Inhibitory Properties of Functional Food Plants on CYP Enzymes and Cree Traditional Medicines on Aldose Reductase

San Nguyen

Thesis submitted to the

Faculty of Graduate and Postdoctoral Studies

University of Ottawa

in partial fulfillment of the requirements for the

M.Sc. degree in the

Ottawa-Carleton Institute of Biology

Acknowledgements

First and foremost, I would like to thank my teacher, my mentor, and supervisor, Dr. John Thor Arnason for the unyielding support and guidance. Dr. Arnason, I am so honored to have had the opportunity to work with you. Words can't express the respect and gratitude that I have for you. Your kindness, generosity and caring nature for your students truly extend beyond the scope of research and academics. For this, I thank you.

The first half of this thesis could not have been realized without the efforts of Dr. Humayoun Akhtar and Dr. Brian C. Foster. In addition, I would like to extend my gratitude towards Dr. Tim Xing and Dr. Doug Johnson for their contribution as committee members. In working with you all, I have learned a great deal and have gained valuable professional experiences. Finally, I would also like to acknowledge my partner and co-author, Huang Huang, for his contributing work towards this thesis as well as his moral support.

The second half of this thesis was an incredible feat within a very short period of time. This accomplishment was the result of an incredible collaborative effort lead by Dr. Pierre Haddad and the CIHR Team in Aboriginal Antidiabetic Medicines. Thank you all for the opportunity to join your team and make my small contribution to your incredible project.

To my lab colleagues, thank you all for the support, knowledge and friendships that has made the last 3 years unforgettable. I owe many thanks to Ammar Saleem, Jose-Antonio Guerrero and Rui Liu for never saying "no". Despite my many requests and questions, you always took the time to provide help and answers with the utmost sincerity.

To my family, this journey would have been much more difficult without you all. Mom and dad, thank you for your unconditional love and support regardless of my trials and mistakes.

To my brother, Tom, thank you for pushing me forward. A guy couldn't ask for a greater brother. To my sisters, Phi and Tiana, thank you for carrying me through the difficult times with your laughter. Finally, to Sophanny and Kimberly, the two love of my life, thank you for embarking on this journey with me. It was a long and rocky road, but we made it.

Abstract

This thesis examines the cytochrome P450 (CYP) drug metabolizing enzyme inhibition and antimicrobial properties of 46 common food plants available in the Canadian Market and the inhibitory properties of 17 traditional Cree antidiabetic medicines on aldose reductase. Inhibitory activity profiles of CYP 3A4, 3A5, 3A7 and 2D6 were created for the 46 samples. The most active plants in the CYP inhibition assay were the spices, belonging to the *Apiaceae* and *Lamiaceae*. Similarly, the most active plants in the antimicrobial assay were also the *Apiaceae* and *Lamiaceae.* Swine lens homogenate was tested as a novel model for the aldose reductase inhibition assay. Several Cree plants selected for the aldose reductase study showed a high activity, primarily in samples which also contained high levels of phenolics. A positive correlation was observed between total phenolics content and aldose reductase inhibition r^2=0.44, p=0.05. Crude extracts of *Rhododendron groenlandicum* exhibited inhibitory activities of 35.11 ± 0.16 %. The subfractionation and HPLC analysis of *R. groenlandicum* revealed high levels of phenolics compounds including, catechin, epicatechin, quercetin and quercetin glycosides. This study found that functional botanicals, consumed as foods or for medicinal purposes, contain phytochemicals that may cause a wide range of biological effects, including beneficial remediation of diabetic complications, or detrimental inhibition of drug metabolizing enzymes and of the gut microflora.

Résumé

Nous avons étudié dans cette thèse les capacités de 46 plantes comestibles, disponibles sur le marché canadien, à inhiber le cytochrome P450 (CYP), enzyme responsable du métabolisme des médicaments, les propriétés antimicrobiennes, et les propriétés inhibitrices de l'aldose réductase à partir de 17 médicaments antidiabétiques traditionnellement utilisés par les Cris. Les profils de l'activité inhibitrice du CYP 3A4, 3A5, 3A7 et 2D6 ont été réalisés pour les 46 plantes à l'étude. Les plantes les plus actives dans le test d'inhibition du CYP furent les épices, plantes appartenant aux familles des *Apiaceae* et *Lamiaceae*. De même, les plantes les plus actives dans le bioessai antimicrobien furent aussi les plantes de ces deux mêmes familles. Un homogénat de cristallin de porc a été utilisé comme modèle nouveau pour le test d'inhibition de l'aldose réductase. Plusieurs plantes, utilisées par les nations Cree, qui ont été sélectionnées pour l'étude, ont montré une forte activité inhibitrice de l'aldose réductase, principalement dans les échantillons qui contenaient des teneurs élevées en composés phénoliques. Une corrélation positive a été observée entre la teneur totale en composés phénoliques et l'inhibition de l'aldose réductase ($r^2 = 0.44$, $p = 0.05$). Des extraits bruts de *Rhododendron groenlandicum* ont montré des activités inhibitrices de 35.11 ± 0.16%. Le sous-fractionnement et l'analyse HPLC de *R. groenlandicum* ont aussi révélé des teneurs élevées de composés phénoliques, incluant la catéchine, l'épicatéchine, la quercétine et les glycosides de quercétine. Cette étude a révélé que les plantes dites fonctionnelles, consommées à des fins alimentaires ou médicinales, contiennent des composés qui peuvent causer un large éventail d'effets biologiques dont la baisse de complications dues au diabète et l'inhibition d'enzymes métabolisant des médicaments ainsi de la microflore intestinale.

TABLE OF CONTENTS

TITLE PAGE ..... **i**

ACKNOWLEDGEMENTS ..... **ii**

ABSTRACT ..... **iv**

TABLE OF CONTENTS ..... **vi**

LIST OF TABLES ..... **ix**

LIST OF FIGURES ..... **x**

LIST OF ABBREVIATIONS ..... **xii**

1.0.0 GENERAL INTRODUCTION AND LITERATURE REVIEW **1**

1.1.0 Foods as medicines versus medicinal plants **4**

1.2.0 Rationale of study **7**

CHAPTER 2 PREFACE **10**

2.0.0 EFFECTS OF FUNCTIONAL FOODS ON HUMAN HEALTH AND WELLNESS: ANTIMICROBIAL AND P450 INHIBITORY PROPERTIES OF COMMON FOOD PLANTS **12**

2.1.0 Introduction **13**

2.2.0 Materials and Methods **18**

2.2.1 Chemicals and reagents **18**

2.2.2 Sample collection **18**

2.2.3 Sample extraction 19

2.2.4 Fluorometric microtitre cytochrome P450 inhibition assays 19

2.2.5 Antimicrobial assay 21

2.3.0 Results 22

2.4.0 Discussion 32

CHAPTER 3 PREFACE 36

3. EFFECTS OF TRADITIONAL ANTIDIABETIC MEDICINES OF THE CREE FIRST NATIONS OF IYOUISTCHEE ON ALDOSE REDUCTASE, AN ENZYME IMPLICATED IN DIABETIC CATARACTOGENESIS 38

3.1.0 Introduction 39

3.2.0 Materials & Methods 43

3.2.1 Plant Collection 43

3.2.2 Plant extraction 44

3.2.3 Materials and chemicals 44

3.2.4 Lens isolation 45

3.2.5 Aldose reductase inhibition assay 45

3.2.6 R. groenlandicum collection 47

3.2.7 Extraction and fractionation of R. groenlandicum for phytochemical analysis 47

3.2.8 Preliminary fractionation of R. groenlandicum 48

3.2.9 Aldose reductase inhibition of R. groenlandicum fractions F1-F9 51

3.2.10 Sub-fractionation of fractions F7 and F8 of R. groenlandicum **51**

3.2.11 Aldose reductase inhibition assay of R. groenlandicum sub-fractions F7 and F8 ..... **55**

3.2.12 HPLC analysis..... **55**

3.3.0 Results **56**

3.3.1 Preliminary screening..... **56**

3.3.2 AR inhibitory potential of R. groenlandicum fractions F1-F9 ..... **56**

3.3.3 AR inhibitory potential of R. groenlandicum of sub-fractions of F7 and F8 ..... **63**

3.3.4 HPLC Analysis..... **63**

3.4.0 Discussion..... **68**

4.0.0 GENERAL DISCUSSION **73**

4.1.0 Main Conclusions..... **74**

4.2.0 Future Work..... **77**

5.0.0 CITED REFERENCES **79**

6.0.0 Appendix..... **86**

LIST OF TABLES & SCHEMATICS

Schematic 3.1. Extraction and primary fractionation scheme for *R. groenlandicum* **.....49**

Table 2.1. Germplasm and spices selected by AAFC for Cytochrome P450 inhibition assays**....16**

Table 2.2. Antimicrobial effects of methanolic and ethanolic extracts of selected *Apiaceae* and *Lamiaceae* (50 mg/ml) against 6 bacterial species **.....31**

Table 3.1. Fractionation by open column chromatography of crude extract from the leaves of *R. groenlandicum* **.....50**

Table 3.2. Fractionation by open column chromatography of active fraction, F7, from the leaves of *R. groenlandicum***.....53**

Table 3.3. Fractionation by open column chromatography of active fraction, F8, from the leaves of *R. groenlandicum***……..54**

Table APP-2.1. Activity of methanolic extracts (50 mg/ml) of samples against Cytochrome P450 3A4, 3A5, 3A7 and 2D6 isozymes expressed as mean percent inhibition (%) ± standard deviation**.....87**

Table APP- 2.2. Activity of aqueous extracts (50 mg/ml) against Cytochrome P450 3A4, 3A5, 3A7 and 2D6 isozymes expressed as mean percent inhibition (%) ± standard deviation **.....89**

TableAPP- 2.3. Antimicrobial effects of selected *Fabaceae* methanolic and ethanolic extracts (50 mg/ml) against 7 bacterial species **.....91**

Table APP-3.1. The inhibitory potential of the top 17 selected Cree antidiabetic plants expressed as a percent inhibition (%) with their respective standard errors and ranking**.....95**

Table APP-3.2. The inhibitory potential of fractions F1-F9 of *R. groenlandicum,* obtained from open column chromatography, against homogenized swine lens isolate **.....96**

Table APP-3.3. Concentration of 10 marker compounds used in HPLC analysis. Quantification was accomplished using a reference table obtained from a known standard mix. Injections were done in duplicates and n=2. Standard error is as shown**.....97**

LIST OF FIGURES

Figure 2.1 Percent inhibition of methanolic (A) and aqueous (B) extracts (50mg/ml) from common food samples on cytochrome P450 3A4 isozyme **.....23**

Figure 2.2 Percent inhibition of methanolic (A) and aqueous (B) extracts (50mg/ml) from common food samples on cytochrome P450 3A5 isozyme **.....24**

Figure 2.3 Percent inhibition of methanolic (A) and aqueous (B) extracts (50mg/ml) from common food samples on cytochrome P450 3A7 isozyme **.....26**

Figure 2.4 Percent inhibition of methanolic (A) and aqueous (B) extracts (50mg/ml) from common food samples on cytochrome P450 2D6 isozyme **.....27**

Figure 2.5 Linear relationship (r^2=0.67) between cytochrome P450 3A5 isozyme inhibition and anti-microbial activity against *Escherichia coli* **.....29**

Figure 2.6 Linear relationship (r^2=0.33) between cytochrome P450 3A7 isozyme inhibition and anti-microbial activity against *Escherichia coli* **.....30**

Figure 3.1 The polyol pathway **.....40**

Figure 3.2. The inhibition potential of *R. groenlandicum, P. balsamifera, K. angustifolia, V. vitis* and *A. balsamea* against aldose reductase acquired from homogenized swine lens **.....58**

Figure 3.3. The inhibition potential of *G. hispidula, L. clavatum, S. decora* and *R. tomentosum* against aldose reductase acquired from homogenized swine lens **.....59**

Figure 3.4. The inhibition potential of *A. incana, J. communis, P. banksiana, S. planifolia* and *P. mariana* against aldose reductase acquired from homogenized swine lens **.....60**

Figure 3.5. The inhibition potential of *S. purpurea* and *L. laricinia* against aldose reductase acquired from homogenized swine lens**.....61**

Figure 3.6. The inhibition by fractions F1-F9 from *R. groenlandicum* of aldose reductase acquired from homogenized swine lens**.....62**

Figure 3.7 Chemical structures of A. catechin, B. chlorogenic acid, C. epicatechin, D. p-coumaric acid, and E. quercetin-3-galactoside selected phenolics used as markers for HPLC analysis**.....64**

Figure 3.8 Chemical structures of F. quercetin-3-rhamnoside, G. quercetin-3-glucoside, H. myricetin, I. rutin and J. quercetin selected phenolics used as markers for HPLC analysis**.....65**

Figure 3.9. The inhibition activity of sub-fractions F7-1 to F8-13 of *R. groenlandicum* against aldose reductase isolated from homogenized swine lens**.....67**

Figure 3.10. A correlative comparison between AR inhibition and total phenolics content **.....69**

LIST OF ABBREVIATIONS

5-MOP – 5-methoxypsoralen

AAFC – Agriculture and Agri-Food Canada

AMMC - 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin

AR – aldose reductase

ARI – aldose reductase inhibition

β-NADPH - β-nicotinamide adenine dinucleotide phosphate, reduced tetra (cyclohexylammonium) salt

CDA – Canadian Diabetes Association

CIHR – Canadian Institutes of Health Research

CIHR-TAAM – Canadian Institutes of Health Research – Team Aboriginal Antidiabetic Medicines

CYP – cytochrome P450

DBF – dibenzylfluoroscein

DPPH - 2,2-diphenyl-1-picrylhydrazyl

EtOAc – ethyl acetate

F3P - fructose-3-phosphate

FC – furanocoumarin

GSH - glutathione

GSSG – glutathione disulfide

HPLC – high performance liquid chromatography

HPLC-DAD – high performance liquid chromatography – diode array detector

IP – inhibitory potential

MeOH – methanol

NAD – nicotine adenine dinucleotide

NHP – natural health product

NHPD – Natural Health Product Directorate

NRP – nutraceutical research program

OD – optical density

PBS – phosphate buffered saline

PI – principal investigator

ROS – reactive oxygen species

TIID – Type II Diabetes

TCM – Traditional Chinese Medicine

UR – uptake rate

WHO – World Health Organization

CHAPTER 1.

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

The use of plants in traditional healing is a cross-cultural practice that is universal in human cultures. One of the oldest records of the medicinal use of plants was written by the Egyptians and Mesopotamians (Retief & Cilliers, 2007) and dates to 2500 BC. Similarly, between 1000 and 2000 BC, Traditional Chinese Medicine (TCM) and Ayurvedic medicine of the Indian subcontinent developed into complex medical systems that entail the therapeutic use of thousands of methodically classified plants, animals and minerals. TCM, in particular, incorporated the study of human anatomy and physiology to map out a network of universal energy channels, "Chi", which provides the foundation for their medical diagnosis. Beginning around 1000 BC, the use of plants for healing and religious purposes is evident in the Mesoamerican archeological record. In this area, healers became specialists in using psychotropic and hallucinogenic plants to communicate with the gods (Schultes & Raffauf, 1990).

Traditional medicine is often intertwined with spirituality and cultural world view. The ancient Mesopotamians believed that illnesses are caused by troubled spirits or ghosts, and require both natural and magical intervention from healers (Koenig *et al*, 2001). The foundations of traditional Chinese medicine are also based heavily on religion and supernatural elements. Elements of Earth, wood, water, wind and fire, taken from Taoism, are used symbolically to categorize the different organs and life giving energies. Deficiencies in any of these elements or obstruction of the flow of Chi throughout the body would lead to illness or disease. Similarly, Maya healers of Central America integrate the use of herbal medicines and spiritual ceremonies to cure *susto,* a culture-bound illness resulting from the loss of the soul after a frightening or

startling event (Bourbonnais-Spear *et al*, 2007). It is believed that if left uncured, patients with *susto* can develop serious, or even fatal, medical conditions.

Today, traditional medicine still serves as the primary health care in as much as 80% of the population in many developing countries (WHO, 2008). Despite the modern day medical advancements, cost and accessibility is still a limiting factor for much of the population living in rural and underdeveloped areas. This is not to say that the use of traditional medicine is only common in these particular areas. In developed countries, as much as 80% of the population has used some form of traditional or herbal medicine. According to the World Health Organization (WHO, 2008), the current global market for herbal medicines is well over $60 billion USD with millions more invested into research to identify novel pharmacologically active compounds isolated from medicinal herbs. According to Craker (2007), the aging American population and the general public's new shift to a worldly and multicultural lifestyle has lead to a surge in the use and consumption of herbal and natural health products from a variety of different origins.

Similarly, a survey from Health Canada (2005) showed that about 71% of Canadians use some form of natural health product (NHP - the legal term in Canada for over the counter natural products sold with claims of health benefits) in a thriving domestic market generating approximately $2.9 billion dollars in revenue (Statistics Canada, 2007). Since the rapid rise in popularity of NHPs, the Natural Health Product Directorate was established in 2004 to regulate and standardize the manufacturing process, safety and efficacy of products sold to the public. Many consumer products regulated include vitamin and mineral supplements, herb and plant-based remedies, traditional medicines (such as Traditional Chinese Medicines or Ayurvedic Medicines), omega-3 and essential fatty acids and probiotics as well as many everyday consumer products, such as certain toothpastes, antiperspirants, shampoos, facial products and

mouthwashes. Over 20,000 products have been processed and entered in to NHPD's database as natural health products (NHPs) possessing health modifying or benefiting properties.

1.1 Foods as medicines versus medicinal plants

In traditional medicinal practices, there is no clear defining line between food and medicine (Heinrich & Prieto, 2008). Although the exact origin of traditional medicine unclear, it is evident from cultural practices such as TCM and Ayurveda, as well as historical documentation, that the use of nutritional foods to promote good health has been taught and passed on for many generations. Today the use of diet and nutrition as a preventative measure to chronic diseases has become very popular. Many individuals have begun to seek out dietary tips and tricks from ethnic foods and medicines to promote and maintain a healthy lifestyle. Historical texts on TCM remedies stress the importance of balancing opposing food groups to obtain optimal health and the detrimental effects of an unbalanced diet (Lee & Shen, 2008). Many research groups have done population-based studies on the influences of dietary patterns on the development of chronic diseases (Fung *et al*, 2001; Hirose *et al*, 2007). These studies not only highlight the health risks and outcomes of certain dietary habits, but also the benefits of traditional preventative dietary patterns as a preventative measure to chronic diseases.

The use of common food plants and herbs for health benefits and remedial application have been seen in homes globally and has recently become an area of considerable scientific interest, known as nutraceutical science or study of functional foods. If examined from a phytochemical standpoint, common food plants contain non-nutritive biologically active compounds (secondary metabolites) that can have a direct effect on human health. For example,

the potential use of food plants for cancer chemoprevention (Béliveau & Gingras, 2006) is an area of intense scientific interest at this time. Unfortunately, considerably less attention is being given to potential adverse effects that may occur with these phytochemicals such as drug interaction effects (Ioannides, 2003).

Traditionally, in indigenous societies, the collection of medicinal plants with no food usage is a ritual that entails careful identification of suitable plants, meticulous harvesting and handling methods that may include ritualistic prayers and offerings performed by trained shamans, healers or trained medical doctors. The knowledge of usage and preparation of medicines is usually passed by oral tradition from teacher to pupil through years of training, practice and guidance. In more organized cultures, traditional medicine is taught as a codified system, through rigorous curriculums based on thousands of years of recorded medical books and literature. Traditional Chinese medicine, perhaps the most well documented and complex system of medicine, is comprised of a series of interconnecting disciplines such as acupuncture, therapeutic massage, cupping, herbal medicine and food therapy that can take years of study to master.

Usage of non-food medicinal plants in indigenous cultures usually requires the experience and expertise of trained healers for several reasons. The first reason entails knowledge of the growth patterns and habitat of the medicinal plant in use. The selection methods for the harvesting of different medicinal plants were based on a variety of traits and conditions such as life stage, ripeness, plant size, habitat, seasonal conditions, soil quality and rainfall. The plant materials collected for medicinal use can be the entire plant or specific parts such as the roots, rhizomes, tubers, stem, buds, leaves, flowers, pollen, seeds and fruits (Halberstein, 2005). American ginseng, or *Panax quinquefolius*, presently endangered due to

over-harvesting, grows in shaded and rich soil heavily populated with deciduous trees, and requires 5-7 years to reach maturation and medicinal potency. Because ginseng is collected for its roots, harvesting a plant would mean destroying it entirely, making ginseng an expensive medicine. With the requirement of an 18 month dormancy period for seed germination, the appropriate and conservative method of harvesting of American ginseng requires the knowledge to be able to identify the maturation stage of the roots based on the characteristic parts of aerial parts. This level of experience is required to aid the propagation of the population of American ginseng as its lengthy life cycle and very particular habitat make it a delicate and difficult plant to sustain.

Toxicity and dosage are also reasons for the requirement of an expert when using some medicinal plants. Foxglove, for instance, is traditionally used to treat skin boils and infections but is highly toxic and can cause nausea, vomiting, diarrhea, confusion, hallucinations, and cardiac arrhythmias (Heinrich *et al*, 2004). The right quantity and dose must be determined by a health practicioner (naturopath or expert herbalist) to prevent potential poisonings. Lastly, preparation and formulation methods are important steps that require the knowledge of a healer. Some remedies require different preparation methods including mastication, grounding, alcoholic extractions, infusions, and decoctions. Phytochemical studies have found that extraction methods using different solvents will draw out different compounds which would result in different physiological and pharmacological effects when ingested. Also the formulation of remedies using a variety of different plants for synergistic or antagonistic effects will also require the knowledge of an expert.

1.2 Rationale of study

Since the early1990's, the use of functional health foods and medicinal plants in North America has seen a surge in popularity. Due to mixing of ethnic foods and cultures, as well as the growth of the global village, complementary and alternative medicines are being introduced into the North American market at an explosive rate. The integration of both functional foods and alternative medicines, for many, is becoming a daily practice as an effort to establish a healthy lifestyle.

The use of alternative medicine and functional foods has also become more of a concern over the past several years as more and more people using them. Many individuals prefer the use of preventative and therapeutic health foods over conventional drugs. This lifestyle is also coupled with the belief that health foods are safer because they are natural and not man-made. In addition, the exchange of dietary and culinary practices in North America between cultures has introduced an abundant diversity of ethnic foods with high levels of plant secondary compounds that can provide either health benefits and/or cause adverse reactions.

Many of these biological activities begin with secondary metabolite- protein interactions. While the possibilities are numerous, this thesis examines two such interrelations. The first study funded by Agriculture and Agri-Food Canada examined to what extent ethnic food plants may inhibit human drug metabolizing enzymes, specifically the CYP P450s, a risk factor for drug interactions. The second study funded by the Canadian Institutes of Health Research team in aboriginal antidiabetic medicines examined the potential benefits of traditional medicines of Canada's indigenous Cree people, as aldose reductase inhibitors which may have potential health benefits in reducing risk of cataract formation in diabetes.

The specific objectives were:

Objective 1: To determine whether selected ethnic food plants on the Canadian market have the potential to inhibit cytochrome P450 enzymes 3A4, 3A5, 3A7 and 2D6. Furthermore, among the 2 principle categories of food plants selected, the herbs and pulses, belonging to the *Fabaceae, Lamiaceae and Apiaceae*, to determine the group that exhibits the highest levels of activity based on our CYP inhibition assay.

Objective 2: To determine whether the selected top 17 antidiabetic medicinal plants from the indigenous Cree people of Northern Québec have the potential to inhibit aldose reductase and to determine which plants have the highest aldose reductase inhibitory potential. Once the most active plant is identified, bioassay guided fractionation will be used to determine the bioactive fractions and quantification of bioactive constituents.

The study in chapter two was conceived as a joint project between Agriculture and Agri-Food Canada (AAFC) functional food group (Principal investigator (PI) Dr. Humayoun Akhtar) and the medicinal natural products group at the Ottawa-Carleton Institute of Biology (PI's Dr. Brian C. Foster, Dr. John T. Arnason, Dr. Tim Xing and Dr. Myron Smith). Teresa Tam provided assistance with developing and validating the method. I completed all the P450 inhibition studies; Huang Huang completed all the antimicrobial studies. The paper was written with joint first authorship by myself and Huang Huang. The paper is being prepared for submission to Pharmaceutical Biology.

The study in chapter three was a collaborative effort with the the Canadian Institute of Health Research – Team in Aboriginal Antidiabetic Medicines, University of Montréal (PI Dr. Pierre Haddad) and the University of Ottawa (PI Dr. John T. Arnason). All AR, analytical chemistry and chromatography work was completed by San Nguyen. Antonio Guerrero, Rui Liu and Ammar Saleem provided assistance with the phytochemical analysis.

PREFACE

The study presented in this chapter is the result of a collaborative effort between the Agriculture and Agri-Food Canada, Health Canada, the Carleton University and the University of Ottawa. The project was lead by Dr. Humayoun Akhtar, Agriculture and Agri-Food Canada, Dr. Brian C. Foster, Health Canada, and Dr. John T. Arnason, University of Ottawa. The goal of this study was to screen selected natural health food products, available to the Canadian public, for any influences on cytochrome P450 enzymes and the gut microflora and create a safety datasheets and CYP inhibition profiles from the generated data. Within a period of two years, CYP inhibition profiles were created for ethanolic and methanolic extracts of 46 different food plant samples against 4 different CYP enzymes. The antimicrobial study also provided activity profiles against 7 species of bacteria. The results from this study provided evidence for CYP inhibition from food plants.

CHAPTER 2.

EFFECTS OF FUNCTIONAL FOODS ON HUMAN HEALTH AND WELLNESS: ANTIMICROBIAL AND P450 INHIBITORY PROPERTIES OF COMMON FOOD PLANTS

2. EFFECTS OF FUNCTIONAL FOODS ON HUMAN HEALTH AND WELLNESS: ANTIMICROBIAL AND P450 INHIBITORY PROPERTIES OF COMMON FOOD PLANTS

San Nguyen[1#], Huang Huang[3#], Brian C. Foster[1,2*], Teresa W. Tam[1], Tim Xing[3], Myron Smith[3], John T. Arnason[1], Humayoun Akhtar[4].

[1]Centre for Research in Biopharmaceutics and Biotechnology, University of Ottawa, 451 Smyth Rd. Ottawa, Ontario, K1H 8M5, Canada.

[2]Therapeutic Products Directorate, Health Products and Food Branch, 1600 Scott St. Ottawa, Ontario, K1A 0K9, Canada.

[3]Carleton University Biology Institute, 209 Nesbitt Building, 1125 Colonel By Drive, Ottawa, Ontario. K1S 5B6, Canada.

[4]Guelph Food Research Centre, Agriculture and Agri-Food Canada, 93 Stone Road West, Guelph, Ontario, N1G 5C9, Canada.

*Corresponding Author: B Foster

#Joint first authors: S. Nguyen and H. Huang

2.1 Introduction

Increased health awareness has led many Canadians to become more vigilant in maintaining good health. Many Canadians have incorporated natural health products (NHPs) and functional foods (foods selected for its beneficial health properties) into their daily lives to achieve optimal health and wellness. A report released by Health Canada in 2005 estimated that 71 % of the Canadian population uses NHPs on a daily basis (Murty, 2007). Both functional foods and NHPs selected for health benefits contain bioactive secondary metabolites but their roles in promoting human health has not been thoroughly studied (Gurib-Fakim, 2006). The high levels of bioactive phytochemicals in some regimens have also raised concerns about possible food-drug and NHP-drug interactions.

Functional foods and NHPs contain bioactive compounds that are metabolized by cytochrome P450 enzymes and may affect drug metabolism thereby resulting in a higher plasma concentration of xenobiotics and drugs exceeding the therapeutic dose and resulting in adverse drug reaction. Some of the major CYP enzymes involved in the metabolism of these products are CYP2D6, CYP3A4, CYP3A5 and CYP3A7. It is well established that grapefruit juice can cause interaction with conventional drugs (Bailey *et al,* 1998; Bailey *et al,* 2000). It was found that furanocoumarins (FC) from grapefruit juice was responsible for mechanism-based inhibition of CYP activity, 6′,7′-dihydroxybergamottin being one of several FCs identified. Recent studies have also reported that star fruit and pomegranate juice also possess active inhibitory effects on drug metabolism (Zhang *et al,* 2007; Faria *et al* 2007).

The human colon contains over 400 species of bacteria and these bacteria produce a wide spectrum of reductive and hydrolytic enzymes that can metabolize xenobiotics (Jain & Jain, 2008). Studies have shown that small amount of drug metabolites produced from the gut

microflora could also alter the P450 enzymes and change the metabolism and toxicity of a drug in the host (Ingelman-Sundberg, 2002). One example of the effect of the microflora on drug disposition is the drug digoxin, which is converted by the gut microflora to reduced metabolites in a higher percentage in a North American population relative to a population from southern India (Nicholson *et al*, 2005). Due to the symbiotic and mutual microflora, and host relationship (Sears, 2005), variability in the composition and concentration of the gut microflora may cause variation in P450 response to drugs and toxins. Therefore, bacteria microflora in the human gut can play an important role in the absorption of drugs and affect both the bioactivity and bioavailability. Foods containing secondary compounds that are antimicrobial may alter the composition of the gut microflora, reduce the metabolism and influence the bioavailability and absorption of drugs. This may further exacerbate the pharmacological action of phytochemicals on the CYP enzymes.

Functional foods are complex products and may contain many pharmacologically active phytochemicals, and these active ingredients may possess multiple biological activities rather than having only one effect on human health. The CYP inhibition and antimicrobial activity data may be correlated by statistical analysis to elucidate the biological activity of the phytochemicals. A strong correlation may suggest that the activity is caused by a particular phytochemical or a class of phytochemicals. In contrast, a weak statistical correlation may indicate the activities are caused by different phytochemicals.

To broaden understanding of drug-food interactions, this study examined a priority group of pulses, spices and herbs on the Canadian market selected by Agriculture and Agri-Food Canada to determine their potential risk for inhibiting human CYP enzymes (Table 2.1) and affecting the gut microflora. Samples were selected from the *Fabaceae* which contain

isoflavones, *Apiaceae* which contain furanocoumarin, and *Lamiaceae* which contain monoterpenes, and tested for potential inhibition against CYP2D6, CYP3A4, CYP3A5 and CYP3A7. Seven representative gut bacterial genera were selected for the antimicrobial screening includes 4 Gram-positive and 3 Gram-negative cultures. By testing many common food samples, the assessment of potential food-drug interactions and antimicrobial activities across a broad spectrum of diets and therapeutic use of functional foods was achieved.

Table 2.1. Germplasm and spices selected by AAFC for Cytochrome P450 inhibition assays.

NRP #	Botanical Name	Common Name	Family	Country of Origin
320	*Glycine max*	Soybean	*Fabaceae*	Canada
321	*Glycine max*	Soybean	*Fabaceae*	Canada
322	*Glycine max*	Soybean	*Fabaceae*	Canada
323	*Glycine max*	Soybean	*Fabaceae*	Canada
324	*Glycine max*	Soybean	*Fabaceae*	Canada
325	*Glycine max*	Soybean	*Fabaceae*	Canada
326	*Glycine max*	Soybean	*Fabaceae*	Canada
327	*Glycine max*	Soybean	*Fabaceae*	Canada
328	*Glycine max*	Soybean	*Fabaceae*	Canada
329	*Glycine max*	Soybean	*Fabaceae*	Canada
330	*Glycine max*	Soybean	*Fabaceae*	Canada
331	*Glycine max*	Soybean	*Fabaceae*	Canada
335	*Phaseolus vulgaris*	Black bean	*Fabaceae*	Unknown
313	*Phaseolus vulgaris*	Black Turtle bean	*Fabaceae*	Canada
314	*Phaseolus vulgaris*	Cranberry bean	*Fabaceae*	Canada
356	*Phaseolus vulgaris*	Great Northern bean	*Fabaceae*	USA
315	*Phaseolus vulgaris*	Dark Red Kidney bean	*Fabaceae*	Canada
316	*Phaseolus vulgaris*	Light Red Kidney bean Var. A	*Fabaceae*	Canada
317	*Phaseolus vulgaris*	Light Red Kidney bean Var. B	*Fabaceae*	Canada
318	*Phaseolus vulgaris*	White Kidney bean Var. A	*Fabaceae*	Canada
319	*Phaseolus vulgaris*	White Kidney bean Var. B	*Fabaceae*	Canada
339	*Phaseolus vulgaris*	White Kidney bean Var. C	*Fabaceae*	Unknown
354	*Phaseolus vulgaris*	White Kidney bean Var. D	*Fabaceae*	Unknown
337	*Phaseolus vulgaris*	Navy bean	*Fabaceae*	Unknown

357	*Phaseolus vulgaris*	Pinto bean	*Fabaceae*	Canada/USA
358	*Phaseolus vulgaris*	Small Red bean	*Fabaceae*	Canada/USA
355	*Lens culinaris*	Eston lentil	*Fabaceae*	Unknown
350	*Lens culinaris*	Green lentil	*Fabaceae*	Canada
359	*Lens culinaris*	Red lentil	*Fabaceae*	Canada
336	*Phaseolus. lunatus*	Lima bean	*Fabaceae*	Unknown
351	*Pisum sativum*	Green pea	*Fabaceae*	Canada
352	*Pisum sativum*	Yellow pea	*Fabaceae*	Canada
338	*Pisum sativum*	Yellow split pea	*Fabaceae*	Unknown
332	*Vigna unguiculata*	Black-eyed pea	*Fabaceae*	Unknown
334	*Vigna unguiculata*	Cow pea	*Fabaceae*	Unknown
353	*Cicer arietinum*	Chick pea	*Fabaceae*	Canada
333	*Cicer cayan*	Congo Pigeon pea	*Fabaceae*	Unknown
341	*Apium graveolens*	Celery seed A	*Apiaceae*	Unknown
342	*Apium graveolens*	Celery seed B	*Apiaceae*	Unknown
343	*Coriandrum sativum*	Coriander	*Apiaceae*	Unknown
344	*Cuminum cyminum*	Cumin	*Apiaceae*	Unknown
345	*Anethum graveolens*	Dill	*Apiaceae*	Unknown
346	*Foeniculum vulgare*	Fennel seed	*Apiaceae*	Unknown
340	*Ocimum basilicum*	Basil leaves	*Lamiaceae*	Unknown
347	*Origanum vulgare*	Oregano leaves	*Lamiaceae*	Unknown
348	*Rosemarinus officinalis*	Rosemary	*Lamiaceae*	Unknown

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

CYP enzymes 3A4 (Human CYP3A4 + reductase, 1 nmol, 500 uL – Cat# 456207), 3A5 (Human CYP3A5 + reductase, 1 nmol, 500 uL – Cat# 456235), 3A7 (Human CYP3A7 + reductase + b5, 0.5 nmol, 500 uL – Cat# 456237), 2D6 (Human CYP2D6*1 + P450 reductase supersomes – Cat# 455117), dibenzylfluoroscein (DBF), and 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC) were obtained from BD Gentest (Franklin Lakes, NJ, USA). All enzymes were stored at -80^{o}C until required. NADPH (β-NADPH reduced tetrasodium salt hydrate – Cat# N7505-1GR), was ordered from Sigma Aldrich (Oakville, ON, Canada) and stored at -20^{o}C in the dark. Ketoconazole was purchased from Calbiochem (Gibbstown, NJ, USA). Methanol was purchased from Fisher Scientific Canada (Ottawa, ON, Canada). Statistical analyses and plots were done on Sigmaplot11.

2.2.2 Sample Collection

All samples come from local supermarkets or farms in the Ottawa and Guelph areas. Each sample was given a Nutraceutical Research Program (NRP) number and all pertinent information such as mass, company name, origin and place of purchase was recorded (see full description and full genus names in main text reference (Table 2.1). Each sample was weighed, recorded and divided into three portions, one portion was stored in the dark in -20 ^{o}C for archiving at the University of Ottawa Herbarium and the other was ground into fine powder using a Thomas-Wiley industrial grinder with a 2 mm pore industrial grade steel mesh filter for consistency. The ground material was then stored in the dark at -4 ^{o}C.

2.2.3 Sample Extraction

To prepare stock extracts of each sample, 1 ml of 80 % methanol was added to 50 mg of ground plant material in a 2 ml centrifuge tube and blended in Fisher Vortex Genie2 at maximum settings for 2 minutes. The sample was then centrifuged in a Fisher Scientific (Ottawa, ON, Canada) Micro12 Centrifuge at 13,000 g for 20 minutes. The supernatant was stored at -4^{o}C in the dark. Aqueous samples were prepared as described above. Extracts were freshly prepared daily.

2.2.4 Fluorometric microtitre cytochrome P450 inhibition assays

A fluorometric microtitre plate assay was used to assess the inhibitory capacities of the plant extracts against CYP3A4, 3A5, 3A7 and 2D6. The procedure used was adapted and modified from Crespi *et al* (1997) and Scott *et al* (2006).

The assays were performed in 96-well plates with white walls and clear, flat bottoms under red-coloured light to minimize the exposure of photosensitive material to light (*i.e.* NADPH, quinidine, substrates, and some extract constituents). The fluorescence was measured using a Cytofluor 4000 Fluorescence Measurement System (Applied Biosystems, Foster City, CA, USA). The percent inhibition for each extract was calculated relative to the CYP activity in the presence of the vehicle control. An amount of 10 µl of each extract was tested in triplicates for all assays. The in well concentration was 2.5 ug/ul. All extracts were freshly made on experimental days and the remainders discarded.

Wells were designated as “control,” “control blank,” “sample,” or “sample blank.” The control represented the MeOH vehicle control, whereas the sample represented the extract or positive control. Solution A contained 1.08 mM NADPH and the substrate in 0.25 M potassium phosphate buffer solution, pH 7.4. Solution B contained the CYP in the 0.13 M buffer solution. Solution C was identical to Solution B but instead contained denatured CYP rather than active enzyme (“blank”). A volume of 100 µl of Solution A was added to each well followed by the addition of 10 µl of the extract. Enzyme was thawed prior to its addition to Solution B or C which were then immediately aliquoted into the wells at a volume of 90 µl. The plate was shaken for three seconds, and the initial fluorescence was measured at various excitation and emission wavelengths depending on the substrate used. The plate was then incubated at 37°C for 20 to 40 minutes depending on the enzyme tested and then final fluorescence was measured.

The concentration of CYP3A4, 3A5, and 3A7 used was 10 µM with DBF as a substrate at a concentration of 1 µM. The positive inhibitor used was ketoconazole at a concentration of 1.9 µM. Samples were read at excitation wavelength of 485 nm and an emissions wavelength of 530 nm with gain set at 50. The concentration of CYP2D6 used was 10 µM with AMMC as a substrate at a concentration of 0.12 µM and quinidine as a positive inhibitor at a concentration of 2 µM. The samples tested against CYP2D6 were read with excitation wavelength of 409 nm and emission wavelength of 460 nm with gain set at 50. The incubation time was 20 minutes for CYP3A4 and 3A5 assays and 40 minutes for CYP3A7 and 2D6 assays.

2.2.5 Antimicrobial assay

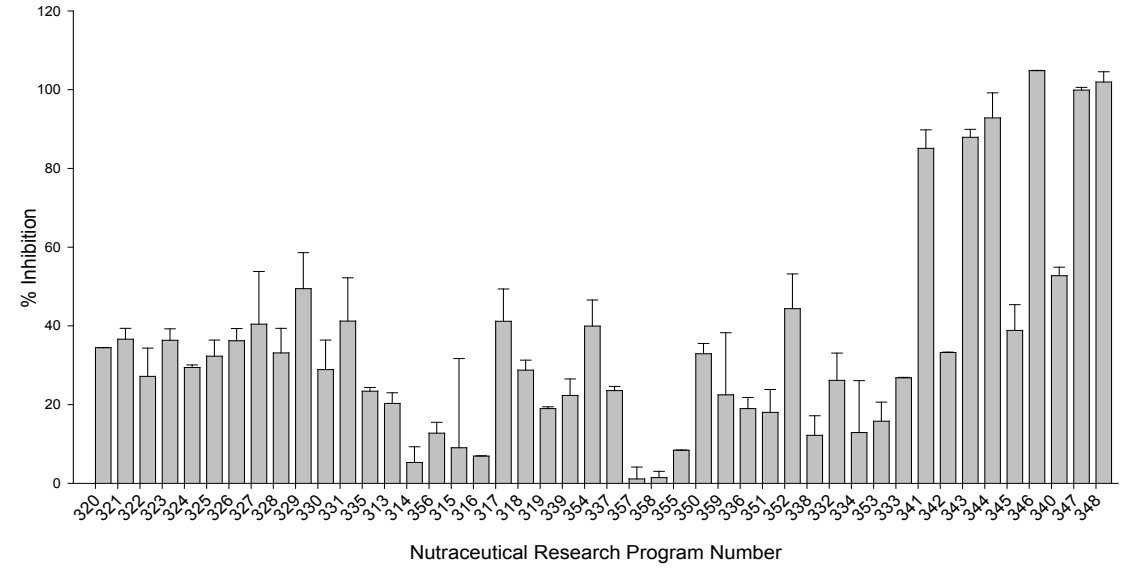
Extracts were examined for antimicrobial activity by using the Kirby-Bauer disc diffusion assay (Omara *et al*, 2000). Both methanolic and ethanolic extracts were tested. A second set of samples were heated in a boiling water bath for 10 minutes to simulate cooking conditions. A total of 7 bacterial species were selected in this study from different genera. There was 3 Gram (+) bacterial species: *Bacillus subtilis*, *Enterococcus faecalis*, and *Listeria innocua*, and 4 Gram (-) bacterial species: *Escherichia coli*, *Pseudomonas putida, Providencia stuartii*, and *Anterobacter calcoaceticus*. Each bacterial species was inoculated in 10 ml of Mueller-Hinton media and cultured over night at 37°C, and then plated using a cotton swab onto Petri dishes containing Mueller-Hinton agar. A 20 ul aliquot of the sample extracts were transferred onto a blank 5 mm bacteria susceptibility disc (Oxoid, Nepean, ON, Canada). Sample discs were then air dried and placed in triplicate on Petri dishes containing the bacterial culture. The Petri dishes were then incubated at 37°C in dark condition and the zones of inhibition were measured at 24 hours. The antibiotic Ciprofloxacin^{TM} was used as the positive control which tested positive against all 7 bacterial species.

2.3 Results

A total of 46 food samples were examined and include 37 *Fabaceae*, 6 *Apiaceae*, and 3 *Lamiaceae*. The inhibitory potential of each sample was categorized as low (<35%), moderate (35-70%) and high (>70%) inhibition. *Apiaceae* and *Lamiaceae* methanolic extracts had the highest CYP3A4 inhibition (Figure 2.1A). Celery seed (var. A), coriander, cumin, and fennel seed of the *Apiaceae* and oregano and rosemary of the *Lamiaceae* inhibited CYP3A5 by over 85%. Among the *Fabaceae*, soybean samples inhibited had low to moderate CYP3A4 inhibition. Remaining *Fabaceae* samples displayed low to moderate inhibition with the exception of light red kidney bean (var. B) and yellow pea having the highest inhibition of 41.2 ± 8.2% and 44.4 ± 8.8 %, respectively. Aqueous extracts had high inhibition (75- 100%) values in numerous samples (Figure 2.1B). Fennel seed, cumin, and celery seed (var. A), rosemary, oregano and basil all displayed high levels of inhibition. Soybean samples all moderately inhibited CYP3A4. The remaining *Fabaceae* samples had low to moderate inhibition with the exception of white kidney bean (var. B) having stronger inhibition at 93.6 ± 8.4%.

Apiaceae and *Lamiaceae* methanolic extracts had high inhibitory levels towards CYP3A5 (Figure 2.2A). Among the *Fabaceae*, soybean samples moderately inhibited CYP3A5 whereas the remaining *Fabaceae* samples displayed low inhibitory levels. Aqueous extracts displayed lower inhibition values in numerous samples (Figure 2.2B). The highest levels of inhibition were observed in cumin, celery seed (var. A) and celery seed (var. B), which were relatively moderate in comparison to the activity observed in the methanolic extracts. Rosemary and oregano were the most active, inhibiting at 99.6 ± 0.5 % and 74.9 ± 3.4%, respectively. Remaining *Fabaceae* samples had low to moderate inhibition.

A



B.

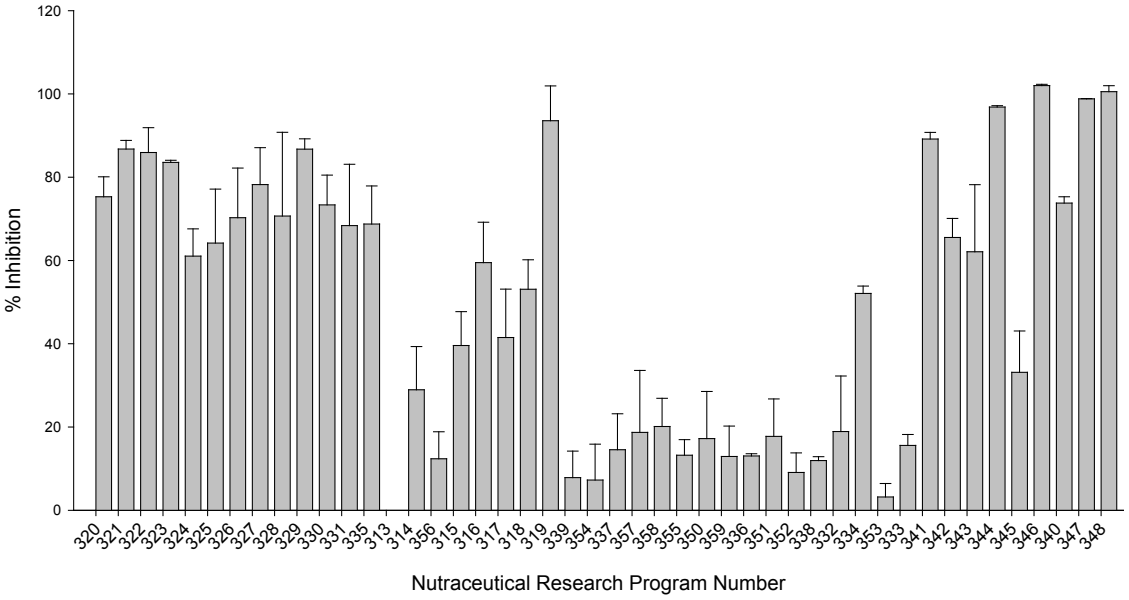
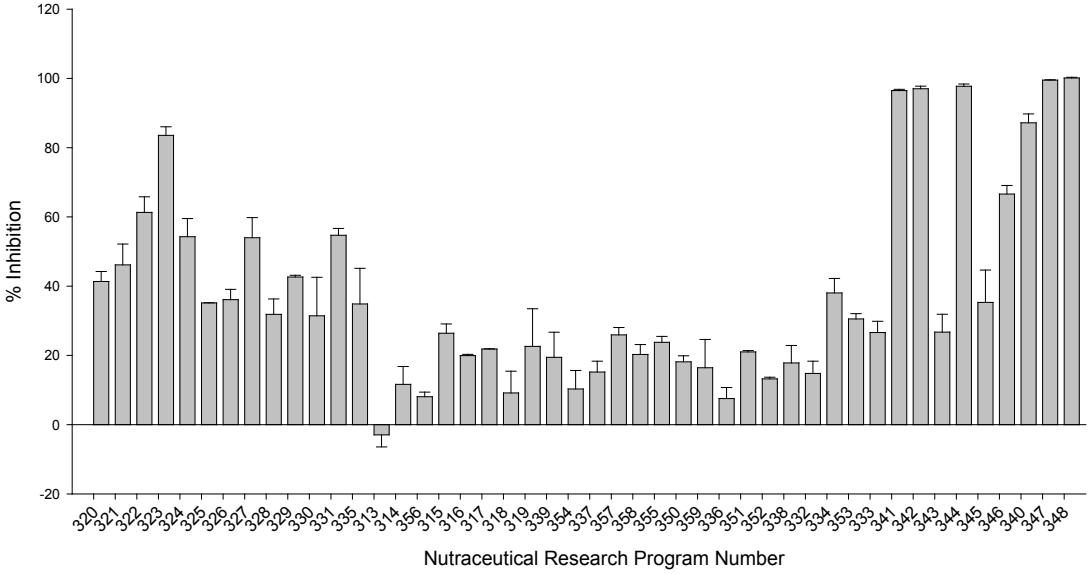


Figure 2.1 Percent inhibition of methanolic (A) and aqueous (B) extracts (50 mg/ml) from common food samples on cytochrome P450 3A4 isozyme. Values are presented as means ± standard deviation of extracts tested in triplicates and repeated twice.

A



B

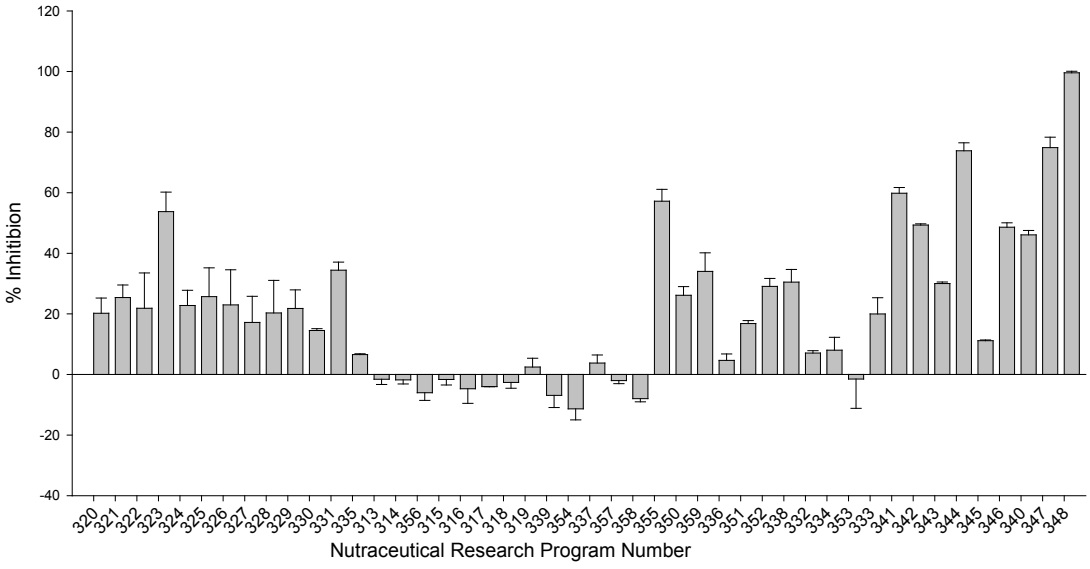


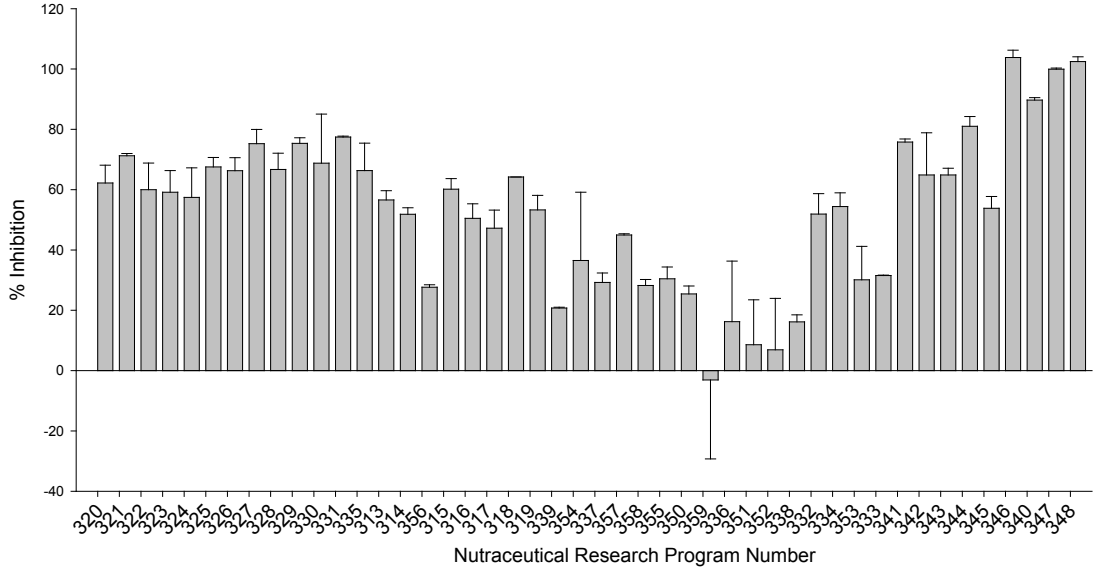
Figure 2.2 Percent inhibition of methanolic (A) and aqueous (B) extracts (50 mg/ml) from common food samples on cytochrome P450 3A5 isozyme. Values are presented as means ± standard deviation of extracts tested in triplicates and repeated twice.

In regards to CYP3A7, the *Apiaceae* and *Lamiaceae* methanolic extracts had the highest inhibition as seen previously in CYP3A5 (Figure 2.3A). The highest levels of inhibition were observed in fennel seed, cumin, and celery seed (var. A). Rosemary and oregano and basil also displayed high inhibitory levels. Soybean samples inhibited CYP3A7 at moderate and high levels whereas the remaining *Fabaceae* inhibited at low to moderate levels. The aqueous extracts generally had lower CYP3A7 inhibition values (Figure 2.3B). High levels of inhibition were observed in fennel seed, dill, and celery seed (var. B). Basil oregano and rosemary had moderate inhibition. The activity levels seen in soybean and the remaining *Fabaceae* samples were low to moderate and were more similar against the CYP3A7 than previously in the CYP3A4 and 3A5 assay.

As previously highlighted from the results of CYP3A4, 3A5 and 3A7 data, the *Apiaceae* and *Lamiaceae* methanolic extracts had the highest CYP2D6 inhibition (Figure 2.4A). The highest levels of inhibition were observed in celery seed (var. A), celery seed (var. B), coriander, oregano and rosemary. Interestingly soybean samples had very low inhibition levels on CYP2D6. The remainder of the *Fabaceae* extracts also had low inhibition with the exception of light red kidney bean (var. B) which inhibited at 94.2 ± 5.6%. Aqueous extracts were similar to methanolic extracts (Figure 2.4B). The highest levels of inhibition were observed in celery seed (var. A), dill, coriander, rosemary and oregano. Basil, on the other hand, had a rather moderately low inhibition activity. Soybean and remaining *Fabaceae* samples also displayed low to moderate levels of inhibition.

The antimicrobial properties of the food samples were examined by the antimicrobial disc-diffusion assay to demonstrate their potential effect on drug disposition by interacting with

A



B

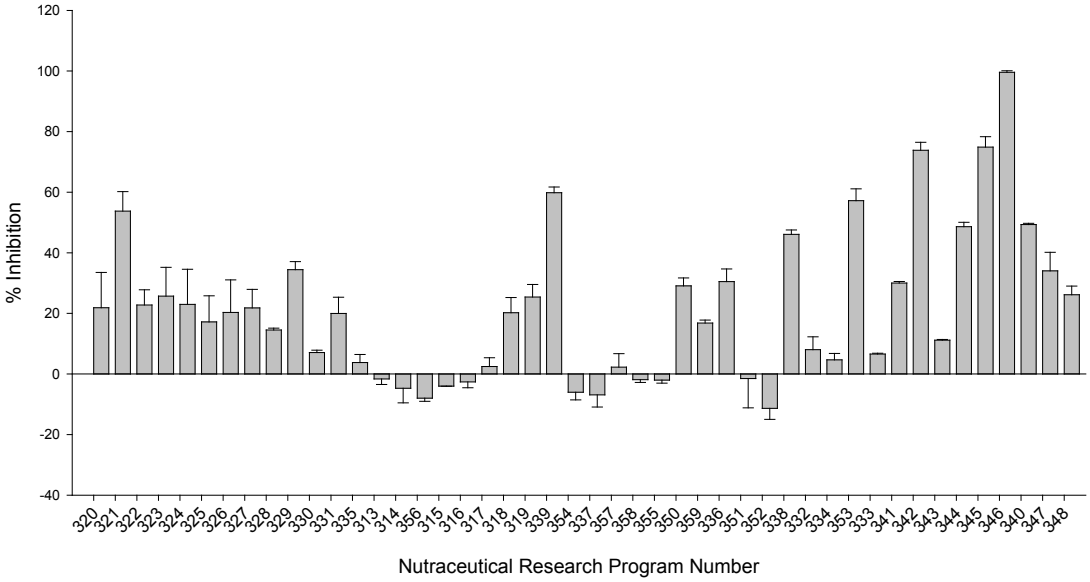
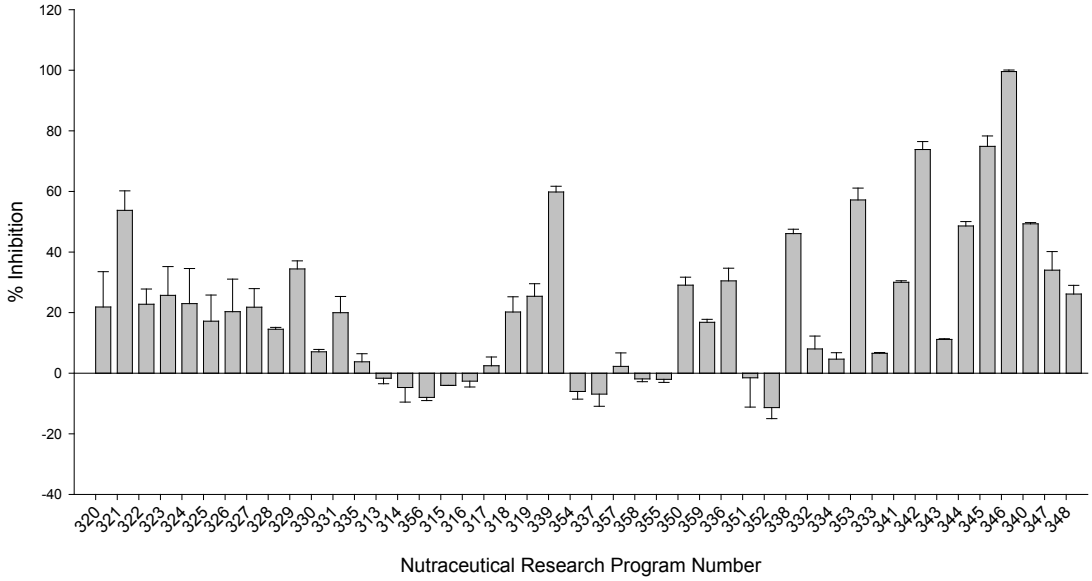


Figure 2.3 Percent inhibition of methanolic (A) and aqueous (B) extracts (50 mg/ml) from common food samples on cytochrome P450 3A7 isozyme. Values are presented as means ± standard deviation of extracts tested in triplicates and repeated twice.

A



B

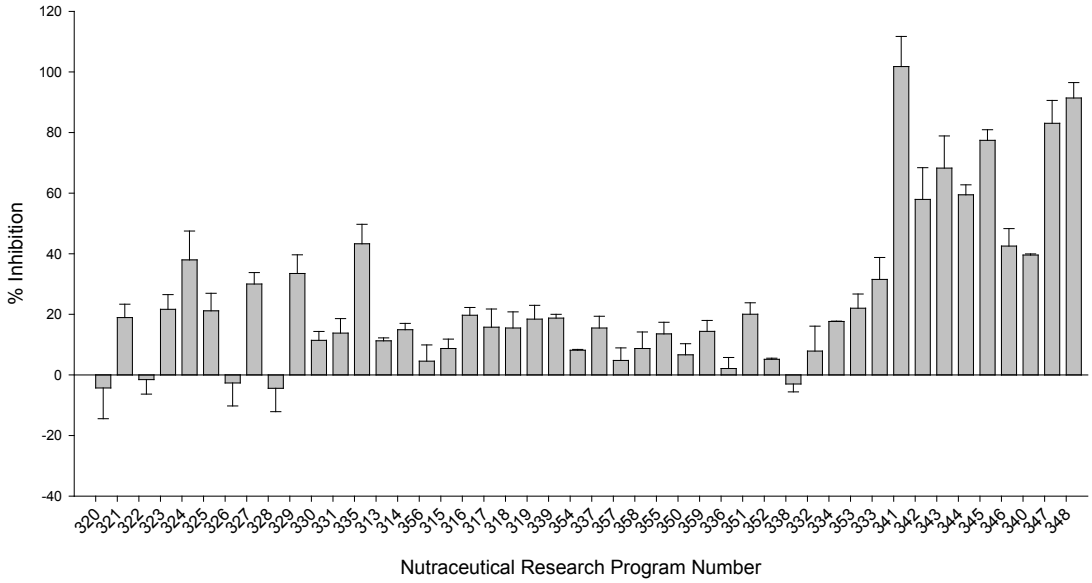


Figure 2.4 Percent inhibition of methanolic (A) and aqueous (B) extracts (50 mg/ml) from common food samples on cytochrome P450 2D6 isozyme. Values are presented as means ± standard deviation of extracts tested in triplicates and repeated twice.

the gut bacterial microflora. The largest zones of inhibition were observed in both methanolic and aqueous extracts of *Apiaceae* and *Lamiaceae* species shown in Table 2.2, suggesting that the microbes were reacting to secondary metabolites in a similar way to the CYP enzymes. Oregano leaves and rosemary demonstrated strong activities against 6 out of the 7 selected bacterial species with the exception of *A. calcoaceticus*. *Apiaceae* extracts including cumin, dill, fennel seed, celery seed, and coriander also displayed relatively strong antimicrobial activities. Fennel seed extract showed the most potent antimicrobial effects with the largest zones of inhibitions in six out of the seven bacteria with the exception of *E. coli*. In comparison, celery seed demonstrated weaker antimicrobial effects and was only effective against *P. putida* and *P. stuartii*. None of the *Apiaceae* extracts were active against *E. coli*. No significant antimicrobial effect was observed in the *Fabaceae* extracts. Few *Fabaceae* samples demonstrated weak activity (less than 8 mm) against *A. calcoaceticus*.

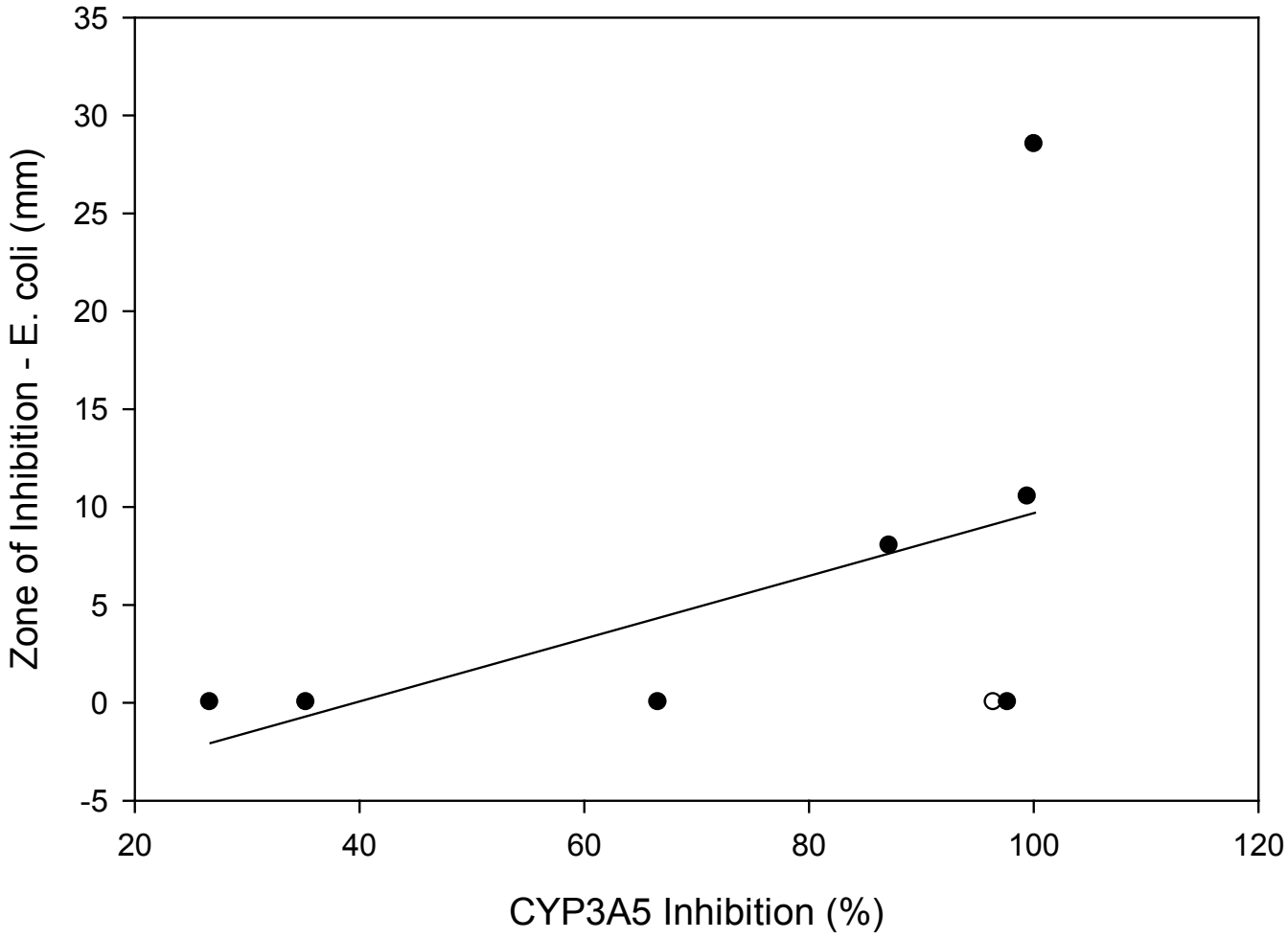


Figure 2.5 Linear relationship between cytochrome P450 3A5 isozyme inhibition and anti-microbial activity against *Escherichia coli*. The r^2=0.23, p=0.23. The equation for the regression line is y=0.16x-6.34.

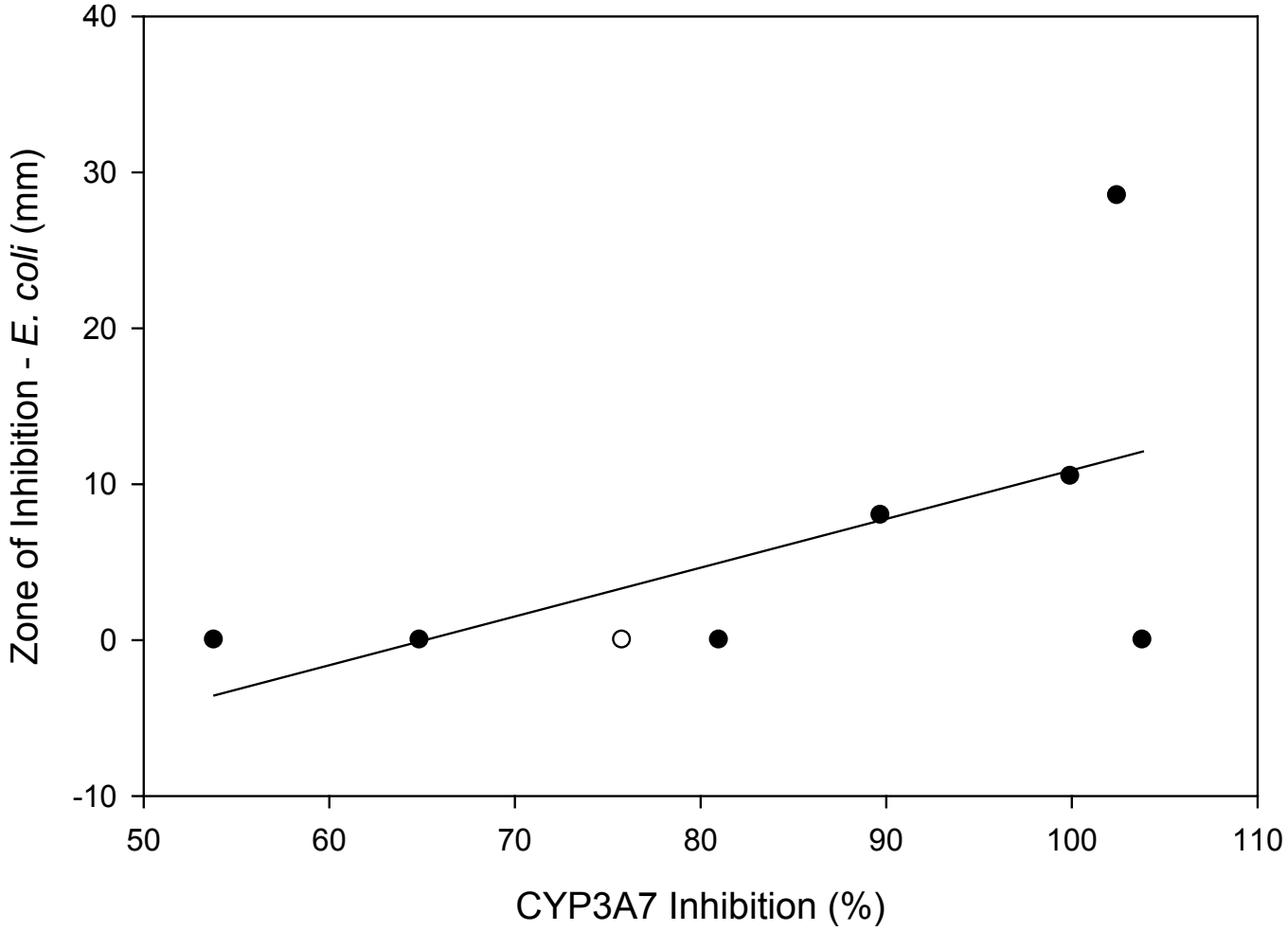


Figure 2.6 Linear relationship between cytochrome P450 3A7 isozyme inhibition and anti-microbial activity against *Escherichia coli*. The r^2=0.33, p=0.14. The equation for the regression line is y=0.31x-20.38.

Table 2.2. Antimicrobial effects of methanolic and ethanolic extracts of selected *Apiaceae* and *Lamiaceae* (50 mg/ml) against 6 bacterial species. Zones of inhibitions are measured according to the Kirby-Bauer disc diffusion assay. Values represent average diameters between triplicates of and measured in millimeters. Ciprofloxacin was used as the positive control. (-) denotes no inhibitory activity or a zone of inhibition of less than 6mm.

Plant Name	NRP No.	*Bacillus subtilis* Gram (+)	*Enterococus faecalis* Gram (+)	*Listeria innocua* Gram (+)	*Escherichia coli* Gram (-)	*Pseudomonas putida* Gram (-)	*Providencia stuartii* Gram (-)	*Acetobacter calcoaceticus* Gram (-)
Cumin	344	8.3 ± 0.6	-	-	-	11.5 ± NA	-	7.5 ± NA
		6.5 ± NA	-	-	-	10.0 ± NA	-	6.3 ± 0.5
Fennel seed	346	10.7 ± 0.8	10.5 ± NA	10.5 ± NA	-	12.0 ± NA	11.0 ± NA	7.0 ± NA
		8.0 ± NA	9.7 ± 0.6	9.0 ± NA	-	9.7 ± 0.6	9.0 ± NA	6.0 ± NA
Dill	345	-	7.7 ± 0.5	11.5 ± NA	-	7.0 ± NA	12.5 ± NA	7.3 ± 0.5
		-	6.3 ± 0.4	10.3 ± 0.8	-	6.0 ± NA	6.5 ± NA	6.0 ± NA
Celery seed	341	8.3 ± 0.6	8.0 ± NA	-	-	11.5 ± NA	7.7 ± 0.5	11.7 ± 0.9
		6.5 ± NA	7.5 ± NA	-	-	9.3 ± 0.6	6.0 ± NA	8.0 ± NA
Coriander	343	-	-	-	-	6.5 ± NA	6.5 ± NA	-
		-	-	-	-	6.5 ± NA	6.0 ± NA	-
Rosemary	348	10.3 ± 0.8	11.5 ± NA	9.7 ± 0.6	8.0 ± NA	10.7 ± 0.8	10.5 ± NA	-
		8.3 ± 0.6	9.0 ± NA	8.5 ± NA	6.0 ± NA	8.0 ± NA	9.3 ± 0.6	-
Oregano	347	7.0 ± NA	12.0 ± NA	9.5 ± NA	10.5 ± NA	10.5 ± NA	11.5 ± NA	-
		6.3 ± 0.5	10.3 ± 0.8	8.0 ± NA	8.0 ± NA	8.0 ± NA	9.0 ± NA	-
Ciprofloxacin		21.5 ± NA	23.0 ± NA	22.7 ± 1.6	28.5 ± NA	31.0 ± NA	27.0 ± NA	29.5 ± NA

2.4 Discussion

Through the evaluation of 46 food-plant samples using 4 different cytochrome P450 enzymes to determine the potential risk of food-drug interactions, the findings provided strong evidence that the selected *Apiaceae* and *Lamiaceae* samples have a higher potential than the *Fabaceae* products examined. The higher levels of activity in spices and herbs may be due to their selection for flavor, which is associated with a high level of phytochemicals (Lampe, 2003). Similar trends were observed in the examination of these plants for their antimicrobial effects with the *Apiaceae* and *Lamiaceae* being the most active.

Some products such as fennel seed, celery seed and cumin exhibited consistently high levels of inhibition among all CYP enzymes tested, which may be attributed to high levels of FCs (Subehan *et al*, 2007). The results obtained from fennel seed are consistent with those of Subehan *et al* (2007) who identified 5-methoxypsoralen (5-MOP) as the mechanism based inhibitor of CYP3A. Coriander and dill of the *Apiaceae* family, although also containing FCs (Cieśla *et al*, 2008), do not express high levels of activity. The varying levels in inhibitory activity may be due to the level of expression of FCs in the plant.

Data obtained suggest that both methanolic and aqueous extracts of *Lamiaceae* plants, oregano and rosemary, exhibit high levels of inhibition towards CYP enzymes. This high level of activity may be attributed to the presence of flavonoids or aromatic monoterpenes. Studies with flavonoid rich food plants, such as pomegranate and rosemary, have also reported high levels of, inhibitory activity against several different CYP enzymes (Offord *et al*, 1995; Faria *et al*, 2007). Given the heightened popularity of antioxidants among the general public, the flavonoid-drug interactions may also be a problem that

will increase. Findings support a collective review by Cermak (2008) who strongly cautions the possibility of flavonoid-drug interactions in functional foods and herbal supplements, and counsels the need for advisory labeling of unregulated products.

Among the *Fabaceae* in this study, the 12 soybeans examined consistently exhibited moderately high inhibition activity against all 4 isozymes. Previous studies have shown that aqueous extracts of soybean have the potential of inhibiting CYP3A4 and CYP3A7 and hydrolyzed soy extracts (50 mg/ml) can reduce CYP3A4 activity to 22.3 ± 5.9 % that of the control (Anderson *et al,* 2003; Foster *et al,* 2003). Lentil and other bean from other genera had lower inhibitory potential.

The antibacterial activities observed were predominantly from the *Apiaceae* and *Lamiaceae*. Among these, the highest and most broadly antibacterial activity, inhibiting 5 out of the 6 bacterial strains, belonged to rosemary and oregano. Previous studies have shown oregano and rosemary to have high antibacterial activity against *E. coli* (Bozin *et al,* 2006; Sagdiç, 2003; Romano *et al*, 2009). *Apiaceae* extracts also produced high antibacterial activity with fennel possessing the strongest and broadest activity. Extracts from the *Apiaceae* family, namely dill, celery, coriander and fennel, have been shown to contain the antibacterial compounds falcarinol and falcarindiol (Christensen & Brandt, 2006). Zones of inhibition observed in this study may be affected by the loss of bioactive volatile phytochemicals and essential oils from the plant material due to processing and drying. A study using fresh plant material will be required to determine their full potential.

The antibacterial activities observed in the *Fabaceae* extracts were relatively low. The majority of the activities were from the *Phaseolus vulgaris* varieties such as the light and dark red kidney bean, the black bean, and black turtle bean. The data obtained corresponds with previous studies and suggests

that *Fabaceae* varieties containing coloured seed coats possessed stronger antibacterial activity as a result of secondary metabolites found in the seed coats (Beninger & Hosfield, 2003). The coloured seed coats were observed to be a potential indication of bioactive secondary metabolites such as tannins and flavonoids (Beninger & Hosfield 2003).

By categorizing samples into families and evaluating their activity, two observations may be made about a food crop's secondary metabolomic content and its dietary selection as either a staple food or a condiment such spice and herb. Firstly, plant family was expected to influence the potential risk of food-drug interactions. Due to the constitutive expression of FCs in *Apiaceae* family (Cieśla *et al*, 2008), CYP and bacterial inhibition was predicted from the *Apiaceae* and supported by the data obtained. Likewise, the high activities in the *Lamiaceae* were also observed as a result of their high levels of secondary metabolites such as terpenoids, phenolics, and flavonoids (Wink, 2003).

Traditionally, spices are used in minute amounts for their flavour and food preserving properties. Food spices and herbs typically contain higher levels of bioactive phytochemicals (Sherman & Geoffrey, 2001). Upon analysis, a linear correlation was derived between the inhibitions of CYP3A5/3A7 (Figures 2.5 & 2.6) versus antibacterial activity against *E. coli* from the spice plants. Therefore, this data further implicate the role of these secondary metabolites, from the selected spices, in the inhibition of drug metabolizing enzymes. On the other hand, staple foods tend to contain a lower bioactive phytochemical content. This however does imply that staple foods, such as pulses, cannot contribute to CYP inhibition as the concentrations may be multiplied as higher volumes are consumed. When consumed in combination alongside with drugs, however, food plants may synergistically affect drug metabolism, either directly on human CYP enzymes or indirectly by disrupting gastrointestinal bacteria flora thereby increasing the pharmacological load on the human system and affect patient's wellness.

With the soaring popularity of NHPs and functional foods, many individuals are consuming larger quantities of these products such as soy, fresh herbs and spices. These products are safe when consumed in reasonable amounts, but when consumed in larger amount or together with other therapeutic products may cause a drug interaction. Food-drug interactions may be the underlying cause for some drug overdoses, drug rejection, and therapeutic failure as a result of direct systemic CYP inhibition or disruption of the bacterial flora. Although the majority of healthy individuals will see very little, if any, effect when consuming common food products, patients undergoing serious medical care should become more aware of potential risks identified with certain foods. The data obtained from the conditions created in this study indicates potential drug interactions between functional foods and other therapeutic products. More work is needed to examine the inhibitory properties of these samples under different sample selection criteria and extraction conditions. Future studies will examine phytochemical content by HPLC in relation to inhibitory activity.

PREFACE TO CHAPTER 3

The study presented in this chapter was a contribution to a major collaborative research project, lead by Dr. Pierre Haddad and the CIHR Team in Aboriginal Antidiabetic Medicines. The project entailed the scientific evaluation of antidiabetic activity of a selected group of traditionally used Cree plants. The funding for this project was provided by CHIR. Previous studies done by CIHR-TAAM reported 17 plants possessing antidiabetic properties. The data obtained from the present study contributed important evidence for the antidiabetic properties of the Cree plants in a new assay, the inhibition of aldose reductase, potentially reducing risk of cataract formation.

CHAPTER 3

EFFECTS OF TRADITIONAL ANTIDIABETIC MEDICINES OF THE CREE FIRST NATIONS OF IYOUISTCHEE ON ALDOSE REDUCTASE, AN ENZYME IMPLICATED IN DIABETIC CATARACTOGENESIS

3. EFFECTS OF TRADITIONAL ANTIDIABETIC MEDICINES OF THE CREE FIRST NATIONS OF EEYOU ISTCHEE ON ALDOSE REDUCTASE, AN ENZYME IMPLICATED IN DIABETIC CATARACTOGENESIS

San Nguyen[1], Jose Antonio Guerero[1], Ammar Saleem[1], Brian C. Foster[2], Pierre Haddad[3], John T. Arnason[1]

[1]Centre for Research in Environmental Genomics, University of Ottawa, 30 Marie Curie, Ottawa, Ontario, K1N 6N5, Canada.

[2]Therapeutic Products Directorate, Health Products and Food Branch, 1600 Scott St., Ottawa, Ontario, K1A 0K9, Canada.

[3]Département de Pharmacologie, Université de Montréal, Montréal QC, Canada.

*Corresponding Author: John.Arnason@uOttawa.ca

3.1 Introduction

The formation of diabetic cataracts is a degenerative complication in the eyes caused by the accumulation of excess glucose in the blood as a result of inadequate insulin signaling. The prevalence of cataracts is five times higher in individuals with diabetes (Obrosova *et al*, 2010). According to the Canadian Diabetes Association (CDA), 33 % of people suffering from type II diabetes (TIID) will develop cataracts in the eyes in later stages. In earlier stages, the formations of cataracts become evident when patients experience difficulty seeing and blurred vision. The progression of cataracts is a slow process that would eventually lead to blindness if no intervention is introduced.

Cataracts are bodies of denatured proteins that form in the lens of the eye as a result of the denaturation from physical or chemical stress. In diabetic patients, the formation of cataracts is believed to be triggered by two factors: a) osmotic stress due to the overproduction of sorbitol by the enzyme aldose reductase (Lee & Chung, 1999) and b) oxidative stress due to increase level of oxidative species in the blood. Aldose reductase is a monomeric cytoplasmic enzyme, belonging to the aldo/keto reductase family (EC 1.1.1.21), which is present in various tissues and organs of the body (Ramasamy & Goldberg, 2010). It is found in high concentrations in the kidneys, eyes, testis and heart and in lower concentrations in the liver, stomach, lung, intestine and colon. Because of its wide distribution, abnormal functions of AR have been linked to a variety of diabetic complications. *In vivo*, AR is responsible for the polyol pathway by which glucose is reduced to fructose for cellular metabolism. In healthy people the expression of AR is moderate and the intermediate alcohol, sorbitol, is produced and reduced by sorbitol dehydrogenase at a steady rate. When blood glucose reaches a critically high level, as seen in diabetic patients, AR will become over-expressed which would result in an overproduction of sorbitol to toxic concentrations (Yabe-Nishimura, 1998). This condition has been linked to nerve and

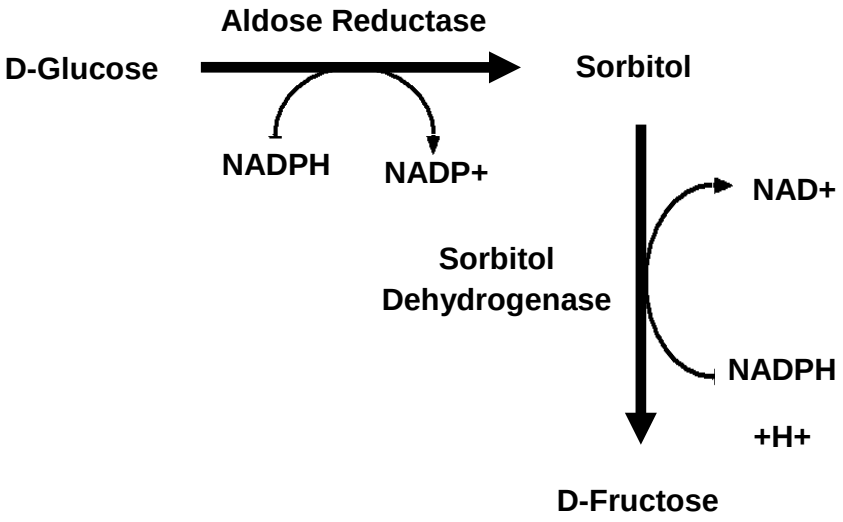


Figure 3.1 Biochemical pathway of aldose reductase. Polyol pathway involving aldose reductase and sorbitol dehydrogenase in the metabolism of D-glucose into D-fructose. NADPH serves as the cofactor to drive the mechanism.

tissue damage such as diabetic neuropathy, retinopathy, cataractogenesis and cardio-vascular disease.

Under normoglycemic conditions in non-diabetics, glucose is metabolized via glycolysis, which involves the phosphorylation of glucose into glucose-6-phosphate by hexokinase. In diabetics, under hyperglycemic conditions, hexokinase becomes saturated with available glucose and thus glucose metabolism shifts towards the polyol pathway (Figure 3.1) (Obrosova, 2010). After the binding of glucose to the anion receptors on the AR enzyme, the reduction of glucose is accomplished, with NADPH as the cofactor, into sorbitol. The secondary step in the polyol pathway involves the NAD-dependent oxidation of sorbitol into fructose. This step is crucial in polyol pathway as it prevents sorbitol from accumulating. The accumulation of sorbitol has been reported to produce osmotic swelling and ionic flux resulting in insolubility of lens protein (Kamei, 1991). The sudden and rapid augmentation in the polyol pathway has also been shown to correlate with the production of fructose-3-phosphate (F3P), a protein glycation agent (Lal *et al*, 1997). The reactive oxygen species (ROS) produced from the resulting advanced glycation end-products contributes to the overall oxidative stress and tissue damage in the lens tissue.

It is known that diabetics experience higher levels of oxidative stress than healthy individuals. Although this phenomenon is a result of the accumulative effects of many pathways disrupted by hyperglycemia, the hyperactivity of AR is also a contributing factor. In diabetics, the levels of ROS produced from the glycosylation of proteins are severely high. One of the body's defenses against ROS involves the glutathione reductase pathway. The removal of oxidative hydroperoxides formed by the reduction of oxidized glutathione (GSSG) into its reduced form (GSH), fueled by NADPH. This common cofactor required for the reduction of ROS is shared with the AR polyol pathway and results in a conflict and flux in the effectiveness of the glutathione reductase pathway. When AR becomes

hyperactive, it consumes more NADPH and diminishes the activity of GSSG, essentially depriving GSSG of its cofactor (Del Corso *et al*, 2008). The overall effect of this occurrence is increase in oxidative stress and formation of cataracts.

Many studies have been conducted to find inhibitors of aldose reductase that would ameliorate and prevent the formation of diabetic cataracts. Quercetin, a well studied flavonoid, has been shown to have 100% inhibition at 10^{-4}M (Head, 2001). This noteworthy inhibitor can slow down the formation of cataracts in diabetics and is now sold as a dietary supplement in the US. Several drugs such as, sorbinil, statil, and tolrestat have been produced but did not show conclusive results due to varying levels of efficacy and toxicity. Due to the nature of its progression, diabetic cataracts are a debilitating complication that affects many individuals with TIID.

In many western countries, cataract surgery and ocular lens replacement is readily available and patients would often regain normal vision with the implant of synthetic lenses. Despite its high rate of success, this invasive procedure is sometimes not preferred due to risk, cost, compliance and personal reasons. With the recent resurgence and popularity of traditional medicines and functional foods, alternative and complementary medicines are being investigated for potential leads to prevent diabetic complications. This study focuses on the traditional medicines of the Cree First Nations of northern Quebec. Past studies by our group have discovered very potent antidiabetic properties in 17 traditionally used medicinal plants (Spoor *et al*, 2006; Harbilas *et al*, 2009). The objective of this research was to test these traditionally used plants and identify the most active species for bioassay guided fractionation to identify active constituents.

3.2 Materials & Methods

3.2.1 Plant collection

Seventeen traditional Cree anti-diabetic plants were chosen for preliminary screening based on their antidiabetic potential as published by Spoor *et al* (2006) and Harbilas *et al* (2009). Briefly, plant samples of *Abies balsamea*, *Alnus incana*, *Larix laricina*, *Picea mariana*, *Pinus banksiana*, *Rhododendron groenlandicum*, *Sarracenia purpurea*, *Sorbus decora*, *Gaultheria hispidula*, *Juniperus communis*, *Kalmia angustifolia*, *Lycopodium clavatum*, *Picea glauca*, *Populus balsamifera*, *Rhododendron tomentosum*, *Salix planifolia*, and *Vaccinium vitis-idaea* were collected with the guide and consent of local Cree elders in Mistissini and Whapmagoostui in northern Québec, Canada**.** At least five samples were collected for each plant. The identification of the plant species was done by Dr. Alain Currier, taxonomist at the Montréal Botanical Garden, and vouchers were prepared and archived in the Marie-Victorin herbarium of the Montréal Botanical Garden in Montréal, Quebec, Canada. Plant materials to be used for biological and chemical analysis were air dried and sent to the University of Ottawa for cleaning, separation by organs, and milling. Dried plant materials were ground into powder form using a Wiley Mill (Arthur H. Thomas, Swedesboro, USA) with a 2 mm screen. The ground plant materials were stored refrigerated in the dark at -20^{o}C until needed.

3.2.2 Plant extraction

Crude extracts were prepared with the ground plant material. Approximately 50 mg of ground material was weighed out on a Sartorius BP210D balance and placed into clean 1000 ml Erlenmeyer flasks. An appropriate volume of 80% ethanol was added to each flask to give a final ratio of 1:10 m/v. The solution was then sealed with Parafilm and rubber stoppers, and then wrapped in aluminum foil to prevent accidental contamination and prevent exposure to direct light. Next, the extracts were removed from the shakers and filtered using a Buchner funnel and 120 mm Whatman filter paper. The filtered extract was collected and stored at 4 ^{o}C while the vegetative residue was air dried and stored at room temperature for archival purposes. To obtain dried plant extracts, the ethanol and water was removed by rotary evaporation on Yamato RE500**.** The sticky residues collected at the bottom of the round bottom flasks were placed in -20 ^{o}C freezers until frozen and placed on EC Super Modulyo at -55^{o}C and10^{-2}mBar until all remaining water evaporated. The remaining dried extracts were then collected and weighed to determine the yield.

3.2.3 Materials and chemicals

The phosphate buffered saline tablets (PBS) (P4417-100TAB), β-nicotinamide adenine dinucleotide phosphate, reduced tetra (cyclohexylammonium) salt (N-5130) (β-NADPH), and DL-glyceraldehyde (G5001) were purchased from Sigma Aldrich. The β-NADPH and DL-glyceraldehyde solutions were prepared using 0.067 M PBS (pH=7.4) at respective concentrations of $25x10^{-5}$M and $5x10^{-4}$ M. The β-NADPH and DL-glyceraldehyde was stored at -20^{o}C in the dark until required.

3.2.4 Lens isolation

Lens isolation method was taken from Hayman and Kinoshita (1964) with several alterations. The lens homogenate was obtained from pig eyes purchased from a health regulated abattoir within 2 hours of slaughtering. The eyes were kept on ice and then transferred dry ice for the duration of the transport and remained on ice for the entire preparation. The eyes were first cleaned of any excess flesh and then immersed in a solution of 1:3 iodine and 0.1M phosphate buffer saline (pH 7.4), respectively, for approximately 3 minutes to sterilize. They were then rinsed in 0.1 M phosphate buffer saline (PBS) to remove the excess iodine and then placed in gauze to dry. A small incision was made in the eye and the lens was removed from the vitreous humor and placed in a sterile falcon tube for weighing. Based on the total mass of the lens obtained, 0.1 M PBS was added to the lens at a ratio of 10:1 w/w, respectively. The lens was then homogenized until uniform. Next, streptomycin was added at 1% to the total volume to prevent bacterial infection. Aliquots of 5 ml of the lens homogenate were placed on temperature regulated centrifuge at 0 ^{o}C for 10 minutes spinning at 10,000 x *g*. The supernatant was collected and stored at –80^{o}C until needed.

3.2.5 Aldose reductase inhibition assay

A colorimetric assay was used to determine the aldose reductase inhibition potentials of the plant extracts against lens isolate. The procedure used was adapted and modified from Halder *et al* (2003).This assay was conducted on ice and in dark lighting conditions. Plant materials and crude extracts were previously collected and prepared by Leduc *et al* (2005) and Spoor *et al* (2006), and stored at 4^{o}C in the dark. The dried crude ethanolic extracts were re-dissolved in 80% ethanol, to a

concentration of 25 mg/ml, in a 2 ml eppendorf tube and vortexed on a Vortex Genie 2 at maximum speed and then sonicated for 20 minutes until no large particulates can be seen. Extracts were then stored at 4^{o}C overnight to allow any suspended solids to settle. The samples were then centrifuged in a Fisher Scientific (Ottawa, ON, Canada) Micro12 Centrifuge at 13,000 g for 20 minutes. The supernatant was collected and stored at 4^{o}C until required.

To prepare the 96-well plates for the bioassay, 45 ul of $5x10^{-4}$ M DL-glyceraldehyde, was loaded into each well of a white clear bottom 96-well Corning plate. Injections of 5 ul of prepared plant extracts were then added to assigned wells and covered to prevent drying. The plates were then quickly moved to a dark working environment where 50 ul of NADPH was added to each well and the plate was then recovered. To initiate the reaction, 100 ul of lens homogenate was added to each well, bringing the final volume to 200 ul, using a multichannel pipetter to reduce lag time in activity. The final concentration of the extract per well was 0.62 mg/ml with an alcohol concentration of 2 %.

The plate was then transferred into a Spectramax M5 spectrophotometer where it was shaken for 5 seconds and read at 340 nm to detect the uptake of NADPH over a period of 20 minutes in 5 minute increments. The absorbance level was plotted against time and the following formula was used to calculate the oxidation of NADPH:

$$\text{Oxidation Rate}_{NADPH} = OD_{t0} - OD_{t20} / 20 \text{ min}$$

To calculate the inhibitory potential (IP) of each sample relative to the positive control, the uptake rate (UR) was used in the following formula:

$$IP_{sample} = 1 - \frac{[UR_{sample} / UR_{control}]}{[UR_{quercetin} / UR_{control}]} \times 100$$

Each sample was tested in triplicate to ensure technical consistency and repeated 4 times. Phosphate buffer was used as the blank control and quercetin (0.75 mg/ml, final concentration) was used as a positive control.

3.2.6 R. groenlandicum collection

Leaves (1.7 kg) of *R. groenlandicum w*ere collected in Mistissini, Québec, in August 2007 and identified by Dr. Alain Currier, a taxonomist at the Montréal Botanical Garden; a voucher specimen (MIS 03-9) was deposited at the Marie-Victorin Herbarium of the Montréal Botanical Garden.

3.2.7 Extraction and fractionation of R. groenlandicum for phytochemical analysis

Dried and shredded leaves (1.7 kg) were milled using a Wiley Mill (Arthur H. Thomas, Swedesboro, USA) with a 2 mm screen. The ground material was then extracted with 80 % ethanol at a ratio of 1:10 *m/v* in a large glass container. The container was wrapped in aluminum foil to reduce light exposure and left to mix for 24 h using a Caframo Stirrer BDC3030**.** To separate the extract from the vegetative residue, the homogenate was filtered using a Buchner funnel and Whatman paper filter. The liquid extract was collected and stored at 4 ^{o}C while the vegetative residue was collected and used for a second extraction. The extraction and filtration method was repeated and the two extracts were pooled.

The combined extracts dried *in vacuo* with a Yamato Rotovaporator at 55 ^{o}C and -800 bar of pressure until a brown residue remains. To dry the remaining water content from the residue, the extract

was frozen at -20 ^{o}C for 4 hours then placed in an EC Super Modulyo**,** until the extract the extract was completely dried. The dried extract was collected, weighed, and the yield (380 g or 22.35 %) was noted. Refer to **Schematic 3.1** for more information.

3.2.8 Preliminary fractionation of R. groenlandicum

A 1.8 L glass column was used for the fractionation of *R. groenlandicum* with 1.5 kg of silica gel. Before adding the silica to the column, 150 gm was set aside and 1.35 kg was added to the column and left to settle. To facilitate the adhering of the crude extract to the silica gel, 100 gm of the extract was solubilized in 100 % methanol and the remaining 150 g of silica was added while mixing, producing a paste. The mixture was left to air dry over night. The following day the extract was carefully added to the column, as to not disturb the silica gel, and a small amount of sand was added over top to prevent disturbance to the silica when solvent was added. The column was then flooded with 1 L hexane and left to soak overnight.

To initiate the elution of the column, the valve was opened and the solvent was collected with 250 ml Erlenmeyer flasks. The gradient of the mobile phase, hexane-EtOAc and EtOAc-MeOH, was adjusted every 2 litres, or every 8 flasks of eluates (Table 3.1). A total of 144 eluates was collected and pooled based on their TLC profiles. Nine fractions, F1-F9, were obtained from the pooled eluates and dried in a desiccator. The dried weight of each fraction was recorded and the fractions were stored in dry cool conditions.

Schematic 3.1. Extraction and primary fractionation scheme for *R. groenlandicum*.

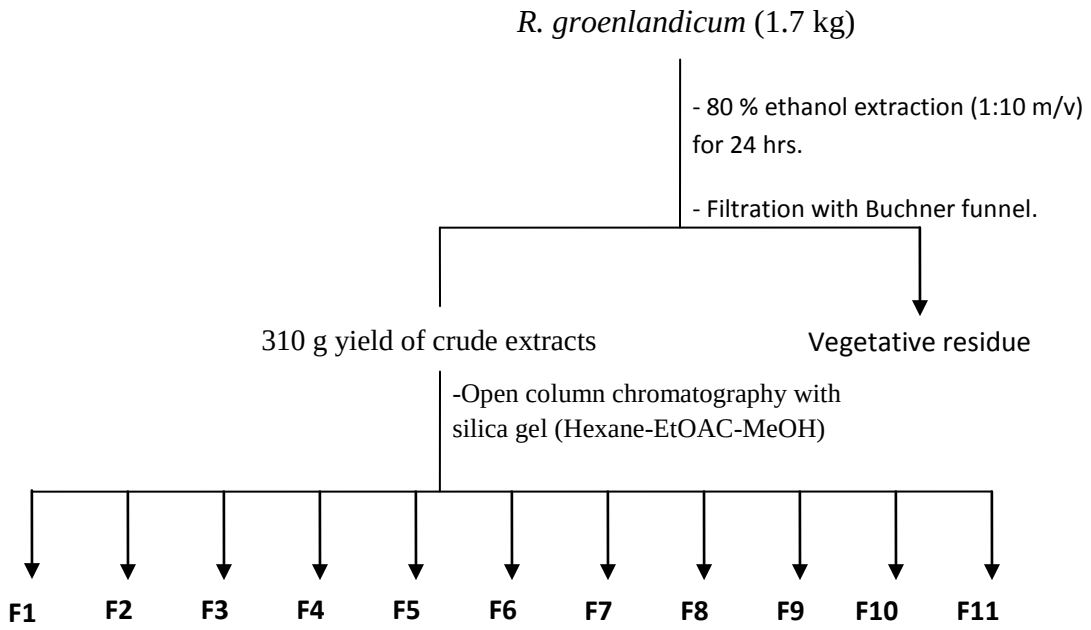


Table 3.1. Fractionation by open column chromatography of crude extract from the leaves of *R. groenlandicum*.

Mobile Phase	Proportions	Eluates	Pooled Fractions	Codes
Hexanes	100	1-8	1-22 (unused)	
Hexanes-EtOAc	90:10	9-16	23-29	**F1**
Hexanes-EtOAc	80:20	17-24	30-35	**F2**
Hexanes-EtOAc	70:30	25-32	36-38	**F3**
Hexanes-EtOAc	60:40	33-40	39-46	**F4**
Hexanes-EtOAc	50:50	41-48	47-57	**F5**
Hexanes-EtOAc	40:60	49-56	58-71	**F6**
Hexanes-EtOAc	30:70	57-64	72-79	**F7**
Hexanes-EtOAc	20:80	65-72	80-90	**F8**
Hexanes-EtOAc	10:90	73-80	91-144	**F9**
EtOAc	100	81-88		
EtOAc-MeOH	95:5	89-96		
EtOAc-MeOH	90:10	97-104		
EtOAc-MeOH	80:20	105-112		
EtOAc-MeOH	70:30	113-120		
EtOAc -MeOH	60:40	121-128		
EtOAc-MeOH	50:50	129-136		
MeOH	100	137-144		

3.2.9 Aldose reductase inhibition of R. groenlandicum fractions F1-F9

The nine fractions obtained from the crude extracts of *R. groenlandicum* were tested for aldose reductase inhibition properties, as described above, to determine the most active fraction. Approximately 10 mg of each fraction were accurately weighed out and the corresponding volumes of 99 % EtOH were added to obtain a concentration of 10 mg/ml. The solubilized extracts were then placed in a Branson200 ultrasonic cleaner for 10 minutes. Five micro litres of each sample were used in each well, bringing the final concentration of the sample in each to 25 ug/ml with an ethanol concentration of 2.5 %. Each assay was performed in triplicates and repeated 3 times. Two of the most active fraction was selected and prepared for further sub-fractionation.

3.2.10 Sub-fractionation of fractions F7 and F8 of R. groenlandicum

Fractions F7 and F8 were selected for further investigations as a result of their high levels of AR inhibition and were fractionated on separate days. A 500 ml column was selected to accommodate a total of 300 mg of silica gel. One tenth of the silica was set aside and 270 mg was used to pack the column. The column was allowed to settle. To prepare the extract, 20 gm of the fraction was solubilized in MeOH and the remaining 30 mg of silica was added while mixing, producing a paste. The mixture was left to air dry over night. The following day the extract was carefully added to the column, so as to not disturb the silica gel, and a small amount of sand was added over top to prevent disturbance to the silica when solvent is added. The column was then flooded with 250 ml of hexane and left to soak overnight.

To initiate the elution of the column, the valve was opened and the solvent was collected with 30 ml Erlenmeyer flasks. The gradient of the mobile phase, hexane-EtOAc and EtOAc-MeOH, was adjusted every 250 ml, or approximately every 8 flasks of eluates (Table 3.2 and 3.3). A total of 260 eluates were collected for F7 and 64 eluates were collected for F8. The fractions were pooled based on their TLC profiles. After the pooling process, 14 sub-fractions was obtained for F7 (F7-1 to F7-14) and 12 sub-fractions was obtained for F8 (F8-1 to F8-12). The sub-fractions were air dried overnight, weighed and stored in cool dry conditions.

Table 3.2. Fractionation by open column chromatography of active fraction, F7, from the leaves of *R. groenlandicum.*

Mobile Phase	Proportions	Eluates	Pooled Fractions	Codes
Hexanes-EtOAc	50:50	1-20	1-28	**F7-1**
Hexanes-EtOAc	40:60	21-40	29-45	**F7-2**
Hexanes-EtOAc	30:70	41-60	46-71	**F7-3**
Hexanes-EtOAc	20:80	61-80	72-81	**F7-4**
Hexanes-EtOAc	10:90	81-100	82-92	**F7-5**
EtOAc	100	101-120	93-95	**F7-6**
EtOAc-MeOH	95:5	121-140	96-114	**F7-7**
EtOAc-MeOH	90:10	141-160	115-147	**F7-8**
EtOAc-MeOH	80:20	161-180	148-167	**F7-9**
EtOAc-MeOH	70:30	181-200	168-173	**F7-10**
EtOAc-MeOH	60:40	201-220	174-178	**F7-11**
EtOAc-MeOH	50:50	221-240	179-189	**F7-12**
MeOH	100	241-260	191-203	**F7-13**
			204-260	**F7-14**

Table 3.3. Fractionation by open column chromatography of active fraction, F8, from the leaves of *R. groenlandicum.*

Mobile Phase	Proportions	Eluates	Pooled Fractions	Codes
EtOAc	100	1-8	1-6	**F8-1**
EtOAc-MeOH	95:5	9-16	7-8	**F8-2**
EtOAc-MeOH	90:10	17-24	9-12	**F8-3**
EtOAc-MeOH	80:20	25-32	13	**F8-4**
EtOAc-MeOH	70:30	33-40	14-15	**F8-5**
EtOAc-MeOH	60:40	41-48	16-20	**F8-6**
EtOAc-MeOH	50:50	49-56	21-25	**F8-7**
MeOH	100	57-64	26-29	**F8-8**
			30-37	**F8-9**
			38-42	**F8-10**
			43-49	**F8-11**
			50-64	**F8-12**

3.2.11 Aldose reductase inhibition assay of R. groenlandicum sub-fractions F7 and F8

Fractions F7 and F8 possessed high levels of activity. As a result, the sub-fractions obtained from F7 and F8 were tested in the aldose reductase inhibition assay, as described above, to determine the most active. Approximately 10 mg of each sub-fraction was weighed out and the corresponding volumes of 99 % EtOH were added to obtain a concentration of 10 mg/ml. The solubilized extracts were then placed in a Branson 200 ultrasonic cleaner for 10 minutes. Five microlitres of each sample was used in each well, bringing the final concentration of the sample in each to 25 ug/ml with an ethanol concentration of 2.5 %. Each assay was performed in triplicates and repeated 3 times.

3.2.12 HPLC analysis

All solvents for the analysis were HPLC grade (Fluka, Oakville, ON, Canada). The samples were re-dissolved at 20 mg/ml in HPLC grade methanol, sonicated for 5 min, filtered through 0.2 micron syringe filters (Chromspec Inc.) and 1 µl was injected on an HPLC-DAD system (1100 series), Agilent Technologies Inc. (Palo Alto, CA, USA). The system consisted of an auto-sampler with 100 µL built in loop, a quaternary pump (maximum pressure limit 400 bars), a photodiode array, a column thermostat. The separations were performed on a Synergi Fusion column – RP 150 mm × 3.00 mm column, 4 micron particle size and pore an average diameter of 80Å (PN 00F –4424-YO, SN 355361-1) at an oven temperature of 50°C at a flow rate of 0.4 ml/min. The elution condition was 10-100 % B in 30 min, the column was washed with 100% B for 2 min, returned to the initial conditions in 0.1 min, and re-equilibrated for 5 min (total runtime 35 min). Standard markers used for quantitative analysis were examined under 280 and 325 nm. The 10 markers selected for detection are: catechin (+), chlorogenic

acid, p-coumaric acid, epicatechin, myricetin, quercetin, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside, rutin, obtained from our laboratory compound library. A standard curve was used to quantify the amount of each standard marker in the samples.

3.3 Results

3.3.1 Preliminary screening

The initial screening of the 17 Cree samples resulted in a very wide range of activity (Figures 3.2-3.5). Because not all the samples could be tested within one experimental run, the samples were randomly grouped into 4 separate experimental runs and the results were standardized against their respective standard control. The highest inhibitory activities were seen in *R. groenlandicum* and *G. hispidula* at 35.11 ± 0.16 % and 36.49 ± 1.62 %, respectively (Appendix Table 3.1 for more detail). Following the top 2 plants were: *K. angustifolia* (26.67± 1.17 %), *P. balsamifera* (26.54 ± 0.93 %), *R. tomentosum* (25.93 ± 3.92 %), *A. incana* (22.20 ± 3.36 %), *P. glauca* (19.55 ± 4.03 %), *L. laricinia* (14.73 ± 1.22 %), *S. decora* (13.57 ± 3.64 %), *V. vitis* (13.19 ± 1.40 %), *J. communis* (12.73 ± 1.41 %), *L. clavatum* (9.71 ± 5.74 %), *A. balsamea* (9.14 ± 0.61 %), *S. purpurea* (8.82 ± 1.82), *S. planifolia* (-1.88± 0.63 %), *P. banksiana* (-8.56± 6.06 %) and *P. mariana* (-30.61± 7.49 %).

3.3.2 AR inhibitory potential of Rhododendron groenlandicum fractions F1-F9

The AR inhibition obtained for fractions F1-F9 produced a bell shaped activity curve (Figure 3.6). The data obtained from each sample were standardized with the control and were ranked with F7 having the highest inhibitory potential (64.35 ± 1.07) followed by F6 (45.00 ± 7.54 %), F9 (36.98 ± 4.37

%), F8 (36.10 ± 0.42 %), F5 (17.16 ± 2.76 %), F3 (8.51 ± 5.06 %), F2 (5.67 ± 2.35 %), F1 (5.66 ± 1.11 %) and F4 (4.41 ± 1.13 %) (Appendix Table 3.2).

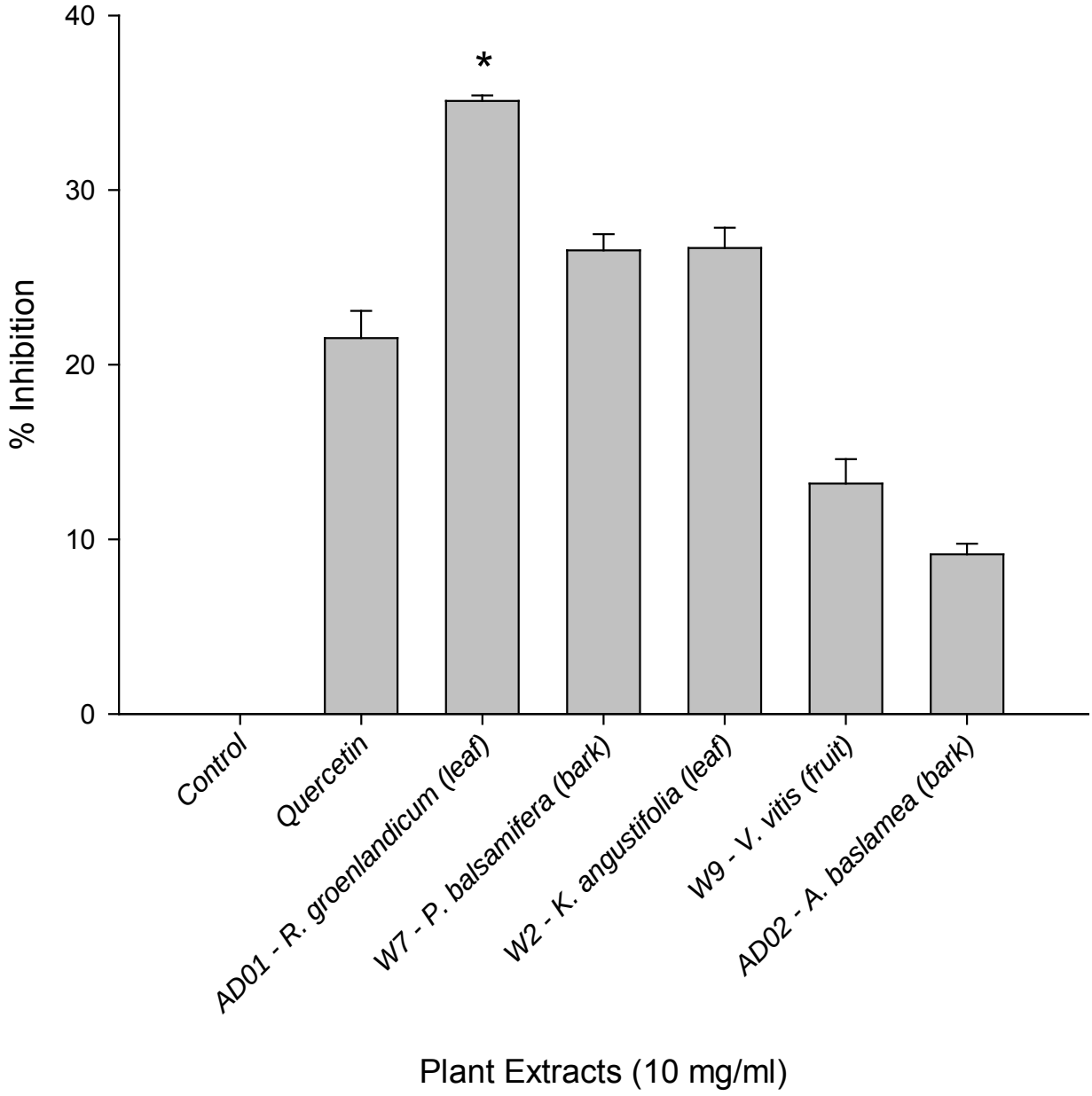


Figure 3.2. The inhibition potential of *R. groenlandicum, P. balsamifera, K. angustifolia, V. vitis* and *A. balsamea* against aldose reductase acquired from homogenized swine lens. The data were standardized with the control to obtain the percent inhibition and compared to quercetin. The n value is 3 and the standard error is as indicated. AD01 displayed significantly higher activity than quercetin and other samples, $p < 0.001$.

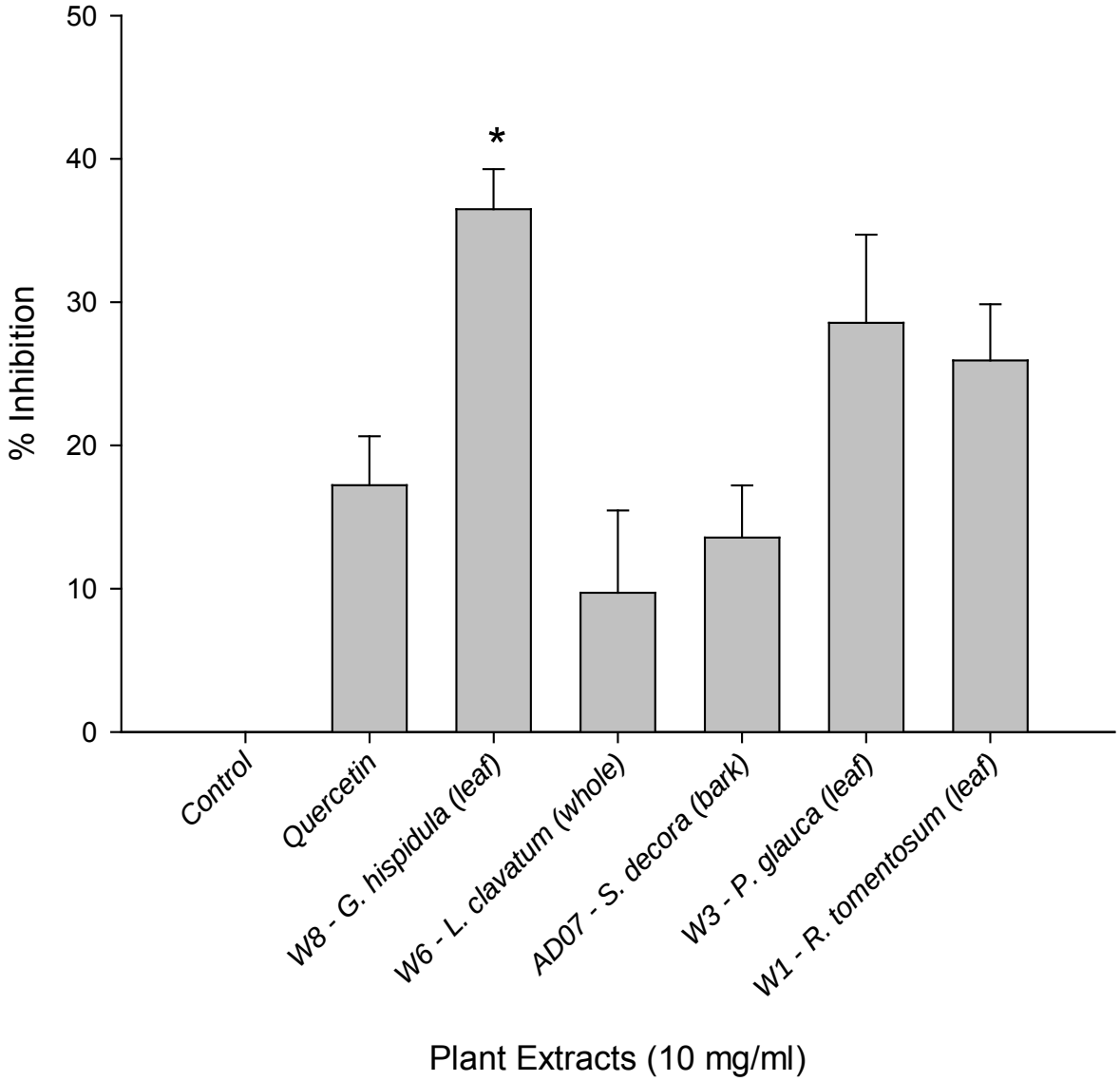


Figure 3.3. The inhibition potential of *G. hispidula, L. clavatum, S. decora* and *R. tomentosum* against aldose reductase acquired from homogenized swine lens. The data was standardized with the control to obtain the percent inhibition and compared to quercetin. The n value is 3 and the standard error is as indicated. The activity of W8 was significantly higher than quercetin, $p = 0.03$.

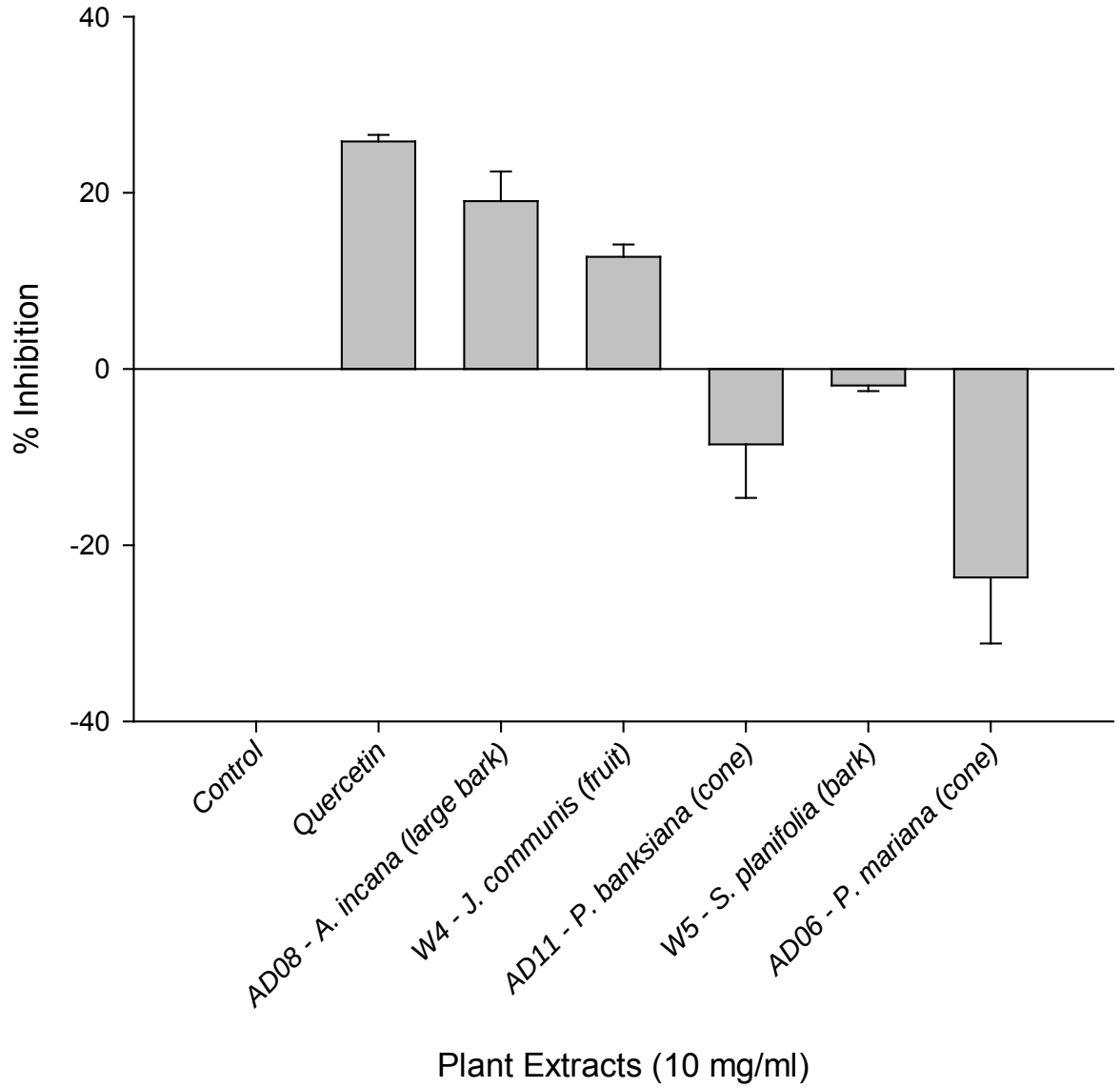


Figure 3.4. The inhibition potential of *A. incana, J. communis, P. banksiana, S. planifolia* and *P. mariana* against aldose reductase acquired from homogenized swine lens. The data was standardized with the control to obtain the percent inhibition and compared to quercetin. The n value is 3 and the standard error is as indicated. No samples displayed higher activity than quercetin.

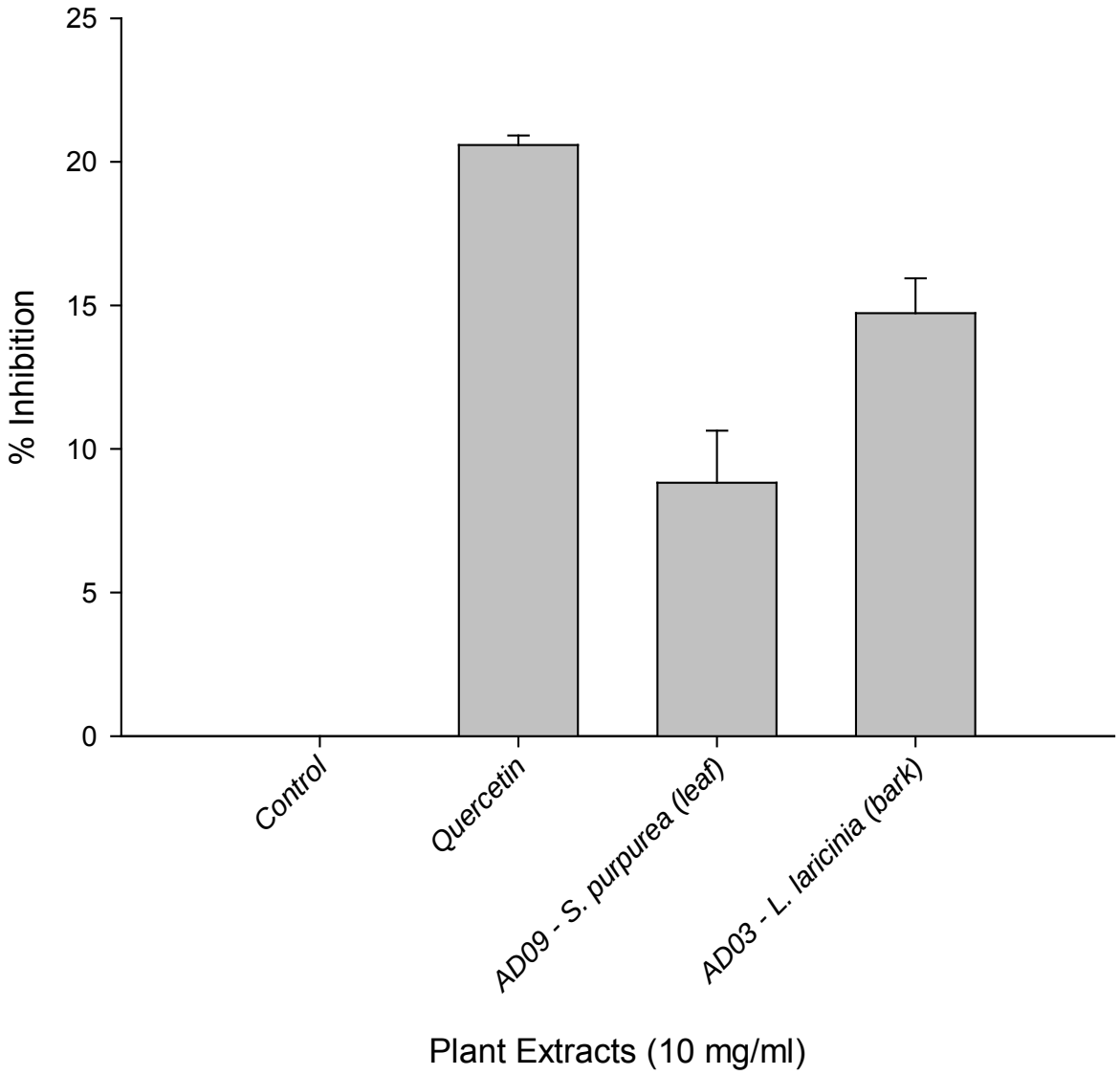


Figure 3.5. The inhibition potential of *S. purpurea* and *L. laricinia* against aldose reductase acquired from homogenized swine lens. The data was standardized with the control to obtain the percent inhibition and compared to quercetin. The n value is 3 and the standard error is as indicated. No samples displayed significantly higher activity than quercetin.

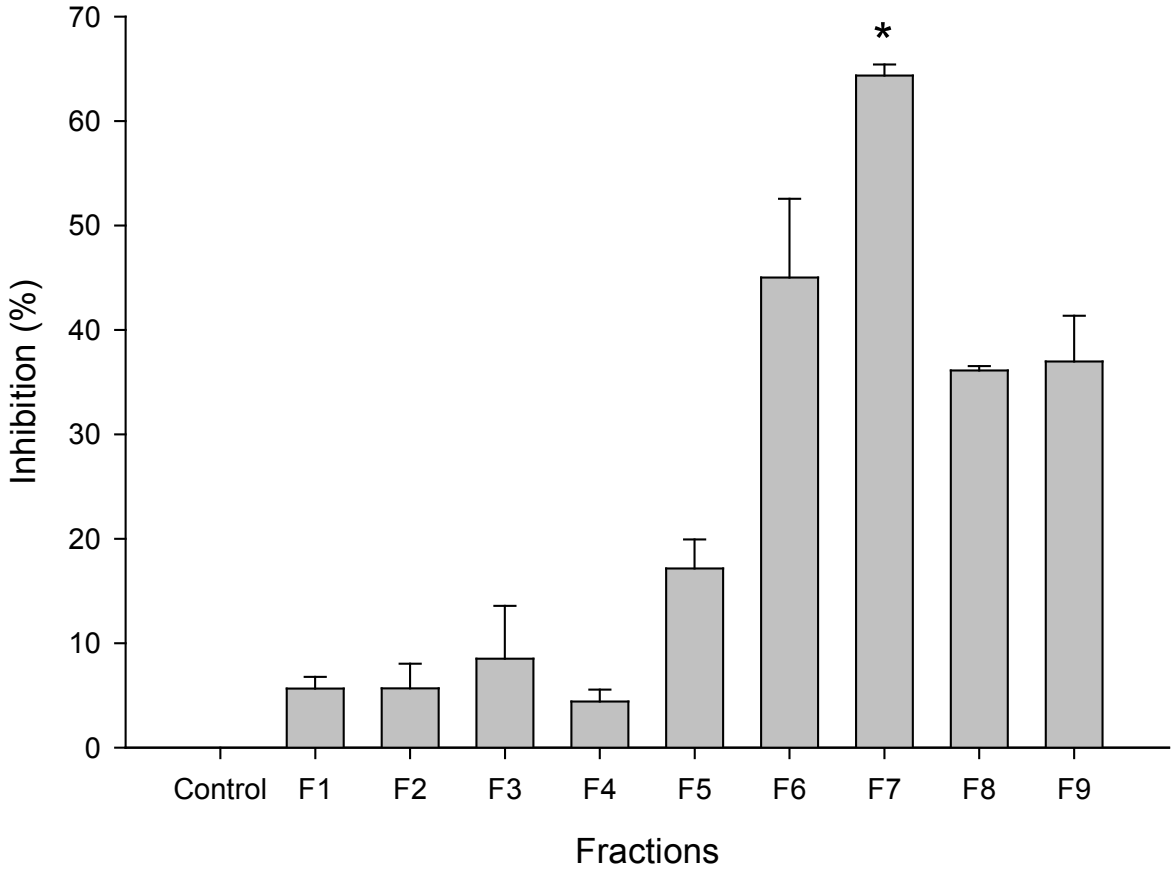


Figure 3.6. The inhibition by fractions F1-F9 from *R. groenlandicum* of aldose reductase acquired from homogenized swine lens. The data was standardized with the control to obtain the percent inhibition. The n value is 3 and the standard error is as indicated. Fraction F7 displayed significantly higher activity than the control, $p = 0.04$.

3.3.3 AR inhibitory potential of R. groenlandicum of sub-fractions of F7 and F8

The AR inhibitory activity of the sub-fractions of F6, F7 and F8 were all relatively high. All sub-fractions displayed inhibition exceeding 70 % inhibition, referenced to quercetin which was standardized to 100 % (Appendix Table 3.3). After a second fractionation, a higher level of inhibitory activity was observed from F7-10 to F8-8 with the apex at F8-2 (Figure 3.9), the highest percentage inhibition (11.49± 0.70 %), followed by F7-13 (11.34 ± 0.87 %) and F8-1 (11.30 ± 0.62 %). The AR inhibitory activity of the sub-fractions falloff on both opposing ends of the F7 and F8 range.

3.3.4 HPLC Analysis

The method used in this analysis was optimized for phenolic detection and quantification. The markers identified as well as previous analysis reports from our lab suggested that the major components of the sub-fractions of F7 and F8 were phenolics. Marker compound identified in the sub-fractions of F7 and F8 include catechin[A], chlorogenic acid[B], epicatechin[C], p-coumaric acid[D], quercetin-3-galactoside[E], quercetin-3-rhamnoside[F], quercetin-3-glucoside[G], myricetin[H], rutin[I] and quercetin[J] (Figures 3.7 and 3.8). Quantification of the marker compounds revealed the presence of quercetin derivatives in very high concentrations in between sub-fractions F7-5 and F8-8. Concentrations of quercetin derivatives were detected and found to be present in relatively high concentration. Quercetin-3-rhamnoside and quercetin-3-galactoside reached 118.26 ± 0.54ug/ml and 621.59 ± 0.00 ug/ml in F7-7 and F8-4, respectively (Appendix Table 3.3). Quercetin and quercetin-3-glucoside were also detected but in lower concentrations at 72.72 ± 0.14 ug/ml and 76.75 ± 1.44ug/ml in sub-fractions F7-9 and F7-1, respectively.

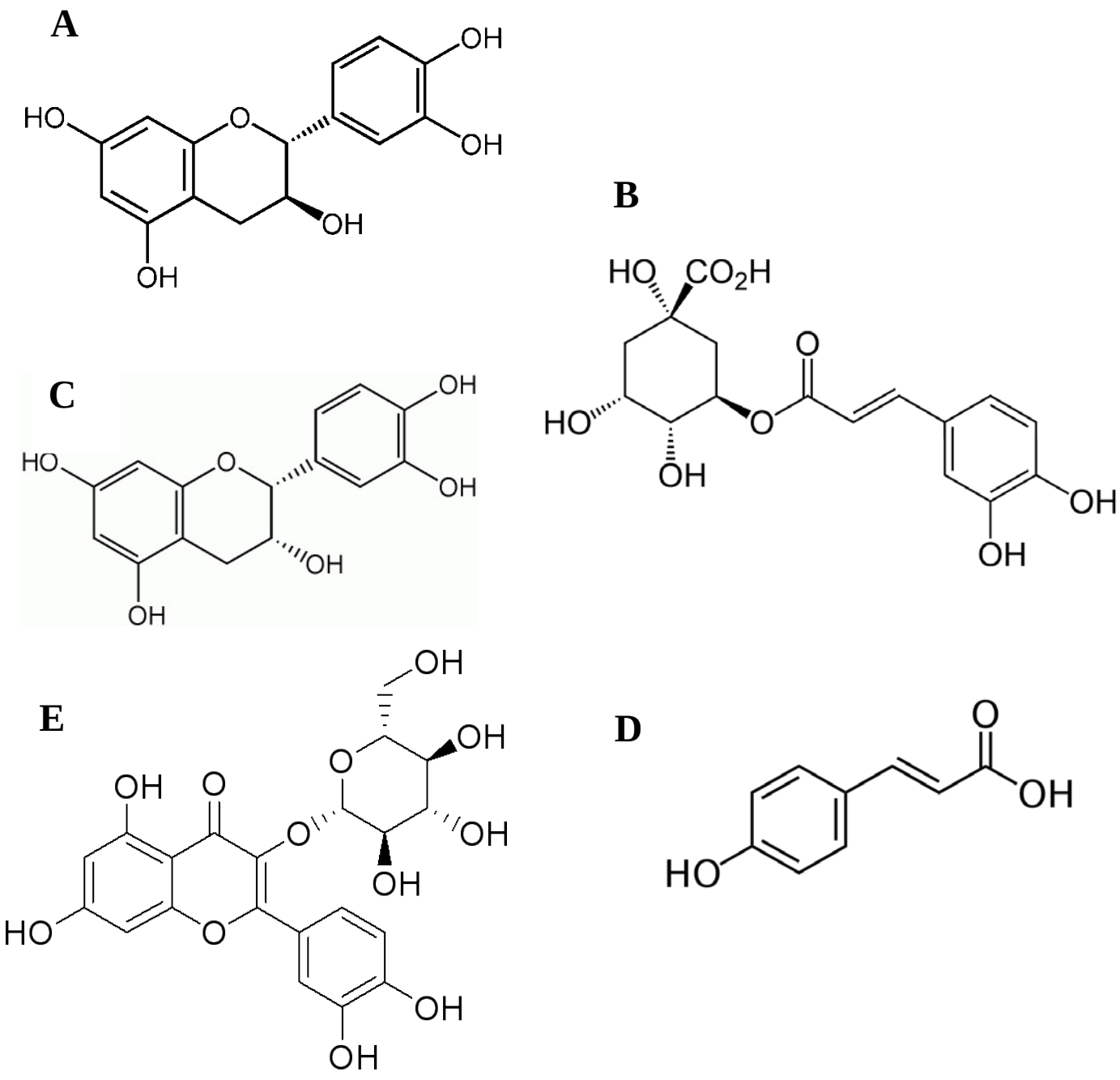


Figure 3.7 Chemical structures of A. catechin, B. chlorogenic acid, C. epicatechin, D. p-coumaric acid, and E. quercetin-3-galactoside selected phenolics used as markers for HPLC analysis.

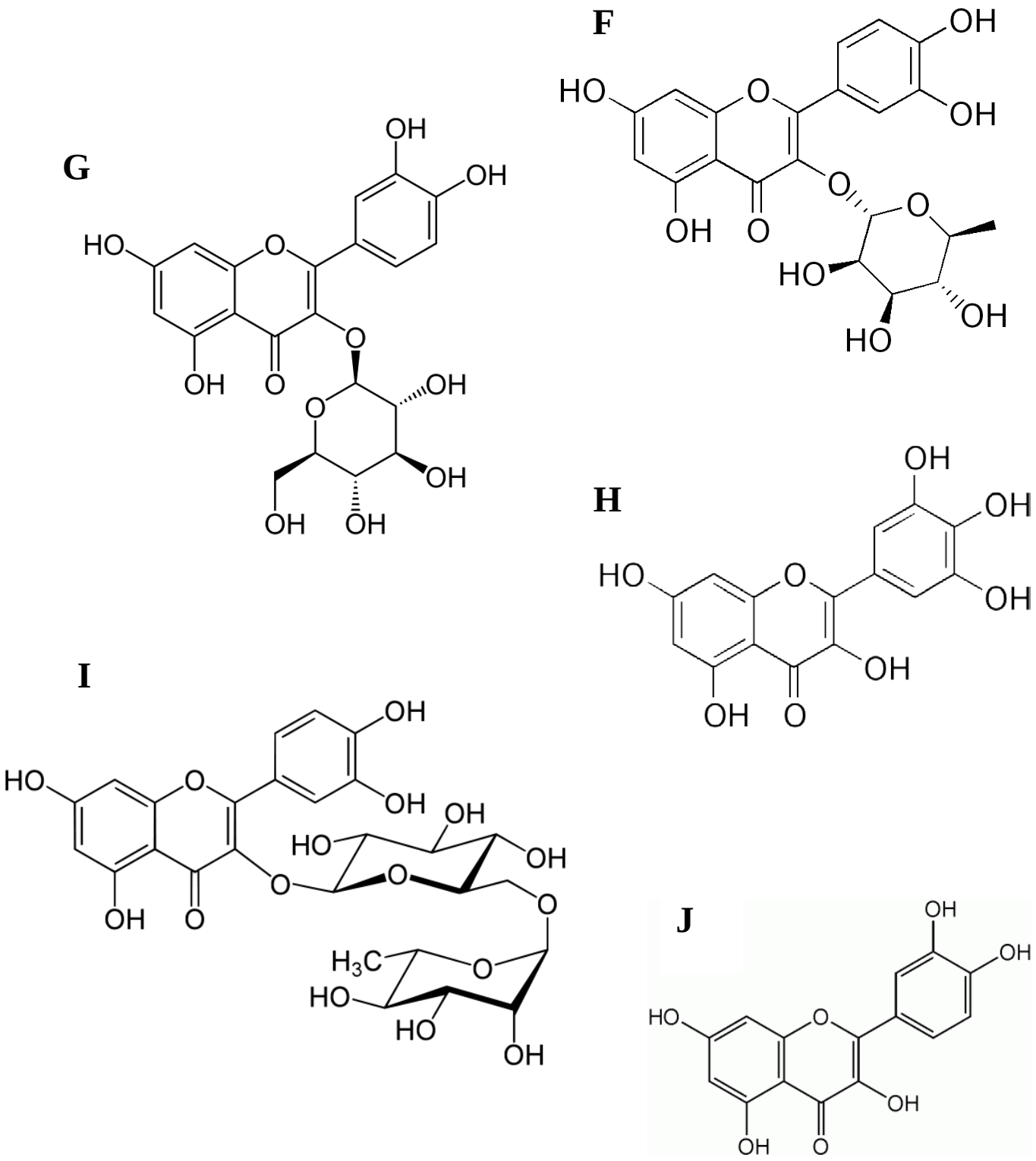


Figure 3.8 Chemical structures of F. quercetin-3-rhamnoside, G. quercetin-3-glucoside, H. myricetin, I. rutin and J. quercetin selected phenolics used as markers for HPLC analysis.

The quantification analysis of p-coumaric acid, rutin and myricetin indicated that they were present in a very broad range but in very low concentrations, < 0.1 ug/ml, in the sub-fractions tested. Chlorogenic acid was detected at higher levels, 0.49 ± 0.00 ug/ml, in the more polar sub-fraction, F8-12. Catechin and epicatechin were detected in great abundance, reaching concentrations of 2.86 ± 0.02 ug/ml and 2.26 ± 0.04 ug/ml in F7-2.

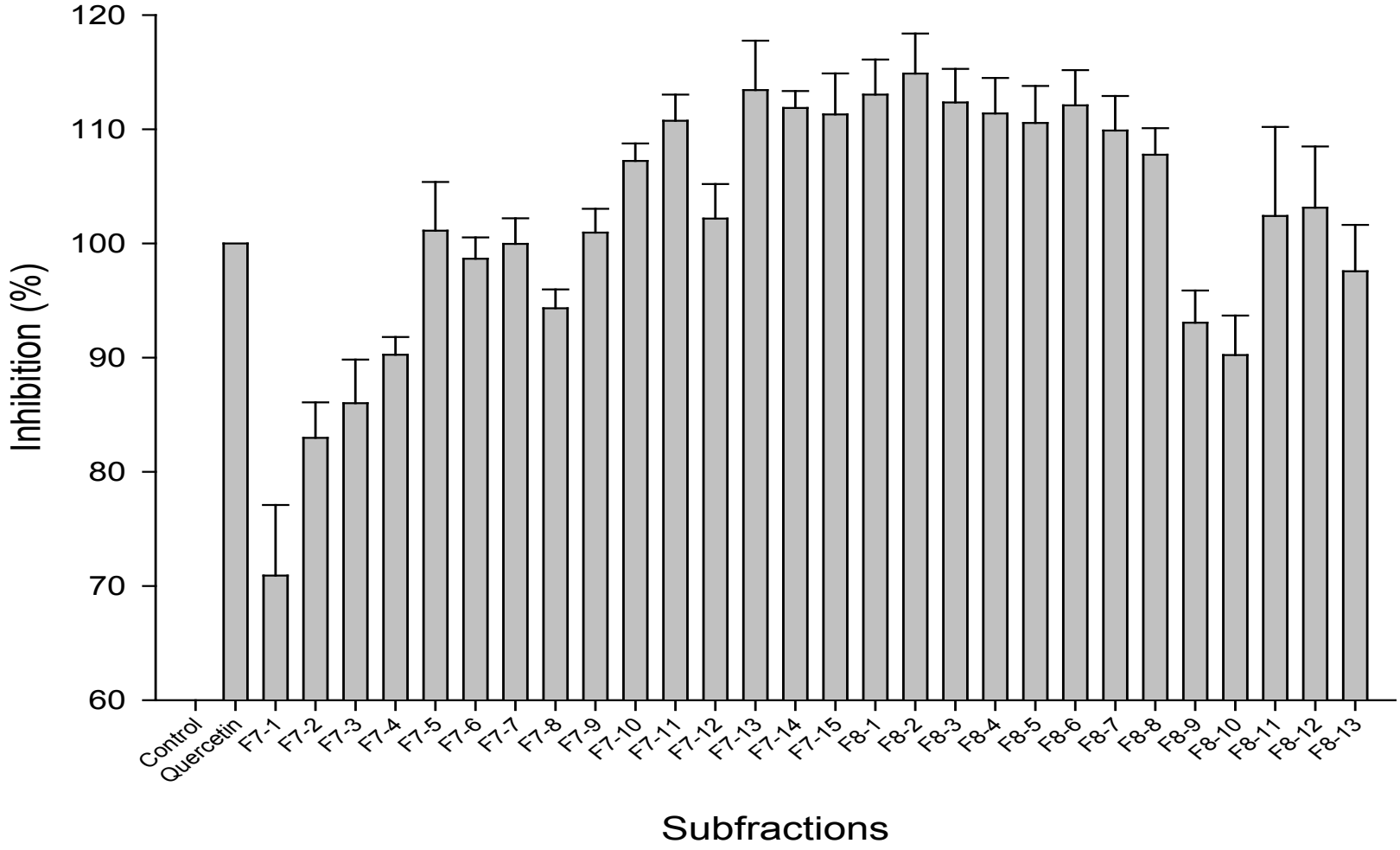


Figure 3.9. The inhibition activity of sub-fractions F7-1 to F8-13 of *R. groenlandicum* against aldose reductase isolated from homogenized swine lens. The data was standardized with the control to obtain the percent inhibition. The n value is 3 and the standard error is as indicated.

3.4 Discussion

The AR inhibition method used in this study is a novel approach to the existing and established ones. Because of the high cost associated with working with human recombinant aldose reductase enzymes, swine eyes have been widely used for high throughput screenings and preliminary investigations. The original manuscripts by Hayman and Kinoshita (1965) described the method adapted here using homogenized lens isolate extracted from calf eyes and rat eyes (Kador *et al*, 1986**).** Swine eyes were selected for this study because they were readily accessible and were a much more cost effective alternative to rat eyes. In addition, these eyes are morphologically much more similar to human eyes than rat eyes and have been used extensively in ophthalmological research (Chinnery *et al*, 2005; Johansson *et al*, 2010; Lalonde *et al*, 2006). The use of swine eyes in aldose reductase inhibition (ARI) research have also been reported by Yadav *et al* (2009), however this approach still requires further investigation. Tests against quercetin as a positive control, with repeatable results, demonstrated the presence and activity of aldose reductase in the lens homogenate. The activity observed in the assays suggested that swine eyes can serve as a model to test aldose reductase inhibition in Cree antidiabetic plants.

The 17 Cree antidiabetic plants were all selected for their traditional use to treat symptoms of diabetes. Previous studies by the CIHR team in antidiabetic traditional medicines have reported the effectiveness of these 17 plants in treating diabetic complications (Harbilas *et al,* 2009). When tested against the AR inhibition assay, several plants also show very high potential in treating diabetic cataracts. Six of the 17 plants tested, which includes *G. hispidula, P. glauca, R. tomentosum, R. groenlandicum, P. balsamifera, and K. angustifolia*, displayed higher inhibitory activity than the

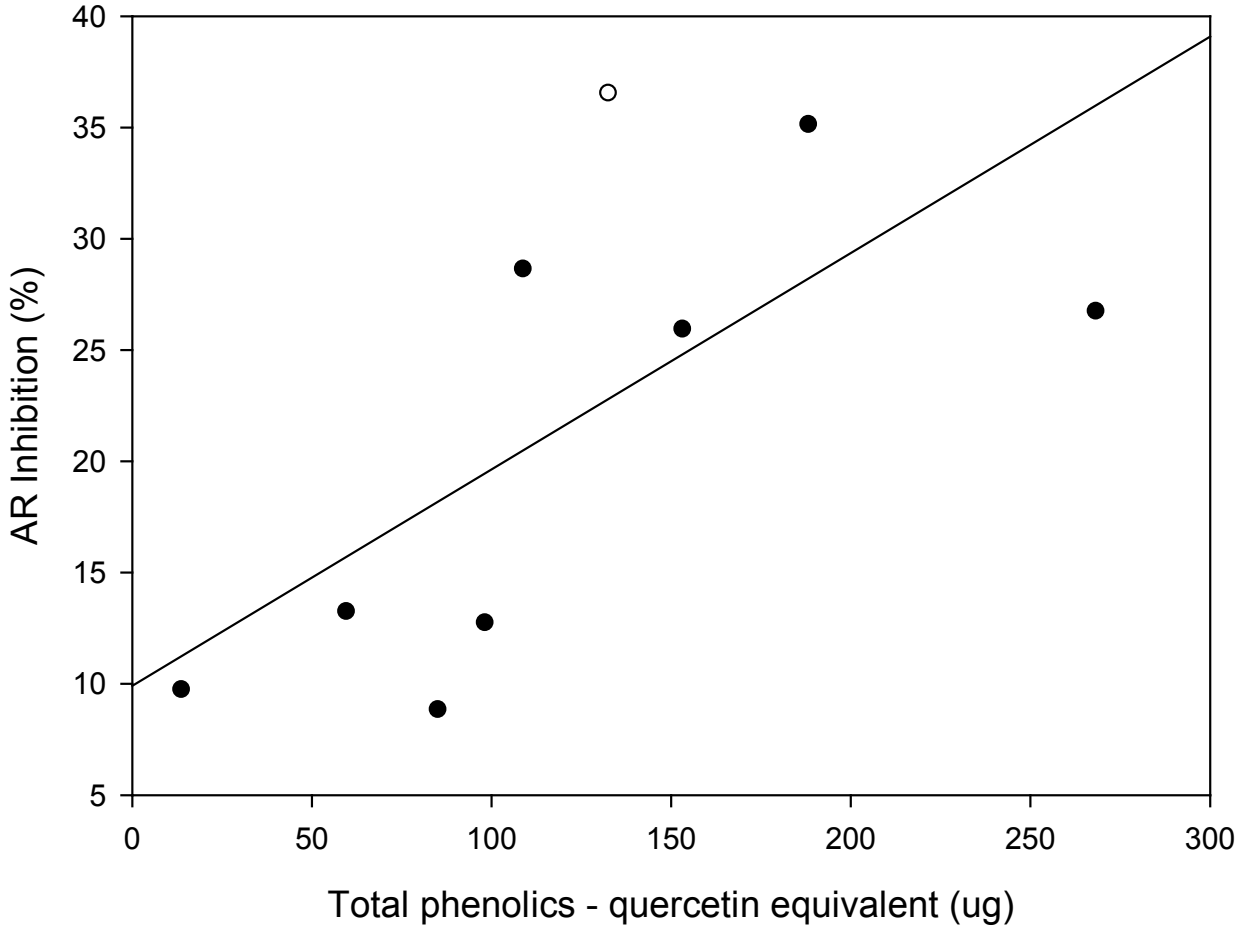


Figure 3.10. A correlative comparison between AR inhibition and total phenolics content. A line of best fit was drawn using a linear regression and the r^2=0.44, p=0.05. The equation of the regression line is y=0.1x+9.92 .

positive control, a pure compound. A direct linear correlation was obtained between total phenolics concentration and AR inhibition, with an R-square value of 0.44 (Figure 3.10).

The linear relationship between AR inhibition and total phenolics content determined from the preliminary screening indicates possible correlation between AR inhibition and antioxidant potential. The total phenolic acid test is, in fact, based on reduction by phenolics and is highly correlated with standard antioxidant assays like DPPH (Spoor *et al*, 2006; Fraser *et al*, 2007). Because oxidative stress is a contributing factor to the development of diabetic cataracts and other diabetic complications, either via protein glycosylation or interference with the glutathione reductase pathway or the polyol pathway, candidate plant samples containing high levels of antioxidants are promising leads for ARI (Rahimi *et al*, 2005). From the data acquired in the preliminary screening, *R. groenlandicum* was selected as the most promising candidate for further study. Spoor *et al* also reported *R. groenlandicum* to contain high levels of phenolics and is an effective antioxidant and thus making it very beneficial as a complementary and alternative medicine to remediate diabetic complications.

In order to further investigate the principle bioactive components of *R. groenlandicum*, it was necessary to produce fractions of its crude extract. The primary fractionation of *R. groenlandicum* produced 9 phytochemically distributed fractions, from which the most active fractions, under the AR inhibition assay, were selected and further fractionated. Fraction F7 showed significantly highest ARI property and, therefore, was chosen for further fractionation. Fraction F8 displayed lower activity ARI activity than F7 but was tested because its subfractions were already prepared. Fractions F6 and F9 were also potentially good candidates but are not as active as F7 and F8, hence were not selected for further investigation in this study. It is important to note that *R. groenlandicum* does contain condensed tannins,

which can bind and cause the precipitation of proteins out of solution (Schofield *et al*, 2001*;* Koleckar *et al*, 2008*)*. Future studies will be investigating the activity of *R. groenlandicum* with tannins removed.

To further identify and validate the active components in F7 and F8, it was necessary to do a phytochemical analysis of their sub-fractions. The marker compounds selected are phenolics known to have antioxidant properties; some are well known AR inhibitors. All 10 markers used were detected and identified in the sub-fractions of F7 and F8**.** The marker compounds identified were: catechin, chlorogenic acid, p-coumaric acid, epicatechin, myricetin, quercetin, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside, rutin. The quantification of the marker compounds revealed that the phenolics detected were quercetin-glycosides. More specifically, all glycoside compounds appeared to be present in higher concentrations between F7-7 and F8-6, the most active region. Much research has been done with AR inhibitors while using quercetin as a positive control (Carbone *et al*, 2009; Patel & Mishra, 2009). The presence of the quercetin and quercetin glycosides in the complex sub-fractions indicates that Labrador tea contains safe and effective active principles for AR inhibition.

The AR inhibition data obtained from the sub-fractions of F7 and F8 all reported positive inhibitory activity throughout (Figure 3.9). When compared to the distribution of marker compound concentration, the highest levels of AR inhibition coincides with the highest concentrations of quercetin and quercetin glycosides in between F7-5 and F8-8. Although catechin and epicatechin also exhibited high concentrations in the sub-fractions, particular in F7-1 and F7-2, their contribution to the AR inhibition was relatively low, comparing to the remaining sub-fractions.

In summary, the 17 Cree plants selected for this study based on their antidiabetic potential determined in previous studies, showed a relatively wide range of AR inhibition, thereby suggesting a

pharmacological basis for their selection in traditional use. *R. groenlandicum,* in particular, exhibited relatively high levels of ARI potential due to the higher concentrations of phenolics that is contained within. As shown, there was a correlation between plant material containing higher levels of phenolics, quercetin and quercetin glycosides in particular, and aldose reductase inhibition. Because complications of diabetes and diabetic cataractogenesis are oxidative stress driven, plant materials containing higher levels of phenolics can play an important role in slowing down their detrimental progression and, therefore, be used as complimentary or alternative medicines.

Lastly, aldose reductase inhibition method developed for this study has proven to be effective for preliminary screenings as well as bioassay guided fractionation and isolation. Further improvements and studies can be made to reduce variability as well as provide quantitative protein quantification for IC50 studies, the latter would require purified swine lens protein isolate or recombinant protein, which would still be more cost effective than human aldose reductase recombinant proteins. Although the use of swine eyes to for other ophthalmological studies is not a novel idea, research is still lacking for aldose reductase inhibition. Due to the physiological and morphological similarities to human eyes, swine eyes have the potential to serve as a very good model for diabetic cataractogenesis as well as aldose reductase inhibition, in general.

CHAPTER 4.

GENERAL DISCUSSION

4.1 Main Conclusions

In recent years, the escalation of cultural mixing and global migration of peoples has created a resurgence of interest in and curiosity for traditional, foreign and exotic plant derived foods and medicines in North America. Alongside the culinary creations intended to stimulate adventurous taste buds, the incorporation of ingredients such as exotic fruits, herbs, spices, teas, pulses and cereals into the North American diet has also introduced many phytochemicals that were not previously present in diets or were only found in minor amounts. This is not to say that consumers are unaware of the health modifying properties of their newly integrated foods. Many have learned of the traditional uses and health benefits of many different ethnobotanical foods and medicines and have tried to incorporate very specific items into their diet in hopes to benefit from them.

The primary objective of the first study was to determine whether common foods, available in the Canadian market possess any potential to inhibit cytochrome P450.The results obtained suggested that common food plant extracts do have activity as inhibitors of CYP enzymes and that certain food groups have a higher inhibitory activity than others. Herbs and spices from the *Apiaceae* and *Lamiaceae* family, in particular, showed very high levels of CYP inhibition in comparison to legumes and pulses. Despite the small quantities that are generally consumed on a daily basis, these flavouring agents contain very high activities, which can be associated with high levels of bioactive phytochemicals, the same phytochemicals responsible for their aroma. Legumes and pulses, on the other hand, have quite low CYP inhibitory potential. Unlike herbs and spices, this food group is often consumed in very large quantities, yet is quite inactive in inhibiting these CYP enzymes.

The secondary objective in this study was to determine if any antibacterial activity was associated with the extracts and if any correlation between CYP inhibition and antimicrobial activity could be established, specifically on bacteria species found in the flora of the gut. Similar to the

activity observed in the CYP inhibition assays, the most active antimicrobial extracts belonged to the *Apiaceae* and *Lamiaceae*. A significant linear correlation was established between several enzyme activities and antibacterial activities. This finding was consistent with the presence of phytochemicals responsible for the CYP inhibition may also be acting as antimicrobial agents, but further work is required to identify specific active phytochemicals.

Prior to this study, the bulk of CYP inhibition investigations have been focused on medicinal plants, new chemical entities, drugs and synthetic compounds. At the time of writing, this was the first study to undertake a large scale screening of common food plants for possible inhibition of CYP 3A4, 3A5, 3A7 and 2D6, as well as one of the very few studies to find a correlation between the well being of the gut micro-flora and drug metabolism. As this study has shown, even common food items do have potential for CYP inhibition. The antimicrobial properties of the extracts tested may also contribute further to CYP inhibition. As reported by Walker (1973)**,** the micro flora in the gut serves as a first line of defense against foreign xenobiotics. When encountering foreign compounds the bacterial flora provide a preliminary barrier by metabolizing some of the compounds. This activity provides serves a support system to reduce the metabolic load on the systemic CYP enzymes. If disturbed by antimicrobial compounds, the microflora may suffer a deficit in concentration, thereby increasing the metabolic load onto the gut's innate CYP system

In the second experimental study on aldose reductase inhibition, one of the specific objectives of the study was to evaluate a new and more accessible experimental model for the AR inhibition assay. The most established model conventionally used for the AR inhibition assay is lens homogenate obtained from rat eyes. Because rat eyes are not always readily available, another accessible and cost effective model was required. In a previous study (unpublished), we proposed using lens homogenate extracted from the lens of pig eyes, purchased from a local abattoir, as a source of AR enzymes. As

reported, the isolated lens homogenate exhibited linear activity during the initial control tests and was able to provide consistent results during the experimental assays. Pig eyes were much more accessible, cost effective and abundant source of AR, which as a meat byproduct do not require the sacrifice of experimental animals.

The rationale behind the aldose reductase study was to determine whether traditional Cree anti-diabetic medicines have inhibitory activity on the AR enzyme, which is a potential target in prevention of diabetic cataracts. The AR study provides suggests that traditionally used Cree plants do have biological effects on the enzyme in vitro. One interesting result obtained from the AR study was the direct correlation between total phenolic content and AR inhibition. Previous AR inhibition studies have found phenolic content in plants to be good indicators of AR inhibition, and that antioxidants are strong inhibitors of AR (Coudert *et al*, 1994; El-Kabbani *et al*, 2004; Termentzi *et al*, 2008).Bioassay guided fractionation lead to the separation and identification of a series of subfractions possessing AR inhibition activity. These subfractions contained high concentrations of phenolics: quercetin, quercetin glycosides, catechin and epicatechin.

Fueled by the ever growing exchange of cultural practices and the demands of individuals to take hold of their own health, complementary and alternative medicines, as well as natural health products, have become very abundant items in the market place. Health Canada has been working to inspect and evaluate manufacturer submissions for licensing and introduction of new products into the market. Furthermore, scientific evaluations by third party and academic laboratories have also contributed valuable reports and publication to further aid in the evaluation of these products.

Lastly, the data and observation obtained from this study provides a means of accrediting the Cree people of Northern Québec for their traditional knowledge of healing. It is important to note that TIID is a relatively new disease to the Cree. Nonetheless, traditional Cree knowledge of naturally

occurring medicinal plants was enough to provide effective remedies for symptoms of TIID. This knowledge is currently on the brink of extinction as new generations have begun to move away from the traditional lifestyle to pursue more urban lifestyles. Study such as these will provide the necessary documentation to retain and save this knowledge from disappearing. According to the World Health Organization, 80 % of the world populations still depend on traditional medicines and 25 % of modern drugs developed for the remaining 20 % of the population are derived from plant sources. Therefore, it is necessary to preserve the knowledge possessed by the indigenous people of Canada and further provide a reliable feedback system with scientific research.

4.2 Future Work

The primary goal for the collaborative project with Agriculture and Agri-Food Canada was to screen common food plants and NHPs for potential CYP inhibition activity. This ambitious project will require a large scale collection of samples, careful cataloguing, and testing to generate a public databank. This databank will serve as a food and NHPs safety sheet where the general public can consult for information on potential food/NHPs versus drugs interaction that may occur with their daily diet. Based on the findings, it is possible that food drug interaction maybe a result of direct inhibition of systemic CYP enzymes or by the killing of the gut microflora. A more thorough evaluation of antimicrobial foods on human microflora bacterial cultures and their xenobiotic metabolism after exposure will provide a better insight to the roles of the microflora.

In the Cree antidiabetic project, the goal was to evaluate the Cree people's pharmacopoeia and its effectiveness on treating complications of diabetes, specifically cataractogenesis. To strengthen the current finding, the swine model will need to undergo a more thorough quantification of enzyme

content and active plants will require IC50 studies to determine dosage. Furthermore, active subfractions and constituents will need to be isolated and reintroduced into the AR inhibition assay to determine potency. It will also be necessary to determine whether any synergistic effects are occurring on the CYP system between the separate constituents and marker compounds isolated from the active plant material. Lastly, study the effectiveness of these active principles in *vivo*, an animal model will be useful to determine the overall pharmacological and pharmacokinetic effects. The findings in future studies may provide significant contributions and applications in medicine.

CHAPTER 5.

REFERENCES CITED

5.0 REFERENCE CITED

Anderson, G. D., Rosito, G., Mohustsy, M. A., Elmer, G. W., 2003. Drug Interaction Potential of Soy Extract and Panax Ginseng. Journal of Clinical Pharmacology 43, 643-649.

Bailey, D. G., Dresser, G. K., Kreeft, J. H., Munoz, C., Freeman, D. J., Bend, J. R., 2000. Grapefruit-felodipine interaction: effect of unprocessed fruit and probable active ingredients. Clinical Pharmacology & Therapeutics 68, 468-477.

Bailey, D. G., Malcolm, J., Arnold, O., Spence, J. D., 1998. Grapefruit Juice-Drug Interactions. British Journal of Clinical Pharmacology 46, 101-110.

Béliveau, R., Gingras, D., 2006. Foods That Fight Cancer: Preventing Cancer through Diet. McClelland & Stewart, Toronto.

Beninger, C. W., Hosfield, G. L., 2003. Antioxidant Activity of Extracts, Condensed Tannin Fractions, and Pure Flavonoids from *Phaseolus vulgaris L.* Seed Coat Color Genotypes. Journal of Agricultural and Food Chemistry 51, 7879-7883.

Bourbonnais-Spear, N., Awad, R., Merali, Z., Maquin, P., Cal, V., Arnason, J. T., 2007. Ethnopharmacological investigation of plants used to treat susto, a folk illness. Journal of Ethnopharmacology 109, 380-387.

Bozin, B., Mimica-Dukic, N., Simin, N., Anackov, G., 2006. Characterization of the volatile composition of essential oils of some lamiaceae spices and the antimicrobial and antioxidant activities of the entire oils. Journal of Agricultural and Food Chemistry 54, 1822-1828.

Carbone, V., Zhao, H. T., Chung, R., Endo, S., Hara, A., El-Kabbani, O., 2009. Correlation of binding constants and molecular modelling of inhibitors in the active sites of aldose reductase and aldehyde reductase. Bioorganic and Medicinal Chemistry 17, 1244-1250.

Cermak, R., 2008. Effect of dietary flavonoids on pathways involved in drug metabolism. Expert Opinion on Drug Metabolism and Toxicology 4, 17-36.

Chinnery, H., Kezic, J., Yeung, S., McMenamin, P. G., 2005. Band-like opacity in the corneas of abattoir-acquired pig eyes. Clinical and Experimental Ophthalmology 33, 668-669.

Christensen, L. P., Brandt, K., 2006. Bioactive polyacetylenes in food plants of the Apiaceae family: Occurence, bioactivity and analysis. Journal of Pharmaceutical and Biomedical Analysis 41, 683-693.

Cieśla, L., Bogucka-Kocka, A., Hajnos, M., Petruczynik, A., Waksmundzka-Hajnos, M., 2008. Two-dimensional thin-layer chromatography with adsorbent gradient as a method of chromatographic fingerprinting of furanocoumarins for distinguishing selected varieties and forms of *Heracleum spp.* Journal of Chromatography 1207, 160-168.

Coudert, P., Albuisson, E., Boire, J. Y., Duroux, E., Bastide, P., Couquelet, J., 1994. Synthesis of pyridazine acetic acid derivatives possessing aldose reductase inhibitory activity and antioxidant properties. European Journal of Medicinal Chemistry 29, 471-477.

Craker, L. E., 2007. Medicinal and Aromatic Plants—Future Opportunities. In: Janick, J., Whipkey, A. (Eds.), Issues in New Crops and New Uses: Proceedings of the Sixth National Symposium Creating Markets for Economic Development of New Crops and New Uses. ASHS Press,, Alexandria, p. 10.

Crespi, C. L., Miller, V. P., Penman, B. W., 1997. Microtiter Plate Assays for Inhibition of Human, Drug-Metabolizing Cytochromes P450. Analytical Chemistry 248, 188-190.

Del Corso, A., Cappiello, M., Mura, U., 2008. From a dull enzyme to something else: Facts and perspectives regarding aldose reductase. Current Medicinal Chemistry 15, 1452-1461.

El-Kabbani, O., Ruiz, F., Darmanin, C., Chung, R. P. T., 2004. Aldose reductase structures: Implications for mechanism and inhibition. Cellular and Molecular Life Sciences 61, 750-762.

Faria, A., Monteiro, R., Azevedo, I., Calhau, C., 2007. Pomegranate Juice Effects on Cytochrome P450s Expression: In Vivo Studies. Journal of Medicinal Food 10, 643-649.

Foster, B. C., Vandenhoek, S., Hana, J., Krantis, A., Akhtar, M. H., Bryan, M., Budzinski, J. W., Ramputh, A., Arnason, J. T., 2003. In vitro inhibition of human cytochrome P450-mediated metabolism of marker substrates by natural products. Phytomedicine 10, 334-342.

Fung, T. T., Rimm, E. B., Spiegelman, D., Rifai, N., Tofler, G. H., Willett, W. C., Hu, F. B., 2001. Association between dietary patterns and plasma biomarkers of obesity and cardiovascular disease risk. American Journal of Clinical Nutrition 73, 61-67.

Gurib-Fakim, A., 2006. Medicinal plants: Traditions of yesterday and drugs of tomorrow. Molecular Aspects of Medicine 27, 1-93.

Halberstein, R. A., 2005. Medicinal plants: Historical and cross-cultural usage patterns. Annals of Epidemiology 15, 686-699.

Halder, N., Joshi, S., Gupta, S. K., 2003. Lens aldose reductase inhibiting potential of some indigenous plants. Journal of Ethnopharmacology 86, 113-116.

Harbilas, D., Martineau, L. C., Harris, C. S., Adeyiwola-Spoor, D. C. A., Saleem, A., Lambert, J., Caves, D., Johns, T., Prentki, M., Cuerrier, A., Arnason, J. T., Bennett, S. A. L., Haddad, P. S., 2009. Evaluation of the antidiabetic potential of selected medicinal plant extracts from the Canadian boreal forest used to treat symptoms of diabetes: Part II. Canadian Journal of Physiology and Pharmacology 87, 479-492.

Hayman, S., Kinoshita, J. H., 1965. Isolation and properties of lens aldose reductase. The Journal of Biological Chemistry 240, 6.

Head, K. A., 2001. Natural therapies for ocular disorders part two: Cataracts and glaucoma. Alternative Medicine Review 6, 141-166.

Heinrich, M., Barnes, J., Gibbons, S., Williamson, E., 2004. Fundementals of Pharmacognosy and Phytotherapy. Churchill Livingstone, London.

Heinrich, M., Prieto, J. M., 2008. Diet and healthy ageing 2100: Will we globalise local knowledge systems? Ageing Research Reviews 7, 249-274.

Hirose, K., Matsuo, K., Iwata, H., Tajima, K., 2007. Dietary patterns and the risk of breast cancer in Japanese women. Cancer Science 98, 1431-1438.

Ingelman-Sundberg, M., 2002. Polymorphism of cytochrome P450 and xenobiotic toxicity. Toxicology 181-182, 447-452.

Ioannides, C., 2003. Drug-phytochemical interactions. Inflammopharmacology 11, 7-42.

Jain, S. K., Jain, A., 2008. Target-specific drug release to the colon. Expert Opinion on Drug Delivery 5, 483-488.

Johansson, U. E., Eftekhari, S., Warfvinge, K., 2010. A battery of cell- and structure-specific markers for the adult porcine retina. Journal of Histochemistry and Cytochemistry 58, 377-389.

Kador, P.F., Kinoshita, J.H., Brittain, D.R., 1986. Purified rat lens aldose reductase. Polyol production in vitro and its inhibition by aldose reductase inhibitors. Biochemical Journal 240, 233-237.

Kamei, A., 1991. Possible process of insolubilization of lens proteins - Direct effect of glucose. Experimental Eye Research 52, 369-374.

Koenig, H. G., McCullough, M. E., Larson, D. B., 2001. Handbook of religion and health. Oxford University Press, New York.

Koleckar, V., Kubikova, K., Rehakova, Z., Kuca, K., Jun, D., Jahodar, L., Opletal, L., 2008. Condensed and hydrolysable tannins as antioxidants influencing the health. Mini-Reviews in Medicinal Chemistry 8, 436-447.

Lal, S., Randall, W. C., Taylor, A. H., Kappler, F., Walker, M., Brown, T. R., Szwergold, B. S., 1997. Fructose-3 phosphate production and polyol pathway metabolism in diabetic rat hearts. Metabolism: Clinical and Experimental 46, 1333-1338.

Lalonde, M. R., Chauhan, B. C., Tremblay, F., 2006. Retinal ganglion cell activity from the multifocal electroretinogram in pig: Optic nerve section, anaesthesia and intravitreal tetrodotoxin. Journal of Physiology 570, 325-338.

Lampe, J.W., 2003. Spicing up a vegetarian diet: chemopreventive effects of phytochemicals. American Journal of Clinical Nutrition 78, 579-583.

Leduc, C., Coonishish, J., Haddad, P., Cuerrier, A., 2006. Plants used by the Cree Nation of Eeyou Istchee (Quebec, Canada) for the treatment of diabetes: A novel approach in quantitative ethnobotany. Journal of Ethnopharmacology 105, 55-63.

Lee, A.Y.W., Chung, S.S.M., 1999. Contributions of polyol pathway to oxidative stress in diabetic cataract. FASEB Journal 13, 23-30.

Lee, M.M., Shen, J.M., 2008. Dietary patterns using Traditional Chinese Medicine principles in epidemiological studies. Asia Pacific Journal of Clinical Nutrition 17, 79-81.

Murty, M., 2007. Postmarket surveillance of natural health products in Canada: clinical and federal regulatory perspectives. Canadian Journal of Physiology and Pharmacology 85, 952-955.

Nicholson, J.K., Holmes, E., Wilson, I.D., 2005. Gut microorganisms, mammalian metabolism and personalized health care. Nature Reviews Microbiology 3, 431-438.

Obrosova, I.G., Chung, S.S.M., Kador, P.F., Diabetic cataracts: Mechanisms and management. Diabetes/Metabolism Research and Reviews 26, 172-180.

Offord, E.A., Mace, K., Ruffieux, C., Malnoe, A., M.A.Pfeifer, A., 1995. Rosemary components inhibit benzo[a]pyrene-induced genotoxicit in human bronchial cells. Carcinogenesis 16, 2057-2062.

Omara, S., Lemonnierb, B., Jonesa, N., Ficker, C., Smith, M.L., Neema, C., Towers, G.H.N., Goel, K., Arnason, J.T., 2000. Antimicrobial activity of extracts of eastern North American hardwood trees and relation to traditional medicine. . Journal of Ethnopharmacology 73, 161-170.

Patel, M. B., Mishra, S. M., 2009. Aldose reductase inhibitory activity of a c-glycosidic flavonoid derived from enicostemma hyssopifolium. Journal of Complementary and Integrative Medicine 6, 5.

Rahimi, R., Nikfar, S., Larijani, B., Abdollahi, M., 2005. A review on the role of antioxidants in the management of diabetes and its complications. Biomedicine and Pharmacotherapy 59, 365-373.

Ramasamy, R., Goldberg, I. J., Aldose reductase and cardiovascular diseases, creating human-like diabetic complications in an experimental model. Circulation Research 106, 1449-1458.

Retief, F. P., Cilliers, L., 2007. Mesopotamian medicine. South African Medical Journal 97, 27-30.

Rickey, T., 2005. Grapefruit juice and medication can be a dangerous mix. Medical News Today. University of Rochester Medical Center, Rochester, New York.

Romano, C. S., Abadi, K., Repetto, V., Vojnov, A. A., Moreno, S., 2009. Synergistic antioxidant and antibacterial activity of rosemary plus butylated derivatives. Food Chemistry 115, 456-461.

Sagdiç, O., Karahan, A. G., Özcan, M., Özkan, G., 2003. Note: Effect of Some Spice Extracts on Bacterial Inhibition. Food Science and Technology International 9, 353-359.

Schofield, P., Mbugua, D. M., Pell, A. N., 2001. Analysis of condensed tannins: A review. Animal Feed Science and Technology 91, 21-40

Scott, I. M., Leduc, R. I., Burt, A. J., Marles, R. J., Arnason, J. T., Foster, B. C., 2006. The Inhibition of Human Cytochrome P450 by Ethanol Extracts of North American Botanicals. Pharmaceutical Biology 44, 315-327.

Sears, C. L., 2005. A dynamic partnership: Celebrating our gut flora. Anaerobe 11, 247-251.

Sherman, P. W., Hash, G. A., 2001. Why vegetable recipes are not very spicy Evolution and Human Behavior 22, 147-164.

Spoor, D. C. A., Martineau, L. C., Leduc, C., Benhaddou-Andaloussi, A., Meddah, B., Harris, C., Burt, A., Fraser, M. H., Coonishish, J., Joly, E., Cuerrier, A., Bennett, S. A. L., Johns, T., Prentki, M., Arnason, J. T., Haddad, P. S., 2006. Selected plant species from the Cree pharmacopoeia of northern Quebec possess anti-diabetic potential. Canadian Journal of Physiology and Pharmacology 84, 847-858.

Subehan, S. Z. F. H., Kadota, S., Tezuka, Y., 2007. Inhibition on Human Liver Cytochrome P450 3A4 by Constituents of Fennel (*Foeniculum vulgare*): Identification and Characterization of a Mechanism-Based Inactivator. Journal of Agricultural and Food Chemistry 55, 10162-10168.

Termentzi, A., Alexiou, P., Demopoulos, V.J., Kokkalou, E., 2008. The aldose reductase inhibitory capacity of Sorbus domestica fruit extracts depends on their phenolic content and may be useful for the control of diabetic complications. Pharmazie 63, 693-696.

Walker, R., 1973. The influence of gut micro organisms on the metabolism of drugs and food additives. Proceedings of the Nutrition Society 32, 73-78.

Wink, M., 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry 64, 3-19.

Yabe-Nishimura, C., 1998. Aldose reductase in glucose toxicity: A potential target for the prevention of diabetic complications. Pharmacological Reviews 50, 21-33.

Yadav, U. C. S., Ighani-Hosseinabad, F., Van Kuijk, F. J. G. M., Srivastava, S. K., Ramana, K. V., 2009. Prevention of posterior capsular opacification through aldose reductase inhibition. Investigative Ophthalmology and Visual Science 50, 752-759.

Zhang, J. W., Liu, Y., Cheng, J., Li, W., Ma, H., Liu, H. T., Sun, J., Wang, L. M., He, Y. Q., Wang, Y., Wang, Z. T., Yang, L., 2007. Inhibition of human liver cytochrome P450 by star fruit juice. Journal of Pharmacy and Pharmaceutical Sciences 10, 496-503.

APPENDIX

Table APP-2.1. Activity of methanolic extracts (50 mg/ml) of samples against Cytochrome P450 3A4, 3A5, 3A7 and 2D6 isozymes expressed as mean percent inhibition (%) ± standard deviation.

NRP No.	3A4	3A5	3A7	2D6
320	34.44 ± 0.06	41.35 ± 2.90	62.22 ± 5.90	18.31 ± 0.67
321	36.61 ± 2.77	46.15 ± 6.01	71.25 ± 0.74	27.71 ± 3.46
322	27.17 ± 7.17	61.30 ± 4.53	60.00 ± 8.80	21.50 ± 3.20
323	36.35 ± 2.89	83.55 ± 2.48	59.15 ± 7.16	27.62 ± 3.28
324	29.42 ± 0.67	54.30 ± 5.23	57.43 ± 9.81	10.76 ± 0.13
325	32.31 ± 4.09	35.15 ± 0.07	67.53 ± 3.15	17.24 ± 3.94
326	36.20 ± 3.12	36.10 ± 2.97	66.29 ± 4.30	16.63 ± 2.29
327	40.43 ± 13.40	54.00 ± 5.80	75.26 ± 4.71	20.36 ± 3.43
328	33.12 ± 6.26	31.85 ± 4.46	66.70 ± 5.40	19.86 ± 2.33
329	49.46 ± 9.14	42.65 ± 0.50	75.34 ± 1.86	20.32 ± 2.98
330	28.89 ± 7.49	31.45 ± 11.10	68.78 ± 16.27	14.91 ± 2.35
331	41.22 ± 10.99	54.70 ± 1.98	77.45 ± 0.30	5.80 ± 4.93
335	23.40 ± 0.96	34.87 ± 10.29	66.33 ± 9.07	15.92 ± 6.12
313	20.30 ± 2.71	-2.95 ± 3.47	56.60 ± 3.07	3.20 ± 2.80
314	5.30 ± 3.97	11.65 ± 5.16	51.84 ± 2.17	16.49 ± 3.64
356	12.74 ± 2.75	8.08 ± 1.32	27.66 ± 0.80	0.66 ± 0.42
315	9.03 ± 22.66	26.40 ± 2.69	60.16 ± 3.49	13.48 ± 3.05
316	6.90 ± 0.11	19.95 ± 0.35	50.49 ± 4.83	9.05 ± 4.46
317	41.17 ± 8.21	21.85 ± 0.07	47.23 ± 6.00	94.23 ± 5.58
318	28.76 ± 2.52	9.15 ± 6.29	64.19 ± 0.02	6.78 ± 0.80
319	19.00 ± 0.45	22.60 ± 10.89	53.29 ± 4.82	21.70 ± 1.37
339	22.32 ± 4.20	19.46 ± 7.26	20.75 ± 0.27	14.46 ± 6.09

354	39.95 ± 6.62	10.33 ± 5.33	36.51 ± 22.63	3.39 ± 5.84
337	23.55 ± 1.07	15.22 ± 3.10	29.24 ± 3.14	7.20 ± 5.78
357	1.12 ± 3.02	25.94 ± 2.11	44.98 ± 0.41	12.22 ± 0.64
358	1.47 ± 1.58	20.27 ± 2.86	28.23 ± 1.99	18.01 ± 18.26
355	8.40 ± 0.08	23.77 ± 1.73	30.46 ± 3.90	11.58 ± 5.50
350	32.91 ± 2.63	18.15 ± 1.73	25.44 ± 2.61	10.14 ± 1.48
359	22.47 ± 15.80	16.45 ± 8.17	-3.10 ± 26.17	26.00 ± 6.65
336	18.99 ± 2.81	7.55 ± 3.18	16.24 ± 20.07	7.33 ± 1.75
351	18.02 ± 5.81	21.01 ± 0.38	8.58 ± 14.90	25.97 ± 9.69
352	44.38 ± 8.81	13.24 ± 0.47	6.89 ± 17.06	18.16 ± 5.07
338	12.20 ± 4.97	17.82 ± 5.06	16.17 ± 2.33	21.44 ± 0.65
332	26.16 ± 6.92	14.80 ± 3.54	51.92 ± 6.74	20.09 ± 2.44
334	12.89 ± 13.19	38.05 ± 4.17	54.40 ± 4.57	27.83 ± 4.29
353	15.77 ± 4.84	30.52 ± 1.55	30.10 ± 11.10	23.88 ± 2.45
333	26.80 ± 0.11	26.60 ± 3.25	31.53 ± 0.14	13.43 ± 4.26
341*	85.09 ± 4.72	96.48 ± 0.37	75.79 ± 1.01	91.63 ± 5.78
342*	33.22 ± 0.11	97.02 ± 0.74	64.87 ± 13.97	84.84 ± 1.00
343*	30.22 ± 39.34	26.74 ± 5.14	64.89 ± 2.23	85.78 ± 3.01
344*	92.84 ± 6.36	97.74 ± 0.64	81.01 ± 3.24	82.43 ± 15.48
345*	10.85 ± 31.07	35.30 ± 9.35	53.81 ± 3.92	79.63 ± 13.71
346*	104.83 ± 0.08	66.62 ± 2.46	103.83 ± 2.40	62.78 ± 9.26
340**	52.75 ± 2.16	87.20 ± 2.55	89.71 ± 0.81	51.57 ± 3.13
347**	99.91 ± 0.68	99.50 ± 0.11	99.94 ± 0.38	105.29 ± 9.47
348**	101.95 ± 2.59	100.11 ± 0.22	102.46 ± 1.59	84.80 ± 6.34

**Apiaceae **Lamiaceae*

Table APP- 2.2. Activity of aqueous extracts (50 mg/ml) of samples against Cytochrome P450 3A4, 3A5, 3A7 and 2D6 isozymes expressed as mean percent inhibition (%) ± standard deviation.

NRP No.	3A4	3A5	3A7	2D6
320	75.29 ± 4.80	20.22 ± 5.01	21.85 ± 11.65	-4.31 ± 10.15
321	86.76 ± 2.08	25.38 ± 4.17	53.77 ± 6.42	18.92 ± 4.43
322	85.94 ± 5.95	21.85 ± 11.65	22.76 ± 5.04	-1.54 ± 4.77
323	83.57 ± 0.48	53.77 ± 6.42	25.69 ± 9.52	21.67 ± 4.82
324	61.06 ± 6.53	22.76 ± 5.04	22.98 ± 11.58	37.95 ± 9.56
325	64.19 ± 12.95	25.69 ± 9.52	17.18 ± 8.63	21.14 ± 5.82
326	70.28 ± 11.91	22.98 ± 11.58	20.32 ± 10.73	-2.65 ± 7.58
327	78.23 ± 8.86	17.18 ± 8.63	21.79 ± 6.12	30.00 ± 3.78
328	70.67 ± 20.11	20.32 ± 10.73	14.53 ± 0.58	-4.40 ± 7.74
329	86.74 ± 2.48	21.79 ± 6.12	34.44 ± 2.65	33.48 ± 6.17
330	73.35 ± 7.14	14.53 ± 0.58	7.06 ± 0.80	11.42 ± 2.94
331	68.36 ± 14.74	34.44 ± 2.65	19.99 ± 5.35	13.81 ± 4.79
335	68.75 ± 9.16	6.58 ± 0.27	3.78 ± 2.66	43.27 ± 6.44
313	58.37 ± 58.44	-1.59 ± 1.71	-1.66 ± 1.82	11.26 ± 0.98
314	28.94 ± 10.37	-1.79 ± 1.35	-4.704.82	14.91 ± 2.09
356	12.36 ± 6.49	-6.04 ± 2.53	-8.00 ± 1.03	4.53 ± 5.39
315	39.57 ± 8.14	-1.66 ± 1.82	-3.99 ± 0.06	8.71 ± 3.11
316	59.47 ± 9.70	-4.70 ± 4.82	-2.62 ± 1.92	19.72 ± 2.54
317	41.49 ± 11.64	-3.99 ± 0.06	2.46 ± 2.91	15.77 ± 5.98
318	53.08 ± 7.08	-2.62 ± 1.92	20.22 ± 5.01	15.49 ± 5.33
319	93.55 ± 8.37	2.46 ± 2.91	25.38 ± 4.17	18.42 ± 4.56
339	7.85 ± 6.36	-6.91 ± 4.00	59.82 ± 1.90	18.76 ± 1.25

354	7.25 ± 8.63	-11.37 ± 3.62	-6.04 ± 2.53	8.15 ± 0.28
337	14.54 ± 8.64	3.78 ± 2.66	-6.91 ± 4.00	15.50 ± 3.88
357	18.70 ± 14.89	-2.03 ± 0.99	2.26 ± 4.43	4.77 ± 4.17
358	20.13 ± 6.77	-8.00 ± 1.03	-1.89 ± 0.90	8.73 ± 5.45
355	13.23 ± 3.74	57.20 ± 3.90	-2.03 ± 0.99	13.56 ± 3.82
350	17.22 ± 11.32	26.13 ± 2.86	29.06 ± 2.64	6.64 ± 3.67
359	12.93 ± 7.30	34.03 ± 6.11	16.80 ± 1.00	14.39 ± 3.61
336	13.05 ± 0.52	4.65 ± 2.11	30.49 ± 4.17	2.12 ± 3.65
351	17.76 ± 8.98	16.80 ± 1.00	-1.53 ± 9.65	20.04 ± 3.78
352	9.09 ± 4.69	29.06 ± 2.64	-11.37 ± 3.62	5.17 ± 0.37
338	11.92 ± 0.94	30.49 ± 4.17	46.08 ± 1.46	-2.99 ± 2.60
332	18.92 ± 13.33	7.06 ± 0.80	8.03 ± 4.22	7.87 ± 8.25
334	52.07 ± 1.78	8.03 ± 4.22	4.65 ± 2.11	17.64 ± 0.11
353	3.19 ± 3.25	-1.52 ± 9.65	57.20 ± 3.90	22.03 ± 4.67
333	15.57 ± 2.63	19.99 ± 5.35	6.58 ± 0.27	31.54 ± 7.22
341*	89.17 ± 1.58	59.82 ± 1.90	30.03 ± 0.48	101.79 ± 9.93
342*	65.52 ± 4.57	49.31 ± 0.40	73.83 ± 2.64	57.93 ± 10.50
343*	62.08 ± 16.14	30.03 ± 0.48	11.14 ± 0.24	68.26 ± 10.64
344*	96.84 ± 0.34	73.83 ± 2.64	48.62 ± 1.44	59.45 ± 3.30
345*	33.14 ± 9.93	11.14 ± 0.24	74.89 ± 3.41	77.44 ± 3.51
346*	101.99 ± 0.30	48.62 ± 1.44	99.56 ± 0.53	42.54 ± 5.77
340**	73.78 ± 1.51	46.08 ± 1.46	49.31 ± 0.40	39.58 ± 0.37
347**	98.81 ± 0.05	74.89 ± 3.41	34.03 ± 6.11	83.06 ± 7.57
348**	100.55 ± 1.44	99.56 ± 0.53	26.13 ± 2.86	91.42 ± 5.09

**Apiaceae **Lamiaceae*

TableAPP- 2.3. Antimicrobial effects of selected *Fabaceae* methanolic and ethanolic extracts (50 mg/ml) against 7 bacterial species. Zones of inhibitions are measured according to the Kirby-Bauer disc diffusion assay. Values represent average diameters between triplicates of and measured in millimetres. Ciprofloxacin was used as the positive control. (-) denotes no inhibitory activity or a zone of inhibition of less than 6 mm.

Common Name	NRP#	*Bacillus subtilis* Gram (+)	*Enterococcus faecalis* Gram (+)	*Listeria innocua* Gram (+)	*Escherichia coli* Gram (-)	*Pseudomonas putida* Gram (-)	*Providencia stuartii* Gram (-)	*Acetobacter calcoaceticus* Gram (-)
Soybean	320	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Soybean	321	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Soybean	322	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Soybean	323	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Soybean	324	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Soybean	325	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Soybean	326	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Soybean	327	-	-	-	-	-	-	-

		-	-	-	-	-	-	-
Soybean	328	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Soybean	329	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Soybean	330	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Soybean	331	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Black bean	335	-	-	-	-	6.0 ± 0.0	-	7.5 ± 0.0
		-	-	-	-	6.0 ± 0.0	-	7.0 ± 0.0
Black Turtle bean	313	-	-	-	6.5 ± 0.0	6.5 ± 0.0	6.7 ± 0.5	6.0 ± 0.0
		-	-	-	6.5 ± 0.0	6.5 ± 0.0	6.5 ± 0.0	6.0 ± 0.0
Cranberry bean	314	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Great Northern bean	356	-	-	-	-	6.5 ± 0.0	-	-
		-	-	-	-	6.5 ± 0.0	-	-
Dark Red Kidney bean	315	-	-	-	-	6.7 ± 0.5	6.5 ± 0.0	-
		-	-	-	-	6.0 ± 0.0	6.0 ± 0.0	-
Light Red Kidney bean Var. A	316	-	6.5 ± 0.0	-	-	6.5 ± 0.0	-	8.7 ± 0.7
		-	6.5 ± 0.0	-	-	6.5 ± 0.0	-	8.0 ± 0.0

Light Red Kidney bean Var. B	317	-	-	-	6.3 ± 0.4	-	-	-
		-	-	-	6.0 ± 0.0	-	-	-
White Kidney bean Var. A	318	-	6.0 ± 0.0	-	-	-	-	8.5 ± 0.0
		-	6.0 ± 0.0	-	-	-	-	8.5 ± 0.0
White Kidney bean Var. B	319	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
White Kidney bean Var. C	339	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
White Kidney bean Var. D	354	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Navy bean	337	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Pinto bean	357	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Small Red bean	358	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Eston lentil	355	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Green lentil	350	-	-	-	-	-	6.5 ± 0.0	-
		-	-	-	-	-	6.5 ± 0.0	-
Red lentil	359	-	-	-	-	-	6.7 ± 0.5	-

		-	-	-	-	-	6.0 ± 0.0	-
Lima bean	336	-	6.5 ± 0.0	-	6.7 ± 0.5	-	-	7.7 ± 0.6
		-	6.0 ± 0.0	-	6.0 ± 0.0	-	-	7.0 ± 0.0
Green pea	351	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Yellow pea	352	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Yellow split pea	338	-	-	-	-	-	-	6.5 ±0.0
		-	-	-	-	-	-	6.3 ± 0.4
Black-eyed pea	332	-	6.3 ± 0.4	-	-	-	-	-
		-	6.0 ± 0.0	-	-	-	-	-
Cow pea	334	-	-	-	-	-	-	-
		-	-	-	-	-	-	-
Chick pea	353	-	-	-	-	-	6.0 ± 0.0	-
		-	-	-	-	-	6.0 ± 0.0	-
Congo Pigeon pea	333	-	-	-	-	-	-	6.5 ± 0.0
		-	-	-	-	-	-	6.0 ± 0.0

Table APP-3.1. The inhibitory potential of the top 17 selected Cree antidiabetic plants expressed as a percent inhibition (%) with their respective standard errors and ranking. The n value for each sample is 3.

Samples	Average	SD (+/-)	SE (+/-)	Ranking
Quercetin	21.29	3.55	1.77	7
W1	25.93	6.79	3.92	5
W2	26.67	2.33	1.17	3
W3	19.55	6.98	4.03	8
W4	12.73	2.837	1.41	12
W5	-1.88	1.25	0.63	16
W6	9.71	9.95	5.74	13
W7	26.54	1.85	0.93	4
W8	36.49	4.85	2.80	1
W9	13.19	2.80	1.40	11
AD01	35.11	0.62	0.31	2
AD02	9.14	1.22	0.61	14
AD03	14.73	2.433	1.22	9
AD06	-30.61	14.98	7.49	18
AD07	13.57	6.301	3.64	10
AD08	22.20	6.72	3.36	6
AD09	8.82	3.63	1.82	15
AD11	-8.56	12.11	6.06	17

Table APP-3.2. The inhibitory potential of fractions F1-F9 of *R. groenlandicum,* obtained from open column chromatography, against homogenized swine lens isolate. The data was standardized with the control to obtain the percent inhibition. The n value is 3 and the standard error is as indicated.

Sample	Average	SD (+/-)	SE (+/-)	Rank
Control	0	0	0	
F1	5.66	1.57	1.11	8
F2	5.67	3.32	2.35	7
F3	8.51	7.15	5.06	6
F4	4.41	1.60	1.13	9
F5	17.16	3.91	2.76	5
F6	45.00	10.67	7.54	2
F7	64.35	1.51	1.07	1
F8	36.10	0.59	0.42	4
F9	36.98	6.17	4.37	3

Table APP-3.3. Concentration of 10 marker compounds used in HPLC analysis. Quantification was accomplished using a reference table obtained from a known standard mix. Injections were done in duplicates and n=2. Standard error is as shown.

	Catechin(+)		Chlorogenic acid		Epicatechin		p-Coumaric acid		Rutin	
Sub-fractions	Average (ug)	SE (±)	Average (ug)	SE (±)	Average (ug)	SE (±)	Average (ug)	SE (±)	Average (ug)	SE (±)
RG-7-1	35.05	0.00	0.00	0.00	15.81	0.05	2.50	0.00	0.00	0.00
RG-7-2	285.92	2.07	0.00	0.00	225.89	3.58	0.00	0.00	0.00	0.00
RG-7-3	14.82	0.05	0.00	0.00	10.12	0.07	0.00	0.00	0.00	0.00
RG-7-4	13.70	0.03	0.00	0.00	17.94	0.03	0.00	0.00	0.00	0.00
RG-7-5	5.52	0.09	0.00	0.00	14.49	0.07	0.00	0.00	0.00	0.00
RG-7-6	7.32	0.15	0.00	0.00	3.19	0.02	0.00	0.00	0.00	0.00
RG-7-7	3.58	0.09	0.00	0.00	2.57	0.07	0.13	0.03	0.00	0.00
RG-7-8	8.86	0.07	0.00	0.00	2.67	0.02	0.00	0.00	2.27	0.01
RG-7-9	7.55	0.10	0.79	0.01	2.73	0.00	0.18	0.07	0.00	0.00
RG-7-10	2.52	0.01	0.00	0.00	1.47	0.02	0.00	0.00	0.00	0.00
RG-7-11	1.34	0.02	0.00	0.00	0.63	0.02	0.00	0.00	0.00	0.00
RG-7-12	0.93	0.02	0.00	0.00	0.30	0.00	0.00	0.00	0.00	0.00
RG-7-13	1.02	0.01	0.00	0.00	0.29	0.00	0.00	0.00	0.00	0.00
RG-7-14	0.79	0.01	0.00	0.00	0.39	0.00	0.00	0.00	0.00	0.00
RG-7-15	1.74	0.01	0.00	0.00	0.41	0.00	0.00	0.00	0.00	0.00
RG-8-1	49.20	1.61	0.00	0.00	34.82	1.17	0.76	0.02	0.00	0.00
RG-8-2	5.81	0.03	0.00	0.00	4.76	0.04	0.25	0.04	0.00	0.00
RG-8-3	9.55	0.01	0.45	0.00	1.37	0.09	0.15	0.00	0.61	0.03
RG-8-4	5.93	0.00	0.00	0.00	158.35	0.00	0.00	0.00	0.00	0.00
RG-8-5	4.16	0.00	0.30	0.00	163.48	0.00	0.00	0.00	0.00	0.00
RG-8-6	3.14	0.10	2.60	0.02	0.98	0.12	0.00	0.00	0.00	0.00
RG-8-7	0.75	0.00	1.81	0.01	0.62	0.00	0.00	0.00	0.00	0.00
RG-8-8	4.39	0.02	0.00	0.00	0.75	0.01	0.00	0.00	0.00	0.00
RG-8-9	7.80	0.00	1.03	0.02	1.39	0.01	0.00	0.00	0.00	0.00
RG-8-10	1.09	0.09	2.29	0.01	0.00	0.00	0.00	0.00	0.00	0.00
RG-8-11	1.12	0.00	13.97	0.05	0.63	0.02	0.34	0.01	0.00	0.00

RG-8-12	0.80	0.00	48.53	0.13	0.30	0.01	0.12	0.00	1.20	0.00
RG-8-13	1.02	0.00	24.05	0.13	0.00	0.00	0.00	0.00	0.00	0.00

Sub-fractions	Quercetin-3-galactoside Average(ug)	SE(±)	Quecertin-3-glucoside Average(ug)	SE(±)	Quecertin-3-rhamnoside Average(ug)	SE(±)	Myricetin Average(ug)	SE(±)	Quercetin Average(ug)	SE(±)
RG-7-1	8.97	0.01	28.59	0.06	0.00	0.00	1.58	0.01	72.72	0.14
RG-7-2	0.00	0.00	4.90	0.02	0.00	0.00	0.00	0.00	45.39	0.21
RG-7-3	0.00	0.00	2.94	0.04	0.00	0.00	0.80	0.01	17.35	0.00
RG-7-4	0.00	0.00	0.00	0.00	0.00	0.00	0.47	0.02	2.56	0.00
RG-7-5	0.00	0.00	0.00	0.00	0.96	0.00	3.32	0.04	1.29	0.01
RG-7-6	0.00	0.00	0.00	0.00	32.61	0.05	5.21	0.12	0.98	0.01
RG-7-7	0.00	0.00	0.00	0.00	118.26	0.54	2.23	0.34	2.69	0.02
RG-7-8	0.00	0.00	31.58	0.20	27.15	0.11	4.76	0.33	1.90	0.04
RG-7-9	184.90	1.28	76.75	1.44	30.82	0.30	7.62	0.09	2.67	0.01
RG-7-10	168.96	0.10	31.13	0.03	10.06	0.69	1.06	0.00	1.91	0.04
RG-7-11	133.01	2.73	27.65	0.55	6.87	0.18	2.28	0.11	0.00	0.00
RG-7-12	108.94	0.53	22.03	0.10	5.81	0.05	1.13	1.02	0.00	0.00
RG-7-13	60.97	0.45	13.58	0.10	3.65	0.00	0.95	0.50	0.00	0.00
RG-7-14	72.15	0.00	14.28	0.01	3.79	0.03	1.83	0.01	0.00	0.00
RG-7-15	23.04	0.04	4.99	0.02	1.47	0.27	0.89	0.01	0.00	0.00
RG-8-1	2.11	0.05	7.97	0.16	0.00	0.00	1.45	0.01	40.99	0.91
RG-8-2	2.62	0.03	6.33	0.09	46.44	0.04	4.71	0.09	3.32	0.01
RG-8-3	142.28	8.77	74.56	0.09	20.75	0.22	7.15	0.07	1.08	0.00
RG-8-4	621.59	0.00	11.94	1.23	2.31	0.12	0.56	0.01	0.00	0.00
RG-8-5	472.61	0.00	0.00	0.00	0.00	0.00	0.22	0.02	0.00	0.00
RG-8-6	67.71	1.00	4.56	0.09	0.00	0.00	1.34	0.01	0.00	0.00
RG-8-7	15.91	0.18	1.06	0.03	0.00	0.00	0.48	0.09	0.00	0.00
RG-8-8	5.75	0.08	0.62	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RG-8-9	8.41	0.04	1.38	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RG-8-10	7.36	0.01	1.42	0.00	2.04	0.01	0.00	0.00	0.00	0.00
RG-8-11	26.66	0.11	2.24	0.02	1.85	0.01	0.47	0.02	0.00	0.00
RG-8-12	8.41	0.06	1.21	0.00	1.40	0.00	0.00	0.00	0.00	0.00
RG-8-13	3.92	0.04	0.00	0.00	1.02	0.01	0.00	0.00	0.00	0.00