IMPACT OF *SUTHERLANDIA FRUTESCENS* ON HEPATIC STEATOSIS IN HIGH-FAT FED RATS

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Doctor of Philosophy

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by

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IMPACT OF *SUTHERLANDIA FRUTESCENS* ON HEPATIC STEATOSIS IN HIGH-FAT FED RATS

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LIST OF ABBREVIATIONS

ACOX-1	Acyl-coenzyme A oxidase-1
AIDS	Acquired immunodeficiency syndrome
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AP-1	Activator protein-1
AST	Aspartate aminotransferase
BMI	Body mass index
CD	Cluster of differentiation
ChREBP	Carbohydrate-responsive element-binding protein
COX-2	Cyclooxygenase-2
Ct	Threshold cycle
CYP	Cytochrome P450
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
ELSD	Evaporative light scattering detectors
ERK	Extracellular signal-regulated protein kinase
FAS	Fatty acid synthase
FFA	Free fatty acids
g	Gram

GABA	γ-Aminobutyric acid
h	Hour
HCC	Hepatocellular carcinoma
HFD	High-fat diet
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HSCCC	High-speed counter-current chromatography
IC	Inhibitory concentration
IFN-γ	Interferon gamma
IL-1β	Interleukin-1 beta
IL-4	Interleukin-4
IL-6	Interleukin-6
IgG	Immunoglobulin G
LC	Liquid chromatography
LFD	Low-fat diet
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein-1
mg	Milligram
mL	Milliliter
mm	Millimeter
mRNA	Messenger RNA
MS	Mass spectrometry
MtSK	Shikimate kinase enzyme of *Mycobacterium tuberculosis*

NAFLD	Nonalcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartic acid
n	Number of animals
ng	Nanogram
NO	Nitric oxide
OVA	Ovalbumin
oz	ounce
PBS	Phosphate buffered saline
pg	Picogram
Pino	Pinosundia
PPAR-α	Peroxisome proliferator activated receptor-alpha
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
ROS	Reactive oxygen species
rt-q PCRs	Quantitative reverse transcription polymerase chain reactions
SEM	Standard error of mean
SREBP-1C	Sterol regulatory element-binding protein-1C
Su	Sutherlandia
SuB	Sutherlandioside B
STAT1	Signal transducer and activator of transcription 1
TG	Triglycerides

TLC	Thin layer chromatography
TLR	Toll-like receptor
TMB	3,3’,5,5’-Tetramethylbenzidine
TNF-α	Tumor necrosis factor-α
TPA	12-O-tetra-decanoylphorbol-13-acetate
T2DM	Type 2 diabetes mellitus
UV	Ultra violet
VLDL	Very low density lipoproteins
w/w	Weight/weight
µg	Microgram
µL	Microliter
µm	Micrometer
°C	Degree centigrade
3β-HSD2	3β-hydroxysteroid dehydrogenase type 2

IMPACT OF *SUTHERLANDIA FRUTESCENS* ON HEPATIC STEATOSIS IN HIGH-FAT FED RATS

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ABSTRACT

Hepatic steatosis is closely associated with development of obesity and type II diabetes mellitus. Western medicine for these metabolic conditions is less accessible to many developing countries because of cost, availability and need for clinical monitoring. These impediments might be addressed by development of evidence-based phytotherapies, especially those that are currently used and for which evidence of safety and efficacy can be provided.

Sutherlandia frutescens (L.) R.Br. (Sutherlandia) is a southern African medicinal plant that is widely used for a number of diseases and health conditions, including obesity and type II diabetes mellitus. Several bioactive compounds have been identified in *S. frutescens*, including L-canavanine, D-pinitol, and gamma-aminobutyric acid, as well as novel components such as the sutherlandiosides A-D (a group of cycloartane triterpenoids) and sutherlandins A-D (a group of flavonoids). Limited evidence from cell culture and animal studies suggests Sutherlandia alters glucose homeostasis and lipid metabolism; however, the molecular mechanisms remain to be established. This research

was conducted to examine the hypothesis that Sutherlandia consumption mitigates hepatic steatosis of rats subjected to a high-fat diet by increasing fatty acid oxidation and/or decreasing *de novo* lipid synthesis in the liver.

The Wistar male rats (12 animals/group) received either a standard low-fat diet (LFD) or a high-fat diet (HFD) *ad libitum*. The high-fat fed rats were treated for 12 weeks with or without Sutherlandia extracts in drinking water or dried Sutherlandia incorporated into feed. The results showed that both Sutherlandia treatments significantly reduced triglycerides and diminished the size of lipid droplets in the livers of HFD-fed rats, which indicates that less lipid was present in the liver cells of these rats. Furthermore, these treatments significantly upregulated fatty acid oxidation-associated genes including the transcription factor peroxisome proliferator-activated receptor α (PPAR-α) and its target genes including acyl-coenzyme A oxidase-1 (ACOX-1) and cytochrome P450, family 4, subfamily A, polypeptide 14 (CYP4A14), but did not considerably alter hepatic transcriptional expression of *de novo* lipid synthesis-related genes such as sterol regulatory element-binding protein-1C (SREBP-1C), carbohydrate-responsive element-binding protein (ChREBP), and fatty acid synthase (FAS).

Sutherlandiosides are unique compounds that have been recently characterized in *S. frutescens.* An indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed in collaboration with the Department of Chemistry of Natural Compounds, Institute of Chemical Technology Prague, Czech Republic with the aim of determining the concentrations of these unique, bioactive compounds in plasma samples of the

rats consuming Sutherlandia. A standard curve using purified sutherlandiosides from the Sutherlandia plant material was successfully established. Quantification of sutherlandiosides in plasma, however, was unsuccessful due to very low levels of sutherlandiosides present in plasma and limited availability of the totally purified sutherlandioside B standard.

Overall, this study provides a better understanding of the beneficial effects of Sutherlandia on hepatic steatosis by showing that consumption of this plant extract reduced HFD-induced accumulation of hepatic triglycerides by a mechanism that may involve increased fatty acid oxidation. This medicinal plant, therefore, may be a promising phytotherapy for hepatic conditions involving excess triglyceride accumulation.

CHAPTER ONE

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is common in both developed and developing countries (1-4). NAFLD is characterized by a wide range of pathological alterations in the liver, ranging from simple steatosis to nonalcoholic steatohepatitis, fibrosis, cirrhosis and hepatocellular carcinoma (5-7). It is also strongly associated with metabolic diseases including insulin resistance, type 2 diabetes mellitus, hypertension, and dyslipidemia (5, 8-10).

Hepatic steatosis (fatty liver), an early and reversible stage of NAFLD (11), is clinically defined when accumulation of intrahepatic triglycerides exceeds 5% of the total liver weight (12, 13). Development of steatosis occurs when the rate of fatty acid uptake and synthesis exceeds the rate of fatty acid oxidation and secretion (14).

Herbal remedies are increasingly employed for ameliorating metabolic diseases (15-17). Among these phytotherapies, a southern African medicinal plant *Sutherlandia frutescens* (L.) R.Br (Sutherlandia), also known as “cancer bush”, is claimed to alleviate numerous chronic diseases including diabetes, cancer, as well as infections (18-23). Several bioactive compounds have been identified in *S. frutescens*, including L-canavanine, D-pinitol, and gamma-aminobutyric acid (24, 25), as well as novel components such as the sutherlandiosides A-D (a group of cycloartane triterpenoids) (26) and sutherlandins A-D (a group of flavonoids) (27, 28).

Previous studies demonstrated that D-pinitol and L-canavanine exhibited anti-diabetic and anti-hyperlipidemic effects (19, 25, 29, 30). Even though there is no scientific evidence about beneficial effects of sutherlandiosides A-D and sutherlandins A-D on metabolic disorders, some studies showed that triterpenoids improved glucose metabolism, enhanced fatty acid oxidation (31), and reduced inflammation in liver and white adipose tissues of mice subjected to a high-fat diet (HFD) (32). Furthermore, other studies have demonstrated that flavonoids alleviated hepatic steatosis in diet-induced obese mice (33, 34).

Importantly, aqueous extracts of *Sutherlandia frutescens* (subsp. *microphylla*) were shown to increase glucose uptake in muscle and adipose tissues in obese rats (21), decrease plasma free fatty acids, prevent the development of insulin resistance in HFD-fed rats (22, 23), and to modulate expression of 27 diabetes-related genes in a fructose-induced insulin resistant human Chang liver cell model (35). Molecular mechanisms responsible for these advantageous affects need to be elucidated. Therefore, this study aims to provide a better understanding of how *S. frutescens* improves lipid metabolism *in vivo*.

Male Wistar rats (12 animals/group) were fed either a standard low-fat diet or a high-fat diet *ad libitum*. The HFD-fed rats were treated for 12 weeks with or without extracts of *S. frutescens* in drinking water (50 mg extract/kg body weight/day) or 1% dried Sutherlandia vegetative material incorporated into the HFD. Liver triglycerides, histology, and transcriptional expression of genes involved in hepatic lipid oxidation and synthesis were examined in order to verify

the hypothesis that *S. frutescens* consumption alleviates obesity-induced hepatic steatosis by decreasing hepatic *de novo* lipogenesis and/or by increasing fatty acid oxidation in the livers of HFD-fed rats.

Chapter two will summarize biological information on NAFLD (including its definition, pathogenesis, diagnosis and prevalence), as well as on bioactive compounds and medicinal properties of *S. frutescens.*

CHAPTER TWO

LITERATURE REVIEW

Hepatic steatosis

Introduction

Obesity refers to a medical condition when there is a substantial increase in caloric intake exceeding the caloric expenditure, leading to an accumulation of fat throughout the body (36). There are about 500 million people worldwide sufferring from obesity (defined as having a body mass index (BMI) of greater than 30 kg per m^2) in 2011, and that these numbers are estimated to double in 2030 (37). Moreover, Ogden et al. (2015) reported that over one-third of adults and 17% of youth in the United States were obese in 2011–2014, and that obesity was more prevalent among women than among men (38). Furthermore, obesity has been also increasing remarkably in developing countries; for instance, more than 29% of men and 56% of women were categorized as overweight (BMI > 25) or obese (BMI > 30) in South Africa (39).

Obesity is a risk factor for developing a number of chronic diseases including high blood pressure, type 2 diabetes mellitus (T2DM), heart disease and stroke (40). More importantly, obesity is associated with hepatic steatosis (fatty liver), the hallmark feature of non-alcoholic fatty liver disease (NAFLD) (41).

Definition of non-alcoholic fatty liver disease (NAFLD)

The defining feature of NAFLD is the accumulation of triglycerides (lipids) within the cytoplasm of hepatocytes, in the absence of excessive alcohol consumption (42), i.e. more than 2 drinks/day by men or more than 1 drink/day by women (a standard drink is equal to 14 g (0.6 oz) of alcohol) (43). NAFLD covers a wide spectrum of liver damage based on histological hepatocellular characteristics, initiating from benign, asymptomatic hepatic steatosis, then transforming to a complex pattern with hepatocellular injury and inflammation called non-alcoholic steatohepatitis (NASH), and ultimately ending with severe liver disease such as fibrosis, cirrhosis and hepatocellular carcinoma (HCC or liver cancer) in some cases (5-7, 44).

Pathogenesis of NAFLD

The liver is one of the largest organs in the human body and is essential for normal physiology, and has a central role in glucose and lipid metabolism in the body (45-48). Hepatocytes are the epithelial component of the liver and perform most of the liver's physiologic functions (49).

Simple hepatic steatosis results from the accumulation of triglycerides within the cytoplasm of hepatocytes in the liver and generally represents a non-progressive disease condition with minimal treatment requirements and low risk of development of end-stage liver disease (44, 50). Hepatic steatosis is clinically defined when triglycerides (TG) exceed 5% of the total liver weight (12, 13). Liver lipid homeostasis is dependent on the balance of the following metabolic pathways: 1) hepatic uptake of plasma free fatty acids (FFA) from lipolysis of

adipose tissue triglycerides, 2) hepatic *de novo* lipogenesis, (3) hepatic fatty acid oxidation, and (4) fatty acid secretion in the form of triglyceride in the core of very low density lipoproteins (VLDL) (51, 52). Changes in the equilibrium of one or more of these processes can lead to hepatic steatosis (14).

Transition from steatosis to NASH occurs when the capacity of hepatocytes to store fat is overloaded by sustained uptake, local synthesis or impaired breakdown of fatty acids, which eventuates in cell death and inflammation (53, 54). It is estimated that about 30% of people having fatty liver can develop NASH (55, 56).

Untreated NASH can progress to end-stage liver disease characterized by fibrosis and cirrhosis through deposition of collagen and scar tissue due to mitochondrial injury, stellate cell activation and microvascular injury (57). Approximately 20-25% of individuals affected by NASH can develop cirrhosis with the subsequent risk of HCC (56, 58).

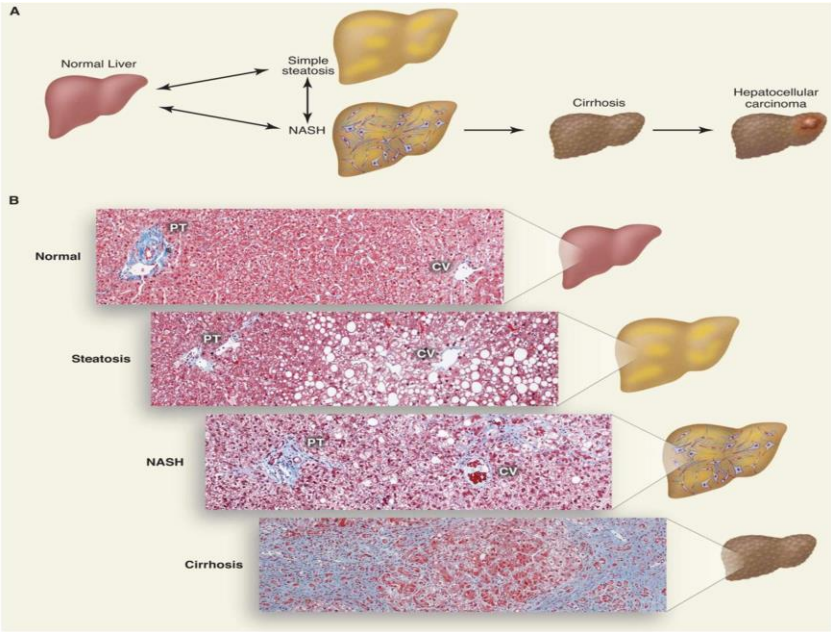


Figure 2.1. Progression of non-alcoholic fatty liver disease (NAFLD) (44).

Diagnosis of NAFLD

The spectrum of NAFLD can be categorized into four different types based on histological hepatocellular characteristics as summarized in Table 2.1 below (59-61).

Table 2.1. Diagnostic criteria for NAFLD

Type	Histological Characteristics	Clinical Information
Type 1	Simple steatosis	Non-progressive
Type 2	Steatosis + lobular inflammation	Benign
Type 3	Type 2 characteristics + ballooning degeneration	Considered NASH without fibrosis and may progress to liver cirrhosis
Type 4	Type 3 characteristics + fibrosis	Considered NASH with fibrosis and may progress to liver cirrhosis/failure

Liver biopsy remains the gold standard for the diagnosis and staging of NAFLD; nevertheless, its widespread use as a screening tool is not feasible due to its invasive nature (62). Physicians, instead, have been employing serum enzyme markers such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) to determine hepatocellular damage (63). ALT (specific for the liver) and AST (found in both liver and muscle cells) will leak out of the cells, under cell membrane damage, into liver sinusoids (small fenestrated capillary-like tubes that extend from the portal veins and arteries and facilitate

exchange of blood and nutrients to the hepatocytes), and then into systemic circulation (64). Yano *et al*. in 2001 showed that the sensitivity of ALT and AST tests to detect fatty liver was 35.7%, which is inferior to the use of the body mass index (65). However, about 70% overweight/obese patients with T2DM were diagnosed with NAFLD even in the presence of normal ALT levels (8, 66, 67). Moreover, currently available serum biomarkers are not sufficiently sensitive and specific to differentiate steatosis from NASH or to stage the presence and extent of liver fibrosis (53, 68). Therefore, there is a need for developing better non-invasive tools and biomarkers to identify the progression of NAFLD (69). Elastography, magnetic resonance elastography and acoustic radiation force imaging are imaging techniques that can distinguish a variety of chronic liver conditions in addition to NAFLD (70-72). Evaluation of changes in the lipid profiles (phospholipids and diacylglycerols) in the blood or urine could be powerful in classifying the progression of NAFLD (73).

Prevalence of NAFLD

NAFLD is the most prevalent form of liver disease worldwide, and is the most common cause of chronic liver disease in both developed and developing countries (1-4). A recent study determined that one-third of the United States adult population has hepatic steatosis (74). Other studies showed that NAFLD impacts 30% of the United States population; 30% of the South American population; 27% of Asian populations; 24% of the European population; and 13% of the African population (5, 75, 76). Approximately 70% of overweight/obese patients with T2DM worldwide have been diagnosed with NAFLD (8, 52, 66). The

elevation of NAFLD is proportional to increasing body mass index (BMI), and is anticipated to become the most prominent grounds for final stage liver disease in the United States by 2020 (77, 78).

Medicinal plants

Medicinal plants have been utilized as food, flavoring, medicine, or perfume to treat a variety of diseases or to improve human health by stabilizing physiological function (79). Herbal medicines have been employed for as dietary supplements for millennia even though there is a lack of understanding about their efficacy and safety (80, 81). Approximately 40% of Americans consume some types of dietary supplement (82, 83).

On one hand, medicinal plants offer several beneficial aspects including relatively low cost, widespread availability, and high acceptability (84). Using traditional medicine, on the other hand, is also associated with a number of disadvantages such as contamination with toxins, limited knowledge on the dosage, poor understanding of the medicinal interactions, lack of regulation, and possible serious physiological effects (79).

A number of interdisciplinary research centers have been funded by two components of the National Institutes of Health – the National Center for Complementary and Alternative Medicine and the Office of Dietary Supplements since 1999 – with the goal of determining mechanisms of action, efficacy and safety of botanicals, performing clinical investigation, and exploring new therapeutic approaches for medical treatment of diseases (81, 85).

Traditional medicine has been widely used in Africa, and therefore play essential roles in culture and life of Africans (86). About 3,000 medicinal plants have been traditionally used in southern Africa for treatment of a wide variety of diseases, including cancer, inflammation, and diabetes (18). However, little research has been conducted to establish the scientific rationale for the use of botanicals in Africa (86).

Phytotherapies are extensively utilized for mitigating obesity (16, 17, 87-90). Among these phytotherapies, *Sutherlandia frutescens* (L.) R.Br (Sutherlandia), a southern African medicinal plant, has been claimed for alleviation of various chronic diseases including diabetes, cancer, as well as infections (18-23).

Development of advanced biological technologies leads to more investigation into the chemical composition, biological functions, as well as underlying mechanisms of medicinal properties of herbs. Not only do these findings offer a better appreciation of herbal medicine, they also initiate novel implications for clinical treatment.

Sutherlandia frutescens

Introduction

Sutherlandia frutescens (L.) R.Br (Sutherlandia) (Figure 2.2) is a South African medicinal plant, which is commonly known as “cancer bush”. The *Sutherlandia* genus is named after James Sutherland who was the first administrator of the Edinburgh Botanic Garden. Sutherlandia belongs to the Fabaceae family, which

features a group of pod-bearing, flowering plants found all over the world (18). There are six species in the genus *Sutherlandia*, which are endemic to Southern Africa. These six species are reduced to two, i.e. *Sutherlandia frutescens* and *Sutherlandia tomentosa*; the former is further divided into three subspecies (subsp.), namely subsp. *frutescens*, subsp. *microphylla*, and subsp. *speciosa*. These subspecies are distinguished from one another by their habitat, the shapes of the pods and leaflets (91). *Sutherlandia frutescens* is a perennial, erect shrub having compound leaves, bladder-like pods and bright red flowers with a height of 0.2-2.5 meters (18). These plants are easily grown from the seeds collected from the pods; moreover, they well tolerate most soil types, are resistant to frost, flourish in full sun and well-drained soils, as well as perform at best when enjoying ample moisture in winter and spring seasons (92).

Figure 2.2. *Sutherlandia frutescens* (18)

Compounds in *Sutherlandia frutescens* extracts

Several bioactive compounds in Sutherlandia have been identifed in extracts of *Sutherlandia frutescens* including: D-pinitol, L-canavanine, gamma-aminobutyric acid (25), sutherlandiosides A, B, C and D (cycloartane glycosides) (26, 93), sutherlandins A, B, C and D (3-hydroxy-3-methylglutaro-yl containing flavonol glycosides) (27, 28), and polysaccharides (94).

Amino acids

D-pinitol was found to be most abundant (18.17 mg/g), followed by GABA (3.48 mg/g), and L-canavanine (0.08 mg/g), in leaf extracts (95).

D-pinitol (3-O-Methyl-D-chiro-inositol) (Figure 2.3 A), one of the common compounds in Sutherlandia leaves, has been reported to reduce blood sugar levels (29). In addition, this compound has been demonstrated to exhibit anti-inflammatory (96) and anti-hyperlipidemic (30) effects.

The non-protein amino acid L-canavanine (2-Amino-4-(guanidinooxy) butyric acid) (Figure 2.3 B) is able to interfere with arginine uptake essential for Nitrogen Oxide (NO) production (19, 25) that can destroy beta cells of the pancreas (97), thus inhibiting transcription and translation of insulin (98).

Gamma-aminobutyric acid (GABA) (Figure 2.3 C), an inhibitory neurotransmitter, has been shown to mitigate anxiety (Smith and van Vuuren, 2013). Significant amounts of GABA present in Sutherlandia shoots may be account for this anti-stress effects of this medicinal plant (18, 99). Furthermore, GABA was shown to suppress tumor cell migration (100), and influence steroid production through the hypothalamic-pituitary-adrenal axis (101, 102).

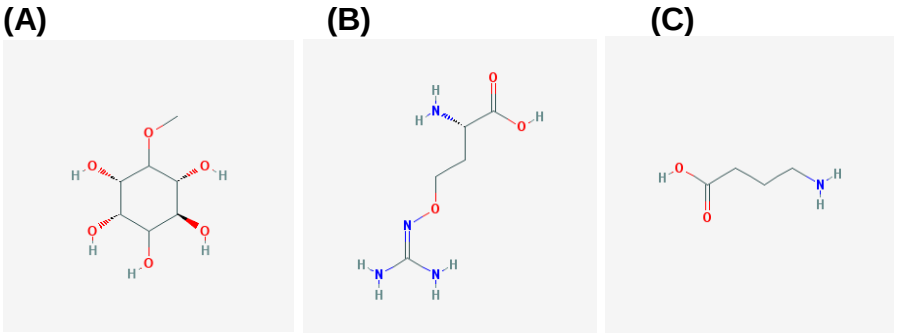


Figure 2.3. Chemical structures of A) D-pinitol[a], B) L-canavanine[b], and C) GABA[c].

[a] National Center for Biotechnology Information. PubChem Compound Database; CID=164619, https://pubchem.ncbi.nlm.nih.gov/compound/164619

[b] National Center for Biotechnology Information. PubChem Compound Database; CID=439202, https://pubchem.ncbi.nlm.nih.gov/compound/439202

[c] National Center for Biotechnology Information. PubChem Compound Database; CID=119, https://pubchem.ncbi.nlm.nih.gov/compound/119

Triterpenoids and flavonoids

Unique compounds of *S. frutescens* including sutherlandiosides A through D (cycloartanol glycosides) (Figures 2.4) and sutherlandins A through D (flavonol glycosides) (Figures 2.5) were characterized in the recent years (26-28).

Sutherlandioside B is the major cycloartene-type tetracyclic triterpene saponin in *S. frutescens* (2.75%, weight by total weight of dry plant sample), followed by sutherlandiosides A (0.61% w/w, C (0.22% w/w) and D (0.64% w/w) (27). Saponins are generally known as non-volatile, surface active compounds that are widely found in the plant kingdom (103, 104). The name ‘saponin’ is derived from the Latin word *sapo*, which means ‘soap’, due to the soap-like foams of saponin molecules in aqueous solutions (105). The triterpene saponins

are featured by a hydrophobic triterpenoid structure (aglycone) containing a hydrophilic sugar chain (glycone) (104), and are grouped into the following classes: oleananes, cycloartanes, dammaranes, tirucallanes, lupanes, hopanes, taraxasteranes, ursanes, lanostanes, and cucurbitanes (105). None of these sutherlandiosides showed anti-microbial and anti-malarial activities (26). However, Lin *et al.* (2016) discovered that sutherlandioside D, the most potent compound in the *S. frutescens* methanol extract, could suppress Gli-reporter in prostate cancer cells (106). Moreover, several studies have established biological impacts of triterpenoid glycosides. These compounds exerted anti-diabetic effects by improving glucose metabolism and enhancing fatty acid oxidation (31). In addition, they were reported to inhibit nociceptive responses (107), and suppress the nuclear factor–kappa light chain enhancer of activated B cells (NF-κB) inflammatory signaling pathway in human TNF-α induced astroglial cells (108) as well as in liver and white adipose tissues of male C57BL/6J mice fed a high-fat diet for 19 weeks (32). Furthermore, triterpenoid glycosides also exhibited anti-cancer effects by hindering abnormal cell proliferation and inducing apoptosis through mitochondrial pro-apoptotic mechanisms in rats initiated by 7,12-dimethylbenz(a)anthracene (DMBA) mammary carcinogenesis (109).

Flavonoids, classified into flavones, isoflavones, flavanols, flavan-3-ols and anthocyanidins (110), are also presentin *S. frutescens* extracts. These compounds are linked to several therapeutic values. Five types of flavonoids (3',4'-dihydroxy-4-methoxydalbergione, 4-methoxydalbergion, isoliquiritigenin, chrysin, and cearoin) displayed anti-inflammatory effects by significantly inhibiting

the NF-κB activity after the onset of lipopolysacharide (LPS)-induced inflammation (111). In addition, quercetin, a well-recognized flavonoid, elicited beneficial effects in alleviation of hepatic steatosis in C57/BL6J mice fed a high-fat, high-cholesterol and high-sucrose Western diet for 20 weeks by reducing hepatic triglycerides, decreasing oxidative stress and improving hepatic lipid metabolism by significantly increasing transcriptional expression of peroxisome proliferator-activated receptor alpha (PPAR-α) as well as suppressing expression of peroxisome proliferator-activated receptor gamma (PPAR-γ), cluster of differentiation 36 (CD36) or fatty acid translocase, as sterol regulatory element-binding protein-1C (SREBP-1c), and fatty acid synthase (FAS) (33). Moreover, kaempferol (3,5,7,4'-tetrahydroxyflavone), another well-known flavonoid, was shown to lower hepatic triglycerides and lipid droplet accumulation by increasing lipid metabolism through upregulation of PPAR-α, acyl-CoA oxidase (ACO), cytochrome P450 isoform 4A1 (CYP4A1), and downregulation of SREBPs in the livers of rats fed a high-fat diet for 10 weeks (34). Although flavonoid compounds within *S. frutescens* extract have been shown to be present in relatively low concentrations (112), Avula et al. (2010) identified and quantified the four flavonol glycosides, namely sutherlandins A-D (0.53%, 0.49%, 0.94%, and 0.54% (w/w), respectively), in the *S. frutescens* extracts (27). Sutherlandins A-D and sutherlandiosides A-D contributed very little to the anti-inflammatory effects in RAW 264.7 murine macrophage cells stimulated by LPS/interferon gamma (IFN-γ) (113).

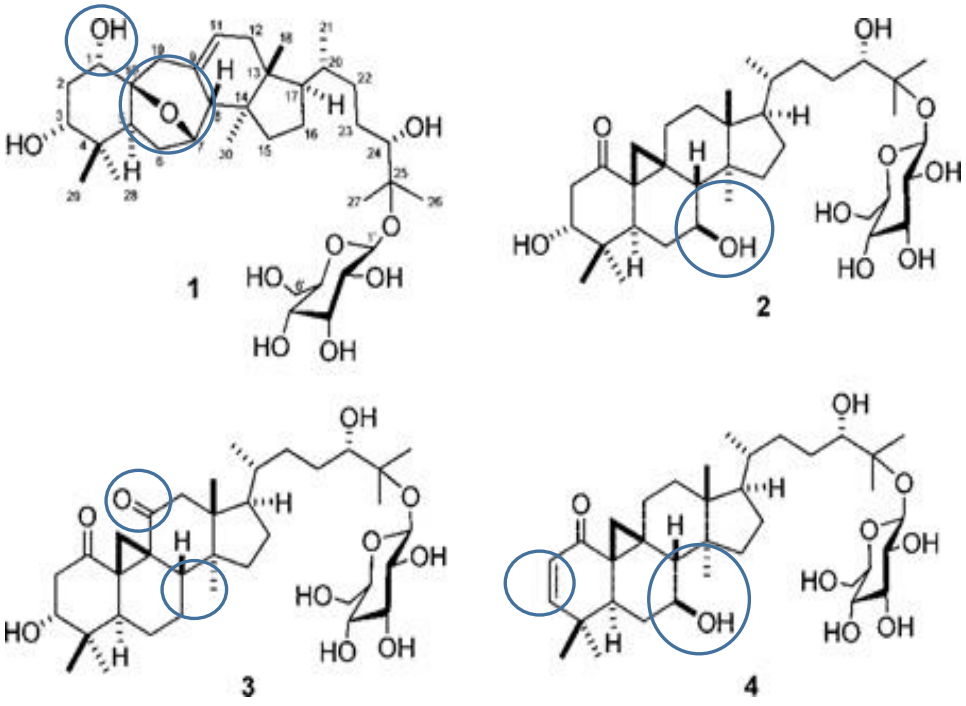


Figure 2-4. Structures of sutherlandiosides A to D (26). The circles indicate the structural differences of sutherlandiosides.

1: Sutherlandioside A, 1S,3R,24S,25-tetrahydroxycycloartane-7S,10S-epoxy-9,10-seco-9,19-cyclolanost-9(11)-ene 25-O-β-D-glucopyranoside,

2: Sutherlandioside B, 3R,7S,24S,25-tetrahydroxycycloartane-1-one 25-O-β-D-glucopyranoside,

3: Sutherlandioside C, 3R,24S,25-trihydroxycycloartane-1,11-dione 25-O-β-D-glucopyranoside,

4: Sutherlandioside D, 7S,24S,25-trihydroxycycloartane-2-en-1-one 25-O-β-D-glucopyranoside.

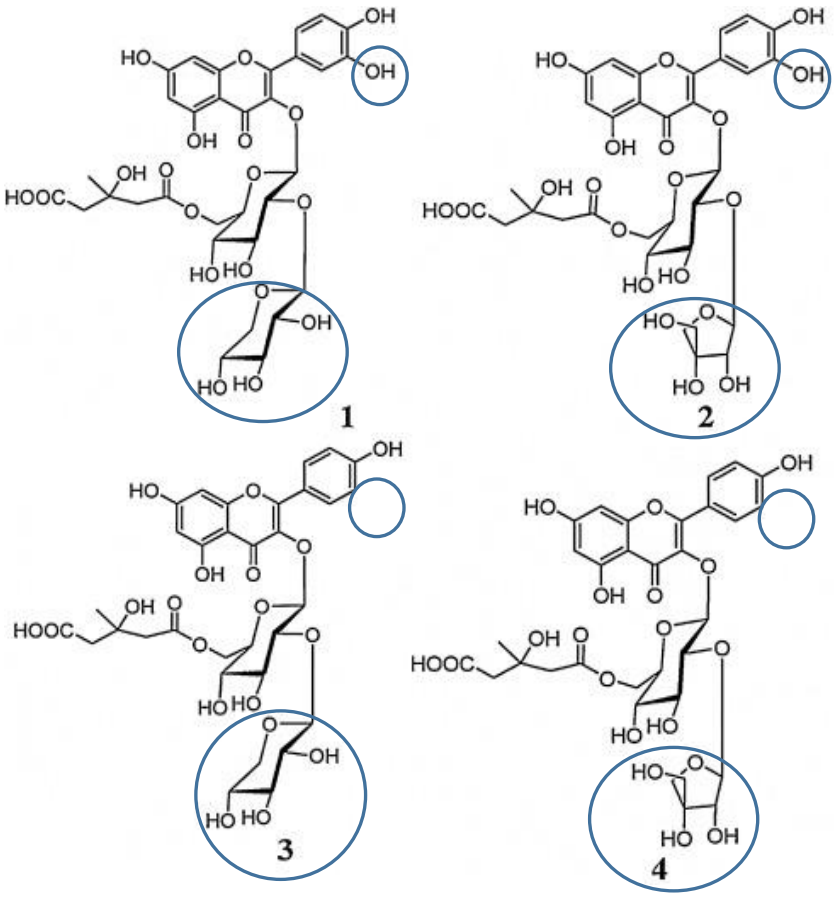


Figure 2-5. Structures of sutherlandins A to D (28). The circles indicate the structural differences of sutherlandins.

1: Sutherlandin A, quercetin 3-O-β-D-xylopyranosyl (1 → 2)-[6-O-(3-hydroxy-3-methylglutaroyl)]-β-D-glucopyranoside,

2: Sutherlandin B, 3R quercetin 3-O-β-D-apiofuranosyl (1 → 2)-[6-O-(3-hydroxy-3-methylglutaroyl)]-β-D-glucopyranoside,

3: Sutherlandin C, kaempferol 3-O-β-D-xylopyranosyl (1 → 2)-[6-O-(3-hydroxy-3-methylglutaroyl)]-β-D-glucopyranoside,

4: Sutherlandin D, kaempferol 3-O-β-D- apiofuranosyl (1 → 2)-[6-O-(3-hydroxy-3-methylglutaroyl)]-β-D-glucopyranoside.

Other bioactive components

First, high levels of polysaccharides of the pectin type were found in the leaves of *S. frutescens* (94). These polysaccharides were shown to display immunomodulatory properties by facilitating complement fixation; more specifically, the complement fixation activity was highly associated with regions rich in galactose and xylose (94).

Second, alpha-linolenic acid was isolated from the dichloromethane: methanol (1:1) extracts *S. frutescens* (114). The authors illustrated anti-tuberculosis effects of *this extract as it* was shown to inhibit recombinant shikimate kinase - an effective drug target for *Mycobacterium tuberculosis.*

Furthermore, cardenolides, a type of cardiac glycosides that has been used to treat congestive heart failure (115), have recently been identified in leaf and seed extracts of *S. frutescens* (112).

Lastly, some other compounds were also reported to be present in *S. frutescens* ethanol extracts, including hexadecanoic acid, γ-sitosterol, and stigmast-4-en-3-one (25).

Medicinal properties and potential mechanisms of *S. frutescens*

The aqueous extract of the stems and leaves of *S. frutescens* has been widely used in alleviating conditions of various chronic diseases such as T2DM, inflammation, cancer, stomach problems, influenza, and HIV/AIDS in southern Africa (18-23). The possible mechanisms for the medicinal properties of this medicinal plant have been evaluated in cell and animal models.

Diabetes

Rodent studies in South Africa have reported that administration of *S. frutescens* (800 mg/kg bodyweight) diminished blood glucose concentrations in streptozotocin-induced diabetic rats and that this beneficial effect was maintained longer than chlorpropamide - a conventional hypoglycemic agent (116). Moreover, traditional aqueous extracts of *Sutherlandia frutescens* (subsp. *microphylla*) increased glucose uptake in muscle and adipose tissues in high-fat fed rats (21). In addition, a commercial aqueous *Sutherlandia frutescens* (subsp. *microphylla*) extract (Pinosundia, Value Added Life Health Products (Pty) Ltd, South Africa; Registered trade mark with application number 2008/19581) at a dose of 50 mg/kg body weight/day decreased plasma free fatty acids and prevented the development of insulin resistance in male Wistar rats fed a high-fat diet for 12-16 weeks (22, 23).

Recently, *S. frutescens* aqueous extracts were shown to alter expression of 27 diabetes-related genes including peroxisome proliferator-activated receptor α (PPAR-α) - one of the key transcription factors responsible for hepatic lipid metabolism - in a fructose-induced insulin resistant human Chang liver cell model (35).

Inflammation

S. frutescens has been used to target several chronic inflammatory conditions, such as rheumatoid arthritis, osteoarthritis, and gout (99). Ojewole (2004) demonstrated that Sutherlandia aqueous extracts at a dose of 800 mg/kg *per os* significantly reduced acute inflammation of paw edema caused by the

fresh egg albumin in Wistar rats (116). A commercial aqueous *S. frutescens* (subsp. *microphylla*) extract (Pinosundia, Value Added Life Health Products (Pty) Ltd, South Africa) at a dose of 50 mg/kg body weight/day, however, did not lead to alteration in circulating plasma cytokine TNF-α and chemokine monocyte chemotactic protein-1 (MCP-1) in high-fat fed Wistar rats (23). Furthermore, no significant modification of host inflammatory response towards either gram negative (*E. coli*) or gram positive (*L. monocytogenes*) bacteria was detected in both C57BL/6 and BALB/c female and male mice fed an AIN-93G basal rodent diet incorporated with 0.25% or 1% w/w ground powder of the vegetative parts of *S. frutescens* for four weeks (117).

Regarding *in vitro* studies, a hot water extract of *S. frutescens* displayed antioxidant and/or anti-inflammatory activities in human neutrophils, Chinese hamster ovary cells, human hepatoma cells, and human pulmonary alveolar carcinoma (19, 118). Moreover, a methanolic extract of this plant was shown to decrease 12-O-tetra-decanoylphorbol-13-acetate (TPA)-induced cyclooxygenase-2 (COX-2) expression in mouse skin by diminishing the activity of extracellular signal-regulated protein kinase (ERK) and activator protein-1 (AP-1) (119). In addition, a 70% ethanol extract of *S. frutescens* did not remarkably suppress nitric oxide production or inhibit mRNA expression of pro-inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in murine macrophage RAW 264.7 cells (25). *S. frutescens* extracts, nonetheless, was reported to modulate the inflammatory responses by altering levels of IL-8 and TNF in HL60 cell lines stimulated by phorbol 12-myristate 13-acetate (24). Also,

ethanolic extracts of *S. frutescens* (20 µg/ml) suppressed N-methyl-D-aspartic acid (NMDA)-induced reactive oxygen species (ROS) production in neurons and LPS- and IFN-γ-induced ROS and nitric oxide production in microglial cells, as well as reduced activation of the ERK and signal transducer and activator of transcription 1 (STAT1) signaling pathways in microglial cells (120). Moreover, Lei et al. (2015) suggested that chlorophyll present in ethanolic extracts of *S. frutescens* (200 µg/ml) contribute to a decrease in TNF-α and IL-6 production, as well as an inhibition of the NF-κB activity in RAW 264.7 mouse macrophage cells induced by lipopolysacharide (LPS) and interferon gamma (IFN-γ) (113). Interestingly, polysaccharide-enriched fraction of *S. frutescens* significantly elevated production of ROS, NO, and TNF-α in RAW 264.7 cells in a dose-dependent manner, and these activities were completely abolished by co-treatment with CLI-095 – a specific inhibitor of toll-like receptor-4 (TLR-4) (121).

Immunodeficiency disorders

S. frutescens has been traditionally employed as an immune-stimulating tonic for human immunodeficiency virus (HIV) infected patients, and this practice has been acknowledged by the South African Ministry of Health (122).

Bessong *et al.* (2005) reported that a methanolic extract of this plant stimulated recombinant HIV-1 reverse transcriptase (RT) activity (123); in contrast, Harnett *et al.* (2005) suggested that tannins, a compound present in *S. frutescens* aqueous extracts, may contribute to inhibition of HIV-1 RT and HIV-1 target enzymes including α- and β-glucosidase (124). In addition, *S. frutescens* may affect anti-retroviral drug metabolism because it was discovered to inhibit

P450 3A4 (CYP3A4), a hepatic cytochrome P450 enzyme involved in drug metabolism (20).

Moreover, Korb *et al.* (2010) suggested that consumption of ethanolic *S. frutescens* extracts may lead to induction of apoptosis in cluster of differentiation (CD) 4+ cells and T lymphocytes in normal human lymphocytes *in vitro*, which would exacerbate the condition of immunocompromised patients as they have low CD4+ cell counts (125). Wilson *et al.* (2015), however, challenged this finding by illustrating that HIV viral load, CD4+ cells and T lymphocyte counts were not significantly different in HIV seropositive individuals with or without *S. frutescens* treatment. Moreover, there was a greater burden of infection in the group receiving *S. frutescens* as two subjects of this group subsequently contracted tuberculosis while undergoing isoniazid preventative therapy, a first line regimen used worldwide for prevention and treatment of tuberculosis (Wilson et al., 2015). This unexpected side effect may be attributed to the indication that *S. frutescens* inhibited expression of genes involved in oxidative stress, inflammatory signaling and toll-like receptor pathways, which can lessen the host's immune response to infection and reactivation of latent *Mycobacterium tuberculosis* (126).

A *S. frutescens* hexane extract was also shown to have strong antibacterial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, and *Escherichia coli* (127). *S. frutescens* and alpha-linolenic acid, isolated from the extracts, impeded the metabolic action of shikimate kinase enzyme (MtSK) of *Mycobacterium tuberculosis* (114). MtSK is the key enzyme in the shikimate biosynthetic pathway from *M. tuberculosis* that only takes place in microbes and

some plants, rendering this shikimate pathway an excellent target for developing novel anti-tuberculosis agents (128). Masoko *et al.* (2016) discovered that alpha-linolenic acid purified from the dichloromethane: methanol (1:1) extract of *S. frutescens* elicited a significant inhibition of MtSK with a half maximal inhibitory concentration (IC_{50}) of 3.7 µg/ml. (114).

Cancer

The potential mechanisms towardcancer of *S. frutescens*, the well-known "cancer bush", have been extensively investigated. *S. frutescens* was shown by Tai *et al.* (2004) to be capable of limiting the growth, proliferation, and mutagenic activities, and of promoting the process of apoptosis in various human cancer cell lines including MCF7, MDA-MB-468, Jurkat, and HL60 (25). These effects of *S. frutescens* may be linkedto its ability to suppress growth, morphology and proliferation of MCF-7 human breast adenocarcinoma cells (129). Also, *S. frutescens* treatment led to typical signs of apoptosis such as cell shrinkage and plasma membrane blebbing in esophageal cancer cells (130).

Moreover, *S. frutescens* ethanolic extracts was found to inhibit approximately 50% of Hedgehog-signaling responsive genes in TRAMPC2 prostate cancer cells (131), which implies that by *S. frutescens* has significant potential in cancer treatments because Hedgehog signaling has been associated with advanced prostate cancer. In addition, Lin et al. (2016) suggested that sutherlandioside D, which was identified in the *S. frutescens* methanol extract, may exert anti-cancer effects by repressing Gli-reporter in prostate cancer cells

and by reducing formation of prostate carcinoma in TRAMP mice fed a diet supplemented with 1% w/w of this plant (106).

Other functions

S. frutescens has been traditionally used for several other diseases/symptoms including the stress-related ailments, shock, trauma, and severe depression (18).

S. frutescens could inhibit glucocorticoid production and mitigate symptoms related to psychological stress through inhibition of the catalytic activity of adrenal steroidogenic P450 enzymes, P450 17α-hydroxylase/17,20-lyase (CYP17A1) and P450 21-hydroxylase (CYP21A2) (132, 133). Moreover, a low dose of that *S. frutescens* aqueous extracts (4mg/kg body weight) modulated steroidogenesis by eliminating the down-regulation of glucocorticoid receptor in Wistar male rats subjected to acute psychological stress (134). More recently, Sergeant *et al.* (2017) reported that sutherlandioside B, a compound unique to *S. frutescens,* significantly inhibited CYP17A1 and 3β-hydroxysteroid dehydrogenase type 2 (3β-HSD2) in monkey kidney COS-1 cells, and decreased cortisol and androgen precursors in an angiotensin-II-responsive steroid-producing adrenocortical H295R cell line (135). In addition, this study also showed that the methanolic *S. frutescens* extracts significantly decreased total steroid production and mineralocorticoid metabolites under forskolin-stimulated steroid conditions.

Ojewole (2008) described the anticonvulsant and anticoagulant properties of *S. frutescens* (99). Recently, dietary *S. frutescens* was reported to decrease

ischemia-induced neuronal cell death and mitigate p47phox and of ERK1/2 phosphorylation in microglial cells (136).

Safety and efficacy

The use of *S. frutescens* has not been linked with severe side effects even though symptoms such as dryness of the mouth, occasional mild diarrhea or mild diuresis, and dizziness in cachectic patients have been documented (20).

The traditional dose of this herb is infusion or decoctions of 2.5 to 5 g of dry material per day, and no ill effect was illustrated after taking a decoction of 5 g of dried material daily (twice a day) for more than six years (18).

Clinical and toxicological studies in South Africa determined that oral consumption of the Sutherlandia leaf powder (800 mg per day) is well tolerated by healthy and HIV-infected adults, and demonstrated no significant clinically relevant effects in biochemical and hematologic measures, with elevated appetites being the most significant difference between the treatment and placebo group (137, 138). Moreover, Wilson *et al.* (2015) observed that the HIV viral load and $CD4_+$ T-lymphocytes were similar in control and treatment groups when *S. frutescens* was administered to HIV seropositive adults (138). Importantly, this study pointed out that the total burden of infection was considerably greater in the group receiving *S. frutescens* with two subjects developing tuberculosis while receiving isoniazid preventative therapy.

Assessment of the safety of *S. frutescens* consumption was also conducted in animal studies. Seier *et al.* (2002) fed vervet monkeys a carefully monitored standard diet containing 0, 1, 3 and 9 times of *S. frutescens* compared

to the recommended daily dose of 9 mg/kg body weight (i.e. 0, 9.0, 27.0, and 81 mg of leaf powder per day, respectively) for three months, and found no clinically significant side effects (139). In addition, BALB/c mice were given graded shoot aqueous extracts of *S. frutescens* (25, 50, 100, 200, 400, 800, and 1600 mg/kg body weight) by intraperitoneal injection for an acute toxicity investigation (116). The results showed that crude extracts of *S. frutescens* are probably relatively safe in mammals based on the calculated median lethal dose (LD50) of 1280 ± 71 mg of extract per kg body weight.

Bioavailability of phytochemicals

Many bioactive compounds have been shown to exert various medicinal effects *in vitro*; nevertheless, it is not uncommon that these effects are not always observed *in vivo.* The discrepancy between *in vivo* and *in vitro* effects can be attributed to the bioavailability of the natural compounds in the target tissues because these compounds would be subjected to digestion, absorption, metabolism, and degradation by gut flora, hepatic enzymes and potential transport within circulation. Consequently, these compounds may not reach their intended target in the active form.

Lipinski (2004) described a guideline for identifying potential new drugs that would be bioavailable after oral intake (140). There are four physicochemical characteristics that should be taken into account, including limitations for molecular weight (≤ 500 Da), lipophilicity (log P ≤ 5) and the number of hydrogen donors and acceptors (H-bond donors ≤ 5 and H-bond acceptors ≤ 10). These features have been employed to predict the aqueous solubility and intestinal

permeability of compounds, and have been associated with about 90% of oral medications that have reached phase II clinical trials.

Compounds with relatively simple chemical structures such as GABA, D-pinitol and L-canavanine in *S. frutescens* extracts are most likely to be readily absorbed from the intestines; however, the more complex molecules such as flavonoids and triterpenoids may be less likely to be absorbed *in vivo* (141). The digestion and absorption of triterpenoids in Wistar rats was investigated by oral administration of mogroside V (a pentaglucose-conjugated mogroside) - the main sweetening component and also a type of triterpenoids found in the traditional Chinese fruit *Siraitia grosvenori* - into rats (142). The authors discovered that mogroside V had been degraded to tetra and triglucose mogroside conjugates by digestive enzymes and intestinal microflora and was excreted in the feces as mogrol and its mono- and diglucosides whereas a trace amount of mogrol and its monoglucoside were detected in the portal blood as sulfates and/or glucuronides conjugates.

Ginsenosides, which are components of *Panax* ginseng and classified into the dammarane-type tetracyclic and oleanane-type pentacyclic triterpene saponin groups (143), were shown to be present in hepatic tissues at much higher concentrations than plasma and other tissues (kidney, stomach and intestine) of Sprague–Dawley rats (144). The addition of small molecule fractions to the dried rhizome *Anemarrhena asphodeloides* extracts improved the bioavailability of steroidal saponins - active compounds in this herb - in rat plasma (145).

In order to increase the bioavailability of natural compounds, a number of advanced technologies have been developed including the application of nano-sized drug carrier particles such as liposomes and micelles, crystal engineering, micronization, and cyclodextration (146-151).

Summary and Conclusion

NAFLD is the most prevalent form of liver disease worldwide, and is associated with obesity, insulin resistance, and type 2 diabetes mellitus. Hepatic steatosis refers to the excessive accumulation of lipids in the liver, is a fundamental feature of NAFLD, and may progress to nonalcoholic steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma.

The great majority of the South African people depend upon traditional medicine for either part of or their entire primary health care. South Africans have extensively used traditional remedies such as *S. frutescens* decoctions regardless of lacking thorough understanding about the impact of the constituents of this plant on the disease conditions. The scientific validation of the use of *S. frutescens* extracts as therapeutic targets for diabetes, depression, inflammation and cancer may foster the use of this herbal remedy by the general public beyond Africa. Unlike western pharmaceuticals, no significant side effects have been connected with the application of *S. frutescens.* This herb, therefore, may provide safe, affordable treatment for the aforementioned ailments.

South Africa is one of the three richest floristic areas in southern Africa; nevertheless, there is still a lack of commercialization of marketable medicinal products in spite of a remarkable increase in the scientific substantiation of the

use of botanicals and of increasing commercial and scientific interest in this area. It is important to support the sustainable development of the natural product industry in both developing and developed countries because the successful integration of science and traditional medicine has great potential for both economic and scientific benefits.

A number of medicinal uses of *Sutherlandia frutescens* have been documented in southern Africa. Previous studies showed that *S. frutescens extracts* reduced plasma free fatty acids and inhibited the development of insulin resistance in high-fat fed rats, and that *S. frutescens extracts* altered diabetes-related gene expression in a fructose-induced insulin resistant human Chang liver cell model. Molecular mechanisms of how Sutherlandia corrects the insult of high-fat diets, prevents insulin resistance and improves lipid metabolsim, however, remain unknown. The experiments described in the following chapters were designed and conducted to determine the impact of *S. frutescens* on obesity-induced hepatic steatosis.

CHAPTER THREE

SUTHERLANDIA FRUTESCENS CONSUMPTION MITIGATES HEPATIC STEATOSIS BY INCREASING EXPRESSION OF FATTY ACID OXIDATION-RELATED GENES IN MALE WISTAR RATS FED A HIGH-FAT DIET

Abstract

Hepatic steatosis (fatty liver) is closely associated with the development of obesity and type II diabetes mellitus, which are dramatically increasing worldwide due to changes in nutrition and lifestyle. The pathophysiology of hepatic steatosis is multifactorial and not well-addressed by current pharmacological treatments, many of which are not accessible to those at greatest risk, because of cost, availability, and need for clinical monitoring. These impediments might be addressed by phytotherapies for which adequate evidence of safety and efficacy can be provided.

Sutherlandia frutescens (L.) R.Br. (Sutherlandia) is a southern African medicinal plant that is widely used for a number of diseases and health conditions, including diabetes. Clinical studies have determined that consumption of Sutherlandia is well tolerated by healthy and HIV-infected adults, and limited evidence from cell culture and animal studies suggests Sutherlandia moderates glucose homeostasis and lipid metabolism; however, the pertinent molecular mechanisms remain unknown.

This study was conducted to determine the effects of Sutherlandia consumption on hepatic steatosis of rats consuming a high-fat diet and to determine the basis for observed changes in lipid metabolism. Male Wistar rats received either a standard low-fat diet (LFD) or a high-fat diet (HFD) *ad libitum*. The HFD-fed rats were treated with or without aqueous extracts of Sutherlandia or Sutherlandia incorporated into feed.

The results exhibited that Sutherlandia treatments significantly reduced liver triglycerides, steatosis and lipid droplet size in rats fed HFD compared to those fed HFD without treatment. These treatments significantly upregulated hepatic fatty acid oxidation-associated genes including the transcription factor peroxisome proliferator-activated receptor alpha (PPAR-α) and its target genes peroxisomal acyl-coenzyme A oxidase-1 (ACOX-1) and Cytochrome P450, family 4, subfamily A, polypeptide 14 (CYP4A14), but did not significantly alter genes associated with *de novo* lipid synthesis such as sterol regulatory element-binding protein-1C (SREBP-1C), carbohydrate-responsive element-binding protein (ChREBP), or fatty acid synthase (FAS).

In conclusion, Sutherlandia reduces HFD-induced accumulation of liver triglycerides in rats by increasing fatty acid oxidation and deserves additional study as a phytotherapy for hepatic steatosis and related conditions.

Introduction

Nonalcoholic fatty liver disease (NAFLD) has become a prominent chronic liver disease in developed nations and also is increasing worldwide (4). This disease impacts 30% of the U. S. population; 30% of South Americans; 27% of Asians; 24% of Europeans and 13% of Africans (5, 75, 76). Approximately 70% of overweight/obese patients with type 2 diabetes mellitus (T2DM) worldwide have NAFLD (8, 52, 66), which is related to increasing body mass index (BMI), and will become the most prominent contributor to final stage liver disease in the United States by 2020 (77, 78). In South Africa, more than 29% of men and 56% of women are categorized as overweight (BMI > 25) or obese (BMI > 30) in South Africa (39), and for whom NAFLD is common (152).

NAFLD begins with asymptomatic hepatic steatosis (fatty liver), which leads to steatohepatitis (fatty liver and inflammation), and may end with liver fibrosis, cirrhosis and hepatocellular carcinoma (5-7). Hepatic steatosis is clinically defined when triglycerides (TG) exceed 5% of the total liver weight (12, 13). *De novo* lipogenesis and fatty acid oxidation are two key processes contributing to the development of hepatic steatosis (13, 51, 153).

Phytotherapies are widely employed for mitigating obesity and other metabolic disorders such as hepatic steatosis (16, 17, 87-90). These remedies are usually more affordable and accessible than pharmaceuticals to many people in developing countries (84). Among the phytotherapies widely used in South Africa, *Sutherlandia frutescens* (L.) R.Br (Sutherlandia), also known as “unwele and cancer bush”, has been claimed to alleviate various chronic diseases

including diabetes, cancer, as well as infections (18-23). Clinical and toxicological studies determined that consumption of Sutherlandia leaf powder is well tolerated by healthy and HIV-infected adults and caused no significant clinically relevant effects in biochemical and hematologic measures (137, 138).

Studies in rodents indicate that aqueous preparations of Sutherlandia decreased plasma free fatty acids and prevented the development of insulin resistance in male Wistar rats fed a high-fat diet (HFD) (21-23). The molecular mechanism(s) for such effects, however, remains to be established. This study tests the hypothesis that Sutherlandia consumption prevents hepatic steatosis in HFD-fed rats by reducing hepatic *de novo* lipogenesis and/or by increasing fatty acid oxidation.

Materials and Methods

Animals and experimental design

Male Wistar rats at 4 weeks of age were purchased from Harlan Laboratories and housed individually at the University of Missouri Animal Sciences Research Center (ASRC). They were maintained at 25^{o}C ± 3^{o}C on a 12h:12h light-dark cycle with free access to food and water. All animal procedures adhered to Institutional Animal Care and Use Committee's guidelines. The rats (12 animals/group) received either a standard 3.5% soybean oil (SBO) low-fat diet (LFD) (Harlan Teklad, TD.06683, Wisconsin, USA: 20.7% protein, 69.8% carbohydrates and 9.5% fat (expressed as % energy per 100g)) or a high-fat diet (HFD) (Harlan Teklad, TD.95217, Wisconsin, USA: 18.8%

protein, 41.4% carbohydrates, and 39.8% fat (34% saturated, 18% trans, 29% monounsaturated and 19% polyunsaturated fat) (expressed as % energy per 100g)) *ad libitum.* The HFD-fed rats were treated with or without Sutherlandia (aqueous extracts in drinking water at a daily dose of 50 mg extract/kg body weight or dried, milled vegetative material in the feed (1%w/w)) for 12 consecutive weeks. Body weight was recorded weekly.

At weeks 1 - 3 after Sutherlandia treatments, all rats were fasted overnight, and blood from tail vein was collected into ethylenediaminetetraacetic acid (EDTA)-containing collection tubes. The collected blood was then centrifuged at 2000 x g for 20 minutes. Plasma was removed and snap frozen in liquid nitrogen and stored at -80°C for further analysis.

After 12 weeks of treatments, all rats were fasted overnight and euthanized by CO_2 inhalation and cervical dislocation. Blood was removed via heart puncture and plasma was collected the same way as above. Liver tissues were removed, weighed, snap frozen in liquid nitrogen and kept at -80°C for further analysis.

Sutherlandia preparation

Dried, milled vegetative material of *S. frutescens* (L.) R.Br. was purchased from Big Tree Nutraceutical (Fish Hoek, South Africa), and its composition was verified by high-performance liquid chromatography (HPLC). Extracts were prepared by mixing 50 g dried, crushed leaves with 2 liters of boiling water, and allowing it to cool overnight. The extract was filtered using a sterilized 0.2 µm nylon filter (Fisher Scientific, Pittsburgh, PA, USA), and then stored in small

aliquots at -20°C until use. A dose of 50 mg extract/kg body weight/day approximates the human equivalent dose of 3 cups of tea per day (21), and was employed in this study. Every morning, fresh Sutherlandia extract was provided to the rats, whose consumption was measured daily (approximately 25 mL per day), and the appropriate amount of Sutherlandia aqueous extract was adjusted according to the body weights of the rats.

Moreover, a commercial aqueous *S. frutescens* extract (ProBetix®, Value Added Life Health Products (Pty) Ltd, South Africa) - a nutritional supplement widely distributed over the counter in South Africa - was also employed in this study. ProBetix® contains 90 mg (39%) of Pinosundia® from the aerial parts of the *S. frutescens* plant and 75 mg (33%) of alpha-lipoic acid (ALA or 1,2-dithiolane-3-pentanoic acid) - a potent biological antioxidant naturally distributed in small amounts in plants and animals (154-156), and 65 mg (28%) of excipient (e.g., inactive fillers and preservatives). An amount of 25.58 grams of the dried powder from ProBetix capsules was added to 2 liters of water. The mixture was filtered using a sterilized 0.2 µm nylon filter (Fisher Scientific, Pittsburgh, PA, USA), and then stored in small aliquots at -20°C until use. Rats received a dose of 50 mg extract/kg body weight/day - the dose that Mackenzie *et al.* claimed to diminish plasma free fatty acids and ameliorate insulin resistance in HFD-fed rats (22, 23).

Sutherlandia (Su) did not appear to alter the amount of liquid or diet consumed, as rats in the LFD control, HFD control, HFD + Su tea, and HFD + Pino tea groups drank similar amounts of liquid (25 mL) per day (Appendix

Figure 3-2), and HFD with or without Sutherlandia was consumed at approximately the amount expected (23 g diet per day) (157).

In addition, this dried, milled vegetative material of Sutherlandia was incorporated into the Harlan Teklad 95217 HFD. The concentration of Sutherlandioside B (SuB: $C_{36}H_{60}O_{10}$) - a cycloartene-type tetracyclic triterpene saponin present in Sutherlandia (26, 93) - in the feed (calculated as 0.12 mg SuB/g diet) was adjusted to be roughly comparable to that consumed by rats when Sutherlandia was incorporated in the drinking water (0.18 mg SuB/mL). In addition, the concentration of SuB in the commercial Pinosundia® is 0.64 mg SuB/mL. High-performance liquid chromatography (HPLC) analyses of SuB in the teas are displayed in Appendix Figure 3-1 B-C.

Determination of plasma glucose, insulin, and triglycerides (TG)

Plasma concentrations of glucose and triglycerides (TG) were determined by Dr. Charles E. Wiedmeyer from the Veterinary Clinical Pathology lab using a Beckman-Coulter AU680 Clinical Chemistry instrument. Plasma insulin was measured using a commercial Rat Insulin enzyme-linked immunosorbent assay (ELISA) kit (ALPCO; Salem, New Hampshire). Duplicate measurements were performed according to the manufacturer's protocols.

Determination of liver weights and TG

Liver from each rat was weighed, and a sample was homogenized and subjected to TG measurement using commercial kits (Wako L-type TG M kit; Wako Diagnostics, Richmond, Virginia) and expressed per gram tissue.

Duplicate measurements were performed according to the manufacturer's protocols.

Liver histological analysis

Frozen liver tissues were sectioned at 10 μm with a cryomicrotome (Leica), and three sections at 50-μm intervals were placed on a slide. Neutral lipids (i.e., triglycerides, diacylglycerols and cholesterol esters) in the liver sections on these slides were stained with Oil Red O (Sigma-Aldrich) as instructed in the Biological Stain Commission guidelines (158), counterstained with Mayer's hematoxylin (Sigma-Aldrich), and cover-slipped using an aqueous mounting media for microscopic examination. Four images from one randomly chosen section per slide were acquired with a Leica DM 5500B microscope fitted with a Leica DFC290 color camera. All images were acquired using a 20x HCX PL Fluotar objective and identical illumination and camera settings. The images were analyzed using Fiji image analysis software (159) by a cytologist who was unaware of treatment allocations. The number and area size of lipid droplets were calculated using a custom-written macro. Images of ORO-stained lipid droplets were separated from blue hematoxylin counterstain using the Color Deconvolution plugin for ImageJ that implements the stain separation method (160). Data from 4 different areas of the same section were averaged and used to calculate group means and standard errors of the mean (SEM).

Determination of mRNA levels

Frozen liver tissues were homogenized in TRIzol solution (Sigma-Aldrich, St. Louis, MO) using a tissue homogenizer (TissueLyser LT, Qiagen, Valencia,

CA). Total RNA was then isolated using an RNeasy Kit (QIAGEN) and analyzed using a Nanodrop spectrophotometer (ThermoScientific, Wilmington, DE) to assess purity and concentration. Complementary DNA (cDNA) was synthesized from 1µg of total RNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Quantitative reverse transcription polymerase chain reactions (rt-q PCRs) were conducted in a 20-µL final reaction volume using SYBR Green Master Mix (Biotool^{TM}) on a QuantStudio real-time PCR system (Applied Biosystems) to measure the relative hepatic mRNA concentrations of genes involved in the following: 1) fatty acid oxidation: peroxisome proliferator-activated receptor α (PPAR-α), acyl-coenzyme A oxidase-1 (ACOX-1) and cytochrome P450, family 4, subfamily A, polypeptide 14 (CYP4A14), 2) *de novo* lipogenesis: sterol regulatory element-binding protein-1C (SREBP-1C), carbohydrate-responsive element-binding protein (ChREBP), and fatty acid synthase (FAS), and 3) inflammation: TNF-α, MCP-1, IL-6 and IL-4. PCR conditions were as follows: 50.0°C for 2 minutes (1 repetition), 95°C for 10 minutes (1 repetition), and 95°C for 15 seconds, followed by 60°C for 45 seconds (40 repetitions). PPAR-α, ACOX-1, CYPA414, ChREBP, FAS, TNF-α, MCP-1, IL-6, IL-4, and 18S rRNA primers have previously been published (161-163), and were synthesized by Integrated DNA Technologies. SREBP-1C primers were designed and synthesized by Sigma-Aldrich. Primer sequences are provided in Table 3-1. All primers were tested for PCR efficiency relative to the internal control (18S rRNA) by using serial dilutions of the cDNA template. The internal control gene was determined based on its comparable expressions across the

treatment groups. All the mRNA data were determined using the ΔΔCT approach (164), comparing expression of target genes relative to the housekeeping gene (18S rRNA), and the data were expressed as fold differences relative to the high-fat diet control group.

Table 3-1. Sequences of primers used for rt-q PCR assays.

Genes	**Primer sequences**
PPAR-α	Forward: 5'-GAGGTCCGATTCTTCCACTG-3' Reverse: 5'-ATCCCTGCTCTCCTGTATGG-3'
ACOX-1	Forward: 5'-TCAGCAGGAGAAATGGATGC-3' Reverse: 5'-TGGAAGTTTTCCCAAGTCCC-3'
CYPA414	Forward: 5'-CTTGATGACACTGGACAC-3' Reverse: 5'-ACTCCATCTGTGTGCTCATG-3'
SREBP-1C	Forward: 5'-AAACCTGAAGTGGTAGAAAC-3' Reverse: 5'-TTATCCTCAAAGGCTGGG-3'
ChREBP	Forward: 5'-CCTGAAGACCCAAAGACCAA-3' Reverse: 5'-AGATGGAGTGCAGGGCTCTA-3'
FAS	Forward: 5'-AGTGAGTGTACGGGAGGGCT-3' Reverse: 5'-GCTGGGACACATGTGATGGT-3'
TNF-α	Forward: 5'-ATTGCTCTGTGAGGCGACTG-3' Reverse: 5'-GGGGCTCTGAGGAGTAGACG-3'
MCP-1	Forward: 5'-CTGTCTCAGCCAGATGCAGTTAA-3' Reverse: 5'-AGCCGACTCATTGGGATCAT-3'
IL-6	Forward: 5'-AGAGACTTCCAGCCAGTTGC-3' Reverse: 5'-AGCCTCCGACTTGTGAAGTG-3'
IL-4	Forward: 5'-CGTGATGTACCTCCGTGCTT-3' Reverse: 5'-GTGAGTTCAGACCGCTGACA-3'
18S	Forward: 5'-GCCGCTAGAGGTGAAATTCTTG-3' Reverse: 5'-CATTCTTGGCAAATGCTTTCG-3'

Statistical analysis

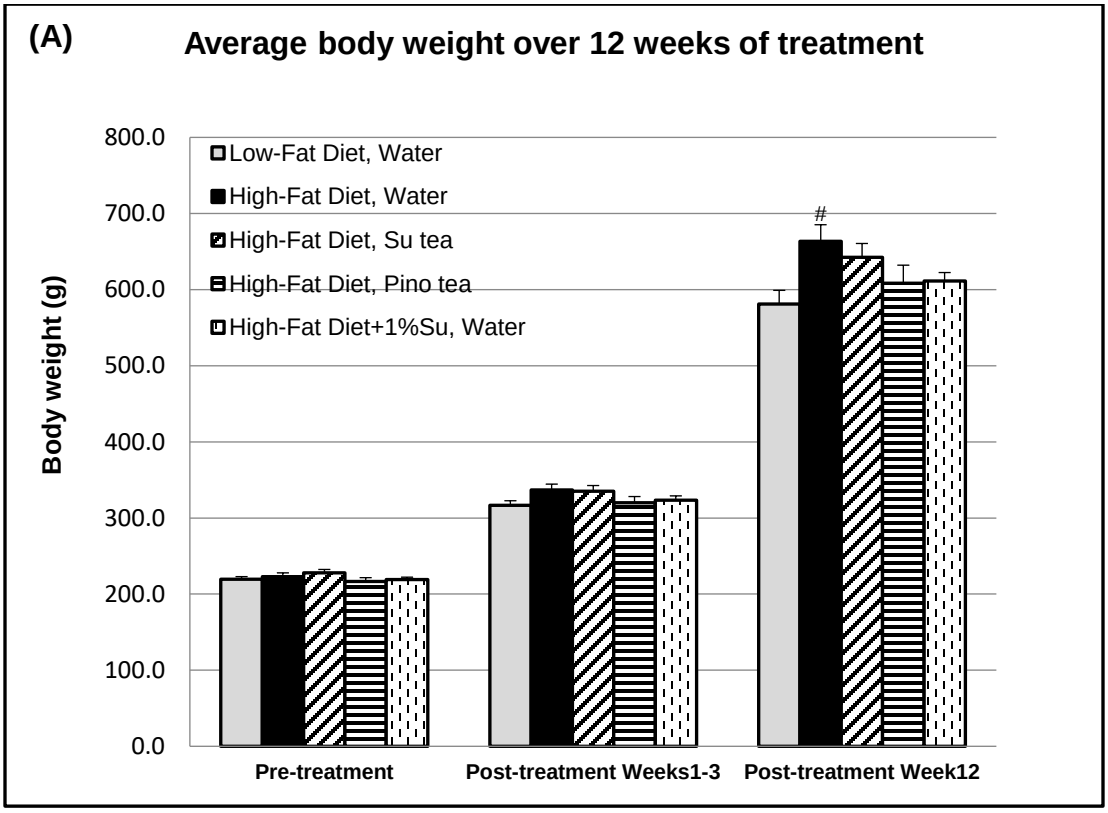
Data are expressed as mean ± standard errors of the mean (SEM). Comparisons between group means were carried out using one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparisons test. The level of significance was set at p-value < 0.05. Minitab statistical software package version 17 (Minitab Inc., State College, PA) was used for all statistical tests.

Results

Effects of Sutherlandia consumption on body weight and liver weight

Wistar, male rats were fed either a 3.5% SBO low-fat diet (LFD: 9.5% fat) or a high-fat diet (HFD: 39.8% fat) *ad libitum*. The HFD-fed rats were treated with or without Sutherlandia (Su) in the aqueous extracts at a daily dose of 50 mg extract/kg body weight (HFD + Su tea or HFD + Pinosundia® (Pino) tea) or in the feed (HFD + 1%Su diet) for 12 consecutive weeks. The average body weight in all animal groups was comparable within the first three weeks of treatment (Figure 3-1 A); however, by the end of the 12-week treatment, the average body weight of the untreated HFD-fed rats was significantly greater than that of the LFD-fed rats (p < 0.025, Figure 3-1 A). Remarkably, Sutherlandia treatments reduced body weight of the rats fed HFD compared to the untreated HFD group, and the average body weights of those rats fed HFD with Sutherlandia (HFD + Su tea, HFD + Pino tea, or HFD + 1%Su diet) were intermediate to those of the LFD and HFD controls (p > 0.05, Figure 3-1 A).

At week 12 of treatment, all of the rats were fasted overnight, euthanized, and several tissues including livers were collected. All three forms of Sutherlandia treatments also reduced fasting liver weight in rats fed HFD compared to those fed HFD alone (Figure 3-1 B). Even though this reduction was not statistically significant ($p > 0.05$, Figure 3-1 B), all Sutherlandia treatments, especially the 1% Sutherlandia powder integrated in the HFD, brought the liver weight down to be comparable with the LFD control group.



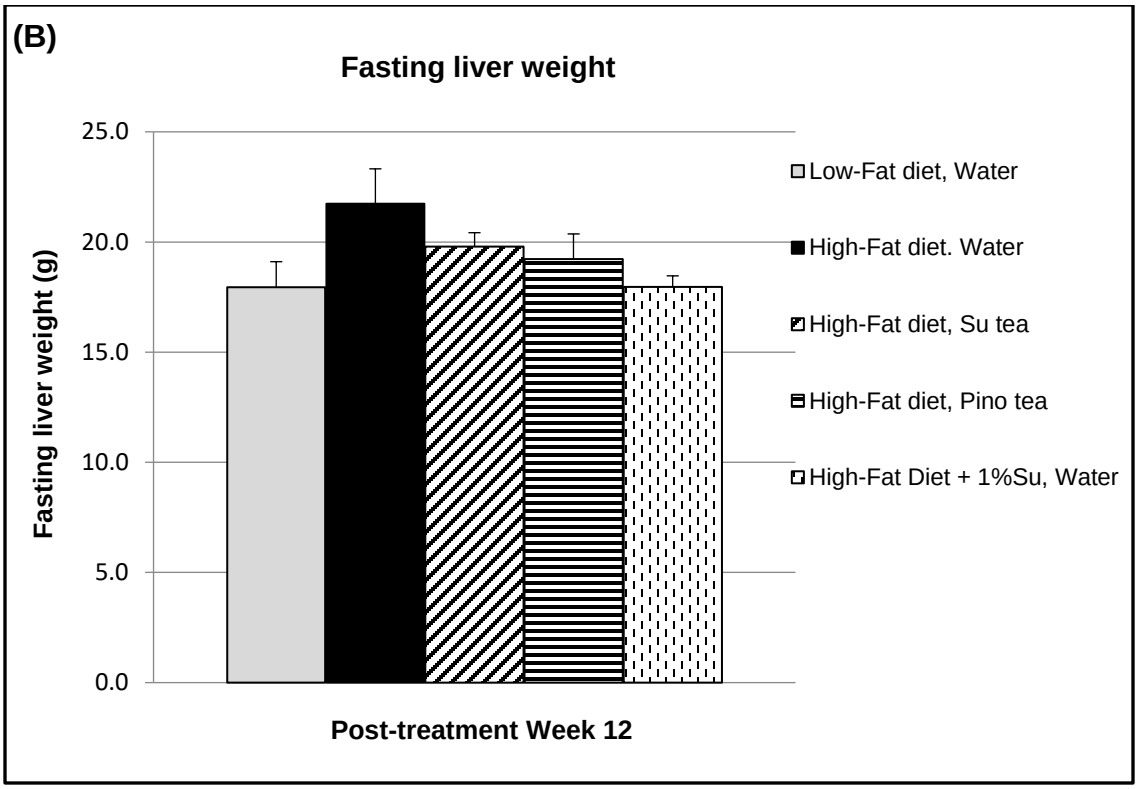
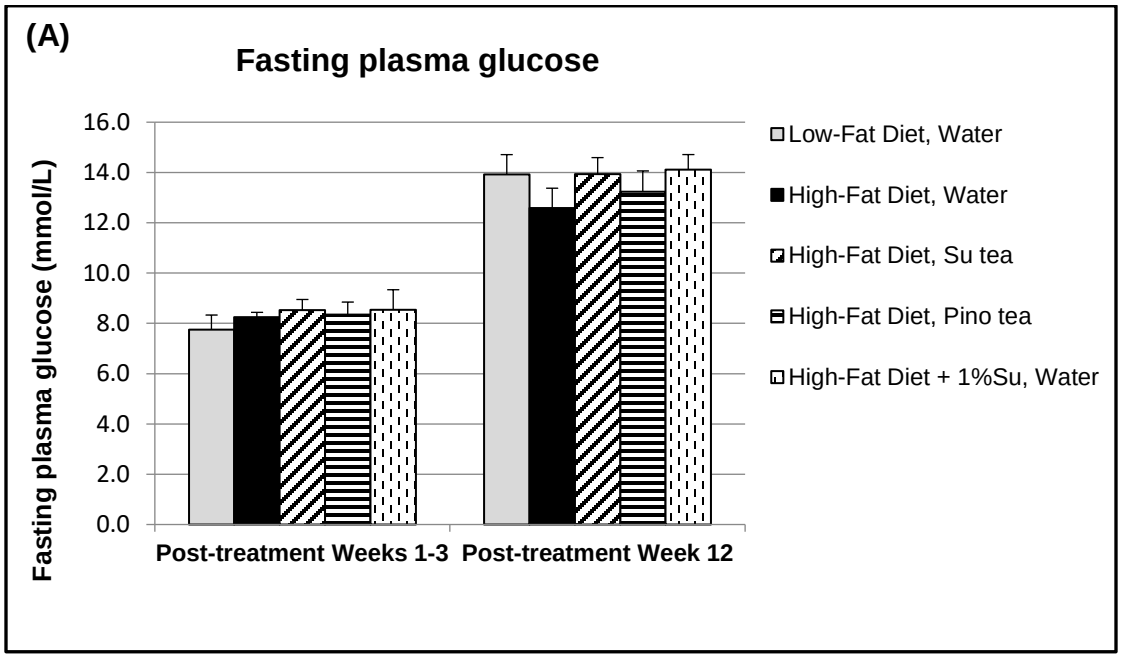


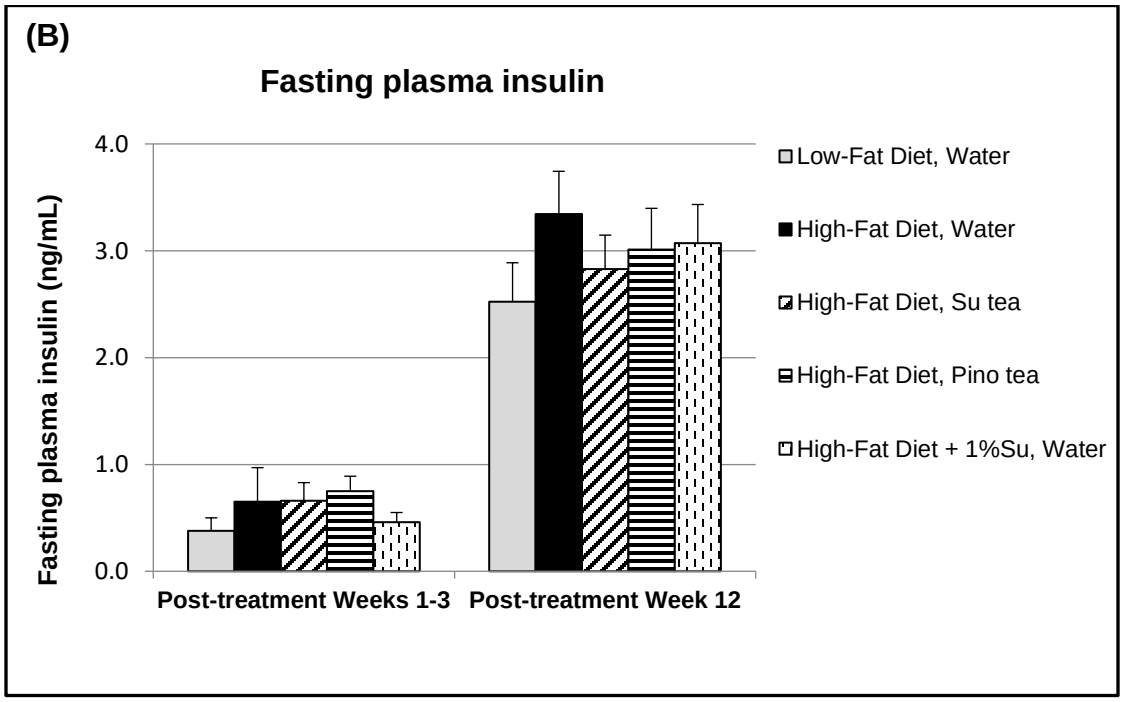
Figure 3-1. Effects of Sutherlandia on body weight (A) and fasting liver weight (B) of HFD-fed rats. Data are expressed as mean ± standard errors of the mean (SEM) of each group; n = 11-12 per group. Comparisons between groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test. $^{\#}p < 0.025$ versus the LFD group.

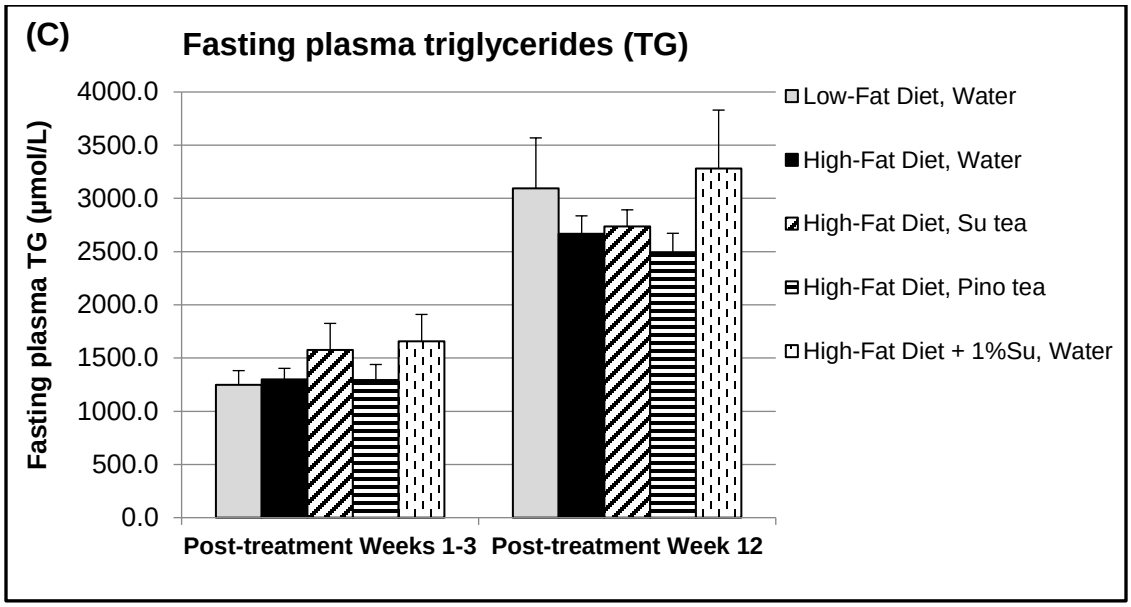
Effects of Sutherlandia consumption on plasma homeostasis

Levels of fasting plasma glucose, insulin, triglycerides (TG), and free fatty acids (FFA) were not of significant difference among five groups at initial and final post-treatment (Figure 3-2 A), indicating that these rats did not develop diabetes or insulin resistance.

A decrease in plasma insulin and FFA in HFD-fed rats medicated with all three forms of Sutherlandia administrations compared to the untreated HFD group was also observed ($p > 0.05$, Figure 3-2 B, D).







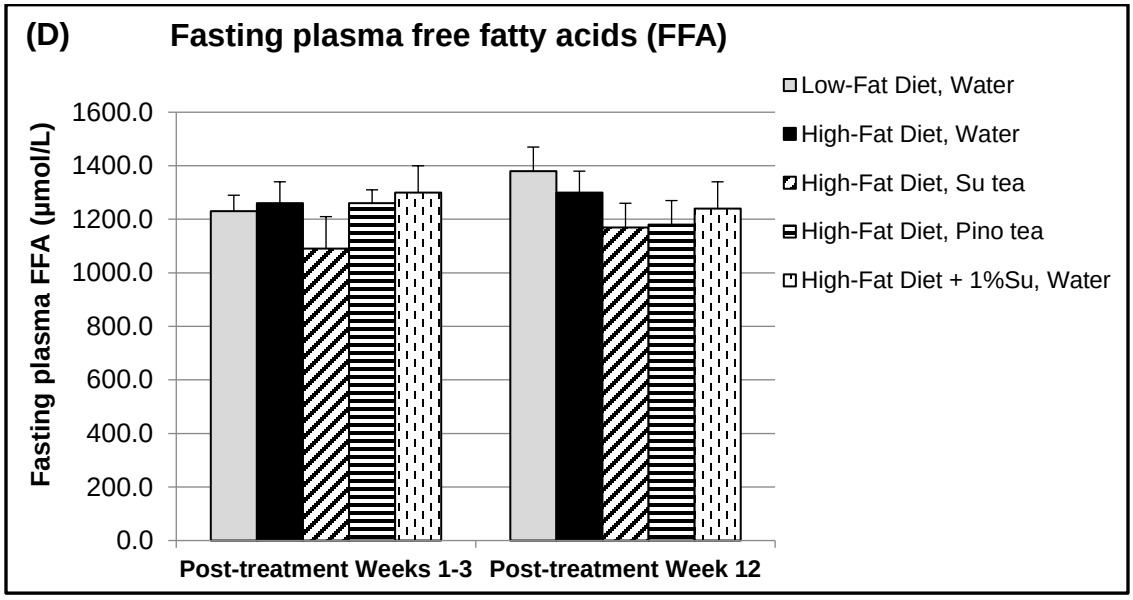
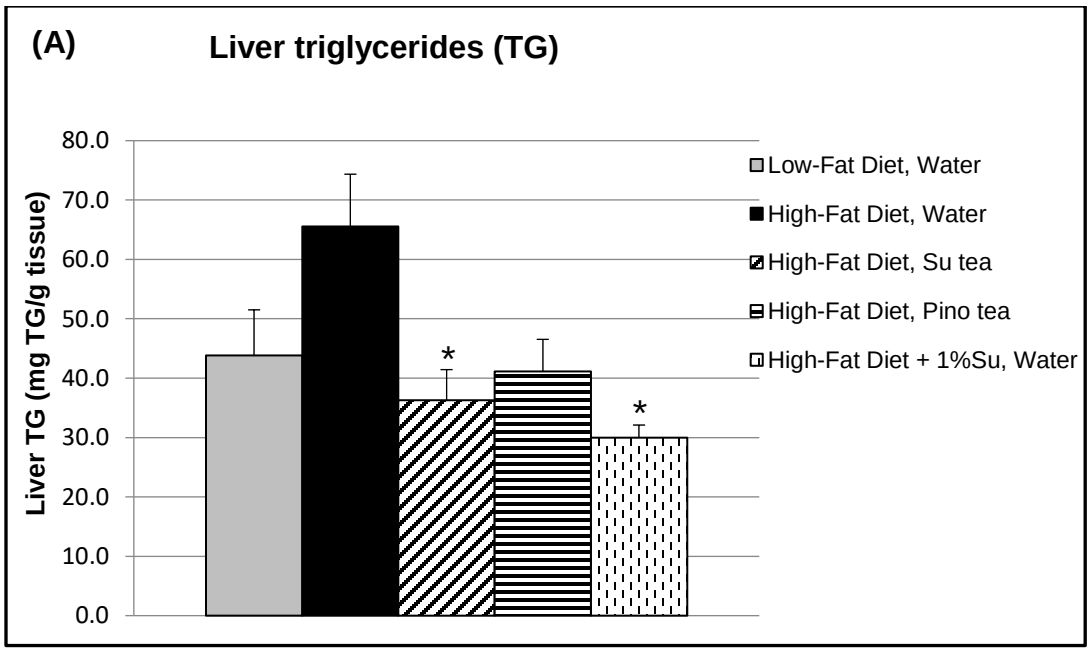


Figure 3-2. Effects of Sutherlandia on fasting plasma glucose (A), insulin (B), triglycerides (C), and free fatty acids (D) of HFD-fed rats. Data are expressed as mean ± standard errors of the mean (SEM) of each group; n = 11-12 per group. Comparisons between groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test. $^{\#}p < 0.025$ versus the LFD group.

Sutherlandia treatments mitigate hepatic steatosis in HFD-fed rats

To determine whether Sutherlandia regulates hepatic steatosis, we investigated hepatic TG content. HFD rats developed hepatic steatosis by week 12 and both Sutherlandia tea and Sutherlandia powder in the feed significantly reduced total liver TG levels (Figure 3-3 A) as well as TG fraction of liver weight (Figure 3-3 B) compared to the untreated HFD-fed rats ($p < 0.05$), showing that Sutherlandia inhibits hepatic steatosis in obese animals. In addition, Pinosunsia tea also diminished total liver TG levels (Figure 3-3 A) as well as TG fraction of liver weight (Figure 3-3 B), albeit this decrease was not statistically significant ($p > 0.05$).



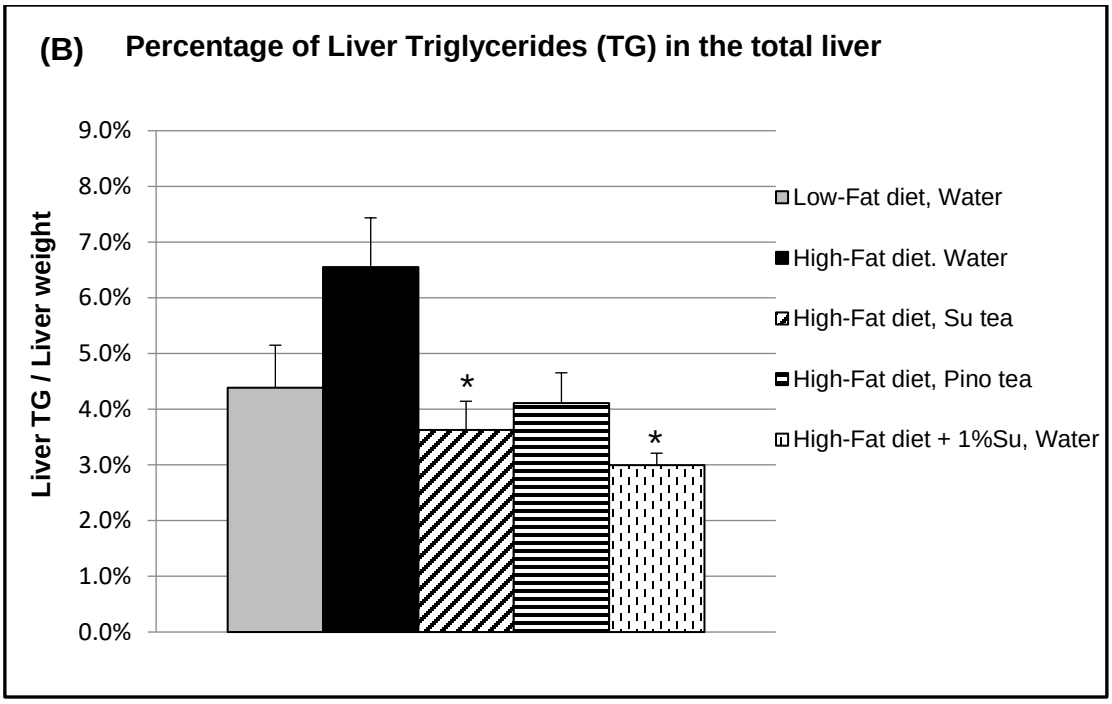


Figure 3-3. Inhibition of hepatic steatosis by Sutherlandia treatments in HFD-fed rats. (A) Liver TG concentrations, and **(B)** Percentage of Liver Triglycerides (TG) in the total liver weight. Data are expressed as mean ± standard errors of the mean (SEM) of each group; n = 11-12 per group. Comparisons between groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test. *p < 0.05 versus the HFD group.

Sutherlandia treatments reduce intrahepatocellular lipid accumulation in HFD-fed rats

Frozen livers were sectioned and stained by Oil Red O (ORO) and counterstained with Mayer's hematoxylin to visualize and assess changes in lipid droplets that comprise neutral lipids (primarily triglycerides and cholesterol esters). Larger, but fewer lipid droplets were observed in the HFD control group compared to the LFD control and the Sutherlandia treatment groups (Figure 3-4).

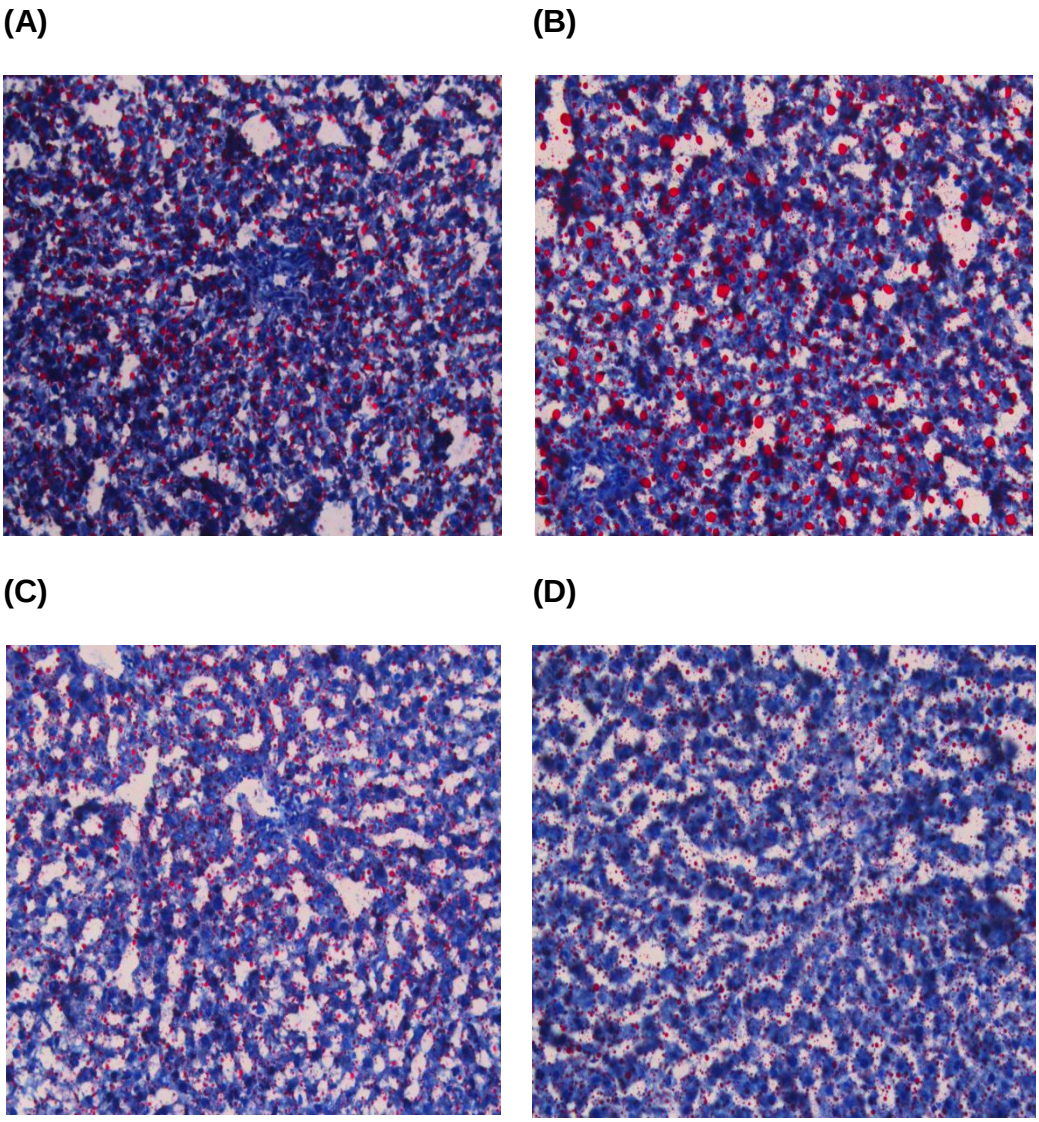
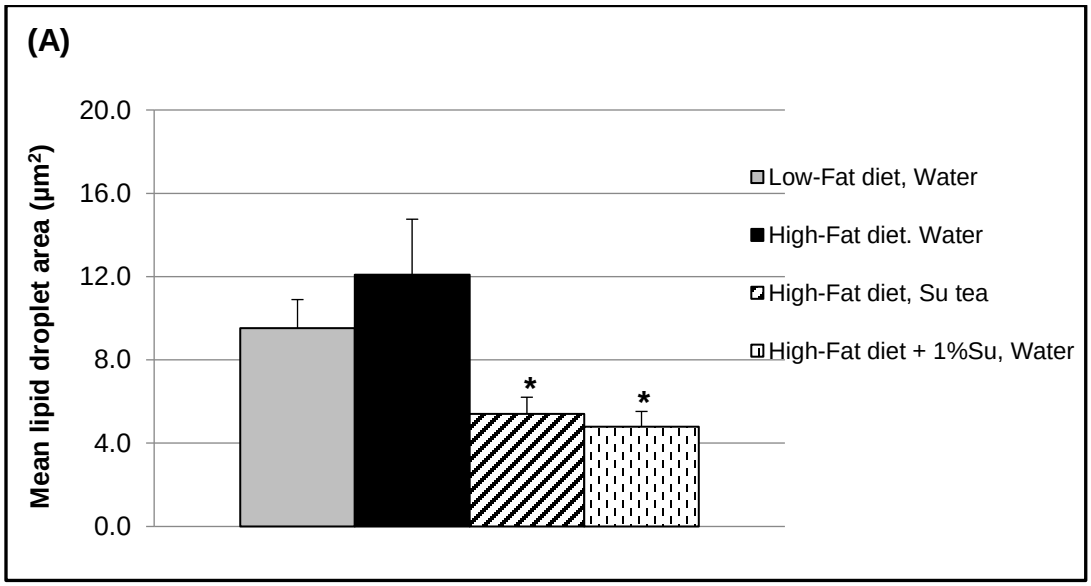


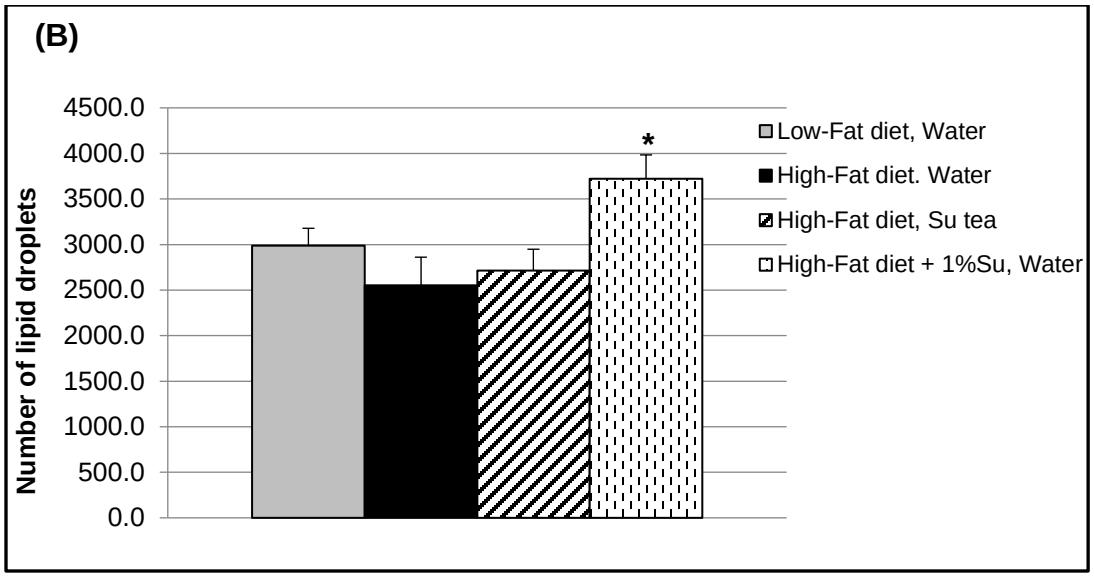
Figure 3-4. Effects of Sutherlandia treatments on morphology of lipid droplets. Representative sections of livers from rats fed **(A)** LFD, **(B)** HFD, **(C)** HFD + Su tea, or **(D)** HFD + 1%Su were stained by ORO and counterstained with Mayer's hematoxylin (original magnification, 20X).

Livers of rats in both the Sutherlandia treatment groups had lipid droplets with significantly smaller area compared to those in the HFD control group ($p < 0.05$, Figure 3-5 A). The smaller lipid droplet size in these two treatment groups was associated with a slightly higher number of lipid droplets in the HFD + Su tea

group and with a remarkably higher number of lipid droplets in the HFD + 1%Su diet (Figure 3-5 B) compared to the HFD control group.

Lipid droplet volume was also evaluated to examine the degree of TG storage in cytoplasmic lipid droplets (165). Total volume was measured by multiplying the number of lipid droplets by the mean volume of lipid droplets (166). The smaller lipid droplet size in both Sutherlandia treatment groups resulted in remarkably less total droplet volume ($p < 0.05$, Figure 3-5 C) even though these two treatment groups accumulated more, smaller lipid droplets compared to the HFD control group. This is consistent with the measurements of hepatic TG (Figure 3-3) and may reflect compositional differences in neutral lipids deserving further study.





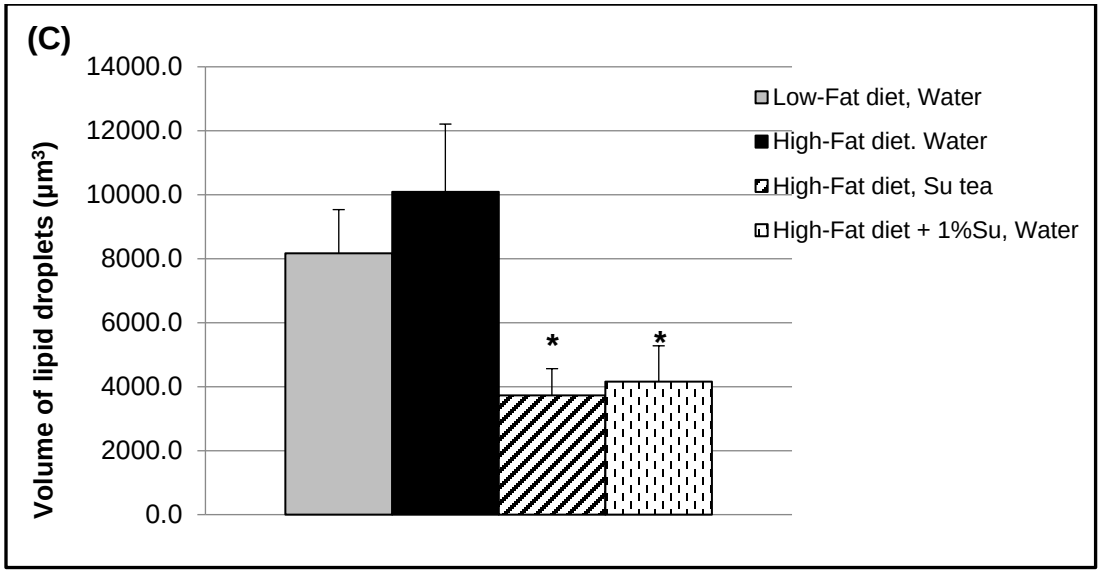


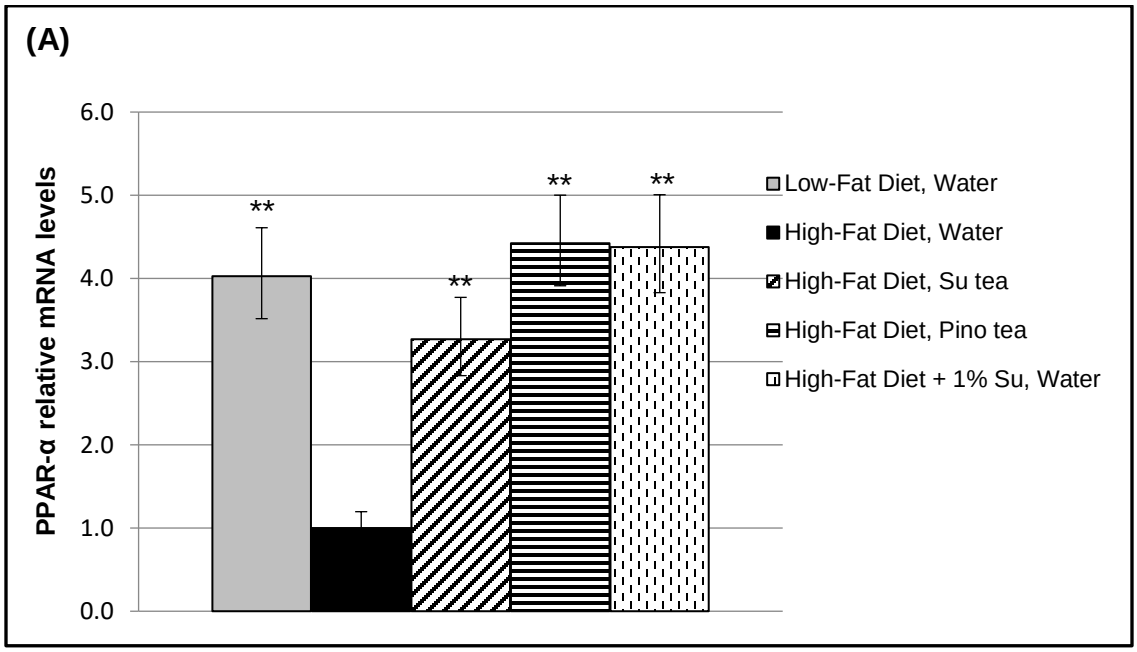
Figure 3-5. Effects of Sutherlandia treatments on size and number of lipid droplets in livers of HFD-fed rats. (A) Mean lipid droplet area. **(B)** Number of lipid droplets. **(C)** Volume of lipid droplets. Data are expressed as mean ± standard errors of the mean (SEM) of each group; n = 8-9 per group. Comparisons between groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test. *p < 0.05 versus the HFD group.

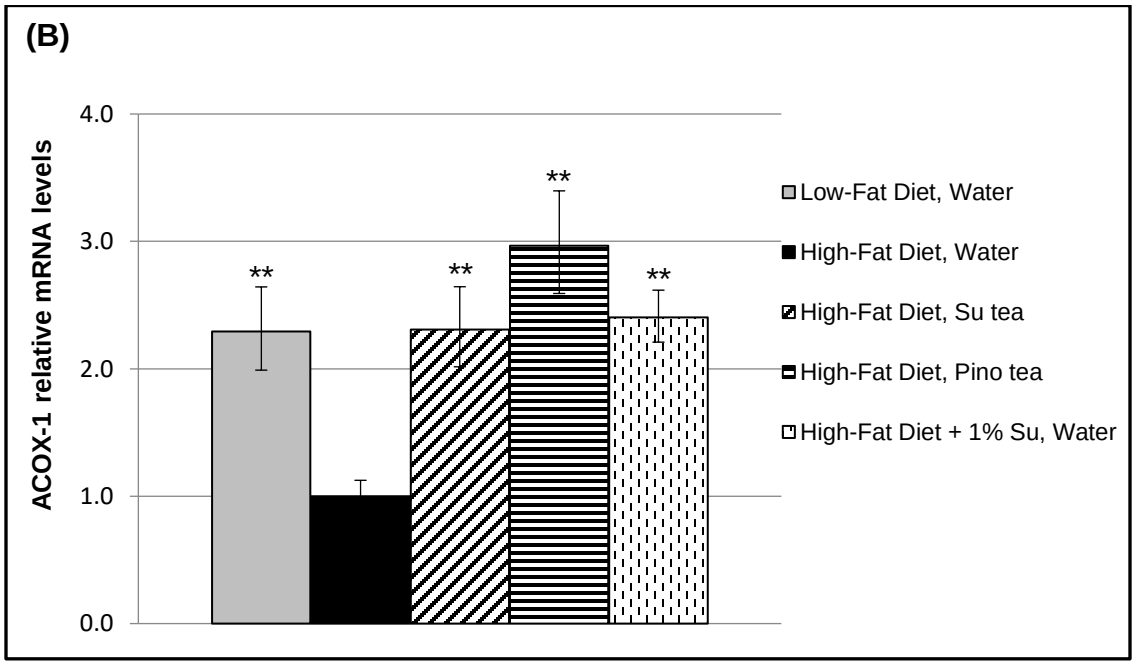
Sutherlandia treatments alter expression of lipid metabolism genes

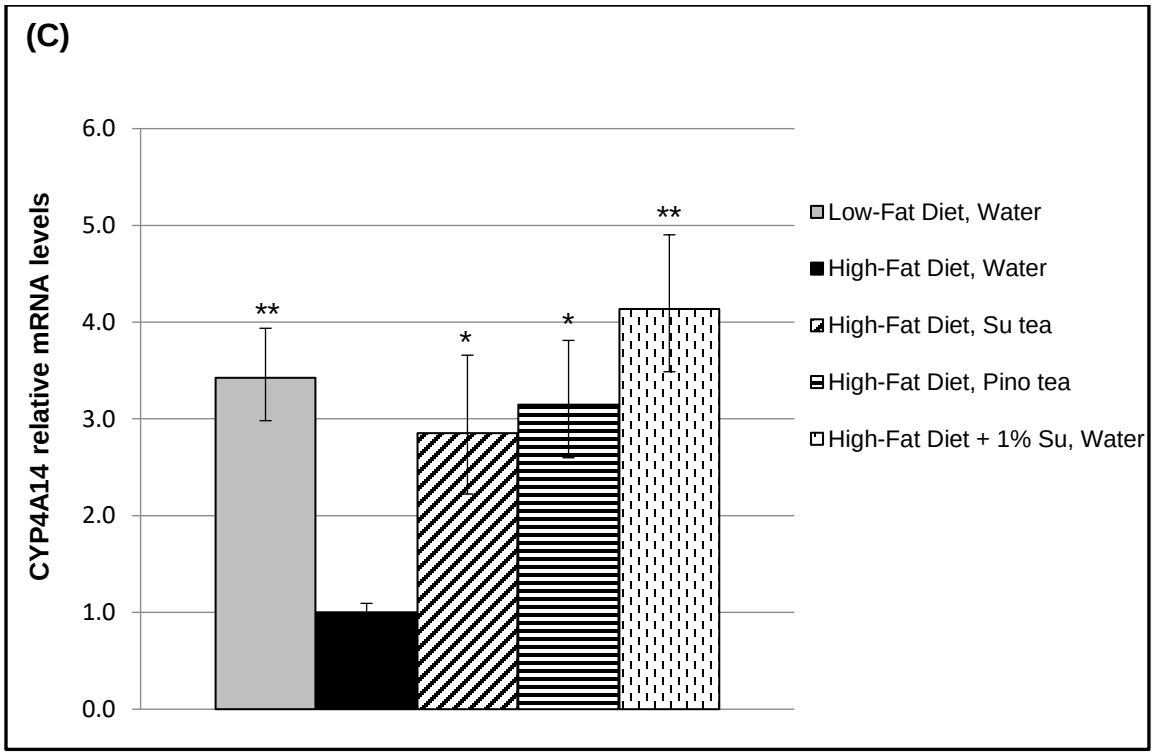
To evaluate whether the effects of Sutherlandia on hepatic steatosis are due to modification of fatty acid oxidation, we measured mRNA levels of the PPAR-α transcription factor as well as its target genes ACOX-1 and CYP4A14 by rt-q PCR. The mRNA expression of PPAR-α, ACOX-1 and CYP4A14 was significantly lower in the HFD control group compared to the LFD control group ($p < 0.01$, Figure 3-6 A-C). All Sutherlandia treatments reversed this negative effect of HFD by significantly increasing hepatic mRNA expression of PPAR-α ($p < 0.01$), ACOX-1 ($p < 0.01$), and CYP4A14 ($p < 0.05$) (Figure 3-6 A-C).

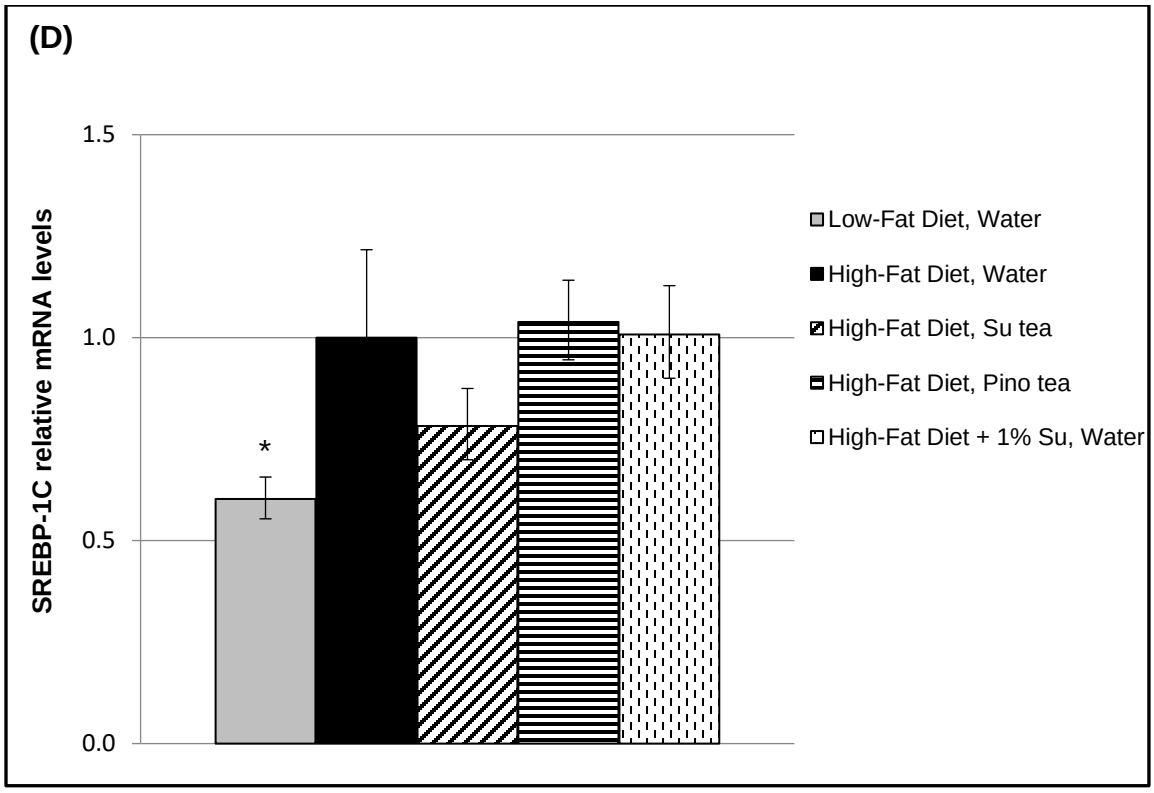
De novo lipogenesis also plays a role in development of hepatic steatosis. Therefore, we quantified mRNA expression of transcription factors sterol regulatory element-binding protein-1C (SREBP-1C) and carbohydrate-responsive element-binding protein (ChREBP) as well as its target gene fatty acid synthase (FAS). Feeding the HFD for 12 consecutive weeks remarkably increased mRNA expression of SREBP-1C ($p < 0.05$, Figure 3-6 D) and ChREBP ($p < 0.01$, Figure 3-6 E), but not FAS ($p > 0.05$, Figure 3-6 F). Treatment with either Sutherlandia or Pinosundia tea caused slightly reduced expression of SREBP-1C, ChREBP, and FAS genes; however, there was no statistically significant difference at $p < 0.05$ (Figure 3-6 D-F). Similarly, dried Sutherlandia in the feed caused slightly decreased expression of ChREBP and FAS, but not SREBP-1C, but this was not statistically significant ($p > 0.05$, Figure 3-6 D-F).

These results suggest that Sutherlandia consumption mitigates hepatic steatosis by upregulating fatty acid oxidation-associated genes without significantly changing *de novo* lipid synthesis.






(C)
CYP4A14 relative mRNA levels
6.0
5.0
4.0
3.0
2.0
1.0
0.0
**
*
*
**
Low-Fat Diet, Water
High-Fat Diet, Water
High-Fat Diet, Su tea
High-Fat Diet, Pino tea
High-Fat Diet + 1% Su, Water


(D)
SREBP-1C relative mRNA levels
1.5
1.0
0.5
0.0
*
Low-Fat Diet, Water
High-Fat Diet, Water
High-Fat Diet, Su tea
High-Fat Diet, Pino tea
High-Fat Diet + 1% Su, Water

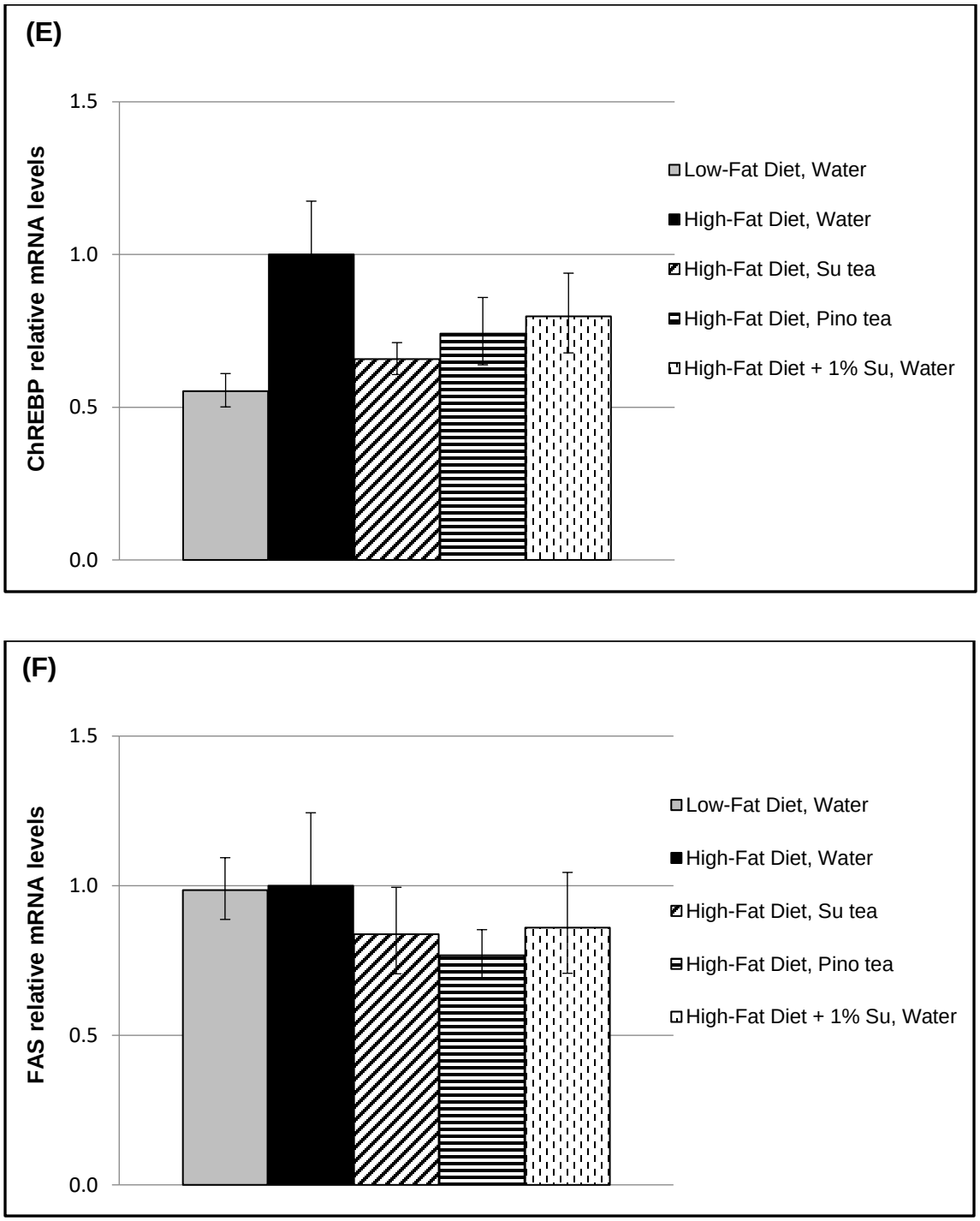
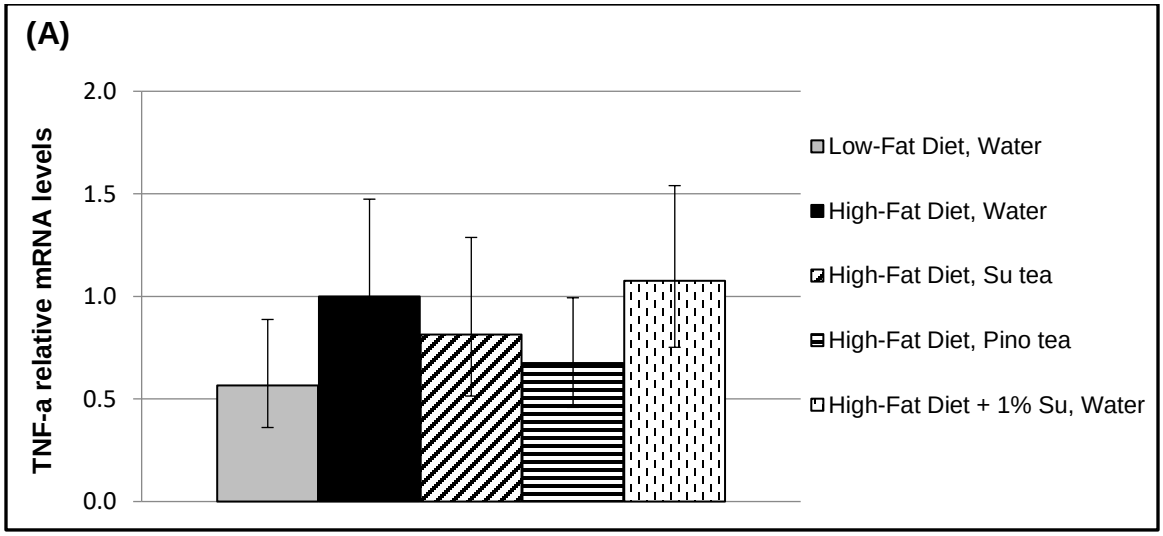


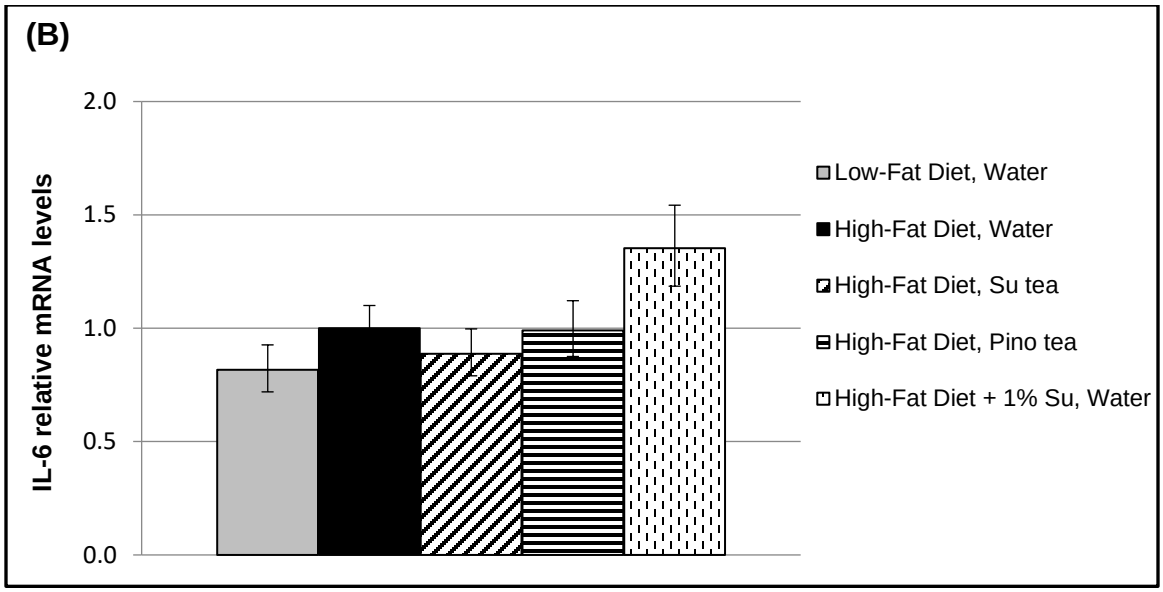
Figure 3-6. Effects of Sutherlandia on mRNA expression of genes involved in hepatic lipid metabolism. mRNA levels of **(A)** PPAR-a, **(B)** ACOX-1, **(C)** CYP4A14, **(D)** SREBP-1C, **(E)** ChREBP, and **(F)** FAS were measured by rt-q PCRs. Data are expressed as mean ± standard errors of the mean (SEM) of

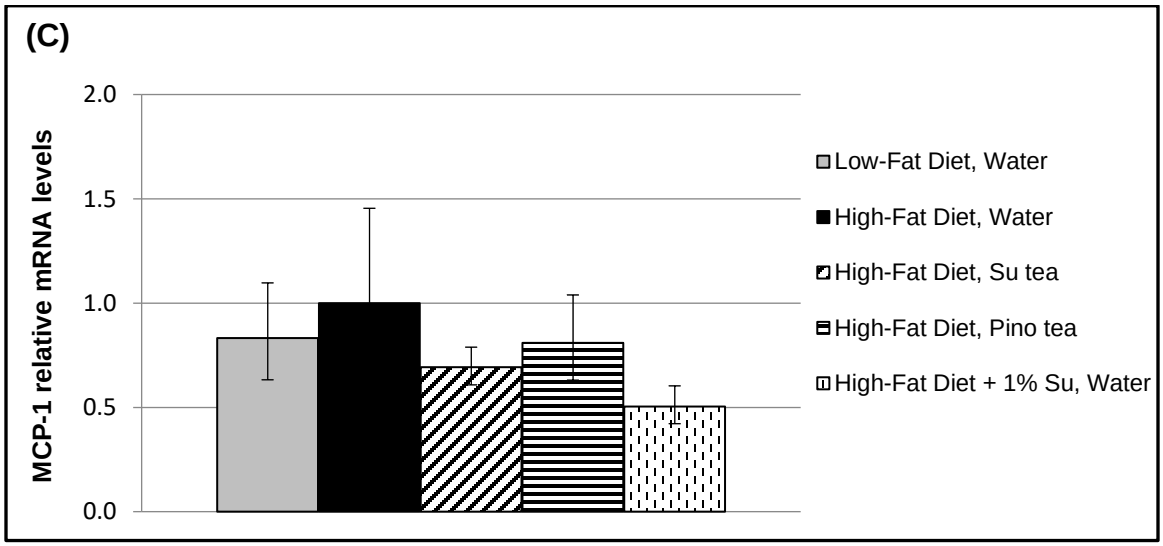
each group; n = 11-12 per group. Comparisons between groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test. *$p < 0.05$ versus the HFD group, **$p < 0.01$ versus the HFD group.

Effects of Sutherlandia treatments on hepatic inflammation

Hepatic steatosis may be accompanied with hepatic inflammation (167, 168), so the impact of Sutherlandia treatments on inflammation was examined. The mRNA levels of pro-inflammatory cytokines TNF-α, IL-6 and MCP-1, and of anti-inflammatory cytokine IL-4 were not considerably different among five groups ($p > 0.05$); moreover, these changes were highly variable (Figure 3-7).




(B)
IL-6 relative mRNA levels
2.0
1.5
1.0
0.5
0.0
Low-Fat Diet, Water
High-Fat Diet, Water
High-Fat Diet, Su tea
High-Fat Diet, Pino tea
High-Fat Diet + 1% Su, Water


(C)
MCP-1 relative mRNA levels
2.0
1.5
1.0
0.5
0.0
Low-Fat Diet, Water
High-Fat Diet, Water
High-Fat Diet, Su tea
High-Fat Diet, Pino tea
High-Fat Diet + 1% Su, Water

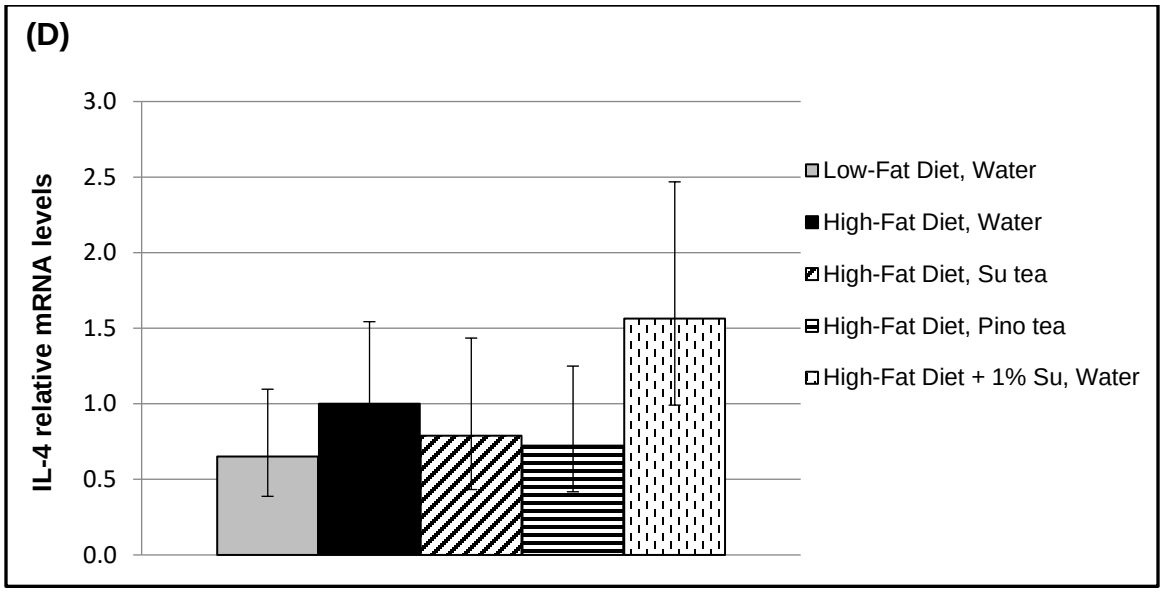


Figure 3-7. Effects of Sutherlandia on mRNA expression of inflammatory genes. mRNA levels of **(A)** TNF-α, **(B)** IL-6, **(C)** MCP-1, and **(D)** IL-4 were measured by rt-q PCRs. Data are expressed as mean ± standard errors of the mean (SEM) of each group; n = 11-12 per group. Comparisons between groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test. *$p < 0.05$ versus the HFD group, **$p < 0.01$ versus the HFD group.

Discussion and Conclusion

NAFLD, pathologically ranging from hepatic steatosis to steatohepatitis, fibrosis, and cirrhosis and hepatocellular carcinoma in some cases, is the most common liver disease in developed nations (5-7), and has been increasing rapidly in the developing world including South Africa (4). Hepatic steatosis, also known as fatty liver, is closely associated with obesity, T2DM, and hyperlipidemia (169, 170). Phytotherapies are increasingly being employed for metabolic

diseases (16, 17, 87-90), and for which Sutherlandia has been extensively used in complementary and alternative medicines for diabetes in South Africa (18).

Studies in rodents have shown that aqueous extracts of Sutherlandia reduced plasma free fatty acids and inhibited the development of insulin resistance (21-23). Recently, Williams et al. (2013) reported that Sutherlandia extracts altered diabetes-related gene expression in a fructose-induced insulin resistant human Chang liver cell model (35). Numerous bioactive compounds in Sutherlandia have been identifed that might be responsible for such effects, including L-canavanine, D-pinitol, gamma-aminobutyric acid (GABA), triterpenoid glycosides, and modified flavonoids (18); but mechanisms of action remain to be elucidated. D-pinitol has been reported to reduce blood sugar levels (29), and L-canavanine has been shown to interfere with arginine uptake essential for Nitrogen Oxide (NO) production (19, 25) that can destroy beta cells (97), thus preventing the inhibition of insulin production (98). Triterpenoid glycosides exerted hypoglycemic effects and enhanced fatty acid oxidation (31). Quercetin, a well-recognized flavonoid, elicited beneficial effects in alleviation of hepatic steatosis in C57/BL6J mice fed a high-fat, high-cholesterol and high-sucrose Western-style diet for 20 weeks by reducing hepatic triglycerides, decreasing oxidative stress and improving hepatic lipid metabolism by significantly increasing transcriptional expression of PPAR-α as well as suppressing expression of PPAR-γ, CD36, SREBP-1c, and FAS (33). Moreover, kaempferol (3,5,7,4'-tetrahydroxyflavone), another well-known flavonoid, lowered hepatic triglycerides and lipid droplet accumulation by increasing lipid metabolism

through upregulation of PPAR-α, acyl-CoA oxidase (ACO), cytochrome P450 isoform 4A1 (CYP4A1), and downregulation of SREBPs in the livers of rats fed a high-fat diet (34).

This study demonstrates anti-steatotic effects of Sutherlandia extracts in HFD-fed rats, including reduced hepatic triglycerides, decreased lipid droplet accumulation, and elevated expression of fatty acid oxidation pathway members. That is, Sutherlandia aqueous extracts delivered in tea and Sutherlandia powder delivered in feed both significantly attenuated liver triglyceride accumulation in HFD-fed rats compared to those fed HFD only.

Hepatocytes are responsible for mobilizing lipids for energy and storing excess lipids in the form of lipid droplets, mainly triglycerides and cheloestrol esters, thereby rendering the liver a crucial organ accounting for lipid homeostasis (171, 172). In this study, Sutherlandia tea and Sutherlandia powder in feed led to increased numbers of hepatic lipid droplets, but the smaller sizes of these droplets reflects the lesser lipid content of the liver. The greater total number of lipid droplets may have been due to augmented lipid efflux; indeed, droplets can undergo fission to expand surface area as well as the amount of lipids accessible to enzymes involved in lipid efflux and lipolysis (171). The smaller lipid droplet phenotype also may be indicative of greater mitochondrial fat oxidation as indicated by gene expression profiling.

Remarkably, the striking improvements observed in the livers of rats treated with Sutherlandia were not paralleled by robust changes in body weight, liver weight, plasma glucose, plasma insulin, plasma triglyceride, and plasma free fatty acids levels, suggesting Sutherlandia presents metabolic effects in a

tissue-specific manner and the effects are not contingent upon weight loss. More specifically, plasma glucose displayed no significant changes in these five groups, which was documented by Chadwick *et al.* (21) and Mackenzie *et al.* (22, 23). In this case, the pancreas is supposed to release higher amounts of insulin to transport the same glucose levels into target tissues so as to maintain normoglycemia (173). Analysis of fasting insulin levels showed slightly higher values in the untreated HFD group compared to other groups, implying that these animals were not insulin resistant. This outcome contrasts the observations from Chadwick *et al.* (21) and Mackenzie *et al.* (22, 23). One reason accounting for the fact that the animals in this study were neither diabetic nor insulin resistant would be because these Wistar rats are outbred, meaning that they possess considerable genetic variation reminiscent of the genetic heterogeneity of the human condition (174).

Peroxisome proliferator-activated receptor α (PPAR-α) is a ligand-activated transcription factor that modulates the expression of several genes responsible for fatty acid oxidation in the liver such as peroxisomal acyl-coenzyme A oxidase-1 (ACOX-1) and microsomal cytochrome P450, family 4, subfamily A, polypeptide 14 (CYP4A14) (175-178). The CYP4 family of cytochrome P450s catalyzes omega hydroxylation of saturated, branched chain, and unsaturated fatty acid; in particular, the cytochrome P450 4A (CYP4A) subfamily is one of the 18 subfamilies that consists of 20 individual forms in 9 different mammalian species (179, 180). The expression of CYP4A14 is selectively induced by PPAR-α (180-182). Our study showed that all three forms

of Sutherlandia treatments significantly increased hepatic mRNA expression of PPAR-α and its target genes including ACOX-1 and CYP4A14 in the liver of the rats fed HFD with Sutherlandia versus those fed HFD alone. Moreover, SREBP-1C and ChREBP control the expression of genes involved in the fatty acid biosynthetic pathway or *de novo* lipogenesis (183). However, in this study, none of the Sutherlandia treatments altered hepatic mRNA expression of SREBP-1C and ChREBP or their target gene, FAS. Taken together, this implies that the anti-steatotic effect of Sutherlandia is likely due to an elevation in hepatic fatty acid oxidation.

Furthermore, fatty liver may be accompanied with hepatic inflammation (167, 168), but this phenomenon was not observed in this study due to the very subtle signs of hepatic steatosis in this rodent model under this diet regimen. In fact, the mRNA levels of pro-inflammatory cytokines TNF-α, IL-6 and MCP-1, and of anti-inflammatory cytokine IL-4 were not substantially different among five groups.

In addition, alpha-lipoic acid was shown to elicit beneficial effects on glucose and lipid metabolism (184-186), as well as attenuate hepatic steatosis in HFD-fed mice (187). Nonetheless, it was not detected in Pinosundia tea by current HPLC analysis (Appendix Figure 3-1 C), which is contrasting to what was shown on the label of the ProBetix® bottle (Value Added Life Health Products (Pty) Ltd, South Africa). Instead, HPLC analysis displayed that Pinosundia tea has a mixture of sutherlandiosides B, C, and D (Appendix Figure 3-1 C). In light of the fact that the standard of alpha-lipoic acid was not included for compare

and contrast in this analysis, further analytical methods are required to fully characterize the metabolite profile of Pinosundia tea.

The findings of this study should be considered in light of its limitations. As mentioned above, hepatic mitochondrial oxidative capacity was not directly measured; rather, histological and gene expression analyses together served as indirect measures of hepatic lipid metabolism. Future *in vivo* and *in vitro* studies should directly assess the effects of Sutherlandia treatment on hepatic lipid oxidation. One way to measure to directly fatty acid oxidation is to assess mitochondrial oxidation levels from the liver cells. In addition, Sutherlandia was administered *ad libitum* and thus specific administered dose could not be determined. However, the fact that all delivery systems (i.e., in tea and in feed) resulted in significant improvements compared to non-treated rats is noteworthy. Moreover, it is unlikely that the effects were due to taste aversion, which would have resulted in lower energy consumption, since there were no significant differences in body weight between the untreated and treated HFD-fed rats. The fact that the treated groups were not statistically different from the LFD control rats suggests a very modest body weight reduction which was consistent across treatment vehicle groups. Nonetheless, future studies should employ methods to accurately assess dose as well as food intake and systemic energy expenditure.

CHAPTER FOUR

QUANTIFICATION OF SUTHERLANDIOSIDES BY INDIRECT COMPETITIVE ELISA

Abstract

Sutherlandia frutescens (L.) R. Br. (Sutherlandia) is a medicinal plant of southern Africa that is frequently employed as a dietary supplement and a traditional remedy for multiple chronic diseases such as diabetes, cancer and infections. Scientific evidence for the these claimed benefits of Sutherlandia, however, is limited. Sutherlandia contains significant quantities of novel and unique cycloartenol glycosides (sutherlandiosides). Furthermore, studies in animal models supported by the University of Missouri (MU) Center for Botanical Interaction Studies and the MU Research Board require determination of the Sutherlandia bioactive compounds in tissues and fluids. Importantly, my research demonstrated that Sutherlandia extracts, which contain sutherlandiosies, ameliorate hepatic lipid accumulation in high-fat diet-induced obese rats. In order to determine the concentrations of the unique sutherlandiosides in plasma samples of these rats, an indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed in collaboration with the Department of Chemistry of Natural Compounds, Institute of Chemical Technology Prague, Czech Republic.

The results demonstrated successful development of a standard curve using purified sutherlandiosides from the Sutherlandia plant material. Quantification of sutherlandiosides in plasma, however, was not successful due to very low levels of sutherlandiosides present in plasma. In the future, a more specific assay is required to reliably and consistently measure concentrations of sutherlandiosides in biological samples for analysis of the mechanism(s) of action of S. *frutescens.*

Introduction

S. frutescens is a multipurpose medicinal plant that is widely used in southern African for a variety of chronic diseases such as T2DM, inflammation, cancer, stomach problems, influenza, and HIV/AIDS in southern Africa (18-23). Recently, sutherlandiosides A through D (cycloartanol glycosides) - unique compounds of *S. frutescens* - have been characterized (Figures 2.4) (26, 27). Sutherlandioside B is the major cycloartene-type tetracyclic triterpene glycoside in *S. frutescens* (2.75%, weight by total weight of dry plant sample) (27). None of these sutherlandiosides showed anti-microbial and anti-malarial activities (26). However, a number of studies showed that Sutherlandia extracts elicited anti-diabetic (21-23, 116), anti-inflammatory (116), anti-prostate cancer (106), and anti-psychological stress (134) effects. In my research, Sutherlandia extracts, which contain sutherlandiosies, were reported to ameliorate hepatic lipid accumulation in high-fat diet-induced obese rats (Chapter 3). Consequently,

there is a need to determine Sutherlandia bioactive compounds in tissues and fluids.

Liquid chromatography (LC)-ultra violet (UV)/evaporative light scattering detectors (ELSD) methods have been developed to measure flavonoids and cycloartanol glycosides from aerial parts of *Sutherlandia frutescens* (27). Nevertheless, the limit of detection (LoD) of 7.5 µg/mL and limit of quantitation (LoQ) of 25 µg/mL (due to the low UV absorption of the sutherlandiosides) render these methods inappropriate for detection and quantitation of the minute amounts present in biological samples such as plasma, sera and tissues. Furthermore, these analytical methods require extensive sample preparation, which is often not possible. To overcome the lack of sensitivity of the currently available analytical methods, we have developed a competitive ELISA immunoassay that can detect sutherlandiosides in a more sensitive and effective way compared to the LC/ELSD method. The validation of the ELISA assay for sutherlandiosides will enable correlations between bioavailability of sutherlandiosides in biological samples and observed changes in RNAs and proteins.

The objective of this study is to develop, optimize, and validate an indirect competitive ELISA assay for quantification of the sutherlandiosides in sera, plasma, urine, and tissues of clinical samples and of animal samples.

Materials and Methods

Purified sutherlandioside B (SuB) coupled to ovalbumin (OVA) was prepared and used to raise polyclonal rabbit antibodies against the

sutherlandioside moiety by Lukáš Huml (Department of Chemistry of Natural Compounds, Institute of Chemical Technology Prague, Czech Republic). The reaction between these antibodies and SuB-OVA coated onto wells of a polystyrene microplate was detected by goat anti-rabbit immunoglobulin G (IgG)-peroxidase and 3,3',5,5'-Tetramethylbenzidine (TMB). In specific, SuB-OVA (0.4 µg/mL) was pipetted into polystyrene microplate wells and incubated overnight for absorption. Then, aliquots of the matrix to be tested or partially purified SuB standard (a dilution gradient ranging from 0.01 – 1,000 ng/mL or 10^1 – 10^6 pg/mL) and the anti-SuB rabbit antiserum (833 ng/mL) were added and incubated. After washing, goat anti-rabbit IgG-peroxidase was added, incubated and washed, and TMB was then added to react with peroxidase. The enzyme reaction was stopped with sulfuric acid and the absorabance at 450nm was measured. (Figure 4-1).

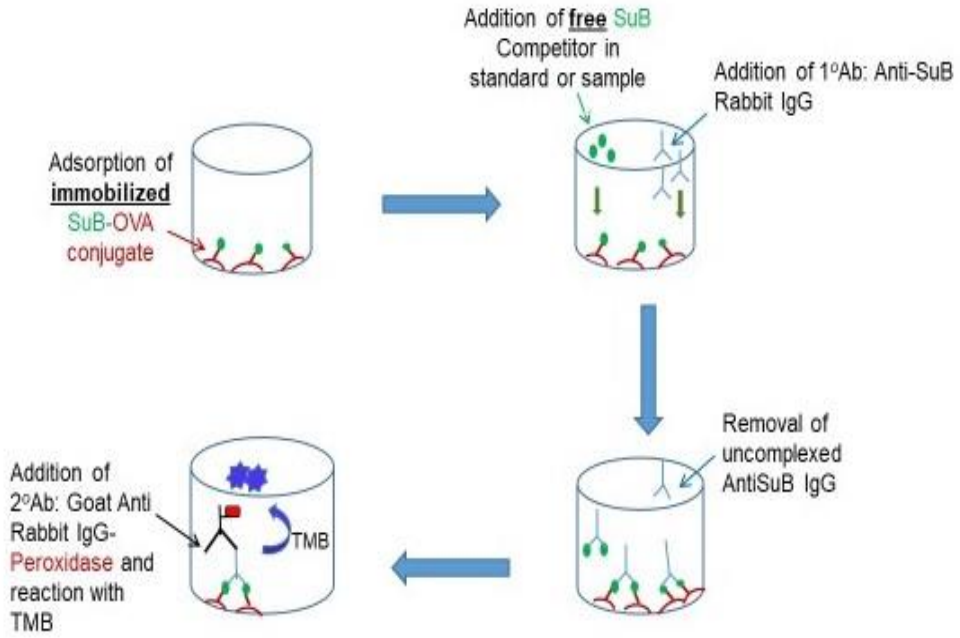


Figure 4-1. Illustration of indirect competitive sutherlandioside ELISA

Results

The standard curves were successfully developed using partially purified sutherlandiosides. The sutherlandiosides were partially purified from the Sutherlandia plant powder as described in Appendix 3-1.

The orange line in Figure 4-2 describes the standard curve using the partially purified sutherlandiosides only while the blue line illustrates the standard curve using the mixture of the partially purified sutherlandiosides and the control rat plasma.

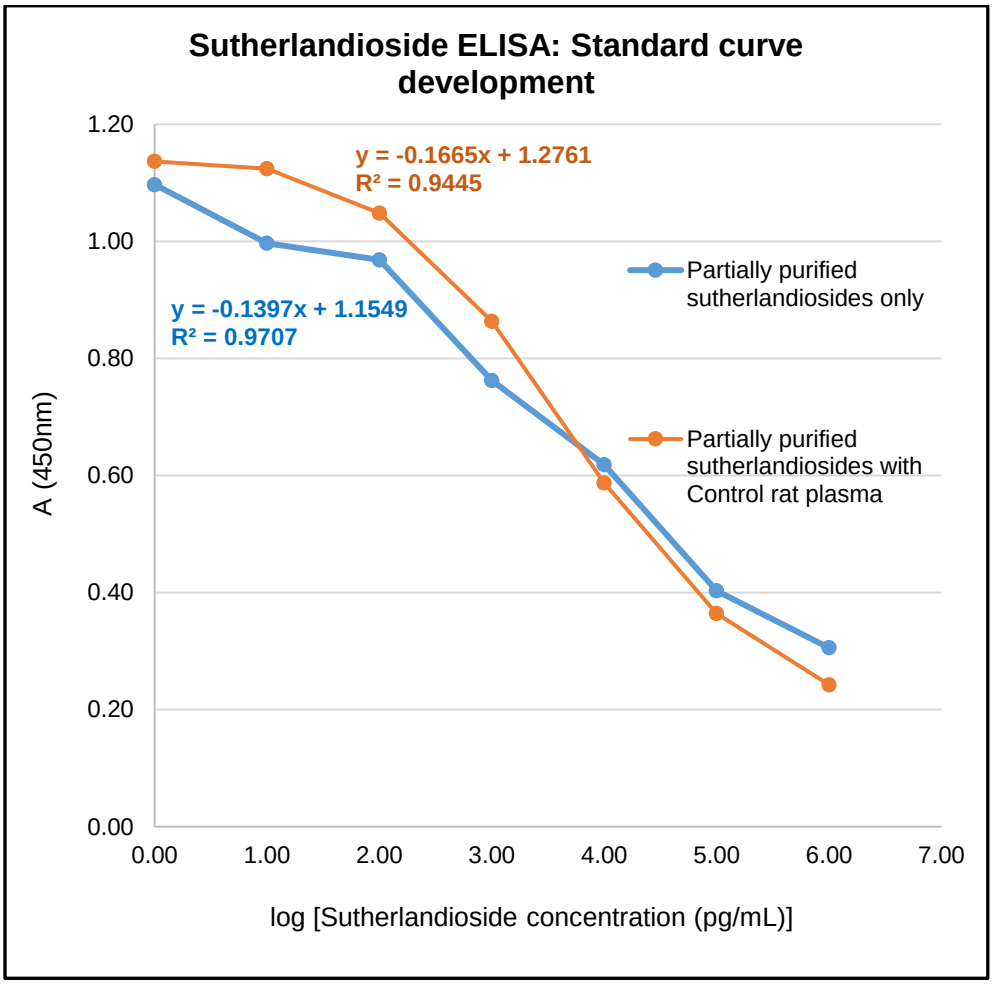


Figure 4-2. Standard curve development of sutherlandioside ELISA

Table 4-1 summarizes concentrations of sutherlandiosides in rat plasma determined by indirect competitive sutherlandioside ELISA at different days. These results displayed inconsistent concentrations of sutherlandiosides of the same sample analyzed at different days. For example, on day 1, the concentration of sutherlandiosides in sample 20 is 6.84 ng/mL, but this concentration dramatically dropped to 0.72 ng/mL on day 3. Another example is that there was a considerable decrease in the concentration of sutherlandiosides in sample 25N from 63.89 ng/mL (day 2) to 19.51 ng/mL (day 3).

Furthermore, it is possible to distinguish between negative and positive samples within the same day of assay analysis. For instance, on day 1, samples 5 and 8 (negative samples from rats having no Sutherlandia treatment) had a remarkably lower concentration of sutherlandiosides, i.e. 0.10 and 0.16 ng/mL, respectively, compared to the positive samples 14 (1.09 ng/mL), 14N (2.30 ng/mL), 20 (6.84 ng/mL), 27 (9.07 ng/mL) and 27N (17.84 ng/mL). Nevertheless, it is impossible to compare the concentrations of negative or positive samples at different times of analysis. To demonstrate, the negative sample 8 was assayed with the concentration of 0.16 ng/mL on day 1, but this number cannot be taken as a standard for negative results for other samples assayed at other times because the positive sample 15 was shown to have 0.18 ng/mL of sutherlandiosides on day 3. Therefore, this ELISA is not a quantitative, but rather a qualitative assay.

Table 4-1. Quantitation of sutherlandiosides in rat plasma samples by indirect competitive sutherlandioside ELISA.

Day	Sample ID	Group	Concentrations of sutherlandiosides (ng/mL)
1	5	LFD	0.10
	8	HFD	0.16
	14	HFD + Su tea	1.09
	14N	HFD + Su tea	2.30
	20	HFD + Pino tea	6.84
	27	HFD+1%Su	9.07
	27N	HFD+1%Su	17.84
2	1	LFD	0.03
	8N	HFD	0.004
	13	HFD + Su tea	0.46
	13N	HFD + Su tea	1.32
	19	HFD + Pino tea	1.43
	19N	HFD + Pino tea	2.40
	25	HFD+1%Su	13.27
	25N	HFD+1%Su	63.89
3	1	LFD	Undetected
	15	HFD + Su tea	0.18
	15N	HFD + Su tea	13.06
	22	HFD + Pino tea	8.85
	22N	HFD + Pino tea	4.94
	20	HFD + Pino tea	0.72
	25N	HFD+1%Su	19.51
	26N	HFD+1%Su	4.35

Discussion and Conclusion

The standard curve of the indirect competitive sutherlandioside ELISA was successfully established using the partially purified sutherlandiosides from Sutherlandia plant powder.

This ELISA assay can differentiate between negative (without Sutherlandia treatment) and positive (with Sutherlandia treatment) rat plasma samples that were analyzed at the same time. Nonetheless, it is impossible for this assay to compare the concentrations of negative or positive samples at different times of analysis or to determine the accurate amounts of sutherlandiosides in each sample due to inconsistent concentrations of sutherlandiosides of the same sample analyzed at different days. One reason accounting for this outcome is because the low bioavailability of sutherlandiosides in biological samples. In fact, these compounds are subjected to digestion, absorption, metabolism, and degradation by gut flora, hepatic enzymes and potential transport within circulation. Another reason is that the primary antibody targeting the sutherlandioside standard (free antigen) in this project was generated from purified sutherlandioside B; in contrast, this sutherlandioside standard was semi-purified because of limited availability of totally purified sutherlandioside B. In fact, it is extremely expensive and time-consuming to isolate and purify this specific compound from the plant powder. As a result, these challenges hinder us from accurately measuring concentrations of sutherlandiosides in plasma samples.

In the future, one way to troubleshoot this problem is to advance the process of producing a completely purified sutherlandioside so that a unique and specific antibody will target and bind to that sutherlandioside only. Moreover, with the advance of liquid chromatography-mass spectrometry (LC-MS) instrumentation and metabolomics, it becomes promising to effectively measure bioactive compounds in biological samples in a reliable manner.

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

Non-alcoholic fatty liver disease (NAFLD) covers a wide spectrum of liver pathology, ranging from simple steatosis to nonalcoholic steatohepatitis, fibrosis, cirrhosis and hepatocellular carcinoma (5-7). It is not only the most prominent chronic liver disease in developed nations in recent years, but also has been increasing in the developing world including South Africa (1-4). Moreover, it has been shown to be associated with metabolic diseases including obesity, insulin resistance, type 2 diabetes mellitus, hypertension, and dyslipidemia (5, 8-10). This dissertation focuses on hepatic steatosis - the first, benign and reversible stage of NAFLD (11).

Sutherlandia frutescens has been traditionally used in southern Africa for a long time (18). Several possible mechanisms of the anti-diabetic, anti-cancer, anti-inflammatory and anti-oxidant properties of *S. frutescens* were determined in both cell and animal models (18-23). Studies in rodents indicate that an aqueous preparation of *S. frutescens* extract at a daily dose of 50 mg/kg body weight led to a reduction in plasma free fatty acids and prevention of diet-induced insulin resistance in male Wistar rats (21-23). In addition, *S. frutescens* aqueous extracts were shown to modulate expression of 27 diabetes-related genes including PPAR-α - one of the key transcription factors responsible for hepatic lipid metabolism - in a fructose-induced insulin resistant human Chang liver cell model (35). The molecular mechanism(s) by which *S. frutescens* alters lipid

metabolism and insulin resistance, however, remain to be examined and established.

In chapter 3, I employed the aqueous extracts of freeze-dried and commercial *Sutherlandia frutescens*, i.e., Sutherlandia and Pinosundia teas, respectively, as well as the dried *S. frutescens* (1%) incorporated into feed to investigate the impact of *S. frutescens* consumption on obesity-induced hepatic steatosis in the livers of HFD-fed rats. The results demonstrated that *S. frutescens* extracts, when fed *ad libitum* either in food or water, significantly improved HFD-induced dysfunction in hepatic lipid metabolism. This improved liver phenotype included reduced hepatic triglyceride levels and lipid droplet accumulation, as well as increased mRNA expression of fatty acid oxidation-related genes PPAR-α, ACOX-1, and CYP4A14. These results illuminate an underlying molecular pathway by which Sutherlandia ameliorates lipid metabolism and provide new insights into the potential use of *S. frutescens* as a phytotherapy for NAFLD. Further examinations of how specific bioactive compounds of *S. frutescens* modulate lipid metabolism via the PPAR-α mechanisms are warranted. Moreover, future *in vivo* and *in vitro* studies should directly evaluate the impact of *S. frutescens* treatment on hepatic lipid oxidation. In addition, the specific dose of *S. frutescens* might be accurately determined by oral gavage or intraperitoneal injection rather than by administering *ad libitum* as described in this study.

The fact that supplementation of *S. frutescens* extracts led to significant improvements in hepatic steatosis compared to non-treated rats is remarkable.

Importantly, it is unlikely that these beneficial effects were due to taste aversion because there were no significant differences in body weight between the untreated and treated HFD-fed rats. An additional point worth mentioning is that physical exercise has been demonstrated as a potent strategy in the prevention of hepatic fat accumulation and metabolic disorders (188-192). Consequently, methods to precisely assess systemic energy expenditure should be employed in future studies.

A substantial effort was made to quantify and compare concentrations of sutherlandiosides - unique bioactive compounds in *S. frutescens* extracts - in plasma samples of untreated and treated rats using indirect competitive ELISA. In chapter 4, this assay was described. The standard curve was successfully developed using the partially purified sutherlandiosides from the *S. frutescens* plant powder. This ELISA is not a quantitative, but rather a qualitative assay, because it can only tell whether sutherlandiosides are present in a particular sample at the same time of analysis; nevertheless, it is unable to determine the exact amounts of sutherlandiosides in plasma samples. Very low amounts of sutherlandiosides are present in plasma because these compounds are subjected to digestion, absorption, metabolism, and degradation by gut flora, hepatic enzymes and potential transport within circulation. Therefore, this challenge hampers accurate measurement of the concentrations of sutherlandiosides in plasma samples. Moreover, there is a strong demand to characterize over-the-counter medications of *S. frutescens* in specific, and of medicinal plants in general. One way of generating a reliable profile of bioactive

compounds in biological samples as well as in plant and commercial extracts is to perform metabolomic analysis.

Furthermore, global expression of lipid metabolism genes using RNA sequencing should be conducted to investigate a comprehensive picture of transcriptome profiling regarding the presence and quantity of RNA in control and *S. frutescens*-treated samples. We should then, based on those results, determine the exact molecular mechanism(s) by which *S. frutescens* modulates lipid metabolism in diet-induced hepatic steatosis.

In general, the findings in this dissertation shed light on the molecular mechanism by which *S. frutescens* alleviates hepatic lipid accumulation HFD-fed rats, and provide novel visions about the use of *S. frutescens* as a phytotherapy. Future studies and experiments should take the above recommendations into account to offer a better understanding of this medicinal plant in terms of pharmacological values.

APPENDIX 3-1

Determination of sutherlandioside B from *S. frutescens plant or from S. frutescens* aqueous extracts

Materials and Methods

Preparation of aqueous extracts of S. frutescens

The aqueous extracts of freeze-dried and commercial *Sutherlandia frutescens*, i.e., Sutherlandia and Pinosundia teas, respectively, were prepared as described in Chapter 3 (pp. 34-35).

Isolation and quantification of sutherlandiosides

Sutherlandiosides were isolated and quantified from *S. frutescens* by Korey Brownstein using modifications of the isolation procedure published by Fu *et al.* (26) and high-speed counter-current chromatography (HSCCC) (93). *S. frutescens* plant material was extracted with methanol and concentrated *in vacuo*. The residue was re-dissolved in water and extracted with hexane, chloroform, followed by *n*-butanol (water saturated). The *n*-butanol fraction was then concentrated *in vacuo*. Ethyl acetate, *n*-butanol, methanol, and water (15:1:3:15 v/v) were included in the two-phase solvent system. The solvents were comprehensively equilibrated in a 500 mL separating funnel and the upper and lower phases were separated before use. The rotor was filled with the upper stationary phase and the lower stationary phase was pumped at 1 mL/min while the HSCCC apparatus (CC Biotech LLC, Rockville, MD) was run at 830 rpm. The

samples were injected and 4-minute fractions were collected. Seventy fractions were collected. Finally, the samples were freeze-dried and examined by thin layer chromatography (TLC) to identify the fractions containing sutherlandiosides.

Fractions were run on Analtech HL silica gel TLC plates by employing the mobile phase chloroform:toluene:methanol (3:2:1) and then visualized by *p*-anisaldehyde spray. Subsequently, the sutherlandiosides from these HSCCC fractions were purified by semipreparative reversed phase high-performance liquid chromatography (HPLC) to quantify concentrations of sutherlandioside B and a mixer of sutherlandiosides A, C and D.

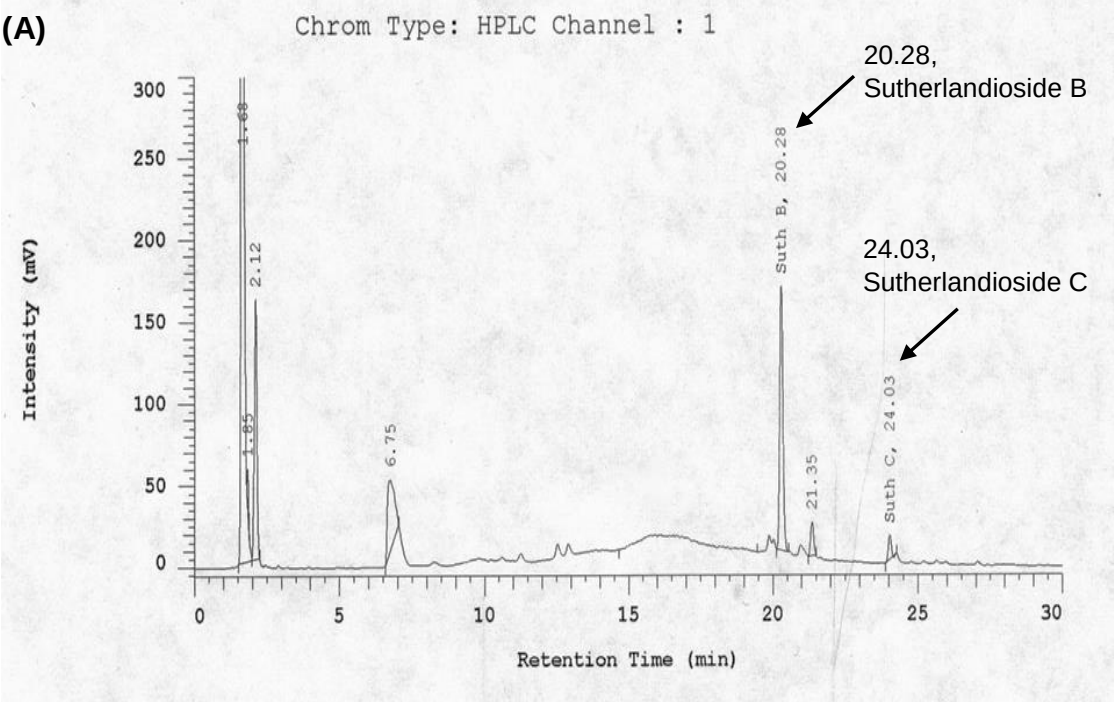
Analysis of sutherlandiosides using LC-ESLD

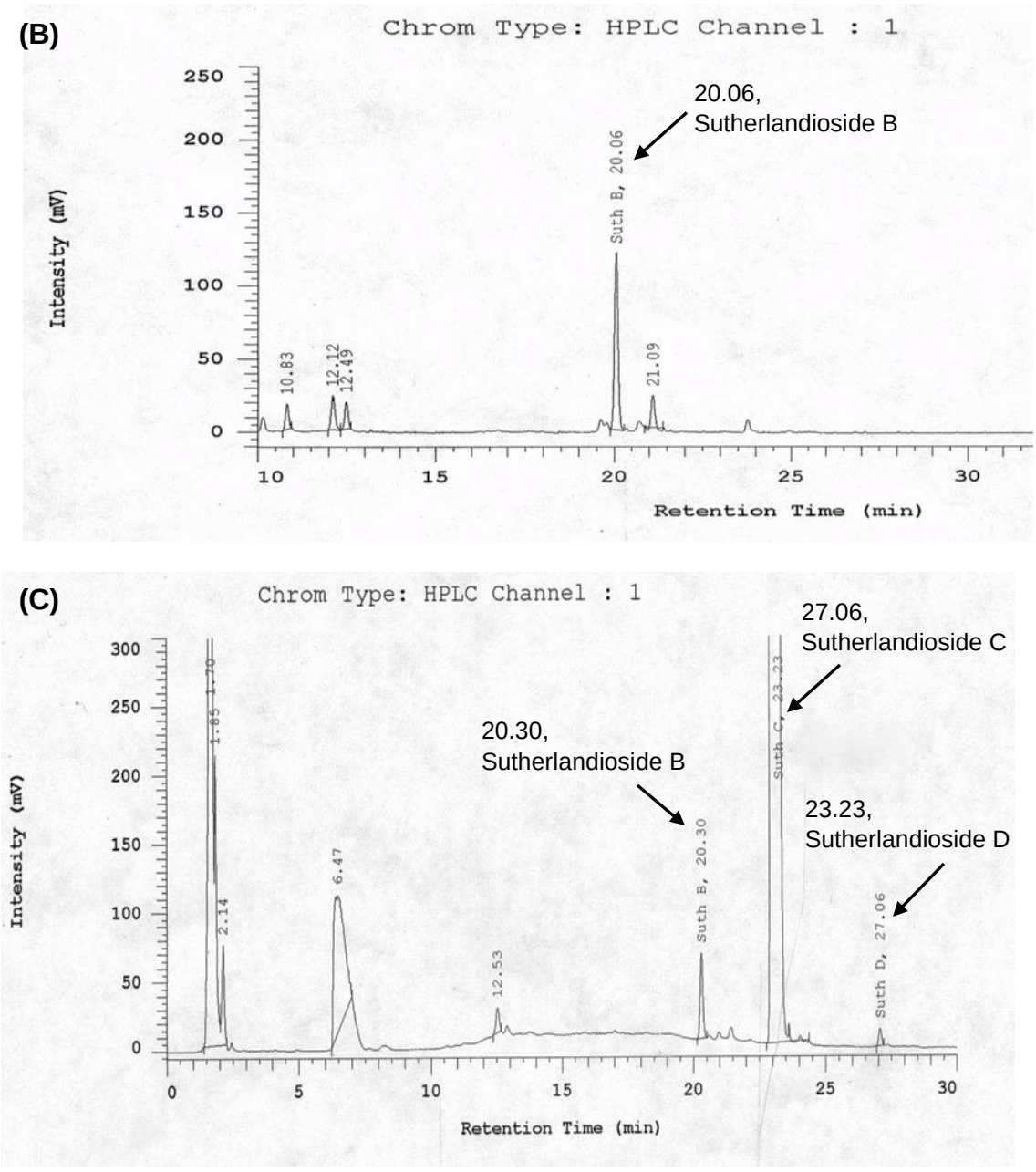
The purity and quantitation of fractions of sutherlandiosides were determined by HPLC-evaporative light scattering detectors (ELSD) as described by Avula *et al.* (27). In specific, twenty microliters of samples were injected and separated in a C18 column (150 mm × 4.6 mm; 5 μm particle size) using a mobile phase that consisted of water/0.1% acetic acid (A) and acetonitrile/0.1% acetic acid (B) at a flow rate of 1.0 mL/minute. A slightly concave gradient elution from 85% A/15% B to 45% A/55% B for 40 minutes was used. Each run was then followed by a 3-minute wash with 100% B and a 10-minute calibration. The ELSD T_1 and T_2 were 35°C and 55°C, respectively. Nitrogen was used as the nebulizer gas at 20 psi. The peaks were assigned by spiking the samples with standard sutherlandioside B and comparison of the retention times.

Results

The *S. frutescens* plant material comprises of a mixture of sutherlandiosides B and C (Appendix Figure 3-1 A).

Sutherlandioside B was detected in the aqueous extracts of Sutherlandia tea (0.5g Su extract/100mL water) (Appendix Figure 3-1 B). Pinosundia tea (0.5g Su extract/100mL water) does not contain alpha-lipoic acid as claimed by the manufacturer (ProBetix®, Value Added Life Health Products (Pty) Ltd, South Africa), but it has a mixture of sutherlandiosides B, C, and D (Appendix Figure 3-1 C).





Appendix Figure 3-1. Chromatograms of purified sutherlandiosides B and C from *S. frutescens* (Big Tree Nutraceutical) (A), sutherlandioside B in Sutherlandia tea (B), and sutherlandiosides B, C, and D in Pinosundia tea detected by the LC-ELSD (C).

APPENDIX 3-2

Validation of taste aversion of *S. frutescens*

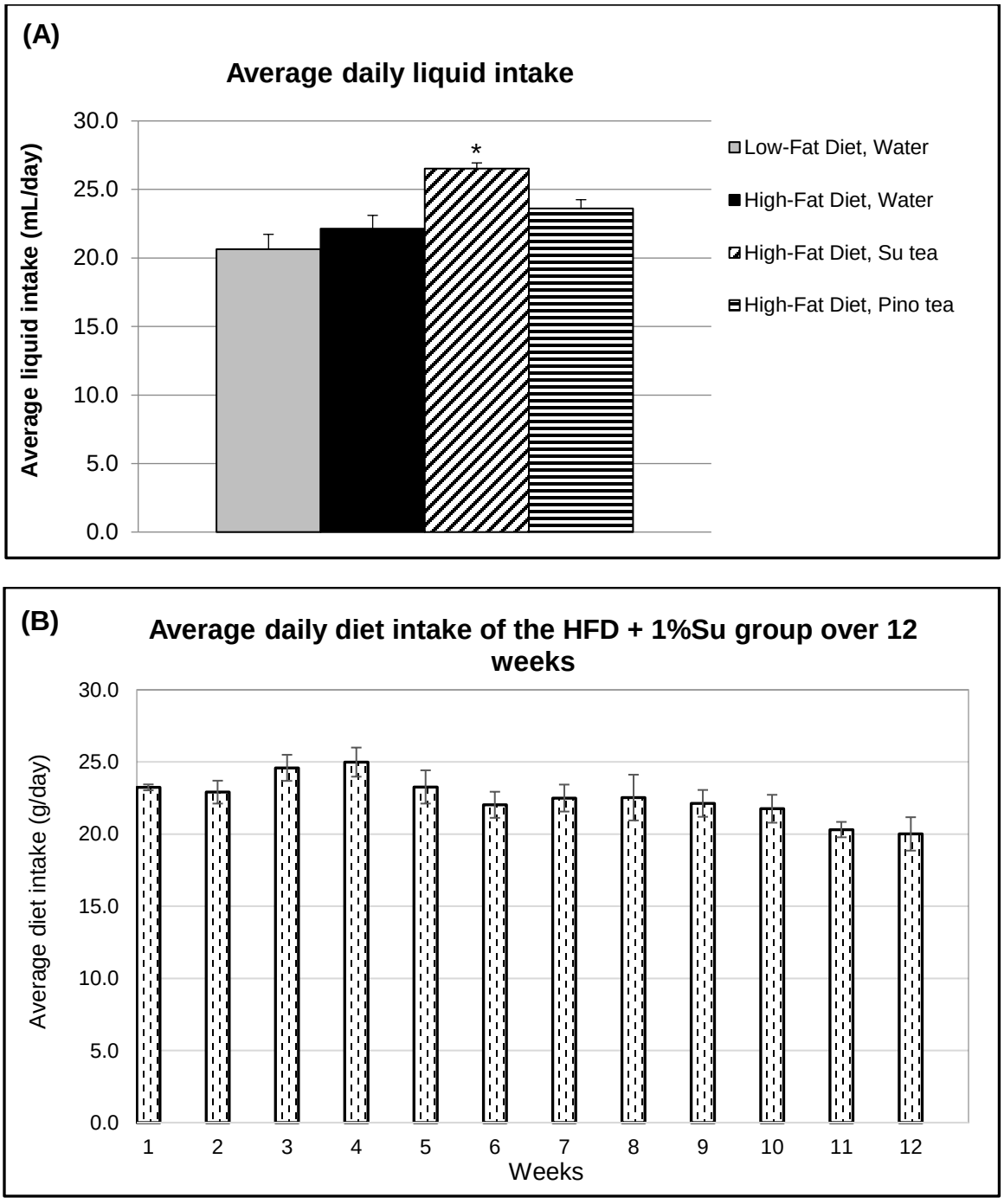
Materials and Methods

The administration of Sutherlandia extracts to rats were illustrated in Chapter 3 (pp. 33-36).

Results

The bitterness of Sutherlandia either in teas or in feed did not alter the amount of liquid or diet the rats consumed compared to the LFD or HFD control groups (Appendix Figure 3-2). In fact, the LFD control, HFD control, and HFD + Pino tea groups drank similar amounts of liquid per day (Appendix Figure 3-2 A). Importantly, the group medicated with Sutherlandia tea consumed a significantly higher amount of liquid compared to the untreated LFD and HFD groups ($p < 0.05$, Appendix Figure 3-2 A), which indicates that the aversion of Sutherlandia did not have a negative impact on the amount of liquid intake in these rats.

Moreover, the rats having dried Sutherlandia incorporated in the HFD consumed approximately the amount expected (23 g diet per day) (157) (Appendix Figure 3-2 B).



Appendix Figure 3-2. Average daily liquid (A) and diet (B) intake of Sutherlandia. Data are expressed as mean ± SEM of each group; n = 11-12 per group. Comparisons between groups used ANOVA followed by Tukey–Kramer multiple comparisons test.

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VITA

Nhu Nguyen was born in Ho Chi Minh City (HCMC, former name: Saigon), Vietnam on September 8th, 1986. After completing her senior high school in one of the top classes for the gifted in natural science, Nhu attended the International University (IU) – a member of the Vietnam National University in HCMC, Vietnam – to pursue a degree in Biotechnology with a focus on biomedical science and obtained her Bachelor's degree in 2010. IU was the first and only university in the southern of Vietnam to employ English as the main language of instruction at that time. Nhu had a precious opportunity to do her internship under supervision of Dr. Sarah Dunstan – group head in human genetics – at the Oxford University Clinical Research Unit (OUCRU) (based in the Hospital for Tropical Diseases in HCMC) during the last six months of her undergraduate studies. After completing her Bachelor's, Nhu worked with Dr. Thuy Le – group leader in HIV research at the OUCRU-HCMC – and with Dr. Peter Horby – former Director of the OUCRU-Hanoi for about two years. Nhu has two peer-reviewed publications on the systematic review of the role of host genetics in susceptibility to influenza with Dr. Horby.

Although there were opportunities for Nhu to conduct doctoral research at the OUCRU, she decided to challenge herself to pursue her Ph.D. in complementary and alternative medicine using medicinal plants – a research area different from her previous training and background – in Dr. William Folk's biochemistry laboratory at the University of Missouri in Columbia, Missouri

starting in August 2012. Part of her financial support for this Ph.D. training was from a two-year fellowship granted by the Vietnam Education Foundation – an independent U.S. Federal Government agency established in 2000 by the U.S. Congress to consolidate the bilateral relationship between the United States and Vietnam through educational exchange in the Science, Technology, Engineering, Mathematics, and Medicine disciplines. This fellowship program based on a national competition offered a unique and tremendous opportunity for young aspiring Vietnamese students to fulfill their academic career goals.

Nhu's initial project was about investigating the effects of *Sutherlandia frutescens* – a southern African herb – on neurocognitive dysfunction in HIV transgenic rats. At the end of 2015, due to changes in funding and her growing interest in molecular nutrition and metabolism, she shifted her focus, with agreement from her Ph. D. adviser and Committee, to do research on the impact of *Sutherlandia frutescens* on fatty liver of high-fat fed rats. During her training at the University of Missouri, she presented multiple posters and received positive feedback at several local and international conferences. Nhu completed her doctoral training and earned her Ph.D. in July 2018.