Comparison of the physicochemical characteristics and flavonoid release profiles of *Sutherlandia frutescens* phytosomes *versus* liposomes

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A thesis submitted in partial fulfillment of the requirements for the degree of Magister Pharmaceuticiae in the School of Pharmacy, Faculty of Natural Sciences at the University of the Western Cape, Bellville, South Africa.

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KEYWORDS

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Liposomes

Phytosomes

Drug release profile

Flavonoids

Sutherlandins

High performance liquid chromatography (HPLC)

ABSTRACT

Sutherlandia frutescens is a traditional plant medicine widely used in South Africa. Traditionally, the leaves of *S. frutescens* are mainly used as a tea, but these traditional dosage forms have several disadvantages, including that they are not particularly convenient to prepare and store, encourage dosage inaccuracy and are highly susceptible to microbial contamination. To solve these problems, dried aqueous extract forms, e.g. freeze dried aqueous extract (FDAE) of *S. frutescens* were prepared, but they, in turn, may still suffer from instability and contain mainly hydrophilic phytoconstituents that are poorly absorbed and delivered for *in vivo* activity. Modified forms of the FDAE, i.e. the active phytopharmaceutical ingredient (API), may be a better solution. Therefore this study sought to prepare liposomes and phytosomes of the freeze dried aqueous extract of *Sutherlandia frutescens*, as a means of increasing the total the surface area of the API, thus improving its release and dissolution in gastrointestinal fluids.

Liposomes and phytosomes of the FDAE of *Sutherlandia frutescens* obtained were prepared using a thin film hydration method at ratios of lecithin: *S. frutescens* (3:1) and phosphatidylcholine: *S. frutescens (*2:1) respectively. The physical characteristics (i.e. particle size, size distribution, zeta potential, and morphology), of flavonoid glycosides (i.e. sutherlandins A to D; API) as well as content and release profiles of each dosage form (i.e. FDAE liposome or phytosomes) at pH 1.2 and pH 6.8 was determined. A validated HPLC assay was used to determine and compare the flavonoid glycoside content and release profiles of the liposomes and phytosomes.

Both liposomes and phytosomes were successfully prepared, in moderate yields (± 30 %, and ± 50 %, respectively), using the thin film hydration method. The liposomes had a significantly smaller size, lower size distribution, higher zeta potential and better stability

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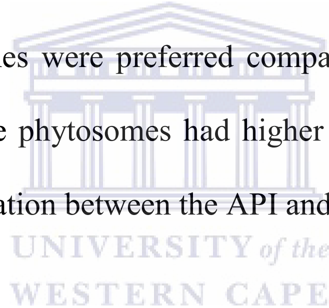
than the phytosomes ($p < 0.05$). The phytosomes, however, had significantly higher flavonoid glycoside encapsulation efficiency than the liposomes (±50 % vs ±26 %; $p < 0.01$). In addition, the release at 120 minutes, of flavonoid glycosides from the liposomes (63%, 58%, 76% and 46% % at pH 1.2, and 78%, 76%, 87% and 89 % at pH 6.8 for sutherlandins A, B, C and D, respectively) was significantly higher and faster than that of the phytosomes (52%, 41%, 51% and 39 % at pH 1.2, and 31% 31%, 33%and 45% % at pH 6.8, for sutherlandins A, B, C and D, respectively). The differences in release were likely due to differences in particle size and size distribution of the two modified API forms.

Overall, liposomes and phytosomes can be considered promising vehicles for delayed delivery of herbal crude extracts. Based on its characteristics (i.e. narrower size distribution, and better stability), the liposomes were preferred compared to the phytosomes offering a better kinetic release profile. The phytosomes had higher encapsulation than the liposomes that may be due to complex formation between the API and the lipid.



DECLARATION

I declare that the thesis entitled Comparison of the physicochemical characteristics and flavonoid release profiles of Sutherlandia frutescens phytosomes *versus* liposomes is my own work, that it has not been submitted before for any degree or examination in any other university, and that all sources used or quoted have been indicated and acknowledged by complete references.

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Signed by:

M. I. Daghman
November 2015

UWC, Bellville

DEDICATION

To Mrs. Suad, Mr. Ibrahim, Hanan, Sara, Heba, for their love, support and encouragement.

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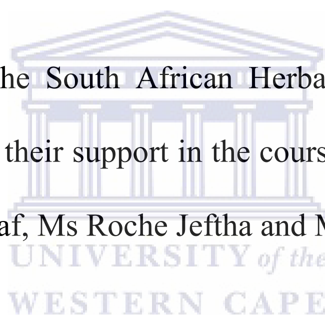

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ABBREVIATIONS AND DEFINITIONS

ANOVA:	Analysis of variance
API:	Active pharmaceutical ingredient
COX:	Cyclooxygenase
DDS:	Drug delivery system
DSL:	Dynamic scattering spectroscopy
EE:	Encapsulation efficacy
FDAE:	Freeze-dried aqueous extract (of S. frutescens)
GABA:	Gamma-amino butyric acid
GUV:	Giant unilamellar vesicles
HPLC:	High performance liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
LUV:	Large unilamellar vesicles
MV:	Multivesicular vesicles
MLV:	Multilamellar large vesicle
MUV:	Medium sized unilamellar vesicles
OLV:	Oligolamellar vesicles
PBS:	Buffer phosphate solution
PC:	Phosphatidylcholine
PCS:	Photon correlation spectroscopy
PDI:	Polydispersity index
SEM:	Scanning Electronic Microscopy
SF:	*Sutherlandia frutescens*
Suth A	Sutherlandin A
Suth B	Sutherlandin B
Suth C	Sutherlandin C
Suth D	Sutherlandin D
SUV:	Small unilamellar vesicles
TM:	Traditional Medicine
TEM:	Transmission Electron Microscopy
WHO:	World Health Organisation
ZP:	Zeta potential

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Chapter One
INTRODUCTION

Traditional medicine (TM) has in the last several years claimed an increasing share of the public's awareness and the agenda of medical researchers (Ghani, 1990). Traditional medicine encompasses knowledge and belief based health practices. It refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being (Wirth, *et al* 2005). According to WHO traditional medicine is the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness (World Health Organization, 1978). Traditional medicine has been used to treat various diseases such as headache, backache, influenza, colds, evaluated blood pressure, diabetes, peptic ulcer, liver problems and HIV. And usually the dosage forms used in traditional medicine are liquids such as teas and decoctions (Sofowora, 1982).

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Sutherlandia frutescens is one such traditional medicine that is popular and widely used in South Africa. It is a small soft wooded shrub with pinnately compound leaves that have a very bitter taste. *Sutherlandia* belongs to the family Fabaceae, and is known locally by several common names, e.g. *Sutherlandia*, Cancer Bush and Balloon Pea, etc. It generally grows naturally throughout the drier parts of southern Africa, in the Western Cape and up the west coast as far north as Namibia

and into Botswana, as well as in the western Karoo to Eastern Cape (Mncwangi, Viljoen 2007). Within this distribution there is much variation in the different parts of the plant that is used and its clinical uses. Indeed, *Sutherlandia* is used in treatment of various diseases, including type II diabetes, cancer, peptic ulcer disease, stomach and liver problems and HIV infection (Ojewole, 2004).

Traditionally, the leaves of *Sutherlandia frutescens* are mainly used and usually as tea dosage forms (Hess, 2010), but these traditional tea dosage forms have several disadvantages. Some of these disadvantages include: time and correct method needed for preparation, difficulty in controlling the exact dose, short duration of effect of the herbal active constituents, and high susceptibility to microbial contamination and product degradation. Due to these disadvantages, there is an ever increasing need to use more appropriate and standardized pharmaceutically acceptable dosage forms of herbal products, such as *S. frutescens*, than the tea dosage form.

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Solid dosage forms of dried herbal extract, e.g. tablets and capsules, can be used as a solution to address some of the challenges seen with the traditional tea dosage form of e.g. *S. frutescens.* For instance, solid dosage forms could provide greater accuracy in dosing and good pharmaceutical product quality and stability in terms of shelf life. In addition, capsules and tablets containing the freeze dried aqueous extracts (FDAE) could provide actions similar to that of the tea dosage form. However, the dried extract itself may also have further disadvantages of its own. For instance the dried extract powder may not have uniform particle size, adequate flow characteristics and might be hygroscopic, all of which may lead to difficulties in the manufacture and poor quality of the final product. The hygroscopicity may even lead to instability and degradation of the active constituents of the herbal product. The use of the FDAE of

S. frutescens to produce a solid oral dosage form of this indigenous medicinal plant may thus not be an appropriate solution. Using modified forms, such as phytosomes and liposomes, of the *S. frutescens* FDAE, could however be a more viable solution.

Liposomes are defined as artificial microscopic vesicles consisting of a central aqueous compartment surrounded by one or more concentric phospholipid layers (Laouini *et al.* 2012). They are primarily used to deliver water-soluble substances to the body, most likely the skin. Liposomes are formed by mixing a water-soluble substance with phospholipids leading to a nanoparticle in which no chemical bonds are formed between the substance and lipid. They are useful for drug delivery systems as they are capable of entrapping both hydrophilic and lipophilic drugs into their aqueous compartment and their lipid bilayer, respectively (Banerjee, 2001). Depending on the solvent used, herbal extracts may contain hydrophilic and lipophilic ingredients and liposomes have been made of such extracts (Saraf, 2010).

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Phytosomes are phospholipid-based drug delivery systems that show great promise for herbal drug delivery. For instance, Raju et al, complexed the polyphenolic phytoconstituents in *Silybummarianum* with phosphatidylcholine to produce a new herbal drug delivery system, the phytosomes, in which the phytoconstituents had enhanced capacity to cross lipid-rich bio-membranes and easily enter the systemic circulation leading to augmented bio-availability compared to that of simple herbal extracts (Raju, *et al* 2011). In phytosomes the targeted phyto-compounds complex with the phospholipid and they have the ability to carry hydrophilic, hydrophobic and amphiphilic compounds and thus the potential of giving a better entrapment efficacy for the array of compounds that could be contained in a herbal extract.

Similar to the liposomes, phytosomes thus also have the ability to carry and deliver water soluble phyto-constituents, e.g. flavonoids, etc., that are poorly absorbed, and increase their absorption and bioavailability. It is however not clear which of the two, liposomes or phytosomes, would be the more effective delivery system for a FDAE of *Sutherlandia frutescens*.

Collectively phytosomes and liposomes have characteristics that could improve the product quality of a FDAE of *S. frutescens*. They have the ability to deliver high concentrations of drugs to disease sites because of their unique size and high drug loading capacities. They also deliver drug that has small particle size which in turn increases the entire surface area of the drug allowing quicker dissolution in the gastrointestinal fluids or blood and a decrease in the dose of the drug required in the formulation (Paolino *et al.* 2006).

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Given the above arguments and disadvantages associated with the traditionally used *Sutherlandia* dosage forms and the advantages that modified forms of the FDAE may hold, the overall aim of this present study was to compare the suitability of liposomes and phytosomes of the freeze dried aqueous extract of *Sutherlandia frutescens* for solid oral dosage form of this medicinal plant. The specific objectives were to prepare and physically characterise liposomes and phytosomes of the FDAE of *S. frutescens* and to determine the drug, i.e. flavonoid, release profile of these liposomes and phytosomes.

CHAPTER TWO
LITERATURE REVIEW

2.1 Introduction.

In this chapter an overview is presented, first, on traditional herbal medicine, focusing on the advantages and disadvantages of their use. Then, the South African traditional medicine, *S. frutescens*, is discussed with emphasis on its traditional uses, pharmacological and toxicological effects, potentially active ingredients and the main traditional dosage forms used. Thereafter, the liposome and phytosome delivery systems are reviewed with focus on their properties, similarities and differences, typical phospholipid constituents and methods of preparation and characterisation.

2.2 Traditional Herbal Medicine.

Traditional Herbal medicine (THM) encompasses knowledge and belief-based health practices. It refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being (Wirth, *et al* 2005).

The use of traditional medicine and natural health products is widespread among those living with several diseases. Many patients take a broad range of natural health products (NHPs) in addition to their conventional therapeutic products. In Africa, traditional herbal medicines are often used as primary treatment for diseases such as HIV/AIDS and diabetes (Elujoba, A 2005). Medicinal plant products in

various forms are available to treat diseases and, most importantly, these plant products are the only means of medical treatment for some African communities.

2.2.1 Advantages and Disadvantages of Traditional Herbal Medicine.

Traditional medicine has remained the most affordable and easily accessible source of treatment in the primary health care system of resource poor communities in Africa. There are numerous advantages and disadvantages of herbal medicine thus it is proper for anyone considering using herbal medicine to treat health conditions to speak with a qualified health professional. Some of the claimed advantages of herbal medicines include:

- **Reduced Risk of Side Effects:** Most herbal medicines are well tolerated by the patient, with fewer unintended consequences than pharmaceutical drugs. Herbs typically have fewer side effects than pharmaceutical medicine, and may be safer to use over time (Subhedar, Goswami 2011; Gyamfuaaosei, Y. 2013).
- **Effectiveness in Chronic Conditions:** Herbal medicines tend to be more effective for long-standing health complaints that don't respond well to allopathic medicinal (Miles 1998).
- **Lower Cost:** Another advantage of herbal medicine is low cost. Herbs typically cost much less than prescription medications (Van Agtmael, *et al.* 1999).
- **Widespread Availability:** Yet another advantage of herbal medicines is their availability. Herbs are available without a prescription. You can grow some simple herbs, such as peppermint and chamomile, at home. In some remote parts of the world, herbs may be the only treatment available to the majority of people (Kumar, 2010).

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Herbal Medicines however, also have many disadvantages. These include:

- **Inappropriateness for Many Conditions:** Modern medicine treats sudden and serious illnesses and accidents much more effectively than herbal or alternative treatments (Bandaranayake, 2006).
- **Lack of Dosage Instructions:** Another disadvantage of herbal medicine is the very real risks of harming yourself through self-dosing with herbs. There's a very real risk of overdose (Knishinsky, 2011).
- **Poison Risk Associated with Wild Herbs:** Users run a very real risk of poisoning themselves if they don't correctly identify the herb, or if they use the wrong part of the plant (Bass, 1990).
- **Medication Interactions:** Herbal treatments can interact with medications, which may decrease the bioavailability of the drug or could break up the active constituent for the drug such as tea interacts with ferrous (Fugh-Berman, 2000).
- **Lack of Regulation:** Because herbal products are not tightly regulated, consumers also run the risk of buying inferior quality herbs (Fetrow and Avila, 2000).

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One of the most popular indigenous South African medicinal plants is *Sutherlandia frutescens* which is widely used as traditional medicine as well as commercial products that may be purchased from pharmacies and health shops.

2.3 *Sutherlandia frutescens.*

Sutherlandia frutescens (SF), a member of the Leguminosae family, is a multipurpose medicinal plant endemic to South Africa. Commonly known as 'cancer bush', it has been used in crude form for years by traditional healers to treat a variety of ailments, including internal cancers, diabetes, uterine disease, influenza, HIV, depression, and

arthritis (Phulukdaree, *et al* 2010). Various doses of SF leaf powder have been administered to humans, and have generally produced no obvious side effects (Johnson, *et al* 2007). However, Mills stated in his paper that known side effects include occasional mild diarrhoea, dry mouth, mild diuresis and dizziness (Mills *et al*. 2005).

2.3.1 Vernacular Names and Nomenclature.

Sutherlandia is the common English name for the single stemmed plant with the botanical name *Sutherlandia frutescens (subs. microphylla)*, a flowering bush type plant that grows wild in Southern Africa. It is known by many other names, a reflection of the many cultures that have discovered its remarkable properties. Some of these common names are listed in table 2.1 (Knowles, C. L. 2005).

Table 2.1: Vernacular names and nomenclature of *S. frutescens*

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Name	Language
Cancer bush	(English)
Duck Plant	(English)
Kankerbos	(Afrikaans)
Wildekeer	(Afrikaans)
Rooikeurtjie	(Afrikaans)
Kalkoenbos	(Afrikaans)
Belbos	(Afrikaans)

Gansies	(Afrikaans)
Unwele	(Zulu)
Insiswa	(Zulu)
Musa-Pelo	(Sotho)
Motlepelo	(Sotho)
Phethola	(Sotho)

2.3.2 Botanical Description and Distribution

Sutherlandia frutescens, is a perennial, short-lived shrub which can grow up to 2.5 meters high and which produces red to orange flowers (figure 2.1) during the flowering season which lasts from Spring to Summer (Collling, 2009). The bitter tasting leaves of this plant are compound, pinnate and have a silvery green-gray colour. After flowering, black seeds are produced in the green to red swollen, bladder-like pods, which grow about 5 cm long and become papery upon drying. The plants growing in the coastal regions are shorter and have light gray leaves, whereas the plants growing in the inland regions are taller and have darker gray leaves. During a survey of medicinal plants used in the Karoo region, Van Wyk *et al*. (2008) reported that the *S. frutescens* was the shorter plant (and is considered to be female and is used for men's problems), whilst the taller plant was *S. microphylla* (considered to be male and is used for women's problems).

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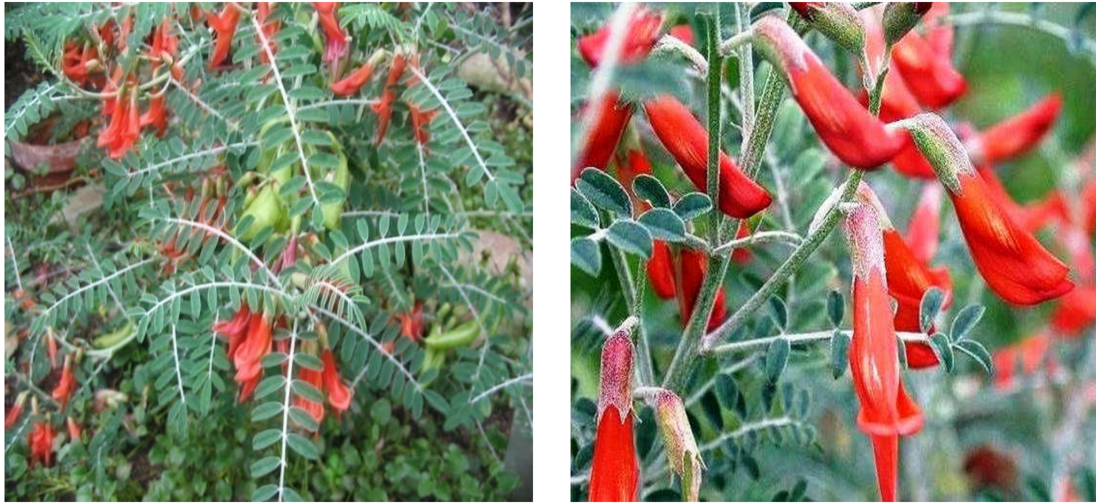

Figure 2.1 ***Sutherlandia frutescens***

The geographic distribution of *Sutherlandia frutescens* is variable and widespread and the plant occurs throughout southern Africa including the drier parts of the Western Cape, Eastern Cape, KwaZulu-Natal, Lesotho, Namibia and the south eastern corner of Botswana. (figure 2.2). According to Xaba and Notten (2003) these plants grow particularly in arid areas where the ground has been disturbed.

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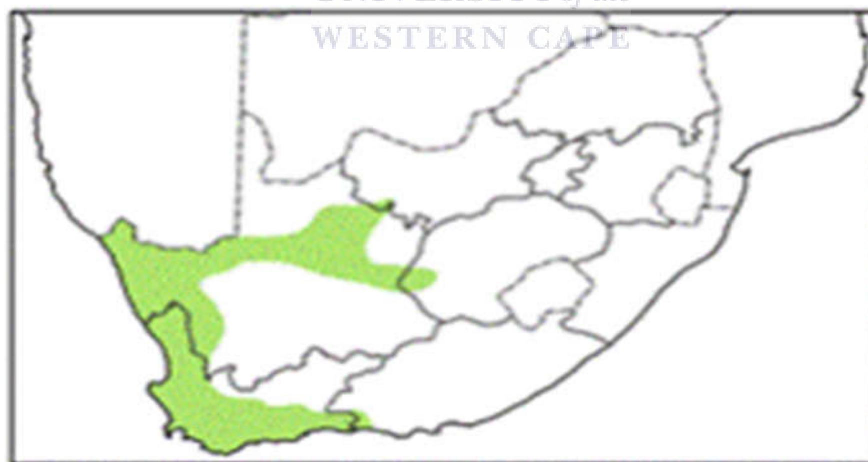

Figure 2.2 **Geographical distribution of *S. frutescens* over Southern Africa adapted from** (Ifeanyi, 2009)**.**

2.3.3 Traditional Uses of *Sutherlandia frutescens.*

Sutherlandia frutescens is among the most multi-purpose and useful of the medicinal plants in Southern Africa. Because of its efficacy and safety as a tonic for diverse health conditions, it has enjoyed a long history of use by all cultures in Southern Africa. The plant helps the immune system to adapt to unfavorable circumstances and is therefore considered an excellent adaptogen (Van Wyk, and Albrecht 2008).

Generally it has been used or advocated for fever, poor appetite, indigestion, heartburn, gastritis, esophagitis, peptic ulcer, dysentery, cancer (prevention and treatment), diabetes (type 2), colds and flu, cough, asthma, chronic bronchitis, kidney and liver conditions, rheumatism, heart failure, urinary tract infections, arthritis, viral hepatitis, Chronic Fatigue Syndrome, stress, anxiety and depression (Kumar, 2010). Thus, tinctures, infusions and decoctions of the leaves and young stems have been used in the Cape from early times, originally by the Khoi and San (Nortje 2011) and the other native groups. The latter include the traditional Tswana who call the plant Phetola or "it changes", meaning that the plant changes the course of many illnesses into a favorable outcome and the North Sotho who name it Lerumo-lamadi i.e. "the spear for the blood" meaning that *Sutherlandia* is a powerful blood-purifier or all-purpose tonic.

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2.3.4 Pharmacological Effects and Toxicology of *Sutherlandia frutescens.*

Over the years several preclinical studies have been conducted to investigate some of the claimed pharmacological activities (e.g. anti-cancer, anti-inflammatory,

antioxidant, and anti-diabetic activities), and the related mechanisms of action, of *S. frutescens.*

Firstly, with regard to the claimed anti-cancer activity it was found that *Sutherlandia* inhibited the proliferation of specific cancer cells by as much as fifty percent (Tai, J. *et al* 2004) and that extracts could kill carcinoma cells. In both cases *Sutherlandia* was only tested *in vitro*, which is to say in a test tube, and not in animals or humans, but these results has led to the conclusion "that these findings warrant further research with a view to develop *Sutherlandia frutescens* extracts for use in anti-cancer therapy" (Chinkwo, K. A. 2005). It should however be noted that these studies only looked at the effect of *Sutherlandia* on cancer cells growing in a laboratory, and not its effect on cancer growing in a human being.

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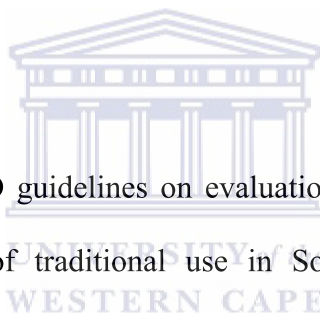
Secondly, polar and non-polar extracts of *S. frutescens* have been demonstrated to possess anti-inflammatory and antioxidant effects. The former effect has been shown *in vitro* and *in vivo* and possibly is mediated via inhibition of cyclooxygenase (COX)-2 expression (Chen, 2007; Katerere and Eloff, 2005; Kundu *et al.*, 2005; Fernandes *et al.*, 2004; Na *et al.*, 2004; Ojewole, 2004), however, researchers at a South African university concluded that the strong anti-oxidant activities of the plant might also explain its reported effectiveness in treating inflammation (Iwalewa, *et al* 2007).

Thirdly, *S. frutescens* is also claimed to have antidiabetic activity. Indeed studies have shown that it decreases blood sugar levels in diabetic rats, possibly due to its pinitol content, although a preliminary study with pinitol in type 2 diabetic individuals did not show encouraging results (Davis *et al.*, 2000). Nevertheless Ojewole (2004) has reported the hypoglycaemic effect of the aqueous extract of the

plant in STZ-induced diabetic rats and demonstrated its ability to normalize insulin levels and glucose uptake in peripheral tissues and to suppress intestinal glucose uptake.

Finally, *Sutherlandia* has shown anticonvulsant and antithrombotic activities (Kee *et al.*, 2008; Ojewole, 2004) and it is also said to have the ability to serve as an adaptogenic agent in stress-related disorders, most likely via attenuation of adrenal P450 enzymes leading to a reduction in glucocorticoid levels (and, by extension, stress symptoms) (Prevoo *et al.*, 2008; Smith and Myburgh, 2004). Collectively, all these preclinical studies have thus presented a reasonable picture of the pharmacological activities of *Sutherlandia* which offers support for its use in the treatment of several ailments.



In accordance with the WHO guidelines on evaluation of herbal medicines and based on its long history of traditional use in South Africa (Addy, 2004) *Sutherlandia* is also generally regarded as having few toxic effects and being safe to use. Moreover, a toxicity study, sponsored by the Medical Research Council of South Africa and the National Research Foundation, has substantiated the safety claims. In this study, monkeys were given 9 times the recommended daily dose (9x 9mg/kg body weight = 81mg/kg body weight) of *Sutherlandia* dry powder over a 3 month period and no changes, compared to the control group, were found in liver, kidneys, muscles, lung, intestine, bone and biochemical parameters (Seier *et al.* 2002).

Apart from the identification of the above-mentioned pharmacological activities of *S. frutescens*, some of its chemical constituents have also been investigated as possible contributors to the effects.

2.4 Active Ingredients of *Sutherlandia frutescens.*

Several active ingredients have so far been found in *Sutherlandia.* For instance, L-canavanine, L-arginine, pinitol, gama-aminobutyric acid (GABA), asparagine and secondary plant metabolites such as saponins (triterpene glycosides) and flavonoids have been isolated from the plant. Of these, flavonoids may be primary candidates for much of the biological and pharmacological effects of this plant.

Sutherlandia frutescens contains several flavonoids which may be responsible for at least some of its biological and pharmacological effects. Some of the flavonoids identified include the flavonol aglycones, quercetin and kaempferol, and their corresponding glycosides, referred to as sutherlandins A, B, C and D (Fu *et al*. 2010). Other glycosides of these aglycones which may be present in *S. frutescens* include rutin, kaempferol-3-O-rutinoside and quercitrin. Sutherlandins A and B, rutin and quercitrin are glycosides of the aglycone, quercetin; while sutherlandins C and D, and kaempferol-3-O-rutinoside are glycosides of the aglycone, kaempferol. The structures of these compounds are shown in figure 2.3.

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The flavonoids found in *S. frutescens* may be responsible for many of the effects of the plant. For instance flavonoids can exert anti-oxidant effects by neutralizing or chelating different types of oxidizing radicals which include the superoxide and hydroxyl radicals (Tobwala *et al*. 2014) Flavonoids have also been shown to demonstrate anti-inflammatory and anti-tumorial properties (Lai *et al*., 2007), and these pharmacological properties have also been reported for *S. frutescens* (Kee *et al*., 2008; Ojewole, 2004). *S. frutescens* shows the ability to serve as an adaptogenic agent in stress-related disorders and this may be due to its flavonoids attenuating

adrenal P450 enzymes leading to a reduction in glucocorticoid levels (and by extension stress symptoms). The presence and levels of flavonoids in any dosage form of *S. frutescens* may thus have a significant influence on the activity or potency of that preparation, whether it is a traditional or modern sophisticated pharmaceutical dosage form of *S. frutescens.*

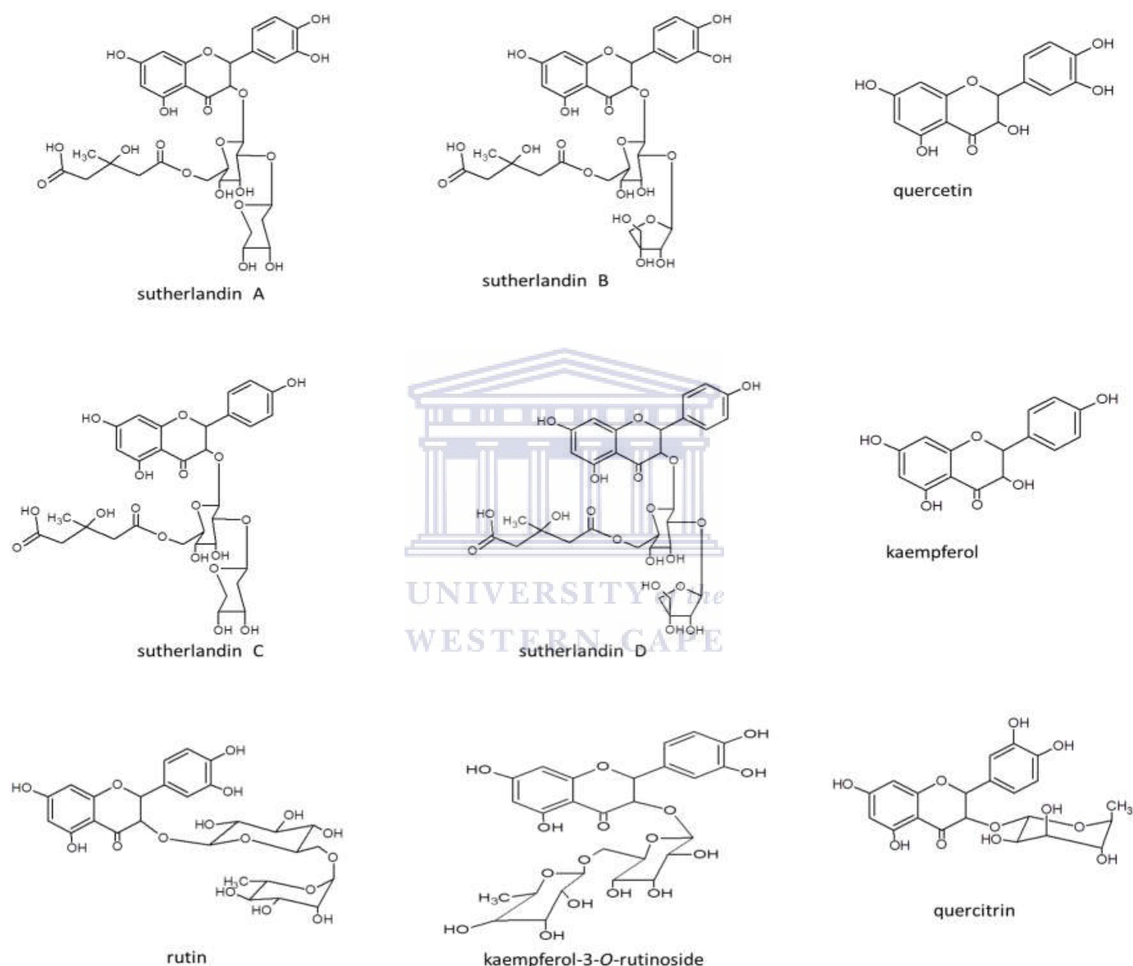

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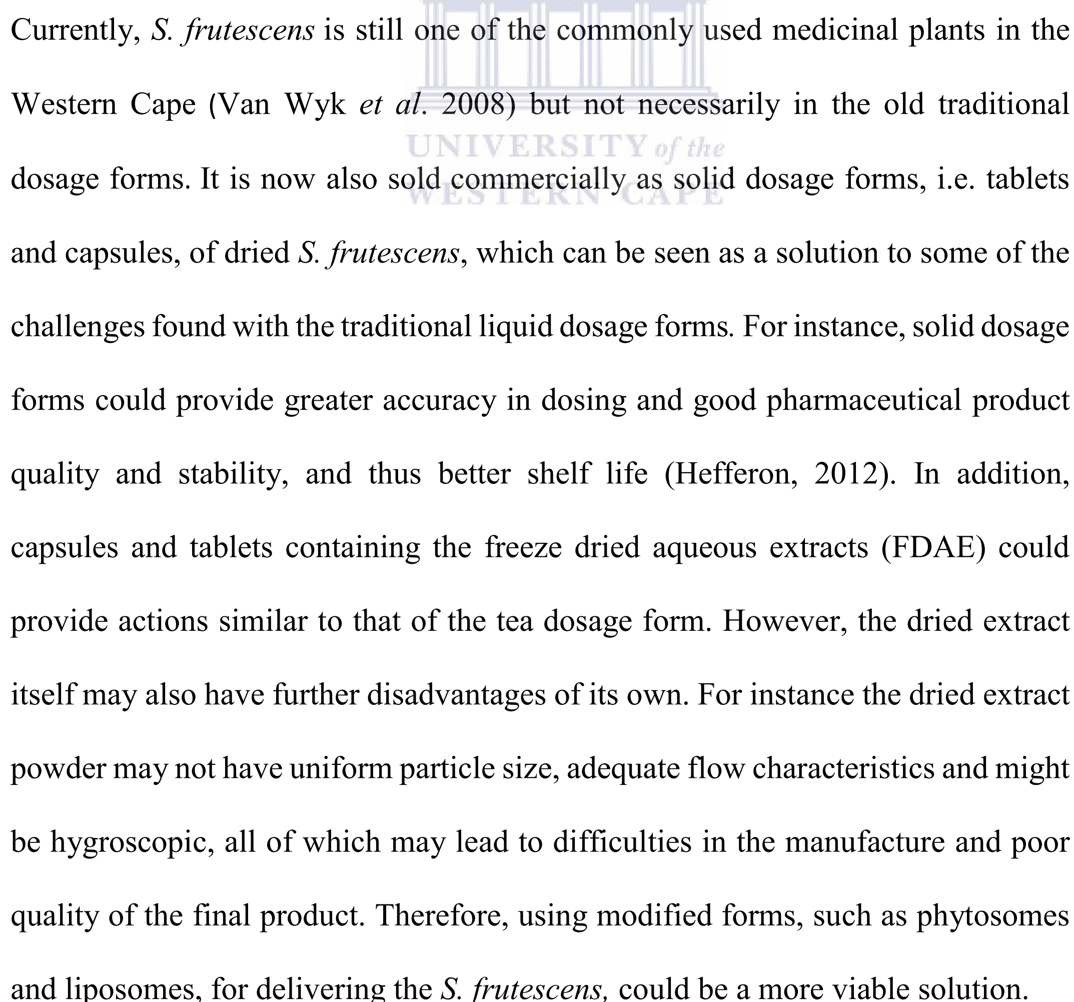
Figure 2.3 ***Structures of the flavonoid glycosides and aglycones.***

2.5 Traditional dosage forms of *S. frutescens*.

Traditionally *Sutherlandia* was used in many ways by the indigenous people of the Cape region, the Khoi, Sotho and Nama. It is used topically for healing cuts and wounds and as a tonic, it serves as an appetite and digestion stimulant (Thring, *et*

al 2006). Generally, a tea made from the leaves is traditionally used for treatment of diseases such as chickenpox, for colds, rheumatism, liver disease and diabetes, while a decoction of *Sutherlandia* is used to wash wounds and the eyes and to reduce fevers, and the infusions from the leaves and stems used to treat cancers, fever, diabetes, kidney and liver problems, rheumatism, and stomach ailments (Aboyade *et al.* 2014). However, these traditional tea and decoction dosage forms have several disadvantages. Some of the disadvantages include: time and correct method needed for preparation, difficulty in controlling the exact dose, short duration of effect of the herbal active constituents, and high susceptibility to microbial contamination and product degradation (Munodawafa, T. 2012).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Currently, *S. frutescens* is still one of the commonly used medicinal plants in the Western Cape (Van Wyk *et al*. 2008) but not necessarily in the old traditional dosage forms. It is now also sold commercially as solid dosage forms, i.e. tablets and capsules, of dried *S. frutescens*, which can be seen as a solution to some of the challenges found with the traditional liquid dosage forms. For instance, solid dosage forms could provide greater accuracy in dosing and good pharmaceutical product quality and stability, and thus better shelf life (Hefferon, 2012). In addition, capsules and tablets containing the freeze dried aqueous extracts (FDAE) could provide actions similar to that of the tea dosage form. However, the dried extract itself may also have further disadvantages of its own. For instance the dried extract powder may not have uniform particle size, adequate flow characteristics and might be hygroscopic, all of which may lead to difficulties in the manufacture and poor quality of the final product. Therefore, using modified forms, such as phytosomes and liposomes, for delivering the *S. frutescens,* could be a more viable solution.

2.6 Drug Delivery Systems.

A drug delivery system (DDS) is a preparation in which a drug is incorporated in a carrier to control the delivery of the drug to the site of action. Examples of DDS include liposomes, ethosomes, niosomes and phytosomes, etc. as shown in figure 2.4. **A** DDS offers the advantages of delivering drugs, including phytoconstituents, at predetermined rate and delivery at the site of action thus minimizing the incidence of toxic effects and increasing the bioavailability of the drugs. This is possible because in DDS technology, control of the distribution of drug is achieved by incorporating the drug in a carrier system or in changing the structure of the drug at molecular level (Dhiman, *et al* 2012). The carrier, firstly, should deliver the drug at a rate as needed by the body over the period of treatment and secondly, it should deliver the actives of the herbal drug to the relevant site of action (Kataoka, *et al* 2001). The use of an effective DDS can thus make herbal drugs less toxic and also enhance their therapeutic effects (Mills, *et al* 2005). Therefore, in this study the use of two such DDS, viz. liposomes and phytosomes for delivery of herbal products, was investigated.

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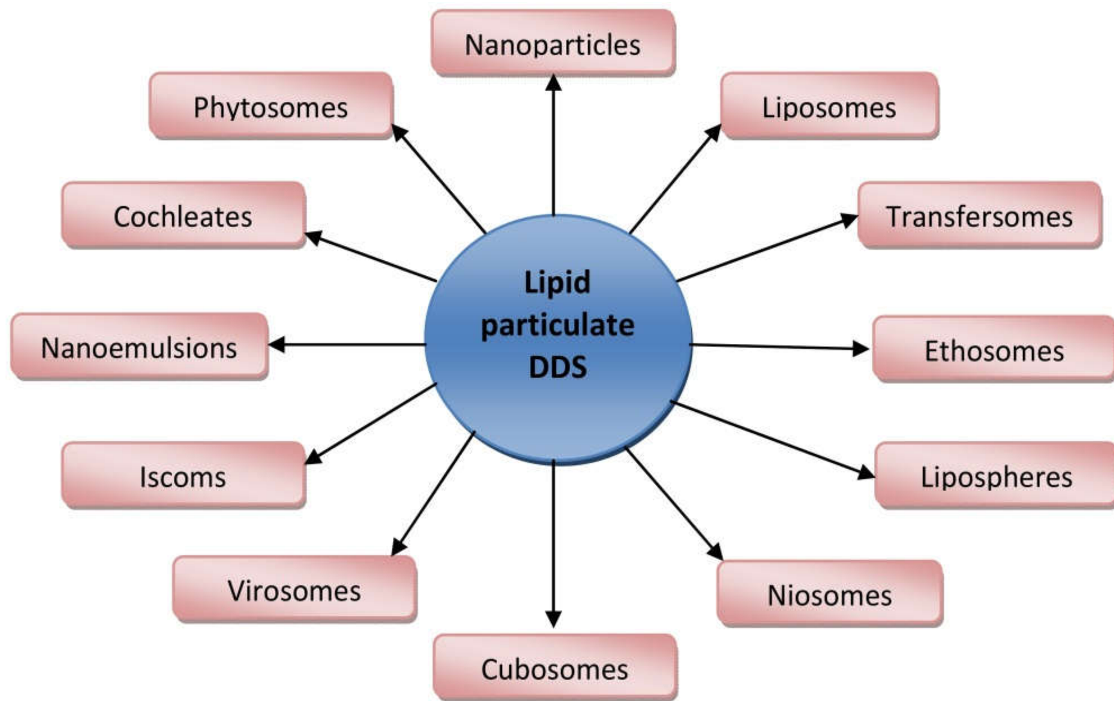

Figure 2.4 Drug delivery systems based on herbal formulation adapted from (Attama, *et al* 2012)

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2.6.1 Liposomes.

A liposome, defined as microscopic spherical-shaped vesicle, consists of an internal aqueous compartment entrapped by one or multiple concentric lipidic bilayers. The liposome's membrane is composed of natural and/or synthetic lipids, which are relatively biocompatible, biodegradable and non-immunogenic material (Laouini, *et al* 2012). This delivery system was first produced in England in the 1960's, by Bangham who was studying phospholipids and blood clotting (Bangham, A. *et al* 1967). According to legend, he was experimenting with new laboratory equipment, and he observed that phospholipids formed closed multilamellar vesicles spontaneously in aqueous solution, an observation which took two years to be fully proved (Laouini *et al*. 2012). Liposomes consist of polar lipids which are characterized by having a lipophilic and hydrophilic group on the same molecule, i.e. are amphiphilic molecules (Gregoriadis, Florence 1993). Upon interaction with

water, the polar lipids self-assemble and form self-organized colloidal particles. A cross-section of a liposome (figure 2.5) depicts the hydrophilic heads of the amphiphile orienting towards the water compartment while the lipophilic tails orient away from the water towards the center of the vesicle, thus forming a bilayer. Consequently, water soluble compounds are entrapped in the water compartment and lipid soluble compounds aggregate in the lipid section. Uniquely, liposomes can encapsulate both hydrophilic and lipophilic materials.

Liposomes have been used to change the pharmacokinetic profile of, not only drugs, but also herbs, vitamins and enzymes. Indeed, a variety of herbal liposomal formulations has been made and studied. Because of their unique properties liposomes are able to enhance the performance of herbal drugs or products by increasing or enhancing solubility (Laouini, et al 2012), bioavailability, intracellular uptake and *in vitro* and *in vivo* stability of the active ingredient as well as alter active ingredient pharmacokinetics and bio-distribution (Jaafar-Maalej, *et al*. 2012). Liposomes, as a drug delivery system, can therefor improve the therapeutic activity and safety of drugs, mainly by optimally delivering them to their site of action and by maintaining therapeutic drug levels for prolonged periods of time. Liposomes of *S. frutescens* could therefore also have these improved qualities.

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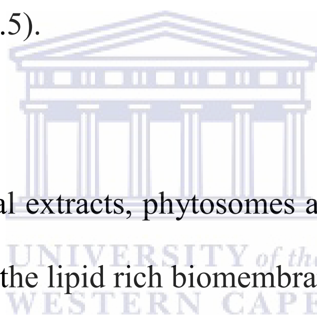
2.6.2 Phytosomes.

Phytosomes are phospholipid-based drug delivery systems that have also been found promising for herbal drug delivery. The term phytosome relates to “phyto”, which means plant; while “some” means cell-like. Phytosomes are advanced forms

of herbal products that are better absorbed and utilized to produce better results than conventional herbal extracts (Choudhury, *et al* 2014). They generally show better pharmacokinetic and therapeutic profiles than conventional herbal extracts (Sindhumol, *et al* 2010).

The production of phytosomes is an intricate process. It involves the incorporation of phospholipids into standardized herbal extracts and the phytosomes are prepared by complexing the poly-phenolic phyto-constituents with phosphatidylcholine in a 1:2 or 1:1 ratio. During this process, a small micro-sphere or cell is produced (Gandhi *et al.* 2012); (figure 2.5).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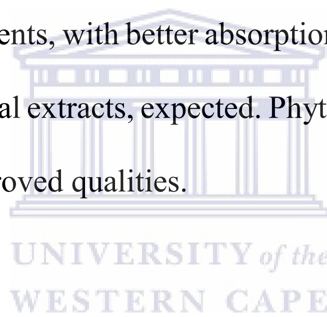
In comparison to simple herbal extracts, phytosomes are more available and have an enhanced capacity to cross the lipid rich biomembranes and finally reaching the blood (Amin, *et al* 2012). Compared to a conventional herbal formulation, a phytosome have following advantages.

- It enhances the absorption of lipid insoluble polar phyto-constituents administered via oral as well as topical routes leading to better bioavailability,
- Besides acting as a carrier, the phosphatidylcholine used in preparation of the phytosome also acts as a hepatoprotective agent hence producing synergistic effect if hepatoprotective substances are to be delivered, and

- Phytosomes can be used for systemic targeting; thus it is widely used in cosmetic technology due to its skin penetration ability and high lipid permeability properties.

The phytosome process has been applied successfully to many popular herbal extracts (Nilesh *et al*., 2010) including *Ginkgo biloba*, grape seed, hawthorn, milk thistle (*Silybum marianum*), green tea (*Thea sinensis*) and ginseng (*Panax ginseng*). The flavonoid and terpenoid components of these herbal extracts are able to directly bind to phosphatidylcholine. Consequently, phytosomes is a particularly exciting new advanced modern dosage formulation technology for delivery of herbal products containing such phytoconstituents, with better absorption and bioavailability than that obtained by conventional herbal extracts, expected. Phytosomes of *S. frutescens* could therefore also have these improved qualities.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2.7 Similarities and Differences between Phytosomes and Liposomes.

Although similar, fundamental differences exist between a phytosome and a liposome. Liposomes are formed by mixing phosphatidylcholine with solution of water soluble substances in a definite ratio. Figure 2.5 shows phosphatidylcholine molecules surrounding the water soluble substance with no chemical bond being formed between them. Usually, there are hundreds or even thousands of phosphatidylcholine molecules surrounding the water-soluble compound (Saraf 2010). In phytosome formation, on the contrary, the plant components and the phosphatidylcholine actually form a 1:1 or a 2:1 molecular complex depending on the plant substance (s) involved. And this leads to the formation of a chemical bond

between the plant constituent and lipid which, in turn, increases the lipophilicity, rate of absorption and bioavailability of the plant constituent in the phytosomes, better than in the case of liposomes. Because of this, the tissue penetrationof drug in phytosomes have generally been found to be superior that for liposomes in topical and skin care products (Bhattacharya 2009).

Another difference between phytosomes and liposomes is their active drug encapsulation efficacies. Phytosomes have a higher drug encapsulation efficacy than liposomes. According to Kareparamban, J. *et al* (2012) this is because the drug itself is conjugated (i.e. bound) with lipids when the vesicles are formed, leading to higher drug levels in the vesicles and, consequently, phytosomes.

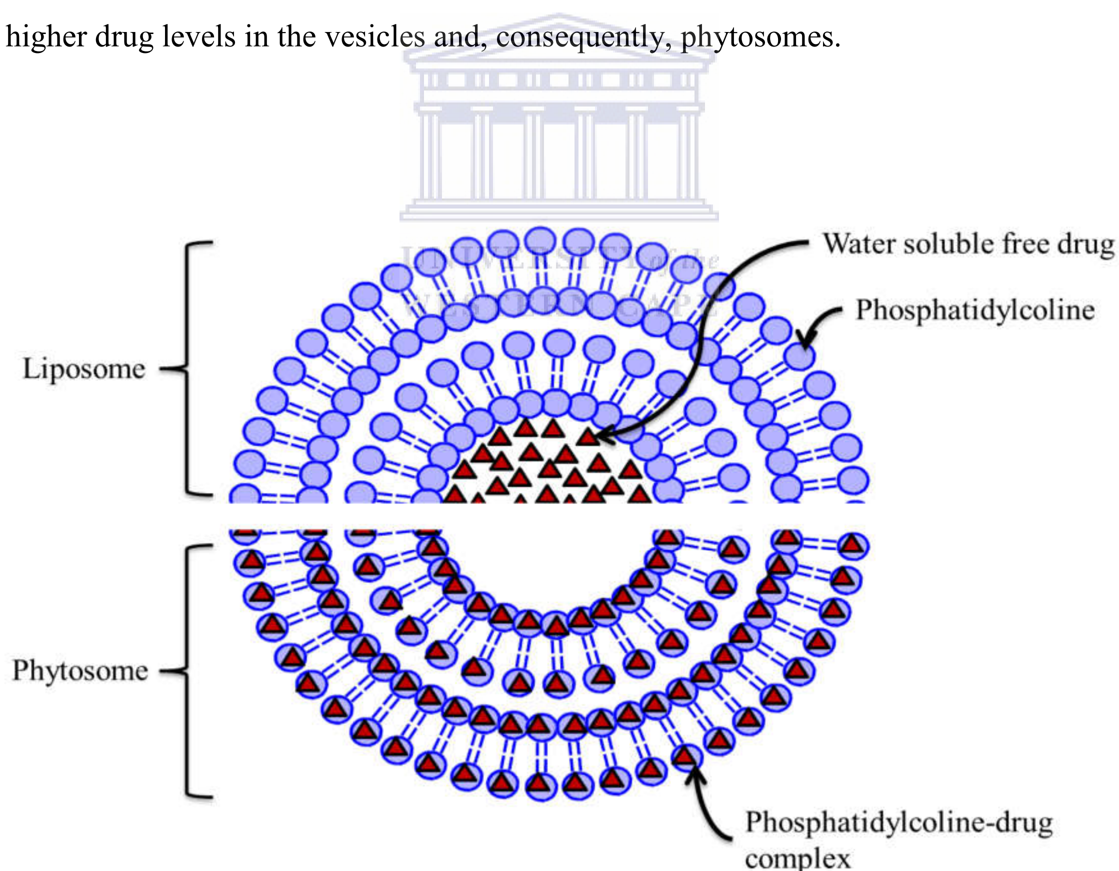
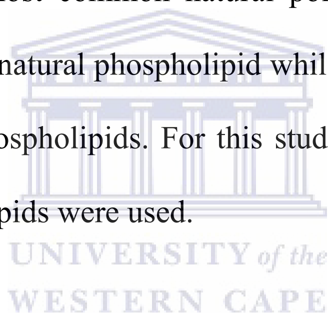


Figure 2.5 Similarities and Difference between phytosome and liposome.

2.8 Phospholipids Used in Preparation of Liposomes and Phytosomes.

Phosphoplipids are amphipathic molecules containing a hydrophilic head group and hydrophobic hydrocarbon chain with glycerol or sphingomyeline as a backbone (figure 2.6) (Thompson 2005). They are found in high levels in all cell membranes of living matter and in aqueous solutions can form into a variety of supramolecular structures through the hydrophobic interactions of the hydrocarbon chains. The amphipathic nature of these molecules in fact gives them the ability to form closed concentric bilayers in the presence of water.

Phospholipids of natural or synthetic origin are used in the production of liposomes. Phosphatidylcholine is the most common natural polar phospholipid used and lecithin another example of a natural phospholipid while the glycerophospholipids are examples of synthetic phospholipids. For this study phosphatidylcholine and lecithin, the natural phospholipids were used.



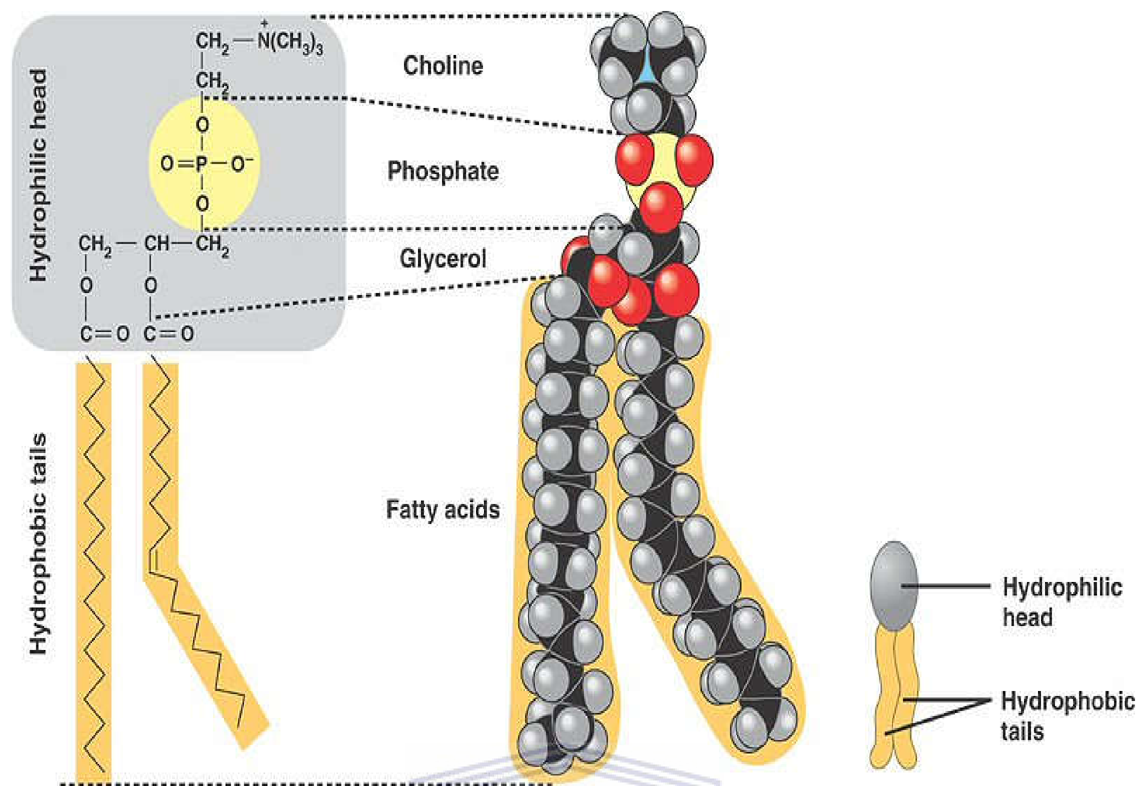


Figure 2.6 **Structure of phospholipids.**

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2.8.1 Lecithin.

Lecithin is a generic term used to designate any group of yellow-brownish fatty substances occurring in animal and plant tissues and is manufactured in the liver from dietary choline. Lecithins are usually phospholipids composed of phosphoric acid with choline, glycerol or other fatty acids, usually glycolipids or triglyceride (i.e. is mostly a mixture of glycolipids, triglycerides, and phospholipids). The glycerophospholipids in lecithin include phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and phosphatidic acid.

Lecithin has for many years been widely used as a pharmacological agent and food supplement. It is most commonly produced by separating and refining of egg yolk

and soy bean. Lecithin has low solubility in water; however in aqueous solution its phospholipids can form liposome bilayer sheets, micelles or lamellar structures, depending on hydration and temperature (Stein, Stein 1969).

2.8.2 Phosphatidylcholine (PC).

Phosphatidylcholine is the most common phospholipid used in the preparation of liposomes and phytosomes, mainly due to its stability and capacity to act against changes in pH (Kulkarn, *et al* 2011). It is also the most important and common lipid found in natural membranes, accounting for 50-90% of the cell membrane (Carmona-Ribeiro 2003). In mammals, PCs are synthesized from the diacylglycerol (DAG) branch of the phospholipid synthetic pathway. Phosphocholine is transferred to carbon-3 via the action of the choline: 1, 2-diacylglycerol choline phosphor transferase. This results in a compound that contains a hydrophilic head group with a quaternary ammonium moiety choline, which is linked to glycerol via a phosphoric ester (Brandl 2001). The permanent positive charge on the choline of the head group counteracts the negative charge of the phosphate to give a neutral hydrophilic head group (Crowell 1997). Phosphatidylcholine is easily obtained from many different sources but most commonly from egg yolk or soybeans through mechanical or chemical extraction using hexane. Phosphatidylcholine is such a major component of lecithin that in some contexts the terms are sometimes used as synonyms. However, lecithin extracts actually consist of a mixture of phosphatidylcholine and other compounds (Adeyeye 2013).

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2.9 Preparation Methods for Phospholipid-based Drug Delivery Systems (DDS).

There are three classical methods of preparation for phospholipid-based drug delivery systems. The difference between the various methods is usually based on the way in which the lipids are dried of organic solvents and then re-dispersed in aqueous media. These steps are performed individually, as described in the following sections.

2.9.1 Hydration of a Thin Lipid Film Method

This is the method which was originally used for liposome production (Bangham, A. *et al* 1967). In this method, the mixture of phospholipid was dissolved in organic solvent for preparation of liposomes, while in the case of phytosomes the lipid phase was dissolved in organic solvent containing the drug substance. Then, the organic solvent was removed by means of evaporation (under reduced pressure using a Rotary Evaporator). Finally, the dry lipid film deposited on wall of the flask was hydrated by adding an aqueous buffer solution under agitation and at temperature above the lipid transition temperature. This resulted in phospholipids dispersed in the aqueous buffer yielding multilamellar liposomes and phytosomes (MLVs) which are heterogeneous in both size (1–5 µm diameter) and shape. This method is widely used and easy to implement. However, to solve the heterogenous size and shape problem new techniques, such as sonication for small unilamellar vesicle (SUV) formation or extrusion through polycarbonate filters for large unilamellar vesicles (LUVs), were introduced to produce smaller and more uniformly sized populations of vesicles (Picard *et al*. 1999).

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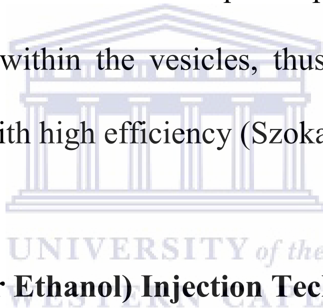
2.9.2 The Reverse-Phase Evaporation (REV) Technique.

In this method a lipidic film is also prepared by evaporating the organic solvent under reduced pressure. The system is then purged with nitrogen and the lipids re-dissolved in a second organic phase, which usually consists of diethyl ether and/or isopropyl ether. Thereafter oligolamellar vesicles are formed when an aqueous buffer is introduced into this mixture. The organic solvent is subsequently removed and the system is maintained under continuous nitrogen.

These vesicles typically have aqueous volume to lipid ratios that are 30 times higher than that of sonicated preparations and 4 times higher than multilamellar vesicles. Most importantly, a substantial fraction of the aqueous phase (up to 62% at low salt concentrations) is entrapped within the vesicles, thus encapsulating even large macromolecular assemblies with high efficiency (Szoka, Papahadjopoulos 1978).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2.9.3 The Solvent (Ether or Ethanol) Injection Technique.

The solvent injection methods involve the dissolution of the lipid into an organic phase (ethanol or ether), followed by the injection of the lipid solution into aqueous media to form liposomes. The ethanol injection method was first described in 1973 (Wagner, Vorauer-Uhl and Katinger 2002) and the main relevance of the ethanol injection method resides in the observation that a narrow size distribution of small liposomes (under 100 nm in size) can be obtained, in one step, by simply injecting an ethanolic lipid solution into water without extrusion or sonication. The ether injection method differs from the ethanol injection method in that the ether is immiscible with the aqueous phase, and the latter heated to remove the solvent from the liposomal product. Specifically, the method involves injection of ether-lipid solutions into aqueous phases warmed at above the boiling point of the ether. The

ether vaporizes upon contacting the aqueous phase, and the dispersed lipid forms into, primarily, unilamellar liposomes. An advantage of the ether injection method, compared to the ethanol injection method, is that this removal of the solvent from the product enables the process to be run for extended periods forming a concentrated liposomal product with high entrapment efficiencies.

For the present study, the thin film hydration technique was chosen because of its simplicity, as well as the fact that it does not introduce any impurities that can affect the phase behavior and release properties of liposomes, as may occur in the case of the reverse phase evaporation method (Akrachalanont, P. 2008)**.**

2.10 Classification of Drug Delivery Systems Based on Size.

Liposomes and phytosomes vary in size usually, depending on the method of production, ranging from 20 nm upwards and may be composed of one or many concentric layers, each with a thickness of roughly 4 nm (figure 2.7) (Barbe *et al*. 2004). Sonication is the most extensively used method for preparation of liposomes in a size range of 200 nm and above. Here the multilamellar vesicles (MLVs) are transformed into SUVs (small unilamellar vesicles) by either probe or bath sonication (Lichtenberg, Barenholz 1988).

For optimal therapeutic use and administration route considerations the size of novel drug systems is an important parameter since size will influence the rate of clearance of the DDS by the reticular endothelial system and its ability to remain in the blood vessels after introduction into the human body. The size also influences the drug loading capacity and the bioavailability of the DDS (Saraf 2010).

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Therefore, novel drug delivery systems are typically classified on the basis of their size and number of bilayers as shown in Table 2.2.

Table 2.2: Size specification of vesicular novel drug delivery systems

Abbreviation	**Type of vesicles**	**Size Specification**
MLV	Multilamellar large vesicle	> 0.5μm
OLV	Oligolamellar vesicles-	0.1 - 1μm
UV	Unilamellar vesicles	No fixed size range (20 nm - >1 μm)
SUV	Small Unilamellar vesicles-	20-100 nm
MUV	Medium sized Unilamellar vesicles	40-80 nm
LUV	Large Unilamellar vesicles	> 100-200 nm
GUV	Giant Unilamellar vesicles-	> 1μm
MV	Multivesicular vesicles-	1μm

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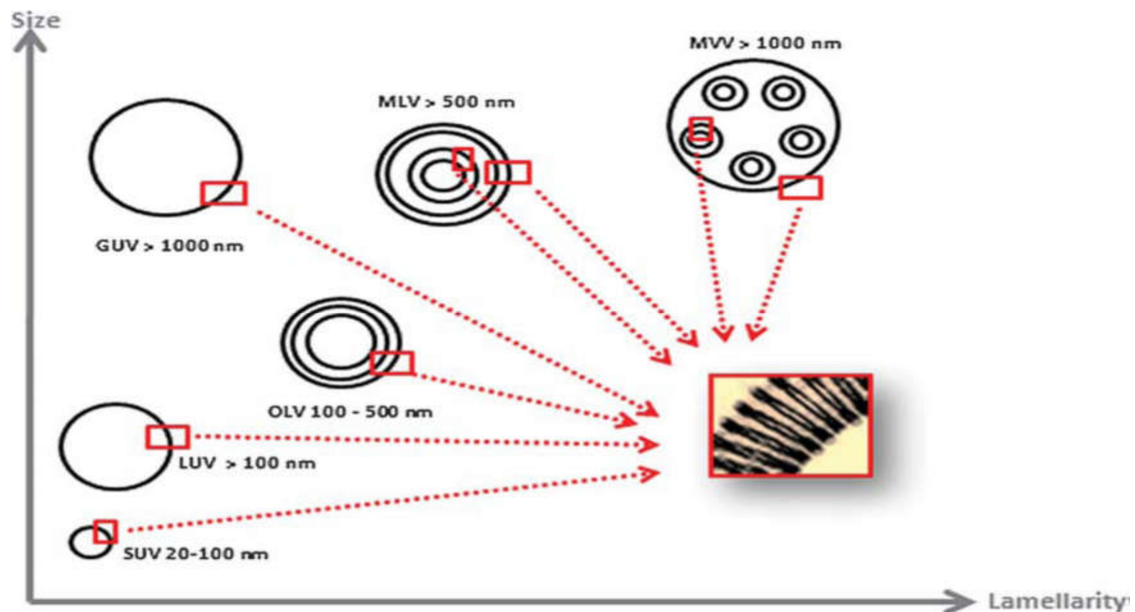

Figure 2.7 Classification of drug delivery systems based on size (Laouini *et al*. 2012)

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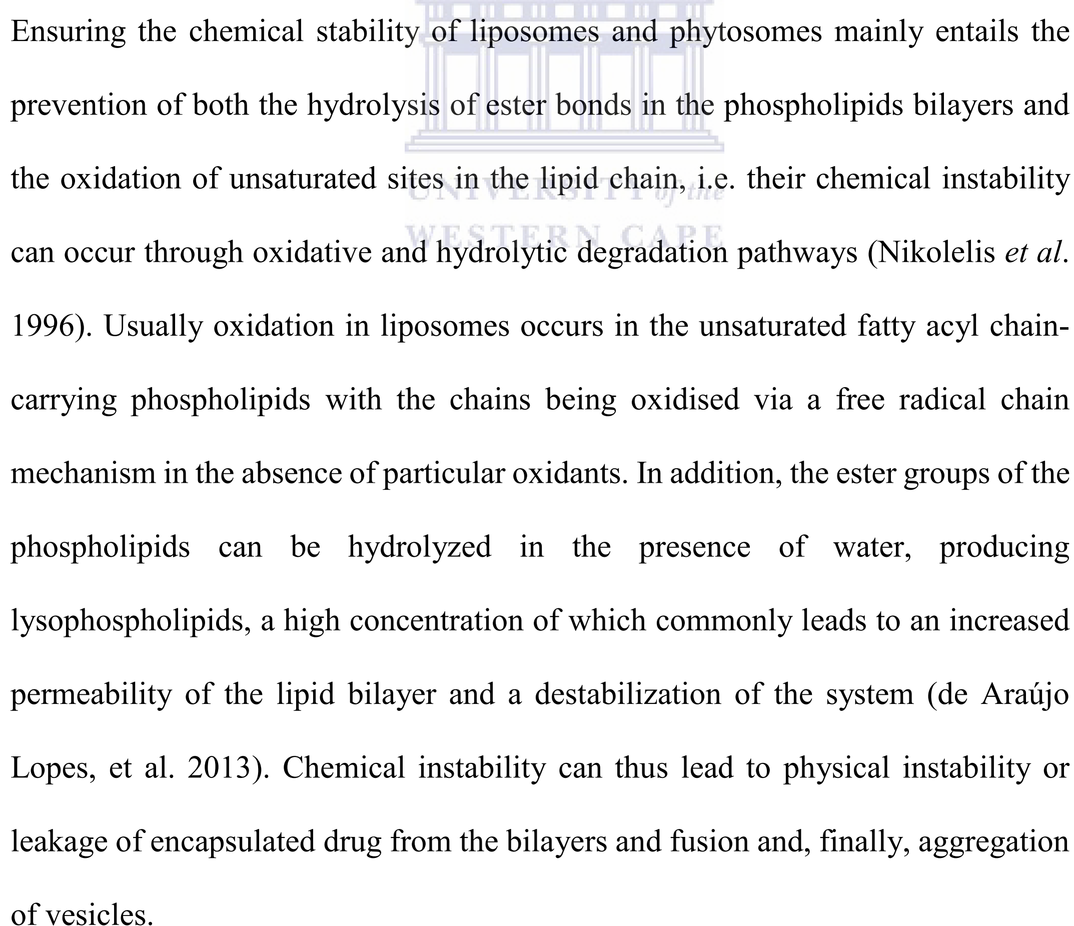
2.11 Novel Drug Delivery System Stability.

The stability of a DDS, such as a liposome or phytosome, is another important, and sometimes complex, pharmaceutical characteristic that must be determined and known. Novel drug delivery system stability entails both physical and chemical stability.

The physical stability of a DDS is mostly assessed by determining the size and charge, and the lipid to active agent ratio used for, the DDS. The charge of a DDS may be measured in terms of Zeta potential (i.e. the difference in electric potential between the dispersion medium and the stationary layer of fluid attached to a dispersed particle) and a low Zeta potential (i.e. in the range between +30mv to -30mv) results in physical instability of such a nano-formulation (Averineni *et al*.,

2012). The charge of the liposome surface should be considered if the possibility of aggregation is considered. The ability of liposomes and phytosomes to maintain their superficial charge for long durations during storage adds to the high physical stability of the formulation and shelf life. Thus, cationic liposomes can be stable at 4°C for long periods of time, if properly sterilized (Tikshdeep, C. *et al*. 2012). Finally, while the lipid to active agent ratio used also affects the physical stability of a DDS, phytosomes also have a better stability profile due to the formation of chemical bonds between phosphatidylcholine molecules and the phytoconstituents (Kareparamban, J. *et al* 2012).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Ensuring the chemical stability of liposomes and phytosomes mainly entails the prevention of both the hydrolysis of ester bonds in the phospholipids bilayers and the oxidation of unsaturated sites in the lipid chain, i.e. their chemical instability can occur through oxidative and hydrolytic degradation pathways (Nikolelis *et al*. 1996). Usually oxidation in liposomes occurs in the unsaturated fatty acyl chain-carrying phospholipids with the chains being oxidised via a free radical chain mechanism in the absence of particular oxidants. In addition, the ester groups of the phospholipids can be hydrolyzed in the presence of water, producing lysophospholipids, a high concentration of which commonly leads to an increased permeability of the lipid bilayer and a destabilization of the system (de Araújo Lopes, et al. 2013). Chemical instability can thus lead to physical instability or leakage of encapsulated drug from the bilayers and fusion and, finally, aggregation of vesicles.

Finally, several approaches can be taken to increase the physical and chemical stability of liposomes, including the following precautions (Kaur, *et al* 2013):

- To reduce oxidation, novel drug delivery systems can be stored at low temperatures, protected from light and antioxidants such as α-tocopherol and butyl hydroxy toluene added. More so, processing with fresh, purified lipids and solvents also helps to reduce oxidation.
- Avoidance of high temperature and excessive shear forces.
- Maintenance of low oxygen potential (i.e. nitrogen purging) by working under nitrogen or argon thereby minimizing the oxidation of lipids during preparation.
- Use of antioxidant or metal chelators (as additional excipients or during preparation).
- The hydrolysis of ester bonds can be reduced by optimising the pH, temperature, ionic strength, chain length and the amount of cholesterol incorporated into the bilayer during (liposome) preparation (Rosen, Kunjappu 2012).
- Formulating a preparation having or at neutral pH.

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Using these strategies DDS with increased or optimal physical and chemical stability can be obtained.

2.12 Characterization of Liposomes and Phytosomes

The characterization of novel drug delivery systems such as liposomes and phytosomes can be divided into three parts: physical, chemical and biological characterization.

Physical characterization mainly consists of evaluating the size, shape, surface features, lamellarity, phase behaviors and drug release profile of the liposome or phytosome. A variety of useful parameters can be determined using the methods and instruments indicated in table 2.3.

Table 2.3: Physical parameters and instrumental methods of analysis for characterization of novel drug delivery systems (e.g. liposomes and phytosomes)

Characterization parameter	**Instrument for analysis**
Vesicle shape, and surface morphology	TEM and SEM
Vesicle size and size distribution	Dynamic light scattering ,TEM
Surface charge	Free flow electrophoresis
Electrical surface potential and surfacePH	Zeta potential measurement and PHsensitive probes
Lamellarity	P31NMR
Phase behaviour	DSC, freeze fracture electron microscopy
Percent capture	Mini column centrifugation, gel exclusion
Drug release	Diffuse cell/ dialysis

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On the other hand, the chemical characterization of liposomes is mostly focused on establishing the level and purity of the various liposomal constituents. This includes the quantification of phospholipids, level of lipid oxidation, pH and the percentage of encapsulated drug (active constituent). These characteristics can be determined using the methods and instruments indicated in table 2.4.

Table 2.4: Parameters and methods used for chemical characterization of lipid-based drug delivery systems.

Characterization parameter	Instrument for analysis
Phospholipids concentration	HPLC/Barrlet assay
Cholesterol concentration	HPLC / cholesterol oxide assay
Drug concentration	Assay method
Phospholipids per oxidation	UV observance
Phospholipids hydrolysis	HPLC/ TLC
Cholesterol auto-oxidation	HPLC/ TLC
Anti-oxidant degradation	HPLC/TLC
PH	PH meter
Osmolarity	Osmometer

Finally, biological characterization of DDS is also important and must be done to establish the safety profiles and suitability of the DDS formulation for therapeutic application. However, by just taking the physical and chemical characteristics into account, a reasonably fair idea of the final *in vitro* and *in vivo* behavior of a new DDS can already be obtained.

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2.13 Drug Release Profile of Drug Delivery System.

Finally, one of the most important characteristics of a drug delivery system is its ability to release drug from the carrier. There are numerous factors that affect drug release, e.g. drug solubility, desorption kinetics of adsorbed drug, diffusion of drug through the nanoparticles, nanoparticle degradation, and the combination of degradation and diffusion (Lockman, P. R., 2012). Typically, in conventional nanospheres, the drug is distributed uniformly and diffusion or erosion of the matrix causes the drug to be released. If diffusion happens faster than matrix erosion, the

release mechanism is controlled by diffusion (Siegel, R. A., and Rathbone, M. J. 2012).

Kinetic experiments are usually done to determine the release characteristics of a DDS and such kinetic experiments are carried out respecting/maintaining sink conditions in the receptor compartment (Ammoury, *et al* 1990). Samples are then taken at various time intervals and assayed for the drug by HPLC, spectrophotometry or any other convenient method. The sample volume is normally replaced with fresh dissolution medium so that the volume of the receptor compartment remains constant. Every kinetic experiment is performed in triplicate and the average values are taken to establish the release profile of the drug from the liposome and phytosomes suspension (Ammoury, *et al* 1990).

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CHAPTER THREE
Plan of Work

3.1 Introduction

In this chapter, the specific objectives, hypotheses and study approach for this study are described.

3.2 Objectives

The overall aim of the study was to establish which of phytosomes or liposomes are the better dosage form for delivery of flavonoid actives from the freeze-dried aqueous extract of *Sutherlandia frutescens*.

The specific objectives of the study were to:

1. Formulate and prepare phytosomes and liposomes containing *Sutherlandia frutescens* freeze dried aqueous extract (FDAE),

2. Determine and compare the physical characteristics i.e., particle shape, size, size distribution (in terms of polydispersity index) and stability (in terms of zeta potential) of the *Sutherlandia* phytosomes and liposomes,

3. Determine the extent of encapsulation of the *Sutherlandia* flavonoids in these phytosomes *versus* liposomes, and

4. Compare the flavonoid release profiles from the *Sutherlandia* containing phytosomes *versus* liposomes.

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3.3 Hypotheses

It was hypothesized that:

1. Synthesized *S. frutescens* phytosomes will have a higher encapsulation efficiency (i.e. *Sutherlandia* flavonoid content), than the synthesized *S. frutescens* liposomes, i.e.

$$EE\%: \quad EE\%_{\text{Phytosomes}} > EE\%_{\text{Liposomes}}$$

Where the encapsulation efficacy (EE %) is given by the following equation.

$$\mathbf{EE\%} = \frac{C_{total} - C_{free}}{C_{total}} \mathbf{X100}$$

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And C_{total} are the relative amounts (i.e. peak area under the curve of sutherlandins A to D) of individual flavonoid in the starting *Sutherlandia* FDAE solution and C_{free} the concentration in the supernatant after separation of the liposomes or phytosomes.

2. At both gastric and intestinal fluid pH, the amount of flavonoid glycosides (i.e. sutherlandins A to D) released from the *S. frutescens*-containing liposomes will be greater than that for the phytosomes, i.e.:

pH 1.2	pH 6.8
$[Suth.A]^{Liposomes} > [Suth. A]^{Phytosomes}$	$[Suth.A]^{Liposomes} > [Suth. A]^{Phytosomes}$
$[Suth.B]^{Liposomes} > [Suth. B]^{Phytosomes}$	$[Suth.B]^{Liposomes} > [Suth. B]^{Phytosomes}$
$[Suth.C]^{Liposomes} > [Suth. C]^{Phytosomes}$	$[Suth.C]^{Liposomes} > [Suth. C]^{Phytosomes}$
$[Suth.D]^{Liposomes} > [Suth. D]^{Phytosomes}$	$[Suth.D]^{Liposomes} > [Suth. D]^{Phytosomes}$

Where $[Suth\ A, B, C, or\ D]^{Liposomes}$ are the relative amount of sutherlandin A. B, C or D in the liposomes. And $[Suth\ A, B, C, or\ D]^{Phytosomes}$ are relative amount of sutherlandin A. B, C or D in the phytosomes.

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The drug release profile (i.e. % released over time) was determined using the following equation to calculate the percentage released at each time point.

$$\textbf{Drug release (\%)} = \frac{\text{Amount of drug released at specified time point}}{\text{Amount of drug loaded in to nano particle}} \times 100$$

3.4 Study approach and methods

To achieve the above objectives, the following were done.

1. Preparation of *S. frutescens* freeze dried aqueous extract liposomes and phytosomes.

2. Characterization of particle size, shape and stability and extent of encapsulation of *S. frutescens* in phytosomes *versus* liposomes
3. Determination of release profile of the flavonoids contained in *S. frutescens* phytosomes *versus* liposomes.

3.4.1 Preparation of liposomes and phytosomes of *S. frutescens* freeze dried aqueous extract.

For this study, the thin film hydration technique was chosen to prepare the liposomes and phytosomes because of its simplicity as well as the fact that it does not introduce any impurities that can affect the phase behavior and release properties of liposomes, as may occur in the case of the reverse phase evaporation method or detergent dialysis methods (Liang, *et al* 2005).

The method involved mixing lipid compositions in an organic solvent to ensure a homogeneous mixture of lipids with or without the inclusion of hydrophilic molecules for the formation of a dry film. The dry film of lipids would then be deposited onto the wall of a round-bottom flask in a rotary evaporator at a temperature above the lipid's phase transition temperature. Thereafter, the thin film in the round bottom flask was to be hydrated by adding a buffer solution containing the hydrophilic drugs or plant compounds to be encapsulated. This method usually yielded a heterogeneous population of multilamellar liposomes (MLVs) in a size range of 200nm and above, therefore liposome and phytosome size reduction techniques, such as sonication for SUVs formation was further required (Laouini *et al*. 2012) and to be performed in this study.

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3.4.2 Characterization of the *S. frutescens* phytosomes and liposomes.

In order to assess the quality of the liposomes or phytosomes and to obtain quantitative measures that allow comparison of differences between the liposomes and phytosomes, various parameters should be monitored. For drug delivery system applications in analytical and bio-analytical fields, the main characteristics to monitor usually include the particle shape, average mean diameter, size distribution (i.e. polydispersity index) and stability and encapsulation efficiency (Pathak, Thassu 2009), and these characteristics were consequently determined for the *S. frutescens* liposomes and phytosomes in this study.

Specifically, the average size and size distribution of liposomes and phytosomes were measured by Photon Correlation Spectroscopy (PCS) based on dynamic light scattering (DLS) technology. Advantages of using this method include its sensitivity, minimal sample requirement and wide range of applications.(Edwards, Baeumner 2006). For the morphology analysis, the pellet of the liposomes and phytosomes were examined photographically with a scanning electron microscope, a standard procedure to determine the shape and size of the particles.

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The zeta potential of a particle, which is the overall charge that a particle acquires in a particular medium, is another physical property which is exhibited by any particle in suspension and is a very good index of the interaction magnitude between colloidal particles (Wang et al. 2006). If the particles have low zeta potential values, then there will be no force to prevent the particles from aggregating and particle suspensions with zeta potentials $> +30$ mV or < -30 mV are normally considered stable (Lee, Lee &

Jeon 2007). This technique was therefore also used to compare the stability of the liposome *versus* phytosomes particles in the present study.

Finally, the prepared liposomes and phytosomes would contain a mixture of encapsulated and un-encapsulated freeze-dried aqueous extract of *S. frutescens* (drug fractions). For this study the glycosides Sutherlandin A to D was chosen as active marker compounds representing the *S. frutescens* flavonoids, especially due to their known presence in the *S. frutescens* FDAE and because their presence and levels would be representative of changes or differences in the active components of the medicinal plant in the different forms and /or under different conditions. The encapsulation efficiencies of the different forms was also to be compared and the first step required for this determination was the effective separation of the encapsulated drug (i.e. that within the carrier) and the free drug (i.e. in FDAE). For this centrifugation was to be used. The latter is a method that separates the material based on the difference in mass of the drug loaded liposomes or phytosomes and the free drug. Thus the drug loaded liposomes or phytosomes would be in the pellet, while the free drug (i.e. Sutherlandin A to D) was in the supernatant. And finally, for the encapsulation efficiency comparison, the free drug in the various fractions had to be quantified and for this an HPLC assay was used (because using a plate reader and non-specific UV detection method for this purpose did not proof successful in this study). This HPLC assay had to be developed and validated –see 3.4.3.

3.4.3 Determination of the drug release profile of *S. frutescens* liposomes *versus* phytosomes.

The purpose of the drug release profile study was to determine and compare the amounts of flavonoids (as active drug marker compounds) the *S. frutescens* liposomes

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and phytosomes contained and for this the HPLC assay was used. This is a commonly used and sensitive technique for the assay of flavonoids in plant samples. The latter assay was also used because of its ability to separate and quantitate multiple components present in plant matrices.

To determine the drug release profiles two different buffer solutions were used to mimic some of the various physiological parts of the gastrointestinal tract. The pH 1.2 medium was used to represent the gastric condition, while pH 6.8 the conditions in the small intestine. Furthermore, samples were taken at specific time intervals (i.e. 5, 15, 30, 60, 90, 120 minutes) to provide a drug release over time profile for both the liposomes and phytosomes which could then be compared using the parameters, AR_{120} (%) i.e. average amount released at 120 min, R_{AR50} i.e. time of the 50 % amount released and f_2, the similarity factor (Martinez, Amidon 2002). The latter, in particular, is a standard parameter used for such comparison purposes.

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CHAPTER FOUR
Preparation and characterization of *S. frutescens* liposomes and phytosomes

4.1 Introduction

One major aim of this study was to prepare improved active phytopharmaeutical (i.e. API) forms of *S. frutescens*. Specifically, liposomes and phytosomes of the freeze-dried aqueous extract of *S. frutescens* were to be prepared. In this chapter the materials, equipment and unique methodology used to prepare and characterise the liposomes and phytosomes of *S. frutescens* are firstly described. In addition to the freeze-dried extract of *S. frutescens,* liposomes and phytosomes of quercetin and rutin, as marker aglycone and glycoside flavonoids, respectively, were also prepared and characterised. As part of the characterisation the encapsulation efficiency of the liposomes and phytosomes were determined. For this an HPLC assay was required and the development and validation of the assay used is described. Finally, the results obtained from all these assessments are presented and discussed.

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4.2 Materials

4.2.1 Chemicals and reagents.

The following chemicals, reagents and equipment were used.

Acetonitrile (HPLC grade, *Burdick and Jackson®, USA*); **methanol** and **chloroform** (analytical grade, AR *Saarchem, Merck Chemicals, PTY Ltd*); **hydrochloric acid 32%** (analytical grade, AR *Saarchem, Merck Chemicals, PTY Ltd*); **formic acid 99%** (analytical grade, *KIMIX Chemicals & laboratory suppliers*, *USA*); **phospholipids** (Lecithin powder, *Warren Chemical, Germany)*

and L-α-phosphatidylcholine (*Avanti Polar Lipids. USA*); ***Sutherlandia frutescens* PE** (freeze dried aqueous extract (FDAE),BN E62265, see appendix 1, *Afriplex Pty, Paarl, South Africa*); **rutin and quercetin di-hydrate** (*Sigma Aldrich*, *Germany*); **sutherlandin A, B, C and, D** *(isolated at School of Pharmacy, UWC* (see O. Mbamalu *et al.*, 2015)) **Triton x100** (*Sigma Aldrich, Germany*); **distilled water** (prepared using a Purite Select Analyst HP water purification system); **Phosphate Buffer Solution of pH 7.4** (Lonza B-4800, Verviers, *Belgium*).

4.2.2 Equipment and instruments

The following equipment and instruments were used.

High Performance Liquid Chromatography (HPLC) System (An Agilent HPLC system 1200 series consisting of a G1311A Quaternary Pump, a G1322A Degasser, a G1315B Diode-array detector and a G1329A Auto Sampler with, Agilent ChemStation software); **C18 reverse phase column** (Phenomenex Luna® C_{18} column, 5µm particle size and column size of 250 × 4.6 mm, *Puerto Rico, USA*); **2cm LC-18 guard column** (Phenomenex 2cm, *Torrance, CA, USA*).

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Rotor evaporator (V- 700/710 (*BUCHI, Switzerland*); pH meter (*Model PL-700PV, Taipei, Taiwan*); **ultra-sonic bath** (Model 702 Voltage 2030v, 100W, *LABOTEC, South Africa*); **sputter coater** (K550X Emitech, England); **weighing balance** (*AR 2140, 210g United Scientific Ltd, Ohaus Corp. Pine Brook, NJ, USA)***;** **ultracentrifuge** (*Beckman Coulter Optima L-80, Beckman***,** *USA*); **vortex mixer** (*Vortex-2G-560E, Scientific Industries, Inc. Bohemia, N.Y 11716 USA*); **Zetasizer** (*Nano series, Malvern instrument, UK*); **Zetasizer software 7.10** (*Nano series, Malvern, UK*); **Disposable cuvettes DTS0012** (*Nano series, Malvern, UK*);

Disposable flooded capillary cell DTS1070 (*Nano series, Malvern, UK*); **non-sterile syringe filter** (0.45micron size, *Pall life Sciences, USA*); **parafilm M sealing film** (Code: 701605, length 38m, *Merck Chemicals, PTY Ltd);* **Millipore centrifuge tube 50** (91015, polypropylene, conical base 50ml, *TPP, Switzerland*).

4.3 Methods

4.3.1 Preparation of liposomes.

The thin film hydration method (Samad, Sultana and Aqil 2007) was used and the steps for the liposome preparation are summarized in figure 4.1.

For the *S. frutescens* liposomes, 600 mg lecithin (i.e. lipid phase) was mixed and dissolved in 20 ml chloroform for 10 minutes in a 50 ml Millipore centrifuge tube. The resultant clear phospholipid solution was transferred to a round bottom flask, the latter attached to a rotatory evaporator and the solution evaporated to dryness at 60 ^{o}C and 50 rpm. A thin lipid film was obtained. For the hydration phase, 200 mg *S. frutescens* was dissolved in 20 ml PBS pH 7.4 kept at 50 ^{0}C i.e. above the phase transition temperature (Tm) of the lipid. One milliliter of this hydration solution was taken for HPLC analysis of the total amount of flavonoids it contain (i.e. A_{total}, see section 4.3.8) while the rest was then added to the thin lipid film, the mixture in the flask vigorously shaken by hand and vortexed for 20 min and then cooled down in fridge for 30 mins. Thereafter, the liposome suspension was vortexed for a further 30 mins. To reduce the size of (i.e. down size) the liposomes, the sonication method was used - see section 4.3.3.

This same method of liposome preparation was also used to prepare the liposomes of the pure compounds, rutin and quercetin, except that in this case 20 mg of the respective flavonoids and 60 mg of the lecithin was used.

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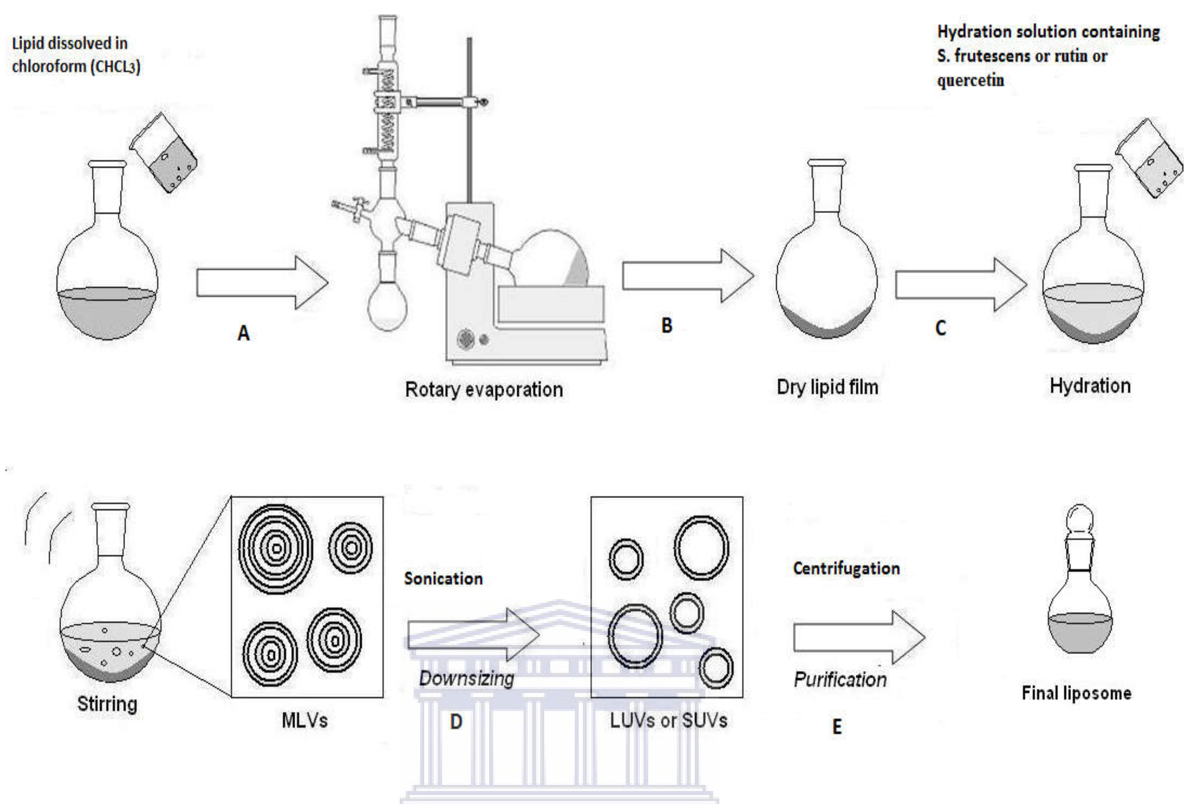

Figure 4.1 Schematic for the preparation of liposomes

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4.3.2 Preparation of phytosomes.

For the phytosome preparation the same method was used and is summarized in figure 4.2. In this case, 200 mg of FDAE and 400 mg of phosphatidylcholine i.e. lipid phase, was first mixed and dissolved in 20 ml of chloroform for 10 min in 50 ml Millipore centrifuge tube and the resultant mixture containing the phosphatidylcholine - *S. frutescens* FDAE complex evaporated to dryness on a rotary evaporator at 60 ^{0}C and 50 rpm. Thereafter, the thin lipid film loaded with crude extract obtained, was hydrated with 20 ml PBS pH 7.4 at 50 ^{0}C, a temperature above the phase transition temperature (Tm) of the lipid, the mixture (i.e. hydrated

solution) vigorously shaken by hand and vortexed for 20 min and then cooled down in a fridge for 30 minutes. The resultant phytosome suspension was vortexed for a further 30 minutes, and to down size the phytosomes, the suspension was sonicated for 45 minutes – see section 4.3.3.

This same method was also used to prepare the phytosomes of the pure compounds, rutin and quercetin, except that in this case 20 mg of the respective flavonoids and 40 mg of the phosphatidylcholine was used.

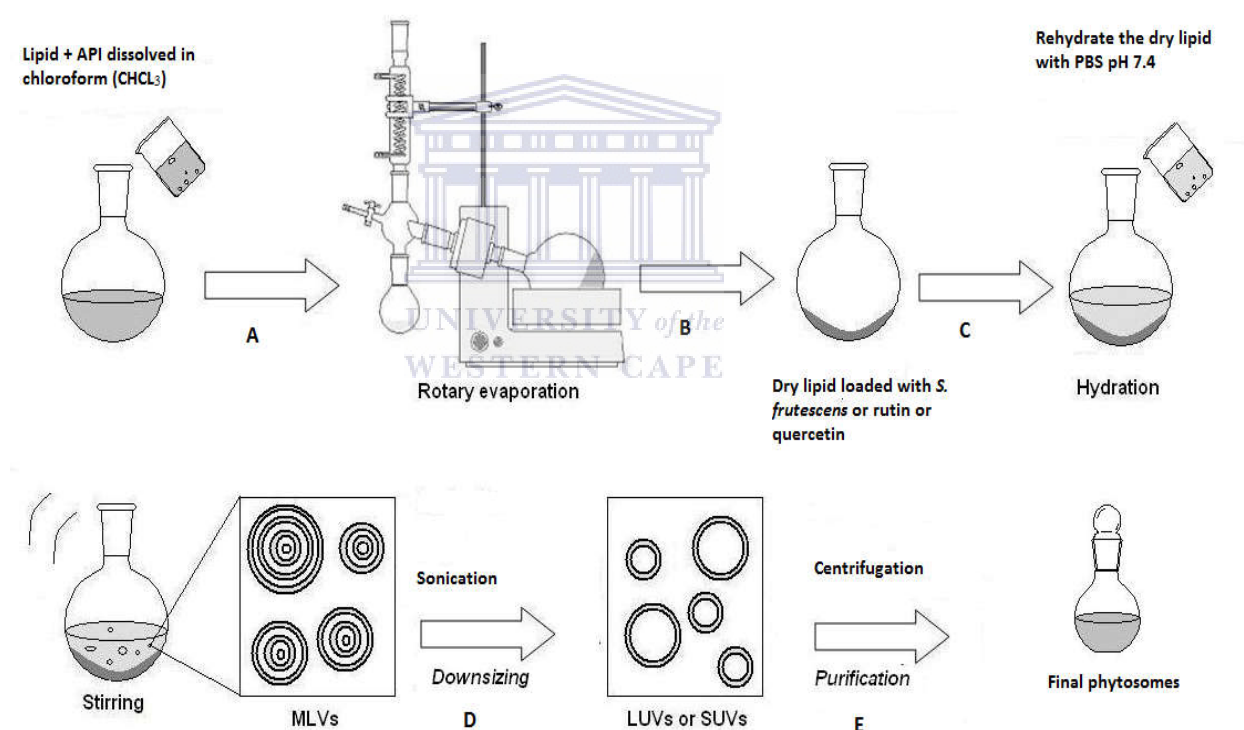

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Figure 4.2 Schematic for the preparation of phytosomes

4.3.3 Reduction of size of liposomes and phytosomes.

The reduction of size of the liposomes and phytosomes were effected using the sonication technique (Huang et al. 2010). For the liposomes, the liposome

suspension **(E)** described in section 4.3.1 was transferred to a round bottom flask, the latter placed in the sonicator bath and sonicated for 1h at 40 ^{0}C. For the phytosomes, the thin film layer was hydrated with PBS pH 7.4 at 50 ^{0}C, and also sonicated for 1h at 40 ^{0}C. After the size reduction process, the liposome and phytosome suspensions were centrifuged for 2 hours at 4 ^{0}C and 30,000 rpm using an ultracentrifuge, to separate unwanted particles leached during the sonication process. Finally, the supernatant was decanted and transferred to a vial for HPLC analysis. The remaining pellet was weighed and stored in its test tube at – 20 °C in freezer until further analysis, i.e. size analysis and distribution by PCS and drug content analysis by HPLC, etc.

4.3.4 Scanning Electron Microscopy (SEM).

To determine the shape and size of the liposomes and phytosomes high resolution scanning electron microscopy (HR-SEM) was used. After ultra-centrifugation **(E)** the supernatant was removed and the pellet rehydrated and used for SEM analysis. A drop of sonicated liposomes or phytosomes was placed on carbon adhesive tape; the latter applied onto an aluminium stub and then dried completely in a fume hood. Thereafter the dried liposome or phytosome nanoparticles were coated with gold palladium using an Emitech K550X (England) sputter coater and viewed using a Auriga F50 HR-Scanning Electron Microscope with working distances of 6.6, 6.7 and 6.8 mm and accelerating voltage of 5 kv as the instrument operating parameters. Photographs of the particles were taken within a range of 5-50 kx and displayed the shape and size characteristics of liposomes and phytosomes.

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4.3.5 Size analysis and distribution of liposomes and phytosomes.

For the size analysis of the liposome and phytosome vesicles the Photon Correlation Spectroscopy (PCS) technique, one that measures the size of particles in the nanometre range, was used to investigate and confirm the vesicular structure of the particles obtained after hydration. Polydispersity of the vesicle size is observed by this technique which gives an idea of the distribution of various sizes of vesicular structures (Goll *et al.* 1982). In addition, a Zetasizer, i.e. an instrument which uses Brownian motion of particles to reflect their size (Dobrovolskaia et al. 2009), was also used for the size and size distribution analysis. In this case, 1 ml of liposome and phytosome suspensions, prepared as described in 4.3.1 and 4.3.2, were placed in a cuvette **(V)** in the Zetasizer and the software facilitated instrument recorded readings obtained. Size and size distribution were presented by the Z-average and polydispersity index (PI) values, respectively. In addition, the dynamic light scattering (DLS) technique, which more accurately measures size of particles in the micrometer size range than particles in the submicron range, was also used. Finally, all the data were used to compare the size and size distribution of the flavonoid-containing liposomes *versus* that for the phytosomes.

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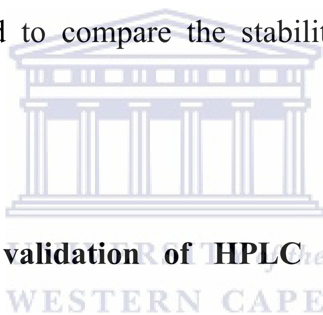
4.3.6 Analysis of stability of liposomes and phytosomes.

A potential exists between the particle surface and the dispersing liquid, which is called the Zeta potential and can be used for measuring the stability of particles (Jiang, *et al* 2003).

For this study measurement of the Zeta potential (ZP) of the liposomes and phytosomes were carried out using the Zetasizer instrument. Before analysis, the disposable folded capillary cell of the instrument was rinsed with distilled water and then 700 µl of the liposome and phytosome suspensions was separately added to the disposable zeta cell, the temperature set at 25°C and the measurements conducted at an angle of 173° for ten cycles at a voltage of 4 mv. The intensity-weighted mean value of three Zeta potential measurements was taken. If the particles had low zeta potential values; there would be no force to prevent the particles from aggregating. Therefore, particle suspensions with zeta potentials $> +30$ mV or < -30 mV are normally considered stable (Lee, Lee & Jeon 2007). This technique was therefore used to compare the stability of the liposome *versus* phytosomes particles.



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4.3.7 Development and validation of HPLC assay for *S. frutescens* flavonoids.

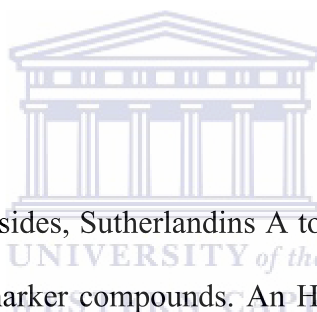
A validated HPLC method, as described below, was used for analysis of the flavonoid content of the liposomes and phytosomes prepared in this study.

4.3.7.1 HPLC system and conditions

The HPLC system used for the acquisition of chromatograms and UV spectra was an Agilent 1200 series system with diode array detection (DAD), and data acquisition and processing was carried out using the OpenLAB™ CDS ChemStation Edition software *(Agilent Technologies, Palo Alto, CA, USA)*.

For chromatographic separation of the flavonoid compounds a reverse phase Phenomenex Luna® C_{18} column (25 cm x 4.6 mm, 5 µm i.d.) with a compatible

guard column, both maintained at 45°C, were used. The mobile phase, consisting of water (0.01 % formic acid) (A) and acetonitrile (0.01 % formic acid) (B), was filtered through a 0.45 µm filter and degassed prior to use. The flow rate of the mobile phase was maintained at 0.8 ml/min, the injection volume was twenty microliters, the wavelength for analysis and detection 370 nm, and peaks were separated by gradient elution with solvents A and B as follows: from 0 to 1 minutes 82% (A) and 18% (B); 15 minutes 75% (A) and 25% (B); 20 minutes 65% (A) and 35% of (B); 25 minutes 40% (A) and 60 % of (B); 26 to 35 minutes 82% of (A) and 18% of (B). The isolated compounds were identified based on their retention times (t_R) and UV-spectra.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4.3.7.2 Validation

Six flavonoids (i.e. five glycosides, Sutherlandins A to D and rutin, and aglycone quercetin) were selected as marker compounds. An HPLC assay was developed, validated and used for the quantification of various flavonoids in *S. frutescens* materials in an earlier study (Mbamalu *et al.*, 2015) and the same assay was now used for the present study and the following parameters determined to validate the HPLC assay according to the ICH (1997) guidelines.

Assay stock solutions were prepared by dissolving specific amounts of the reference standards in methanol: water (50:50) and diluting them further to obtain calibration and quality control sample solutions in concentration ranges between 0.5 and 2 mM for the various marker compounds.

To establish the linear concentration range for each marker compound a calibration curve was developed by injecting 20 µl samples of reference standard solution into

the HPLC, measuring the peak area and plotting concentration *versus* peak area. Triplicate samples over a six sample concentration range were used to establish the curve. The linearity of the calibration curve for each of the reference standards was assessed by linear regression analysis of these plots of peak area against concentration using GraphPad Prism™ version 6.0.

For the Limit of Detection (LOD) and Limit of Quantification (LOQ) determinations, the peak area was used as the signal response and the mean baseline noise taken as = 0.4 mAU (n = 3). The LOD and LOQ were determined using an analyte response 3 and 10 times that of the noise (i.e. signal to noise ratio of 3:1 and 10:1), respectively, and the mean baseline noise = 0.4 mAU; n = 3. The percentage bias was determined as the difference between the mean concentration measured and the prepared concentrations as a percentage of the prepared concentration using n = 6 samples.
The precision of the analytical method was determined by assaying 6 spiked samples at the lower and upper limits of the concentration range studied for each of the marker compounds. The accuracy of the method was determined from the mean concentrations obtained for the replicates and the percentage difference. The relative standard deviations (% RSD) were then calculated for the reference standard samples at the lower and upper limits of the concentration range.
Finally, system suitability parameters were calculated from the chromatograms obtained for each of the reference standards during the studies using the Agilent ChemStation software.

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4.3.7.3 Quantification of levels of marker compounds in *S frutescens* (extract)

In this study liposomes and phytosomes of *S frutescens* were made and the levels of flavonoid marker compounds in the *S frutescens* extract used needed to be determined. For the latter a modification of the method described by Avula *et al.* (2010) was used (as further described by O. Mbamalu *et al*, (2015)).

Briefly, about 100 mg of accurately weighed FDAE *S. frutescens* material was added to 2 ml of 50 % aqueous methanol, the mixture sonicated for 30 minutes and then centrifuged at 3500 rpm for 15 minutes. The extraction procedure was repeated thrice, the respective supernatants combined, the final volume adjusted to 8 ml with 50% aqueous methanol and mixed thoroughly. Prior to injection into the HPLC, appropriate dilutions were made, and the final solutions filtered using a 0.45 µm nylon membrane filter. The first 1.0 ml of filtrate was discarded and the remaining volume collected in an LC sample vial. Twenty microliters of each sample solution was analysed using the validated HPLC method previously described under section 4.7, and the peak areas of the flavonoids of interest noted. Triplicate samples of the FDAE solutions were assayed and the average peak areas calculated to quantify and compare flavonoid content in the *S. frutescens* extract. Data was presented as average percentage content (i.e. mg flavonoid * 100 /mg plant material) ± SD (n = 3).

4.3.8 Determination of drug entrapment efficiency of liposomes and phytosomes.

The efficiency (EE) of the entrapment of *S. frutescens* extract (i.e. the API) or rutin or quercetin in the phytosomes and liposomes were determined after HPLC analysis of samples of the free drug (i.e. A_{total} in FDAE hydrated solutions (see section 4.3.1)

and the encapsulated (loaded) drug within the carrier (liposomes and phytosomes). For the latter, centrifugation was used to separate the encapsulated API and free drug (i.e. API) (Fan *et al.* 2007) and the liposomes and phytosomes analysed were those prepared as described in sections 4.3.1 and 4.3.2.

Briefly, the liposome and phytosome suspensions obtained were centrifuged at 4 ^{0}C and 30,000 rpm, for 2 hours. Thereafter the clear supernatant were collected, filtered (0.45 micron filter), transferred to HPLC vials and aliquots of the latter analyzed to give the levels of free i.e. un-encapsulated API (*S. frutescens* extract, or rutin or quercetin) (A_{free}). Similarly, samples of the *S. frutescens* and flavonoid hydration solutions (see section 4.3.1) were analysed to give the total API levels

The entrapment efficacy was then determined using the following equation:

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$$\mathbf{EE\ \% = (A_{total} - A_{free} / A_{total})*100}$$

where A_{total} is the relative amount (i.e. area under chromatographic peaks) of individual flavonoid (i.e. sutherlandins A to D or rutin or quercetin) in the chromatograms of starting *Sutherlandia* FDAE, or rutin or quercetin solutions and A_{free} the relative amount of these flavonoids in the supernatant after separation of the liposomes or phytosomes. These assays were done in triplicate (n = 3) and the EE (%) results for the liposomes and phytosomes compared using GraphPad Prism and ANOVA.

4.4 Results and discussion

4.4.1 Preparation of liposomes and phytosomes

Both liposomes and phytosomes were successfully prepared using the thin film hydration method. This method requires the formation of a thin film on the surface of a round bottom flask, a hydration step, hand shaking and vortexing, all at a temperature above the phase transition temperature (Tm). The yield of *S. frutescens* phytosomes was 53 %, while that for the *S. frutescens* liposomes was only 32 % (i.e. approximately 40 % lower than that for phytosomes).

It took 60 minutes to obtain a yellowish-white thin film for the phytosomes, but a longer time of approximately 120 minutes to produce a yellow-brownish thin lipid film for the liposomes,. Both of the lipids used worked fine to provide effective thin films for the phytosomes and liposomes. The hydration of the thin films for the phytosomes required 10 to 20 minutes (i.e. less than that for liposomes) of hand shaking and vortexing to form the phytosomes suspension, while it took 30 to 45 minutes to form liposomes suspension. Overall, the thin film hydration method thus worked well to produce both phytosomes and liposomes of *S. frutescens* FDAE. However, the phytosomes needed less time to form and was produced in a higher percentage yield than the liposomes.

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4.4.2. Particle shape, size, size distribution and stability of the liposomes and phytosomes

High Resolution Scanning Electron Microscopic (HR-SEM), Photon Correlation Spectroscopy (PCS) and the Zetasizer were used to determine the shape, size, size distribution and stability of the liposomes and phytosomes.

4.4.2.1 Scanning Electron Microscopy (SEM).

As reported by Mainardes *et al* (2005), the sonication method used to downsize nano-particles usually produces particles that differ in size range and shape. The SEM images obtained for the present study are given in figure 4.3 and shows that the particles had a size of ≥ 200 nm. Morphologically the liposomes were mostly spherical, while the phytosomes were mainly rod-shaped.

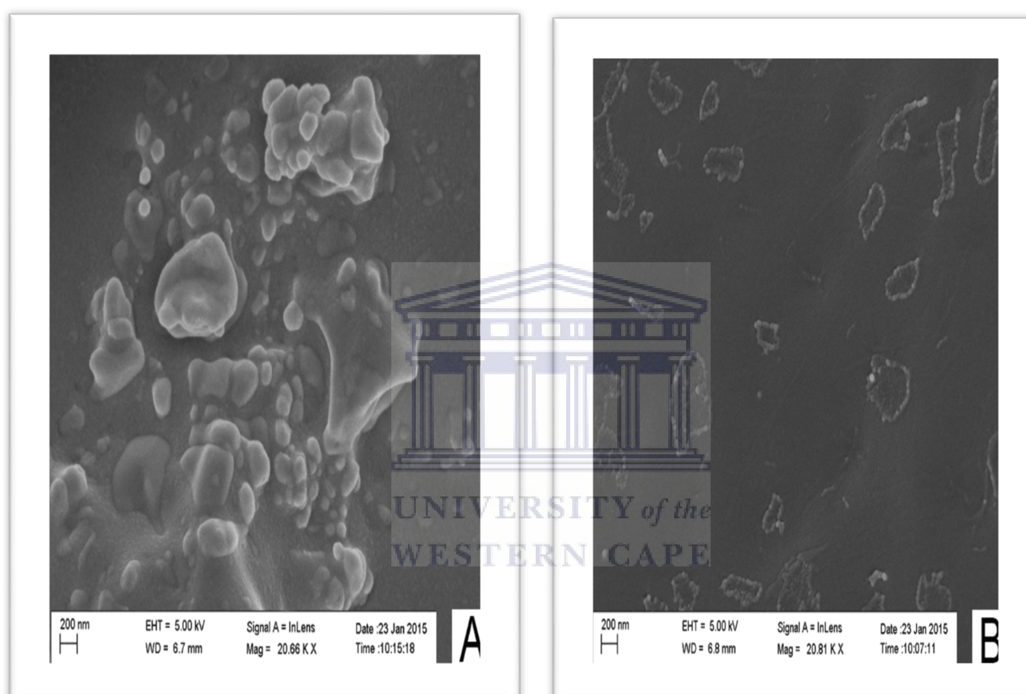

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Figure 4.3 The Scanning Electron Microscopy images showing the shape and size of *S. frutescens* liposomes (A) and phytosomes (B).

4.4.2.2 Photon Correlation Spectroscopic (PCS) Analysis

The mean particle size and size distribution of the prepared liposomes and phytosomes were also determined by PCS. The primary goal in the present study was to obtain liposomes and phytosomes of sufficiently small (i.e. nano particle) size and the results obtained are shown in figure 4.4. Generally, nanoscale particles were obtained with the mean sizes of the *Sutherlandia* extract, quercetin and rutin

liposomes being 230.8 ± 38.4, 451.05 ± 69.79 and 359.6 ± 56.05 nm, respectively, and significantly (**$p < 0.01$** for *S. frutescens,* **$p < 0.05$** for quercetin) greater than the mean phytosomal sizes of 675.23± 32.17, 811.4± 41.02 and 291.17 ± 45.07 nm, respectively. The difference in the rutin liposome *versus* phytosome sizes were not significant (**$p > 0.05$**). The High Resolution Scanning Electron Microscopic (HR-SEM) and Photon Correlation Spectroscopy (PCS) results thus indicated similar sizes for the liposomes and phytosomes.

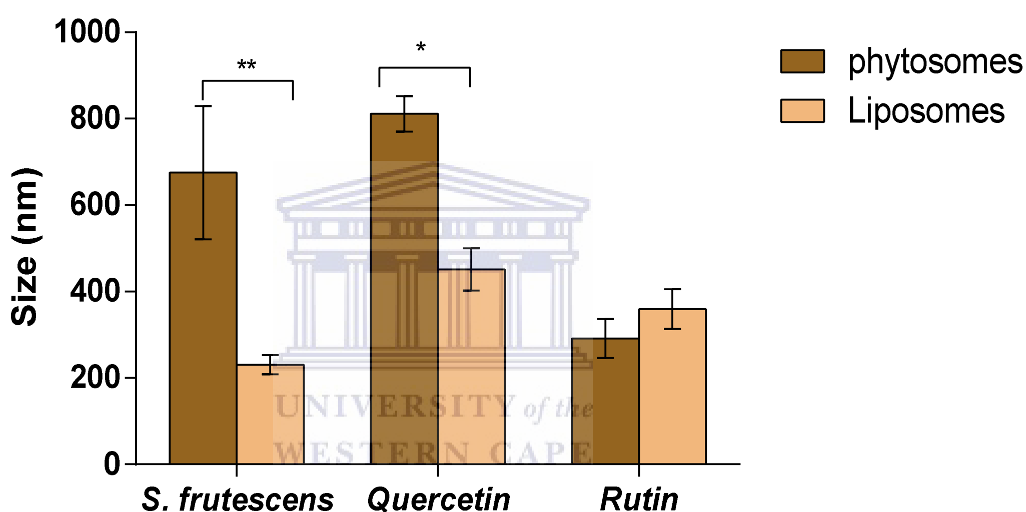

Figure 4.4 **Size of the liposomes and phytosomes containing *S. frutescens*, quercetin and rutin (n = 3 ± std).**

Generally, liposomes and phytosomes of the sizes obtained in the present study are considered to be promising vehicles for delivery of herbal medicine (Zhang *et al.* 2013). Moreover, particles greater than 100 nm, as obtained in this study, are classified as large unilamellar vesicles (LUV) and this size of vesicles have been reported to be more suitable and accepted for food applications (Akbarzadeh *et al.* 2013). The quercetin and rutin (its glycoside) liposomes were approximately

similar in size and had comparable sizes to that obtained by Goniotaki in his formulations of liposomes (Goniotaki et al. 2004).

Overall, the results indicated that, after sonication, liposomes and phytosomes of *S. frutescens*, quercetin (flavonoid aglycone) and rutin (flavonoid glycoside) of sufficiently small sizes were obtained and these could be acceptable formulations for further *in vivo* and *in vitro* studies.

Photon Correlation Spectroscopic (PCS) analysis was also used to assess the particle size distribution, presented as the polydispersity index (PDI), and the results obtained are given in figure 4.5. The PDI of the *Sutherlandia* extract and quercetin liposomes were 0.366 ± 0.1 and 0.890 ± 0.12, respectively, and significantly (**p < 0.01**) different than the 0.94± 0.1 and 0.105± 0.1 values, respectively, for the phytosomes. The PDI of the rutin liposomes (i.e. 0.458± 0.006) and phytosomes (i.e. 0.453± 0.021) was however not significantly different (**p > 0.05**).

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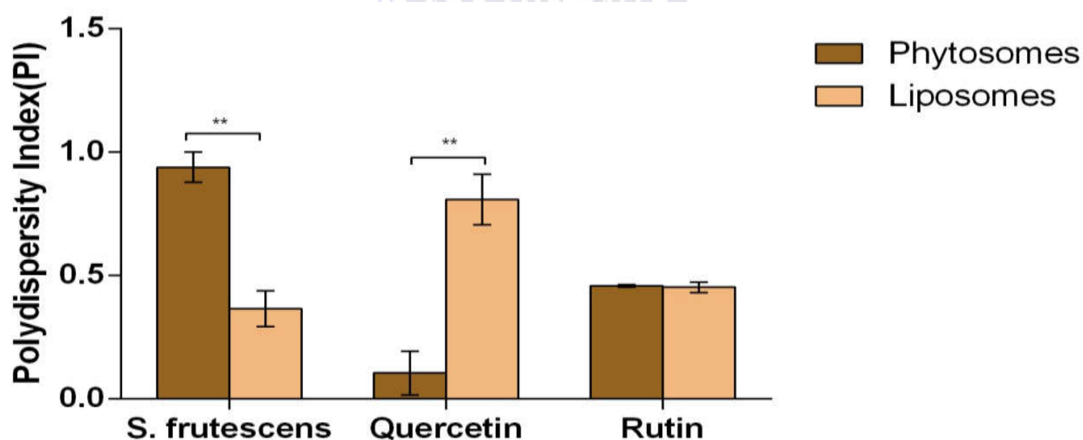

Figure 4.5 Polydispersity Index (PI) of the liposomes and phytosomes containing *S. frutescens*, quercetin and rutin (n =3± std).

The polydispersity index (PDI) reflects the homogeneity of the sizes of particles in a formulation with values of 0 indicating monodisperse and 1 polydisperse

populations while a value of > 0.5 (more than 0.5) indicate broad distributions and < 0.5 homogenous distributions (Iqbal *et al*. 2012). The results obtained suggested that the liposomes of the *Sutherlandia* extract and rutin and phytosomes of the quercetin and rutin all had homogeneous size distributions (**PDI < 0.5**), while the quercetin liposomes and *S. frutescens* extract phytosomes showed broad size distributions (**PDI > 0.5**). The sonication method of size reduction was therefore clearly effective but worked better for the liposomes than the phytosomes of *S. frutescens* extract in this study.

4.4.2.3 Zeta potential analysis of liposomes and phytosomes.

Zeta potential (ZP) analysis was used to determine the stability of the phytosome and liposome formulations and the results that were obtained are given in figure 4.6.

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All the ZP values were negative because the lipids used were neutral (ONG YUNG SHENG 2007). The values for the *Sutherlandia* extract, quercetin and rutin liposomes were -8.55 ± 0.26 , -16.6 ± 0.35 and -5.15 ± 0.77 mv, respectively and significantly (**p < 0.01** for *S. frutescens*, quercetin and rutin) different than the -0.457± 0.06, -2.63± 0.68 and -3.18± 0.16 mv respective ZP values for the phytosomes. The zeta potential for the phytosomes prepared with phosphatidylcholine was around 0 mv, indicating a neutral surface charge. On the other hand the liposomes, prepared using lecithin, had greater negative zeta potential values suggesting better stability than that of the phytosomes.

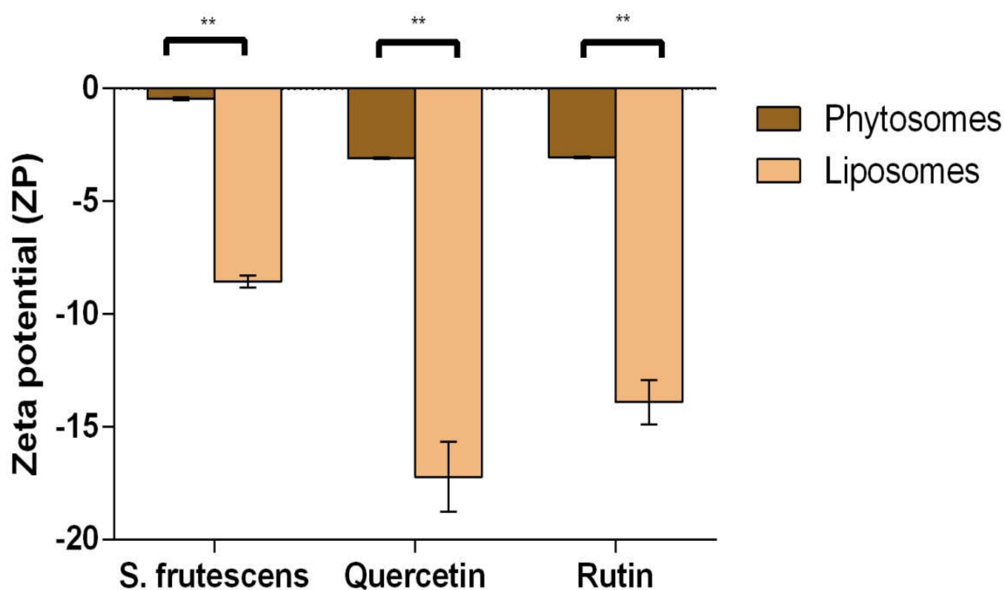

Figure 4.6 Zeta potential of the liposomes and phytosomes containing *S. frutescens*, quercetin and rutin (n = 3 ± std).

The zeta potential reflects the electric repulsion between particles and, in general, nanoparticles form a stable dispersion when the absolute value of zeta potential is above + 30 mv or below – 30 mv (Yan, *et al*. 2012). Although the absolute zeta potential values for the liposomes and phytosomes prepared in the present study were between + 30 mv and – 30 mv, the liposomes showed a relatively higher ZP value suggesting that it had better physical and chemical stability than the phytosomes.

Overall, the liposomes and phytosomes obtained were both of sufficiently small (i.e. nano particle) size. In addition, the liposomes had a narrower size distribution, and better stability than the phytosomes (i.e. broad size distribution), suggesting that they can better deliver precise amounts of API for better cell penetration.

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4.4.3 HPLC assay method

A reversed phase HPLC separation method combined with DAD detection was developed for the assay of *S. frutescens* flavonoids (e.g. glycosides and aglycones), quercetin and rutin in liposomes and phytosomes. With the developed method, the compounds of interest were separated and detected within 35 minutes using a C18 column, gradient elution with acetonitrile and formic acid (ACN formic acid) and UV detection by DAD. Representative chromatograms from the assay are shown in figures 4.7, 4.8 and 4.9. They showed that the retention times for sutherlandin A, B, C and D were 12.3, 13.5, 16.03 and 16.88 minutes, respectively, and that for quercetin 24.6 minutes and for rutin 10.7 minutes.

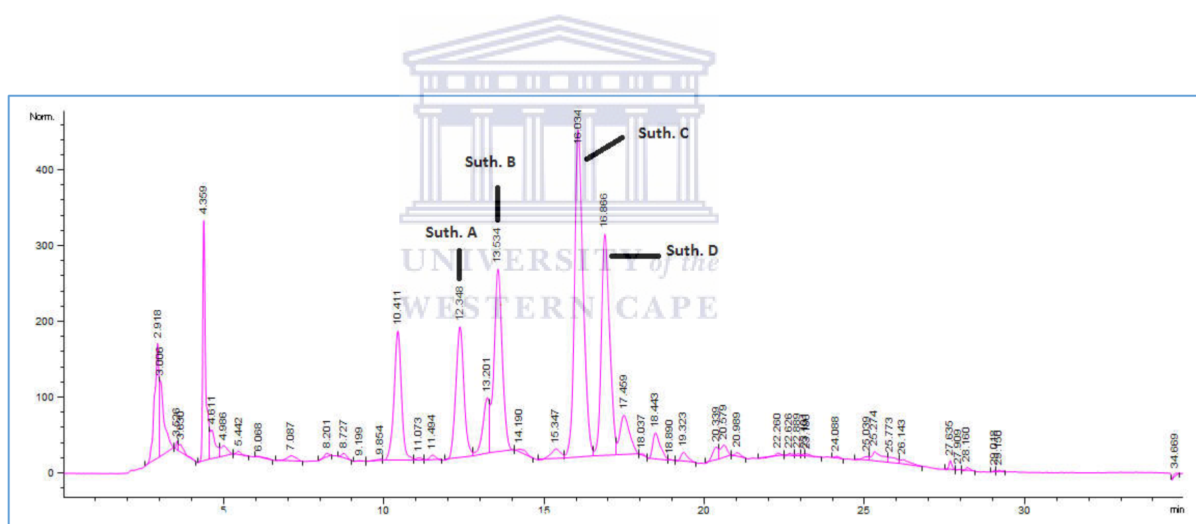

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Figure 4.7 HPLC chromatogram of aqueous *S. frutescens* solution measured at λ = 370 nm. Concentration of *S. frutescens* solution was 6.66 mg/ml, the injection volume 20 µl and Suth A, B, C, and D equal to Sutherlandins A to D.

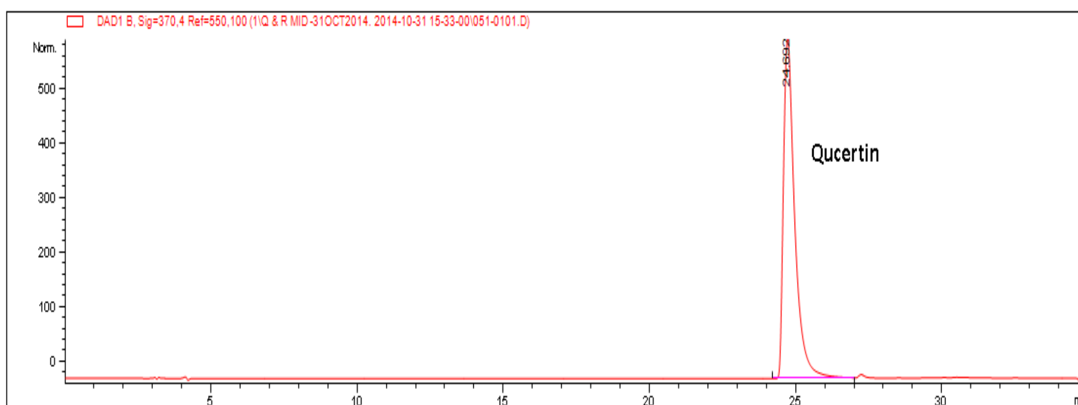

Figure 4.8 **HPLC chromatogram of quercetin measured at λ = 370 nm. Concentration of solution was 2 mg/ml, and injection volume 20 µl.**

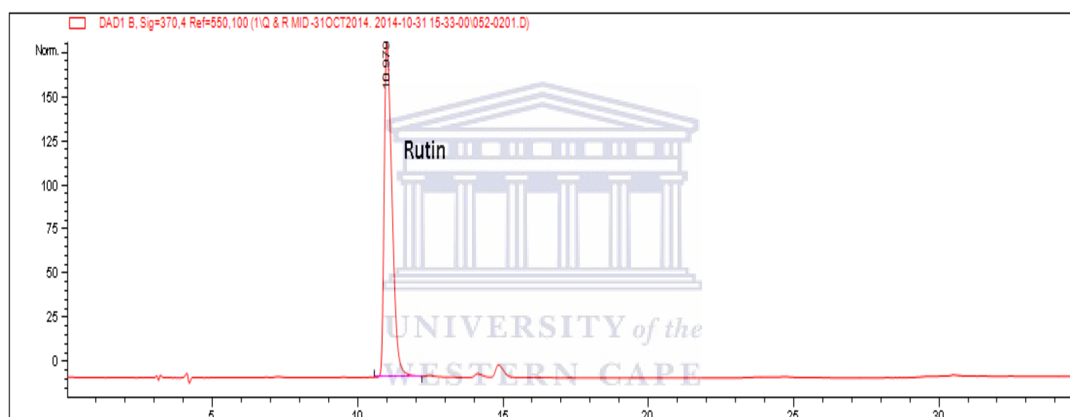

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Figure 4.9 HPLC chromatogram of rutin measured at λ = 370 nm. Concentration of solution was 2 mg/ml, and injection volume 20 µl.

4.4.3.1 Validation of HPLC assay

The following are the results of the validation of the HPLC assay for flavonoids in solution.

4.4.3.1.1 Calibration curves and linearity

The calibration curves representing the reference compounds are presented in Appendix 2 and the results of the curve analyses in Table 4.1. The six point curves for all the reference compounds showed strong linear correlations, i.e. $r^2 > 0.99$, between concentration and peak area of the detector response (Table 4.1).

Table 4.1: Summary of parameters for HPLC assay of flavonoid glycosides and aglycones.

Flavonoid	Regression equation	Concentration range (μg/ml)	r^2	LOD (ug/ml)	LOQ (ug/ml)
Sutherlandin A	y = 22.252x – 9.5909	4.0 to 180.0	0.9998	2	4
Sutherlandin B	y = 14.543x + 13.149	4.0 to 200.0	0.9996	4	4
Sutherlandin C	y = 17.890x + 11.763	4.0 to 200.0	0.9990	4	15
Sutherlandin D	y = 17.45x - 1.5755	4.0 to 180.0	0.9996	2	4
Quercetin	y = 108.51x + 104.95	4.0 to 120.0	0.9967	< 2	9
Rutin	y = 27.88x + 35.835	4.0 to 120.0	0.9988	< 2	15

LOD = Limit of detection and LOQ = Limit of quantitation

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4.4.3.1.2 Limit of detection (LOD) and limit of quantitation (LOQ)

The values for the LOD and LOQ are presented in Table 4.1. The LOQ was lowest for sutherlandins A, B and D, and highest for sutherlandin C and rutin. The results obtained suggested that the proposed method was sensitive enough for the assay of flavonoid glycosides (i.e. sutherlandins A to D and rutin), and aglycones (i.e. quercetin) in the manufactured liposomes and phytosomes.

4.4.3.1.3 Precision and accuracy

Precision and accuracy data for the reference standards are shown in Tables 4.2. The % RSD for most of the reference standards were less than 3%, suggesting that the proposed method was precise enough for the assay of flavonoid glycosides in

Sutherlandia. Exceptions were sutherlandin B and sutherlandin C for which % RSD at lower concentrations were 5.75 and 3.80, respectively. Precision data less than 5% RSD may be acceptable (Costa *et al*, 2011), more so for herbal materials where variation in actives may be quite considerable (Ghosh *et al*. 2012; Gao and Hu, 2010). The interday and intraday assay precision was also consistent, with % RSD less than 1% for all the reference standards.

Overall, the HPLC method developed was thus simple but had relatively good separation, accuracy and precision and sufficiently wide linear concentration ranges to determine the levels of the flavonoid aglycone (e.g. quercetin), and glycosides (i.e. sutherlandins A, B, C and D, and rutin) in, or released from, the *S. frutescens,* quercetin and rutin containing liposomes and phytosomes.

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Table 4.2: Precision and accuracy data for quantification of the reference standards at 370 nm.

	Reference compound	**% RSD**
Lower conc. (ug/mL): 15 ug/mL	Sutherlandin A	2.01
Higher conc. (ug/mL): 33 ug/mL	Sutherlandin A	2.54
Lower conc. (ug/mL): 15 ug/mL	Sutherlandin B	5.75
Higher conc. (ug/mL): 35 ug/mL	Sutherlandin B	2.83
a		
Lower conc. (ug/mL): 15 ug/mL	Sutherlandin C	3.80
Higher conc. (ug/mL): 35 ug/mL	Sutherlandin C	2.14
Lower conc. (ug/mL): 15 ug/mL	Sutherlandin D	2.36
Higher conc. (ug/mL): 33 ug/mL	Sutherlandin D	2.29

Lower conc. (ug/mL): 8 ug/mL	Quercetin	2.84
Higher conc. (ug/mL): 35 ug/mL	Quercetin	1.28
Lower conc. (ug/mL): 9 ug/mL	Rutin	2.39
Higher conc. (ug/mL): 35 ug/mL	Rutin	2.21

4.4.4 Flavonoid encapsulation efficiency of liposomes and phytosomes.

The levels of flavonoids encapsulated in liposomes and phytosomes were determined by HPLC assay and percentage EE calculated. And the results obtained are summarized in appendix 3 and figures 4.10 and 4.11.

The % EE value of the flavonoids in *the Sutherlandia* extract containing phytosomes were 47.62± 3.76, 48.83± 3.75, 43.46± 3.44, and 46.75± 5.01 % for sutherlandins A, B, C and D, respectively, and this was significantly (**$p < 0.001$** for sutherlandins A and B and **$p < 0.01$** for sutherlandins C and D) greater than the liposomal EE % of 26.93± 3.56, 28.51± 2.3, 26.62± 3.18, and 32.53± 8.51 %, respectively. The greater sutherlandin flavonoid EE % of the phytosomes was probably as a result of complex formation between the lipid and drug as reported by Hou (Hou et al. 2012). On the other hand, the flavonoid EE % of the liposomes was rather low, most likely due the low affinity for, and solubility of the *Sutherlandia* extract flavonoids in, the lipid constituting the liposomal

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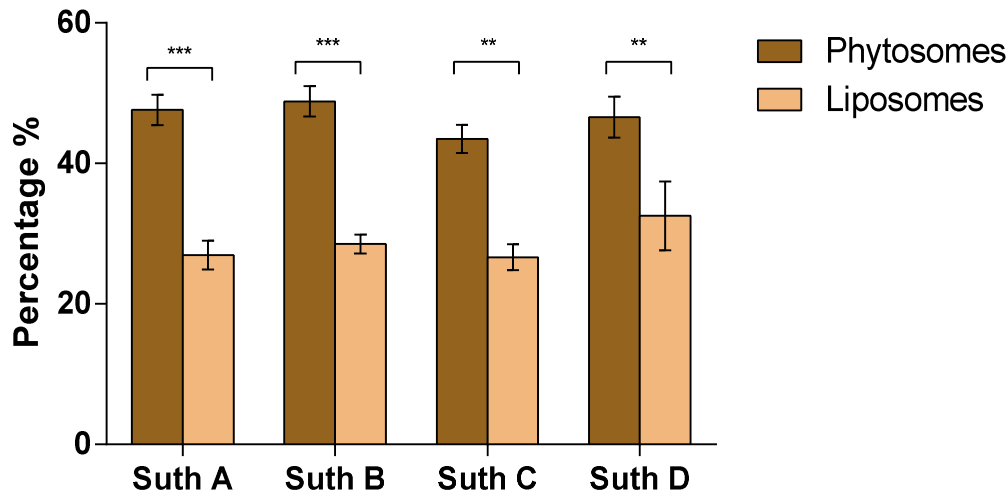

Figure 4.10 Flavonoids glycoside encapsulation efficiency of liposomes and phytosomes containing *S. frutescens* (n = 3 ± std).

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membrane which then decreased the chances of the extract (and thus sutherlandins) being entrapped in the aqueous compartment. The lower amounts of extract entrapped then resulted in the lower individual flavonoid EE % (Fahr, Liu 2007). In the phytosomes the extract constituents could however bind to the lipid bilayer which prevented the drug from escaping and produced the higher flavonoid EE % observed in the phytosomes (Maya et al. 2013). Overall, these results confirmed the first hypothesis *viz*. that the entrapment efficacy of the phytosomes will be higher than that of the liposomes.

For the quercetin (i.e. the flavonoid aglycone) the % EE in the phytosome was 85.94 ± 1.87 % and significantly (**$p < 0.05$**) greater than the 48.75 ± 11.8 % for liposome. This finding may be due to complex formation between quercetin and the phosphatidylcholine or because quercetin, due to the it's planar configuration that can

easily locate into the organized structure of the phospholipids within the phytosomes membranes, had a higher affinity for the phytosomes (Rasaie *et al*. 2014).

The EE % for rutin in the liposomes and phytosomes, i.e. 69.15 ± 0.33 % *versus* 66.39 ± 0.05 %, was however not statically different (**$p > 0.05$**). And this was most likely due to the ability of this glycoside to bind in both the core and lipid bilayer of the nanoparticles. Rutin is a water soluble compound which, in liposomes, has a chance of being entrapped in the aqueous compartment, while in the phytosome, uniform binding of rutin and phosphatidylcholine is possible (Das, Kalita 2014).

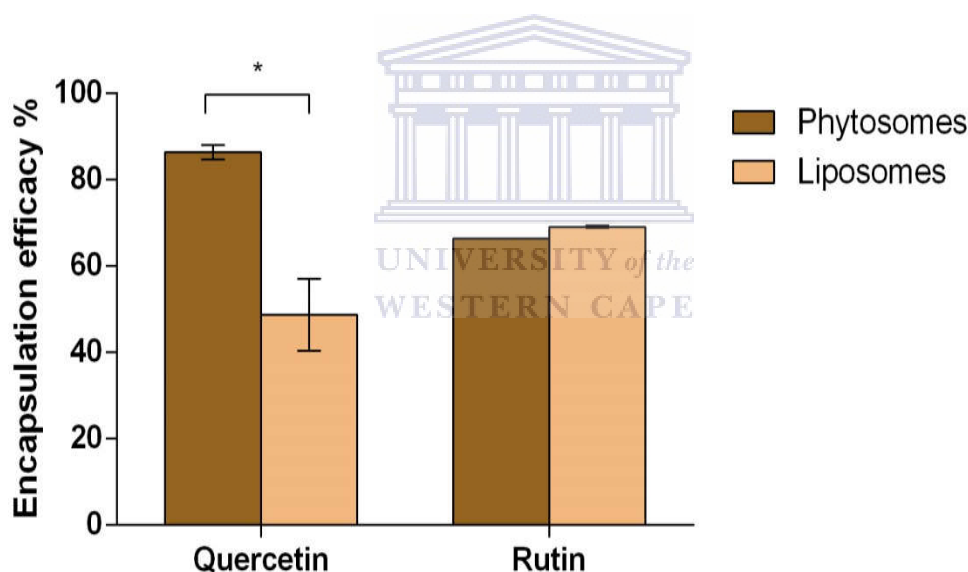

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Figure 4.11 Encapsulation efficiency (%) of rutin and querctin containing liposomes *versus* phytosomes (n = 3 ± std).

Overall, the EE % for rutin (the glycoside) in the liposomes and phytosomes were high and not statically different (i.e. 69 *versus* 66 %), for quercetin (the aglycone) significantly better in phytosomes than liposomes (i.e. 85 *versus* 48 %) and for the sutherlandins (i.e. glycosides) contained in the FDAE of *S frutescens,* significantly

higher in phytosomes than liposomes (i.e. 50 *versus* 26 %). All of this can possibly be attributed to the fact that the FDAE of *Sutherlandia* also contained a matrix of other constituents, such as sugars i.e. polysaccharides, etc., which were also (or even more) water soluble than the sutherlandins and could compete in the entrapment process resulting in the low EE % of sutherlandins in the liposomes, while complex formation between the lipid and sutherlandins in the phytosomes could be a reason for the higher encapsulation efficacy in this nanoparticle. Overall, these results confirm the first hypothesis *viz*. that the entrapment efficacy of the phytosomes will be higher than that of the liposomes.

4. 5 Conclusion

In this present study, a successful method was developed to obtain preparations of the *S. frutescens* liposomes and phytosomes. The sonication method was effective to obtain small particle size (i.e. nano range) for both the liposomes and phytosomes. However, the liposomes had a smaller size; narrower size distribution; higher zeta potential and better stability than the phytosomes, suggesting that they can better deliver precise amounts of API for better cell penetration. In addition, the phytosomes, however, had significantly higher encapsulation than the liposomes (±50 % *versus* ±26 %) suggesting that, most probably due to the complex formed between the API and the lipid, but suggesting that phytosomes can serve as the more promising vehicles to carry different active constituents of the herbal extract. Moreover, this latter finding confirmed the first hypothesis, *viz*. that the entrapment efficacy of the phytosomes would be higher than that of the liposomes.

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In general, the results indicated that the prepared liposome and phytosome formulations could indeed be promising vehicles for delivery of hydrophobic and hydrophilic compounds found in herbal extracts, might be able to facility their rapid absorption and this needs to be investigated further in *in vivo* and *in vitro* studies.

CHAPTER FIVE
Flavonoid release profile of *S. frutescens* containing liposomes *and* phytosomes

5.1 Introduction

One of the main objectives of this study was to investigate and compare the *in vitro* release profile of *S. frutescens* flavonoids (as active phytopharmaceutical ingredient or API) from with liposomes *versus* that from phytosomes. Firstly, to quantitate the amount of flavonoids released, an HPLC assay was used. Secondly, pH 1.2 and 6.8 buffers solutions were used to simulate the release that would occur under gastrointestinal conditions and samples were taken at specific time intervals to provide a drug release over time profile for both the liposomes and phytosomes. Finally, the percentage API released after 120 minutes was determined and used as a parameter to compare profiles of flavonoid released from the liposomes and phytosomes. In this chapter the chemicals, materials and equipment, as well as the methods used are described, and the results obtained presented and discussed.

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5.2 Chemicals, Materials, and Equipment.

All the chemicals, materials and equipment used in this part of the study are given in section 4.2. In addition, pH 1.2 and 6.8 buffer solutions were used and these were prepared using methods found in the BP (2014). Briefly, for the pH 1.2 buffer solution, 9.7 ml of concentrated hydrochloric acid (concentration, 32%) was diluted with distilled water up to a volume of 1000 ml. For the pH 6.8 buffer solution, 21.72 g of anhydrous dibasic sodium phosphate was dissolved in 1 L distilled water and the pH adjusted with a few drops of 2 M hydrochloric acid solution. Finally, the liposomes and phytosomes evaluated were those prepared as was explained in section 4.3.1 and 4.3.2.

5.3 Determination of *in vitro* flavonoid release.

The release profile of *S. frutescens* flavonoids from the liposomes and phytosomes were examined using the method of Kadare *et al.* (Kadare et al. 2014) and two different buffer solutions to mimic conditions obtained in different sections of the gastrointestinal tract (GIT). The medium at pH 1.2 represented gastric conditions while the medium at pH 6.8 represented prevalent pH conditions in the small intestine.

For the flavonoid release studies, 2 ml of either buffer solution were added to six numbered (i.e. 1 to 6) 10 ml Eppendorff tubes containing the pellets of liposomes or phytosomes (Note: the pellets were prepared as described in section 4.3.3), and time recorded as time equal to zero. Three replicates (n=3) of the 6 tubes were used. The eppendroff tubes were placed in a water bath maintained at 37± 0.5 ^{o}C and at specific time intervals (i.e. 5, 15, 30, 60, 90 and 120 minutes, respectively), the tubes representing the time interval (i.e. tube # 1 at 5 minutes, tube number two at 15 minutes, etc.) were removed, 1 ml sample drawn from each one and the withdrawn samples centrifuged at 20,000 rpm for 30 minutes. The supernatant was removed with a syringe, filtered through a 0.45 µm syringe filter, transferred to HPLC vials and analysed using the validated HPLC method (Mbamalu *et al.*, 2015) described in sections 4.3.7 and 4.4.3.

From the HPLC chromatograms the peak areas for the individual sutherlandins (A to D) were obtained and the percentage release calculated using the following formula:

$$\textbf{Drug release (\%)} = \frac{\text{Peak area of flavonoid released at specified time point}}{\text{Peak area of flavonoid loaded into pellet}} \textbf{ X 100}$$

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The peak area of flavonoid released at specified time point was determined as described above. To obtain the total peak area of flavonoid loaded into pellet, 200 µl of Triton X_{100} was added to samples of pellet contained in 2 ml pH 1.2 or 6.8 buffer and the mixture vortexed for 10 minutes to break up and dissolve the phospholipid. Then, 1 ml sample was withdrawn from each tube and the withdrawn samples centrifuged at 20,000 rpm for 30 minutes, the supernatant removed with a syringe, filtered through a 0.45 µm syringe filter and the filtered solution transferred to HPLC vials from which 20 µl was injected unto the HPLC column. The areas of the relevant sutherlandin peaks were then obtained from the chromatogram, taken as the total amount of flavonoid loaded into pellet and used to determine the percentage drug release.

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The percentage drug released *versus* time data was plotted, using GraphPad Prism, to obtain the drug release *versus* time profile for each of the sutherlandins from the liposomes and phytosomes. Several parameters were used to compare the release profiles. First, the average amount released at 120 minutes (i.e. AR_{120}) and average time required for 50 % release (i.e. T_{AR50}) were determined and compared using the student t-test. Next, each combination of the flavonoid release profiles was compared using the similarity factor calculated using the following equation:

$$f_2 = 50 \cdot \log \{[1 + (1/n)\Sigma_{t=1}^{n} (R_t - T_t)^2]^{-0.5} \cdot 100\}$$

Where R_t and T_t are the average percent released at each time point for the reference (liposome) and test (phytosome) products, respectively, and n is the number of observations. An f_2-value between 50 and 100 was taken to indicate similarity of

two release profiles while values below 50 indicate dissimilarity (Shah et al. 1999) and this criterion was used to compare the product profiles. All assessments were carried out in triplicate.

5.4 Results and discussion

5.4.1 Sutherlandin release in pH 1.2 buffer solution

The total amounts (AR_{120}) and rates (T_{AR50}) of sutherlandins (A to D) released from the liposomes and phytosomes found are presented in table 5.1, figure 5.1 and Appendix 4 (i.e. the data from which the graphs were plotted).

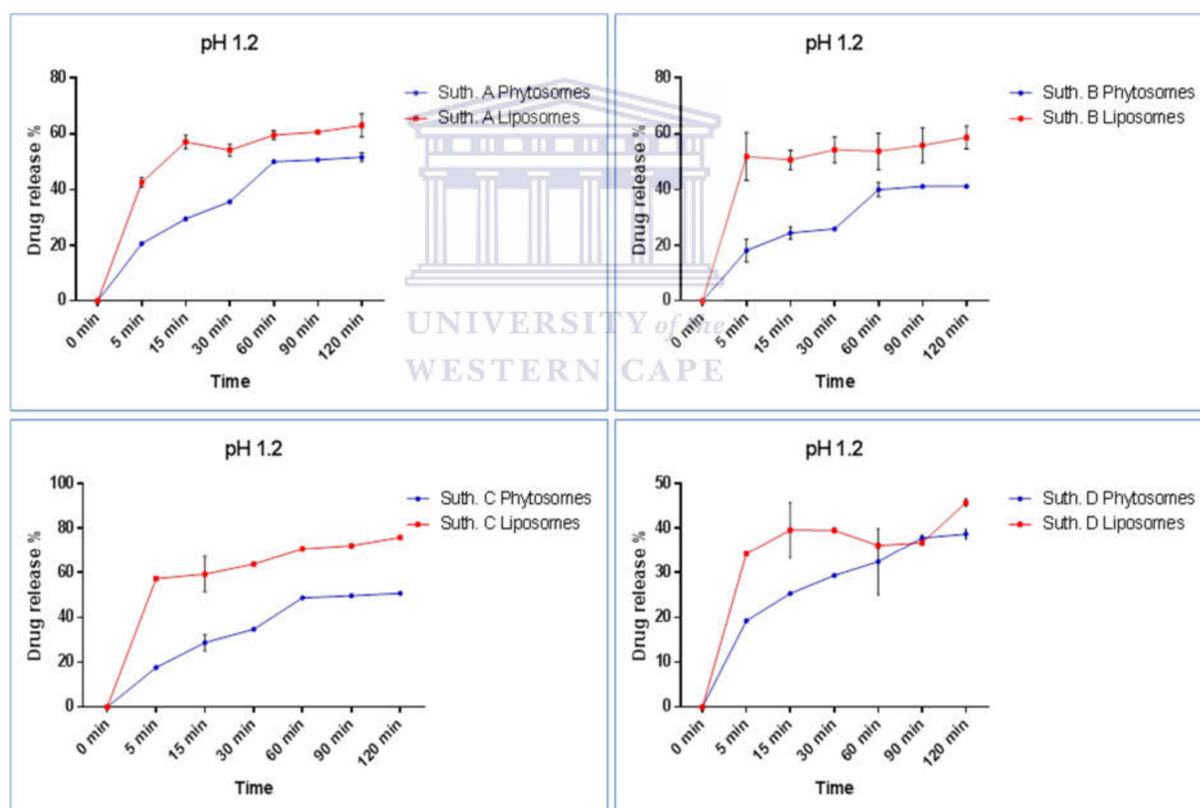

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Figure 5.1: Release profiles of sutherlandins A, B, C and D from *S. frutescens* liposomes and phytosomes at pH 1.2.

Neither the liposomes nor the phytosomes released all (i.e. 100 %) of the individual sutherlandins after 120 mins in pH 1.2 buffer (i.e. $AR_{120} < 100$ %) and the amounts released at all time points were significantly (**i.e. p = 0.0033** for sutherlandins A, **p**

= **0.0021** for sutherlandin B, **p** = **0.001** for sutherlandin C, and **p** = **0.0145** for sutherlandin D) higher from the liposomes than that from the phytosomes. The amounts of sutherlandins A, B, C, and D released from the liposomes at 120 mints, as a percentage of the total amount in the encapsulated *S. frutescens* material, were 62.96, 58.09, 75.74 and 45.74%, respectively, and 51.66, 41.33, 50.74 and 38.75 %, respectively for the phytosomes. Despite the higher flavonoid encapsulation efficiency of the phytosomes, established in chapter 4, the liposomes still released higher amounts of each flavonoid, after 120 mins in pH 1.2 buffer, than the phytosomes.

The sutherlandins were released at different rates from the liposomes with the time for 50% release (i.e.T_{AR50}) being 5 minutes for sutherlandin B and C, 30 minutes for sutherlandin A and > 120 minutes for sutherlandin D. These rates were however faster than that for the phytosomes for which the T_{AR50} for sutherlandins A and C phytosomes were 60 minutes and > 120 minutes for sutherlandins B and D.

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Table 5.1: The percentage release (AR_{120}) and rate of release (T_{AR50}) of the sutherlandins from *Sutherlandia* containing liposomes and phytosomes at pH 1.2

Sutherlandins	**Liposomes**		**Phytosomes**	
	AR_{120} (%)	**T_{AR50} (Minutes)**	**AR_{120} (%)**	**T_{AR50} (Minutes)**
Sutherlandin A	62.96	30	51.66	60
Sutherlandin B	58.09	5	41.33	> 120
Sutherlandin C	75.74	5	50.74	90
Sutherlandin D	45.74	None of time intervals	38.75	> 120

The individual sutherlandin release profiles from the liposomes and phytosomes were also compared and the similarity factors (f_2) for sutherlandins A, B, C and D release

from the liposomes versus phytosomes were 40, 33, 27 and 50, respectively, clearly indicating the dissimilarities of the release profiles of each sutherlandin from the liposomes *versus* the phytosomes. The f_2 value for sutherlandin D was 50, indicating similarity; however, this value is at the margin of similarity.

The faster release of the APIs from the liposomes compared to the phytosomes is in agreement with previous studies which reported that API release profiles from liposomes characteristically show an initial faster API release followed by slower rates which may be due to the large specific surface area and higher API content located on the liposomal surface. It may also be caused by API adsorption on the nano particle surface of the liposome (Lockman *et al*. 2002).

Compared to the liposomes, API release from the phytosomes was however more constant and sustained right from the initial stages of API release. This may be due to an increase in diffusional distance and the hindering effects of the surrounding solid lipid shell found in the phytosome (Sadiq, *et al* 2014). It may also be due to complex formation between the API and phospholipid (i.e. phosphatidylcholine) in the phytosome preparation *(*Patel *et al.* 2009).

It was interesting to note that the percentages of sutherlandins A, B, C, and D released from the phytosomes in the pH 1.2 buffer was increased at 24 h, while their percentages released from the liposomes stayed the same as the AR_{120} values. This suggests that the phytosomes exhibited sustained flavonoid glycoside release in acidic medium.

For quercetin, i.e. the flavonoid aglycone, no release was detected from the phytosomes containing quercetin at 120 minutes in pH 1.2 buffer, but 49.5 %

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release in the 24 hour samples. In addition, no quercetin release was detected from the liposomes at 24 hours, suggesting that the phytosomes (but not the liposomes) has the ability to serve as carriers for both flavonoid glycosides and aglycones.

To determine whether one or all of the sutherlandins would be required if one wishes to compare the API release character or quality of *Sutherlandia* containing liposomes or phytosomes at pH 1.2, the release profiles and data presented in figure 5.2 and table 5.1 were considered.

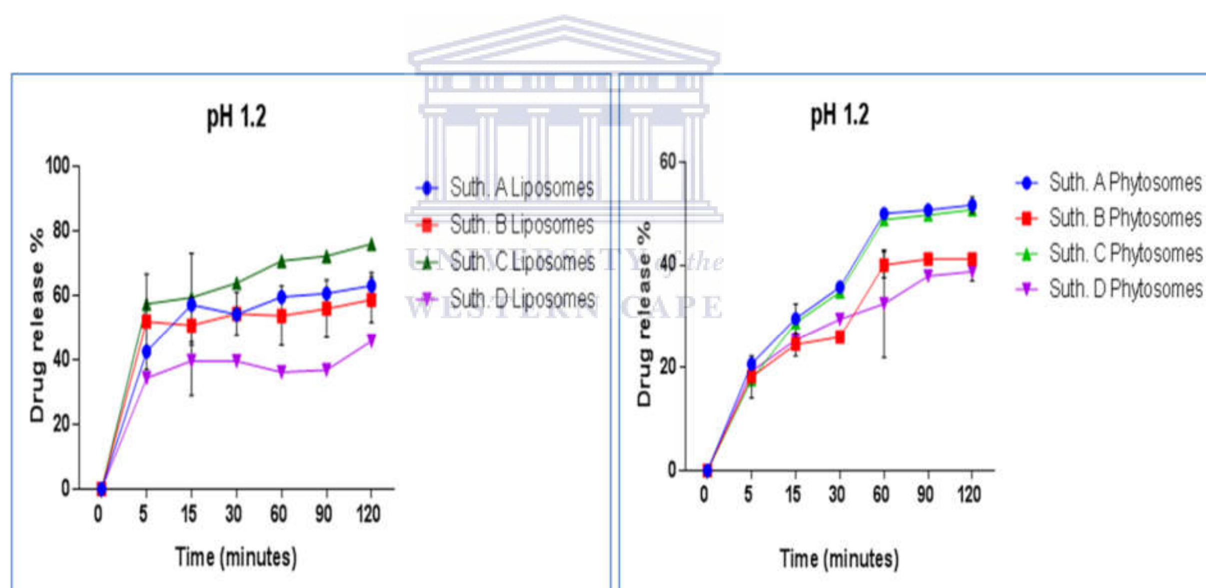

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Figure 5.2 Release profiles of individual sutherlandins A, B, C and D from *S. frutescens* liposomes and phytosomes at pH 1.2.

Table 5.2: Comparison of release profile of individual sutherlandins A, B, C, and D from liposomes at pH 1.2

Sutherlandin comparison	Similarity factor (f_2)	Inference

Suth A vs Suth B	**61**	**Release profile similar**
Suth A vs Suth C	**46**	**Release profile dissimilar**
Suth A vs Suth D	**39**	**Release profile dissimilar**
Suth B vs Suth C	**41**	**Release profile dissimilar**
Suth B vs Suth D	**43**	**Release profile dissimilar**
Suth C vs Suth D	**27**	**Release profile dissimilar**

For sutherlandin release from the liposomes at pH 1.2, the f_2 values were all below 50 with the exception of sutherlandin A *versus* sutherlandin B ($f_2 = 61$). This implies that the release profiles of sutherlandins A and B were similar but dissimilar for all the other comparisons (i.e. A *versus* C, A *versus* D, B *versus* C, B *versus* D, and C *versus* D). This could be because sutherlandins A and B are glycosides of the same aglycone, quercetin. However, if this is so, then we expected the release profiles of sutherlandins C and D to be similar as well because they are glycosides of the same aglycone, viz. kaempferol. This was not the case though. It may be that glycosides of quercetin are more similar than glycosides of kaempferol, and the difference in the sugar moieties of sutherlandins C and D could also contribute to the dissimilarity of their release profiles. No evidence to substantiate or refute this proposition could however be found in literature.

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For the phytosomes, the results of the release profile comparisons of the different sutherlandins at pH 1.2 are presented in figure 5.2 and table 5.3.

Table 5.3: Comparison of release profile of individual sutherlandins A, B, C, and D from phytosomes at pH 1.2

Sutherlandin comparison	Similarity factor (f_2)	Inference
Suth A vs Suth B	**54**	**Release profile similar**
Suth A vs Suth C	**87**	**Release profile similar**
Suth A vs Suth D	**50**	**Release profile similar**
Suth B vs Suth C	**54**	**Release profile similar**
Suth B vs Suth D	**50**	**Release profile similar**
Suth C vs Suth D	**74**	**Release profile similar**

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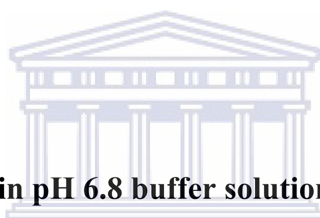
In this case the f_2 values were all above 50, implying similarity of the release profiles of all the sutherlandins from the phytosome preparations. This is different from what was obtained with the liposomes where most of the sutherlandins showed dissimilarity in drug release profiles. It could well be that the phytosome preparation masks the characteristics of individual sutherlandins, so that their release depend on the enclosing material while in the liposome preparation the individual characteristics of the sutherlandins are preserved leading to differences in sutherlandin release from the liposomes but not from the phytosomes. These observed differences could also be due to the bonding characteristics of the sutherlandins being different within the phytosomes but not within the liposomes. No confirmation of these possibilities was however found in the literature.

Collectively, the above results indicated that there were significant differences in the release profiles of the *S. frutescens* flavonoid glycosides from liposomes *versus* phytosomes at pH 1.2 i.e. in gastric conditions, a characteristic shown by all the individual sutherlandins. The differences observed might reflect differences in the sutherlandin encapsulation efficacy or surface area in the liposomes and phytosomes. In addition, for comparison of API release from different phytosome preparations of Sutherlandia, as a product quality control specification, any of the 4 sutherlandins can be considered, but not for liposome preparations, in which case all 4 marker compounds should be used.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5.4.2 Sutherlandin release in pH 6.8 buffer solution

The total amounts (AR_{120}) and rates (T_{AR50}) of sutherlandins (A to D) released from the liposomes and phytosomes are presented in table 5.4, figure 5.3 and Appendix 4 (i.e. the data from which the graphs were plotted).

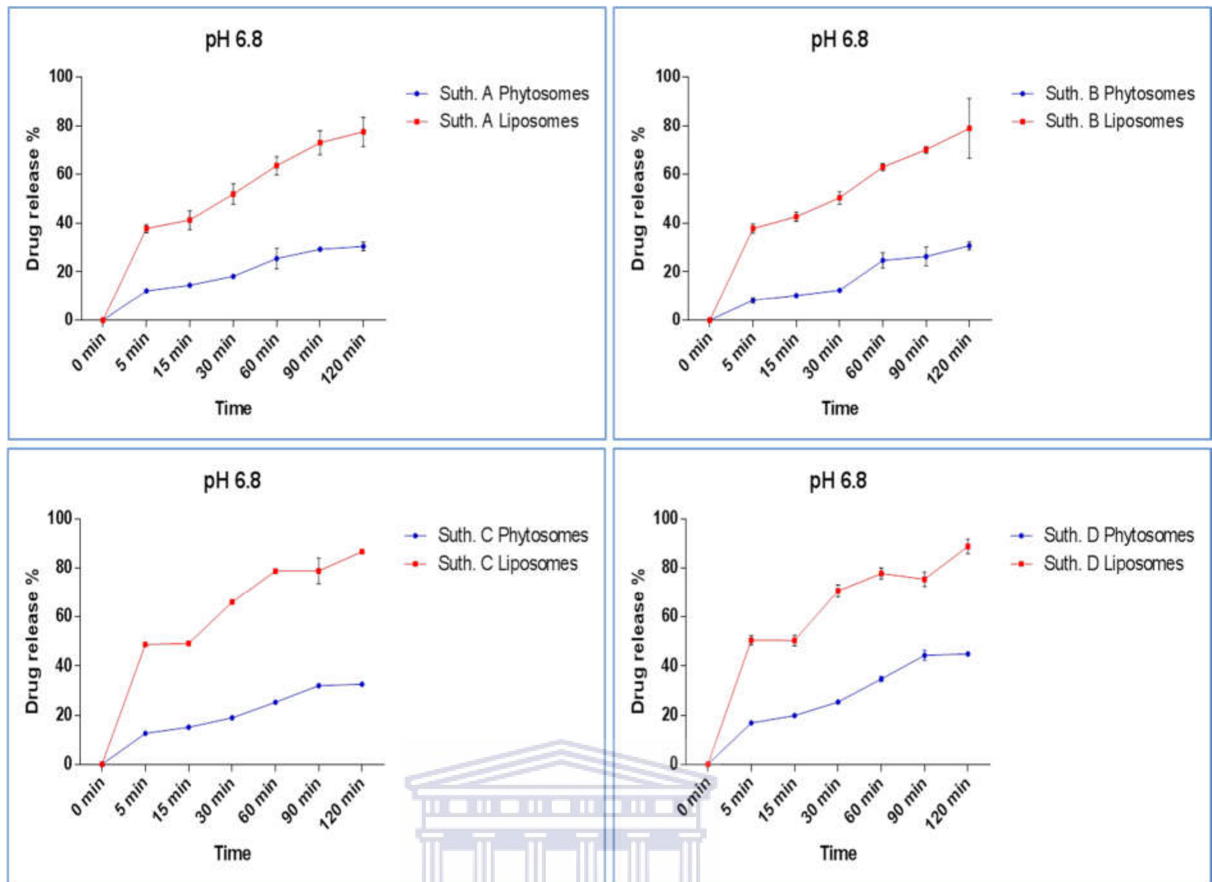

Figure 5.3: Release profiles of sutherlandins A, B, C and D from *S. frutescens* liposomes *versus* phytosomes at pH 6.8.

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Neither the liposomes nor the phytosomes released all (i.e. 100 %) of the individual sutherlandins after 120 mins in pH 6.8 buffer (i.e. $AR_{120} < 100$ %) and the amounts released at all time points were significantly (**p = 0.001** for sutherlandins A and **p = 0.0008** for sutherlandin B, C and D), higher for the liposomes than that from the phytosomes. The amounts of sutherlandins A, B, C, and D released from the liposomes at 120 mins, as a percentage of the total amount of the encapsulated *S. frutescens* material, were 77.47, 75.78, 86.76 and 88.86%, respectively, and 30.55, 30.76, 32.65 and 44.93%, respectively for the phytosomes. Despite the higher flavonoid encapsulation efficiency of the phytosomes, established in chapter 4, the

liposomes still released higher amounts of each flavonoid, after 120 minutes in pH 1.2 buffer, than the phytosomes.

The sutherlandins were released at different rates from the liposomes with the time for 50% release (i.e.T_{AR50}) being 5 minutes for sutherlandin D and 30 minutes for sutherlandins A, B and C. These rates were however faster than that for the phytosomes for which the T_{AR50} for all sutherlandins of the phytosomes > 120 minutes.

Table 5.4: The percentage release (AR_{120}) and rate of release T_{AR50} form the liposomes and phytosomes at pH 6.8

Sutherlandins	Liposomes		Phytosomes	
	AR_{120} (%)	T_{AR50} (Minutes)	AR_{120} (%)	T_{AR50} (Minutes)
Sutherlandin A	77.47	30	30.55	> 120
Sutherlandin B	75.78	30	30.76	> 120
Sutherlandin C	86.76	30	32.65	> 120
Sutherlandin D	88.86	5	44.93	> 120

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The individual sutherlandin release profiles from the liposomes and phytosomes were also compared and the similarity factors (f_2) for sutherlandins A, B, C and D release from the liposomes *versus* phytosomes were 22, 19, 17 and 21, respectively, clearly indicating the dissimilarities of the release profiles of each sutherlandin from the liposomes *versus* the phytosomes.

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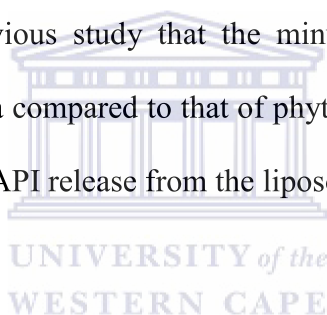
Generally, the liposomes showed faster flavonoid release than the phytosomes. Release of the sutherlandins was also faster at pH 6.8 than at pH 1.2, probably due

to the enhanced solubility of liposomes at higher pH values. The phytosomes, on the other hand, exhibited slower sutherlandin (API) release at pH 6.8. Release of API from solid dosage forms is a complex operation influenced by a number of factors such as differences in surface area, stability, particle size and size distribution (Chiou, *et al* 1971). The significant enhancement of API release from the liposomes compared to the phytosomes could be due to the greater physical stability of the liposomal preparation (confirmed by zeta potential values for liposomes of - 8.55) than that of phytosomes (zeta potential value of - 0.457) (Lim, Kim 2002).The smaller size of the liposome vesicles could be another factor contributing to the increased API release of *S. frutescens* flavonoids. Indeed, it has been demonstrated in a previous study that the minute size of the liposomes provided a bigger surface area compared to that of phytosomes (Ait-Oudhia, *et al.* 2014), and that this hastened API release from the liposomes.



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To determine whether one or all of the sutherlandins would be required if one wishes to compare the API release character or product quality of *Sutherlandia* containing liposomes or phytosomes at pH 6.8, the release profiles and data presented in figure 5.4 and table 5.3 were considered.

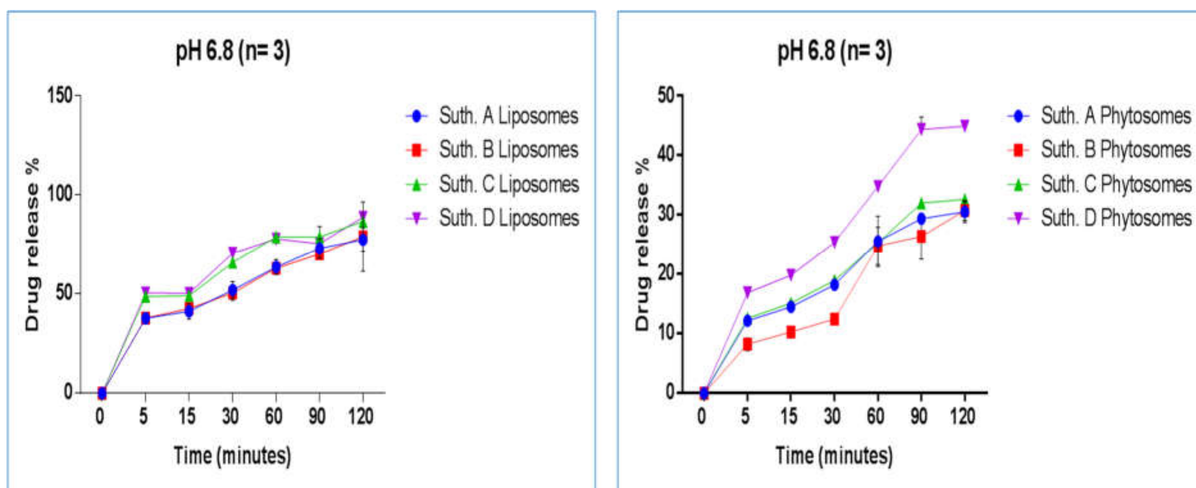

Figure 5.4 Release profiles of individual sutherlandins A, B, C and D from *S. frutescens* liposomes and phytosomes at pH 1.2.

Table 5.5: Comparison of release profile of individual sutherlandins A, B, C, and D from liposomes at pH 6.8

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Sutherlandin comparison	Similarity factor (f_2)	Inference
Suth A vs Suth B	**75**	**Release profile similar**
Suth A vs Suth C	**48**	**Release profile dissimilar**
Suth A vs Suth D	**45**	**Release profile dissimilar**
Suth B vs Suth C	**51**	**Release profile similar**
Suth B vs Suth D	**48**	**Release profile dissimilar**
Suth C vs Suth D	**56**	**Release profile similar**

Generally the f_2 – values for sutherlandin release from the liposomes at pH 6.8 were higher than those obtained at pH 1.2. At the higher pH the release profiles of sutherlandins A *versus* B, B *versus* C and C *versus* D were similar, but dissimilar

for all the other comparisons (i.e. A *versus* C, A *versus* D, and B *versus* D). This could have been because sutherlandins A and B are glycosides of the same aglycone, quercetin, while sutherlandins C and D are glycosides of the aglycone kaempferol, or it might be also due to increased stability of the liposomes at the higher pH.

The results for the release profile comparisons of the different sutherlandins from the phytosomes at pH 6.8 are presented in figure 5.4 and table 5.6.

Table 5.6: Comparison of release profile of individual sutherlandins A, B, C, and D from phytosomes at pH 6.8

Sutherlandin comparison	**Similarity factor (f_2)**	**Inference**
Suth A vs Suth B	**72**	**Release profile similar**
Suth A vs Suth C	**88**	**Release profile similar**
Suth A vs Suth D	**50**	**Release profile similar**
Suth B vs Suth C	**88**	**Release profile similar**
Suth B vs Suth D	**50**	**Release profile similar**
Suth C vs Suth D	**52**	**Release profile similar**

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The f_2 values were all above 50, implying similarity of the release profiles of all the sutherlandins from the phytosome preparations. It could well be that in the phytosome preparation the structural characteristics of individual sutherlandins were masked, so that their release depended on the enclosing material while in the liposome preparation the individual characteristics of the sutherlandins were

preserved leading to differences in sutherlandin release from the liposomes but not from the phytosomes. The observed differences could also be due to the bonding characteristics of the sutherlandins which might also be different within the phytosomes but not within the liposomes. No confirmation for either of these possibilities was however found in the literature.

Collectively, the above results indicated that there were significant differences in the release profiles of the *S. frutescens* flavonoid glycosides from liposomes *versus* phytosomes at pH 6.8 i.e. in intestinal fluid, a characteristic shown by all the individual sutherlandins. The differences observed might reflect differences the encapsulation efficacy or surface area of the API in the liposomes and phytosomes. In addition, for comparison of API release from different phytosome preparations of *Sutherlandia*, (e.g, for product quality control purposes) any of the 4 sutherlandins can be considered, but not for liposome preparations, in which case similarity was only found for *Sutherlandia* A *versus* B, sutherlandin B *versus* C and sutherlandin C *versus* D, and preferably all 4 should be monitored.

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5.4.3 Effect of pH on flavonoid release from liposomes and phytosomes.

Finally, the effect that pH had on flavonoid release was also assessed and the results of comparison of sutherlandins A, B, C and D release from the *Sutherlandia* liposomes and phytosomes at pH 1.2 and 6.8 are presented in figures 5.5 and 5.6.

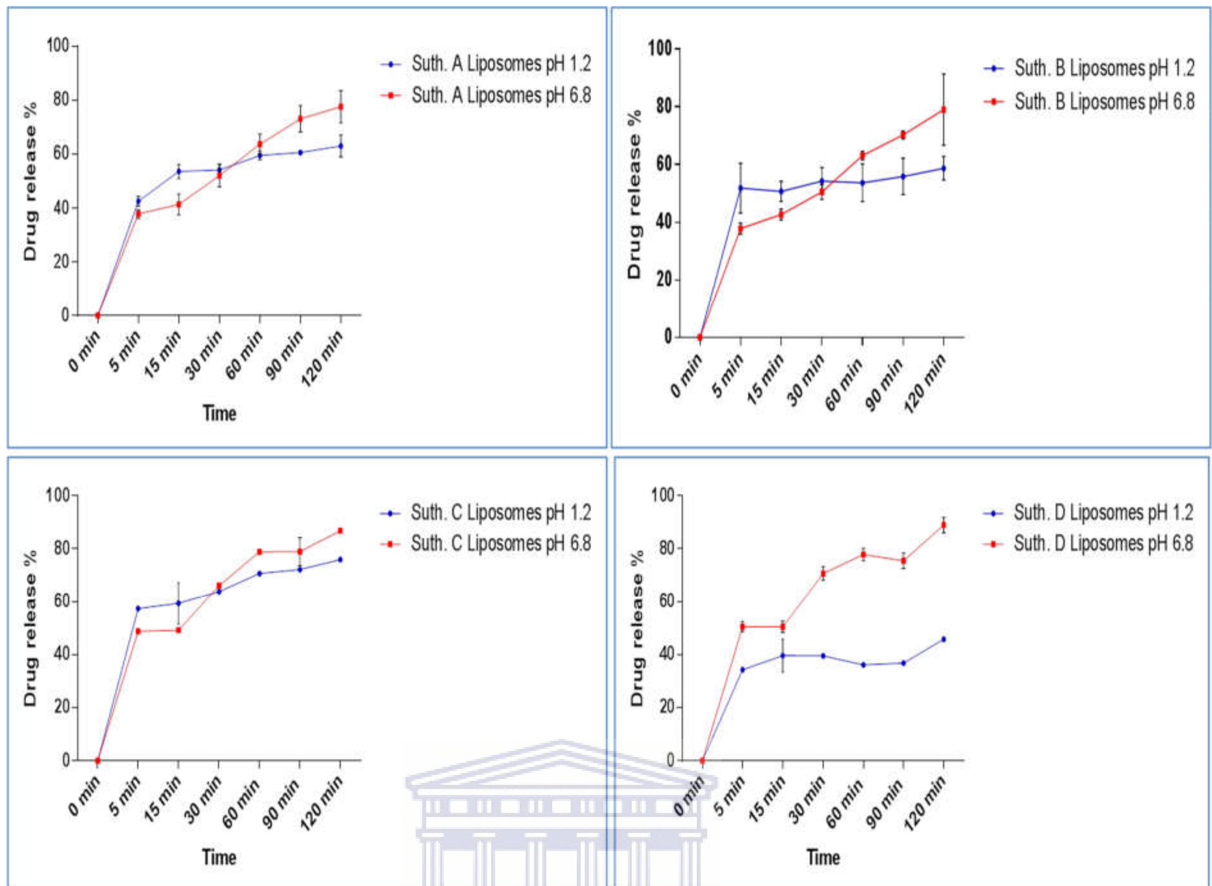

Figure 5.5: Effect of pH on release profiles of sutherlandins A, B, C and D from *S. frutescens* liposomes

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The release profile for sutherlandins A and C from *Sutherlandia* liposomes at pH 1.2 *versus* 6.8 were similar with f_2 values of 52, and 54 respectively and pH therefore did not significantly affect the release profile of these sutherlandins. However, the release of sutherlandin B and D from *Sutherlandia* liposomes at pH 1.2 and 6.8 was not similar with f_2 values of 42 and 24, respectively. In addition, for sutherlandin B the release at the 2 pH's was similar up to 60 minutes (with f_2 value 52), but after 60 minutes, it became dissimilar. Finally, the release of sutherlandin D was clearly dissimilar at the 2 pH's and this was most likely due to instability of this API in the media.

For phytosomes, the release profile for sutherlandins A, B and C (with the exception of sutherlandin D, $f_2 = 65$) from *Sutherlandia* phytosomes at pH 1.2 *versus* 6.8 were dissimilar with f_2 values of 22, 44 and 39, respectively. Clearly pH affected the release profile of these 3 flavonoid glycosides from *Sutherlandia* phytosomes Overall, sutherlandin release from the phytosomes **was** more rapid at pH 1.2 than pH 6.8, i.e. release rate was greater increased at the lower simulated gastric pH, while for liposomes change of pH (from 1.2 to 6.8) did not significantly change the sutherlandin release profiles.

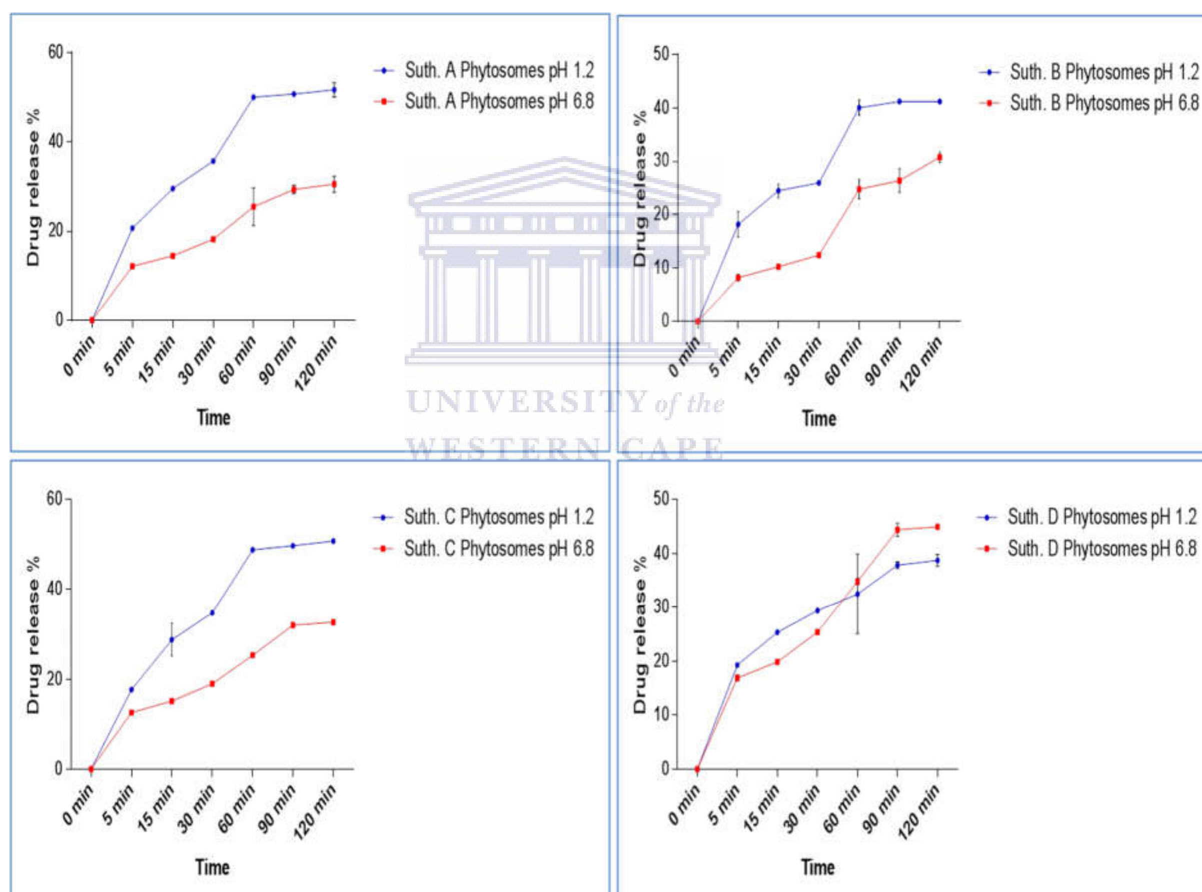

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Figure 5.6: Effect of pH on release profiles of sutherlandins A, B, C and D from *S. frutescens* phytosomes

Overall, the release of sutherlandins A, B, C and D, i.e. potential active compounds of *S. frutescens*, was significantly higher and faster, from the liposomes compared to the phytosomes, at both stomach (1.2) intestinal (6.8) pH, confirming the second

hypothesis tested in this study, *viz.* that sutherlandin release would be faster from the liposomes than from the phytosomes. In addition, for product quality control purposes, any of the 4 sutherlandins can be considered for comparison of API release from different phytosome preparations of *Sutherlandia* at both pH 1.2 and 6.8, but not for liposome preparations.

5.5 Conclusion

The objective of this section of the study was to compare the *in vitro* release profiles of flavonoids from *S. frutescens* liposomes *versus* phytosomes. The release profiles of sutherlandins A, B, C and D showed significant differences with higher and faster release rates from the liposomes compared to the phytosomes at both pH 1.2 and 6.8. This may be due to the smaller particle size and narrower particle size range (as confirmed by the polydispersity index) of the liposomes compared to the phytosomes, attributes that facilitated the release of the API upon penetration of the buffer medium through the preparation matrix. Such penetration then resulted in rapid API diffusion. The phytosomes, with a larger size and broad particle size distribution, however released the API more slowly resulting in more delayed release. Finally, for product quality assessment any of the 4 sutherlandins can be considered for comparison of API release from different phytosome preparations of *Sutherlandia* at both pH 1.2 and 6.8, but not for liposome preparations. In the latter all 4 sutherlandins might have to be used to obtain the best assessment.

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CHAPTER SIX
Conclusions and Recommendations

The primary aim of this study was to prepare and compare liposomes and phytosomes of *S. frutescens* freeze dried aqueous extract (FDAE) for solid oral dosage form application. The specific objectives were to prepare and physically characterise liposomes and phytosomes of the *S. frutescens* FDAE and to determine the drug release profiles of flavonoids from these liposomes and phytosomes.

From the results obtained in this study, the following major conclusions could be drawn:

1. Both phytosomes and liposomes of *S. frutescens* FDAE can be efficiently prepared using the thin film hydration method. In addition, sonication was an effective method for size reduction of both types of particles, but, in this study, worked better for the liposomes than for the phytosomes of *S. frutescens* FDAE.

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2. The optimized liposomes and phytosomes had an appropriate vesicle size and carried a sufficient amount of drug. However, the prepared liposomes had smaller particle size and size distribution as well as a relatively higher zeta potential value than the phytosomes, suggesting that the liposomes had better physical and chemical stability profiles.
3. Phytosomes can encapsulate significantly higher amounts of *S. frutescens* FDAE than do liposomes confirming the first hypothesis, *viz*. that the entrapment efficacy of the phytosomes will be higher than that of the liposomes, tested in this study, and also further strongly indicate that the phytosomes are promising vehicles for delivery of different active constituents of the herbal extract.

4. Finally, the release of sutherlandins A, B, C and D, potential active compounds of *S. frutescens*, was significantly faster, from the liposomes compared to the phytosomes, at both stomach (1.2) and intestinal (6.8) pH, confirming the second hypothesis tested in this study, *viz.* that sutherlandins release would be faster from the liposomes than from the phytosomes.

Collectively, the results obtained strongly suggest that phytosomes could be a very viable particle form for the delivery of flavonoids, and possibly other actives, (i.e. active phytopharmaceutical material) from an aqueous extract of *S. frutescens.*

Overall, this study provides valuable preliminary information on the preparation and evaluation of liposomes and phytosomes of *S. frutescens* aqueous extract. Particularly the phytosomes may be the administration and delivery form to use for systemic delivery of *S. frutescens* and may most likely also be a useful delivery form for dried aqueous extracts of other South African indigenous medicinal plants. More detailed studies on the physical and pharmaceutical properties (e.g. flavonoid or other active compound dissolution profile; stability, etc.) of such products are however needed to confirm this potential usefulness. In addition, *in vitro* and *in vivo* pharmacokinetic studies of *S. frutescens* phytosomal and liposomal products will also be needed. In the immediate future, we however aim to first investigate the optimization of the stability and lamellarity of the phytosomal preparations and the effect which cholesterol, as a formulation (and drug release) stabilizer, may have.

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Appendix 1: **Certificate of analysis for FDAE *S. frutescens*.**

CERTIFICATE OF ANALYSIS

Product Code	00814
Batch No.	62265
Product	Sutherlandia PE
Expiry date	May 2015

Characteristic	Specification	Result
Plant material	*Sutherlandia frutescens* herba sicc	Pass
Appearance	A hygroscopic powder with a mustard brown colour	Pass
Odour & Taste	Characteristic with a bitter taste	Pass
Country of origin	South Africa	Pass
Solubility	≥95% Soluble in water	Pass
Moisture	<7% (m/m)	6% (m/m)
Bulk Density	0.2 – 0.5 g/ml	0.4 g/ml
Identity (FT-IR)	≥95.00% similarity when compared to reference	97.57%
Heavy metals		
Lead	<5.0 mg/kg	Not Detected
Cadmium	<0.5 mg/kg	Not Detected
Mercury	<0.1 mg/kg	Not Detected
Arsenic	<1.00 mg/Kg	Not Detected
Microbiological		
Total viable aerobic count	<2000cfu/g	1305 cfu/g
Yeast & Moulds	<100cfu/g	No growth/g
Escherichia coli	Absent/g	No growth/g
Staph. aureus	Absent/g	No growth/g
Salmonella	Absent/25 g	Absent/25 g

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STORAGE REQUIREMENTS
Closed container, cool (10 - 25°C) and dry conditions

PAARL, 20 Jun 2013

Afriplex
Quality Control Department

Afriplex
PO Box 3186, Paarl 7620, South Africa Tel: +27 21 872 4976 Fax: +27 21 872 4930

Appendix 2: Flavonoid calibration curves and regression equations.

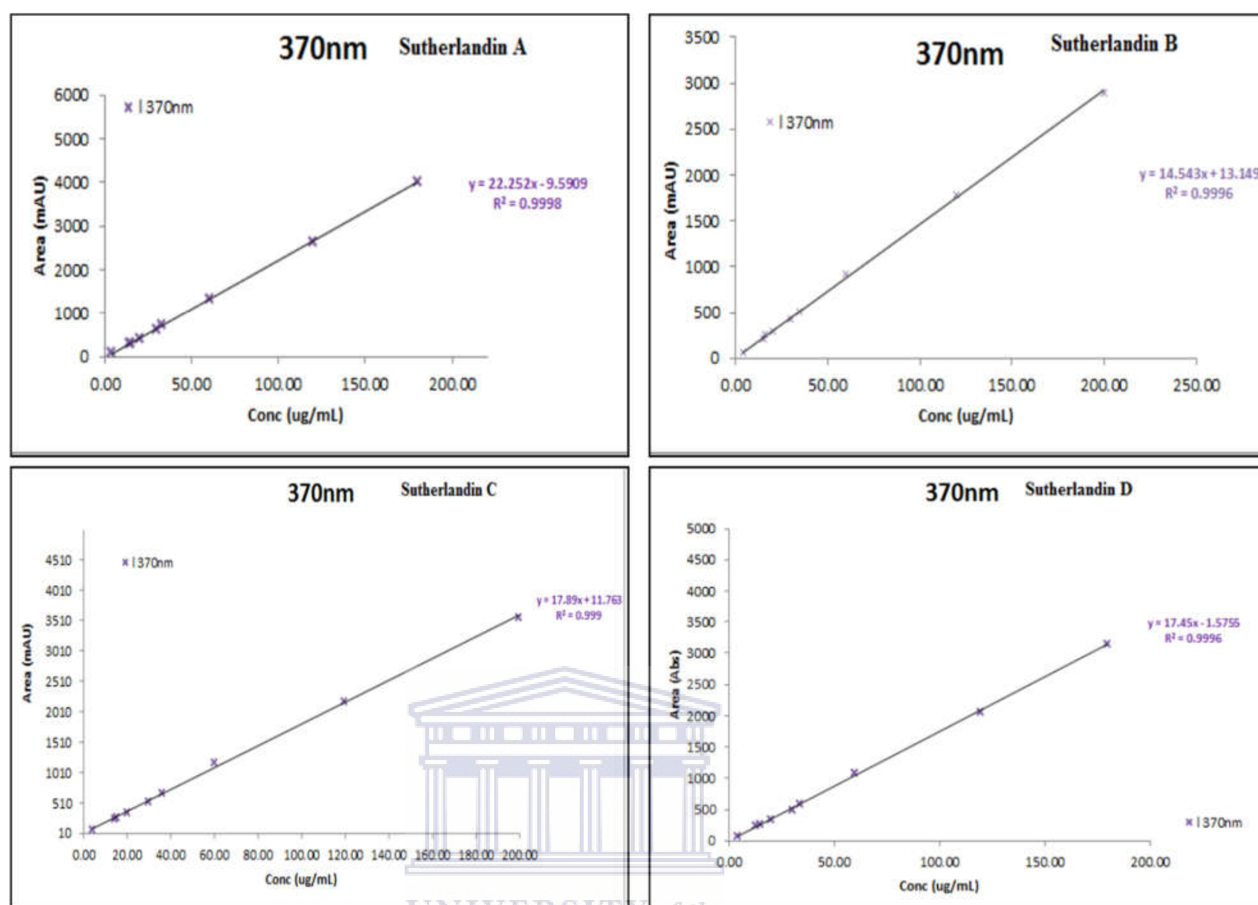

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Figure A 2.1: Calibration curves of sutherlandins (A to D) at wavelengths 370 nm.

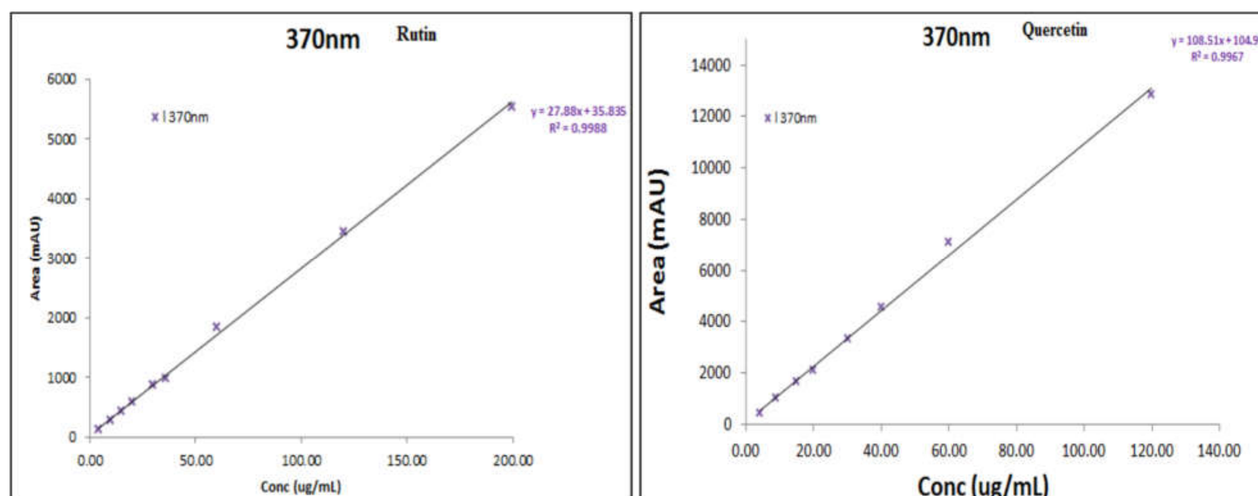

Figure A 2.2: Calibration curves of flavonoid glycoside (rutin) and aglycone (quercetin) at wavelengths 370 nm.

Appendix 3: HPLC peak areas of individual sutherlandins (A to D) released from liposomes and phytosomes and used to calculate entrapment efficacies. The peaks were obtained using method described in section 4.3.8 and detected at 370 nm

Table A3.1: Peak area of individual sutherlandins released from liposomes

Sutherlandin A		Sutherlandin B	
Peak area of starting solution (mAUFs)	Peak area of supernatant (mAUFs)	Peak area of starting solution (mAUFs)	Peak area of supernatant (mAUFs)
723.2	441	1116	814.2
721.8	540	1112.8	816.1
851.2	584	1246	861.5
Sutherlandin C		**Sutherlandin D**	
Peak area of starting solution (mAUFs)	Peak area of supernatant (mAUFs)	Peak area of starting solution (mAUFs)	Peak area of supernatant (mAUFs)
1569.5	918.7	1168.7	995
1568.6	1188.6	1108.5	775.8
1715	1097.2	1105.9	640.5

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Table A3.2: Peak area of individual sutherlandins released from phytosomes

Sutherlandin A		Sutherlandin B	
Peak area of starting solution (mAUFs)	Peak area of supernatant (mAUFs)	Peak area of starting solution (mAUFs)	Peak area of supernatant (mAUFs)
851.2	478.8	1246	697.3
851.2	423	1246	619.6
851.2	422	1246	615
Sutherlandin C		**Sutherlandin D**	
Peak area of starting solution (mAUFs)	Peak area of supernatant (mAUFs)	Peak area of starting solution (mAUFs)	Peak area of supernatant (mAUFs)
1715.9	1043.1	1168.7	691.5
1715.9	941.4	1168.7	590.8
1715.9	941	1168.7	588.9

Appendix 4: Sutherlandin release *versus* time profiles for FDAE *Sutherlandia* phytosomes and liposomes

Table 4.1: Release versus time profile for sutherlandin A from phytosomes of FDAE Sutherlandia at pH 1.2 and 6.8

	Percentage sutherlandin A released at pH 1.2 (%)		Percentage sutherlandin A released at pH 6.8	
Time (minutes)	AVE ± SD	%CV	AVE ± SD	%CV
0	0 ± 0	0	0 ± 0	0
5	46.560 ± 0.551	1.183	27.400 ± 1.058	3.862
15	66.430 ± 0.321	0.484	32.667 ± 0.115	0.353
30	80.300 ± 0.500	0.623	40.967 ± 0.862	2.105
60	112.460 ± 0.153	0.136	57.400 ± 9.440	16.446
90	114.000 ± 0.436	0.382	66.033 ± 2.155	3.263
120	116.100 ± 3.378	2.909	68.667 ± 4.007	5.835

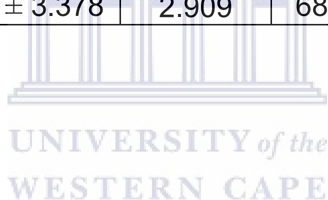

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Table 4.2: Release versus time profile for sutherlandin B from phytosomes of FDAE Sutherlandia at pH 1.2 and 6.8.

	Percentage sutherlandin B released at pH 1.2		Percentages sutherlandin B released at pH 6.8	
Time (minutes)	AVE ± SD	%CV	AVE ±SD	%CV
0	0 ± 0	0	0 ± 0	0
5	73.600 ± 16.639	22.607	33.3 ± 4.099	12.298
15	98.967 ± 8.271	8.358	41.7 ±0.608	1.459
30	104.900 ± 1.868	1.781	50.4 ± 0.520	1.031
60	161.633 ± 9.059	5.605	100.1 ± 12.002	11.990
90	166.450 ± 3.465	2.082	106.6 ± 14.880	13.959
120	166.950 ± 2.758	1.652	124.233 ± 6.799	5.473

Table 3: Drug release versus time profile for sutherlandin C from phytosomes of FDAE Sutherlandia at PH 1.2 and 6.8

	Percentage sutherlandin C released at pH 1.2		Percentage Sutherlandin C released at pH 6.8	
Time (minutes)	**AVE ± SD**	**%CV**	**AVE ± SD**	**%CV**
0	0 ± 0	0	0 ± 0	0
5	84.633 ± 0.702	0.830	60.233 ± 0.603	1.001
15	137.633 ± 17.960	13.049	72.400 ± 0.954	1.318
30	166.400 ± 0.400	0.240	90.867 ± 1.266	1.394
60	233.533 ± 0.208	0.089	121.067 ± 0.404	0.334
90	237.867 ± 0.850	0.358	153.133 ± 0.850	0.555
120	242.767 ± 0.611	0.252	156.233 ± 1.922	1.230

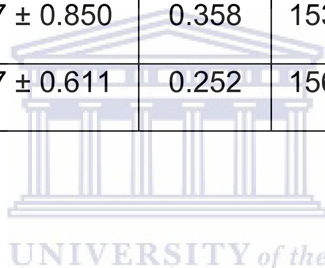

Table 4: Drug release data for sutherlandin D from phytosomes of FDAE Sutherlandia at PH 1.2 and 6.8

	Percentage sutherlandin D released at pH 1.2		Percentage sutherlandin D released at pH 6.8	
Time (minutes)	**AVE ± SD**	**%CV**	**AVE ± SD**	**%CV**
0	0 ± 0	0	0 ± 0	0
5	53.900 ± 1.217	2.257	47.300 ± 2.042	4.317
15	71.000 ± 1.900	2.676	55.567 ± 1.422	2.560
30	82.367 ± 2.316	2.812	71.067 ± 0.839	1.180
60	97.867 ± 24.999	25.544	97.400 ± 1.153	1.184
90	105.300 ± 3.666	3.482	124.267 ± 4.500	3.622
120	108.467 ± 4.932	4.547	125.800 ± 1.308	1.039

Table 5: Drug release data for sutherlandin A from liposomes of FDAE Sutherlandia at PH 1.2 and 6.8

	Percentage Sutherlandin A released at pH 1.2		Percentage sutherlandin A released at pH 6.8	
Time (minutes)	**AVE ± SD**	**%CV**	**AVE ± SD**	**%CV**
0	0 ± 0	0	0 ± 0	0
5	72.9 ± 2.1	1.6	64.800 ± 1.637	2.526
15	83.06 ± 27.1	32.7	70.933 ± 7.569	10.671
30	92.7 ± 1.15	1.24	89.133 ± 5.346	5.998
60	102.03 ± 0.76	0.74	109.167 ± 7.081	6.487
90	102.150 ± 0.071	0.069	125.233 ± 7.022	5.607
120	107.9 ± 4.078	3.7798	132.767 ± 6.732	5.071

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Table 6: Drug release versus time profile for sutherlandin B from liposomes of FDAE Sutherlandia at PH 1.2 and 6.8

	Percentage Sutherlandin B released at pH 1.2		Percentage sutherlandin B released at pH 6.8	
Time (minutes)	**AVE ±SD**	**%CV**	**AVE ±SD**	**%CV**
0	0 ± 0	0	0 ± 0	0
5	130.733 ± 21.150	16.178	101.667 ± 19.19	18.883
15	131.867 ± 30.033	22.775	120.800 ± 9.180	7.600
30	132.050 ± 2.475	1.874	138.400 ± 4.004	2.893
60	133.400 ± 0.424	0.318	170.867 ± 5.865	3.433
90	135.600 ± 2.546	1.877	194.000 ± 1.414	0.729
120	149.800 ± 4.386	2.928	195.400 ± 9.334	4.777

Table 7: Drug release versus time profile data for sutherlandin C from liposomes of FDAE Sutherlandia at PH 1.2 and 6.8

	Percentage sutherlandin C released at pH 1.2		**Percentage sutherlandin C released at pH 6.8**	
Time (minutes)	**AVE ± SD**	**%CV**	**AVE ± SD**	**%CV**
0	0 ± 0	0	0 ± 0	0
5	175.200 ± 1.473	0.841	149.100 ± 0.173	0.116
15	181.633 ± 42.812	23.571	151.000 ± 0.000	0.000
30	195.100 ± 1.493	0.765	201.933 ± 0.709	0.351
60	216.067 ± 0.833	0.385	241.067 ± 0.115	0.048
90	220.533 ± 0.231	0.105	241.267 ± 18.3	7.585
120	231.967 ± 1.531	0.660	265.100 ± 0.529	0.200

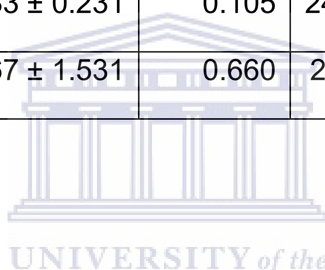
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Table 8: Drug release versus time profile for sutherlandin D from liposomes of FDAE Sutherlandia at PH 1.2 and 6.8

	Percentage Sutherlandin D released at pH 1.2		**Percentage Sutherlandin D released at pH 6.8**	
Time (minutes)	**AVE ± SD**	**%CV**	**AVE ± SD**	**%CV**
0	0 ± 0	0	0 ± 0	0
5	78.833 ± 2.237	2.837	115.900 ± 0.141	0.122
15	90.933 ± 24.194	26.607	114.550 ± 2.051	1.790
30	91.0 ± 1.947	2.139	162.500 ± 3.704	2.279
60	83.067 ±2.150	2.589	179.000 ±0.000	0.000
90	84.533 ± 1.361	1.610	173.667 ± 2.517	1.449
120	105.200 ± 1.970	1.872	204.367 ± 1.704	0.834

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