, and TNF- α . Taken together, we demonstrated that a novel maple syrup extract exhibited anti-inflammatory, anti-hyperglycemic and anti-lipogenic activities *in vitro*. The current study adds to the growing body of *in vitro* and *in vivo* data supporting the biological effects and potential health benefits of the novel curcumin formulation Longvida® and the natural sweetener, maple syrup.

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DEDICATION

This thesis is dedicated to my family, my husband and my son who have always stood by me and dealt with all of my absence from many family occasions with a smile. Their immeasurable support, encouragement, and constant love have sustained me throughout this wonderful journey of graduate school.

PREFACE

The following dissertation titled “Evaluation of in vitro anti-inflammatory, anti-diabetic and anti-lipogenic activity of natural polyphenolic extracts and their pure constituents” is presented in manuscript format. There are three manuscripts in this dissertation. First chapter is introductory and serves as a general background for manuscript one, two and three. Manuscript one is formatted in Journal of Medicinal Food (J Med Food) journal style, second manuscript is in Journal of Functional Foods (J Funct Foods) style, and the third is in The Journal of the Federation of American Societies for Experimental Biology (FASEB J.) format. Manuscripts one and two are published in peer-reviewed journals as of March 2015.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
DEDICATION	vi
PREFACE	vii
TABLE OF CONTENTS	viii
LIST OF TABLES	xii
LIST OF FIGURES	xiii
INTRODUCTION	1
MANUSCRIPT 1	12
1.1 Abstract	13
1.2 Introduction	15
1.3 Materials and methods	18
1.3.1 Solid Lipid Curcumin Particle (SLCP) Formulations and Curcumin Extract	18
1.3.2 Cell culture	19
1.3.3 Cytotoxicity Assay	19
1.3.4 Nitrite determination	20
1.3.5 PGE ₂ determination	20
1.3.6. Determination of IL-6	21
1.3.7. Transient transfection and luciferase assay	21

1.3.8 Statistical analysis.....	22
1.4 Results	22
1.4.1 Aqueous solubility of SLCP formulations vs. curcumin.....	22
1.4.2. Effect of SLCP formulations on LPS-induced NO production.	23
1.4.3. Effect of SLCP on PGE ₂ production.....	24
1.4.4. Effects of SLCPs on the levels of IL-6.....	24
1.4.5. Effects of SLCP formulations on NF- κ B activation.....	25
1.5 Discussion.....	26
1.6 Figure legends.....	30
1.7 Tables.....	31
1.8 Figures.....	32
1.9 References.....	36
MANUSCRIPT 2	40
2.1 Abstract.....	41
2.2 Introduction.....	43
2.3 Materials and methods.....	47
2.3.1 Phenolic-enriched maple syrup ethyl acetate extract.....	47
2.3.2 Isolation and identification of pure compounds.....	47
2.3.3 Cell culture and treatment.....	48
2.3.4. Cytotoxicity Assay.....	49
2.3.5. Nitric oxide (NO) activity.....	49
2.3.6. Prostaglandin E ₂ release.....	50
2.3.7. RNA isolation and qRT-PCR.....	50

2.3.8. Western blotting.....	51
2.3.9. Transient transfection and luciferase assay.....	52
2.3.10. Statistical analysis.....	53
2.4 Results and Discussion.....	53
2.4.1. MS-EtOAc extract inhibit NO production in LPS-stimulated RAW 264.7 cells.....	53
2.4.2. MS-EtOAc inhibits PGE ₂ production in LPS-stimulated RAW 264.7 cells.....	54
2.4.3. Pure maple syrup isolates inhibit NO and PGE ₂ production in LPS-stimulated RAW 264.7 cells.....	55
2.4.4. Effect of MS-EtOAc extract on iNOS and COX-2 mRNA and protein expression in LPS- stimulated RAW 264.7 macrophages.....	58
2.4.5. MS-EtOAc extract decreases NF- κ B response element-luciferase reporter construct activity.....	61
2.5 Conclusion.....	62
2.7 Figure legends.....	64
2.8 Tables.....	67
2.9 Figures.....	68
2.6 References.....	74
MANUSCRIPT 3	81
3.1 Abstract.....	82
3.2 Introduction.....	84
3.3 Materials and methods.....	87

3.3.1 Chemicals.....	87
3.3.2. HepG2 Cell culture and treatment.....	88
3.3.3. Adipocyte culture and treatment.....	88
3.3.4. Glucose Consumption Assay.....	90
3.3.5. Oil red O staining and quantification.....	90
3.3.6. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis.....	90
3.3.7. Western blot.....	91
3.3.8. Statistical analyses.....	92
3.4 Results and Discussion.....	92
3.4.1. MSX increase glucose consumption in HepG2 cells by downregulating gluconeogenic genes via AMPK activation.....	92
3.4.2. MSX imparts lipid-lowering effects in mature mouse and human adipocytes.....	94
3.4.3. MSX downregulated lipogenic targets in mature 3T3-L1 adipocytes.....	95
3.5 Figure legends.....	99
3.6 Figures.....	102
3.7 References.....	109
SUMMARY AND CONCLUSION.....	113

LIST OF TABLES

MANUSCRIPT 1:

Table 1. Solubility of curcumin extract and the SLCP formulations in water...31

MANUSCRIPT 2:

Table 1: Effect of pure compounds isolated from an ethyl acetate extract of Canadian maple syrup (MS-EtOAc) on NO and PGE₂ levels in LPS-induced RAW 264.7 macrophages..... 67

MANUSCRIPT 3: None

LIST OF FIGURES

MANUSCRIPT 1:

Fig. 1: Effects of SLCP formulations on: a) Nitrite and b) PGE ₂ production in LPS-stimulated RAW 264.7 cells.....	32
Fig. 2: Effect of SLCP formulations on LPS-induced production of pro-inflammatory cytokines including IL-6 in RAW 264.7 cells.....	33
Fig. 3: Effect of SLCP formulations on NF-κB activation in stimulated RAW 264.7 macrophages.....	34

MANUSCRIPT 2

Fig. 1 Compounds isolated from an ethyl acetate extract of Canadian maple syrup (MS-EtOAc)	68
Fig. 2 Effect of MS-EtOAc extract on NO (a) and PGE ₂ (b) production in LPS-stimulated RAW 264.7 cells.....	69
Fig. 3 Effects of MS-EtOAc on iNOS mRNA and protein levels in LPS treated macrophages.....	70
Fig. 4 Effects of MS-EtOAc on COX-2 mRNA and protein expressions in LPS-stimulated RAW264.7 macrophages.....	71
Fig. 5 Effects of MS-EtOAc on LPS induced NF-κB luciferase activity in macrophages.....	72

MANUSCRIPT 3

Fig. 1. Effect of MSX extract on glucose levels in the media in HepG2 hepatoma cells.....	102
---	-----

Fig. 2. Effect of MSX extract on mRNA expression of genes involved in gluconeogenesis, namely G6pase (A) and Pepck (B) in HepG2 hepatoma cells.....	103
Fig. 3. Effect of MSX extract on protein expression of pAMPK, pACC and Pepck in HepG2 hepatoma cells	104
Fig. 4. Effect of MSX extract on lipid accumulation by ORO staining and quantification in mature 3T3-L1 and human visceral adipocytes.....	105
Fig. 5. Effect of MSX extract on protein expression of lipogenic targets after MSX treatment in mature 3T3-L1 cells.....	106
Fig. 6. Effect of MSX extract on gene expression of inflammatory markers in mature 3T3-L1 adipocytes.....	107
Fig. 7. MSX hypoglycemic and anti-lipogenic effect in HepG2 cells and 3T3-L1 cells.....	108

Introduction

The rapid growth of health foods, also known as nutraceuticals, has immensely impacted consumers. Though variety of oral drug formulations is available, there is an increasing trend towards using nutraceuticals for prevention of diseases or as adjuncts to conventional therapies (1). Botanical extracts, produced from medicinal plants and plant foods, are widely consumed worldwide as dietary supplements for the promotion and maintenance of human health. Herbal natural products constitute a major approach of Complementary and Alternative Medicine (CAM) that is widely utilized by consumers. Recently, market statistics show the growing attention and confidence that consumers place in the herbal dietary supplements. In 2013 alone, herbal dietary supplements sales were increased by 7.9%, and reported a sales figure of six billion dollars for the herb market. The 20 top-selling herbal supplements in the natural and health foods channel in the United States includes turmeric, flax seed/oil, wheat or barley grass and cranberry etc. (2). These natural products are typically 'whole extracts' constituting complex mixtures of multiple substances found within the plant rather than a single compound alone. This has arisen out of a basic underpinning of the biological effects of botanical extracts, namely, 'the sum of the whole is greater than its parts' due to synergy, additivity and/or complementary effects among multiple constituents.

A growing body of research suggests that plant natural products, both in purified or extract forms, have anti-inflammatory properties and may impart beneficial effects against diseases with an inflammatory component (3-6). Dietary intake of bioactive class of compounds 'phenolics' with antioxidant and anti-inflammatory properties represents one mechanism to combat inflammation and promote overall human health. Polyphenols play a significant role in human nutrition and health, as these potent bioactive constituents are the most abundant phytochemicals in our diet (7-10). Possible efficacy of phenolics including biological extracts such as grape seed extracts, cocoa extracts, etc or pure constituents like resveratrol, curcumin. on inflammation, glucose and lipid homeostasis has been well published in cell culture *studies*, animal models and some clinical trials (14,11-13)

It is now well accepted that inflammation and associated pro-inflammatory processes are centrally linked to several chronic human diseases including cancer, diabetes, cardiovascular and neurodegenerative diseases (15-17). Regulatory mechanisms that monitor metabolism and immunity overlap with each other at many stages (18). The factors contributing to metabolic diseases like diabetes and obesity are insulin resistance, impaired glucose and lipid metabolism (19). As stated earlier, along with chronic inflammation, altered balance of pro-inflammatory and inflammatory factors are also involved in the pathogenesis of these metabolic diseases. Multiple complex molecular pathways that involve JNK, NFκB, SOCS, AMPK and PPAR family members

are responsible for development of hepatic insulin resistance by inflammation (18).

The Nuclear factor kappa B (NFκB) signaling pathway has been well characterized and is considered to be a predominant signaling pathway in inflammation (15, 16). The activated form of NFκB has been reported to be involved in chronic inflammatory diseases such as cancer, atherosclerosis, myocardial infarction, diabetes, arthritis, Alzheimer's disease, osteoporosis, and other (20-22). Thus, agents that can suppress NFκB activation, such as plant natural products can potentially prevent, delay the onset, or treat NFκB - linked diseases (23-25). Moreover, many well-known antioxidants that are currently being marketed in nutraceutical preparations, such as resveratrol, curcumin, and pomegranate, are thought to elicit anti-inflammatory effects through downregulation of the NFκB signaling pathway in macrophages (26, 27). Hence, in manuscript two and three we have demonstrated the anti-inflammatory activities of standardized curcumin formulation Longvida® and polyphenolic maple syrup extract in RAW 264.7 macrophage model.

In the first manuscript, '**Anti-inflammatory effects of novel standardized solid lipid curcumin formulations**', we have investigated *in vitro* biological effects of Longvida®, a commercially available standardized novel curcumin formulation, on the NFκB signaling pathway which is a major molecular pathway involved in inflammation (15, 16). The investigations carried out in last few decades, confirmed that curcumin (the most active constituent of the curry spice turmeric) targets many transcription factors (NFκB, ATF3, AP-1,

STAT-3), inflammatory mediators (NO, PGE₂, cytokines), enzymes, growth factors, protein kinases and cell-cycle regulatory proteins (27-30). Despite the promising health benefits of curcumin, water insolubility, low bioavailability and poor absorption are the limiting factors for the use of curcumin as a potential preventive and therapeutic agent (29, 31). Lately, very promising results were observed in an *in vivo* single-dose pharmacokinetic study of standardized novel solid lipid curcumin particle (SLCP) preparation (Longvida®), which reported 65 fold increased bioavailability of the preparation relative to generic curcumin extract and suggests the potential for sustained release dosage form (32). Furthermore an acute and subchronic toxicity study in rats and mice demonstrated the safety of SLCP and the No Observed-Adverse-Effect Level (NOAEL) was determined to be 720 mg/kg bw/day, the highest dose tested (33). Moreover, a recent study demonstrated that a low dose of a Longvida® (80 mg/ day) can produce a variety of potentially health promoting effects in healthy middle-aged people (34). Yet the effects of this bioavailable curcumin preparation Longvida® on inflammation remains unknown and detail investigation is needed. Thus, in the first manuscript we focused our attention on evaluating anti-inflammatory effects of this novel nutraceutical formulation Longvida®.

In the second manuscript, '**Phenolic mediated anti-inflammatory properties of a maple syrup extract in Raw 264.7 murine macrophages**', we demonstrated that maple syrup extract attenuated lipopolysaccharide induced inflammation via NFκB pathway in the same murine macrophage model that

was used in manuscript 1. Herein we have also investigated anti-inflammatory activities of fifteen pure compounds isolated from maple syrup extract. Maple syrup is a widely consumed natural sweetener with well-studied chemistry. Canada and eastern North America are chiefly responsible for the major commercial production of available maple syrup. Being the world's largest maple syrup supplier, this industry plays a critical role in the economy of this region. Maple syrup is a very interesting food to investigate, primarily due to its unique chemical profile. This natural sweetener constitutes mainly of carbohydrates (sucrose) along with various nutrients such as minerals, vitamins, amino acids, organic acids, phytohormones (abscisic acid, phaseic acid and their metabolites) and most importantly various phytochemicals (majorly phenolics belonging to the sub-classes lignan, phenolic acid, stilbene, coumarin, and flavonoid). The chemistry of maple syrup has been extensively studied; however there are very few biological studies that have been conducted. Thus, in this study we investigated the mechanistic insights into the anti-inflammatory activity of maple syrup extract and its pure constituents. We believe that accomplishing this project has led to increased scientific knowledge on the 'matrix' effects of multiple constituents exerting positive biological effects to this sweetener beyond sucrose alone. This project is thus necessary along the research continuum on this natural product.

Our maple syrup research has led to the development of the first nutraceutical grade botanical extract from this food (named MSX) (35). Initially, the laboratory scale maple syrup extract project was focused on inflammatory

pathways but gradually it evolved, and then we started exploring the mechanisms involved in metabolic diseases like diabetes, obesity etc. that has chronic inflammatory component using food grade extract MSX. In the third manuscript titled, '***In vitro* hypoglycemic and lipid lowering effects of a standardized food grade maple syrup extract (MSX)**', we have explored glucose lowering and lipid lowering potential of a nutraceutical grade MSX extract using hepatocytes and adipocytes respectively.

Searching for a signaling mechanism for the hypoglycemic action of MSX extract, we have explored its effect on 5' adenosine monophosphate-activated protein kinase (AMPK) activation in human HepG2 hepatoma cell model. AMPK, a heterotrimeric serine/threonine kinase, is a vital metabolic regulator of fatty acid and glucose homeostasis. It is emerging as a potential target for the treatment of diabetes and chronic inflammatory diseases (19). Numerous studies have reported that the activation of AMPK can inhibit NFκB signaling indirectly, a master regulator of inflammation, both *in vitro* and *in vivo* (36). Recently, activation of AMPK by metformin, a first-line anti-diabetic drug, has been shown to decrease glucose production and increase fatty acid oxidation in the liver (37, 38). It has been reported that AMPK activation by polyphenols may be at least partially responsible for their therapeutic benefits on hyperlipidemia in diabetes (39).

Moreover, there is a strong interplay between inflammation, diabetes and obesity. Adipocyte dysfunction has been linked to the development of type 2 diabetes, insulin resistance along with sub-clinical inflammation. Polyphenolic

compounds such as catechins, resveratrol etc. have modulatory effects on many complex pathways in adipose tissue including upregulation of fatty acid oxidation and reduction in the expression of the transcription factors, enzymes and genes involved in lipogenesis e.g. peroxisome proliferator-activated receptor γ (PPAR γ), CCAAT enhancer-binding protein- α (C/EBP- α), Sterol-regulatory element-binding protein 1-c (Srebp-1c), fatty acid binding protein (FABP4), fatty acid synthase (FAS), and perillipin, etc. (14). A natural polyphenol, resveratrol has been shown to attenuate adipogenesis and lipogenesis in both murine and human adipocytes including mature and differentiating adipocytes cell models. Resveratrol has been used in several clinical trials and exhibited its effectiveness in inflammatory conditions and metabolic diseases. Thus, in this third manuscript, we investigated MSX effects on lipogenic pathways and adipo/lipogenic transcription factors in mature murine and human adipocytes. This study has provided data that will support the design of future preclinical trials with maple bioactives.

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Anti-inflammatory effects of novel standardized solid lipid curcumin formulations

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Running Title: Anti-inflammatory effects of curcumin formulations

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1.1. Abstract

Inflammation and the presence of pro-inflammatory cytokines are associated with numerous chronic diseases such as type-2 diabetes mellitus, cardiovascular disease, Alzheimer's disease, and cancer. An overwhelming amount of data indicates that curcumin, a polyphenol obtained from the Indian spice turmeric, *Curcuma longa*, is a potential chemopreventive agent for treating certain cancers and other chronic inflammatory diseases. However, the low bioavailability of curcumin, partly due to its low solubility and stability in the digestive tract, limits its therapeutic applications. Recent studies have demonstrated increased bioavailability and health promoting effects of a novel solid lipid particle formulation of curcumin (Curcumin SLCP, Longvida®). The goal of the current study was to evaluate the aqueous solubility and *in vitro* anti-inflammatory effects of SLCP formulations using lipopolysaccharide (LPS)-stimulated RAW 264.7 cultured murine macrophages. SLCPs treatment significantly decreased nitric oxide (NO) and prostaglandin-E₂ (PGE₂) levels at concentrations ranging from 10-50 µg/mL, and reduced Interleukin-6 (IL-6) levels in a concentration-dependent manner. Transient transfection experiments using a nuclear factor-kappa B (NF-κB) reporter construct indicates that SLCPs significantly inhibit the transcriptional activity of NF-κB in macrophages. Taken together, these results show that in RAW 264.7 murine macrophages, SLCPs have improved solubility over unformulated curcumin, and significantly decrease the LPS-induced pro-inflammatory mediators NO, PGE₂ and IL-6 by inhibiting the activation of NF-κB.

Keywords: Curcumin, Longvida[®], Inflammation, Interleukin-6, Interleukin-1 β , NF- κ B, Nitric oxide, Prostaglandin-E₂

Abbreviations

Arbitrary units (AU); Dulbecco's modified Eagle's medium (DMEM); Enzyme-immunoassay (EIA); Fetal bovine serum (FBS); Interleukin-1 β (IL-1 β); Interleukin-6 (IL-6); Lipopolysaccharide (LPS); Nuclear factor kappa B (NF- κ B); Nitric oxide (NO); No Observed-Adverse-Effect Level (NOAEL); Prostaglandin-E₂ (PGE₂); Quantitative real-time polymerase chain reaction (qRT-PCR); Relative light units (RLU); Solid lipid curcumin particle (SLCP); Tumor necrosis factor- α (TNF- α).

1.2. Introduction

Inflammation and associated pro-inflammatory processes are centrally linked to several chronic human diseases including cancer, diabetes, obesity, arthritis, cardiovascular and neurodegenerative diseases¹⁻⁶. During inflammation, macrophages play a critical role in managing various immunopathological phenomena, including the overproduction of inflammatory markers such as nitric oxide (NO), prostaglandin-E₂ (PGE₂), tumor necrotic factor α (TNF α) and cytokines like interleukin-6 (IL-6) and interleukin-1 β (IL-1 β). A number of inflammatory stimuli, for e.g. lipopolysaccharides (LPS) and pro-inflammatory cytokines, activate immune cells to produce inflammatory mediators, and these are therefore useful targets in the development of novel anti-inflammatory drugs and in the evaluation of the molecular mechanisms of potential anti-inflammatory drugs^{7, 8}. Thus, dietary agents that can suppress inflammatory markers, such as plant natural products, can potentially prevent, delay the onset, and/or treat inflammation and inflammatory-mediated diseases. A growing body of research suggests that plant phenolic compounds which possess antioxidant and anti-inflammatory properties offer an attractive dietary strategy to combat inflammation and promote human health and wellness^{9, 10}.

Curcumin (diferuloylmethane) is derived from the ground rhizomes of the *Curcuma longa* L. plant and is the most active curcuminoid in the Indian curry spice, turmeric^{11, 12}. Curcumin is a lipophilic, water insoluble, low molecular weight polyphenol (MW = 368 g/mol), which has been used for

culinary applications in many parts of the world. In Ayurveda, turmeric is widely used to treat a variety of conditions ranging from acute infections, wounds, and injuries in addition to chronic diseases like diabetes, asthma, and various inflammatory diseases¹³⁻¹⁵. A vast number of published research studies support a wide range of pharmacological effects of curcumin including anti-oxidant, anti-cancer, anti-Alzheimer's disease and anti-inflammatory effects both *in vitro* and *in vivo*^{13, 16}. On a molecular level, curcumin targets many transcription factors (including NF- κ B, AP-1, STAT-3), inflammatory mediators (PGE₂, cytokines), enzymes, growth factors, protein kinases and cell-cycle regulatory proteins^{13, 17-19}

Despite the promising health benefits of curcumin, its low water solubility limits its oral delivery in aqueous-based formulations which are popular among consumers and widely used in the nutraceutical and functional food industries. Moreover, the poor bioavailability and extensive phase-II metabolism of curcumin are limiting factors for the oral dosage of unformulated curcumin which limits its potential as a preventive and/or therapeutic agent^{19, 20}. Various clinical trials have reported low systemic bioavailability of curcumin even after oral administration of doses up to 12 g/kg/day^{16, 21}. Several research strategies have been undertaken to improve the bioavailability of curcumin, including blocking non-specific metabolic pathways, as well as novel drug delivery systems and formulations like liposomes, nanoparticles and phospholipid complexes^{13, 22}. In a clinical study, a combination of curcumin and piperine, a nonspecific CYP-450 and (UDP-glucuronosyltransferase) UGT

inhibitor, resulted in increased curcumin bioavailability, but with an absorption half-life of only 7 minutes²³. Other *in vitro* studies suggest that polymer-based nanoparticle formulations of curcumin, also known as 'nano-curcumin', exhibit pharmacological activity at slightly lower concentrations than that of pure curcumin in human pancreatic cancer cell lines²⁴. However, the potential increase in bioavailability of these latter formulations is offset by the lack of data and clinical trials on the safety and metabolism of nanoparticle formulations in humans.

Recently, a single-dose human pharmacokinetic study of a standardized novel solid lipid curcumin particle (SLCP) preparation (commercially available as Longvida®) reported increased bioavailability compared to a generic curcumin extract, suggesting the potential for sustained release dosage forms of this natural product²⁵. Moreover, acute and sub-chronic animal toxicity studies by Dadhaniya et al. (2011) demonstrated the safety of the SLCP and the No Observed-Adverse-Effect Level (NOAEL) was determined to be 720 mg/kg bw/day which was the highest test dose²⁶. In addition, DiSilvestro et al. (2012) demonstrated that a low dose of a Longvida® (80 mg/day) imparted potentially health promoting effects in healthy middle-aged humans²⁷. Also, in a recent investigation, Longvida® selectively suppressed soluble Tau dimers and corrected molecular chaperone, synaptic, and behavioral deficits in transgenic mice suggesting that this curcumin formulation may have potential beneficial effects against Alzheimer's disease²⁸. However, to date, the effects of this bioavailable curcumin preparation, namely, Longvida®, on inflammation

and inflammatory biomarkers remains unknown.

The goal of the present study was to evaluate the effects of two novel SLCP preparations using LPS-stimulated RAW 264.7 macrophages, a well-established *in vitro* anti-inflammatory model. LPS increases inflammatory markers such as NO, PGE₂ and IL-6 and we hypothesized that the standardized SLCP formulations would block the expression of pro-inflammatory mediators.

1.3. Materials and Methods

1.3.1. Solid Lipid Curcumin Particle (SLCP) Formulations and Curcumin Extract

The solid lipid curcumin particle (SLCP) preparation (Longvida®, SLCP-1), a solution-dispersible SLCP preparation (Longvida® SD, SLCP-2), and a curcumin extract (containing 95 % curcuminoids) were provided to our laboratory by Verdure Sciences (Noblesville, IN, USA). The SLCP extracts were produced using patent-pending methodology as described previously^{25, 27} and were standardized to contain approximately 20 % curcumin^{25, 26}. SLCP-1 is a granular powder used for tablets and capsules, and SLCP-2 is a fine powder intended for use in other dosage forms. Briefly, turmeric root extract was mixed with pure phosphatidylcholine, vegetable stearic acid, ascorbic acid (vitamin C) palmitate, and other inert ingredients. The formulations were manufactured under cGMP standards and meet internal and external specifications for precise chemical and physical characteristics. The solubility

of the two SLCP formulations and the curcumin extract are shown in Table 1 (data provided by Verdure Sciences).

1.3.2. Cell culture

RAW 264.7 mouse macrophage cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). RAW 264.7 cells were routinely cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % v/v fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL) (Sigma-Aldrich, St. Louis, MO) and maintained at 37 °C with 5 % CO₂ humidified air. Stimulated RAW 264.7 cells (1×10^5 /100 µL) were treated with 50 ng/mL LPS (Sigma-Aldrich, St. Louis, MO) with or without different concentrations of test samples (10, 25, and 50 µg/mL) for 24 h. All test samples were solubilized in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) with a final DMSO concentration < 0.1% in the culture medium. Cell culture supernatants were collected for nitrite (NO), PGE₂, and cytokine assays.

1.3.3. Cytotoxicity Assay

The viability of RAW 264.7 macrophages after 24 h of continuous exposure to the test compounds was determined by performing colorimetric MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymeth-oxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium salt] assay (Promega, Madison, CA) according to the protocol described previously but with minor modifications²⁹. Briefly, after 24 h of

treatment, 20 μ L of MTS reagent was added to each reaction well (in a 96-well format). After 2 h of incubation, the absorbance was measured at 490 nm using a spectrophotometer (SpectraMax M2, Molecular Devices Corp, Sunnyvale, CA).

1.3.4. Nitrite determination

RAW 264.7 macrophages were plated in a 96-well plate (1×10^5 cells/100 μ L) and incubated at 37 °C for 24 h. After 24 h, the medium was replaced and the cells were co-treated with 50 ng/mL LPS and different concentrations of the SLCP formulations (10, 25 and 50 μ g/mL) for 24 h at 37 °C. Cells that were not treated with LPS served as a negative control. After 24 h, the cell culture supernatants were collected and incubated (1:1, v/v) with modified Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water) (Sigma-Aldrich, St. Louis, MO) for 20 min at room temperature as previously described³⁰. The absorbance at 540 nm was measured using a spectrophotometer (SpectraMax M2, Molecular Devices Corp, Sunnyvale, CA). The nitrite concentration was quantified by comparison with a sodium nitrite standard curve. The assay was performed in triplicate for each concentration.

1.3.5. PGE₂ determination

To evaluate the effects of various concentrations of the SLCP-1 and SLCP-2 formulations on PGE₂ levels, PGE₂ metabolites accumulated in the cell

supernatants were measured using a PGE2 enzyme-immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's protocol. The assay was performed in triplicate for each concentration.

1.3.6. Determination of IL-6

The effects of the SLCP-1 and SLCP-2 formulations on the production of pro-inflammatory cytokine IL-6 was determined by Bio-Plex® Multiplex Immunoassays (BioRad Laboratories, Hercules, CA), as described by the manufacturer's instructions. The experiment was performed in quadruplicate.

1.3.7. Transient transfection and luciferase assay

LPS-induced NF- κ B upregulation accounts for a part of LPS-mediated activation of variety of inflammatory genes and hence it is important to identify the effects of SLCP formulations on the transcriptional activity of NF- κ B³¹. To monitor these effects of the two SLCP formulations, RAW 264.7 macrophages were transiently co-transfected with pNF- κ B and pRL-CMV reporter vectors (Promega, Madison, CA) using GenePORTER® 3000 Transfection Reagent (Genlantis, San Diego, CA) according to the manufacturer's protocol. Briefly, the cells were seeded into 96-well plates (1.2×10^4 cells/100 μ L) in complete medium without antibiotics (DMEM +10 % FBS) and incubated for 24 h (~50-70 % confluency). Cells were then treated with the transfection complexes and incubated for 72 h before treatment with each SLCP formulations (10, 25, and 50 μ g/mL) for 2 h. LPS was added after 2 h (1 μ g/mL). After 5 h, cells were

lysed with 20 μ L passive lysis buffer and NF- κ B activity was measured as relative firefly/renilla luciferase activity using a Dual-Luciferase® Reporter Assay System (Promega, Madison, CA) with a GloMax™ 20/20 Luminometer (Promega, Madison, CA) according to the manufacturer's protocol. All samples were tested in quadruplicate. Luciferase activity was recorded as relative light units (RLU) and expressed as fold change relative to LPS-treatment control and the assay was performed using three replicates for each concentration.

1.3.8. Statistical analysis

All statistical analyses were carried out using the software program GraphPad Prism Version 5.0 (GraphPad Software, La Jolla, CA). Experimental data were grouped by one variable and were analyzed by one-way ANOVA followed by a Dunnett's multiple comparison test. A value of $p < 0.05$ was considered significant.

1.4. Results

1.4.1. Aqueous solubility of SLCP formulations vs. curcumin

The SLCP-1 and SLCP-2 formulations were both soluble in water, with SLCP-1 possessing ca. 14 % solubility and SLCP-2 having ca. 76 % solubility (see Table 1). On the other hand, the curcumin extract exhibited significantly less solubility in water which was in agreement with the previous data published by Kurien et al. (2007)³². Given this solubility data as well as the previously published data supporting the increased bioavailability of the SLCPs

compared to a generic curcumin extract in humans,²⁵ we proceeded to further evaluate the SLCP formulations in targeted *in vitro* bioassays (described below).

1.4.2. Effect of SLCP formulations on LPS-induced NO production

NO and PGE₂ are secreted into cell culture supernatant by RAW 264.7 murine macrophages when they are treated with LPS. Since NO is highly unstable, the accumulation of nitrite (a stable oxidized product of NO) in culture media is often used as a biomarker for NO production in LPS-activated macrophages³³. A nitric oxide assay was used to evaluate the effect of the SLCP formulations on NO production as described in section 2.4. RAW 264.7 cells were incubated with or without LPS (50 ng/mL) in the presence or absence of test samples (10, 25 and 50 µg/mL) for 24 h. In LPS-stimulated macrophages, nitrite levels increased significantly to 88.7 ± 6.2 µM as compared to the solvent control (Fig. 1A). In Fig. 1A, treatment with SLCP formulations suppressed nitrite concentration in LPS-activated macrophages in a concentration-dependent manner. At 25 µg/mL, both SLCP formulations significantly decreased medium nitrite to 33.5 ± 4.2 µM and 61.6 ± 2.0 µM respectively. At 50 µg/mL, SLCP-1 and SLCP-2 both inhibited LPS-induced NO production in macrophages by 100 %. No nitrite production could be detected in cells treated with test compounds without LPS (data not shown).

1.4.3. Effect of SLCP on PGE₂ production

We next evaluated the effects of SLCP on LPS-induced PGE₂ synthesis in RAW 264.7 cells. The same supernatants were used for measurement of PGE₂. After stimulation with LPS (50 ng/mL), PGE₂ was released into the culture medium and rapidly converted into its metabolite. As shown in Fig. 1B, at 10 µg/mL SLCP-1 and SLCP-2 lowered LPS-induced PGE₂ production to about 31 and 25 %, respectively. Treatment with 25 µg/mL of SLCP-1 and SLCP-2 significantly suppressed PGE₂ levels by about 66 and 64 % respectively, whereas both the test compounds at 50 µg/mL concentration reduced PGE₂ levels to about 96 % as compared to control. The SLCP formulations did not significantly increase PGE₂ levels in cells which were not treated with LPS (data not shown). Thus our results show that SLCPs inhibited PGE₂ production in a concentration-dependent manner in LPS-stimulated cells. The test compounds were not responsible for altering the viability of activated macrophages as determined by MTS assay (see Supplementary Fig.1). Hence, the observed reduction in NO and PGE₂ production by the SLCP treatments was not attributed to cytotoxic effects.

1.4.4. Effects of SLCPs on the levels of IL-6

LPS induction in macrophages causes the up-regulation of pro-inflammatory cytokines such as IL-6³⁴. Consequently, the effect of varying concentrations of the test compounds on this cytokine was evaluated. LPS treatment significantly elevated levels of IL-6 (6043 ± 589.94) in the media (Fig. 2). IL-6

levels in macrophages treated with the test compounds alone, without LPS, were negligible (data not shown). The SLCP formulations suppressed IL-6 levels in a concentration-dependent manner. At 25 and 50 µg/mL of SLCP-1 treatment, IL-6 levels were significantly reduced by 29.4 and 97.4 % respectively. Similarly, treatment with 10, 25 and 50 µg/mL of SLCP-2 decreased IL-6 production by about 2, 13 and 95 %, respectively. This data suggested that, both SLCP formulations were effective in lowering IL-6 levels in LPS-stimulated macrophages, with SLCP-1 being slightly more effective than SLCP-2 at higher concentrations.

1.4.5. Effects of SLCP formulations on NF-κB activation

The activation of the transcription factor, NF-κB is a key-step in stimulating pro-inflammatory signals. Thus, we next investigated the effect of the SLCP formulations on NF-κB activity, by transient transfection of macrophages followed by luciferase assay. After treatment with 1 µg/mL LPS, the NF-κB activity of the macrophages was significantly increased as compared to control (Fig. 3). Treatment with the SLCPs formulations significantly reduced NF-κB transcription activity in a concentration dependent manner, as shown in Fig. 3. NF-κB luciferase activity was decreased to almost 10-fold with 10 µg/mL of SLCP-1 treatment, whereas with 10 µg/mL of SLCP-2, the activity was reduced to about 6-fold as compared to control.

1.5. Discussion

Inflammation is a key component in multiple disease states³⁵. Based on a review of the literature, the NF- κ B signaling pathway is the predominant upstream molecular signaling pathway that causes inflammation through enhanced cytokine, nitric oxide, and prostaglandin production³⁶. Several studies in different animal models and in human trials support the diverse pharmacological effects of curcumin including anti-proliferative, anti-angiogenic, anti-oxidant, anti-inflammatory, anti-microbial, hepato- and nephro-protective properties etc.²². The SLCPs are novel proprietary curcumin formulations with improved solubility and bioavailability compared to generic curcumin.²⁵ In this study, we investigated the inhibitory effects of the SLCP treatments on the inflammatory response initiated by LPS in RAW 264.7 macrophages.

In inflammation, macrophages undergo sequential steps to release pro-inflammatory mediators like cytokines, NO and PGE₂. These molecules recruit other immune cells to the sites of inflammation. Therefore inhibition of the release of these chemicals is a good strategy for monitoring inflammatory diseases³⁵.

In the current study, we reported that LPS-activated RAW 264.7 murine cells treated with improved-solubility curcumin formulations, namely, SLCP-1 and SLCP-2, exhibited concentration-dependent down regulation of NO and PGE₂ production. These observations were in agreement with the previous studies, which reported the inhibition of NO through the suppression of

inducible, NO synthase gene and protein expression by curcumin in LPS and IFN- γ activated RAW 264.7 macrophages^{37, 38}. Our study further revealed that the SLCP treatments on LPS-activated macrophages decreased IL-6 levels in a concentration dependent manner. It has been demonstrated previously that there is a reduction in LPS-induced IL-6 levels with curcumin treatment in macrophages^{39, 40}. Moreover, it was reported that curcumin blocked the LPS-induced NF- κ B activation through the prevention of Inhibitor κ B degradation in RAW 264.7 cells³⁸. Thus, the down regulation of NF- κ B activation can be one of the many mechanisms underlying the anti-inflammatory effects of the SLCPs.

The improved solubility of SLCP formulations compared to regular curcumin (see Table 1) may be due to the amphiphilic nature of SLP formulations that utilize phospholipids and other lipids to solubilize curcumin in aqueous solutions. In previous studies, similar types of formulations contributed to the creation of micelle type physical structures that permit increased dissolution of lipophilic compounds. SLCP-1 and SLCP-2 differ with respect to powder size, and the differential solubility between them (Table 1) may be due to the relative amount of surface area exposed to the aqueous medium. In this study, it was found that SLCP-2 possessed greater water solubility than SLCP-1, likely due to its decreased powder size and thus increased surface area exposed to the aqueous solution. However, both SLCPs possessed similar activity *in vitro* on inflammatory mediators. Future research may investigate the dissolution and micellar characteristics of these

formulations and their interactions with cytokines on a molecular level. Overall, the increased aqueous solubility of the SLCP formulations compared to natural curcumin leads to a broader scope of possible formulations which can be utilized by the nutraceutical and functional food industries.

In toto, solid lipid curcumin particle (SLCP) formulations dose-dependently mitigated the LPS-induced inflammatory response in macrophages, by down regulation of the production of the inflammatory markers NO, PGE₂ and IL-6 through inhibition of NF-κB activation. SLCP-1 was slightly more effective at inhibiting the anti-inflammatory response than SLCP-2, and this may be due to concentration-dependent effects. The current findings suggest the use of increased-water soluble curcumin formulations such as SLCPs as potential therapeutic agents to combat chronic inflammatory diseases. Further investigations should explore the potential use of SLCP in the prevention and/or therapy of inflammation-linked diseases but future *in vivo* studies on SLCP are warranted to determine the clinical efficacy of these formulations of curcumin.

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Author Disclosure Statement

The authors declare no competing financial interests.

1.6. Figure legends

Fig. 1: Effects of SLCP formulations on: a) Nitrite and b) PGE₂ production in LPS-stimulated RAW 264.7 cells. Cells were co-treated with LPS (50 ng/mL) and samples (10, 25, 50 µg/mL) for 24 h. SLCP-1 and SLCP-2 inhibited NO and PGE₂ levels in LPS-stimulated macrophages in a concentration-dependent manner. Data are expressed as mean values ± SD. ^a*P* < 0.001 and ^b*P* < 0.01 indicate a significant difference as compared with the LPS-treated group.

Fig. 2: Effect of SLCP formulations on LPS-induced production of pro-inflammatory cytokines including IL-6 in RAW 264.7 cells. The SLCP formulations were able to suppress IL-6 levels in a concentration dependent manner. Data are expressed as mean values ± SD. ^a*P* < 0.001 and ^c*P* < 0.05 indicate a significant difference as compared with the LPS-treated group.

Fig. 3: Effect of SLCP formulations on NF-κB activation in stimulated RAW 264.7 macrophages. The SLCP formulations significantly inhibited the transcriptional activity of NF-κB in LPS-activated murine macrophages at tested concentrations. Data are expressed as mean values ± SD. ^a*P* < 0.001 indicate a significant difference as compared with the LPS-treated group.

1.7. Tables

Table 1. Solubility of curcumin extract and the SLCP formulations in water.

Test samples	Solubility in water (%)	Solubility in water (µg/mL)	Fold improvement
Curcumin (unformulated)	0.00006	0.6	1
SLCP-1 (Longvida®)	14	140,000	233,000
SLCP-2 (Longvida®)	76	760,000	1,270,000

1.8. Figures

Fig. 1: Effects of SLCP formulations on: a) Nitrite and b) PGE₂ production in LPS-stimulated RAW 264.7 cells.

Fig. 2: Effect of SLCP formulations on LPS-induced production of pro-inflammatory cytokines including IL-6 in RAW 264.7 cells.

Fig. 3: Effect of SLCP formulations on NF- κ B activation in stimulated RAW 264.7 macrophages.

Supplementary Fig.1

1.9. References

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**Phenolic mediated anti-inflammatory properties of a maple syrup extract
in Raw 264.7 murine macrophages**

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2.1. Abstract

The *in vitro* anti-inflammatory effects of a phenolic-enriched Canadian maple syrup ethyl acetate extract (MS-EtOAc) and 15 purified phenolic constituents were evaluated in a LPS-stimulated RAW 264.7 murine macrophage cell model. MS-EtOAc decreased nitric oxide (NO) and prostaglandin-E₂ (PGE₂) production at 10-100 µg/mL concentrations. The observed NO inhibition was a direct result of reduced nitric oxide synthase (iNOS) protein and gene expression through suppression of NF-κB transcriptional activation. In addition, MS-EtOAc upregulated cyclooxygenase-2 (COX-2) mRNA and protein expression. Among the 15 pure isolates, (*E*)-3,3'-dimethoxy-4,4'-dihydroxystilbene was most effective in decreasing both NO and PGE₂ levels. However, 4-acetylcatechol, tyrosol, and protocatechuic acid only reduced PGE₂ levels. Thus, the potential anti-inflammatory activity of MS-EtOAc can be attributed to its unique combination of compounds and not as a result of a single purified phenolic constituent alone. Future research on the purified phenolic compounds will be useful in understanding the overall *in vitro* anti-inflammatory effects of maple syrup.

Keywords: Maple syrup extract; Phenolics; Inflammation; RAW 264.7 Macrophages; Lipopolysaccharide

Abbreviations

Nuclear factor kappa B (NF- κ B); nitric oxide (NO); prostaglandin-E₂ (PGE₂); prostaglandins (PGs); tumor necrosis factor- α (TNF- α); interleukin-1 (IL-1); interleukin-6 (IL-6); cyclooxygenases (COX); nitric oxide synthase (NOS); cyclo-oxygenase-2 (COX-2); inducible nitric oxide synthase (iNOS); Maple syrup ethyl acetate extract (MS-EtOAc); lipopolysaccharide (LPS); fetal bovine serum (FBS); Dulbecco's modified Eagle's medium (DMEM); Quantitative real-time polymerase chain reaction (qRT-PCR); enzyme-immunoassay (EIA); phosphate buffered saline (PBS); tris-buffered saline with Tween 20 (TBST); relative light units (RLU)

2.2. Introduction

Functional foods and nutraceuticals, consumed for human health promotion and disease risk reduction, are among the fastest growing sectors of the modern food industry (Hardy, 2000). This has led to increased interest into the discovery and applications of new functional ingredients, from both marine and terrestrial sources, by several research laboratories (Marete, Jacquier, & O’Riordan, 2011; Vo & Kim, 2013). Similarly, our group has been involved in the identification of bioactive compounds from maple (*Acer*) species and maple syrup (Li & Seeram, 2010, 2011a, 2011b; González-Sarrías, Li, & Seeram, 2011; Apostolidis, Li, Lee, & Seeram, 2011). Notably, our research efforts in this area have contributed, in part, to the recent commercial development and utilization of maple sap/water as a functional beverage (Yuan, Li, Zhang & Seeram, 2013; <http://ilovemaple.ca/products/maple-water>). In the current project, we have now focused our attention on investigating the biological activities of a ‘phenolic-enriched and sugar-reduced’ extract derived from maple syrup (further explained below) for future potential applications as a functional food ingredient.

Maple syrup is a natural sweetener produced by concentrating the watery sap collected from maple species including the sugar maple (*Acer saccharum*) and red maple (*Acer rubrum*) species, which are both native to North America (Li & Seeram, 2010; Perkins & van den Berg, 2009). Maple syrup contains sugars (mainly as sucrose), organic acids, amino acids, minerals, and phytochemicals, the latter of which occur primarily as phenolics

(Abou-Zaid, Nozzolillo, Tonon, Coppens, & Lombardo, 2008; Ball, 2007; Li & Seeram, 2010). As mentioned above, our laboratory has previously isolated and identified more than fifty phenolics from maple syrup belonging to the lignan, coumarin, stilbene, and phenolic derivative sub-classes (Li & Seeram, 2010, 2011a, 2011b).

Published studies have shown that phenolic-enriched extracts of maple sap and syrup have *in vitro* anti-oxidant, anti-proliferative, anti-radical and anti-mutagenic activities (Legault, Girard-Lalancette, Grenon, Dussault, & Pichette, 2010; Theriault, Caillet, Kermasha, & Lacroix, 2006). In addition, our group has shown that phenolic-enriched maple syrup extracts and their purified constituents inhibit the growth of human colon cancer cell lines mediated by cell cycle arrest (González-Sarriás, Li, & Seeram, 2011). We also reported that maple syrup extract inhibits the activities of α -glucosidase and α -amylase enzymes using *in vitro* models (Apostolidis, Li, Lee, & Seeram, 2011). However, until now, the anti-inflammatory activity and the associated molecular mechanisms elicited by phenolic-enriched maple syrup extract have not been thoroughly studied. Furthermore, the activities of the purified phenolic compounds isolated from maple syrup that may be responsible for any observable anti-inflammatory activities have not been reported.

Inflammation is implicated in multiple chronic human diseases, such as cancer, diabetes, cardiovascular and neurodegenerative diseases. Overwhelming data suggests that the nuclear factor kappa B (NF- κ B) signaling pathway is a predominant upstream molecular signaling pathway

that causes inflammation through enhanced cytokine, nitric oxide (NO), and prostaglandin (PG) production (Guo, Kong, & Meydani, 2009; He & Karin, 2011; Santangelo, et al., 2007; Vallabhapurapu & Karin, 2009). NF- κ B, a homo- or hetero-dimeric inducible transcription factor, resides in the cytoplasm in an inactive state and when activated, translocates to the nucleus (Valovka & Hottiger, 2011). In the nucleus, the activated form of NF- κ B induces the transcription of genes encoding pro-inflammatory mediators such as prostaglandin-E₂ (PGE₂), NO, and other inflammatory mediators such as tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1) and interleukin-6 (IL-6) in response to pathologic stimuli (Park, Lee, Lee, & Kim, 2013). NO and PGE₂ are widely used as anti-inflammatory activity markers for high throughput biologically active compound screening (Djoko, Chiou, Shee, & Liu, 2007). Both NO and PGs are synthesized by inducible isoforms of the enzymes, nitric oxide synthase (NOS) and cyclooxygenases (COX), respectively (Chiu & Lin, 2008; Korhonen, Lahti, Kankaanranta, & Moilanen, 2005; Posadas, et al., 2000). Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) have critical roles in the response of tissues to injury or infectious agents. These inducible enzymes are essential components of the inflammatory response, injury repair, and carcinogenesis (Posadas, et al., 2000; Vane & Botting, 1998). The activated form of NF- κ B has been found to mediate cancer, atherosclerosis, myocardial infarction, diabetes, arthritis, Alzheimer's disease, osteoporosis, and other inflammatory diseases (Kaltschmidt & Kaltschmidt, 2009; Morgan & Liu, 2011). Thus, dietary agents that can

suppress NF- κ B activation, such as plant natural products, including phenolics, can potentially prevent, delay the onset, or treat NF- κ B mediated diseases (Lau, Joseph, McDonald, & Kalt, 2009; Mortensen, et al., 2008; Park, et al., 2012; Saracino & Lampe, 2007). Currently, many well-known dietary plant phenolics, such as resveratrol, curcumin, and quercetin, are thought to elicit anti-inflammatory and health promoting effects through downregulation of the NF- κ B signaling pathway in macrophages (Kang, et al., 1999; Kang, Heng, Yuan, Baolin, & Fang, 2010; Korhonen, et al., 2005; Rasheed, et al., 2009). Moreover, previous research has shown that plant extracts and their purified compounds inhibit the pro-inflammatory COX-2 enzyme and the transcription factor, NF- κ B (Henry, et al., 2009; Rettig, et al., 2008).

In the present study, we examined the potential for a phenolic-enriched maple syrup ethyl acetate extract (MS-EtOAc) to reduce inflammation endpoints in lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages *in vitro*. The inflammatory markers, NO and PGE₂, along with NF- κ B activity, were measured. Lastly, 15 individual phenolic compounds (structures shown in Fig. 1 and identities shown in Table 1), previously isolated from maple syrup by our group (Li & Seeram, 2010, 2011b), were evaluated for their effects on NO and PGE₂ production in the LPS-stimulated macrophages.

2.3. Materials and Methods

2.3.1. Phenolic-enriched maple syrup ethyl acetate extract (MS-EtOAc)

Maple syrup was donated to our laboratory by the Federation of Maple Syrup Producers of Quebec and was extracted with ethyl acetate as previously reported (González-Sarrías, Li, & Seeram, 2011). The extract was standardized to phenolic content by the Folin–Ciocalteu method as 34% gallic acid equivalents as previously reported (González-Sarrías, Li, & Seeram, 2011).

2.3.2. Isolation and identification of pure compounds from maple syrup

The isolation and identification of compounds from maple syrup have been previously reported (Li & Seeram, 2010, 2011b). The maple syrup isolates are predominantly phenolics belonging to lignan, coumarin, stilbene, and phenolic derivative sub-classes. Unfortunately, since many of the compounds were isolated in small quantities, and we were limited by sample availability, we were only able to pursue studies on 15 of the pure compounds (identities are shown in Table 1; chemical structures are shown in Fig. 1). In addition, because of limited quantity of these pure samples, we were only able to evaluate each sample once (in a 96-well format) but at three different concentrations for the bioassays described below.

2.3.3. Cell culture and treatment

RAW 264.7 mouse macrophage cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS), penicillin G (100 U/mL), and streptomycin (100 µg/mL) (Sigma-Aldrich, St. Louis, MO) and maintained at 37 °C in 5% CO₂ humidified air. RAW 264.7 cells were seeded (1×10^5 cells/100 µL) in 96-well plates. After 24 h incubation, RAW 264.7 cells were stimulated with 50 ng/mL LPS (Sigma-Aldrich, St. Louis, MO), followed by treatment with varying concentrations of MS-EtOAc extract (10, 50 and 100 µg/mL) or pure compounds (10, 25, 50 µM) for 24 h. MS-EtOAc extract and the pure compounds were solubilized in DMSO by sonication. The final DMSO concentration in the culture medium was < 0.3%. Negative controls were not treated with LPS, MS-EtOAc extract or pure compounds. Resveratrol (50 µg/mL) (Sigma-Aldrich, St. Louis, MO) was used as a positive control for the assays, as it has been previously described to decrease LPS-induced NO and PGE₂ induction in RAW 264.7 cells through NF-κB downregulation (Djoko, et al., 2007; Tsai, Lin-Shiau, & Lin, 1999). After 24 h incubation, cell culture supernatants were collected for NO and PGE₂ measurements. Cell lysates were collected for both gene expression and protein expression analyses. For gene expression studies, RAW 264.7 cells were seeded in a 24-well plate at 1×10^6 cells/500 µL density. For Western blot analysis, cells were plated in a 6-well plate at $2 \times$

10⁶ cells/mL density. Cell supernatants and lysates were stored at -80°C until use.

2.3.4. Cytotoxicity Assay

The viability of RAW 264.7 macrophages after 24 h of continuous exposure to MS-EtOAc extract (10-100 µg/mL) or the pure compounds (10 - 100 µg/mL), in the presence or absence of LPS (50 ng/mL), was determined. The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium salt] assay (Promega, Madison, CA) was performed as described previously (Li, Henry, & Seeram, 2009) with modifications. Briefly, after 24 h of treatment, 20 µL of the MTS reagent in combination with the electron-coupling agent, phenazine methosulfate, were added to each reaction well. The cells were incubated at 37°C in a humidified incubator for 2 h and the absorbance was measured at 490 nm using a spectrophotometer (SpectraMax M2, Molecular Devices Corp, Sunnyvale, CA).

2.3.5. Nitric oxide (NO) activity

The nitrite levels in the cell culture supernatants were assayed as an indicator of NO production, according to the Griess reaction as previously described by Legault, Girard-Lalancette et al. (2010). Briefly, cell culture supernatants were incubated (1:1) with modified Griess reagent (Sigma-Aldrich, St. Louis, MO) for 20 min at room temperature. The optical densities were measured at 540

nm and NO concentration was determined by comparison to a standard curve. The assay was performed in triplicate and repeated at least 4 times.

2.3.6. Prostaglandin E₂ release

The Prostaglandin E₂ Metabolite (PGE₂) assay is based on the conversion of all major PGE₂ metabolites into a single stable derivative, which is measured by using an enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI). Briefly, the protocol was followed using the manufacturer's protocol: (<https://www.caymanchem.com/app/template/Product.vm/catalog/514010>) whereby cell culture supernatants were used to examine the effects of the test compounds on PGE₂. The experiment was performed with three individual samples per treatment.

2.3.7. RNA isolation and qRT-PCR

For gene expression studies, RNA was extracted from RAW 264.7 cells after 24 h treatment with MS-EtOAc. Briefly, the medium was removed and the cells were washed with phosphate buffered saline (PBS). Total RNA was isolated with TRIzol (Invitrogen, CA, USA) according to the manufacturer's instructions. The RNA concentration was determined by measuring UV absorbance of the sample at 260 nm using a NanoDrop (Wilmington, DE, USA). RNA samples were stored at -80 °C until use. Total RNA (1 µg) was reverse transcribed to cDNA using the Transcriptor Reverse Transcriptase cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN). According to

the manufacturer's website (<http://www.roche-applied-science.com/shop/products/transcriptor-high-fidelity-cdna-synthesis-kit>), the kit contains a Transcriptor High Fidelity Reverse Transcriptase which is a blend of a recombinant reverse transcriptase and a proofreading mediating enzyme as a key component. Gene expression was measured in qRT-PCR reactions using SYBR-green with gene-specific oligonucleotide primers (Life Technologies, Grand Island, NY) using LightCycler[®] 480 (Roche Applied Science, Indianapolis, IN). Relative mRNA expression of each gene was normalized to the β -actin housekeeping gene. The experiment was performed in triplicate.

2.3.8. Western blotting

To extract protein for Western blot analyses, cells were harvested and washed with PBS. The whole cell protein extracts were prepared using RIPA buffer. The protein lysates were separated by 10% SDS-PAGE. The proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA) prior to blocking for 1 h in Blotto (Santa Cruz biotechnology, Santa Cruz, CA) and then incubated with primary antibodies specific to iNOS, COX-2, or β -actin (Santa Cruz biotechnology, Santa Cruz, CA) for 2 h. Membranes were washed 3 times with tris-buffered saline with 0.1% Tween 20 (TBST) and then incubated with respective anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (Sigma-Aldrich, St. Louis, MO) for 45 min. Bands were visualized on X-ray films using ECL chemiluminescence

detection kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's manual.

2.3.9. Transient transfection and luciferase assay

LPS-induced NF- κ B upregulation accounts for a part of LPS-mediated activation of variety of other inflammatory genes. Hence it is important to identify the effects of MS-EtOAc extract on the transcriptional activity of NF- κ B (Djoko, et al., 2007). To monitor these effects of the MS-EtOAc extract, RAW 264.7 macrophages were transiently co-transfected with pNF- κ B and pRL-CMV reporter vectors (Promega, Madison, CA) using GenePORTER® 3000 Transfection Reagent (Genlantis, San Diego, CA) according to the manufacturer's protocol. Briefly, the cells were seeded into 96-well plates (1.2×10^4 cells/100 μ L) in complete medium without antibiotics (DMEM +10%FBS) and incubated for 24 h (~50-70% confluency). Cells were then treated with the transfection complexes and incubated for 72 h before treatment with MS-EtOAc extract (10, 50 and 100 μ g/mL) for 2 h. LPS was added after 2 h (1 μ g/mL) for 24h. After 5 h, cells were lysed with 20 μ L 1x passive lysis buffer and NF- κ B activity was measured as relative firefly/renilla luciferase activity using a Dual-Luciferase® Reporter Assay System (Promega, Madison, CA) with a GloMax™ 20/20 Luminometer (Promega, Madison, CA) according to the manufacturer's protocol. All samples were tested in quadruplicate. Luciferase activity was recorded as relative light units (RLU) and expressed as

fold change relative to LPS-treatment control and the assay was performed using three biological replicates.

2.3.10. Statistical analysis

All statistical analyses were carried out using the software program GraphPad Prism Version 5.0 (GraphPad Software, La Jolla, CA). Experimental data were grouped by one variable and were analyzed by unpaired two-tailed t-test or one-way ANOVA followed by a Dunnett's multiple comparison test. A value of $p < 0.05$ was considered significant.

2.4. Results and Discussion

2.4.1. MS-EtOAc extract inhibit NO production in LPS-stimulated RAW 264.7 cells

In order to study the anti-inflammatory activity of maple syrup phenolic-enriched extract, a well-known inflammatory marker, namely NO, was selected. The effects of MS-EtOAc extract on NO production was determined by the measurement of nitrite released into the culture supernatants by LPS stimulated RAW 264.7 cells using the Griess reagent (Guevara, et al., 1998; Legault, et al., 2010). Nitrite levels in unstimulated RAW 264.7 cells were at baseline levels ($2.8 \pm 0.2 \mu\text{M}$). However, upon stimulation with LPS, nitrite accumulation in the culture supernatant increased ~22-fold to $60.83 \pm 2.2 \mu\text{M}$. Resveratrol, a known inhibitor of NO activity (Djoko, et al., 2007; Wadsworth & Koop, 1999), was used as a positive control for comparing the activity of MS-

EtOAc. Resveratrol (10 µg/mL) inhibited NO release by 88.67% in LPS-stimulated RAW 264.7 macrophages. Similar activity was obtained with MS-EtOAc (Fig. 2a), which significantly reduced nitrite production by 84.9% (at 50 µg/mL) and 94.3% (at 100 µg/mL) as compared to the nitrite production by stimulated macrophages. Thus our results show that MS-EtOAc inhibited nitrite production in a concentration-dependent manner in LPS-stimulated cells. These findings are in agreement with the previous study where NO inhibition in LPS-induced RAW 264.7 macrophages was significantly higher with a maple syrup ethyl acetate extract in comparison to that of maple sap with a mean of about 75% NO inhibition per 25 µg/mL of extract, which corresponds to a portion of 0.18 mL of pure maple syrup (Legault, et al., 2010).

2.4.2. MS-EtOAc inhibits PGE₂ production in LPS-stimulated RAW 264.7 cells

PGE₂ also functions as a mediator of inflammation, which is produced by metabolism of arachidonic acid by COX enzymes at inflammatory sites (Posadas, et al., 2000). The inhibitory effects of MS-EtOAc extract on PGE₂ levels in LPS-stimulated macrophages were evaluated. As shown in Fig. 2b, PGE₂ production increased following LPS treatment (14 ± 1.4 ng/mL), whereas in the absence of LPS, PGE₂ levels were close to baseline or were undetectable. PGE₂ levels were reduced to 0.8 ng/mL by resveratrol treatment. Treatment with 100 µg/mL of MS-EtOAc extract markedly reduced PGE₂ levels 85.2% in LPS-stimulated RAW 264.7 cells, to 2.1 ng/mL. However,

lower concentrations of MS-EtOAc extract only reduced PGE₂ levels by approximately 6% as compared to LPS-induced macrophages.

Taken together, these results suggest that MS-EtOAc extract is capable of reducing NO and PGE₂ LPS-induced inflammatory effects in stimulated RAW 264.7 macrophages. None of the tested concentrations of extract inhibited the growth of RAW 264.7 macrophages (Supplementary Fig. 1). Hence, the observed reduction in NO and PGE₂ production by the MS-EtOAc treatment was not attributed to cytotoxic effects.

2.4.3. Pure maple syrup isolates inhibit NO and PGE₂ production in LPS-stimulated RAW 264.7 cells

Because MS-EtOAc is a complex mixture of compounds that could account for its NO and PGE₂ inhibitory effects, we tested 15 of its pure isolates for their ability to reduce NO and PGE₂ production. These compounds were previously isolated from maple syrup and their structures were elucidated by NMR methods (Li & Seeram, 2010, 2011b). In the MS-EtOAc phenolic-enriched extract, compound 1 is the only stilbene, whereas compound 5 is the only coumarin among the tested samples. Compounds 9, 10, 12, 14 and 15 were all lignans. All of the other compounds were phenolic derivatives and are briefly categorized as follows: compound 13 contained a C₆ unit; compound 11 contained a C₆-C₁ unit; compounds 2, 3 and 7 contained a C₆-C₂ unit and compounds 4, 6 and 8 contained a C₆-C₃ unit. At the test concentrations, the

pure compounds did not exhibit any cytotoxicity in the RAW 264.7 cells (data not shown).

Among all of the pure isolates, the only stilbene, (*E*)-3,3'-dimethoxy-4,4'-dihydroxystilbene (compound 1), was the most active compound and significantly inhibited NO as well as PGE₂ production in a concentration dependent manner (Table 1). At 50 μ M, compound 1 decreased the nitrite level by 92.5% and PGE₂ level by 89.5% in media. These results were similar to the effects of the well-known stilbene, resveratrol (50 μ M; used as a positive control), on the above inflammatory markers (data not shown).

As shown in Table 1, apart from compound 1, the majority of the other pure compounds were not effective in modulating NO production in LPS-stimulated macrophages. However, compounds 3, 7, and 11 reduced PGE₂ levels in a concentration-dependent manner. At 50 μ M concentrations, compounds 3, 7 and 11 were the most effective in lowering PGE₂ levels with 95, 72.6, and 55.3% decrease, respectively. Interestingly, in this assay, the two most active isolates, compounds 3 and 7, both contained a C₆-C₂ structural motif. However, compound 2, which also contained the same C₆-C₂ structural motif, did not show any activity against the inflammatory markers. Based on structure activity related observations, the active compound 3 contained two aromatic hydroxyl groups while the inactive compound 2 contained three aromatic hydroxyls. Moreover, the active compound 7 only contained one aromatic hydroxyl group. Therefore, it would appear that $\leq$ two aromatic hydroxyls are required for inhibition of PGE₂ levels but further studies

on a larger number of structurally diverse compounds would be required to confirm this. Interestingly, compound 7, protocatechuic acid, has been shown to have anti-oxidant, anti-inflammatory and anti-cancer activity *in vitro and in vivo* (Lende, et al., 2011; Nakamura, et al., 2000; Reis, et al., 2010; Robbins, 2003).

Our data with compound 11, tyrosol, a well-known constituent of olive oil, was in agreement with a previous study where the reported IC₅₀ value for tyrosol for lowering NO level was >100 μ M (Choe, et al., 2012). Tyrosol (at 1, 2 and 4 mM concentrations) has been shown to inhibit iNOS and COX-2 expression in RAW 264.7 cells stimulated with IFN- γ and gliadin through reduction in NF- κ B, interferon regulatory factor-1 (IRF-1) and signal transducer and activator of transcription-1 α (STAT-1 α) activity (De Stefano, et al., 2007). However, this is the first report to show that tyrosol (at 50 μ M test concentration) inhibits PGE₂ level in RAW 264.7 cells stimulated by LPS.

Based on the above observations (detailed in Sec. 3.1 - 3.3), our findings suggest that the unique combination of phenolic phytochemicals in MS-EtOAc extract may be responsible for its anti-inflammatory activity. It is possible that these compounds work complementarily, additively, and/or synergistically *in toto* to abate inflammatory processes. Unfortunately, several of the previously reported pure compounds from MS-EtOAc were not isolated in sufficient quantities to facilitate further biological assaying (Li & Seeram, 2010, 2011b). Thus, future studies to evaluate the effects of all of the purified phenolic compounds from MS-EtOAc are warranted.

During transformation of maple sap into syrup, unique phenolic and non-phenolic compounds are formed due to the intensive heating process. For example, Quebecol, a novel process derived phenolic compound has been reported in maple syrup (Li & Seeram, 2011a). It has been demonstrated that transformation of maple sap in syrup improves NO inhibition activity. The heating process involved in the transformation of maple sap in syrup induces oxidation of the phenolics, suggesting its implication in the activity (Legault, et al., 2010). Further research is needed to identify these compounds as they may contribute to the observed biological effects of maple syrup.

2.4.4. Effect of MS-EtOAc extract on iNOS and COX-2 mRNA and protein expression in LPS- stimulated RAW 264.7 murine macrophages

In macrophages exposed to LPS, iNOS and COX-2 mRNA and protein expression are modulated, which ultimately results in overproduction of NO and PGE₂. In order to assess whether the inhibitory effects of MS-EtOAc extract on NO and PGE₂ were related to the alteration of iNOS and COX-2 enzymes, we examined their relative gene and protein expression by qRT-PCR and western blot analysis, respectively. iNOS and COX-2 mRNA and proteins were barely detectable in un-stimulated cells but were strongly expressed in lysates of LPS-stimulated RAW 264.7 cells. Resveratrol significantly decreased iNOS mRNA and protein levels at 10 µg/mL, which is in agreement with the previous reports wherein gene activation of iNOS was inhibited by resveratrol (Tsai, et al., 1999). MS-EtOAc decreased iNOS mRNA

expression in a concentration-dependent manner, by 51% and 72%, at 50 and 100 µg/mL, concentrations, respectively (Fig. 3a). Not surprisingly, MS-EtOAc extract also inhibited iNOS protein expression in a concentration-dependent manner (Fig. 3b). These results suggest that MS-EtOAc extract likely decreases nitrite accumulation through decreased iNOS gene and protein expression.

The acquired data clearly indicates that MS-EtOAc extract did not inhibit COX-2 mRNA (Fig. 4a) and protein levels (Fig. 4b) but inhibited PGE₂ production as discussed previously. Since COX-2 gene expression was upregulated by MS-EtOAc extract treatment, the inhibitory effects observed in regards to decreased PGE₂ levels may potentially occur at the translational or post-translational levels. The reduced PGE₂ levels could be due to the inhibition at the enzymatic level by MS-EtOAc treatment. Resveratrol has also been shown to have a direct inhibitory effect on PG production by suppressing COX-1 and COX-2 enzyme activity in a concentration-dependent manner (Jang, et al., 1997; Subbaramaiah, et al., 1998).

MS-EtOAc demonstrated a similar mechanism of action common to non-steroidal anti-inflammatory drugs (NSAIDs) in inhibiting PGE₂ production. In the previous studies, NSAIDs were reported to upregulate COX-2 expression in rodents (Davies, Sharkey, Asfaha, Macnaughton, & Wallace, 1997; Minghetti, Polazzi, Nicolini, Creminon, & Levi, 1997; Pang & Hoult, 1996). Indomethacin was reported to increase COX-2 protein expression at a concentration where it also inhibited NO synthesis and iNOS protein

expression in LPS-induced murine macrophages (Pang & Hoult, 1996). Similarly, ajoene, a natural product present in garlic, was also reported to increase COX-2 gene and protein expression but it inhibited NO, PGE₂ release and iNOS expression (Dirsch, Kiemer, Wagner, & Vollmar, 1998; Dirsch & Vollmar, 2001). In murine macrophages prostaglandins are suggested to act as negative feedback regulators of COX-2 expression (Davies, et al., 1997; Minghetti, et al., 1997; Pang & Hoult, 1996). Hence, inhibition of prostaglandin production may cause upregulation of COX-2 expression as shown in our study.

There is a complex 'cross-talk' between the iNOS and COX-2 pathways as has been evidenced by numerous studies. There are reports suggesting that NO seems to decrease COX-2 expression, however, not all published reports are consistent. Inhibitors of nitric oxide synthase activity such as *N*^G-monomethyl-L-arginine (L-NMMA) have been demonstrated to increase COX-2 protein expression with decrease in NO production in rodent macrophages (Habib, et al., 1997; Patel, Attur, Dave, Abramson, & Amin, 1999; Swierkosz, Mitchell, Warner, Botting, & Vane, 1995). Reduced NO levels may contribute to the observed increased COX-2 protein and mRNA expression with MS-EtOAc treatment. Besides this negative regulation of COX-2 expression, other potential modes of action could be an effect of MS-EtOAc on COX-2 mRNA stability or effects at the transcriptional level. Therefore, further investigation is required to explore how MS-EtOAc affects the COX pathway and whether it inhibits COX activity at the enzymatic level.

2.4.5. MS-EtOAc extract decreases NF- κ B response element-luciferase reporter construct activity

Inflammation is associated with high levels of pro-inflammatory mediators like NO and PGE₂, which are produced by the inducible isoforms of enzymes iNOS and COX-2, respectively (Chung, et al., 2008; Nathan, 1992). The transcription factor NF- κ B, which regulates the expression of iNOS and PGE-2, plays a crucial role in inflammatory and immune responses (Chen, Castranova, Shi, & Demers, 1999). Thus, modulating NF- κ B activating signals is one of the widely used strategies for the treatment of diseases associated with inflammation. Hence in order to measure NF- κ B transcriptional activity, the RAW 264.7 cells were transiently transfected with pNF- κ B-luc and pRL-CMV-renilla and reporter constructs were used to determine the effects of MS-EtOAc extract on cells challenged with LPS. As expected, LPS treatment increased activity of the NF- κ B promoter construct by about 20-fold as compared to control (Fig. 5). The NF- κ B activity was decreased by approximately 50% with 10 and 50 μ g/mL concentrations of MS-EtOAc. Moreover, 100 μ g/mL of MS-EtOAc decreased NF- κ B reporter construct activity by ~ 75% (Fig. 5). The data herein supports the hypothesis that MS-EtOAc extract may interfere with NF- κ B transcriptional activity. LPS-induced iNOS gene activation has been linked to LPS-mediated NF- κ B activation (Lowenstein, et al., 1993). Thus downregulation of iNOS expression by MS-EtOAc may be through reduced NF- κ B transcriptional activity.

NF- κ B is also involved in transcriptional control of IL-1-induced COX-2 gene expression (Newton, Kuitert, Bergmann, Adcock, & Barnes, 1997). However, inflammation involves multiple complex pathways and these pathways interfere with each other. Thus, NF- κ B is not the only transcription factor in LPS-mediated COX-2 gene expression in the mouse macrophages. It has been reported previously that NF- κ B site is not essential for LPS-mediated COX-2 gene expression in RAW 264.7 macrophages (Wadleigh, Reddy, Kopp, Ghosh, & Herschman, 2000). This might explain the elevated COX-2 gene and protein expression.

On the basis of current results, we propose that the anti-inflammatory effect of maple syrup extract could be attributed to the inhibition of inflammatory modulators NO and PGE₂ through suppression of NF- κ B transcription.

2.5. Conclusion

The current study is the first report to investigate the mechanisms underlying the anti-inflammatory activity of a phenolic-enriched maple syrup extract and several of its purified constituents. We found that the maple syrup extract and its purified compounds significantly inhibited the production of LPS-stimulated inflammatory markers NO, PGE₂ and iNOS through the direct inhibition of NF- κ B transcriptional activity in RAW 264.7 macrophages. However, the upregulation of COX-2 gene and protein levels remain unexplained. Overall, the cellular mechanisms underlying these multiple effects may be attributed to

the unique combination of pure compounds present in the extract, which appear to act *in toto*. More detailed research is needed to get further insights into the exact anti-inflammatory mechanism of the MS-EtOAc extract.

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Conflict of interest

The authors declare no conflicts of interest.

2.6. Figure legends

Fig. 1 Compounds isolated from an ethyl acetate extract of Canadian maple syrup (MS-EtOAc).

Fig. 2 Effect of MS-EtOAc extract on NO (a) and PGE₂ (b) production in LPS-stimulated RAW 264.7 cells.

The cells were co-incubated with LPS (50 ng/mL) and different concentrations of MS-EtOAc extract ranging from 10 -100 µg/mL for 24 h. The supernatants were then collected for the measurement of NO and PGE₂ production using Griess reagent and an EIA kit, respectively. MS-EtOAc extract significantly inhibited NO (2a) and PGE₂ (2b) levels in LPS-stimulated macrophages. The values are expressed as the means ± S.D. of three individual samples. ***P < 0.001 as compared with the LPS-treated macrophages; significant differences between groups were determined using a one-way ANOVA test followed by a Dunnett's multiple comparison test. #P < 0.05 solvent control compared with the LPS-treated cells; significant difference was determined using unpaired student-t test.

Fig. 3 Effects of MS-EtOAc on iNOS mRNA and protein levels in LPS treated macrophages.

RAW 264.7 cells were co-treated with LPS (50 ng/mL) and various concentrations of MS-EtOAc extract (10, 50,100 µg/mL) for 24 h. (3a) iNOS mRNA levels were determined as described in Section 2. iNOS mRNA

transcript levels were normalized to β -actin expression. MS-EtOAc extract inhibited iNOS mRNA expression in a concentration-dependent manner. Data are expressed as the means $\pm$ S.D. of three different samples. (3b) iNOS and housekeeping gene GAPDH expressions were monitored as described in Section 2.8. MS-EtOAc extract inhibited iNOS protein expression in a concentration dependent manner. ***P < 0.001 as compared with the LPS-treated group; significant differences between groups were determined using a one-way ANOVA test followed by Dunnett's multiple comparison test. #P < 0.05 solvent control compared with the LPS-treated cells; significant difference was determined using unpaired student-t test.

Fig. 4 Effects of MS-EtOAc on COX-2 mRNA and protein expressions in LPS-stimulated RAW264.7 macrophages.

(4a) RAW 264.7 cells were co-treated with LPS (50 ng/mL) and various concentrations of MS-EtOAc extract (10, 50, 100 μ g/mL) for 24 h. 1 μ g of total cellular RNA was subjected to RT-PCR followed by SYBR-green quantitative real-time PCR. COX-2 mRNA transcript levels were normalized to β -actin expression. MS-EtOAc extract increased COX-2 mRNA expression in a concentration dependent manner. Data are expressed as the means $\pm$ S.D. of three different samples. (4b) COX-2 protein expression was measured as described in section 2.8. MS-EtOAc extract treatment increased COX-2 protein expression. ***P < 0.001 as compared with the LPS-treated group; significant differences between groups were determined using a one-way

ANOVA test followed by Dunnett's multiple comparison test. [#]P < 0.05 solvent control compared with the LPS-treated cells; significant difference was determined using unpaired student-t test.

Fig. 5 Effects of MS-EtOAc on LPS induced NF- κ B luciferase activity in macrophages.

RAW 264.7 cells were transiently co-transfected with pNF- κ B and pRL-CMV reporter vectors. NF- κ B activity was measured using a Promega dual luciferase assay system. MS-EtOAc significantly reduced the LPS-induced increase in NF- κ B-dependent luciferase enzyme expression in a concentration-dependent manner. Data is the mean $\pm$ S.D. of three different samples. *P < 0.05, **P < 0.01 and ***P < 0.001 as compared with the LPS-treated group; significant differences between groups were determined using a one-way ANOVA test followed by Dunnett's multiple comparison test. [#]P < 0.05 solvent control compared with the LPS-treated cells; significant difference was determined using unpaired student-t test.

2.7. Tables

Table 1: Effect of pure compounds isolated from an ethyl acetate extract of Canadian maple syrup (MS-EtOAc) on NO and PGE₂ levels in LPS-induced RAW 264.7 macrophages.*

No	Name	% NO inhibition at 50 μ M	% PGE ₂ inhibition		
			10 μ M	25 μ M	50 μ M
1	(<i>E</i>)-3,3'-Dimethoxy-4,4'-dihydroxystilbene	92.5	30.2	76.5	89.5
2	3',4',5'-Trihydroxyacetophenone	0	0.0	0	7.5
3	4-Acetylcatechol	6.5	56.0	88.6	95.0
4	2,3-Dihydroxy-1-(3,4-dihydroxyphenyl)-1-propanone	1	0.0	0.0	0.0
5	Isofraxidin	0	0.0	0.0	0.0
6	2,3-Dihydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone	0	0.0	0.0	0.0
7	Tyrosol	1.6	0.0	45.0	72.6
8	3-Hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)propan-1-one	0	0.0	0.0	0.0
9	Isolariciresinol	1.7	0.0	0.0	0.0
10	5-(3'',4''-dimethoxyphenyl)-3-hydroxy-3-(4'-hydroxy-3'-methoxybenzyl)-4- (hydroxymethyl) dihydrofuran-2-one	0	0.0	2.0	6.2
11	Protocatechuic acid (3,4-Dihydroxybenzoic acid)	0	13.8	31.5	55.3
12	Threo-guaiacylglycerol- β -O-4'-dihydroconiferyl alcohol	0	0.0	0.0	0.0
13	4-Hydroxycatechol (hydroxyhydroquinone)	0	0.0	0.0	0.0
14	Sakuraresinol	6.4	0.0	5.8	28.1
15	Syringaresinol	2.2	0.0	7.5	23.8

*The cells were incubated with 50 ng/mL LPS and 10, 25 and 50 μ M of pure compounds (1-15). After 24 h, the supernatants were collected for determination of NO by Griess reaction and PGE₂ levels by EIA assay. Data is the mean $\pm$ S.D. of three different samples.

2.8. Figures

Fig. 1 Compounds isolated from an ethyl acetate extract of Canadian maple syrup (MS-EtOAc).

Fig. 2 Effect of MS-EtOAc extract on NO (a) and PGE₂ (b) production in LPS-stimulated RAW 264.7 cells.

Fig. 3 Effects of MS-EtOAc on iNOS mRNA and protein levels in LPS treated macrophages.

Fig. 4 Effects of MS-EtOAc on COX-2 mRNA and protein expressions in LPS-stimulated RAW264.7 macrophages.

Fig. 5 Effects of MS-EtOAc on LPS induced NF- κ B luciferase activity in macrophages.

Supplementary Fig. 1

Effect of MS-EtOAc extract on cell viability in LPS-induced RAW 264.7 cells. The cells were co-incubated with LPS (50 ng/mL) and different concentrations of MS-EtOAc extract ranging from 10-100 μ g/mL for 24 h. The MTS-PMS reagent (20 μ L) was then added and the optical densities were measured after 2 h. The values are expressed as the means $\pm$ S.D. of three individual samples.

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***In vitro* hypoglycemic and lipid lowering effects of a standardized food grade maple syrup extract (MSX)**

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3.1. Abstract

Pure maple syrup is highly regarded as a natural sweetener. Our laboratory has conducted extensive chemical and biological studies characterizing maple syrup and we have developed a food grade standardized maple syrup extract (named, MSX), which is well tolerated in rats. In this project, firstly we explored mechanism involved in hypoglycemic effects of MSX in HepG2 cells. Glucose lowering effects of MSX (at 100 µg/mL) was attributed to increased AMPK phosphorylation and lowered gluconeogenic gene expression as compared to control. Secondly, we also evaluated the anti-lipogenic effect of MSX treatment in mature differentiated 3T3-L1 murine adipocytes and human visceral adipocytes. MSX treatment (at 50, 100 µg/mL concentrations) to mature adipocytes decreased lipid accumulation compared to control in both murine and human adipocytes. In 3T3-L1 adipocytes, this effect was associated with downregulation of adipo/lipogenic protein expression (e.g. PPAR γ , SREBP-1). We also observed reduced mRNA expression of the pro-inflammatory mediators, namely IL-6, and TNF- α . The current study adds to the growing body of *in vitro* and *in vivo* data supporting the biological effects and potential health benefits of the natural sweetener, maple syrup.

Keywords: Maple syrup; Phenolics; 3T3L1; HepG2; AMPK; gluconeogenesis; lipogenesis

Abbreviations

Type 2 diabetes mellitus (T2DM); 5' Adenosine monophosphate-activated kinase (AMPK); Insulin resistance (IR); Peroxisome proliferator-activated receptor γ (PPAR γ); CCAAT-enhancer-binding protein (C/EBP α); sterol-regulatory element-binding protein (SREBP-1); Nuclear factor kappa B (NF- κ B); Dulbecco's modified Eagle's medium (DMEM); Hank's balanced salt solution (HBSS); Fetal bovine serum (FBS); Fetal calf serum (FCS); Oil Red O (ORO), Isobutylmethylxanthine (IBMX); Dimethyl sulfoxide (DMSO); Phosphate buffered saline (PBS); Quantitative real-time polymerase chain reaction (qRT-PCR); Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); Polyvinylidene difluoride (PVDF) membranes; Tris buffered saline/Tween-20 (TBST); Phosphoenolpyruvate carboxykinase (PEPCK); Glucose-6-phosphatase (G6pase); Acetyl-CoA carboxylase-1 (ACC-1); Glucose transporter (GLUT4), Fatty-acid-binding protein (FABP4); Lipoprotein lipase (LPL); Fatty acid synthase (FAS); Tumor necrosis factor- α (TNF- α); Interleukin-6 (IL-6)

3.2. Introduction :

In last few decades, the prevalence of type 2 diabetes mellitus (T2DM) has radically increased. Insulin resistance (IR) along with hyperglycemia and hyperinsulinaemia is the hallmark of T2DM. In T2DM, hyperglycaemia is predominantly caused by atypical increase in hepatic gluconeogenesis, leading to drastic increase in glucose levels (1, 2). Metformin, a well-known anti-diabetic drug, primarily corrects hyperglycaemia and hyperinsulinaemia by lowering hepatic gluconeogenesis via 5' adenosine monophosphate-activated kinase (AMPK) activation (3).

There is also an established link between obesity and diabetes due to related inflammatory processes (4). Obesity leads to increased circulation of pro-inflammatory markers leading to 'metabolic inflammation' which has been linked to the pathogenesis of IR and T2DM in humans and animal models (4). The de-lipidating effects of a well-studied polyphenol, resveratrol, via peroxisome proliferator-activated receptor γ (PPAR γ), CCAAT-enhancer-binding protein (C/EBP α) and sterol-regulatory element-binding protein (SREBP-1) etc., is well established (5). Phenolics have attracted significant research attention due to their diverse biological functions and potential positive effects on human health (6-8). Plant natural products, especially those rich in phenolics, have been shown to exert anti-inflammatory properties and therefore may have beneficial effects against diseases with an inflammatory component such as diabetes, obesity, cancer, cardiovascular disease, and arthritis(9-14).

Despite the availability of a wide variety of sweeteners, there is increasing consumer demand for *natural sweeteners* among which pure maple syrup is highly regarded. Maple syrup has different grades (ranging from light to amber to dark, based on light transmittance) and is predominantly used for table consumption. Notably, the maple syrup industry generates large volumes of dark and 'off-flavored' grades of maple syrup, which are not suitable for table consumption and are regarded as industrial 'by-products'. Similar to other food industries, for e.g. cranberry extracts being produced from the squeezed fruit pulp left over after juice extraction, these maple by-products are viable starting materials for the maple syrup industry to use to develop a food grade maple syrup-derived botanical extract. Recently, our laboratory has conducted extensive chemical and biological studies on a laboratory-scale phenolic-enriched maple syrup extract. This work led to development of a nutraceutical grade maple syrup-derived extract (named, MSX) starting from by-products of the industry (15). MSX is chemically standardized and well tolerated by rats (non-toxic at doses of up to 1000 mg/kg/day), has a reduced sugar content compared to maple syrup (ca. 50% vs. 66%; constituting sucrose and *complex polysaccharides*) and maintains *the natural profile of constituents* (minerals, vitamins, organic acids, amino acids, phytohormones and phytochemicals) found in the food. Dietary compounds 'phenolics' are predominantly present among other phytochemicals in maple syrup extract. Given the beneficial health effects of polyphenols, herein we propose that polyphenol-rich MSX with unique chemical matrix would exert potential positive biological effects.

Our recent published studies have shown that a laboratory scale phenolic-enriched extract of maple syrup decreases nuclear factor kappa B (NF- κ B) activity and downstream inflammatory targets in murine macrophages (16). Moreover, maple syrup extracts have been shown to have antioxidant (6), α -glucosidase enzyme inhibitory (17), anticancer (18) and anti-inflammatory (19) properties *in vitro*. In addition, maple syrup has been shown to have liver protective effects (20), improve metabolic response (21), and ability to reduce plasma glucose level compared to a sucrose solution alone (22, 23) in animal models. Recently, St-Pierre et al., (2014) reported that maple syrup produced low glycaemic and insulinaemic responses *in vivo*, and thus represents a natural sweetener alternative to refined sugar (21). Recently, our lab has reported MSX treatment lowered media glucose levels as compared to control in human HepG2 cells (15). However, the mechanism behind hypoglycemic effect of MSX is still unknown. Therefore, given all of above published *in vitro* and animal data, we believe that further investigation into demonstrating the hypoglycemic and hypolipidemic effects of this novel MSX extract is warranted.

In this investigation, we hypothesized that nutraceutical grade MSX extract lowered glucose levels via AMPK phosphorylation in hepatocytes. Moreover, we postulate the de-lipidating effects of MSX extract by downregulating the lipogenic targets in mature adipocytes.

3.3. Materials and Methods :

3.3.1. Chemicals

The cell lines HepG2 and 3T3-L1 preadipocytes were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Human visceral adipocytes and the reagents for maintaining and differentiating them were purchased from Lonza biotechnology. Cell culture reagents such as Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS) and fetal calf serum (FCS) were obtained from Lonza biotechnology. Trypsin-EDTA (ethylenediaminetetraacetic acid) and penicillin–streptomycin were bought from Gibco BRL (Gaithersburg, MD, USA). Metformin, resveratrol, Oil Red O (ORO), isobutylmethylxanthine (IBMX), dimethyl sulfoxide (DMSO), primary antibody anti- β -actin and peroxidase-conjugated secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). All primary antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA) except anti-SREBP1 and anti-PEPCK antibodies were purchased from Abcam. ECL™ detection reagent was bought from GE Healthcare (Buckinghamshire, UK). Glucose assay kit was purchased from Eton Bioscience (San Diego, CA, USA). High-capacity cDNA reverse transcription kit was from Applied Biosystems (Foster City, CA, USA). SYBR Green PCR Master Mix was obtained from Roche Applied Science (Indianapolis, IN, USA). Protein assay kit was purchased from Bio-Rad. Immobilion polyvinylidene difluoride (PVDF) membranes were bought from Millipore EMD Corporation (Billerica, MA, USA). Zhang et al. (2014) had

previously described the preparation and chemical composition of the maple syrup nutraceutical extract (named, MSX), which was used in this study (15).

3.3.2. HepG2 Cell culture and treatment

HepG2 cells were maintained in a high-glucose (4.5 g/L) DMEM supplemented with 10% FBS, 2 mM glutamine, 1000 U/L penicillin and 100 mg/L streptomycin at 37 °C, 5% CO₂, as described previously (15). The cells were seeded into a 12-well plate at a density of 4×10^4 cells/well and cultured for 6-8 h. The cells were incubated with the low-glucose (1 mg/L) DMEM detection media supplemented with 2 mM glutamine and 1% FBS. After overnight incubation, the cells were treated with metformin (1 mM) or MSX extract (at 50 and 100 µg/mL; stock solutions made in DMSO) diluted in the low glucose detection medium. The final DMSO concentration was 0.1% (v/v) in the test extracts. The cell supernatants and the cell lysates were collected after 24 h treatment.

3.3.3. Adipocyte culture and treatment

3T3-L1 preadipocytes were maintained in DMEM containing 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin to confluence at 37°C. The cells were seeded in 6-well and 24-well plates Adipocyte differentiation was induced at 2 days post-confluence (Day 0), using a mixture of IBMX (0.5 mM), dexamethasone (0.25 µM), and insulin (5 µg/mL) in DMEM containing 10% of FBS. On day 2, day 4 and day 6, this medium was replaced with DMEM

containing 10% of FBS and insulin only. The medium was switched to DMEM containing 10% of FBS on day 8 for 24 h. On day 9, the cells were treated with MSX extract (at 50 and 100 µg/mL) and after 48 h the cell culture media and cell lysates were collected and stored at -80°C until analysis. To put the data from our assays into context, other therapeutics known to impact adipocyte function were included, such as metformin (4 mM) or resveratrol (50 µM/10 µg/mL) in these *in vitro* assays. These concentrations were chosen based upon previous studies that have reported the effective concentrations of the above-mentioned positive controls in the adipocytes for lowering the lipid content (24, 25).

Human visceral adipocytes were cultured and differentiated as per the manufacturer's instructions (Lot # 0000313366). Briefly, human primary preadipocytes were grown to confluence in Preadipocyte growth medium-2 provided in 5% CO₂ at 37°C. When the cells reached confluence, the medium was replaced with Preadipocyte differentiation medium supplemented with a cocktail of insulin, dexamethasone, indomethacin and isobutyl-methylxanthine to Preadipocyte Growth Medium-2 for 10 days and cells were monitored for lipid droplet formation. After 10 days of culture, the adipocyte medium was withdrawn and cells were incubated with the MSX extract (100 µg/mL) and positive controls. After 24 h, the cell culture media was removed and the cells were stained with ORO solution for visualizing lipid droplets.

3.3.4. Glucose Consumption Assay

The glucose concentration in the HepG2 cell supernatants was determined using a glucose assay kit as per the manufacturer's instructions. Absorbance was measured at 490 nm using a spectrophotometer (SpectraMax M2, Molecular Devices Corp, Sunnyvale, CA, USA), and the assay was performed in triplicates.

3.3.5. Oil red O staining and quantification

During differentiation of pre-adipocytes into adipocytes, intracellular lipid droplets are accumulated. ORO dye is used for staining these lipid droplets (26, 27). Briefly, after incubation with test extracts for given period of time the cells were washed twice with phosphate buffered saline (PBS); followed by fixing with 10% formalin for 1 h. The cells were washed twice with PBS and stained with filtered ORO solution (150 mg/ 50 mL Oil Red O and 60% (v/v) isopropanol in distilled water) for 15 min. The cells were then thoroughly washed with distilled water before imaging under an optical microscope at x10 magnification (EVOS FL Auto, Life technologies, Carlsbad, CA, USA).

Then the Oil Red O retained in the cells was extracted with isopropanol, the absorbance was spectrophotometrically determined at 570 nm using SpectraMax M2 (Molecular Devices Corp, Sunnyvale, CA, USA).

3.3.6. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted using a Trizol reagent and 1 µg of the extracted RNA

was reverse transcribed into cDNA using a cDNA reverse transcription kit. Then the mRNA expression levels were quantified by quantitative real-time PCR using SYBR Green PCR Master Mix and the Lightcycler 480 Real Time PCR system (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. The quantification of gene expression with real-time PCR data was calculated relative to β -actin gene expression.

3.3.7. Western blot

The cells were washed once with phosphate-buffered saline and lysed in RIPA buffer. Protein concentration was measured using a bichonic protein assay kit. Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. After 1 h incubation in blocking solution (5% skim milk), the membranes were incubated overnight with primary antibodies in cold room. The membranes were washed thrice with Tris buffered saline/Tween-20 (TBST) for 10 min each followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membranes were again washed three times with TBST and then developed on X-ray films using ECL[™] detection reagent. The bands were quantified using the Image J program (NIH, Washington, DC, USA).

3.3.8. Statistical analyses

All statistical analyses were carried out using the software program GraphPad Prism Version 5.0 (GraphPad Software, La Jolla, CA, USA). Results are expressed as the means $\pm$ the standard error of the mean (S.E.). The experimental data was analyzed by unpaired one-tailed t-test (for comparing differences between two individual groups) or one-way ANOVA (for comparing differences between all groups together) followed by a Dunnett's multiple comparison test. $p < 0.05$ was considered significant.

3.4. Results & Discussion:

3.4.1. *MSX increase glucose consumption in HepG2 cells by downregulating gluconeogenic genes via AMPK activation*

It has been reported that natural products can regulate glucose and lipid metabolism in HepG2 liver hepatocytes (28). Similarly, in our recent MSX study (15) in HepG2 cells, MSX lowered glucose levels in the media along with clinical anti-diabetic drug metformin, after brief incubation for 7 h. Given that MSX decreased glucose levels in HepG2 cells through increased glucose consumption, it was investigated whether MSX constituents are activators of the AMPK pathway, which suppress gluconeogenesis (29), similar to metformin (30). Herein, the treatment was extended further for 24 h in HepG2 cells as contrast to our recent study in which the cells were treated only for 7 h. Briefly, HepG2 cells were incubated with low glucose media overnight followed by treatment with MSX (50 and 100 $\mu\text{g/mL}$) or metformin (100 μM or

1 mM) for 24 h. The cell culture media was collected for measurement of glucose. As shown in Fig. 1, MSX (100 µg/mL) reduced media glucose levels to 70% of DMSO treated control cells. Next, the expression of gluconeogenic rate limiting enzymes, (e.g. phosphoenolpyruvate carboxykinase [PEPCK] and glucose-6-phosphatase [G6pase], was evaluated (31). After 24 hrs MSX (100 µg/mL) treatment, G6pase mRNA expression was reduced to 68% of control. However, PEPCK mRNA expression was similar to vehicle-treated controls (Fig. 2).

In order to conduct further investigation into gluconeogenic pathway, upstream regulator AMPK protein expression was studied. AMPK activation is commonly detected by monitoring its phosphorylation at Thr-172 and phosphorylation of downstream target acetyl-CoA carboxylase-1 (ACC-1), using phospho-specific antibodies. ACC-1 is a key enzyme involved in fatty acid synthesis, which is phosphorylated and inactivated by AMPK (32). MSX (100 µg/mL) slightly increased the ratio of pAMPK/ AMPK and pACC-1/ ACC-1 by about 40% and 26% of control, respectively (Fig. 3).

Moreover, MSX reduced the expression of PEPCK protein to about 55% and 43% of control at 50 µg/mL and 100 µg/mL concentration, respectively. Thus, MSX could be inhibiting PEPCK at translational level (Fig. 3) but not transcriptionally (Fig. 2).

Thus, glucose-lowering effect of MSX at higher concentration (100 µg/mL) could be attributed to downregulation of G6pase gene expression and PEPCK protein expression through AMPK activation. These results supported the

published *in vivo* studies with maple syrup; which reported reduced plasma glucose levels compared to a sucrose solution alone in an anti-diabetic animal model (22, 23). However, this is a preliminary study and further more detailed *in vivo* studies are needed to support this data.

3.4.2. MSX imparts lipid-lowering effects in mature mouse and human adipocytes

Next, MSX was evaluated for anti-lipogenic effects in mature mouse 3T3-L1 and human visceral adipocytes. Figs. 4A and 4C depict ORO staining of lipid droplets in 3T3-L1 adipocytes and human visceral adipocytes treated with DMSO vehicle (0.1%), resveratrol (50 μ M), metformin (1- 4 mM), or MSX (50 and/or 100 μ g/mL). Metformin and resveratrol were selected because of documented anti-lipogenic activity in both differentiated 3T3-L1 and human adipocytes (24, 25, 33). Resveratrol, at the concentration selected, reduced the staining to 78% of vehicle-treated control adipocytes (Fig. 4B). In contrast, metformin and MSX (50 and 100 μ g/mL) decreased the ORO staining by approximately 40-50% of control (Fig. 4B). To put our data in context with human health perspective, we tested MSX in mature adipocytes from a human donor. Metformin and MSX treatment reduced ORO staining in human adipocytes to 64% and 43% of vehicle-treated controls, respectively (Fig. 4D).

3.4.3. MSX downregulated lipogenic targets in mature 3T3-L1 adipocytes.

In metabolic syndrome, adipocytes play crucial role in lipid storage and metabolism (34). In adipocytes, several transcription factors act in concert during the adipogenesis and lipogenesis phase, including PPAR γ , C/EBP α , and SREBP-1 (34-36). In order to explore the molecular mechanisms involved in MSX anti-lipogenic effects, the cell lysates were collected after 48 h treatment for evaluating lipogenic target protein expression in mature 3T3-L1 adipocytes. Fig. 5 illustrates that MSX downregulated adipo/lipogenic transcription factors (i.e. PPAR γ , C/EBP α and SREBP-1 protein expression). PPAR γ and C/EBP α regulate the expression of variety of genes that are involved in different pathways including insulin sensitivity (glucose transporter GLUT4), lipogenesis (fatty-acid-binding protein, FABP4) and lipolysis (lipoprotein lipase, LPL, perilipin) and the secreted factors (adiponectin and leptin) (37). Here, we investigated the effect of MSX treatment on two targets, FABP4 and perilipin, in differentiated adipocytes. FABP4 is involved in fatty acid uptake, transport, and metabolism (38), whereas perilipin is localized at the periphery of lipid droplets and plays a pivotal role in lipid storage (39). MSX treatment downregulated the expression of these downstream targets in a concentration dependent manner. SREBP-1 induces the expression of lipogenic enzymes, including ACC-1 and fatty acid synthase (FAS) (34). MSX decreased protein expression of both ACC-1 and FAS in a concentration dependent manner. These observations depict decreased protein expression of adipo/lipogenic

targets after MSX treatment, which are consistent with reduced lipid staining in MSX-treated mature adipocytes depicted in Fig 4.

It is now well accepted that inflammation and associated pro-inflammatory processes are centrally linked to several chronic human diseases including obesity and diabetes (40). Hence, after exhibiting anti-lipogenic effect, MSX also demonstrated specific effects on mature adipocytes that likely contribute to its overall anti-inflammatory effects. TNF- α and IL-6 are two key mediators of the inflammatory response in mature 3T3-L1 adipocytes that have been linked to obesity-related inflammation. Thus, MSX effect on adipocyte inflammation was explored by measuring TNF- α and IL-6 mRNA expression (Fig. 6). At 48 hours, resveratrol reduced TNF- α or IL-6 mRNA expression to 60% and 40% of control respectively. In addition, MSX treatment at 50 μ g/mL also reduced TNF- α and IL-6 to about 20% of control, however 100 μ g/mL MSX treatment reduced both inflammatory targets to 36-47%. This is in agreement with our previous research where we have exhibited anti-inflammatory effects of MSX (15) and maple syrup ethyl acetate extract (16) in lipopolysaccharide-induced macrophages. Moreover, in this study we have demonstrated inhibitory effect of MSX treatment on inflammatory markers in mature adipocytes. This is not surprising as MSX contains high levels of polyphenols, which are well-known antioxidants and anti-inflammatory agents (41). Interestingly, MSX contains stilbenes which are structurally related to resveratrol, a well-known polyphenol, thus we could anticipate MSX anti-lipogenic and anti-inflammatory effects (15).

MSX also contains high quantity of phytohormone abscisic acid (ABA), which has been proposed to improve insulin sensitivity and obesity-related inflammatory diseases through a PPAR γ -dependent mechanism. ABA is structurally similar to thiazolidinediones (drugs prescribed for diabetic management) (42). Dietary abscisic acid has been reported to improve glucose tolerance and obesity-related inflammation in db/db mice fed high-fat diet (43). MSX is particularly rich in lignans and these polyphenolic compounds are also found in flaxseed. Interestingly, flaxseed lignan have been shown to inhibit fat accumulation and induce adiponectin expression in high-fat diet-induced mouse model (44).

In conclusion, as depicted in Fig. 7 the lowered glucose levels after MSX treatment were attributed to AMPK activation in HepG2 hepatoma cell model. However, the effect of MSX-treatment were not drastic in HepG2 cells. Moreover, we demonstrated delipidating effects of MSX extract treatment by downregulating protein expression of adipo/lipogenic transcription factors and their downstream targets in differentiated 3T3L1 adipocytes (Fig. 6). In future, it would be interesting to explore mechanistic insights into obesity-linked inflammatory pathway using MSX-treated human adipose tissue explants.

This study, using in vitro models, provided preliminary data necessary to guide and support future in vivo studies investigating the potential use of a novel food grade maple syrup extract (MSX) as a nutraceutical preparation for positive impact on overall health.

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Conflict of interest

The authors declare no conflicts of interest.

3.5. Figure legends

Fig. 1. Effect of MSX extract on glucose levels in the media in HepG2 hepatoma cells. Cells were treated with MSX (50 and 100 $\mu\text{g/mL}$) or metformin (100 μM) in a low glucose media for 24 h and the cell supernatants were collected. MSX extracts inhibited glucose levels in the media in a concentration-dependent manner. The values are expressed as the means $\pm\text{SE}$ of three individual samples. “*” indicates $P < 0.05$ as compared with the control.

Fig. 2. Effect of MSX extract on mRNA expression of genes involved in gluconeogenesis, namely G6pase (A) and Pepck (B) in HepG2 hepatoma cells. Cells were treated with MSX (50 and 100 $\mu\text{g/mL}$) or metformin (1 mM) in a low glucose media for 24 h and the cell lysates were collected. MSX extracts downregulated the gene expression of G6pase (A) and Pepck (B). The values are expressed as the means $\pm\text{SE}$ of three individual samples. “*” indicates $P < 0.05$ as compared with the control.

Fig. 3. Effect of MSX extract on protein expression of pAMPK, pACC and Pepck in HepG2 hepatoma cells. Cells were treated with MSX (50 and 100 $\mu\text{g/mL}$) or metformin (1 mM) in a low glucose media for 24 h and the cell lysates were collected. MSX extracts upregulated the expression of pAMPK, pACC, and Pepck.

Fig. 4. Effect of MSX extract on lipid accumulation by ORO staining and quantification in mature 3T3-L1 (A,B) and human visceral adipocytes (C,D). Cells were treated with MSX (50 and 100 µg/mL) or metformin (1 or 4 mM) or resveratrol (50 µM) for 48 h and ORO staining was performed to stain and quantify lipid droplets in differentiated murine adipocytes (4A & 4B) and in mature human visceral adipocytes (4C & 4D). ‘*’ indicates $P < 0.05$ and ‘**’ indicates $P < 0.01$ as compared with the control.

Fig. 5. Effect of MSX extract on protein expression of lipogenic targets after MSX treatment in mature 3T3-L1 cells. Cells were treated with MSX (50 and 100 µg/mL) or metformin (1 or 4 mM) or resveratrol (50 µM) for 48 h and the cell lysates were collected after 48 h MSX treatment in mature 3T3-L1 adipocytes for protein expression. MSX extracts downregulated the protein expression of adipo/lipogenic targets.

Fig. 6. Effect of MSX extract on gene expression of inflammatory markers in mature 3T3-L1 adipocytes. Cells were treated with MSX (50 and 100 µg/mL) or resveratrol (50 µM) for 48 h and the cell lysates were collected for mRNA expression. MSX extracts reduced the expression of inflammatory genes TNF- α and IL-6 in differentiated 3T3-L1 cells. The values are expressed as the means $\pm$ SE of three individual samples. Significant differences between groups were determined using a one-way ANOVA test followed by Dunnett’s multiple comparison test. ‘*’ indicates $P < 0.05$ as compared with the control.

Fig. 7. MSX hypoglycemic and anti-lipogenic effect in HepG2 cells and 3T3-L1 cells.

3.6. Figures :

Fig. 2. Effect of MSX extract on mRNA expression of genes involved in gluconeogenesis, namely G6pase (A) and Pepck (B) in HepG2 hepatoma cells.

Fig. 3. Effect of MSX extract on protein expression of pAMPK, pACC and Pepck in HepG2 hepatoma cells.

Fig. 4. Effect of MSX extract on lipid accumulation by ORO staining and quantification in mature 3T3-L1 (A,B) and human visceral adipocytes (C,D).

Fig. 5. Effect of MSX extract on protein expression of lipogenic targets after MSX treatment in mature 3T3-L1 cells.

Fig. 6. Effect of MSX extract on gene expression of inflammatory markers in mature 3T3-L1 adipocytes.

Fig. 7. MSX hypoglycemic and anti-lipogenic effect in HepG2 cells and 3T3-L1 cells.

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SUMMARY AND CONCLUSION

1. In this thesis, we have demonstrated that bioactive polyphenol-rich extracts namely- Longvida® and food grade maple syrup extract exert positive health benefits beyond basic nutrition, thus impacting overall health and wellness.
2. A novel curcumin formulation Longvida® showed improved solubility over unformulated curcumin, and significantly decreased the LPS-induced pro-inflammatory mediators in macrophages.
3. Maple syrup extract and its purified compounds significantly inhibited the production of inflammatory markers through the direct inhibition of NF-kB transcriptional activity in stimulated macrophages. Overall, the cellular mechanisms underlying these multiple effects may be attributed to the unique combination of pure compounds present in the extract, which appear to act *in toto*.
4. We demonstrated delipidating effects of food-grade MSX extract treatment by downregulating protein expression of adipo/lipogenic transcription factors and their downstream targets in differentiated murine 3T3L1 adipocytes. MSX treatment also decreased lipid accumulation in human adipocytes. MSX extract could also have potential application in obesity-linked inflammation.
5. In conclusion, this study, using in vitro models, provided preliminary data necessary to guide and support future in vivo studies investigating the potential use of a novel curcumin formulation Longvida® and food grade maple syrup extract (MSX) as a nutraceutical preparation for positive impact on overall health.