BIOACTIVITIES OF SELECTED *SUTHERLANDIA FRUTESCENS* (L.) R. BR. LEAF EXTRACTS

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Master of Science

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by
YI-CHUN CHEN

Dr. Ingolf U. Grün, Thesis Supervisor

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

BIOACTIVITIES OF SELECTED *SUTHERLANDIA FRUTESCENS* (L.) R. BR. LEAF EXTRACTS

Presented by Yi-Chun Chen

A candidate for the degree of Master of Science

And hereby certify, that in their opinion, it is worthy of acceptance.

_____

Dr. Ingolf U. Grün
Associate Professor, Department of Food Science

_____

Dr. Andrew D. Clarke
Associate Professor, Department of Food Science

_____

Dr. Mark R. Ellersieck
Research Associate Professor, Department of Statistics

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CHAPTER 1

INTRODUCTION

There has been considerable amount of effort and research focused on finding novel antioxidants from natural sources such as tea, fruits, vegetables, herbs and spices (Kanazawa and Sakakibara, 2000). While fruits and vegetables have shown great antioxidant potential in several studies, there is plenty of evidence suggesting that a great number of aromatic, spicy, medicinal and other plants contain chemical compounds exhibiting antioxidant properties (Kuo et al., 1999; Miliauskas et al., 2004). Previous studies also suggest that the natural antioxidants found in fruits and vegetables may reduce the level of oxidative stress and lower the risk of degenerative ailments. For example, based on epidemiological evidence, a diet rich in fruit and vegetables may be protective against coronary heart disease (Heinonen et al., 1998; Weisburger 1999).

Besides the interest in finding novel antioxidants from natural sources, there has been a worldwide move towards the use of traditional medicines due to concerns over the invasive, expensive and potentially toxic mainstream practices (van Wyk, 2002; Ernst 2002). In places like Africa, Asia and Latin America, traditional medicines have been used for centuries to meet their primary health care needs. As a matter of fact, traditional medicine is the only source of treatment for many people in those areas since they cannot afford modern medications (Harnett et al., 2005). Plants and plant derivatives are taken as remedies to treat ailments including coughs, diarrhea, and other medical conditions. Although pharmaceutical science has relied on bacterial and fungal sources for antimicrobial activity, plants are rich in secondary metabolites that have been shown to

have in vitro antimicrobial and antiviral activity (Ernst, 2002; Waladkhani and Clemens 2001).

Sutherlandia frutescens (L.) R. Br. (family: Fabaceae), commonly known as cancer bush, has been used to treat various ailments by traditional healers in South Africa. Extracts of this plant have been used to treat stomach cancer; decoctions have been consumed as a useful bitter tonic and to treat other ailments like rheumatism, piles, liver problems and influenza (van Wyk, 1997). More recently, the plant has even been used to improve the overall health in HIV/AIDS patients (World Health Organization, 2002; Bessong et al., 2006). However, there is only very little scientific data on the mechanism through which *Sutherlandia frutescens* acts on the immune system. The objective of this study was to examine the bioactivity of *Sutherlandia frutescens* extract by employing the fractionation technique with different solvents and to provide a start for gaining some basic knowledge about the health aspects of this plant.

CHAPTER 2

LITERATURE REVIEW

2.1 Plant based remedies

Since ancient times, humans have derived many benefits from natural plants (Cho et al., 2003). Previous research suggests that high consumption of fruits and vegetables is associated with lower risk of degenerative diseases such as cancer, cardiovascular disease, arthritis, cataract and brain and immune dysfunction (Gordon, 1996; Halliwell, 1996; Feskanich et al., 2000). Plants from the tropical and subtropical climates have also been associated with many medicinal properties (Cho et al., 2003). Many of these plants are taken as remedies for coughs, intestinal bleeding, diarrhea and other human ailments (Leong and Shui, 2002). For example, pineapple juice has been taken to alleviate sore throat and seasickness (Leong and Shui, 2002). Tea, on the other hand, has been used for preventing and treating cancer and cardiovascular diseases (Hollman et al., 1999; Higdon and Frei, 2003).

Traditional Chinese herbal medicine has been widely used by the Chinese population for the treatment of various diseases, and it is recognized as a valuable alternative to conventional medicine (Shen et al., 2003). However, for thousands of years, these herbal medicinal prescriptions have been helping people without being systematically tested by modern scientific methods. Furthermore, while remedies using various traditional herbs with medicinal functions have been developed, there is very little scientific evidence to support these functions (Cho et al., 2003).

In Africa, herbal medicines are often used as primary treatment for HIV/AIDS and for HIV-related problems including nausea, depression, insomnia, and weakness (Mills et al., 2005). Many patients take a broad range of natural health products in addition to their conventional therapeutic products. Furthermore, most Africans cannot afford the modern medicines for treating HIV/AIDS and have no choice but to take the traditional medicines for treatment. However, the same troublesome issues beleaguering Chinese traditional herbal medicine exist; the African herbal medicines are not well-researched and in addition poorly regulated (Mills et al., 2005).

2.1.1 The medicinal use of *Sutherlandia frutescens* in South Africa

Sutherlandia frutescens (L.) R. Br. (family: Fabaceae) is a small, perennial woody shrub of up to one meter in height. In South Africa, the plant is often referred to as "cancer bush" in English and "kankerbos" in Afrikaans. The leaves of this herb are divided into numerous small leaflets, which are slightly to densely hairy, often giving the plant a silvery appearance. The large, red flowers of the plant develop into characteristic balloon-like seed pods (Ojewole, 2004).

An overview of the recorded medicinal uses of *Sutherlandia frutescens* suggested that it is one of the most widely used but underrated medicinal plants of South Africa (Ojewole, 2004; Atawodi, 2005). The medicinal use of *Sutherlandia frutescens* probably originated from the Kohi and Nama people for treating cancers internally and sterilizing external wounds. It is an old remedy for stomach problems and internal cancers in the Cape region, which is the Southern coast region of South Africa. Mainly the leaves are used, but all above-ground parts are often included as well (van Wyk, 2002). The

medicinal virtues of the plant extend to relieving the symptoms of colds, fevers, influenza, chicken pox, poor appetite, indigestion or constipation, dysentery and other gastrointestinal tract problems, coughs, asthma, chronic bronchitis, heartburn, gastritis, inflammatory conditions such as rheumatoid arthritis, osteoarthritis and gout, diabetes mellitus, varicose veins, epilepsy and convulsion, kidney and liver problems, urinary tract infections, and body aches and pains (van Wyk 1997, Dalvi 2003; Na et al., 2004; Ojewole, 2004; Reid et al., 2006; Chadwick et al., 2007). The plant has also been used as a supportive treatment in mental and emotional stress including irritability, anxiety and depression (Prevoo et al., 2004) However, little scientific evidence is available to support the veracity of these claims.

The recommended therapeutic dose of *Sutherlandia frutescens* for human is 9 mg/kg bodyweight per day (Seier et al., 2002). However, no scientific data has been documented on the pharmacology and mechanism by which this plant could act on the immune system. Despite the paucity of data, the South African Ministry of Health and member states currently recommend the use of this herbal plant as remedy for HIV/AIDS treatment (World Health Organization, 2002).

2.1.2 Potential compounds and their health effects in *Sutherlandia frutescens*

Previous research indicated that *Sutherlandia frutescens* contains bioactive chemicals including GABA (gamma-aminobutyric acid), L-canavanine and pinitol. The plant also contains amino acids, small amounts of saponin and no alkaloids (van Wyk, 1997). The research conducted by Tai et al. (2004) also confirmed the presence of canavanine, GABA and arginine in commercial *Sutherlandia frutescens* tablets which

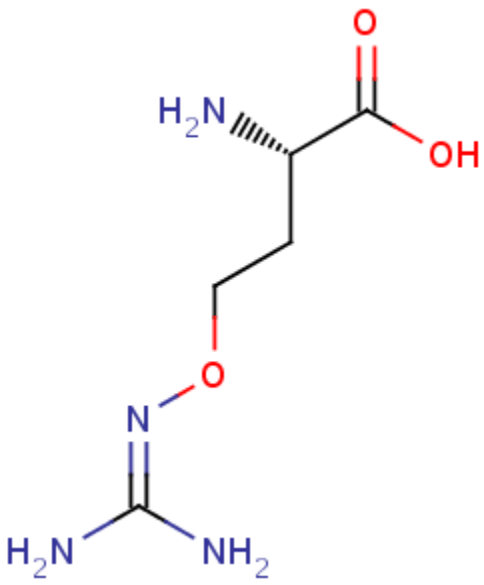
they analyzed by employing gas chromatography/mass spectrometry (GC-MS) and liquid chromatography/mass spectrometry (LC-MS).

L-canavanine exists in certain leguminous plants as the major non-protein amino acid (Rosenthal, 1998). It is the also the structural analogue and antimetabolite of L-arginine. This natural product has been reported to have antiviral activity against influenza and HIV and antineoplastic activity against a number of human cancers (Rosenthal, 1998). Crooks and Rosenthal (1994) developed a new anticancer drug by using this bioactive compound and registered it for a patent (patent number: 5,552,440).

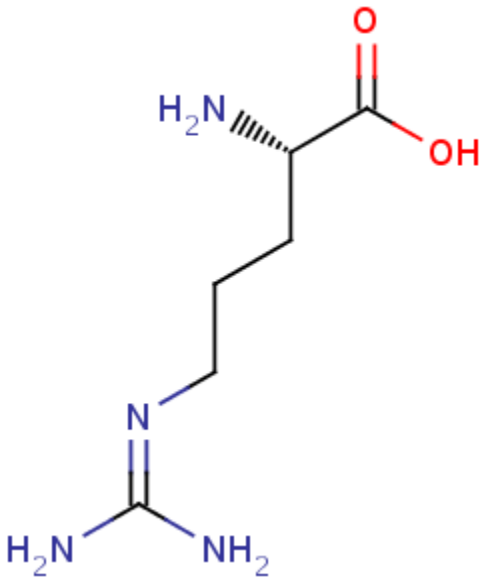
D-pinitol is a type of sugar that occurs in many types of legume. Ostlund and Sherman (1996) received a patent (patent number: 5,550,166) for the use of D-pinitol and its derivatives in treating the wasting syndrome in cancer and AIDS patients.

2.1.2.1 L-Canavanine

L-Canavanine (l-2-amino-[4-guanidinooxy]-butanoic acid), a naturally occurring non-protein amino acid produced by certain leguminous plants, is a potent arginine antimetabolite that bears strong structural analogy to its protein amino acid counterpart. Found in seeds of many common plants, hundreds of legumes (beans) and other arginine-rich foods such as garlic and onions, L-canavanine is a natural toxin that serves as a growth inhibitor and a natural defense against predators. It is also a selective inhibitor of inducible nitric oxide synthase, making it potentially applicable in the treatment of shock and chronic inflammation (Dalvi, 2003). Previous studies indicated that this natural product has demonstrative antitumor activity against a number of human cancers (Bence et al., 2003; Rosenthal, 1998; Worthen et al., 1998).



L-canavanine



L-arginine

Figure 2.1. Chemical structures of L-canavanine and L-arginine.

2.2 Lipid Oxidation

2.2.1 Mechanism of lipid oxidation

Fats, oils and lipid-based foods deteriorate through several degradation reactions both upon heating and long term storage. The main deterioration processes are oxidative reactions and the decomposition of oxidation products, which result in decreased nutritional value, sensory quality and shelf life (Pokorny, 2001).

Oxidative reactions, sometimes referred to as autoxidation, proceed in three distinct stages as shown in Figure 2.2 (Gordon, 2001). In the initiation stage, the fatty acids in the triglycerides can produce free radicals when they react with an external energy source such as heat, light or radiation. In addition, promoters such as metal, pigments and reactive oxygen species (i.e. singlet oxygen) can reduce the amount of energy needed to start autoxidation. Lipid autoxidation is initiated by the abstraction of a hydrogen atom from a fatty acid creating a fatty acid radical.

Propagation involves the continuation and acceleration of the chain reaction begun in initiation. In this stage, the reactions commonly involve abstraction of a hydrogen atom from an adjacent fatty acid to produce an alkyl radical (R•) or addition of oxygen to form a peroxy radical (ROO•). The enthalpy of these reactions is relatively low compared to that of initiation reactions, and therefore propagation reactions occur more rapidly than initiation reactions. Under normal atmospheric pressure of oxygen, the reaction of alkyl radicals with oxygen is very rapid, and the peroxy radicals are present at much higher concentrations than the alkyl radicals. Hence, the peroxy radicals are the primary products of lipid oxidation reactions.

Finally, in the termination stage, the free radicals produced from initiation and propagation states begin to react with each other and form non-radical species which do not feed propagation reactions.

Initiation: $RH \rightarrow R\bullet$

Propagation: $R\bullet + O_2 \rightarrow ROO\bullet$

$ROO + RH \rightarrow ROOH + R\bullet$

Termination: $R\bullet + R\bullet \rightarrow RR$

$ROO\bullet + R\bullet \rightarrow ROOR$

$ROO\bullet + ROO\bullet \rightarrow ROOR + O_2$

Figure 2.2. Mechanism of lipid oxidation (Gordon, 2001).

2.2.2 Free radicals and diseases

A free radical is generally defined as a molecule or an atom with an unpaired electron. By virtue of their unpaired electron, free radicals tend to be unstable and highly reactive (Yoshikawa et al., 1997). It is believed that reactive oxygen species or free radicals (Table 2.1) are closely involved in various biological reactions.

Since most molecules formed under physiological conditions do not have unpaired electrons, free radicals abstract an electron from a stable compound and transform it into a new free radical. Therefore, free radical reactions usually occur as chain reactions. The most-studied free radical chain reaction in living systems is lipid peroxidation, which is

believed to be an important cause of cell membrane destruction and cell damage (Yoshikawa et al., 1997).

Lipids, with the major class being triglycerides, exist in fat storage cells of plants, and animals, as well as occur in biological membranes in the form of phospholipids (Gordon, 2001). Besides lipids, enzymes, nucleic acids and proteins are also important target molecules of free radicals. Both superoxide anion and hydroxyl radical (HO•) have the potential to react with biological macromolecules, thereby inducing tissue damage. In addition, reactive oxygen metabolites and free radicals are involved in many pathogenic conditions via DNA damage, inactivation of nitric oxide (NO•) and oxidation of LDL (low density lipoprotein).

Despite various negatives associated with free radicals, they are naturally produced by some systems within the human body and have beneficial effects. Under normal circumstances, antioxidant defense systems within the body can easily handle the free radicals that are produced naturally. However, when free radical generation exceeds the capacity of the antioxidant defense systems, the resulting oxidative stress can make a significant contribution to the pathogenesis of many human diseases.

Table 2.1 Reactive oxygen species and free radicals

$O_2^{\bullet -}$	superoxide radical
HO•	Hydroxyl radical
HOO•	Hydroperoxyl radical
RO•	alkoxyl radical
ROO•	Alkylperoxyl radical
NO•	Nitric oxide
NO_2•	Nitrogen dioxide
ClO	Hypochloride ion
$Fe^{4+}O$	Ferryl ion
$Fe^{5+}O$	Periferry ion
H_2O_2	Hydrogen peroxide
$^1O_2$	Singlet oxygen
O_3	ozone

Source: Yoshikawa et al. (1997).

2.3 Antioxidants

2.3.1 Introduction

Lipid oxidation is a major cause of quality deterioration in many types of natural and processed foods. Lipid oxidation is usually undesirable in most foods because it leads to the development of rancidity (off-flavors) and potentially toxic reaction products (McClements and Decker, 2000). One of the most effective means of retarding lipid oxidation in foods is to incorporate antioxidants (Reische et al., 1998). Antioxidants have also drawn attention from biochemists and health professionals because they may help protect the body from damage caused by reactive oxygen species (Shahidi, 2000).

The term “antioxidant” must be carefully applied because some substances that retard lipid oxidation under one set of conditions actually promote it under a different set (Reische et al., 1998). In foods, antioxidants are defined as substances that in small quantities are able to retard or to prevent the oxidation of oxidizable materials such as fats (Frankel and Meyer, 2000). In biological systems, the definition is being extended to “any substance that if present at low concentration when compared with that of an oxidizable substrate can significantly delay or prevent the oxidation of that substrate” (Halliwell, 1990). However, neither of these two definitions relates to the antioxidant mechanism. It is considered useful to classify antioxidants as either primary or secondary antioxidants according to the mechanism of their actions (Reische et al., 1998).

2.3.2 Antioxidant mechanism

Ingold (1968) classified all antioxidants into two groups, namely primary or chain-breaking antioxidants and secondary or preventive antioxidants. The primary or chain-breaking antioxidants can react with lipid radicals to convert them into more stable products, while the secondary or preventive antioxidant can reduce the rate of lipid oxidation by a variety of mechanisms (Gordon, 1990). However, it is noteworthy that certain kinds of substances possess more than one mechanism of antioxidant activity (McClements and Decker, 2000).

2.3.2.1 Primary (chain-breaking) antioxidant

A primary antioxidant, also known as “chain-breaking” antioxidant, is a substance that can accept free radicals and further delay the initiation step or interrupt the

propagation step of autoxidation (Reische et al., 1998). Primary antioxidants (AH) can react with lipid and peroxyl radicals and convert them into more stable radicals or non-radical products as shown in the following equations.

$R\bullet + AH \rightarrow RH + A\bullet$

$RO\bullet + AH \rightarrow ROH + A\bullet$

$ROO\bullet + AH \rightarrow ROOH + A\bullet$

The antioxidant radicals (A•) produced by this process are much less reactive than lipid or peroxyl radicals, and therefore do not promote oxidation as lipid or peroxyl radicals do. These antioxidants radicals, in fact, can also terminate the lipid oxidation reaction by reacting with peroxyl radicals, alkoxyl radicals and other antioxidants as shown in the following equations (McClements and Decker, 2000).

$RO\bullet + A\bullet \rightarrow ROA$

$ROO\bullet + A\bullet \rightarrow ROOA$

$A\bullet + A\bullet \rightarrow AA$

2.3.2.2 Secondary antioxidant

Secondary antioxidants can retard lipid oxidation through a variety of mechanisms, including chelation of transition metal ions, oxygen scavenging, replenishing hydrogen to primary antioxidants, absorbing UV radiation and deactivation of reactive species (Reische et al., 1998; Gordon, 1990). The main difference between primary and secondary antioxidants is that secondary antioxidants do not transform or convert free radical species into more stable products. Secondary antioxidants usually

only delay oxidation by interfering with the prooxidant system, such as metals, radiation etc. Many of them show antioxidant activity only if a minor prooxidative component is present in the system. For instance, sequestering agents are only effective in presence of metal ions, and reducing agents such as ascorbic acid are effective in presence of tocopherols or other phenolic antioxidants (Gordon, 1990).

2.3.3 Types of antioxidants

2.3.3.1 Synthetic antioxidant

Some of the most used synthetic antioxidants are phenolic compounds such as butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ) and propyl gallate (PG). They are used widely in the food industry because of their effectiveness and generally being less expensive than natural antioxidants. Concerns regarding toxicological effects and carcinogenic potential of synthetic antioxidants have prompted the need for natural alternatives in the last few decades (Thompson and Trush 1988a, 1988b; Thompson and Moldeus 1988). Since about 1980, natural antioxidants have appeared as a healthier and safer alternative to synthetic antioxidants (Yanishlieva, 2001).

2.3.2.2 Natural antioxidant

Due to the increasing concerns regarding safety issues of using synthetic antioxidants, research has focused on the development and utilization of antioxidants from natural sources. The empirical use of natural compounds as antioxidants is very old. The popularity of smoking and spicing in the home for preservation of meat, fish and

other fat-rich foods may have be due to the recognition of the rancidity-retarding effect of these treatments (Yanishlieva, 2001). Natural antioxidants are found in almost all plants, microorganisms, fungi, and even in animal tissues (Pokorny, 2001). The majority of natural antioxidants are phenolic compounds, and the most important groups of natural antioxidants are the tocopherols, flavonoids and phenolic acids. The mechanisms of these natural antioxidants on autoxidation control or rancidity prevention may be different. However, their presence in live plants may be for the sake of protecting tissues from injurious damage. Furthermore, the beneficial effects of consuming plant food have been ascribed, at least in part, to the presence of antioxidants in the plant and are associated with lowering the risk of most cardiovascular diseases and cancer, among other degenerative diseases of aging (Cuppett et al., 1997).

2.3.4 Phytochemicals

The fact that the diets in industrialized societies, which are deficient in fruits and vegetables, can effectively double the risk of developing many different types of cancer has focused renewed attention on the beneficial properties of these foods. Plant foods are rich in micronutrients, but they also contain an immense variety of biologically active secondary metabolites providing color, flavor and natural toxicity to pests and sometimes humans. The chemistry and classification of such substances is still a matter for much research and debate, but this has not prevented attempts to isolate and exploit substances that have variously been termed “protective factors”, “phytoprotectants” and “nutraceuticals”. Commercial applications tend to be confined to the health food market at the present time. The non-nutrient carotenoids fall into that category, as do a host of

compounds containing phenol rings, phytosterols, sulphur-containing compounds found in onions and their relatives, and another group of sulphur compounds, the glucosinolates from brassica vegetables.

2.3.4.1 Phenolic compounds

The most active dietary antioxidants belong to the family of phenolic and polyphenolic compounds (Shahidi, 2000). Most phenolic compounds are found in vegetables, fruits, spices and herbs (Vinson et al., 1998; Kahkonen et al., 2001; Hu et al., 2003). Many of these phenolic compounds are effective natural antioxidants.

The term “phenolic” or “polyphenol” can be defined chemically as a substance which possesses an aromatic ring bearing one or more hydroxyl substituents, including functional derivatives e.g. esters, methyl ethers, glycosides etc. Most phenolics have two or more hydroxyl groups and are bioactive substances that occur widely in plants. The phenolic compounds which are commonly found in food materials can be classified into three groups including simple phenols and phenolic acids, hydroxycinnamic acid derivatives and flavonoids (Ho, 1992).

Phenolic compounds are ubiquitous in plant foods and therefore are an integral part of the human diet. They are closely associated with the sensory and nutritional quality of fresh or processed plant foods. The antioxidant activities of phenolic compounds have been recognized for decades. Recent studies *in vitro* also confirm that many polyphenols exhibit antioxidant and free radical scavenging properties (Kahkonen et al., 2001; Cheung et al., 2003; Shon et al., 2003). Research on and development of the practice of using natural substances or food ingredients containing phenolic antioxidants will

continue to be of great interest to the food industry. In addition, other biological activities, beside being antioxidants, of phenolic compounds have also become well known in recent years. The most important biological activity of phenolic compounds is probably their inhibitory effect on mutagenesis and carcinogenesis (Ho, 1992).

2.3.5 Measuring antioxidant activity

Antioxidants are used in a wide variety of food products, and their activity may vary depending on various factors such as temperature, food composition, food structure and availability of oxygen. In other words, the activity of antioxidant can vary significantly in different food systems. For example, Ragnarsson et al. (1977) claimed that antioxidants were less effective at elevated temperature than at ambient temperature. Temperatures at which antioxidant activity may be required range from 180-200 ^{o}C for frying oils to as low as 5 ^{o}C for margarine or mayonnaise that are stored in the fridge.

In principle, one could consider measuring the loss of lipid starting material, i.e. fatty acids or triglycerides, or the formation of oxidation products as a method of monitoring oxidative deterioration or antioxidant activity. In practice, measuring the formation of oxidation products is a much more sensitive method of monitoring oxidation (Gordon, 2001). However, the assessment of antioxidant activity by monitoring the formation of oxidation products is not a trivial task. Since a complex mixture of oxidation products is formed and the relative amounts of these products depend on a variety of variables including temperature, metal ion content and other components, such as water, deciding which factors to monitor is an important and difficult decision. Monitoring antioxidant activity under high temperatures may require other products to be monitored

than if the activity is to be assessed under ambient conditions. Thus, hexanal formation can be used to monitor oxidative deterioration in ambient stored products, but cannot be used in frying oils (Gordon, 2001).

Most assessments of antioxidant activity have been performed in oil. This commonly gives sensible predictions for the activity in oil or water-in-oil emulsions, such as margarine, but the data may be misleading for oil-in-water emulsions. It is commonly observed that a non-polar antioxidant such as α-tocopherol is relatively ineffective in oil but is strongly effective in an oil-in-water emulsion. In contrast, a polar antioxidant such as ascorbic acid is more effective in oil than in an emulsion. This has been described as the polar paradox (Porter, 1993; Frankel et al., 1994).

2.3.5.1 Diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity

The inactivation of free radicals is an effective method of controlling oxidation. Therefore, determining the radical scavenging activity is a mean for evaluating antioxidant potential of a compound. The DPPH (α,α-diphenyl-β-picrylhydrazyl) radical is one of few organic nitrogen radicals, which bears a deep purple (violet) color. When it reacts with a substance that can donate a hydrogen atom, the deep purple color will be decolorized and the color starts to fade as it transforms into the non-radical diphenylpicrylhydrazine form (Figure 2.3). The assay is based on the measurement of the reducing ability of antioxidants toward DPPH radicals, and it can be evaluated by electron spin resonance (EPR) or by measuring the decrease in absorbance at 515 nm with a spectrophotometer. Reacting with the antioxidants (AH) or a radical species (R•) would result in the decline of DPPH radicals (Gordon, 2001).

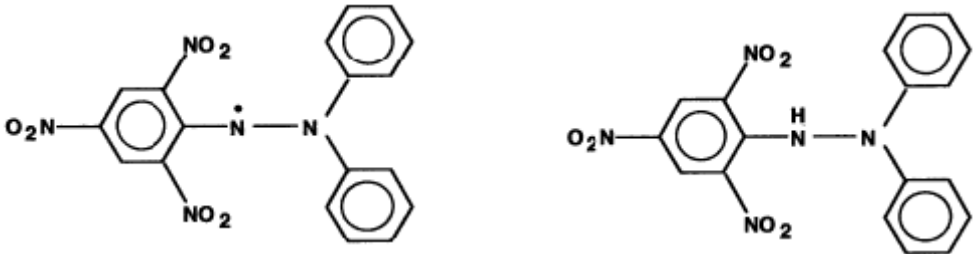
$DPPH\bullet + AH \rightarrow DPPH\text{-}H + A\bullet$

$DPPH\bullet + R\bullet \rightarrow DPPH\text{-}R$

(Violet) (Decolorized)

The absorbance measurement is usually preferred due to simplicity and equipment requirement.

Fast reaction of DPPH radicals occurs with some phenols, but slow secondary reactions may cause a progressive decrease in absorbance, so that the steady state may not be reached for several hours. Most research articles in which the DPPH method has been used report the scavenging after 15 or 30 min reaction time (Gordon, 2001). The data is commonly reported as EC_{50}, which is the concentration of antioxidant required for 50% scavenging of DPPH radicals in the specified time period. The biggest advantage of the DPPH test is that it is simple and rapid and needs only a UV-vis spectrophotometer to be performed.



Diphenylpicrylhydrazyl (free radical) Diphenylpicrylhydrazine (non-radical)

Figure 2.3. Chemical structure of diphenylpicrylhydrazyl (free radical) and diphenylpicrylhydrazine (non-radical).

2.4 Extraction

Extraction can be defined as the removal of soluble components from solid or liquid materials by means of an immiscible solvent (Segado, 1995). In the preparation of a sample for analysis, it is common practice to first extract the analyte away from the bulk of the matrix material and then to remove potentially interfering coextractives by one or more cleanup steps. In the simplest application, it is desired to remove some solute or group of solutes from an insoluble matrix (e.g., animal or plant tissue).

2.4.1 Solvent extraction

Solvent extraction is one of the oldest and most widely used techniques in the preparation of samples for qualitative and quantitative analysis. In its simplest form, extraction has been used since ancient times. According to Chinese legend, emperor Shen-Nung first brewed tea in 2737 B.C. when a few leaves accidentally fell into boiling water. Coffee has been brewed in Arab countries for centuries, and both coffee and tea were introduced to Europe in the seventeenth century (Segado, 1995).

Solvent extraction is defined as the process of separating one constituent from a mixture by dissolving it into a solvent, in which it is soluble while the other constituents of the mixture are not, or are at least less soluble (Holden, 1999). The technique helps scientists to remove as much of the analyte from the matrix as practical, and with a minimum extraction of extraneous materials that might interfere in the analysis if the proper solvent is chosen and used during the process. There are three reasons for using solvent extraction: (1) to isolate a component or analyte of interest; (2) to remove potential interferents from a matrix; and (3) to preconcentrate an analyte prior to measurement (Holden, 1999).

The extraction of an analyte from one phase into a second phase is dependent upon two main factors: solubility and equilibrium. The chemical form of an analyte has a fundamental effect on the efficiency of an extraction and is dependent upon the equilibria of the system. The successful selectivity of a solvent extraction process relies upon the judicious manipulation of the chemical equilibria to produce a species of solute which is preferentially soluble in one of the two solvent phases, usually either organic or aqueous.

The very basic principle of solvent extraction is “like dissolves like”. Removing a nonpolar constituent from a sample matrix requires employing nonpolar solvents. If the solute is charged, it is usually best to form an ion-pair with a counter ion and extract the newly formed neutral complex into a nonpolar solvent (Holden, 1999). Likewise, a polar solvent should be used in the extraction process in order to remove a polar solute from a solution. The major problem in extracting polar solutes into polar solvents is the miscibility of polar solvents with water, which is the main matrix for many samples.

Qualitative predictions could be made for an extraction by considering the polarity of the desired analyte and of the solvents used. The solvent should be able to provide the highest solvency for the solutes of interest, and this solvent should also be matched in terms of polarity and selectivity to the solutes. While selective separation of the solute from another is desired during solvent extraction, the solvent must be selected to maximize the solubility of the desired solute, and to minimize the solubility of other solutes. In some cases, complete extraction of a desired constituent from an insoluble sample matrix requires effective wetting of the matrix by the solvent. Thus, the extraction of hydrophilic samples such as animal tissue is best done with water-miscible solvents such as methanol or acetone, modified if necessary by the mixture of other solvents into

the solvent mixture. Similarly, the extraction of oily solids such as chopped nuts is best done with water-immiscible solvents such as benzene or chloroform (Snyder, 1978). Analytes will dissolve into the separate phase based on their partition coefficient for the solvents used.

Depending on if the analyte is part of a solid or liquid matrix, one distinguishes between solid-liquid and liquid-liquid extraction systems, respectively. For liquid-liquid extraction, solvent extraction involves the distribution of sample components between two immiscible liquid phases. In its most classic form, solvent extraction is performed in a separatory funnel by agitating the mixture to disperse drops of one liquid into the other with carefully releasing the pressure by inverting the funnel and opening the tap when necessary, then discontinuing agitation to allow drop coalescence and separating the bulk liquid phases from one another (Scheibel 1978; Cantwell and Losier, 2002). Finally, by opening the tap of the separatory funnel, both liquid phases can be collected separately. If the extraction procedure is performed only once, then it is termed a single extraction method. Nevertheless, the extraction process may be repeated up to five times using fresh solvent each time to extract the majority of the solute from the solution; this is termed as multiple extraction. The separate extracts could then be combined if the concentration of the analyte in the final extract solution was too low. In addition, some of the solvent could be evaporated to reduce the volume in order to improve analyte concentration. The procedure of solvent extraction is simple, rapid and quantitative, requiring the minimum of apparatus (Holden, 1999).

Table 2.2 Physical constants of solvents used in the study, listed in order of decreasing E_T^N value, as empirical parameter of solvent polarity

Name	**Formula**	**Formula weight[(a)]**	**Density[(a)]**	**Boiling point[(a)]**	**Solubility in 100 parts solvent[(a)]**	**$E_T^{N(b)}$**
Water	H_2O	18.02	1	100		1
Methanol	CH_3OH	32.04	0.7913	64.7	Misc qe, alc, bz, chl, eth	0.762
1-Butanol	C_4H_9OH	74.12	0.8097	117.7	7.4 aq; misc alc, eth	0.586
Chloroform	$CHCl_3$	88.11	1.484	61.7	0.82 aq	0.259
Ethyl acetate	$CH_3COOC_2H_5$	119.39	0.9006	77.1	9.7 aq; misc alc, acet, chl, eth	0.228

Source : (a) Dean (1992) (b) Reichardt (2003)

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample preparation

Sutherlandia frutescens was purchased from Big Tree Health Products (Cape Town, South Africa). The product (see Appendix A for more information from the original label) was stored at room temperature in the dark prior to use and analysis. The plant powder was removed from the original capsules and ground into smaller particles with a pestle and mortar. The extraction scheme used in this study is similar to a South Korea patent used for extracting antioxidant from steamed red ginseng (Ryu et al., 2006; patent number: 2006108268). The flowchart (Figure 3.1.) illustrates the extraction process of *Sutherlandia frutescens*. A 25 g sample of *Sutherlandia frutescens* powder was mixed with 500 mL of methanol in the ratio of 1:20 (powder: methanol) and further extracted with a reflux system above a hot sand bath at 125^{o}C for two hours. The mixture was then filtered through a Whatman No. 1 filter paper on a Büchner funnel with suction and the methanolic solution was concentrated with a rotary vacuum evaporator at around 40^{o}C to a volume of 50 mL or slightly less. The volume of the concentrate was adjusted to 50 mL with methanol and 50 mL of distilled water was then added for a 1:1 ratio of methanol:water.

The mixture was then extracted with 400 mL of chloroform in a separatory funnel by shaking vigorously for 20 seconds and allowed to stand for 30 minutes. The lower

phase was filtered and collected in a rotary vacuum flask and labeled “Chloroform Extract”.

The remaining solution (upper phase) was further extracted with 200 mL of ethyl acetate, again by shaking the mixture solution vigorously for 20 seconds and allowing it to stand for 30 minutes. The resulting upper phase was obtained and labeled “Ethyl Acetate Extract”.

The remaining solution was then extracted with 200 mL of butanol, and both, the “Butanol Extract” and the “Water Extract” were collected at this time.

The collected layers were concentrated at 40^{o}C with a rotary vacuum evaporator until the concentrates became sticky, and left to cool for the remaining solvent to evaporate in a fume hood at room temperature.

Another methanol extract (see Fig 3.1. Flowchart) was prepared separately without the fractionation process. This extraction process was essentially identical to the fractionation process, except that the amount of methanol and *Sutherlandia frutescens* plant powder sample used in preparing the methanol extract were only half of the amounts used for the fractionation.

All extractions, including the separate methanol extraction, were replicated in triplicate.

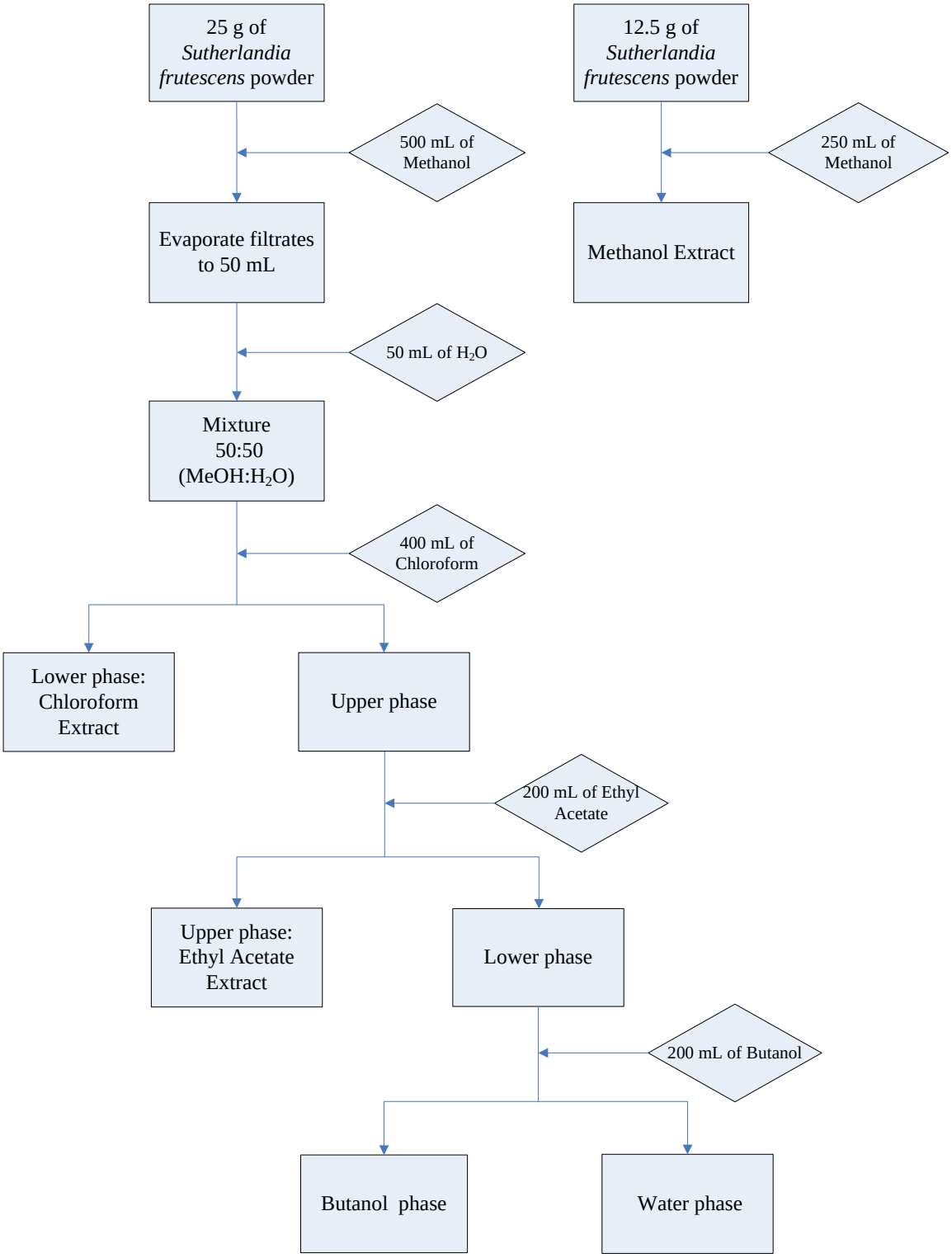


Figure 3.1. Flowchart of extraction process of *Sutherlandia frutescens*.

3.2 Determination of total polyphenols

The total phenolic content was determined by the Folin-Ciocalteu method described by Wu et al. (2003) with some minor modifications. A 0.3 mL aliquot of the methanolic sample solution was added to 1.5 mL of Folin-Ciocalteu phenol reagent and 1.2 mL of 7.5 % (w/v) sodium carbonate solution in test tubes. The tubes were vortexed for 15 seconds and allowed to stand for 30 minutes at room temperature. Absorbance was measured at 765 nm with the Spectronic 20+ spectrophotometer (Bausch & Lomb, Rochester, NY, USA). Results were expressed as milligram of catechol equivalent per gram of extract weight. The preparation of the reagent solutions is shown in Appendix B and the raw data are shown in Appendix C.

3.3 α,α-Diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity

This assay was based on the procedure described by Brand-Williams et al. (1995) with some modifications. A 1 mg of *Sutherlandia frutescens* extract was dissolved in 1 mL of methanol and mixed with 2 mL of a 1 x 10^{-4} M methanolic DPPH solution in round cuvettes. The decrease in absorbance was measured at 515 nm after 60 minutes using a Milton Roy Spectronic 20+ (Bausch & Lomb, Rochester, NY, USA) spectrophotometer. The scavenging activity of sample extract was expressed as the inhibition of DPPH radical and calculated according to the following formula with BHT as the control:

$$\text{Scavenging Activity (\%)} = [(A_{control} - A_{sample)} / A_{control}]*100$$

where $A_{control}$ is the absorbance of the blank, and A_{sample} is the absorbance of the sample solution.

3.4 Inhibitory effects on human prostate tumor cells

The inhibitory effects of different *Sutherlandia frutescens* sample extracts on human prostate tumor cells assay was conducted by Dr. Nader Shenouda (University of Missouri-Columbia), and the procedures are described in his published paper (Shenouda et al., 2004). Briefly, PC3 and LNCaP cells were plated at 1 x 10^4 cells per well in 24-well culture dishes with a RMPI 1640 complete culture medium, and cells were grown to 50% confluence. After 2 days, the media were replaced with complete media containing each *Sutherlandia frutescens* sample extract at different concentrations (0, 2.5, 5, 10 μl/ml) and incubated for 72 hours. The solvent free extracts were reconstituted at different concentrations in HEPES buffer. The total cellular protein concentration was determined by DC Bio-Rad assay using the manufacturer's protocol (Bio-Rad Labratories, Hercules, CA).

3.5 L-Canavanine content analysis

3.5.1 Experiment reagents

Reagent solutions used in L-canavanine content analysis:

Solution A: 0.1 M sodium phosphate buffer, pH 7.4.

Solution B: 0.5 mM PLP (Pyridoxal 5'-phosphate) in 0.1 M Tris-HCl buffer (pH=7.4). The solution was prepared daily and an aluminum foil was used to shield it from light.

Solution C: 1 mg/mL arginase in solution A.

Solution D: 2.5 mM stock solution of canavanine was prepared by dissolving 44 mg of L-canavanine in 100 mL of distilled water. Dilutions from the stock solution were made with solution A.

The sodium phosphate buffer was made by mixing 95 mL of 0.2 M monobasic sodium phosphate (NaH_2PO_4) solution with 405 mL of 0.2 M dibasic sodium phosphate (Na_2HPO_4) solution in a 1000 mL volumetric flask, and diluting the mixture with distilled water to volume. The pH value was measured with a pH/Ion meter (Fisher Accumet Model 230 A) to ensure that the pH value was 7.4.

Pyridoxal 5'-phosphate, NaH_2PO_4, 0.1 M Tris-HCl buffer (pH=7.4), arginase, and L-canavanine were purchased from Sigma Chemical Co. (St. Louis, MO). Na_2HPO_4 was obtained from Fisher Scientific Co. (Fair Lawn, NJ).

3.5.2 Sample treatment

Ten milligram of each sample extract was mixed with 10 mL of hot solution A and 0.3 g of activated carbon in stoppered test tubes. The test tubes were then sonicated for 6 hours and solutions were filtered through a Whatman No.1 filter paper on a Büchner funnel with suction, followed by centrifugation at 7000 x g for 15 minutes. The solution was then filtered again and the filtrate was ready for further analysis in this experiment.

3.5.3 Measurement of L-Canavanine

The L-canavanine content was measured by using the colorimetric method described by Korpela et al. (1982) with some modifications. The experiment was divided into three sets as standard, sample, and blank. The formula for each set is shown in Table 3.1.

Table 3.1 Solution composition for the sample, standard, and blank sets used in L-canavanine content analysis

Solution / Set	Sample	A	B	C	D
Sample	**0.8 mL**	**--**	**0.1 mL**	**0.05 mL**	**-**
Standard	**-**	**-**	**0.1 mL**	**0.05 mL**	**0.8 mL**
Blank for sample	**0.8 mL**	**0.05 mL**	**0.1 mL**	**-**	**-**
Blank for standard	**-**	**0.05 mL**	**0.1 mL**	**-**	**0.8 mL**

A standard curve was made by adding 0.1 mL of solution B and 0.05 mL of solution C to 0.8 mL of solution D at different concentration levels of 0, 10, 20, 30, 40, and 50 μmole/L in the cuvettes. Sample analysis was performed by mixing 0.1 mL of solution B and 0.05 mL of solution C with 0.8 mL of sample solution. Finally, blanks were prepared for each standard concentration and each sample extract. This was done by adding 0.1 mL of solution B and 0.05 mL of solution A to either 0.8 mL of sample extract solution or 0.8 mL of solution D at the corresponding concentrations. A control

was prepared for each standard concentration and each sample because the sample and control solutions both possessed a certain degree of color even after the decoloration treatment. The cuvettes were incubated in a closed water bath at 38^{o}C in the dark for 24 hours. The absorbance was measured at 405 nm against distilled water with 1.0 mL disposable UV/Vis plastic cuvettes using a Cary 50 Bio UV/Vis (Varian, Palo Alto, CA, USA) spectrophotometer.

The changes in absorbance from standard and sample extract were calculated according to the following equations:

$$\Delta A_{standard} = A_{blank} + A_{arginase} - A_{standard}$$

$$\Delta A_{sample} = A_{blank} + A_{arginase} - A_{sample}$$

where $\Delta A_{standard}$ is the absorbance change of standard before and after the reaction, A_{blank} is the absorbance of corresponding control, $A_{arginase}$ is the absorbance of arginase, $A_{standard}$ is the absorbance of standard solution, ΔA_{sample} is the absorbance change of sample before and after the reaction, and A_{sample} is the absorbance of the sample. Basically, the absorbance was calculated as the reading of blank plus the absorbance of arginase thereafter minus the reading of the corresponding standard or sample. The absorbance of arginase was taken into consideration because the arginase solution contributed a slight increase in absorbance to the reading. Therefore, the real change in

absorbance from sample extract and standard should be adjusted prior to the L-canavanine content analysis.

3.6 Statistical analysis

The data were presented as mean ± standard deviation of analyses on three replicate extracts of *Sutherlandia frutescens*. All data were analyzed using the Statistical Analysis System (SAS 8.2, 2001) program. Statistical analyses were performed using a one-way analysis of variance (ANOVA). The level of confidence required for significance was selected at $p < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

Although *Sutherlandia frutescens* has been used for treating various ailments in South Africa, the mechanism regarding the healing effects of this plant is still not clearly understood. The sample, *Sutherlandia frutescens* leaf powder, was extracted with five different solvents in order to attempt to isolate the bioactive compounds from the plant by polarity differentiation. The obtained *Sutherlandia frutescens* extracts were used for various bioactivity and antioxidant activity analysis, including DPPH assay, inhibitory effects on human prostate tumor cells, and total L-canavanine and polyphenol contents in this study.

4.1 Total polyphenols of selected *Sutherlandia frutescens* extracts

The total phenolic content of selected *Sutherlandia frutescens* extracts was determined by the Folin-Ciocalteu method as described by Wu et al. (2003) with some minor modifications. Results were expressed as catechol equivalents in milligram per gram of extract weight and are shown in Table 4.1.

As can been seen from Table 4.1, the ethyl acetate extract contains most of the polyphenols, whereas the water extract contains the fewest phenolic compounds. The polyphenols found in the butanol, chloroform and methanol extract do not differ significantly ($p>0.05$) from each other. However, the ethyl acetate extract has a significantly higher concentration of polyphenols than the other four extracts, while the

water extract contains a significantly lower concentration of polyphenols among all of the extracts.

Table 4.1 Total polyphenols in selected solvent extracts of *Sutherlandia frutescens* determined by Folin-Ciocalteu method. The results are presented as mean ± standard deviation (n=3)

Extracts*	Total polyphenols (mg catechol equivalents/ g extract)**
Ethyl acetate	$49.01^{a} \pm 2.71$
Methanol	$25.98^{b} \pm 1.07$
Chloroform	$22.62^{b} \pm 0.95$
Butanol	$22.42^{b} \pm 2.68$
Water	$7.09^{c} \pm 1.08$

*The concentration of each *Sutherlandia frutescens* extract used in this analysis was 1 mg/mL (1000 ppm)

**Means with different superscript letters are significantly different at $p<0.05$

Traditional knowledge of medicinal plants has always been traced to the occurrence of natural products with medicinal properties and has guided the search for new cures. In spite of the advent of modern high throughput drug discovery and screening techniques, traditional knowledge systems have given clues to the discovery of valuable drugs. Although their efficacy and mechanisms of action have not been tested scientifically in most cases, these simple medicinal preparations often mediate beneficial

responses due to their bioactive constituents such as phenolic compounds. Medicinal plant parts (roots, leaves, stems, barks, flowers and fruits) are commonly rich in phenolic compounds, such as flavonoids, phenolic acids, tannins, coumarins, lignans and lignins (Larson, 1988; Cai et al., 2004). These constituents also commonly exhibit biological effects such as antioxidant activity. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to acts as reducing agents, hydrogen donators, singlet oxygen quenchers, and metal chelators (Morel et al., 1994; Rice-Evan et al., 1997).

Surveswaran et al. (2007) investigated total phenolic contents and antioxidant capacities of 133 Indian medicinal plants and found positive correlations between antioxidant activities and total phenolic contents. Four of these medicinal plants, *Mucuna pruriens*, *Abrus precatorius*, *Psoralea corylifolia* and *Dolichos biflorus* belong to Fabaceae family, which *Sutherlandia frutescens* belongs to as well. These four species contain polyphenols expressed as gallic acid equivalents in the amounts of 6.15, 3.97, 2.53, 0.35 g per 100 g dry weight, respectively. Xu and Chang (2007) investigated total phenolic contents of eight legume (Fabaceae family) materials by using six different extraction solvents (50% acetone, 80% acetone, acidic 70% acetone, 70% methanol, 70% ethanol and 100% ethanol) and Folin-Ciocalteu method. Their results suggested that solvents with different polarity had significant effects on determining the total phenolic contents, extracted components, and antioxidant activities. Turkmen et al. (2006) studied the effects of using water and several organic solvents at various concentrations on the total polyphenol content and antioxidant activity of black tea and mate tea. They also concluded that solvents with different polarity had significant effects on polyphenol

content and antioxidant activity, and found a high correlation between polyphenol content and antioxidant activity of tea extracts.

Khan et al. (2005) investigated the total polyphenol content and DPPH radical scavenging activity of *Paeonia emodi*, a medicinal plant, by using a similar extraction scheme to that in this study. Their results indicated the ethyl acetate fraction contained the most polyphenols, while the water fraction had the least polyphenols. Furthermore, the crude ethanolic extract, chloroform and butanol fractions obtained in their study were not significantly different in amounts of polyphenols, which is in agreement with our findings.

4.2. α,α-Diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging activity

There is increasing interest in free radicals and their deleterious role in food and lipids rancidity, tissue injury and disease. It is known and supported by various studies that free radicals are involved in the propagation of lipid oxidation, and many reactive radical species (e.g. HO•, LO•, LOO• etc.) are formed during the process. Relatively stable radicals such as DPPH•, are often preferred in the assessment of radical scavenging activity. This radical has been widely used in various studies of plant extracts and foods (Koleva et al., 2002; Lee et al., 2003). The popularity of using the DPPH free radical method for estimating free radical scavenging activity may be due to its simple, rapid and economic characteristics.

A modification of the method used by Brand-Williams et al. (1995) was adopted to evaluate DPPH radical scavenging activity of selected *Sutherlandia frutescens* extracts and the results are reported as the mean value of three replicates ± standard deviation in

Table 4.2. The ethyl acetate extract demonstrated the highest DPPH radical scavenging activity among all five extracts and exhibited very good scavenging activity closed to the control (BHT) at the 0.5 mg/mL (500 ppm) concentration, even though they were significantly different. The water extract showed the poorest DPPH radical scavenging activity at both 0.1 and 0.5 mg/mL (100 and 500 ppm). The methanol, butanol and chloroform extracts exhibited similar scavenging activity with no significant difference ($p>0.05$) at 0.1 mg/mL; however, when the concentration level was increased to 0.5 mg/mL, the scavenging activity found in these three extracts differed significantly from each other. Thus, as expected, all *Sutherlandia frutescens* extracts with the exception of the water extract showed DPPH radical scavenging activity in a dose dependent manner.

Table 4.2 The scavenging activity (%)* of selected *Sutherlandia frutescens* extracts and BHT (butylated hydroxytoluene) toward α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical at 0.1 and 0.5 mg/mL (100 and 500 ppm) concentration levels. The results are presented as mean ± standard deviation (n=3)

Extracts	Concentrations	
	0.1 mg/mL (100ppm)	0.5 mg/mL (500ppm)
Control (BHT)	$_{B}83.77^{a} \pm 2.75$	$_{A}89.53^{a} \pm 0.90$
Ethyl acetate	$_{B}32.81^{b} \pm 5.28$	$_{A}82.36^{b} \pm 4.26$
Methanol	$_{B}11.43^{c} \pm 0.97$	$_{A}43.76^{c} \pm 1.67$
Butanol	$_{B}8.80^{cd} \pm 0.12$	$_{A}28.72^{d} \pm 0.55$
Chloroform	$_{B}5.98^{cd} \pm 1.43$	$_{A}17.15^{e} \pm 3.03$
Water	$_{A}4.62^{d} \pm 0.75$	$_{A}8.05^{f} \pm 1.31$

*DPPH radical scavenging activity (%) = [(absorbance of control at 515 nm)-(absorbance of sample at 515 nm)] / (absorbance of control at 515 nm) x 100

$_{A-B}$ Means with different subscript letters within a row are significantly different at $p<0.05$

$^{a-f}$ Means with different superscript letters within a column are significantly different at $p<0.05$

Many medicinal plants extract are proposed to have antioxidant and free radical scavenging activities which is supported by various studies (Atawodi, 2005; Lee et al., 2003; Miliauskas et al., 2004; Surveswaran et al., 2007). Fernandes et al. (2004) analyzed the antioxidant potential of *Sutherlandia frutescens*. The inhibitory effects of hot water extract of *Sutherlandia frutescens* on both luminal and lucigenin enhanced

chemiluminescence of neutrophils stimulated by L-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), as well as its superoxide and hydrogen peroxide scavenging properties in a cell free system, were investigated in the study. Their results suggested that *Sutherlandia frutescens* hot water extract significantly decreased both the luminal and lucigenin enhanced chemiluminescence responses of neutrophils stimulated by FMLP in a dose related manner. In addition, the *Sutherlandia frutescens* hot water extract demonstrated superoxide and hydrogen peroxide scavenging activity at concentrations as low as 10 μg/mL and 2.5 μg/mL, respectively. It was proposed by Fernandes et al. (2004) that the antioxidant activity of *Sutherlandia frutescens* hot water extract could possibly be related to phenolic compounds.

Katerere and Eloff (2005) investigated the antibacterial and antioxidant activity of *Sutherlandia frutescens* leaf using two different extraction schemes. The first extraction scheme used hexane as the first extraction solvent followed by dichloromethane, acetone and ethyl acetate. The other extraction scheme was obtained by extracting three portions of 5 g of leaf sample with acetone, ethanol and water separately. The fractions and extracts were separated by TLC, and DPPH scavenging activity of the polar and non-polar constituents of each extract were determined by spraying the TLC plate with DPPH radicals. In general, the DPPH scavenging activity was lower in the first extract scheme fractions than in the more polar second extraction scheme extracts. The fractions obtained from the first extraction method showed DPPH radical scavenging activity only after 30 min in the acetone, dichloromethane and ethyl acetate fractions, while little activity was observed for the hexane fraction. Polar compounds extracted in both the acetone and ethyl acetate extracts, showed distinct activity while non-polar compounds extracted with

dichloromethane, ethyl acetate and hexane only showed some minor DPPH scavenging activity. The more polar fractions of the second extraction scheme all showed significant DPPH scavenging activity, and TLC separated two antioxidant components in the acetone and ethanol extracts while the aqueous fraction possessed three.

Because the radical scavenging activity was more substantial in the more polar solvent extracts, Katerere and Eloff (2005) proposed that the radical scavenging activity exhibited in the study might be associated with polar constituents such as phenolic compounds, which are known to be able to scavenge radicals readily. However, with a very similar extraction scheme, Koleva et al. (2002) presented quite different results in their study. Based on their findings, the methanol, ethyl acetate and 1-butanol extracts showed the highest radical scavenging activity while the aqueous extract exhibited the lowest DPPH radical scavenging activity. They also suggested that the activity of ether and aqueous extracts was much lower and was influenced by the origin of the plant sample but not by the polarity. Their semi-quantitative TLC tests showed lower amounts of phenolic components in these more polar extracts. Koleva's et al. (2002) results, who used an extraction scheme similar to ours, concur with our results. Differences in radical scavenging activities of solvent extracts of plant material are most likely depending on which part of the plant was extracted as well as the specific extraction scheme used.

By comparing the results exhibited in Table 4.1 to that in Table 4.2, it is suggested that the DPPH radical scavenging activity is generally associated with total phenolic content in selected *Sutherlandia frutescens* extracts. The total polyphenols content determined in the butanol and chloroform extracts does not differ significantly from each

other. Furthermore, there is also no significantly different DPPH scavenging activity found in these two extracts for the 0.1 mg/mL (100 ppm) concentration level.

4.3 L-canavanine content analysis

The L-canavanine content of selected *Sutherlandia frutescens* extracts was determined by a photometric method described by Korpela et al. (1982) with some minor modifications in reagent concentrations, and the results are reported in Table 4.3. The butanol extract contains a higher amount of L-canavanine than the other four extracts. However, it does not differ significantly from the ethyl acetate extract. The chloroform extract contains the least amount of L-canavanine, which, does not differ significantly from the water extract.

Tai et al. (2004) determined there is 3 mg of L-canavanine with in each *Sutherlandia frutescens* tablet sample (300 mg) by LC-MS, which indicates the presence of ten milligram of L-canvanine in one gram of the plant material they used (10 mg/g). By comparing their findings with our results, the L-canavanine content Tai et al. (2004) determined is about two to three times higher than our findings.

Table 4.3 The L-canavanine content determined in five selected solvent extracts of *Sutherlandia frutescens*. The results are expressed as mean ± standard deviation (n=3)

Extracts*	L-canavanine Content (mg L-canvanine/g extract)**
Butanol	$1.219^{a} \pm 0.040$
Ethyl acetate	$0.953^{ab} \pm 0.243$
Methanol	$0.857^{b} \pm 0.068$
Water	$0.462^{c} \pm 0.030$
Chloroform	$0.345^{c} \pm 0.015$

*The concentration of each *Sutherlandia frutescens* extract used in this analysis was 1 mg/mL (1000 ppm)

**Means with different superscript letters are significantly different at $p<0.05$

The results exhibited in Table 4.3 are not close to the Tai's study (2004), which may be the result of both the extraction scheme and the analysis method. Tai et al. (2004) extracted 0.5 g of crushed *Sutherlandia frutescens* tablets with 5 ml of methylene chloride and employed LC-MS ACPI (atmospheric pressure chemical ionization) and SIM (selected ion monitoring) to accomplish quantitation. In this study, however, the extraction was done with methanol and further fractionated with other solvents, which each possess different levels of polarity, and the L-canavanine content was determined by a photometric method.

Chinkwo (2005) investigated the apoptosis activity of aqueous whole plant extract (leaves, stems and flowers) of *Sutherlandia frutescens* on several human tumor cell lines. The plant material in the study was obtained from three regions within South Africa. The

material obtained from Western Cape Province was more active than the other two regions (Northern Province and Orange State). This result suggests that certain environmental conditions may stimulate plants to synthesize different or new compounds in different areas, and which makes them distinct from each other even if the plants belong to the same species. In other words, the different compounds or the same compound at different levels present in the same species could be attributed to distinct soil composition and environmental factor from different regions (e.g. high UV), which ultimately leads to synthesis and accumulation of these secondary metabolites (Chinkwo, 2005; Reichling, 1999).

L-canavanine (2-amino-4-guanidinooxy butanoic acid) is found widely distributed in the plant kingdom. While emphasis has been placed on its occurrence in the leguminous plants, it is also present in other plants such as the common onion. L-canavanine is a potent non-protein amino acid and L-arginine antagonist with documented anti-viral, anti-bacterial, anti-fungal, and anti-cancer activities. Rosenthal (1998) indicated that L-canavanine was shown to exhibit significant antineoplastic activity against MIAPaCa-2, a human pancreatic cancer. Another study conducted by Bence et al. (2003) also proved that L-canavanine is a promising novel anticancer agent and equally cytotoxic for both MDR-positive and MDR-negative neoplastic cells. Therefore, the *Sutherlandia frutescens* fractions were investigated for their inhibitory effects on two human prostate tumor cell lines.

4.4 Inhibitory effects of selected *Sutherlandia frutescens* extracts on human prostate tumor cells

Sutherlandia frutescens has been used for treating internal cancers and other various human ailments by traditional healers in South Africa. In this study, growth inhibition effect of selected *Sutherlandia frutescens* extracts at different concentrations (2.5, 5, and 10 µL/mL) on both PC-3 (androgen-insensitive) and LNCaP (androgen-sensitive) human prostate tumor cells were investigated. The growth responses, as determined by total cellular concentration, to the varying concentrations of five selected *Sutherlandia frutescens* extracts are shown in Figure 4.1 for the PC-3 cell line and in Figure 4.2 for the LNCaP cell line.

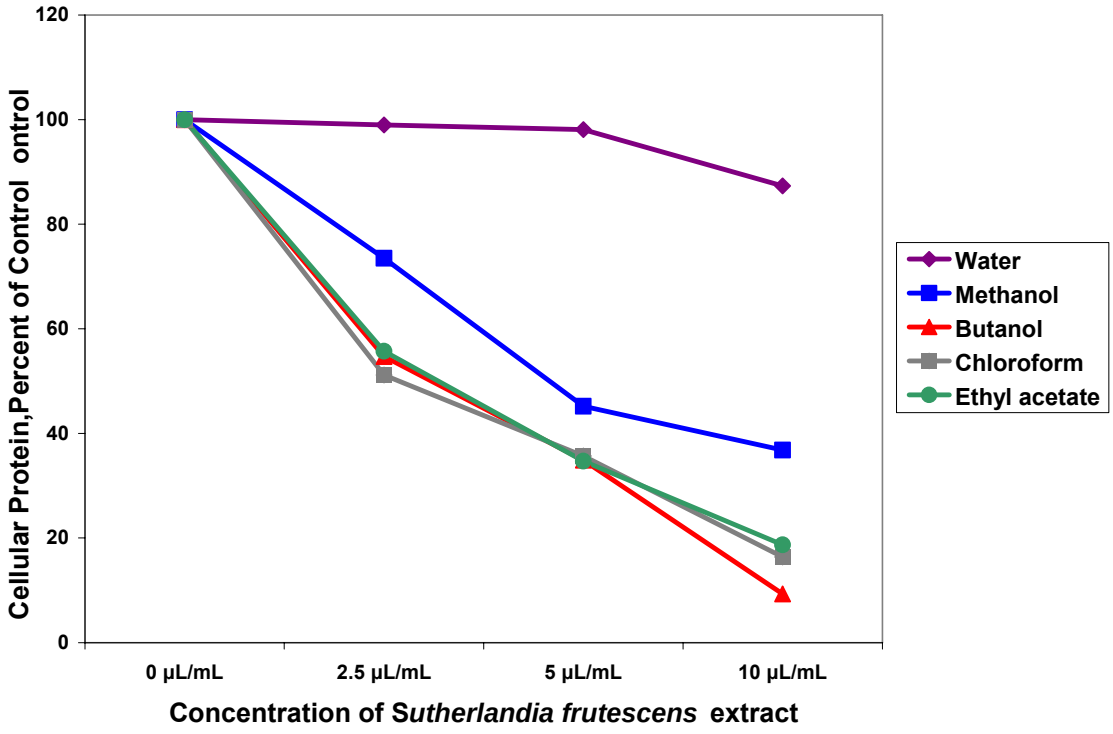


Figure 4.1. Inhibition effect of five different *Sutherlandia frutescens* extracts on PC-3 cells at 0, 2.5, 5 and 10 µL/mL concentrations.

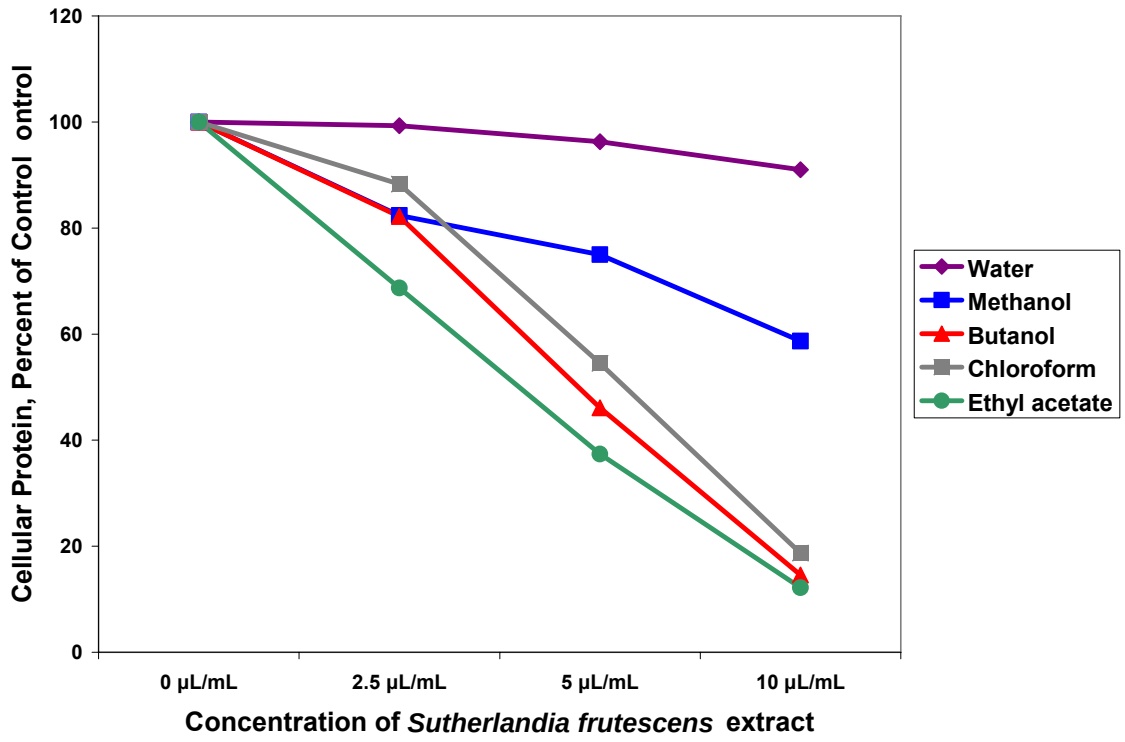


Figure 4.2. Inhibition effect of five different *Sutherlandia frutescens* extracts on LNCaP cells at 0, 2.5, 5 and 10 µL/mL concentrations.

The growth inhibition effect of selected *Sutherlandia frutescens* extracts on PC-3 and LNCaP cell lines was determined by measuring the reductions in growth responses compared to the untreated control, and the results are expressed as the mean value of three replicates as shown in Table 4.4 and Table 4.5.

Table 4.4 Growth inhibition effects (% reduction of protein increase) of selected *Sutherlandia frutescens* extracts at different concentrations on PC-3 cell line

Extracts	**0 μL/mL**	**2.5 μL/mL**	**5 μL/mL**	**10 μL/mL**
Butanol	0	45.3	65.1	90.7
Chloroform	0	48.84	64.3	83.6
Ethyl acetate	0	44.3	65.3	81.3
Methanol	0	26.5	54.8	63.2
Water	0	1	1.9	12.7

Table 4.5 Growth inhibition effects (% reduction of protein increase) of selected *Sutherlandia frutescens* extracts at different concentrations on LNCaP cell line

Extracts	**0 μL/mL**	**2.5 μL/mL**	**5 μL/mL**	**10 μL/mL**
Ethyl acetate	0	31.3	62.6	87.8
Butanol	0	17.8	53.9	85.4
Chloroform	0	11.7	45.5	81.3
Methanol	0	17.6	25	41.3
Water	0	0.7	3.7	9

As can be seen from Table 4.4 and Table 4.5, the growth inhibition effect of selected *Sutherlandia frutescens* extracts on targeted tumor cells showed positive linear relationship with the increase in concentration. For the PC-3 cell lines, the butanol extract at the 10 μL/mL concentration demonstrated the strongest growth inhibition effect.

However, the growth inhibition effects of the chloroform, ethyl acetate, and butanol extracts were similar at each concentration level. The chloroform extract showed slightly better growth inhibition effect at the lowest concentration level (2.5 μL/mL), but no specific trend can be observed.

For the LNCaP cell lines, the ethyl acetate extract showed the best growth inhibition effect among all *Sutherlandia frutescen* extracts at all three concentrations. In opposite to the results for the PC-3 cell line, the chloroform extract showed the weakest growth inhibition effect, which was even weaker than the methanol extract, at the 2.5 μL/mL concentration; however showed considerably higher growth inhibition effects than the methanol extract at the higher concentration levels.

Overall, all *Sutherlandia frutescens* extracts used in this study showed a concentration dependent growth inhibition effect on both PC-3 and LNCaP tumor cells. In addition, the aqueous extract of *Sutherlandia frutescens* exhibited the poorest growth inhibition effect on both PC-3 and LNCaP tumor cells at all three experimental concentrations.

Ojewole (2004) investigated the anti-inflammatory effect of an aqueous extract of *Sutherlandia frutescens* shoots with young adult Wistar rats as subjects. A 0.5 mL/kg of fresh egg albumin was injected into the subplantar surface of the right hind paw of each rat as the phlogistic agent to induce edema as a model of acute inflammation. Increases in the linear diameter of the right hind paws were measured and taken as an indicator of paw edema. The results demonstrated that the aqueous shoot extract of *Sutherlandia frutescens* (800 mg/kg p.o.) produced significant reductions in the fresh egg albumin induced acute inflammation of the rat hind paw.

A study conducted by Tai et al. (2004) indicated that ethanolic *Sutherlandia frutescens* extract showed a concentration dependent antiproliferative effect on several tumor cell lines, with 50% inhibition (IC_{50}) of proliferation of MCF7, MDA-MB-468 (breast tumor cells), Jurkat and HL60 cells (leukemia cell lines) at 1/250, 1/200, 1/150 and 1/200 dilutions of original extract (300 mg of herb powder/2.2mL of ethanol), respectively.

Chinkwo (2005) observed cytotoxicity induced by crude aqueous extract of the whole *Sutherlandia frutescens* plant (leaves, stems, and flowers) to neoplastic cells (cervical carcinoma) and CHO (Chinese Hamster Ovary) cell lines at different concentrations (1.5, 2, 2.5, 3.5, 4, 4.5, 5 mg/mL). However, in this research, the aqueous whole plant extract of *Sutherlandia frutescens* exhibited concentration dependent effect on CHO cells at low concentrations (1.5, 2 and 2.5 mg/mL), but did not at higher concentrations (3.5, 4, 4.5 and 5 mg/mL).

There are large varieties of herbal products available in natural health product stores as dietary supplements for cancer patients. Even though many of them have been used traditionally for cancer treatment for a long time, few peer-reviewed reports are available about their active principle, mode of action, side effects and possible adverse interactions with conventional anti-tumor drugs. Therefore testing of herbal agents in vitro on cancer cell lines to understand their mechanism of action may be helpful to enhance their effectiveness and prevent their undesirable effects.

Although the precise nature of the active components of *Sutherlandia's* anti-tumor activity is not clear, L-canavanine may be one of the important bioactive compounds. Based on the results exhibited in this study (Tables 4.3, 4.4 and 4.5), the butanol extract,

which contained the most amount of L-canavanine, showed high inhibition of PC-3 cell growth but did not show the strongest growth inhibition effect on LNCaP human prostate cells. The chloroform extract comprised the least amount of L-canavanine, but demonstrated stronger growth inhibition effect than three and two other extracts on PC-3 and LNCaP cell lines, respectively. Furthermore, the water extract showed the poorest growth inhibition effect on both PC-3 and LNCaP cell lines, but it was not the lowest in L-canavanine content. The comparison of these results suggests that L-canavanine is very likely to be only one of many factors contributing to the anti-tumor activity of *Sutherlandia frutescens* in vitro. This observation is supported by a study conducted by Tai et al. (2004) when they found adding 1mM of L-arginine to MCF7 cells could significantly reduce the antiproliferative activity of L-canavanine, but the same treatment did not have the same effect at other concentration levels (1/200 to 1/800 dilutions). Since L-canavanine is an L-arginine antagonist, Tai et al. (2004) also proposed that L-canavanine may be one of many factors contributing to the anti-tumor activities of *Sutherlandia frutescens* (ethanolic) extract *in vitro*, which is in agreement with the results in this study.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Based on the results found in this study, the following conclusions can be made:

1. All *Sutherlandia frutescens* extracts obtained in this study contain phenolic compounds.
2. All *Sutherlandia frutescens* extracts obtained in this study demonstrated concentration dependent DPPH radical scavenging activities at both 0.1 and 0.5 mg/mL concentration levels.
3. The DPPH radical scavenging activity shown by *Sutherlandia frutescens* extracts obtained in this study is very likely to be associated with phenolic compounds.
4. All *Sutherlandia frutescens* extracts obtained in this study contain L-canavanine to different extents.
5. All *Sutherlandia frutescens* extracts obtained in this study exhibited growth inhibition effects on both PC-3 and LNCaP human prostate tumor cell lines at 2.5, 5, 10 μL/mL concentration levels in a dose dependent manner.
6. L-canavanine is most likely only one of the constituents of *Sutherlandia frutescens* contributing to the plant's growth inhibition effects on both PC-3 and LNCaP tumor cells in this study.

Sutherlandia frutescens extracts have anti-carcinogenic activities, that may in part be related to containing L-canavanine and polyphenols, which are most likely for the antioxidant activities of the extracts.

APPENDIX A

PRODUCT LABEL FROM ORIGINAL CONTAINER


8 55352 00001 3
SUGGESTED USE:
1 capsule twice daily
or as directed by a
healthcare practitioner
Do not use during pregnancy
or while breast feeding.
Keep out of reach of
children. Store in a cool dry
place. Do not use if seal is
broken or missing.
ORGANICALLY GROWN
NON-IRRADIATED
*These statements have
not been evaluated by
the Food and Drug
Administration.
This product is not
intended to diagnose,
treat, cure or prevent
any disease.
Big Tree
AFRICAN GINSENG™
SUTHERLANDIA FRUTESCENS
SUBSP. MICROPHYLLA ELITE PN1™ CHEMOTYPE
African Ginseng™ is a powerful
adaptogen and provides support for the
proper functioning of the immune system*
60 x 400 mg VEGE-CAPSULES
Dietary Supplement
Supplement Facts
Serving Size 1 Capsule
Servings Per Container 60
AMOUNT PER SERVING
%DAILY VALUE
Sutherlandia Frutescens
Subsp. Microphylla (leaves) 400mg **
** DAILY VALUE NOT ESTABLISHED
OTHER INGREDIENTS:
Vegetarian Capsule Shell 90mg
(Hydroxypropylmethylcellulose)
Free of corn, yeast, wheat, soy, salt, sugar and dairy
products and formulated without the use of preservatives,
artificial colours or flavours. Suitable for vegetarians.
For more information visit www.bigtreehealth.com
Carefully manufactured by:
BIG TREE HEALTH PRODUCTS
Cape Town 8001, South Africa • info@bigtreehealth.com
See underside of container for batch number and best before date.

APPENDIX B

PREPARATION OF CHEMICAL SOLUTIONS USED IN THE STUDY

1. Reagent solutions for DPPH radical scavenging activity assay

DPPH methanolic solution (1×10^{-4} M)

4 mg of DPPH (Sigma Chemical Co., St. Louis, MO; FW: 394.32) was weighed out on an analytical balance and transferred into a 100-mL volumetric flask. The flask was then brought to volume with methanol.

BHT (butylated hydroxytoluene) standard solution (0.5 mg/mL)

50 mg of BHT was weighed and transferred into a 100-mL volumetric flask. The flask was then brought to volume with methanol. Thus, the final concentration of BHT stock solution for DPPH scavenging activity was 500 ppm.

2. Reagent solutions for total polyphenols content analysis

Sodium carbonate solution (7.5 % w/v)

7.5 g of sodium carbonate was weighed and dissolved with 80 mL of distilled water in a beaker with a stirring bar. The solution was then transferred to a 100-mL volumetric flask with 3 times rinsing and brought to volume with distilled water.

Catechol stock solution (0.1mg/mL)

0.01 g of catechol (Sigma Chemical Co., St. Louis, MO) was weighed out on an analytical balance, and transferred to a 100-mL volumetric flask. The flask was then brought to the volume with methanol. Thus, the final concentration of catechol stock solution for total polyphenol content analysis was 100 ppm.

3. Reagent solutions for L-Canavanine

Monobasic sodium phosphate solution (0.2 M)

13.9 grams of sodium phosphate monobasic (Sigma Chemical Co., St. Louis, MO; FW: 120) was weighed and dissolved in a beaker with 400 mL of distilled water. The solution was then quantitatively transferred to a 500-mL volumetric flask and diluting the solution to volume with distilled water.

Dibasic sodium phosphate solution (0.2 M)

28.4 grams of anhydrous sodium phosphate dibasic (Fisher Scientific Co., Fair Lawn, NJ; FW: 141.96) was weighed and dissolved in a beaker with distilled water. After dissolving, the solution was quantitatively transferred to a 1000-mL volumetric flask and brought to volume with distilled water.

Sodium phosphate buffer solution, pH 7.4 (0.1 M)

95 mL of 0.2M monobasic sodium phosphate solution was mixed with 405 mL of 0.2M dibasic sodium phosphate solution in a 1000-mL volumetric flask, and diluting the mixture with distilled water to volume. A pH/Ion meter (Fisher Accumet Model 230 A) was used to ensure that the pH value of the buffer was 7.4.

PLP (Pyridoxal 5'-phosphate) solution (0.5 mM)

26 mg of PLP (Pyridoxal 5'-phosphate) (Sigma Chemical Co., St. Louis, MO; FW: 265.17) was dissolved with 0.1 M Tris-HCl buffer (pH=7.4, Sigma Chemical Co., St. Louis, MO) in a 100-mL volumetric flask, and the mixture was diluted to volume with

0.1 M Tris-HCl buffer solution (pH=7.4). An aluminum foil was used to shield the solution from light during the storage in the refrigerator, and this 1 mM solution was diluted with Tris-HCl buffer solution (pH=7.4) at 1:1 volumetric ratio to make a 0.5 mM solution prior to analysis.

Arginase solution (1 mg/mL)

18 mg of arginase was weighed and mixed with 18 mL of 0.1 M sodium phosphate buffer solution (pH=7.4) in a capped vial. A stirring bar was put into the vial to help dissolve the arginase.

L-canavanine stock solution (0.25 mM)

44 mg of L-canavanine ($C_5H_{12}N_4O_3$) (Sigma Chemical Co., St. Louis, MO; FW: 176.2) was weighed and transferred to a 100-mL volumetric flask, then brought to volume with distilled water. A stirring bar was put into the volumetric flask to help dissolve L-canavanine. This solution (2.5 mM L-canavanine solution) was further diluted to 0.25 mM by transferring 10 mL of the solution (2.5 mM L-canavanine solution) to a 100-mL volumetric flask with a pipet and then brought to volume with distilled water, and is considered the standard stock solution for the standard curve.

APPENDIX C

PREPARATION OF STANDARD CURVE AND CALCULATIONS FOR TOTAL POLYPHENOLS CONTENT ANALYSIS

Standard Curve (0, 10, 50, 100 ppm)

The standard curve was prepared by making dilutions from the catechol stock solution (see Appendix A) with methanol. Dilutions were made by pipetting 10, 5, 1 and 0 mL of catechol stock solution into capped glass tubes with 0, 5, 9 and 10 mL of methanol, respectively. The tubes were then vortexed for 10 seconds to ensure the solution was well mixed. The absorbance was measured at 765 nm with a Spectronic 20+ spectrophotometer (Bausch & Lomb, Rochester, NY, USA) and the results are shown in Table B.1.

Table B.1. Absorbance of catechol solution at different concentrations

Concentration (mg/L)	**Absorbance at 765 nm**
0	0.134
10	0.187
50	0.452
100	0.815

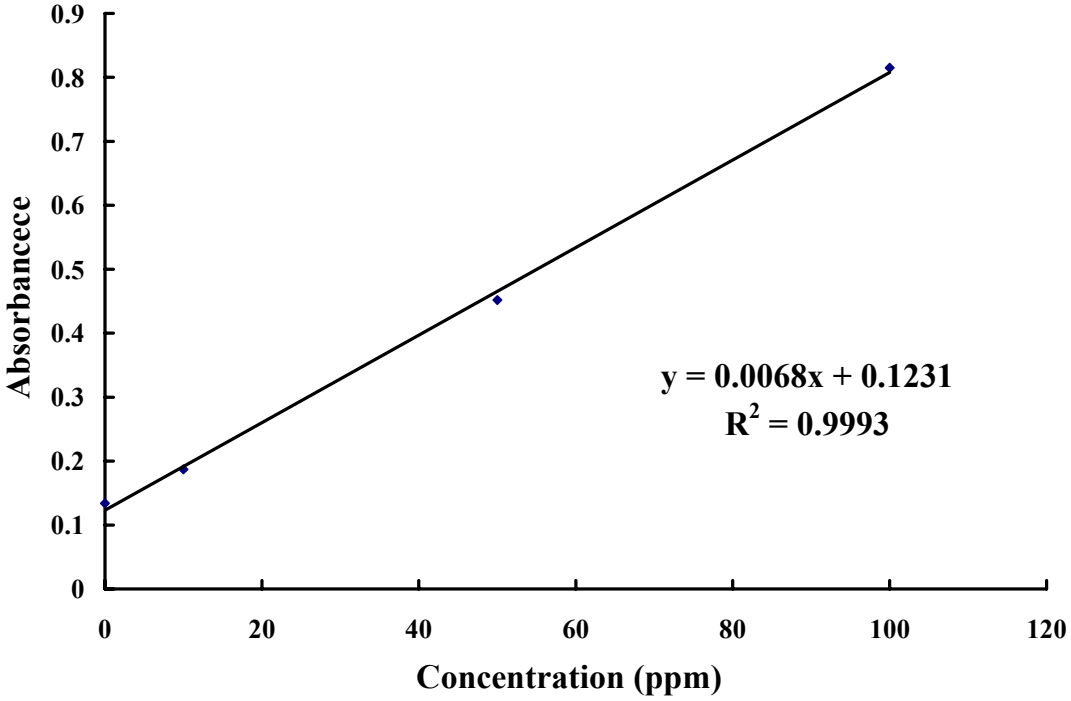


Fig B.1 Standard curve for total polyphenols analysis

Table B.2. Absorbance of selected *Sutherlandia frutescens* extracts at 765 nm in triplicate in total polyphenols content assay

Extracts\ Replicate	**1**	**2**	**3**
Ethyl acetate	0.452	0.484	0.440
Methanol	0.301	0.310	0.292
Chloroform	0.278	0.270	0.286
Butanol	0.282	0.252	0.296
Water	0.163	0.181	0.171

Calculations for polyphenol contents

From standard curve, $Y = 0.0068X + 0.1231$

Hence, the polyphenol content X = (absorbance of sample extract – 0.1231)/0.0068

APPENDIX D

DATA FOR DPPH RADICAL SCAVENGING ACTIVITY

Table C.1 The absorbance of selected *Sutherlandia frutescens* extracts at 0.1 mg/mL concentration level under 515 nm in DPPH radical scavenging activity assay

Extracts\ Replicate	**1**	**2**	**3**
Control	0.758		
BHT	0.142	0.133	0.094
Ethyl acetate	0.460	0.558	0.510
Methanol	0.672	0.662	0.680
Butanol	0.692	0.692	0.690
Chloroform	0.728	0.704	0.706
Water	0.730	0.716	0.723

Table C.1 The absorbance of selected *Sutherlandia frutescens* extracts at 0.5 mg/mL concentration level under 515 nm in DPPH radical scavenging activity assay

Extracts\ Replicate	**1**	**2**	**3**
Control	0.758		
BHT	0.082	0.086	0.070
Ethyl acetate	0.102	0.202	0.178
Methanol	0.444	0.420	0.415
Butanol	0.536	0.546	0.539
Chloroform	0.659	0.604	0.621
Water	0.710	0.686	0.695

The DPPH radical scavenging activity (%) was calculated by the following equation:

DPPH radical scavenging activity (%) = [(absorbance of control at 515 nm)-(absorbance of sample at 515 nm)] / (absorbance of control at 515 nm) x 100.

APPENDIX E

PREPARATION OF STANDARD CURVE AND CALCULATIONS FOR L-CANAVANINE CONTENT ANALYSIS

Standard Curve (10, 20, 30, 40, 50 μmole/L)

The standard curve was prepared by making dilutions from the L-canavanine stock solution (0.25 mM) with 0.1 M sodium phosphate buffer (pH=7.4) according to the volumes shown in Table D.1.

Table D.1. Dilutions from 0.25 mM L-canavanine solution (Appendix A)

Concentration (μmole/ L)	0	10	20	30	40	50
0.25 mM L-canavanine stock solution (mL)	0	0.2	0.4	0.6	0.8	1
0.1 M sodium phosphate buffer (mL)	5	4.8	4.6	4.4	4.2	4
Total volume (mL)	5	5	5	5	5	5

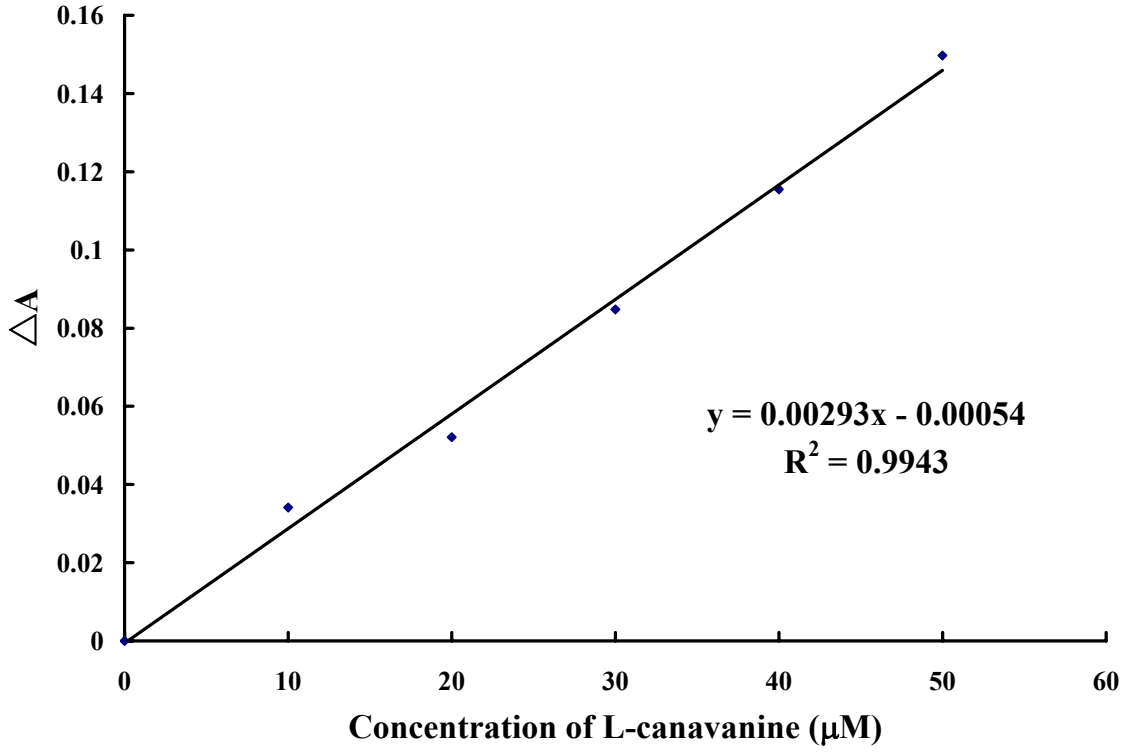


Fig D.1. Standard curve for L-canavanine content analysis at concentrations of 10, 20, 30, 40, 50 μM.

Table D.2 Absorbance and absorbance changes of different concentrations of L-canavanine standard solution at 405 nm

Concentration (μM)	**Absorbance at 405 nm**	**ΔA**
0	0.2892	0
10 μM blank	0.2885	
10 μM	0.2704	0.0341
20 μM blank	0.2706	
20 μM	0.2345	0.0521
30 μM blank	0.2612	
30 μM	0.1924	0.0848
40 μM blank	0.2520	
40 μM	0.1525	0.1155
50 μM blank	0.2455	
50 μM	0.1118	0.1497

Table D.3 Absorbance and absorbance changes of different *Sutherlandia frutescens* extracts at 405 nm in triplicate

Sample extracts\ Replicate	**A_1**	**ΔA_1**	**A_2**	**ΔA_2**	**A_3**	**ΔA_3**
Methanol blank	0.4374		0.4373		0.4325	
Methanol	0.4412	0.0122	0.4384	0.0149	0.4345	0.0140
Chloroform blank	0.3116		0.3225		0.3287	
Chloroform	0.3221	0.0055	0.3333	0.0052	0.3398	0.0049
Ethyl acetate blank	0.6959		0.5312		0.6139	
Ethyl acetate	0.6917	0.0202	0.5369	0.0103	0.6145	0.0154
Butanol blank	0.3739		0.3765		0.3664	
Butanol	0.3711	0.0188	0.3724	0.0201	0.3621	0.0203
Water blank	0.3852		0.3194		0.3162	
Water	0.3942	0.0070	0.3288	0.0066	0.3244	0.0078

The absorbance change ($\triangle A$) was calculated by the following equations:

$$\triangle A_{standard} = A_{blank} + A_{arginase} - A_{standard}$$

$$\triangle A_{sample} = A_{blank} + A_{arginase} - A_{sample}$$

where $\triangle A_{standard}$ is the absorbance change of standard before and after the reaction, A_{blank} is the absorbance of corresponding control, $A_{arginase}$ is the absorbance of arginase (0.016), $A_{standard}$ is the absorbance of standard solution, $\triangle A_{sample}$ is the absorbance change of sample before and after the reaction, and A_{sample} is the absorbance of the sample. Basically, the absorbance was calculated as the reading of blank plus the

absorbance of arginase thereafter minus the reading of the corresponding standard or sample. The reason for such calculations was already expressed in Chapter 3.

Calculation for L-canavanine content

From L-canavanine standard curve, Y=0.00293x - 0.00054.

Thus, L-canavanine concentration of sample extracts (C)

= ($\triangle$A+0.000540)/0.00293

In addition, from C, L-canavanine content of each *Sutherlandia frutescens* extract can be calculated by the following equation and was expressed as catechol equivalents in milligram per gram of extract weight.

$$\text{L-canavanine content} = [C \times 10^{-6} \times 0.8 \times 10^{-3} \times 176.2 \times 10^{3}] / 8 \times 10^{-4}$$

Where C = L-canavanine concentration of sample extract

10^{-6} = Convert μM to M

10^{-3} = Convert mL to L

176.2 = Molecular weight of L-canavanine

10^{3} = Convert gram into milligram

8×10^{-4} = Weight of each sample extract used in analysis

APPENDIX F

SAS PROGRAM FOR DATA PROCESSING

```
title1 'ANOVA of Polyphenol';
data pphenol;
input sample$ rep score;
datalines;
Water 1 5.823321555
Water 2 8.45229682
Water 3 6.991755006
Butanol 1 23.20376914
Butanol 2 18.8221437
Butanol 3 25.24852768
EthylA 1 48.03297998
EthylA 2 52.70671378
EthylA 3 46.2803298
Chloroform 1 22.61955241
Chloroform 2 21.45111896
Chloroform 3 23.78798587
Methanol 1 25.97879859
Methanol 2 27.29328622
Methanol 3 24.66431095
;
proc anova;
class sample rep;
model score=sample rep;
means sample rep/lsd;
run;
```

```
title1 'ANOVA of DPPH';
data DPPH;
input sample$ rep score;
datalines;
BHT100 1 0.142
BHT100 2 0.133
BHT100 3 0.094
Meth100 1 0.672
Meth100 2 0.662
Meth100 3 0.68
Chlo100 1 0.728
Chlo100 2 0.704
Chlo100 3 0.706
EthylA100 1 0.46
EthylA100 2 0.558
EthylA100 3 0.51
But100 1 0.692
But100 2 0.692
But100 3 0.69
```

```
Wat100      1   0.73
Wat100      2   0.716
Wat100      3   0.723
BHT500      1   0.082
BHT500      2   0.086
BHT500      3   0.07
Meth500     1   0.444
Meth500     2   0.42
Meth500     3   0.415
Chlo500     1   0.659
Chlo500     2   0.604
Chlo500     3   0.621
EthylA500   1   0.102
EthylA500   2   0.202
EthylA500   3   0.178
But500      1   0.536
But500      2   0.546
But500      3   0.539
Wat500      1   0.71
Wat500      2   0.686
Wat500      3   0.695
;
proc anova;
class sample rep;
model score=sample rep;
means sample rep/lsd;
run;


title1 'ANOVA of DPPH';
data DPPH;
input sample$ rep score;
datalines;
BHT500      1   0.082
BHT500      2   0.086
BHT500      3   0.07
Meth500     1   0.444
Meth500     2   0.42
Meth500     3   0.415
Chlo500     1   0.659
Chlo500     2   0.604
Chlo500     3   0.621
EthylA500   1   0.102
EthylA500   2   0.202
EthylA500   3   0.178
But500      1   0.536
```

```
But500      2    0.546
But500      3    0.539
Wat500      1    0.71
Wat500      2    0.686
Wat500      3    0.695
;
proc anova;
class sample rep;
model score=sample rep;
means sample rep/lsd;
run;


title1 'ANOVA of L-Canavanine';
data canavanine;
input sample$ rep score;
datalines;
Butanol     1    1.163325531
Butanol     2    1.24151063
Butanol     3    1.253539107
EthylA      1    1.247524868
EthylA      2    0.652115272
EthylA      3    0.958841428
Methanol    1    0.766385801
Methanol    2    0.928770236
Methanol    3    0.874642091
Water       1    0.453645407
Water       2    0.429588453
Water       3    0.501759313
Chloroform  1    0.363431831
Chloroform  2    0.345389116
Chloroform  3    0.327346401
;
proc anova;
class sample rep;
model score=sample rep;
means sample rep/lsd;
run;
```

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