Preparation, characterisation and evaluation of *Artemisia afra* phytosomes with modified release properties

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Key words

Artemisia afra

Phytosomes

Complex formation efficiency

Luteolin

Dissolution

Modified release

HPLC

Summary

Dissolution studies on various dosage forms (powder, tablets, teabags and alginate beads) of the *Artemisia afra* freeze dried aqueous extract (FDAE) all exhibit a rapid release profile. Generally, such a release profile may be therapeutically undesirable as it may affect absorption and hence the therapeutic outcome. In addition, also associated with rapid release profiles, is frequent dosing required (to maintain therapeutic plasma concentrations) and unavoidable fluctuations in plasma drug concentrations, leading to under and or over dosing. Based on the aforementioned shortcomings, there may be need to modify the dissolution profile of the phytoconstituents of *A. afra*. Phytosomes, which are complexes of phospholipids with phytoconstituents, offer a very viable dosage form option for *A. afra* as they could afford advantages of increased lipophilicity hence a decreased dissolution rate and improved absorption or permeability of the phytoconstituents.

The objectives of this study were to; (1) prepare and characterise an *A. afra* freeze dried aqueous extract and the phytosomes thereof, (2) determine factors that influence the complex formation efficiency (CFE) between phytoconstituents and phosphatidylcholine in the preparation of *A. afra* phytosomes and (3) compare the phytoconstituent release properties, in terms of dissolution profiles, of FDAE *versus* the phytosomes, in simulated gastric and intestinal fluid, using luteolin as the marker compound. It was hypothesised that (1) the phytosome drug delivery technology can be successfully used on the *A. afra* FDAE to produce phytosomes with acceptable pharmaceutical physiochemical characteristics; (2) *A. afra* phytoconstituents in the phytosome complex are more lipophilic than the phytoconstituents in the FDAE and therefore the phytosome complex has a higher apparent solubility in *n*-octanol and (3) the dissolution rate of phytoconstituents from the FDAE is higher than that of phytoconstituents from the *A. afra* phytosomes.

To realise these objectives, an *A. afra* freeze dried aqueous extract was prepared and characterised. Then, using a 2^3 full factorial experimental design, different batches of phytosomes were prepared from the FDAE to establish the factors that affected the complex formation efficiency. The independent variables investigated were stirring speed, type of organic solvent and mass ratio of FDAE to phospholipids, and the dependent variable was complex formation efficiency. From the experimental design results, the optimised

preparation variables were used to prepare phytosomes which were then characterised in terms of pharmaceutical physicochemical parameters. Finally, the dissolution profiles of the FDAE and the phytosomes were determined in simulated gastric (pH 1.2) and intestinal (pH 6.8) fluids and compared using Q-release values, mean dissolution time values and mathematical models.

A moderate yield (26.16 ± 2.90%) of the FDAE was obtained and the extract had good aqueous solubility, acceptable moisture content (2.47 ± 0.15%), a high flavonoid content (34.1 ± 6.4 mg QE/g) and contained moderate levels of the selected marker compound, luteolin (1.92 ± 0.01 µg/mg), mostly in the conjugate form (76.6%). However, the FDAE was hygroscopic and poorly lipid soluble (log P < 2).

The solvent evaporation mechanical dispersion method was suitable for the preparation of *A. afra* phytosomes and yielded phytosomes with a moderately good complex formation efficiency (21.2 to 68.7%). Results from the experimental design showed that the complex formation efficiency was significantly affected by the phosphatidylcholine amount ($p = 0.0049$) and stirring speed ($p = 0.0086$), where the CFE varied directly with the former and inversely with the latter. Furthermore, the phytosomes possessed suitable pharmaceutical physicochemical characteristics with smooth, spherical, mono-dispersed (PDI = 0.201 ± 0.076), nano-sized particles (487 ± 35 nm) which had good colloidal stability (-54.6 ± 0.2 mV) and significantly improved lipid solubility ($p = 0.001$). Lastly, comparison of the dissolution profiles of the FDAE *versus* phytosomes exhibited significantly different ($p < 0.05$) Q_{75} values, of 16 and 366 minutes, respectively, and mean dissolution time values of 18.9 and 269.4 minutes, respectively. Also, the release mechanisms of luteolin from the FDAE and phytosomes were dissimilar with release from the former being by Fickian diffusion and from the latter by a complex mechanism.

In summary, the results showed that phytosome technology can be successfully used on the freeze dried aqueous extract of *A. afra* to modify the release of phytoconstituents from the extract. Moreover, the technique is also capable of producing phytosomes with acceptable pharmaceutical physicochemical properties suitable for drug delivery.

Declaration

I declare that the thesis **Preparation, characterisation and evaluation of *Artemisia afra* phytosomes with modified release properties** is my own work, that it has not been submitted before for any degree or examination in any other university and that all the sources I have used or quoted have been indicated and acknowledged by means of complete referencing.

Nyashadzashe G. Bepe
April, 2017

Signed: UWC, Bellville

Dedication

I dedicate this master's thesis to my wife, Tapiwa and our late son, Mukundi Immanuel Bepe. You will forever be remembered Mukundi.

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List of abbreviations and units

A. afra	*Artemisia afra*
µl	Microlitre
ATM	African traditional medicine
BP	British Pharmacopeia
CAM	Complementary/alternate medicine
CFE	Complex formation efficiency
DLS	Dynamic light scattering
DSC	Differential scanning calorimetry
Eqn	Equation
FDAE	Freeze dried aqueous extract
FT-IR	Fourier transform infrared
G	Grams
HPLC	High performance liquid chromatography
L	Litres
LOD	Limit of detection
LOQ	Limit of quantification
mcg or µg	Micrograms
MDT	Mean dissolution time
Mg	Milligrams
Min	Minute
Ml	Milli-litres
mV	Millivolts
Nm	Nanometres
NMR	Nuclear magnetic resonance
^{o}C	Degrees Celsius
PC/PL	Phosphatidylcholine/phospholipids
PDI	Poly dispersity index

PSD	Particle size distribution
Rpm	Rotations per minute
SEM	Scanning electron microscopy
TFC	Total flavonoid content
USP	United States Pharmacopeia
UV	Ultra violet
WHO	World Health Organisation

Chapter 1

Introduction

The use of herbal products in the management of various ailments has gained popularity in recent years. With the advancements in herbal product technology, plant-based drugs have received substantial attention from the medical community as well as patients (Barnes *et al.*, 2004; Capasso *et al.*, 2000). Despite the increased attention, the clinical use of herbal products is however often still limited due to the poor oral bioavailability of the bioactive phytoconstituents, which typically have high molecular weights, poor aqueous or lipid solubility, and low plasma membrane permeability (Teng *et al.*, 2012; WHO, 2004). This challenge has encouraged researchers to formulate these phytoconstituents in delivery forms that improve the ease of administration, ensure sufficient bioavailability and have desirable aesthetic properties. The development of modern drug delivery systems for phytoconstituents has therefore become an essential element of pharmaceutical research.

In recent years, the technique of complexing plant extracts with phospholipids to produce phytosomes, a drug delivery system, has emerged as one of the most successful methods to improve the delivery of hydrophilic and hydrophobic phytoconstituents (Khan *et al.*, 2013). Indeed, this drug delivery system i.e. phytosomes, has been used to improve the biopharmaceutical properties of herbal extracts, leading to enhanced bioavailability (Bombardelli, 1991; Bombardelli *et al.*, 1989; Habbu *et al.*, 2013; Kidd, 2009; Yanyu *et al.*, 2006; Yue *et al.*, 2010), modified release (Khan *et al.*, 2013; Singh *et al.*, 2012) and improved stability (Khan *et al.*, 2014; Saraf, 2010) of phytoconstituents. Examples of such successful commercial phytosomal herbal products on the market include Silybin Phytosome™, Green Tea Phytosome™ and Ginkgo Biloba Phytosome™ (Bhattacharya, 2009; Dhir *et al.*, 2016; Karimi *et al.*, 2015). The phytosome delivery system has also been extended to conventional drugs such as aspirin (Semalty *et al.*, 2010c), diclofenac (Khazaeinia & Jamali, 2003), aceclofenac (Semalty *et al.*, 2010a) and rifampicin (Singh *et al.*, 2014), but generally, the technique's main application remains in the delivery of plant phytoconstituents.

Artemisia afra (common names; African wormwood; wilde-als; umhlonyane) is a medicinal plant widely used in southern Africa for numerous ailments, including colds, coughs, diabetes mellitus, heartburns, bronchitis and asthma among others (Watt & Breyer Brandwijk, 1962; Van Wyk & Gericke, 2000). Chemical analyses conducted on extracts of *A. afra* have reported the species to contain acetylenes, coumarins, flavonoids, terpenoids and volatile oils (Van Wyk & Gericke, 2000; Watt & Breyer Brandwijk, 1962; Avula *et al.*, 2009; Asfaw *et al.*, 2005). However, most of these bioactive constituents, especially flavonoids and their glycosides (Willcox, 2009; Mukinda *et al.*, 2010; Tikiso, 2015), are water-soluble and limited in their effectiveness because of poor absorption and bioavailability when taken orally (Komperlla, 2004; Manach *et al.*, 2004; Khan *et al.*, 2013; Nkengla, 2014) and this may be a significant problem if the aqueous liquid or dried extracts of *A. afra* are used.

Indeed, various dosage forms (e.g. powder, tablets, teabags and alginate beads) of the *A. afra* freeze dried aqueous extract (FDAE) have thus far been made which all exhibited a rapid dissolution profile (Komperlla, 2004; Dube, 2006; Max, 2007; Nkengla, 2014), characteristic of water-soluble phytoconstituents. However, as Costa and colleagues (2011) have highlighted, such rapid phytoconstituent release and dissolution may be therapeutically undesirable as it may affect absorption and hence the therapeutic outcome (Costa *et al.*, 2011). Also, with such products frequent dosing is then required to maintain therapeutic plasma concentrations and unavoidable fluctuations in drug concentrations which may, in turn, lead to under and or over dosing. Thus there may be a need to modify the rapid dissolution profile of the FDAE phytoconstituents.

Phytosomes may also be a very viable dosage form option to help solve these problems associated with *A. afra* FDAE. It could help increase the lipophilicity, decrease the dissolution and release rate, and improve absorption or cellular permeability of the water soluble phytoconstituents of this widely used herbal (traditional) medicine.

Given the aforementioned arguments, the main objectives of the present study were to (i) prepare and characterise an *A. afra* freeze dried aqueous extract and the phytosomes thereof, (ii) determine factors that influence the complex formation efficiency (CFE) between phytoconstituents and phosphatidylcholine in the preparation of *A. afra* phytosomes, and (iii) compare the phytoconstituent release properties, in terms of dissolution profiles, of FDAE *versus* the phytosomes, in simulated gastric and intestinal fluid, using luteolin as the marker

compound. It was hypothesised the phytosome preparation method would yield phytosomes having acceptable physicochemical pharmaceutical characteristics and that the dissolution rate of phytoconstituents (using luteolin as a marker compound) from the phytosomes would be less than that of the freeze dried aqueous extract.

Chapter 2

Literature Review

2.1 Introduction

In this chapter, the herbal drug delivery phytosome system is discussed, including the various preparation methods and characterisation techniques. An overview of the pharmacognostic aspects of *Artemisia afra* and the shortcomings of its oral dosage formulations are detailed. Finally, a review of dissolution methods and methods for dissolution profile comparisons are also be presented.

2.2 Drug delivery of herbal products

Herbal products have gained immense attention and access to the medicine markets throughout the globe as safer and effective substitutes of modern synthetic medicines which are considered to be full of adverse and toxic interactions. In under developed and developing nations all over the world plant drugs in traditional forms have been supposed to satisfy the primary healthcare needs of about 80% of the population and even in developed nations these medicines are being utilized by about 65% of the population (Khan *et al.*, 2013).

However, the bioavailability of the plant phytoconstituents has become an issue of concern for researchers because many of them have poor oral bioavailabilities, specifically those containing polyphenolic rings in their structures which are water soluble such as flavonoids, terpenoids and tannins (Manach *et al.*, 2004). The reasons for the poor bioavailability of these substances are low aqueous or lipid solubility, high molecular weight/size and poor plasma membrane permeability (Karakaya, 2004; Manach *et al.*, 2005; Teng *et al.*, 2012). Moreover, orally administered plant extracts are also subject to degradation or destruction in the presence of gastric fluids (Bhattacharya, 2009). This has therefore restricted the use of pharmacologically effective polyphenolic plant actives for treating various disorders.

In order to address these challenges and enhance the effectiveness of herbal therapy several novel delivery systems have been trialled in the recent time. These delivery systems include liposomes, niosomes and transfersomes (Pandita & Sharma, 2013) with liposomal formulations being the most popular.

2.2.1 Phytosomes

These are sometimes referred to as pharmacosomes, naturosomes or phospholipid-complexes. In recent years, the technique of complexing plant drugs with phospholipids has emerged as a challenging but one of the most successful methods for improving bioavailability and therapeutic efficacy of a number of poorly absorbed plant constituents. This technique uses phospholipid molecules containing phosphatidylcholine in their structure to form complexes (phytosomes) with standardized herbal extracts and/or the specific active pharmaceutical ingredient of the plant. As a result, the phytosome complex improves the membrane permeability, oil-water partition coefficient and hence the systemic bioavailability of these drugs (Raju, Reddy & Reddy, 2011; Loguercio *et al*., 2012).

Incorporation of water soluble drugs into phospholipid complexes considerably enhances bioavailability of the phytoconstituents by increasing penetration through the lipoidal plasma membrane while the phospholipid complexation of poorly water soluble drugs, increases bioavailability by improving solubility in gastric fluids (Shivanand & Kinjal, 2010; Saraf, 2010). The phyto-phospholipid complexation technique in recent years has made it possible to administer highly efficacious plant actives with an improved biological profile.

Some of the advantages of phyto-phospholipid complexes over pure plant drugs are presented in (Figure 2.1).

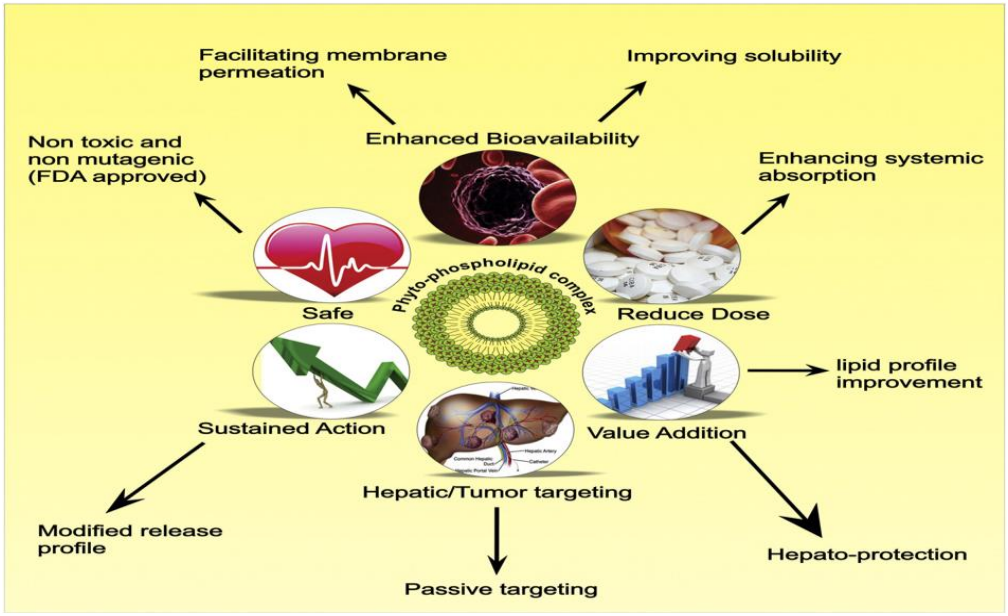

Figure 2.1: Advantages of phytosomal drug delivery system (Khan *et al.*, 2013)

2.2.2 Background of phytosomes

Phytosomes were first developed by Indena® in 1988, an Italian nutraceutical company (Indena®, 2016; Simms, Quinn & Wendel, 2000). Within the framework of their studies, after observation that phospholipids had a marked affinity for flavonoids, they developed a new series of compounds designated "Phytosome®" which were essentially complexes from the complexation of polar botanical derivatives and phospholipids. These complexes were considered to be novel entities on the basis of their physicochemical and spectroscopic characteristics. Their characterisation revealed that they were solid lipophilic substances, with a defined melting point, freely soluble in aprotic solvents, moderately soluble in fats and insoluble in water. Interestingly, when treated with water the phytosomes assumed a micelle arrangement, similar to that of liposomal dispersions.

However, due to the close similarity of phytosomes and liposomes, it is important to structurally clearly differentiate the two (Figure 2.2)

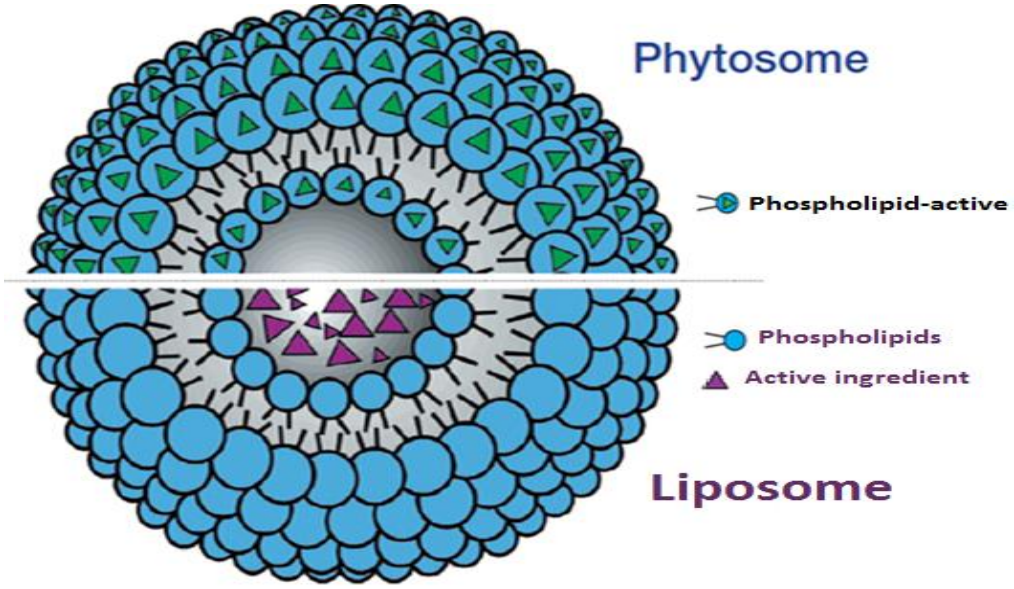

Figure 2.2: Comparison between liposomes and phytosomes (Indena®, 2016)

The fundamental difference is that in liposomes the active principle is the aqueous cavity enclosed by the phospholipids whereas in the phytosome the active principle is an integral part of the phospholipid through molecular interactions. The aforementioned structural differences are the basis of the advantages of the phytosomes over the liposomes. For instance, due to the presence of molecular interactions in phytosomes, the complex is more stable than the liposome which is mainly prone to structural leakages (Singh *et al.*, 2011a). Also, on the basis of the chemical bonds present in the phytosome, the improved bioavailability is more pronounced than that with liposomes. The natures of the interactions present in the phytosome complex are dealt with in detail in the next section.

2.2.3 Phytoconstituent - phospholipid interactions

Phytoconstituents interact uniquely with phospholipid molecules through chemical bonds with them. This has been established by characterisation techniques involving (Nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC) and Fourier transform infrared (FT-IR) spectroscopy, where analysis of drug–phospholipid complexes were compared with respect to analyses of pure drug and their physical mixture with phospholipids (Bombardelli *et al.*, 1989; Bombardelli, 1991).

Bombardelli and colleagues (1989) thoroughly characterised the catechin–phospholipid complex using the aforementioned techniques. They found in the ^{1}H-NMR that signals from protons belonging to the flavonoid moiety were so broad that the protons could not be

revealed. For the phospholipid there was broadening of all signals, while the singlet corresponding to N-$(CH_3)_3$ of choline underwent an extensive up-field shift. And in the ^{13}C-NMR spectra, all the flavonoid carbons were practically invisible, signals corresponding to the glycerol and choline portion of the phospholipid broadened and some shifted while most resonances of the fatty acid chains retained their original sharp lines shape.

All the mentioned physical and physicochemical features, revealed a strong interaction between the flavonoid molecule and the polar head of the phospholipid, with the two aliphatic chains wrapped around the flavonoid to produce a lipophilic envelope that allows the complex to dissolve in low polarity solvents. Moreover, inspection of the Dreiding molecular models supports this hypothesis as the average length of flavonoids was 13A^o which fitted well with the polar region of the lipid, while the two C18 carbon fatty acid chains sufficiently enclosed the complex (Bombardelli *et al.*, 1989). A molecular representation of the complex is shown in Figure 2.3.

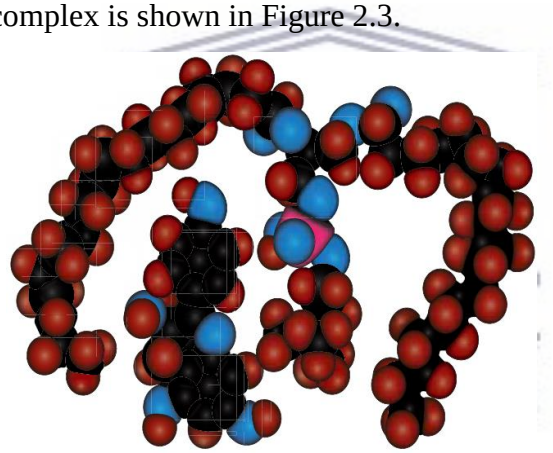

Figure 2.3: Schematic of phytosome molecular complex (Kidd, 2009)

Indirect evidence of the formation of the complex was confirmed through FT-IR and DSC (Bombardelli, 1991). The FT-IR spectra of the complex, physical mixture and pure drug were all unique thus revealing an interaction as per NMR results. Similarly, DSC thermograms of the previously mentioned samples were also different from each other thus also confirming formation of the complex.

The interaction has been attributed to formation of hydrogen bond and/or hydrophobic interaction between the two molecules (Bombardelli *et al.*, 1989; Bombardelli, 1991; Pathan & Bhandari, 2011). The -OH group of phenolic rings existing in the structure of the drug molecule is suggested to be involved in the formation of hydrogen bonding and the formation

of Vander Waals forces between the two moieties has also been suggested by some researchers in studies on phospholipid complexes of luteolin (Khan *et al.*, 2014), *Centella* extract (Saoji *et al.*, 2016), tetrandrine (Zhao *et al.*, 2013) and rutin (Das & Kalita, 2014).

2.2.4 Preparation methods

Various methods are used for production of phytosomes including anti-solvent precipitation (Maiti *et al.*, 2007; Semalty *et al.*, 2010b; Gupta & Dixit, 2011; Arora, Sharma & Kaur, 2013; Habbu *et al.*, 2013; Keerthi, Pingali & Srinivas, 2014; Saoji *et al.*, 2016), solvent evaporation (Yanyu *et al.*, 2006; Sikarwar *et al.*, 2008; Zhao *et al.*, 2013; Keerthi, Pingali & Srinivas, 2014; Khan *et al.*, 2014; Yue *et al.*, 2010), precipitation (Singh *et al.*, 2012) and anhydrous co-solvent lyophilization (Awasthi, Kulkarni & Pawar, 2011). The main methods, namely, anti-solvent and solvent evaporation, will be discussed.

2.2.4.1 Solvent evaporation

In the solvent evaporation method, the drug and the phospholipids are placed in the same flask containing a suitable solvent system such as tetrahydrofuran or ethanol or in separate flasks with different organic solvents. The reaction is allowed to be carried out by sonication, refluxing or stirring at suitable fixed temperature for a fixed duration of time to get maximum possible yield and drug entrapment. The solvent is then evaporated under vacuum or at room temperature to yield a residue which is then flushed with nitrogen gas. In a modified form of the solvent evaporation technique, Sikarwar *et al.* formulated marsupsin–phospholipid complex using mechanical dispersion oriented liquid anti-solvent precipitation process (Sikarwar *et al.*, 2008). They dissolved soy lecithin in diethyl ether by sonication and marsupsin in double distilled water. The drug solution was then added drop-wise to the phospholipid solution with sonication. The resultant formulation was then refrigerated and on analyzing the complex showed 44% entrapment of marsupsin.

2.2.4.2 Anti-solvent precipitation

The traditional anti-solvent precipitation technique is the most utilised by many researchers. It incorporates n-hexane as the anti-solvent to precipitate out the drug–phospholipid complex from the organic solvent (Maiti *et al.*, 2007; Semalty *et al.*, 2010b; Gupta & Dixit, 2011). In this method, both the plant extract/active and phospholipids are dissolved in the same solvent, and refluxed for a fixed time at a fixed temperature resulting in a clear solution. To this clear

solution, n-hexane is then added to precipitate the phytosomes which are either filtered and flushed with nitrogen gas or simply just left to dry in a desiccator.

2.2.4.3 Preparation parameters and variables

There are numerous preparation parameters and variables that affect the characteristics of the final phytosomal products. These include, but are not limited to: preparation method, type and purity of phospholipids, type of organic solvents used, ratio of drug to phospholipids, stirring speed (depending on preparation method), reaction time and concentration of reactants. Only a few of these are discussed in the proceeding sections.

2.2.4.3.1 Type of phospholipids

Phospholipids are indispensable components of all cellular and sub-cellular membranes, they can arrange as bilayer membranes. They are widely distributed in humans, animals and plants.

These molecules possess a hydrophilic head group and hydrophobic acyl chains are linked to an alcohol. The variation in head groups, aliphatic chains and alcohols leads to the existence of a wide variety of phospholipids. Examples include phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine, sphingolipids and cardiolipin, which are predominantly found in eukaryotic cell membranes (Li *et al.*, 2015). In addition, the different sources of phospholipids also enhance the species of phospholipids. Various phospholipids, such as soybean phosphatidylcholine, egg phosphatidylcholine, or synthetic phosphatidylcholine, as well as hydrogenated phosphatidylcholine, are commonly used in different types of formulations.

Phosphatidylcholine and phosphatidylethanolamine are most abundantly present in the lipid fraction of most biological membranes and they mainly constitute the matrix of these membranes. Table 2.1 shows the percentage contributions of different lipids in the mammalian cell membrane.

Table 2.1: Contribution of different lipids in typical phospholipid structure (Khan *et al.*, 2013)

Lipid	Sub-type	Percentage contribution
Glycerolipids	Phosphatidylcholine	45–55
	Phosphatidyl ethanolamine	15–25
	Phosphatidyl inositol	10–15
	Phosphatidyl serine	5–10
Sphingolipids	Sphingomyelin	5–10
	Glycosphingolipids	2–5
Sterols	Cholesterol	10–20

Phospholipids are amphipathic molecules having considerable solubility in aqueous and oily mediums. They have a polar and a non-polar portion in their structures (Acharya, Parihar & Acharya, 2011). Naturally occurring phospholipids incorporate an unsaturated fatty acid (such as oleic acid, linoleic acid or arachidonic acid) in position 2 and a saturated one (such as stearic acid or palmitic acid) in position 1. The most commonly used phospholipids (Figure 2.4) are those derived from soya bean containing higher proportions that is about 76% of phosphatidylcholine with a high content of polyunsaturated fatty acids like linoleic acid about 70%, linolenic acid and oleic acid (Scholfield, 1981).

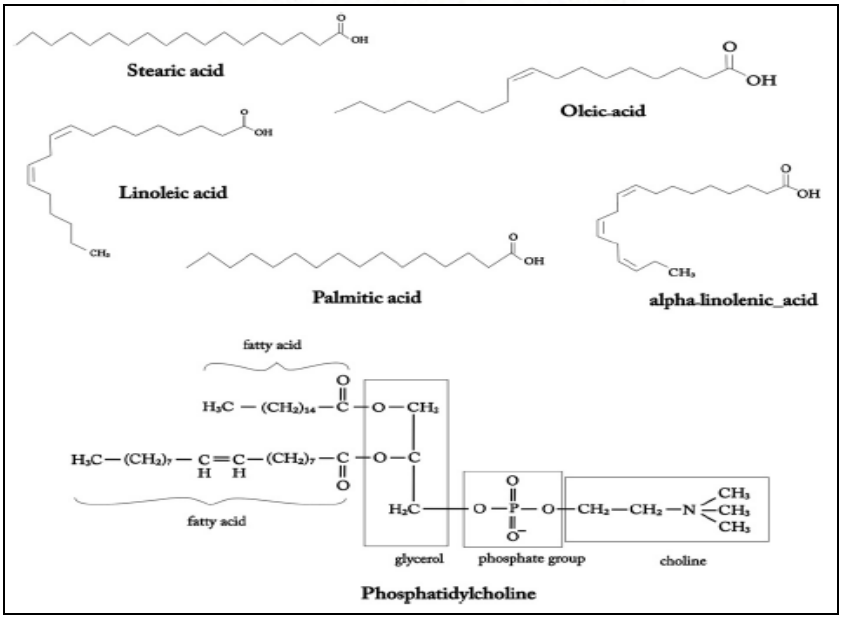

Figure 2.4: Different fatty acids constituting phosphatidylcholine (Khan *et al.*, 2013)

Lastly, phytosomes formed using lower purity grades of phospholipids are greasy in nature and form large aggregates while those prepared using lipids of more than 90% purity grade show susceptibility to degradation due to oxidation, which affects complex stability. So, 80% purity grade is the commonly used phospholipid grade (Pandita & Sharma, 2013).

2.2.4.3.2 Solvents used in the preparation of phytosomes

In contrast to the phospholipids, where phosphatidylcholine is the main lipid used in the preparation of phytosomes, a wide range of solvents have been used as reaction mediums for formulating phytosomes.

Aprotic solvents such as tetrahydrofuran (Yue *et al.*, 2010; Zhao *et al.*, 2013), dichloromethane (Maiti *et al.*, 2007; Semalty *et al.*, 2010c; Arora, Sharma & Kaur, 2013; Habbu *et al.*, 2013; Keerthi, Pingali & Srinivas, 2014), diethyl ether and chloroform (Mali *et al.*, 2014) have been used. However, of late, protic solvents, especially ethanol (Yanyu *et al.*, 2006; Peng *et al.*, 2010; Pathan & Bhandari, 2011; Khan *et al.*, 2014; Saoji *et al.*, 2016) have largely replaced the use of the aprotic solvents. Though most preparation techniques involve the use of a single solvent, several authors have used mixed solvent systems whereby the phospholipids are dissolved in a different solvent from that of the drug/extract. Mixed solvent systems utilised include methanol and dichloromethane (Das & Kalita, 2014), diethyl ether and water (Sikarwar *et al.*, 2008) and dichloromethane and ethanol (Keerthi, Pingali & Srinivas, 2014).

Lastly, in as far as the anti-solvent preparation method is concerned; the precipitation of the phytosomes has almost exclusively been mediated by n-hexane, as previously stated.

2.2.4.3.3 Ratio of drug to phospholipids

Complexation of phytoconstituents and phospholipids has been performed in varying mass/molar ratios varying from 1:0.5 to 1:3. In most research works, a stoichiometric ratio of 1:1 has been considered most suitable for the preparation of the complex (Jena *et al.*, 2014; Semalty *et al.*, 2010c; Arora, Sharma & Kaur, 2013; Changediya, Khadke & Devdhe, 2011; Gandhi *et al.*, 2012). Khan *et al*, Saoji *et al*, Pathan and Bhandari, and, Yue *et al* optimised the preparation method of luteolin, *Centella* extract, embelin and oxymatrine phospholipid complexes, respectively (Khan *et al.*, 2014; Yue *et al.*, 2010; Saoji *et al.*, 2016; Pathan &

Bhandari, 2011). All authors noted that an increase in the phospholipid content resulted in an increase in the encapsulation efficiency, with their optimal ratios of drug to phospholipid being 1:2.33, 1:3, 1:3 and 1:3, respectively. On the contrary, Qin *et al* also performed an optimisation study and obtained an optimal ratio of 1:0.9 (Qin *et al.*, 2010). These conflicting findings therefore suggest that optimal ratios actually vary depending on the particular drug/extract and probably other factors such as preparation method and solvents used.

2.2.5 Characterisation techniques

Methods of characterisation of phytosomes for use immediately after preparation and upon storage are required for adequate quality control of the product. The methods have to be reproducible, precise and rapid, in the context of their use in the industrial setting.

2.2.5.1 Complex formation efficiency

This is also referred to as encapsulation/entrapment efficiency or drug entrapment. It is a very critical characteristic of the final complex as it reflects the suitability of the preparation method and the various preparation parameters and variables. Drug content is usually quantified by UV spectrophotometry (Sikarwar *et al.*, 2008; Keerthi, Pingali & Srinivas, 2014; Das & Kalita, 2014) or by HPLC (Jena *et al.*, 2014; Pathan & Bhandari, 2011). The two main methods used in the determination of the CFE are the direct and indirect methods. There are several variations of these methods, some of which are highlighted below.

Both methods generally involve centrifugation to separate the complexes from the uncomplexed constituents in the medium. In the direct method, the final phytosomes in solid form are solubilised by an aprotic solvent such as chloroform or protic solvent such as methanol so as to release the phytoconstituents from the complex. Alternatively, in the case of phytosomes prepared from the anti-solvent technique which yields phytosomes in the solid form inclusive of the uncomplexed drug, the mixture can be dissolved in an aprotic solvent such as chloroform that selectively dissolves the complex while the uncomplexed drug sediments (Saoji *et al.*, 2016). The CFE can therefore be determined by analysis of the drug content in the supernatant.

In contrast, with the indirect method, the amount of uncomplexed drug is determined by analysis of the supernatant, after centrifugation (Das & Kalita, 2014). The

encapsulated/complexed drug can be determined by removing the supernatant, washing the pellet and then either lysing the pellet with a surfactant such as Triton X-100 or simply dissolving the pellet in an organic solvent such as methanol/ethanol (Peng *et al.*, 2010). A more accurate indirect method involves determination of total drug in the suspension by lysing the suspension with a surfactant.

2.2.5.2 Particle analysis (particle size and distribution)

The average size and size distribution of phytosomes are important parameters which have effects on the overall performance of the product. Drug dissolution rate, absorption rate, dosage form content uniformity and stability are all, to varying degrees, dependent on particle size, size distribution and interactions of solid surfaces (Aulton & Taylor, 2013). In many cases, for both drugs and additives, particle size reduction is required to achieve the desired physiochemical characteristics.

Several methods exist for the determination of particle size but the main methods used in phytosome formulations are dynamic light scattering (DLS) and scanning electron microscopy. The DLS is the most popular for this use as it is a non-invasive, well-established technique for measuring the size and size distribution of molecules and particles typically in the submicron region and, with the latest technology, lower than 1nm. The technique measures Brownian motion (random movement of particles in a liquid due to the bombardment by the molecules that surround them) and relates this to the size of the particles. It does this by illuminating the particles with a laser and analysing the intensity fluctuations in the scattered light (Malvern Instruments, 2004).

2.2.5.3 Colloidal stability

The magnitude of the zeta potential is an indication of the potential stability of the colloidal system. A colloidal system is when one of the three states of matter: gas, liquid and solid, are finely dispersed in one of the others. For this technique the main states of interest are of: a solid dispersed in a liquid, and a liquid dispersed in a liquid, i.e. an emulsion. The zeta potential is determined by calculating the electrophoretic mobility. The electrophoretic mobility is determined by performing an electrophoresis experiment on the sample and measuring the velocity of the particles using Laser Doppler Velocimetry (LDV) (Malvern Instruments, 2016).

2.2.5.4 Particle surface morphology

Transmission electron microscopy (TEM) or Scanning electron microscopy (SEM) can be used to visualize the phytosome. These techniques can be utilised for studying the surface order of the complexes. The purity grades of the lipid being used and few variables observed during operation (method of preparation, vacuum assigned and rotational speed) alter the shape and size of phytosomes.

2.2.5.5 Partition coefficient

If a third substance is added to a system of two immiscible liquids in equilibrium, the added component will distribute itself between the two liquid phases until the ratio of its concentrations in each phase attain a certain value: the distribution constant or partition ratio. The octanol–water distribution ratio, *Ko/*w, is the accepted physicochemical property measuring the hydrophobicity of chemicals (Sangster, 1997). The technique can also be used to evaluate the solubilities and apparent solubilities of substances.

2.2.5.6 Complexation confirmatory analyses

Part of the characterisation of the phytosomes involves confirmatory analyses which verify complexation between the phytoconstituents and phospholipids. One such analysis, NMR, has been discussed in detail in section 2.2.3 and will therefore be excluded from this section. Other techniques, FT-IR and DSC, also highlighted in section 2.2.3, will be briefly discussed.

2.2.5.6.1 Spectroscopic properties

This can be determined by FT-IR and has been described in section 2.2.3. Comparison between the spectrum of the complex and spectrum of the individual components and their mechanical mixtures confirms the complex formation by infrared spectroscopy.

2.2.5.6.2 Thermal analysis

Determination of this characteristic has been described in section 2.2.3. DSC thermal analysis apparatus measures the temperature variation of physical properties of a sample against time (Haynie, 2001). In other words, the instrument determines the temperature and heat flow associated with material transitions as a function of time and temperature (Haines, Reading & Wilburn, 1998). During a change in temperature, DSC measures the amount of heat, which is

radiated or absorbed by the sample by measuring the difference in temperature between the sample and reference (Haynie, 2001; Haines, Reading & Wilburn, 1998).

2.2.5.6.3 Crystalline nature

The crystalline nature of drug can be determined using X-ray powder diffraction technique. The method hinges on the generation of X-rays which are focused on the sample. The sample then diffracts the rays and these are detected (Dutrow & Clark, 2014). A pivotal aspect of all diffractions, is the angle between the incident and reflected rays, which can be regulated through the scan angle. The overall combined intensity of all reflection peaks is projected by area under curve of X-ray powder diffraction pattern that specifies the product properties (Pandita & Sharma, 2013).

2.3 *Artemisia afra*: An overview

This section covers the pharmacognostic aspect of the species where the taxonomy, geographical distribution, traditional uses, pharmacological activity and phytochemical composition are reviewed. In addition, the various dosage formulations of the extract and their shortcomings are also reviewed.

2.3.1 Taxonomy (Tropicos, 2016)

Family: Asteraceae Bercht. & J. Presl

Family homonym: Compositae Giseke

Genus: *Artemisia* L.

Species: *Artemisia afra* Jacq. Ex Willd.

Common names: Wild wormwood, African wormwood and Wilde-als (Patil, Dass & Chandra, 2011)

Indigenous South African names: Umhlonyane (Xhosa), Mhlonyane (Zulu), Lanyana (Sotho) and Lengana (Tswana) (Burits, Asres & Bucar, 2001)

Figure 2.5: *Artemisia afra* plant

2.3.2 Distribution

The species grows in the South and Eastern regions of the African continent and has been located in Ethiopia, Kenya, Namibia, Tanzania, Democratic Republic of Congo, Zambia, Zimbabwe, Angola and the Republic of South Africa (Watt & Breyer Brandwijk, 1962; Liu, Van der Kooy & Verpoorte, 2009).

2.3.3 Traditional uses of *A. afra*

The species is administered in a variety of ways including as enemas, poultices, infusions, body washes and lotions and smoked, snuffed or drunk as a tea for the treatment and management of various ailments (Patil, Dass & Chandra, 2011).

2.3.3.1 Use in respiratory tract related conditions

The *A. afra* species is primarily used in common cold, cough, sore throat, influenza and asthma (Watt & Breyer Brandwijk, 1962; Graven *et al.*, 1990; Hutchings *et al.*, 1996; Van Wyk & Wink, 2004). The leaves are heated and the vapours inhaled to alleviate symptoms of colds and flu (Watt & Breyer Brandwijk, 1962; Bhat & Jacobs, 1995). It is also used to clear the blocked nasal passage by inserting fresh leaves in the nostrils or by using as snuff; to relieve pain in the throat in scarlet fever, either the hot infusion being used as gargle or the throat exposed to vapors (Watt & Breyer Brandwijk, 1962; Taylor *et al.*, 2001). The leaves are also smoked by some tribes to help release phlegm, to ease and soothe a sore throat and coughing at night (Roberts, 1990). For cold and chest problems in infants, fresh leaves are placed in a flannel bag and hung around baby's neck (Van Wyk, De Wet & Van Heerden, 2008).

2.3.3.2 Other traditional uses of *A. afra*

The species is also used in several other conditions which include gynaecological, gastrointestinal, topical, pyretic and inflammatory ailments. Gynaecological ailments include dysmenorrhea (Van Wyk & Gericke, 2000), amenorrhea and menstrual cramps (Steenkamp, 2003). Gastrointestinal ailments include indigestion, colic, constipation, flatulence, gastritis, dry dyspepsia and intestinal worms infestations (Jansen, 1981; Buchbauer & Silbernagel, 1989; McGaw, Jäger & Van Staden, 2000; Van Wyk & Wink, 2004).

Watt and Brandwiijk reported topical use of the species where the extract is applied topically to ease the pain and hasten bursting of boils, carbuncles, large acne pimples; hot baths in which the decoction is used to bring out the rash in measles, mumps, chicken pox and an infusion or decoction is used to bathe haemorrhoids, herpes and venereal sores (Watt & Breyer Brandwijk, 1962).

Other miscellaneous uses include that in rheumatism (Burits, Asres & Bucar, 2001), gout (Van Wyk & Wink, 2004), fever (Watt & Breyer Brandwijk, 1962; Fowler, 2006), malaria (Fowler, 2006), epilepsy (Yineger *et al.*, 2008), haematuria and to alleviate stabbing pain (Abebe & Ayehu, 1993), as anti-fertility agent (Desta, 1994), in diabetes mellitus (Watt & Breyer Brandwijk, 1962; Deutschländer, Lall & Van De Venter, 2009) and in tinea captitis (Abebe & Ayehu, 1993).

Besides its beneficial medicinal properties, the *A. afra* species may also be used by the communities as a preservative in food stuffs (Muyima *et al.*, 2002; Ashebir & Ashenafi, 1999). In addition, it can be used as an insecticide to reduce pest pressure on crops which is achieved by planting it as a border surrounding vegetable plants and crops (Patil, Dass & Chandra, 2011).

2.3.3.3 Pharmacological actions of *A. afra*

Despite the traditional uses, researches have gone on to establish the *in vitro* pharmacological activity of the species. Activities reported include anti-fungal and anti-bacterial (Graven *et al.*, 1992; Libbey & Sturtz, 1989; Mangena & Muyima, 1999), anti-diabetic (Afolayan & Sunmonu, 2011; Sunmonu & Afolayan, 2013), bronchodilatory (Mulubwe, 2007; Mjiqiza, Syce & Obikeze, 2013), anti-cancer (Fouche *et al.*, 2008; Nibret & Wink, 2010), anti-

inflammatory (Ntutela *et al.*, 2009), anti tuberculotic (Ntutela *et al.*, 2009), anti-spasmolytic (Mulatu & Mekonnen, 2007) and anti-malarial (Liu *et al.*, 2010) activities.

2.3.4 Phytochemical constituents of *Artemisia afra*

Artemisia afra is rich in phytoconstituents, non-volatile and volatile secondary metabolites. These are shown in Tables 2.2 and 2.3.

Table 2.2: Volatile *A. afra* phytoconstituents

	Constituent	**References**
Monoterpenoids	e.g. artemisia alcohol, artemisia ketone, camphor, 1,8-cineole, limonene, β-thujone	(Chagonda, Makanda & Chalchat, 1999; Burits, Asres & Bucar, 2001; Muyima *et al.*, 2002; Viljoen *et al.*, 2006; Asekun, Grierson & Afolayan, 2007; Vagionas *et al.*, 2007)
Sesquiterpenes	Bicycloelemene, α-bisabolol, β-caryophyllene, germacrene D-4-ol, β-selinene, (Z)-β-farnesene	(Chagonda, Makanda & Chalchat, 1999; Viljoen *et al.*, 2006)

Table 2.3: Non-volatile *A. afra* phytoconstituents

	Constituent	References
Sesquiterpenes	β-farnesene	(Jakupovic *et al.*, 1988)
Triterpenes	α-amyrin, β-amyrin, friedelin, squalene	(Silbernagel, Spreitzer & Buchbauer, n.d.; Jakupovic *et al.*, 1988)
Coumarins	Ouebrachitol, umbelliferone derivatives, scopoletin, isofraxidin, 12-hydroxy-α-cyperone	(Goodson, 1922; Bohlmann & Zdero, 1972; Jakupovic *et al.*, 1988)
Glaucolides	artemisia glaucolide, 1α-hydroxyafraglaucolide, 12-hydroxy-α-cyperone	(Jakupovic *et al.*, 1988)
Guaianolides	11,13-dehydromatricarin, guaianolides 3 (11 derivatives), guaianolides 1 (2 derivatives)	(Jakupovic *et al.*, 1988; Kraft *et al.*, 2003)
Flavonoids	Acacetin, apigenin, chrysoeriol, diosmetin, genkwanin, 7-methoxyacacetin, quercetin, kaempferol, tamarixetin, luteolin	(Wollenweber & Mann, 1989; Tang *et al.*, 2000; Kraft *et al.*, 2003; Waithaka, 2004; Avula *et al.*, 2009)

2.3.5 Selection of chemical marker compounds for herbal product quality control

The European Medicines Agency (EMEA) defines chemical markers as chemically defined constituents or groups of constituents of a herbal medicinal product which are of interest for quality control purposes regardless of whether they possess any therapeutic activity (Li *et al.*, 2008). The EMEA further classified the chemical markers into active markers which are constituents or groups of constituents known to contribute to therapeutic activities and analytical markers which are the constituents or groups of constituents that serve solely for analytical purposes.

An ideal chemical marker should exhibit therapeutic effects of the herbal medicinal product. Herbals have a multitude of compounds but only a limited number have been shown to possess pharmacological activity and as such other chemical components that may not exhibit pharmacological effects may also be employed as markers (Li *et al.*, 2008). In the case of

Artemisia afra the flavonoids have been shown to be partially responsible for the therapeutic activity of the extract (Mukinda *et al.*, 2010) and, of particular interest, luteolin which has been identified in sizeable quantities compared to the other flavonoids (Waithaka, 2004).

2.3.5.1 The flavonoids

Flavonoids belong to a vast group of polyphenolic compounds that are widely distributed in all plants. Plant polyphenols have been of interest to scientists for decades, originally owing to their importance in plant physiology, specifically for their roles in plant pigmentation and flavour. Polyphenols are involved in plant growth and reproduction, provide resistance to pathogens and predators, and protect crops from disease and pre-harvest seed germination (Ross & Kasum, 2002). Recently, interest in the possible health benefits of polyphenols (particularly flavonoids) has increased owing to their antioxidant and free-radical scavenging abilities observed *in vitro*.

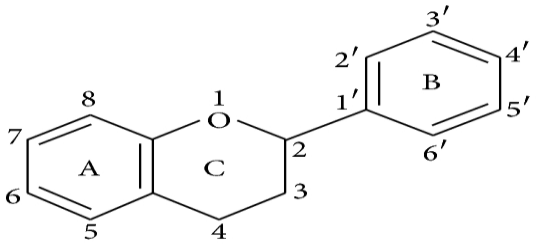

Figure 2.6: Basic structure of flavonoids

They are produced as the result of the secondary metabolism of plants and are frequently found attached to sugars (glycosides), thus tending to be water-soluble. Occasionally, polyphenols also occur in plants as aglycones.

Flavonoids can be divided into eight subclasses based on their molecular structure (Figure 2.6). The structure of the subclasses differ in their arrangement of hydroxyl, methoxy and glycosidic side groups and in the conjunction between A and B rings (Sandhar *et al.*, 2011). A variation in C ring provides division of subclasses. The six major subclasses of flavonoids include the flavones (e.g., apigenin, luteolin), flavonols (e.g., quercetin, myricetin), flavanones (e.g., naringenin, hesperidin), catechins or flavanols (e.g., epicatechin, gallocatechin), anthocyanidins (e.g., cyanidin, pelargonidin), and isoflavones (e.g., genistein, daidzein).

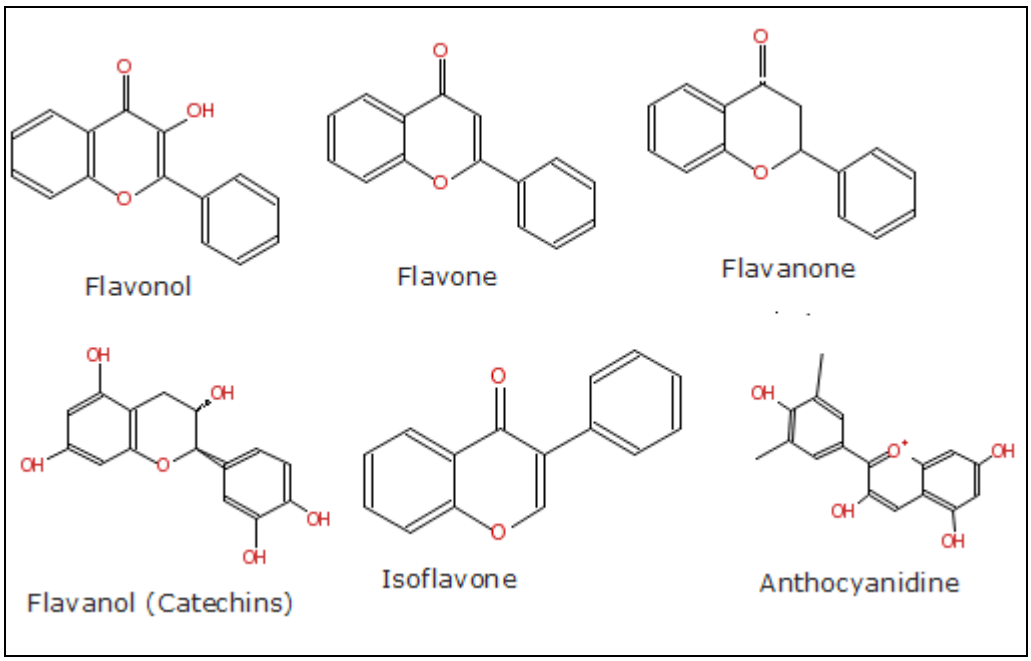

Figure 2.7: The six major subclasses of flavonoids (Lakhanpal & Rai, 2007)

Flavonols and flavones are the most widely occurring flavonoids; of these luteolin, quercetin, kaempferol, myricetin, chrysin and apigenin are widely distributed. The flavanones, flavanols, dihydroflavones and dihydrochalcones, are considered minor flavonoids because of their limited natural distribution (Di Carlo *et al.*, 1999)

Most herbal medicinal plant materials including *A. afra*, are usually prepared as aqueous-based decoctions and infusions. In these formulations, different active phytoconstituents such as the aqueous-soluble flavonoid glycoside compounds will be released. Thus, the flavonoid glycosides represent the form in which actives from many traditional medicinal products (such as *A. afra*) are released when prepared for oral consumption, and are inherently more abundant than the poorly aqueous soluble flavonoid aglycones. These flavonoid glycosides, due to their hydrophilicity, are generally poorly absorbed (Ross & Kasum, 2002; Manach *et al.*, 2004).

Despite their abundance, it is difficult to quantify individual flavonoid glycosides due to their inherently large number in plant samples. However these glycosides are derived from a limited number of flavonoid aglycones, and have actually been described as "sustained-release natural pro-drugs of their aglycones"(Chao, Hsiu & Hou, 2002).

Due to their abundance, glycosides, in quantification studies are usually acid-hydrolysed to the aglycones (Figure 2.8) and glycoside levels quantified in terms of the aglycone content.

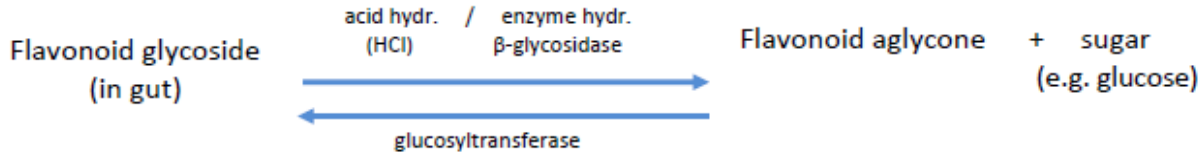

Figure 2.8: Illustration of flavonoid glycoside hydrolysis

Several analytical techniques are available for the identification, separation and quantification of the flavonoid aglycones, the most commonly used being chromatographic methods e.g. HPLC (Komperlla, 2004; Dube, 2006; Max, 2007; Avula *et al.*, 2009; Nkengla, 2014).

2.4 Dissolution

Dissolution is defined as the transfer of molecules or ions from a solid state into solution (Aulton & Taylor, 2013). Dissolution studies are an indispensable tool to assess quality and predict to some extent the absorption and bioavailability of the actives and consequently, the therapeutic outcome of a drug (or pharmaceutical) product (or dosage form). Drug absorption from solid dosage forms after oral administration depends on the release of the drug substance from the drug product, the dissolution or solubilisation of the drug under physiological conditions, and the permeability across the gastrointestinal tract. Because of the critical nature of the first two of these steps, *in vitro* dissolution may be relevant to the prediction of *in vivo* performance.

2.4.1 Dissolution of herbal products

Dissolution testing is the customary measure of release from a dosage form. The technique has been applied to herbal products and the focus has been extended to its use in the prediction of bioavailability and bioequivalence. (Wang *et al.*, 2015).

Guidelines for dissolution testing of oral dosage forms for orthodox medicines have been developed by the Food and Drug Administration (FDA) (FDA, 1997). However, the application of such guidelines to herbal products is not so straight forward. For the vast majority of the herbal products, there are no compendial methods on dissolution. Also, because of their multi-component nature whereby they consist of numerous phytoconstituents which may be active or may have a synergistic effect on each other, the selection of a suitable

marker compound can be quite complicated. This, coupled with the general lack of standardisation of these herbal products, results in large variations in the quality and quantity of active components in such products.

The complexity associated with the selection of a marker compound is evident in a study by Nair and Kanfer on the release of hypoxoside and sterols from African potato (*Hypoxis hemerocallidea*) commercial products. The authors, using the USP basket apparatus, reported a minimum of 75% release of hypoxoside and sterols in one hour, in dissolution studies conducted at pH 1.2 and fed state simulation fluid at pH 5.0, respectively (Nair & Kanfer, 2008). Due to the different physicochemical characteristics of the marker compounds, different dissolution media had to be utilised thus complicating the standardisation of a dissolution method for the plant extract.

Despite such difficulties, dissolution tests can of course clearly show differences in herbal products and/ dosage forms. For instance, in one of the few studies done so far, dissolution tests on *Passiflora* products demonstrated 50% and almost 100% dissolution of actives from capsules containing the powder and capsules containing the extract, respectively, in 10 minutes (USP paddle method at a speed of 50 rpm, using water at 37 ± 0.5°C and UV spectrophotometric detection at 340 nm) (Costa *et al.*, 2011).

2.4.1.1 Dissolution of *Artemisia afra* formulations

Several pharmaceutical dosage forms of *A. afra* have been investigated. These include tablets (Komperlla, 2004), encapsulated FDAE (Max, 2007), tea bags (Dube, 2006) and alginate and polymethylmethacrylate coated FDAE (Nkengla, 2014). The tablet and encapsulated formulations were subjected to compendial dissolution methods in water and pH 1.2 buffer, respectively. Both authors reported rapid/immediate release profiles for their preparations with more than 75% of the luteolin marker compound released with 45 minutes. The infusion study on the tea bag also revealed a similar release profile. In addition, although the release study was conducted in a beaker, the alginate and polymethylmethacrylate forms of the *A. afra* FDAE, interestingly also showed an immediate release profile in pH 6.8 medium where over 75% of the marker compound was released within 30 minutes. This was not expected as these polymers should be able to modify the release profile of the constituents.

The above-mentioned rapid release profiles are however disadvantageous as they can result in sharp increases in drug concentrations potentially leading to toxic levels (Freiberg & Zhu, 2004). Also, the therapeutic levels are poorly maintained as frequent re-administration is required to maintain the therapeutic plasma levels. Costa *et al.* also highlighted the undesirability of rapid release profiles, suggesting that in some instances, e.g. herbal products, it may be necessary to determine whether the organism is capable of readily absorbing all the active principles released at the beginning of the dissolution process without affecting the therapeutic outcome (Costa *et al.*, 2011). Modification of the release of the phytoconstituents of *A. afra* will greatly assist in reducing the dosing frequency and amount, produce more reproducibility, uniform drug release and absorption, reduction of local irritations and reduced dose dumping (Das *et al.*, 2015). These are all characteristics that may be attained through complexation of the phytoconstituents with phospholipids.

2.4.1.2 Dissolution of phospholipid-complexes (phytosomes)

Several studies on the dissolution of phytosomes have been conducted (Yanyu *et al.*, 2006; Semalty *et al.*, 2010c; Arora, Sharma & Kaur, 2013; Jena *et al.*, 2014; Keerthi, Pingali & Srinivas, 2014; Saoji *et al.*, 2016). Various dissolution parameters were used including different volumes ranging from 500 – 900 ml, stirring speeds 50 – 100 rpm, dissolution apparatus USP type I or II, dissolution media pH 1.2 - 7.4 and durations of dissolution tests ranging from 2 – 12 hours. Most studies were conducted in a phosphate buffer medium at pH 6.8. Interestingly most of the dissolution profiles showed approximately a 100% release after about 12 hours. Arora *et al.*, described the dissolution profile of their phytosomal tablet formulation as sustained release (Arora, Sharma & Kaur, 2013), which was similar to that of most phospholipid complex dissolution studies.

Of note, in all these phospholipid-complex dissolution studies, the objective of complexation was to improve aqueous solubility of poorly-water soluble constituents hence in all the studies there is improved dissolution efficiency. Here, the authors utilised the amphiphilic nature of phospholipids which render the characteristic to the constituents. Incipiently, development of phytosomes was mainly targeted at improving lipophilicity of hydrophilic constituents so as to improve bioavailability. According to the innovator of the technique these complexes are insoluble in water (Bombardelli, 1991), which is contrary to the findings of the dissolution studies. This may be explained by the fact that complexing hydrophilic constituents will result in a complex with significantly less aqueous solubility whereas

complexation of a hydrophobic constituent results in a complex with improved water solubility properties. All this is attributed to the amphiphilic nature of the phospholipids.

2.4.2 Development of dissolution methods

The absorption of a drug is controlled/determined by its dissolution such that differences in dissolution profiles are indicative of different bioavailabilities, which in turn can lead to variations in the therapeutic and toxic effects (Ngo, 2007). It is therefore important that in the development of a dissolution method, the conditions in the gastrointestinal tract, where the dissolution and absorption occur, are closely mimicked.

The USP suggests that for botanicals, compliance with dissolution testing is performed by testing six or more dosage units individually in each vessel and measuring one or more index marker compounds or the extract specified in the individual monograph (Nair & Kanfer, 2008).

Typical media for dissolution may include the following: diluted hydrochloric acid; buffers (phosphate or acetate) in the physiologic pH range of 1.2–7.5; simulated gastric or intestinal fluid (with or without enzymes) and water. For some drugs, incompatibility of the drug with certain buffers or salts may influence the choice of buffer. The molarity of the buffers and acids used can influence the solubilising effect, and this factor may have to be evaluated.

For poorly soluble drugs, a percentage of a surfactant may be added to the aqueous solutions (acidic or buffer solutions) to enhance the solubility of the drug. FDA approved surfactants include, but are not limited to, sodium dodecyl sulfate (SDS), sodium lauryl sulfate (SLS), polysorbates 20 and 80, cetyltrimethylammonium bromide (CTAB), lauryl dimethylamine oxide and polyoxyethylene lauryl ether (Brij35) (U.S. Food and Drug Administration, 2016). The surfactants selected for the solubility investigations should cover all common surfactant types, i.e., anionic, nonionic, and cationic. When a suitable surfactant has been identified, different concentrations of that surfactant should be investigated to identify the lowest concentration needed to achieve sink conditions. Typically, the surfactant concentration is above its critical micellar concentration (CMC) (USP 36, 2004).

For compendial Apparatus 1 (basket) and Apparatus 2 (paddle), the volume of the dissolution medium used can vary from 500 to 1000 ml, with 900 ml as the most common volume. The

choice of apparatus is based on knowledge of the formulation design and the practical aspects of dosage form performance in the *in vitro* test system. In general, a compendial apparatus should be selected. For solid oral dosage forms, Apparatus 1 and Apparatus 2 are used most frequently. When Apparatus 1 or Apparatus 2 is not appropriate, another official apparatus may be used.

Apparatus 1 (baskets) at 50–100 rpm or Apparatus 2 (paddles) at 50 or 75 rpm are used most commonly. Other agitation speeds are acceptable with appropriate justification. Rates outside 25–150 rpm for both the paddle and the basket are usually not appropriate because of mixing inconsistencies that can be generated by stirring too slow or too fast. Agitation rates between 25 and 50 rpm are generally acceptable for suspensions.

2.4.3 Methods for comparison of dissolution profiles

Another important area in dissolution data analysis is assessment of the similarity between dissolution profiles. Several approaches have been developed for comparing dissolution profiles. The main methods for comparing dissolution profiles are shown in Table 2.4.

Table 2.4: Categories of methods to compare dissolution profiles (Polli *et al.*, 1997)

Approach	Method	Parameter/equation
ANOVA based	Multivariate ANOVA	-
	Multiple univariate ANOVA	-
Model-independent	Ratio test procedures	Ratio percentage dissolved
		Ratio of AUC(dissolution curves)
		Ratio of mean dissolution time
	Pair-wise procedures	Difference factor (f_1)
		Similarity factor (f_2)
		Index of Rescigno (ξ_1 and ξ_2)
Model-dependent	See Table 2.5	See Table 2.5

2.4.3.1 Model dependent methods for dissolution profile comparisons

Model dependent methods (Table 2.5) for dissolution profile analysis and comparison attempt to determine the release of the active from a formulation by employing mathematical equations and use fitted models to describe dissolution and the kinetics of drug release from a

product in different areas of the gastrointestinal tract, each section signified by its appropriate dissolution medium.

Table 2.5: Mathematical equations for models describing release of active pharmaceutical ingredient (API) from its matrix (Zhang *et al.*, 2010)

Model	**Equation**	**Parameters**
Zero order	$F = k_0*t$	k_0
First order with F_{max}	F = Fmax*[1 - Exp(- k1*t)]	k_1, F_{max}
Weibull_1	F = 100*{1-Exp[-((t-Ti)^β)/α]}	α, β, T_i
Weibull_2	F = 100*{1-Exp[-(t^β)/α]}	α, β
Weibull_3	$F = F_{max}*\{1-Exp[-(t^\beta)/\alpha]\}$	α, β, F_{max}
Weibull_4	$F = F_{max}*\{1-Exp[-((t-Ti)^\beta)/\alpha]\}$	α, β, T_i, F_{max}
Makoid-Banakar with T_{lag}	$F = k_{MB}*(t-T_{lag})^n*Exp[-k*(t-T_{lag})]$	k_{MB}, n, k, T_{lag}
Peppas-Sahlin 1 with T_{lag}	$F = k_1*(t-T_{lag})^m+k_2*(t-T_{lag})^{(2*m)}$	k_1, k_2, m, T_{lag}
Peppas-Sahlin 2 with T_{lag}	F = k1*(t - Tlag)^0.5 + k2*(t - Tlag)	k_1, k_2, T_{lag}
Probit_2	F = Fmax*Φ[α+β*log(t)]	F_{max}, α, β
Logistic_2	$F=F_{max}*Exp[\alpha+\beta*log(t)]/\{1+Exp[\alpha+\beta*log(t)]\}$	α, β, F_{max}
Logistic_3	$F = F_{max}/\{1+Exp[-k*(t-\gamma)]\}$	k, γ, F_{max}
Korsmeyer-Peppas	$F = k_{KP}*t^n$	k_{KP}, n
Korsmeyer-Peppas with T_{lag}	$F = k_{KP}*(t-T_{lag})^n$	k_{KP}, n, T_{lag}

2.4.3.1.1 Zero order kinetics

This is dissolution that occurs in dosage forms that do not disintegrate but release the drug slowly (assuming that area does not change and no equilibrium conditions are obtained). It may be represented mathematically by the equation below and graphically by Figure 2.9

$$F = K_0 t \qquad \text{Eqn 2.1}$$

Where, **F** is the fraction of drug dissolved in time **t** and K_0 is the zero order release constant.

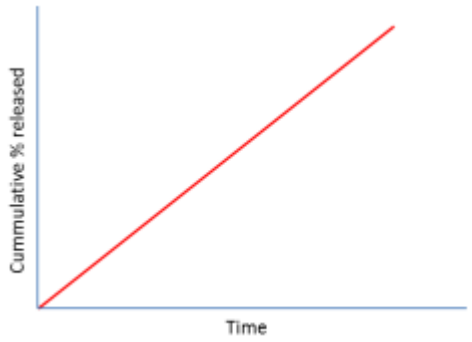

Figure 2.9: Graphical representation of zero order release kinetics

Zero order release kinetics is usually exhibited by modified release dosage forms, transdermal systems, matrix tablets and osmotic systems. These systems release a constant amount of drug per unit time which is ideal to achieve prolonged therapeutic effects (Costa & Lobo, 2001).

2.4.3.1.2 First order kinetics

Here, the rate of drug release is dependent upon the concentration gradient between the concentration in the static liquid layer next to the solid surface and concentration in the bulk medium at a time (**t**) (Singhvi & Singh, 2011). This release is represented by Equation 2.2 below and Figure 2.10.

$$F = 100 * [1 - e^{-k1*t}] \quad \text{Eqn 2.2}$$

Where k_1 is the first order release constant.

The dosage forms following this dissolution profile include water soluble drugs in porous matrices such that release of drug will be proportional to the amount of drug remaining in the matrix.

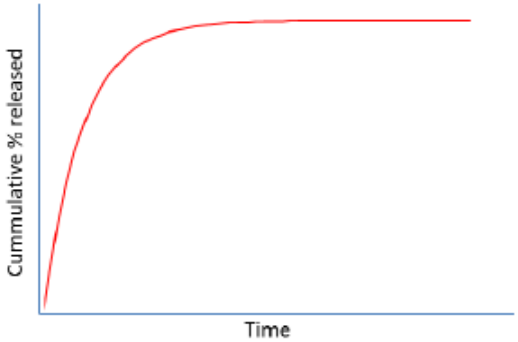

Figure 2.10: Graphical representation of first order release kinetics

2.4.3.1.3 Weibull model

This model has been successfully applied to almost all kinds of dissolution curves (Costa & Lobo, 2001). The model expresses the accumulated amount of drug (**F**) in solution at time (**t**) by the Equation 2.3

$$F = \left[1 - e^{-\frac{(t-T_i)^{\beta}}{\alpha}}\right] \qquad \textbf{Eqn 2.3}$$

Where, **α** is a scale parameter that describes the time scale of the process, $\mathbf{T_i}$ represents the lag time before onset of dissolution. The term **β**, is a shape parameter that describes the shape of the curve as either exponential (β = 1) (Case 1), sigmoid (S-shaped) with upward curvature followed by a turning point (β > 1) (Case 2), or parabolic (β < 1) (Case 3), with a higher initial slope and after that consistent with the exponential.

There are four different modifications of the Weibull model which generally differ in terms of the presence or absence of parameters such as T_i and F_{max}. The model has been subject to criticism as it is an empiric model without any kinetic fundament that characterises the dissolution kinetic properties of the drug. But of late studies have shown that the β, can be used to as an indicator to describe the mechanism of release of the drug through a polymer matrix (Papadopoulou *et al.*, 2006) (Table 2.6).

Table 2.6: Interpretation of release mechanisms from polymeric systems using the Weibull function (Mbamalu, 2015)

Time exponent, β	Solute release mechanism
β ≤ 0.75	Fickian diffusion in fractal or Euclidian spaces
0.75 < β < 1	Combined release mechanism
β > 1	Complex release mechanism

2.4.3.1.4 Logistic models

These are also empirical models, and lack significant kinetic properties. These models are valid only when the value of time ***t*** exceeds zero (Mbamalu, 2015). There are three variations of this model which relates the fraction **F**, of drug released at time **t** but for purposes of this study only Logistic models 2 and 3 (Logistic_2 and Logistic_3) will be described. They are represented by the respective Equations 2.4 and 2.5;

$$F = F_{max} \cdot \frac{e^{[\alpha + \beta * \log(t)]}}{1 + e^{[\alpha + \beta * \log(t)]}} \quad \text{Eqn 2.4}$$

and,

$$F = F_{max} \cdot \frac{1}{\{1 + e^{[-k*(t-\gamma)]}\}} \quad \text{Eqn 2.5}$$

where, **α** is the scale factor and **β** is the shape factor in Logistic_2 model. In the Logistic_3 model **k** is the shape factor, **γ** is the time at which $F = F_{max}/2$ and F_{max} is the maximum fraction of the drug released at infinite time.

2.4.3.1.5 Peppas-Sahlin with T_{lag}

Peppas and Sahlin in 1989 developed an equation that described drug release governed by diffusion and/ relaxation (Peppas & Sahlin, 1989). The Equations 2.6 and 2.7 describe the fraction, **F**, of drug released at time **t**;

$$F = k_1 * (t - T_{lag})^m + k_2 * (t - T_{lag})^{2m} \quad \text{Eqn 2.6}$$

and

$$F = k_1 * (t - T_{lag})^{0.5} + k_2 * (t - T_{lag}) \quad \text{Eqn 2.7}$$

where, k_1 is the constant related to the Fickian kinetics, k_2 is the constant related to Case-II relaxation kinetics, **m** (which usually ranges between 0 and 1) is the diffusional exponent for a device of any geometric shape which exhibits controlled release and T_{lag} is the lag time prior to drug release. These models differ from the original Peppas-Sahlin model, simply by the inclusion of the T_{lag} parameter.

2.4.3.1.6 Makoid-Bakanar with T_{lag}

The Makoid-Banakar function is represented by:

$$F = k_{MB} * (t - T_{lag})^n * e^{[-k * (t - Tlag)]} \quad \text{Eqn 2.8}$$

where, k_{MB}, n and k are empirical parameters of the model. These parameters are limited by the fact that total drug /marker compound dissolution must occur at the same time as the maximum value of the Makoid-Banakar function (Pais, 2001). The Makoid-Banakar with T_{lag} model differs from the original Makoid-Banakar model by the inclusion of the parameter, T_{lag}, which represents the time before onset of drug release.

2.4.3.1.7 Korsmeyer-Peppas with T_{lag}

This is a semi-empiric model that exponentially relates drug release to the time (**t**) elapsed. It was developed in by Korsmeyer and colleagues (Korsmeyer *et al.*, 1983) and further modified by Peppas (Peppas, 1985). It has generally been used in the analysis of drug release from dosage forms where the mechanism of release is not well elucidated or the release involves more than one type of release mechanism. The generic equation is shown below

$$\frac{M_t}{M_\infty} = k t^n \qquad \text{Eqn 2.9}$$

where M_t / M_∞ is the fraction of drug released, **k** is the rate constant and **n** is the release exponent.

Peppas (1985) utilised the release exponent **n**, to characterise various release mechanisms depending on the shape of the dosage form, either slab e.g. circular flat topped tablets or cylindrical e.g. capsules. The interpretation of **n** for slabs was as follows; n = 0.5 represents Fickian diffusion and higher values of n, between 0.5 and 1.0, or n = 1.0 indicate mass transfers following non-Fickian diffusion. Table 2.7 describes the interpretation of the release exponent in polymeric systems which are cylindrical in shape.

Table 2.7: Interpretation of the release exponent in cylindrical polymeric systems (Singhvi & Singh, 2011)

Release exponent (n)	Drug release mechanism	Rate as a function of time
0.45	Fickian diffusion	$t^{-0.5}$
0.45 < n < 0.89	Anomalous transport	t^{n-1}
0.89	Case II transport	Zero order release
n > 0.89	Super Case II transport	t^{n-1}

Several models have been discussed but no model universally fits all the different types of dissolution/release data and it is therefore necessary to select the best model that best fits and describes the data.

The most common method used to assess the fit of a model equation is the coefficient of determination, R^2 (Costa & Lobo, 2001). It however has a weakness of the R^2 increasing with an increase in the number of parameters in the model irrespective of the significance of the variable added to the model and hence it is only useful when comparing models with the same number of parameters. In order to compare models with different numbers of parameters, the adjusted coefficient of determination, R^2_{adj}, is used. The best model is selected based on the value of the R^2_{adj}, where the highest value indicates best fit.

The Akaike Information Criterion (AIC) has become a standard tool in model fitting (Costa & Lobo, 2001). It measures goodness of fit based on maximum likelihood, where the model with best fit for a given set of data is the model with the lowest AIC value. This tool is only applicable in comparisons of models with similar weighting schemes.

2.4.3.2 Model independent methods for dissolution profile comparisons

As shown in Table 2.4, these can be further differentiated as ratio tests and pair-wise procedures. The ratio tests are relations between parameters obtained from the release assay of the reference formulation and the release assay of the test product at the same time and can range from a simple ratio of percent dissolved drug (*t*) to a ratio of area under the release or dissolution curve (AUC) or even to a ratio of mean dissolution time (MDT).

The most commonly used pair-wise procedures are the difference factor and similarity factor (Moore & Flanner, 1996) and these are therefore now briefly discussed.

The contrast between the two is that difference factor (f_1) measures the percent error between two curves over all time points (Eqn 2.10) whereas the similarity factor (f_2) is a logarithmic reciprocal square root transformation of one plus the mean squared (the average sum of squares) differences of drug percent dissolved between test and reference (Eqn 2.11) (Costa & Lobo, 2001).

$$f_1 = \left\{ \frac{\sum |R_t - T_t|}{\sum_{t=1}^{n} R_t} \right\} \times 100 \qquad \textbf{Eqn 2.10}$$

where ***Rt*** and ***Tt*** are the mean percent dissolved at each time point for the reference and test products, respectively, and ***n*** is the number of observations /dissolution sample times being

considered for the computation. An f_1 value between 0 and 15 indicates similarity of two dissolution profiles while values outside this range indicate dissimilarity.

$$f_2 = 50 \log \left(\frac{100}{\sqrt{\left[1 + \frac{1}{n} \sum_{t=1}^{n} W_t \ (R_t - T_t)^2\right]}} \right) \qquad \textbf{Eqn 2.11}$$

where, the parameters, ***Rt***, ***Tt*** and ***n*** are as described for the difference factor, f_1 and $\mathbf{W_t}$ is an optional weight factor.

The f_2 is more sensitive than the f_1 in the determination of dissolution profile similarity and is therefore the method of choice advocated by the FDA for purposes of the assessment of similarity between two *in-vitro* dissolution profiles. The similarity factor ranges from 0 to 100, where 100 indicates the profiles are identical and 0 for dissimilar profiles. Limits for similarity are values between 50 and 100 whereas dissimilar profiles will be any value less than 50. The method is suitable for dissolution profile comparisons when there are more than 3 dissolution time points.

2.4.3.3 Statistical methods for dissolution profile comparisons

The methods based on the analysis of variance can also be divided into two, viz. the one-way analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA). These statistical methods assess the difference between the means of two drug release data sets in single time point dissolution (ANOVA or *t*-student test) or in multiple time point dissolution (MANOVA) (Zhang *et al.*, 2010).

These statistical methods may seem good for dissolution profile comparison of active compounds from herbal materials, where within batch variations may be quite significant, as it takes note of the within-batch variation in analysed samples. However, statistical methods for dissolution profiles comparison are not ideal as each dissolution time point is treated as a separate entity, independent of other points, which is not necessarily so. As such, the difference in profiles may be significant at some points and not at others, making interpretation difficult.

Moreover, ANOVA-based methods answer whether the profiles are statistically indistinguishable from each other but this does not necessarily equate to the profiles being pharmaceutically or practically indistinguishable.

Chapter 3

Work Plan

3.1 Introduction

This chapter details the study objectives, hypotheses tested and the study approach implemented to meet the objectives. The chapter further elucidates rationales for, and approaches followed in, the different aspects of the study.

3.2 Study objectives

The overall objectives were to determine whether;

1) phytosome technology could be successfully applied to *A. afra* to produce phytosomes possessing an acceptable pharmaceutical quality and,
2) *A. afra* phytosomes prepared from the FDAE would have different dissolution properties of the phytoconstituents *versus* the extract.

To realize these objectives, the following were to be done;

(i) Preparation and characterisation of a FDAE of *A. afra*,

(ii) Preparation of *A. afra* phytosomes from the FDAE using the solvent evaporation mechanical dispersion method,

(iii) Determination of factors that influence the efficiency of complex formation between phytoconstituents and phosphatidylcholine in the preparation of *A. afra* phytosomes,

(iv) Determination of physicochemical pharmaceutical characteristics (particle size and distribution, particle morphology, colloidal stability, apparent solubility) of *A. afra* phytosomes and

(v) Comparison of the dissolution profiles of *A. afra* phytosomes *versus* the FDAE, in simulated gastric and intestinal fluid, using luteolin as the marker compound.

3.3 Hypotheses

The following hypotheses were to be tested;

Hypothesis 1: The drug delivery phytosome technology can be successfully used on the FDAE of *A. afra* to produce phytosomes having the following acceptable physiochemical pharmaceutical characteristics, i.e.

Poly dispersity index ≤ 0.2

-30 mV ≥ Zeta potential ≥ 30 mV

Hypothesis 2: *A. afra* phytoconstituents in the phytosome complex (AAP) are more lipophilic than the *A. afra* FDAE phytoconstituents and therefore have a higher apparent solubility (AS) in octanol, i.e.

$$\mathbf{AS_{oct}^{AAP} > AS_{oct}^{FDAE}}$$

And the converse; the FDAE phytoconstituents have a higher apparent solubility in water compared to the phytosome phytoconstituents, i.e.

$$\mathbf{AS_{water}^{AAP} < AS_{water}^{FDAE}}$$

where, $\mathbf{AS_{oct}^{AAP}}$ and $\mathbf{AS_{oct}^{FDAE}}$ are the apparent solubilities, measured in terms of the marker compound luteolin, of phytosomes and FDAE in 1-octanol, respectively; and $\mathbf{AS_{water}^{AAP}}$ and $\mathbf{AS_{water}^{FDAE}}$ the apparent solubilities of the phytosomes and FDAE in water, respectively.

Finally, usually increased lipophilicity results in a decrease in aqueous solubility, which directly results in a decrease in the dissolution rate (drug release). Therefore, the final hypothesis to be tested was:

Hypothesis 3: The dissolution rate (D) of luteolin from the FDAE is higher than that of luteolin from the *A. afra* phytosomes, i.e.

$$\mathbf{D_{FDAE} > D_{AAP}}$$

where, $\mathbf{D_{FDAE}}$ and $\mathbf{D_{AAP}}$ were the dissolution rates, based on levels of marker compound, luteolin, of FDAE and the phytosomes, respectively.

and

$$Q_{75}^{FDAE} < Q_{75}^{AAP}$$

Where, Q_{75}^{FDAE} and Q_{75}^{AAP} is the time for 75% of the luteolin to be released into solution from the FDAE and phytosomes, respectively.

3.4 Study approach

The study questioned whether the phytosome drug delivery technology could be successfully applied to *A. afra* to produce phytosomes of an acceptable pharmaceutical quality and whether the *A. afra* phytosomes prepared from the FDAE would modify (decrease) the release properties of the extract's phytoconstituents into physiologic fluids.

In quest to answer these questions, firstly, an *A. afra* freeze dried aqueous extract was to be prepared. Then, from the FDAE, phytosomes were to be prepared based on a 2^3 full factorial experimental design to identify the factors that affect the efficiency of complex formation. In the phytosome preparation, the selected independent variables to be investigated included stirring speed, type of organic solvent and mass ratio of FDAE to phospholipids, and the dependent variable was the CFE (complex formation efficiency). From the results obtained, the optimised preparation variables were to be selected and used to prepare the final phytosomes and the latter characterised in terms of physicochemical pharmaceutical parameters such as particle size, morphology and shape, particle size distribution, colloidal stability and solubility. Subsequently, the dissolution tests were to be carried out in simulated gastric (pH 1.2) and intestinal (pH 6.8) fluids to determine and compare the phytoconstituent release profiles from the FDAE *versus* phytosomes, using luteolin as the selected marker compound. Lastly, statistical and model dependant and independent approaches were to be used to compare the dissolution profiles.

3.4.1 Rationale for modifying the release properties of *Artemisia afra* phytoconstituents

In a report on dissolution studies carried out on tea bags of the FDAE (Dube, 2006), the author described the release profile as 'rapid', with 85% of the constituents released within 15 minutes, and in another study on encapsulated *A. afra* FDAE, the preparation was also

described ‘rapidly dissolving’ (Max, 2007). This phytoconstituent release pattern was however fully expected since the FDAE was prepared by aqueous extraction and its actives, consequently, highly water soluble.

However, as previously mentioned, the rapid phytoconstituent release is particularly problematic in circumstances where the active constituents are poorly absorbed, as is the case with *A. afra* (Komperlla, 2004). In addition, challenges associated with a rapid dissolution of poorly absorbed substances is that frequent dosing is required to maintain therapeutic plasma concentrations and unavoidable fluctuations in drug concentrations may lead to under and or over dosing. But a modification in the release profile was expected to result in a reduction of dosing interval, more reproducibility and uniform drug release and absorption, reduction of local irritations and minimal dose dumping (Das *et al.*, 2015). All these considerations motivated the basis for the study.

3.4.2 Why luteolin as marker compound?

For this study and for several of the characteristics and parameters to be measured, a suitable marker compound had to be selected. Herbal products are generally quite complex, consisting of complex components that may be active individually or in combination. The “actives” are usually defined to be the whole herbal preparation, e.g. the extract in its entirety. For characterisation purposes the European Agency for the Evaluation of Medicinal Products (EMEA) proposes three categories of herbal medicinal products based on the characterisation of their active components (Nair & Kanfer, 2008). Relevant to *A. afra*, Category A products consist of standardised extracts for which an active moiety/moieties has/have been definitively identified, and in this case, the flavonoid luteolin, as it has been identified to be partly responsible for the therapeutic activity of the extract (Mjiqiza, Syce & Obikeze, 2013; Tikiso, 2015). The aforementioned luteolin, has also been found to be the major flavonoid constituent in crude extracts of *A. afra* (Waithaka, 2004), several studies involving *A. afra* have successfully used it as a marker compound (Komperlla, 2004; Dube, 2006; Max, 2007; Nkengla, 2014; Tikiso, 2015) and finally, much is known on the extraction, detection and quantification of luteolin in plant matrices. All this thus made luteolin a most suitable marker compound to characterise *A. afra* preparations and for use in the present study.

Chapter 4

Preparation and characterisation of *Artemisia afra* preparations: FDAE and phytosomes

4.1 Introduction

The overall goal of the work reported in this chapter was to test the hypothesis that phytosome drug delivery technology can be successfully used on *Artemisia afra* FDAE to produce phytosomes with acceptable pharmaceutical physicochemical characteristics. The equipment, materials and methods used to prepare and characterise *Artemisia afra* preparations are presented, and the results obtained reported and discussed.

4.2 Equipment and materials

4.2.1 Equipment

The following equipment was used:

-86°C ultralow freezer (*Nuaire, Model NU 9668E, Nuaire, Japan),* **heating mantle** (*MRC, Model MN-500C),* **oven** *(Labotech, Model LDO-080F, DaihanLabotech Co Ltd, Korea),* **analytical balance** *(Ohaus, Model PA413, Ohaus Corporation, USA),* **square hot plate magnetic stirrer** *(Dragonlab, Model MS7-H550 Pro, Dragon Laboratories, China),* **ultrasonic bath** *(Scientech, Model 702, Labotec, South Africa),* **vortex mixer** *(KK, Model VM-300, Germany Industrial Corp, Taiwan),* **vacuum pump** *(Buchi, Model V-500, BuchiLarbotechnik AG, Switzerland),* **rotavapor** *(Buchi, Model R-11, BuchiLarbotechnik AG, Switzerland)* **connected to a vacuum pump** *(Buchi, Model V-700, BuchiLarbotechnik AG, Switzerland),* **centrifuge** *(Baxter, 13 Microcentrifuge, Heraeus Thermo),* **freeze drier** *(Virtis TM mobile freeze-dryer, model 125L),* **magnetic stir bar** *(5 cm),* **ceramic mortar**, **Buchner funnel** *(funnel base 210 mm),* **desiccator, Whatman No. 1 paper filter, nylon syringe filters** *(25 mm diameter, 0.45 µm pore size),* **micropipettes, HPLC filter unit** (*Millipore Cameo 25 AS, DDA 02025So MSI: Micro separation Inc., USA),* **membrane filters** *(Durapore, 0.45 µm HV, Millipore),* **FTIR instrument fitted with UATR and controlled with Spectrum® software version 6.3.5.0176** *(Perkin-Elmer 100,*

Perkin-Elmer, USA), **Fine Coat Ion Sputter** *(JFC-1100, JEOL, Tokyo, Japan),* **Auriga CrossBeam microscope** *(Zeiss, Germany),* **Zetasizer nano-series** *(Malvern instruments, Model Nano-ZS90, United Kingdom),* **Zetasizer software** *(version 7.11, Malvern Instruments Ltd),* **rotator shaker**, **HR 73 halogen moisture analyser** *(Mettler Toledo, Model H73,United States of America),* **cuvette** and **folded capillary zeta cell.**

The **HPLC system** (*Agilent, Model 1200 series, Chemetrics*) used consisted of a **vacuum degasser** *(Agilent 1200 series, Model G1322A, Germany),* **autosampler** (*Agilent 1200 series, Model G1329A, Germany),* **thermostatted column compartment** *(Agilent 1200 series, Model G1316A, Germany),* **a quaternary pump** (*Agilent 1200 series, Model G1311A, Germany*), **a diode array detector (DAD)** (*Agilent 1200 series, Model G1315B, Germany),* **a PC with Chemstation® software** (*Agilent, OpenLAB CDS Chemstation Edition LC and CE version A.01.04(033))* and a **reverse phase column** *(Luna® 5 µm, C18, 250 x 4.60 mm, Phenomenex, United States of America).*

4.2.2 Chemicals

The following chemicals and reagents were used:

Acetonitrile and **methanol** *(HPLC grade, Burdick & Jackson, United States of America),* **ethanol, sodium nitrate** and **aluminium chloride** *(Merck, Darmstadt, Germany),* **formic acid** and **sodium acetate** *(BDH Chemicals Ltd, England),* **hydrochloric acid 32% $^{w}/_{v}$** *(Kimix, USA),* **monobasic potassium phosphate**, **distilled water** (prepared with Millipore filtration system 40/35), **ethyl acetate** *(Saarchem, South Africa),* **sodium hydroxide**, **polymethylmethacrylate**, **acetone**, **dry ice**, **dichloromethane**, **tetrahydrofuran**, **triton X-100**, **polyvinyl alcohol**, **cholesterol**, **α-L phosphatidylcholine**, **1-octanol** and **reference standard luteolin** *(Sigma Aldrich, Germany).* All other materials and reagents were either of analytical grade or of the highest purity and used as received.

4.2.3 Materials

Dried *A. afra* leaves were purchased from local industrial herbal supplier Grassroot Group (Pty) Limited (UWC voucher number 6867, Groenvlei Farm, South Africa), placed in plastic

bags and stored in a temperature controlled laboratory room at 20°C in a dark place away from light.

4.3 Methods

4.3.1 Preparation and characterisation of *A. afra* freeze dried aqueous extract (FDAE)

4.3.1.1 Preparation of freeze dried aqueous extract (FDAE) powder from *Artemisia afra* leaf material

The aqueous extract was prepared by adding boiling distilled water to dried *A. afra* leaves in a ratio of 1:20 of mass (in grams) of dried leaves to volume (measured in millilitres) of distilled water (Mukinda *et al.*, 2010). The mixture was boiled for 30 minutes, cooled at room temperature and then filtered under vacuum using a Buchner funnel with Whatman No. 1 filter paper. The filtrate was transferred into a 250 ml round bottom flask, shell frozen and thereafter freeze-dried under vacuum (Virtis TM mobile freeze-dryer, model 125 L) for 48 hours. The FDAE powders from the different batches were combined into a single final homogenous batch and weighed to determine the percentage yield. Finally, the FDAE powder was placed in stoppered amber glass bottles to protect it from light and stored at room temperature in a desiccator until used.

4.3.1.2 Characterisation and evaluation of the *Artemisia afra* freeze dried aqueous extract powder

4.3.1.2.1 Determination of organoleptic properties

4.3.1.2.1.1 Colour

To determine the colour of the FDAE, 0.2 g of the material was placed against a white background in diffuse daylight, viewed by eye and its colour described accordingly.

4.3.1.2.1.2 Odour

To determine the odour of the FDAE, 0.4 g of the material was placed in a 5 cm diameter watch glass, left for 15 minutes and thereafter the air above the sample was inhaled slowly and repeatedly. The strength of the odour was determined by classifying it as either non-existent, weak, distinct or strong and the odour sensation described as either aromatic, fruity, musky, mouldy or rancid (WHO, 2011).

4.3.1.2.1.3 Taste

For the taste determination, the FDAE of *A. afra* was dispersed in distilled water at a concentration of 0.01 g/ml, stirred and allowed to stand for 10 minutes before the researcher tasted 0.5 ml of the mixture by mouth. The taste was described in terms of none, sweet, sour or bitter.

4.3.1.2.2 Determination of extractable matter and solubility

The amount of extractable matter and degree of solubility of the FDAE in distilled water and buffers pH 1.2 and pH 6.8 were determined at room temperature. Accurately weighed 0.2 g of the FDAE in a glass-stoppered 10 ml test tube, added 5 ml of the solvent and mixed for 6 hours, by hand shaking for 1 minute every 30 minutes. Then, the mixture was allowed to stand for a further 18 hours, before being rapidly filtered using a vacuum pump and a previously weighed Whatman No.1 filter paper (WHO, 2011). The filter paper containing the residue was then dried in an oven at 35 °C for 6 hours, placed in a desiccator for 30 minutes to equilibrate to room temperature and immediately thereafter weighed.

To calculate the weight of the residue, the weight of the empty filter paper was subtracted from the combined weight of the dried filter paper with residue and the extractable matter, which also resembles solubility, determined using Equation 4.1 and expressed in terms of mg per gram of plant material.

$$\text{Extractable matter} = \frac{\text{Initial weight of plant material} - \text{weight of residue}}{\text{Weight of initial plant material}} \qquad \text{Eqn. 4.1}$$

In addition, the solubility was determined using the following equation (Eqn. 4.2):

$$\text{Solubility} = \frac{\text{Initial weight of plant material} - \text{weight of residue}}{\text{Volume of solvent}} \times 100 \qquad \text{Eqn. 4.2}$$

Finally, the solubility of the material was described using the common descriptive phrases of solubility and the corresponding quantitative solubility ranges given in the BP 2013 (Table 4.1) and expressed in terms of "parts", which represented the number of millilitres (ml) of the solvent, in which 1 g of the solid was soluble.

Table 4.1: Common descriptive phrases of solubility and the corresponding quantitative solubility ranges (BP, 2013)**.**

Descriptive phrase	Approximate quantities of solvent by volume for 1 part of solute by weight
Very soluble	Less than 1 part
Freely soluble	From 1 to 10 parts
Soluble	From 10 to 30 parts
Sparingly soluble	From 30 to 100 parts
Slightly soluble	From 100 to 1000 parts
Very slightly soluble	From 1000 to 10, 000 parts
Practically insoluble	More than 10, 000 parts

4.3.1.2.3 Determination of moisture content of *A. afra* FDAE

The moisture content of *A. afra* FDAE was determined using a gravimetric method which involved the determination of a loss in mass on drying (Souza, Bott & Oliveira, 2007). For this, an HR73 Halogen Moisture Analyser was used. Samples of a minimum of 0.1 g FDAE were evenly spread on the sample pan and inserted into the analyser. The moisture analyser automatically weighed and heated (105 °C) the sample and calculated the percentage loss on drying. The equations below illustrate how the moisture analyser determined the percentage moisture content.

Moisture weight= Initial weight (Before drying) – Final weight (After drying) **Eqn. 4.3**

$$\text{Moisture content} = \frac{\text{Moisture weight}}{\text{Initial weight}} \times 100 \qquad \text{Eqn. 4.4}$$

4.3.1.2.4 Determination of the Total Flavonoid Content of *A. afra* FDAE

The aluminium chloride colorimetric method (Barku *et al.*, 2013), with minor modifications, was used to measure the flavonoid content of the FDAE. Briefly, an aqueous solution of the FDAE (0.25 ml, 1 mg/ml) was added to 1.25 ml of distilled water. Sodium nitrate solution (0.075 ml, 5% $^{w}/_{v}$) was then added to the mixture followed by incubation at room temperature for 5 minutes after which 0.15 ml of 10% $^{w}/_{v}$ aluminium chloride was added. The mixture was allowed to stand for 6 minutes at room temperature before 0.5 ml of 1 M sodium

hydroxide was added and, finally, the mixture diluted with 0.275 ml distilled water. The absorbance of the reaction mixture was immediately measured at 350 nm with an HPLC Diode Array Detector. Quercetin was used as the standard and standard solutions of 12.5, 25, 50, 80 and 100 µg/ml of quercetin in 80% methanol for the calibration curve. From the UV absorbance of the FDAE test sample, the flavonoid concentration (in terms of quercetin equivalents, QE) was determined from the calibration curve and the total flavonoid content (TFC) determined according to Equation 4.5 and expressed as mg QE/g dry weight (D.W.).

$$\mathbf{TFC = QE \times (V/M)} \qquad \mathbf{Eqn.\ 4.5}$$

where **TFC** is the total flavonoid content expressed in mg QE/g; **QE** is quercetin equivalent concentration (in mg/ml) of quercetin solution established from the calibration curve; **V** is the volume (in ml) of the extract solution and **M** is mass of the plant extract (in g). Three replicate samples were assayed and the TFC reported as average $^{+}$/- standard deviation value.

4.3.1.2.5 Development and validation of HPLC assay for luteolin

In addition to its TFC, the *A. afra* FDAE was also characterised in terms of its luteolin content, the selected marker compound for the study, and for this an HPLC method was developed. The method developed (and eventually used) was based on previous gradient elution methods used by Nkengla (2014) and Tikiso (2015), which were simply modified to suit the current study.

Briefly, separation of the marker compound was effected on a Phenomenex Luna® C-18 column (250 x 4.6 mm, 5 µm) kept at temperature of 25^{o}C and a mobile phase consisting of 0.1% $^{v}/_{v}$ formic acid aqueous solution (solvent A) and 0.1% $^{v}/_{v}$ formic acid in acetonitrile (solvent D) used. Both solvents were filtered and degassed by membrane filtration prior to use. The elution gradient used was: 0-22 min 15% D; 22-27 min 100% D; 27-35 min 15% D; solvent flow rate 0.8 ml/min; the sample injection volume 20 µl and the luteolin peaks detected at 350 nm.

To validate the assay, the linear concentration range, assay specificity and precision, and limits of detection and quantification were determined. For this, a stock solution of luteolin, 165 µg/ml of 75% methanol, was prepared and from this standard solutions in concentration range 2.6 to 82.5 µg/ml 75% methanol were used to plot the calibration curve.

The linearity of the calibration curve for the reference standards was assessed in triplicate over a six sample concentration range, the calibration curves constructed as plots of peak area against concentration (ICH, 2005), subjected to linear regression and the correlation coefficient (R^2) determined using GraphPad Prism 6.

The detection and quantification limits (i.e. LOD and LOQ, respectively) were determined using the standard deviation of the response and the slope of the curve, and the following equations (ICH, 2005);

$$\mathbf{LOD} = \frac{\mathbf{3.3\,\sigma}}{\mathbf{S}} \qquad \textbf{Eqn. 4.6}$$

$$\mathbf{LOQ} = \frac{\mathbf{10\,\sigma}}{\mathbf{S}} \qquad \textbf{Eqn. 4.7}$$

where **σ** is the standard deviation of *y*-intercepts of regression lines and **S** is the slope of the calibration curve.

To determine the specificity of the assay, firstly, 150 µl of luteolin standard (165 µg/ml) was spiked with 150 µl of components of the phytosome matrix. The latter matrix was prepared by lysing extract free (unloaded) phytosomes with Triton X-100. The sample was prepared in triplicate and analysed on the HPLC system. Using the Chemstation® peak analysis function, peak purity tests were used to show that the luteolin chromatographic peak was not attributable to more than one compound. Also, the chromatograms were compared to identify any interference between the blank phytosome matrix with luteolin. Secondly, to determine the specificity of the assay for luteolin in the plant extract, chromatograms of standard luteolin sample (165 µg/ml) and FDAE solution were compared for peak retention time and the peak in the chromatogram of the FDAE solution subjected to peak purity analysis using the Chemstation® software.

Lastly, to determine the precision of the assay, 3 replicates of the low, medium and high concentration standard solutions were assayed three times a day, for three consecutive days and on each occasion the mean peak area, standard deviation and % relative standard deviation (RSD) calculated.

4.3.1.2.6 Quantification of luteolin content in *Artemisia afra* FDAE

The afore-mentioned validated assay was subsequently used to determine the levels of luteolin, the marker compound, in the *A. afra* FDAE. Specifically, the FDAE was evaluated for total, free and conjugated luteolin.

Briefly, a 3 ml solution of 15 mg/ml (50% methanol) of the FDAE was prepared, filtered using a 0.45 µm syringe filter and the filtrate divided into two 1 ml samples for analysis of unhydrolysed (free) luteolin and hydrolysed (total) luteolin. For the unhydrolysed luteolin, 20 µl of the sample was injected onto the HPLC system for analysis. For the assay of the hydrolysed luteolin however, 0.5 ml of 2 N hydrochloric acid and 0.5 ml of methanol were added to the 1 ml FDAE sample, the mixture vortexed for 30 seconds, left to 'hydrolyse' in a water bath at 80°C for 40 minutes, thereafter allowed to cool under room temperature and then, finally, centrifuged for 10 minutes at 5 000 rpm. The supernatant (20 µl) was then injected onto the HPLC system for analysis.

To identify luteolin in the plant samples, the UV spectra and retention times of the peaks obtained from the chromatograms of the plant samples were compared to that obtained with the pure luteolin standard solutions. Finally, the area of the luteolin peak was noted and the levels of free or total luteolin in each sample determined from the standard curve regression equation. The level of conjugated luteolin was determined by subtracting the free (unhydrolysed) luteolin level from the total (hydrolysed) luteolin level. Three replicates of each preparation were assayed.

4.3.1.2.7 Determination of oil/water partition coefficient (log P) of the FDAE

The partition coefficient was determined using the shake flask method (Berthod & Carda-Broch, 2004) with two different solvent systems. The first solvent system consisted of 1-octanol and 0.1 N hydrochloric acid (pH 1.2), and was designed to simulate gastric conditions while the second solvent system was a combination of 1-octanol and phosphate buffer (pH 6.8) and designed to simulate intestinal conditions.

Equal volumes of each solvent system [i.e. 1-octanol (10 ml) and phosphate buffer (10 ml), or 1-octanol (10ml) and 0.1 N hydrochloric acid (10 ml)] were equilibrated in a pear-shaped separatory funnel by shaking for 24 hours in order to co-saturate the two phases and then left to separate for 12 hours before being stored at room temperature for further use.

To determine the log P, 75 mg of FDAE was dissolved in 3 ml of the pre-saturated aqueous solvent. To this solution, 3 ml of the pre-saturated 1-octanol was added, the mixture shaken continuously for 24 hours, then centrifuged at 5 000 rpm for 10 minutes, the two phases separated by means of a pipette and finally, the samples thereof transferred to HPLC sample vials and each injected (20 µl) into the HPLC system to determine the concentrations of luteolin. The experiment was done in triplicate and the ratio of the luteolin concentration in the 1-octanol phase to the luteolin concentration in the respective aqueous phase was used to calculate the log P value using Equation 4.8 (Van Zyl, 2012).

$$\textbf{Log P} = \log\left[\frac{\textbf{Concentration of luteolin in organic phase (1-octanol)}}{\textbf{Concentration of luteolin in aqueous phase}}\right] \qquad \textbf{Eqn. 4.8}$$

4.3.2 Preparation and characterisation of *A. afra* phytosomes

4.3.2.1 Preparation of the *A. afra* phytosomes

The phytosomes were prepared using the aqueous mechanical dispersion solvent evaporation method of Sikarwar, Sharma *et al.* (2008), with minor modifications. Based on a factorial design, varying masses (100 and 200 mg) of phosphatidylcholine (PC) and a fixed mass of the FDAE (100 mg) were weighed on the analytical balance *(Ohaus, PA413)* and dissolved in 20 ml of organic solvent (tetrahydrofuran or dichloromethane) and 20 ml of distilled water, respectively. Using 0.45 µm nylon membrane syringe filters the mixtures were separately filtered, into a 50 ml glass beaker (FDAE) and a 100 ml Erlenmeyer flask (PC).

A 5 cm magnetic stirring bar was then placed in the flask containing the PC solution and, with the aid of a clamp, the flask secured onto the magnetic stirrer as shown below (Figure 4.1). Thereafter, the FDAE solution was added drop-wise over 5 minutes, via a 20 ml syringe, into the PC solution which was continuously stirred (800 – 1000 rpm) at room temperature. The mixture was stirred for a total of 60 minutes and immediately after mixing, it was transferred to a 100 ml round bottom flask, rota-vaporated under vacuum at 40^{o}C for 30 minutes (to remove any residual organic solvent), vortexed for 3 minutes, sonicated in an ultrasonic bath for 30 minutes before filling three Eppendorf tubes (T1, T2 and T3) with 1 ml of the mixture for analysis of complex formation efficiency and further characterisation.

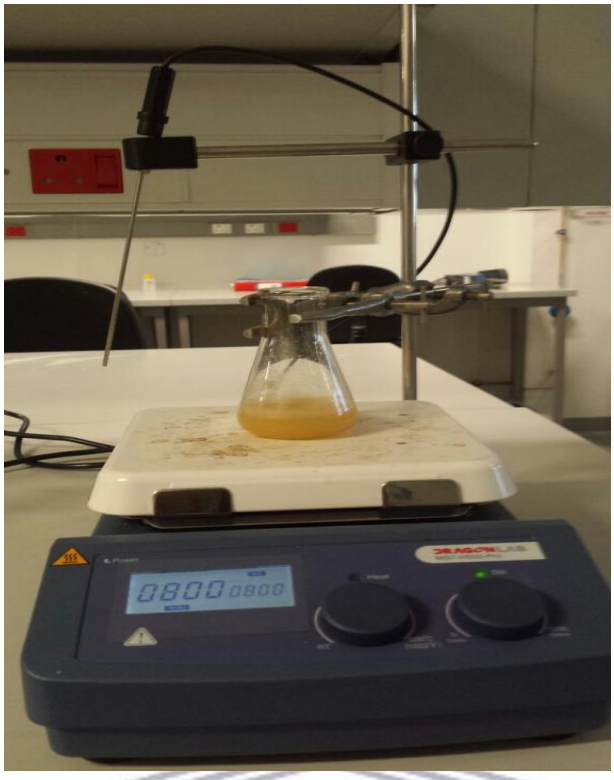

Figure 4.1: Set up of the equipment for the preparation of *A. afra* phytosomes

4.3.2.2 Experimental design for determination of factors affecting complex formation efficiency.

To determine the effect of various parameters on the efficiency of the complex formation (CFE) between the phospholipids and phytoconstituents in the *A. afra* FDAE phytosomes, a 2^3 full factorial experimental design (Bhalerao & Raje Harshal, 2003; Joshi *et al.*, 2012; Shah & Pathak, 2010) was implemented. For this analysis Design-Expert® version 8 software was used.

Three independent variables, namely, mass ratio of FDAE to phospholipids (X_1), stirring speed (X_2) and type of organic solvent (X_3) were taken at two levels: low and high, which were represented by transform (coded) values of −1 and +1, respectively. Values used for these selected variables are shown in Table 4.2.

Table 4.2: Key to the 2^3 factorial design

Factor	Parameter	Levels	
		Low (-1)	**High (+1)**
X_1	Mass ratio of FDAE: PC	1:1	1:2
X_2	**S**tirring speed	800 rpm	1000 rpm
X_3	Organic solvent	Dichloromethane	Tetrahydrofuran

Sixteen batches (eight formulations duplicated) were prepared according to the experimental design (shown in Table 4.3) and were evaluated for complex formation efficiency (CFE), Y, (dependent variable). Fitting a multiple linear regression model to the factorial design gave a predictor equation which was a first order polynomial, having the form,

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \qquad \textbf{Eqn. 4.9}$$

where, **Y** was the dependent variable; b_0, an intercept representing the arithmetic mean response of all quantitative outcomes of sixteen runs, and b_i the estimated coefficient for the factor X_i.

In order to ensure a good model, tests for significance of the regression model, significance on individual model coefficients and for lack-of-fit were performed using analysis of variance (ANOVA) and regression coefficients (Vali *et al.*, 2008). The model was then used to identify the parameters that significantly affected CFE, with level of significance set at $p < 0.05$.

Table 4.3: Coded factorial design set up

Formulation	X_1 (mass ratio)	X_2 (stirring speed)	X_3 (organic solvent)
F1	+1	- 1	- 1
F2	+1	+1	- 1
F3	- 1	- 1	- 1
F4	- 1	+1	- 1
F5	- 1	+1	+1
F6	+1	- 1	+1
F7	- 1	- 1	+1
F8	+1	+1	+1
F9	+1	- 1	- 1
F10	+1	+1	- 1
F11	- 1	- 1	- 1
F12	- 1	+1	- 1
F13	- 1	+1	+1
F14	+1	- 1	+1
F15	- 1	- 1	+1
F16	+1	+1	+1

4.3.2.3 Characterisation of the *A. afra* phytosomes

4.3.2.3.1 Determination of phospholipid-phytoconstituent complex formation efficiency (CFE)

The efficiency of complex formation between the *A. afra* FDAE phytoconstituents and phosphatidylcholine was determined using luteolin as marker compound and Equation 4.10. Briefly, to determine the uncomplexed (free) luteolin content, sample T1 was centrifuged at 10 000 rpm for 20 minutes after which 0.5 ml of the supernatant was carefully withdrawn by pipette, without disrupting the pellet, and transferred to an HPLC vial for analysis on the HPLC system *(Agilent 1200 series)*. To determine the total (i.e. complexed plus uncomplexed) luteolin content, Triton X-100 (0.075 ml; to lyse the phytosomes) was added to the T2 sample, the mixture vortexed for 5 minutes, centrifuged at 6 000 rpm for 10 minutes

(Baxter, 13 Microcentrifuge, Heraeus Thermo) and, finally, 0.5 ml of the supernatant solution transferred to an HPLC vial for luteolin content analysis on the HPLC system. The complex formation efficiency was then calculated using Equation 4.10.

$$\textbf{Complex formation efficiency \%} = \frac{\textbf{Total luteolin} - \textbf{Free luteolin}}{\textbf{Total luteolin}} \times 100 \qquad \textbf{Eqn. 4.10}$$

4.3.2.3.2 Determination of particle size and distribution and, colloidal stability

To determine the particle size, particle size distribution and colloidal stability, the T3 sample was centrifuged at 10 000 rpm for 20 minutes *(Baxter, 13 Microcentrifuge, Heraeus Thermo)*, the supernatant removed, 1 ml of distilled water added, the mixture vortexed for 1 minute to re-disperse the pellet and then centrifuged at 10 000 rpm for 20 minutes. This washing of the pellet was repeated twice. Thereafter the pellet was re-dispersed in distilled water by vortexing for 3 minutes, sonicated for 10 minutes in an ultrasonic bath and finally aliquots of this suspension were pipetted into a cuvette and folded capillary zeta cell, for analysis of particle size and distribution, and zeta potential, respectively, on the Zetasizer *(Malvern instruments, Model Nano-ZS90)*. The particle size and distribution analyses were conducted at 25^{o}C and the zeta potential measurement at pH 6.43 and 25^{o}C. Attenuator setting and measurement duration were set to automatic and number of runs and measurement angle were set at 3 runs per sample and 90^{o}, respectively.

4.3.2.3.3 Fourier Transformation Infrared (FTIR) spectra

Infra-Red spectra of the phytosomes, phospholipids, FDAE and a physical mixture of the FDAE and phospholipids were obtained using a Perkin- Elmer 100 FTIR instrument fitted with UATR and controlled with Spectrum® software version 6.3.5.0176. Samples in powder form were analysed over the wavelength range 400 - 4000 cm^{-1} and the percentage transmittance recorded against frequency.

4.3.2.3.4 Determination of the phytosome particle surface morphology

The surface morphology of the phytosome particles was determined using the scanning electron microscopy technique. First, the phytosomes were coated with gold in a Fine Coat Ion Sputter JFC-1100. Then the stub with the coated phytosomes was placed in the Zeiss

AURIGA® CrossBeam scanning electron microscope and surface morphology viewed and photographed.

4.3.2.3.5 Determination of the apparent solubility of *A. afra* phytosomes and FDAE

To determine the apparent solubility of luteolin in the preparations, an excess of the phytosomes or FDAE was added to 5 ml of water or n-octanol in sealed glass containers at room temperature (Khan *et al.*, 2014). The liquid was agitated for 24 hours on rotator shaker, centrifuged for 20 minutes at 4 000 rpm, the supernatant (0.5 ml) filtered through 0.45 µm nylon membrane filter, transferred into an HPLC sample vial for analysis on the HPLC to quantify amount of luteolin present and finally, the concentration of luteolin determined for each preparation in each solvent. All experiments were conducted in triplicate and average values compared using the Student t-test with level of significance set at $p < 0.05$.

4.4 Results and discussion

4.4.1 Preparation and characteristics of *A. afra* FDAE

4.4.1.1 Yield of freeze-dried aqueous extract

The aqueous extract was prepared in a way that closely mimicked the method traditional herbal practitioners use to extract their plant medicines. This was done to minimise variations in the phytochemical content of the FDAE compared to the traditional decoction. In the present study a moderate yield of 26.2% (Table 4.4 and Appendix 1) was obtained. This was however similar to yields obtained in other *A. afra* studies, viz. the 21.8, 21.96 and 28.4% by Nkengla (2014), Dube (2005) and Komperlla (2004), respectively. This may be attributed to similar extraction methods used.

Table 4.4: Characteristics of *A. afra* FDAE

Characteristic		Result
Yield (%)		26.16 ± 2.90
Moisture content (%)		2.47 ± 0.15
Total flavonoid content (mg QE/g)		34.1 ± 6.4
Luteolin content	**Un-hydrolysed (µg/mg)**	0.45 ± 0.01
	Hydrolysed (µg/mg)	1.92
	Conjugated (%)	76.6

4.4.1.2 Organoleptic properties

Usually, an examination of organoleptic characteristics is the first step towards establishing the identity and the degree of purity of plant materials. If, in terms of colour, consistency, odour or taste, a plant material sample is found to be significantly different from the set specifications, it is considered as not fulfilling the requirements. The results obtained for the FDAE of *A. afra* are summarized in Table 4.5.

Table 4.5: Organoleptic properties of *A. afra* FDAE

Organoleptic property	Description
Colour and appearance	Pale brown coloured powder which turns to a dark brown deliquescent gum on prolonged exposure to the air
Odour	Aromatic
Taste	Extremely bitter

The *Artemisia afra* had a characteristic aromatic odour which emanated from its essential oils. Compounds such as α- and β-Thujone (52.1 - 39.8%), camphor (14.4 - 8.2%), 1, 8-cineole (21.8–13.1%) and borneol (7.8 - 2.7%) are known to be the major components responsible for the characteristic flavour of the species (Asekun, Grierson & Afolayan, 2007).

Colour can be used as a means of identifying a particular substance and several Pharmacopoeias include the colour of the substance as part of the substances monograph. The FDAE produced in the current study was light brown in colour but turned dark brown on extended exposure to air (Figure 4.2). The change in colour and appearance was most likely due to the hygroscopic nature of the FDAE as was also reported in several other studies (Komperlla, 2004; Dube, 2006; Nkengla, 2014).

Figure 4.2: Physical appearance of *A. afra* FDAE

Finally, the FDAE had an extremely bitter taste. Bitterness is usually quantified with a so-called bitterness value and determined by comparison with quinine hydrochloride, for which the bitterness value is set at 200 000. Olivier and Van Wyk (2013) recorded a bitterness value of 26 393 ± 29 759 for *Artemisia afra*, to some extent confirming the bitter taste obtained in the present study (Olivier & van Wyk, 2013). Several other studies have also obtained a similar qualitative severity of bitterness associated with this species (Dube, 2006; Nkengla, 2014; Wagner & Wiesenauer, 2003). For instance, Dube (2006) recorded a bitterness of 600 000, triple that of the quinine standard. The difference in the bitterness values obtained by Olivier and Van Wyk (2013) and Dube (2006), and, the large standard deviation documented by former authors are however evidence that this test, because it is based on an individuals' sensory functions, is generally prone to large errors. Nevertheless, the qualitative description of the taste was consistent in all previous studies and the present one.

4.4.1.3 Extractable matter and solubility

This characteristic gives an indication of the amount of active constituents extracted with solvents from a given herbal material. It is generally employed for materials for which no suitable chemical or biological assay exists as yet (WHO, 2011).

The results (Appendix 2) obtained for the *A. afra* FDAE in the present study are summarised in Table 4.6.

Table 4.6: Extractable matter and solubility of *A. afra* FDAE in various solvents

Solvent	Extractable matter* (mg/g)	Solubility* (Parts)	Solubility* mg/ml	Description
Water	888 ± 62	27.9 ± 1.4	35.9 ±1.8	soluble
pH 1.2 buffer	847 ± 2.6	27.1 ± 0.1	36.9 ± 0.1	soluble
Phosphate buffer (pH6.8)	983 ± 3.2	24.8 ± 0.3	40.4 ± 0.2	soluble

*n = 2

According to the common descriptive phrase of the BP 2013, the *A. afra* FDAE was soluble in all the solvents investigated. This was as predicted, given that the extract was derived by aqueous extraction. In contrast to the finding of this study, Nkengla (2014) and Komperlla (2004) reported their extracts as being sparingly soluble and requiring 38.7 and 40 ml of water, respectively, to dissolve 1 g of extract. The solubility and amount of extractable matter in the buffer systems was of interest as the buffer conditions used were similar to those found in the gastrointestinal tract and the results would indicate the extent of availability of extract for absorption in the stomach (pH 1.2) and small intestines (pH 6.8). Of note, the solubility was not pH dependant and the good solubility of *A. afra* actives ingredients in the present study was thus a good finding.

4.4.1.4 Moisture content

Moisture content is one of the most important factors influencing quality and storability of plant materials. Pharmacopoeias generally recommend the use of the oven drying method to determine the moisture content of plant materials but in this study a halogen moisture analyser was used because it was quick and efficient. The moisture content of the FDAE was found to be 2.47 ± 0.15% (Table 4.4 and Appendix 3). This low moisture level was desirable because it discourages hydrolysis and microbial growth and thereby ensures preservation of the extract. Indeed, residual moisture levels > 1 to 5% are generally regarded as acceptable for solid oral dosage forms (FDA, 1990). In addition, the result obtained in this study was also lower than that obtained by other authors, e.g. 8.62% by (Dube, 2006) and 10.68% by (Nkengla, 2014).

Overall, the low moisture content result obtained may have been attributed to the shell freezing technique, which ensured quick and uniform drying of the sample, but,

unfortunately, this reason cannot be asserted as the aforementioned authors did not detail their respective freezing techniques. In addition, the low moisture may also be attributed the analysis method used, as the halogen moisture analyser, minimises human error unlike the oven drying method.

4.4.1.5 Total Flavonoid Content (TFC) of the FDAE

The TFC of the extract was determined using the aluminium chloride colorimetric method, with quercetin (QE) used as the standard and a calibration curve of absorbance peak area *versus* quercetin concentration constructed (Figure 4.3). Linearity (R^2 = 0.999) was established within the concentration range of 12.5 – 100 µg/ml. The flavonoid content was expressed in terms of quercetin equivalent (mg of QE) per g of extract, and the results obtained are shown in Table 4.4.

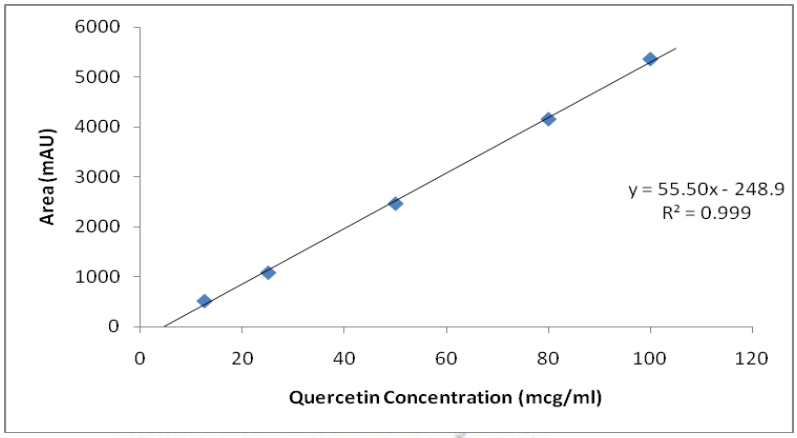

Figure 4.3: Standard curve of quercetin concentration *versus* absorbance area at 350 nm used for the quantification of the total flavonoid content

The TFC of the *A. afra* FDAE was found to be 34.1 ± 6.4 mg QE/g, a level fairly similar to that found in other species; e.g. 32.317 ± 0.629 mg QE/g in *Pseuderanthemum palatiferum* (Nees) radlk, (Nguyen & Eun, 2011; Zhishen, Mengcheng & Jianming, 1999) and 26.6 mg QE/g in *Morus alba* L., (Zhishen, Mengcheng & Jianming, 1999). Nkengla (2014) also determined the TFC of a FDAE of *A. afra* but used a different method from the one used in the present study and obtained a level of 24.5 mg LUT/g (Nkengla, 2014). Although the latter finding is reasonably similar to that found in the present study, the two results can however not be directly compared because of differences in the method used.

Overall, the FDAE had a reasonably high TFC, which was expected, as the genus, *Artemisia* L. has been documented to contain over 160 flavonoids (Avula *et al.*, 2009) and the

presence of high and quantifiable flavonoid content, therefore also makes the TFC a potential quality control parameter for *A. afra* preparations.

4.4.1.6 Validation of HPLC assay for luteolin

A reverse phase HPLC method was developed for the quantification of luteolin, the selected marker compound for the study. The assay developed showed luteolin peaks with good symmetry, retention time of 28.60 ± 0.11 minutes (Figure 4.4) and UV spectrum (Figure 4.4 inset) showing maximal absorption around 350 nm as expected of flavonoids.

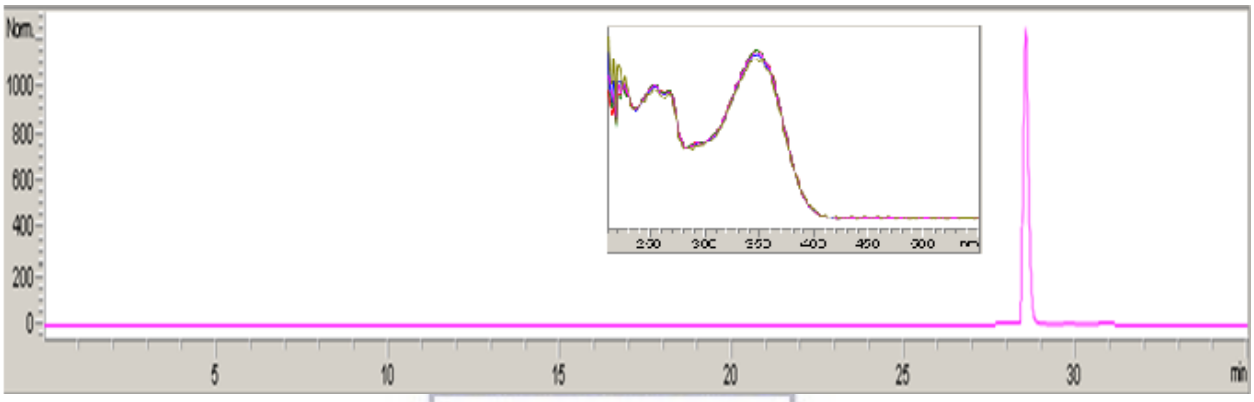

Figure 4.4: Typical HPLC chromatogram for the luteolin reference standard at 350 nm. Inset: UV/Vis spectrum for the reference compound at ~28.6 min

The results of the validation are summarized in Table 4.7. The six-point peak area *versus* concentration of luteolin standard curve was linear in the range of 2.58 - 82.5 µg/ml (i.e. 51.6 – 1650 ng of luteolin on column) (Figure 4.5), described by the equation $y = 90.01x + 13.86$ (where y = peak area, x = luteolin concentration in µg/ml) and had a regression correlation coefficient, R^2 value of 0.999.

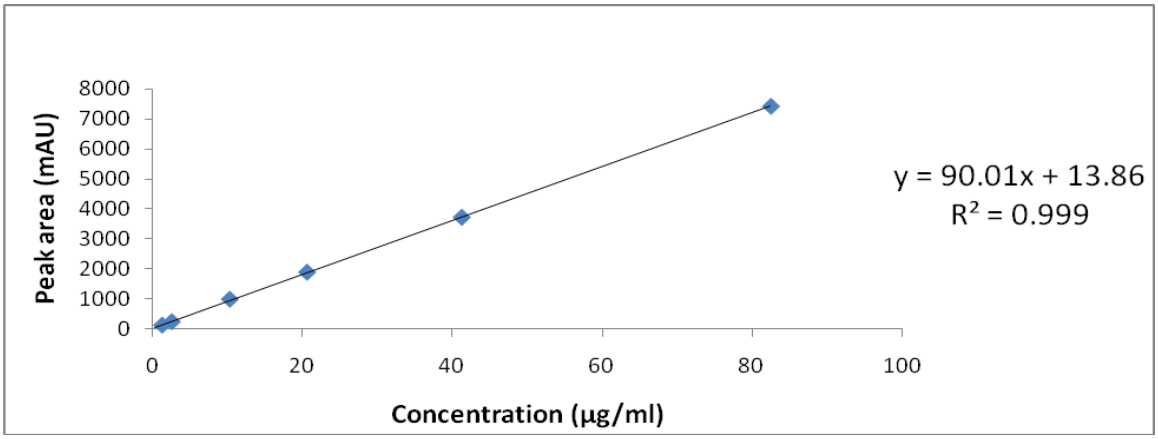

Figure 4.5: Calibration curve of mean peak area against the concentration of replicate samples of luteolin at 350 nm

The average intra-day and inter-day precision was 0.986 ± 0.728% and 1.202 ± 0.672%, respectively. The assay method was therefore deemed as precise since the %RSD was less than 2% (FDA, 2000). The limits of detection and quantification were 0.03 µg/ml (0.61 ng on column) and 0.09 µg/ml (1.84 ng on column), respectively.

Table 4.7: Summary of luteolin HPLC assay validation results

Validation parameter		**Acceptability criteria**	**Result**
Linearity		$R^2 \geq 0.997$ (ICH, 2005)	0.999
Precision	Inter-day	%RSD ≤ 2% (FDA, 2000)	0.986 ± 0.728%
	Intra-day		1.202 ± 0.672%
LOD		-	0.03 µg/ml
LOQ		-	0.09 µg/ml
Specificity		Peak purity test (ICH, 2005)	No interference
Accuracy		Infered if linearity, precision and specificity are established (ICH, 2005)	Infered

The specificity of the assay was confirmed by the peak purity test (UV spectra insert A in Figure 4.6) which showed that the luteolin chromatographic peak was only attributable to one compound and that there was therefore no interference with the elution of luteolin. In addition, the chromatograms showed that there was no co-elution with any component of the blank phytosome matrix at the luteolin retention time.

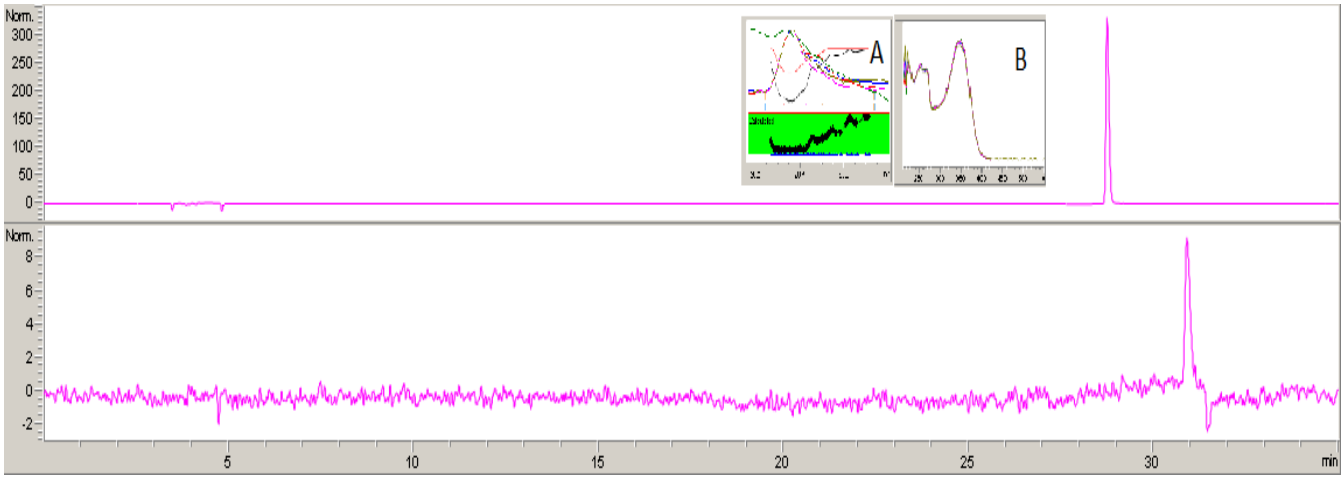

Figure 4.6: HPLC chromatogram for spiked (top) and blank phytosome matrix (lower). Inset A and B represent the peak purity test and UV spectrum at 350 nm

Collectively, the validation results showed acceptable validity and reproducibility and hence the HPLC assay for luteolin was deemed suitable for use.

4.4.1.7 Identification and quantification of luteolin in *A. afra* FDAE

Using the aforementioned validated HPLC assay (section 4.4.1.6), levels of luteolin in the FDAE were quantified. Representative chromatograms of un-hydrolysed and hydrolysed samples of *A. afra* FDAE are shown in Figures 4.7 and 4.8, respectively, and the luteolin levels in the FDAE are depicted in Tables 4.4 and 4.8.

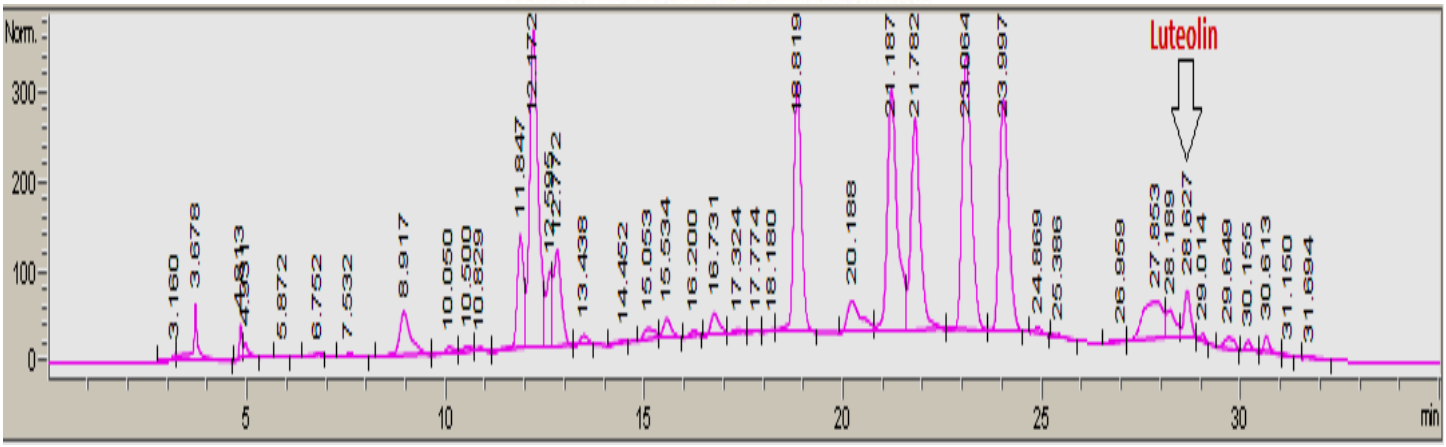

Figure 4.7: Representative HPLC chromatogram of unhydrolysed FDAE of *A. afra*. The retention time of luteolin at 28.627 minutes

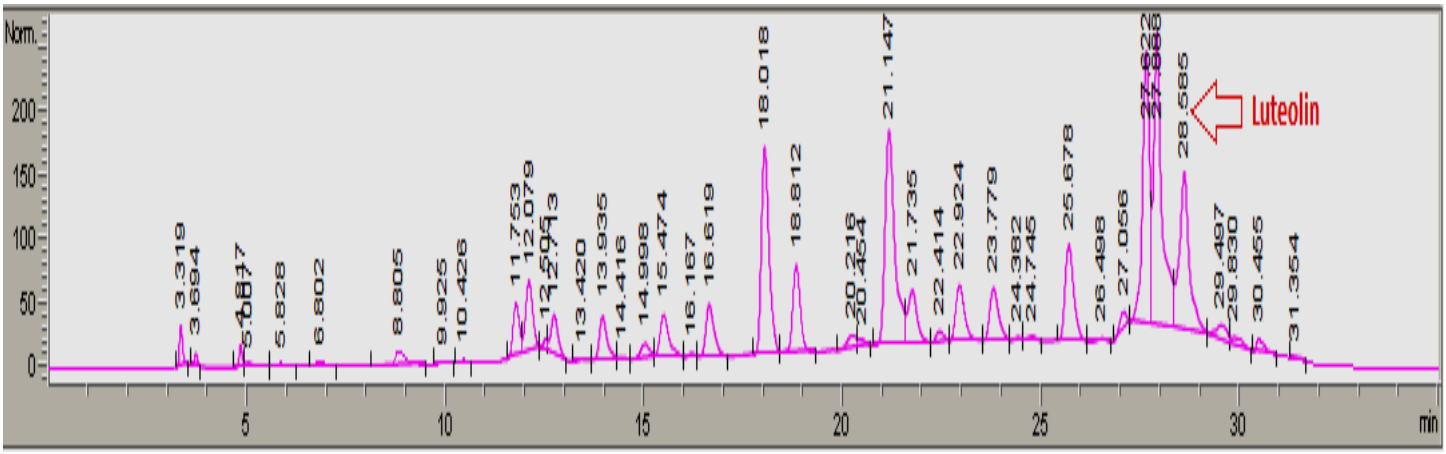

Figure 4.8: Representative HPLC chromatogram of hydrolysed FDAE of *A. afra*. The retention time of luteolin at 28.585 minutes

The chromatograms of the hydrolysed and un-hydrolysed *A. afra* samples showed peaks of several other compounds, of which most had a UV/Vis spectrum similar to that of flavonoids. These peaks therefore most likely indicated the presence of other flavonoids and or glycosides of luteolin. However, the luteolin peak (retention time 28.5± 0.255 min) was confirmed by spiking samples and by UV spectral analysis done using the diode array detector and HPLC Chemstation® software.

Overall, the FDAE contained 0.45 ± 0.01, 1.92 ± 0.01 and 1.47 ± 0.01 µg/mg of free, total and conjugated luteolin (Table 4.8). Most (76.6%) of the luteolin was in the conjugated form as expected. Normally, the majority of flavonoids present in the leafy parts of plants are in the form of O- and C- glycosides (e.g. glucosides, rutinosides, glucopyranoside) and this was confirmed by the results of the present study where after acid hydrolysis of the FDAE, the luteolin levels quadrupled in comparison to the unhydrolysed sample. The conjugates were generally unstable and could be easily hydrolysed to the aglycone form, and this characteristic can thus be potentially also used as a quality control marker of stability, i.e. if the unhydrolysed extract showed an increased level of aglycone luteolin concentration then it may be an indication of degradation of the plant material.

Table 4.8: Luteolin levels in FDAE of *A. afra*

Luteolin content	**Unhydrolysed (µg/mg)**	0.45 ± 0.01
	Hydrolysed (µg/mg)	1.92 ± 0.01
	Conjugated (%)	76.6

Other authors have reported varying concentrations of luteolin in *A. afra* FDAEs. For instance, Nkengla (2014) obtained 0.185 ± 0.24 and 0.235 ± 0.026 µg/mg, while, in contrast, Dube (2006) had higher levels, 7.0880 ± 0.4751 and 13.870 ± 1.2460 µg/mg of free and total luteolin, respectively. These variations in phytochemical concentrations are quite common and pose major challenges in the standardisation and quality control of herbal products. The variations mainly arise from differences in season and geographical location of harvest (Asekun, Grierson & Afolayan, 2007; Viljoen *et al.*, 2006) and therefore should be factored in when standardising herbal products.

All in all, luteolin was successfully identified and quantified by the validated HPLC assay and of note; the FDAE contained significantly more conjugated luteolin than the aglycone form.

4.4.1.8 Oil/water partition coefficient (log P) of the FDAE

The octanol–water partition coefficient is the accepted physicochemical property for measuring the lipophilicity of substances (Sangster, 1997) and is also used to predict the potential absorption of substances. The log P values obtained for the FDAE in the present study are given in Table 4.9 and showed that, in terms of both luteolin and TFC, the FDAE was more lipophilic under gastric (pH 1.2) than intestinal (pH 6.8) conditions.

Table 4.9: Oil/water partition coefficient (log P) values of FDAE in pH 1.2 and pH 6.8

	Partition coefficient	
	pH 1.2	**pH 6.8**
Luteolin	1.52 ± 0.004	0.44 ± 0.004
Total flavonoid content	-0.35 ± 0.06	-1.10

Under gastric and small intestinal conditions, luteolin was more lipophilic than the total flavonoids. Despite the differences in lipophilicity under different conditions, all the log P values indicated that the phytoconstituents would be poorly absorbed (i.e. log P < 2) under both pH conditions. A log P value of between 3 to 4.6 is generally predictive of good

absorption of the compound (Leeson & Springthorpe, 2007), while values less than 2 are reflective of hydrophilic compounds which are poorly absorbed.

There are advantages and disadvantages associated with the use of luteolin and TFC as marker compounds in the determination of log P values. The use of luteolin is more representative of the aglycones (e.g. for quercetin, log P = 1.5; apigenin, log P = 1.7; genkwanin, log P = 2.1) (Pubchem, 2016) which are known to be more lipophilic than the glycosides (e.g. luteolin 7-glucoside, log P = 0.5) (Pubchem, 2016). As shown by the previous results in section 4.4.1.7, the FDAE contained mostly flavonoid glycosides (76%) which are known to be hydrophilic (Evans, 2009), hence the aglycone log P value is not an accurate representation of the measure of lipophilicity of the whole extract. On the contrary, TFC depicts a more holistic measure of lipophilicity of the FDAE as it incorporates all flavonoids, which are known to be responsible for the extracts' pharmacological action. The present study therefore portrays one of the disadvantages of using the aglycone marker compound for quality control purposes for an extract that contains numerous phytoconstituents.

All summed up, as expected, the FDAE proved to be hydrophilic and therefore had a high potential to be poorly absorbed as evidenced by the low log P values.

4.4.2 Preparation and characteristics of *A. afra* phytosomes

Artemisia afra phytosomes were prepared using an aqueous based solvent evaporation mechanical dispersion method. This method is seldom used since the solvent evaporation and reflux techniques (Arora, Sharma & Kaur, 2013; Das & Kalita, 2014; Habbu *et al.*, 2013; Khan *et al.*, 2014; Mali *et al.*, 2014; Yue *et al.*, 2010) are generally preferred. These latter methods are applicable where the extract is soluble in an organic solvent. In this study, preliminary solubility studies revealed that the FDAE of *A. afra* was insoluble in dichloromethane, tetrahydrofuran, methanol, ethanol and diethyl ether, which therefore rendered the commonly used preparation methods unsuitable for preparation of the *A. afra* phytosomes. In addition, from a pharmaceutical production perspective, the aqueous based mechanical dispersion method minimises the amount of organic solvents used (which may be cumbersome to remove) and would be rapid and amenable to scale up procedures due to its simplicity.

In the preparation of the phytosomes, mass ratios of FDAE to phospholipids were used instead of the molar ratios due to the complexity of the phytoconstituents of the extract which are difficult to quantify in terms of molarity. In addition, the phospholipids contained ≥ 40% phosphatidylcholine; therefore, any stoichiometric molar ratio used would have been inaccurate.

All in all, the preparation method was simple to use and presented no major challenges throughout all the preparation steps.

4.4.2.1 Complex formation efficiency (CFE)

To successfully develop a phytosomal drug delivery system, complex formation (encapsulation) efficiency is key as it represents the efficiency and acceptability of the preparation method followed (Khan *et al.*, 2014). In this study the CFE was determined using an indirect method that took into account losses of excipients that incurred during the preparation of the phytosomes. The CFE results obtained are given in Table 4.10 and the sixteen batches showed a wide variation, from 21.2 to 68.7%, in CFE. The variation was generally similar to the 38.8 to 75.3% that Khan et al (2014) obtained in the preparation of luteolin phospholipid complexes. This therefore confirmed the suitability of the preparation technique.

Table 4.10: Complex formation efficiency of sixteen phytosome formulations

Batch code	X_1 (mass ratio)	X_2 (stirring speed)	X_3 (Organic solvent)	Y (CFE %)
F1	+1	- 1	- 1	68.7
F2	+1	+1	- 1	56.8
F3	- 1	- 1	- 1	43.2
F4	- 1	+1	- 1	43.8
F5	- 1	+1	+1	41.0
F6	+1	- 1	+1	56.0
F7	- 1	- 1	+1	51.3
F8	+1	+1	+1	38.6
F9	+1	- 1	- 1	51.2
F10	+1	+1	- 1	51.1
F11	- 1	- 1	- 1	43.0
F12	- 1	+1	- 1	33.2
F13	- 1	+1	+1	21.2
F14	+1	- 1	+1	57.1
F15	- 1	- 1	+1	42.2
F16	+1	+1	+1	37.9

Based on the aforementioned findings, the experimental variables that gave the highest CFE i.e. dichloromethane, 800 rpm and ratio of 1:2, were used to prepare another batch of phytosomes which was then lyophilised to produce the phytosomes in solid form. These lyophilised phytosomes were pale cream-yellow in colour, sticky non-free flowing powders (Figure 4.9). These characteristics were similar to those obtained by Das (2014) in the preparation of rutin phytosomes.

Figure 4.9: Physical appearance of *A. afra* phospholipid complex

The poor flow properties of the phytosomes were expected to pose a major challenge when encapsulating them for dissolution studies. Ideally, reformulation by addition of other excipients such as silicone dioxide and magnesium stearate was required so as to counteract the sticky nature of the phytosomes and thereby improve its flow properties.

Suitability of phytosomes as potential drug carriers of the phytoconstituents of the FDAE was partly dependent upon the ability of PC to complex with most of the phytoconstituents. As previously stated, luteolin was the marker compound selected for the determination of the complex formation efficiency. However, luteolin was just one of the phytoconstituents present in the FDAE and it was therefore also necessary to qualitatively assess the complex formation in terms of the numerous other constituents. The chromatograms illustrated in Figure 4.10, show peaks representing phytoconstituents complexed to the PC. From visual comparison of chromatograms of the unhydrolysed FDAE (Figure 4.7) and unhydrolysed phytoconstituents present in the phytosome (Figure 4.10A), it can be noted that the unhydrolysed FDAE had about 29 compounds while the unhydrolysed phytosomes had

approximately 21 compounds. From this comparison it was evident that most of the phytoconstituents of the FDAE interacted with the PC to form the phytosome complex.

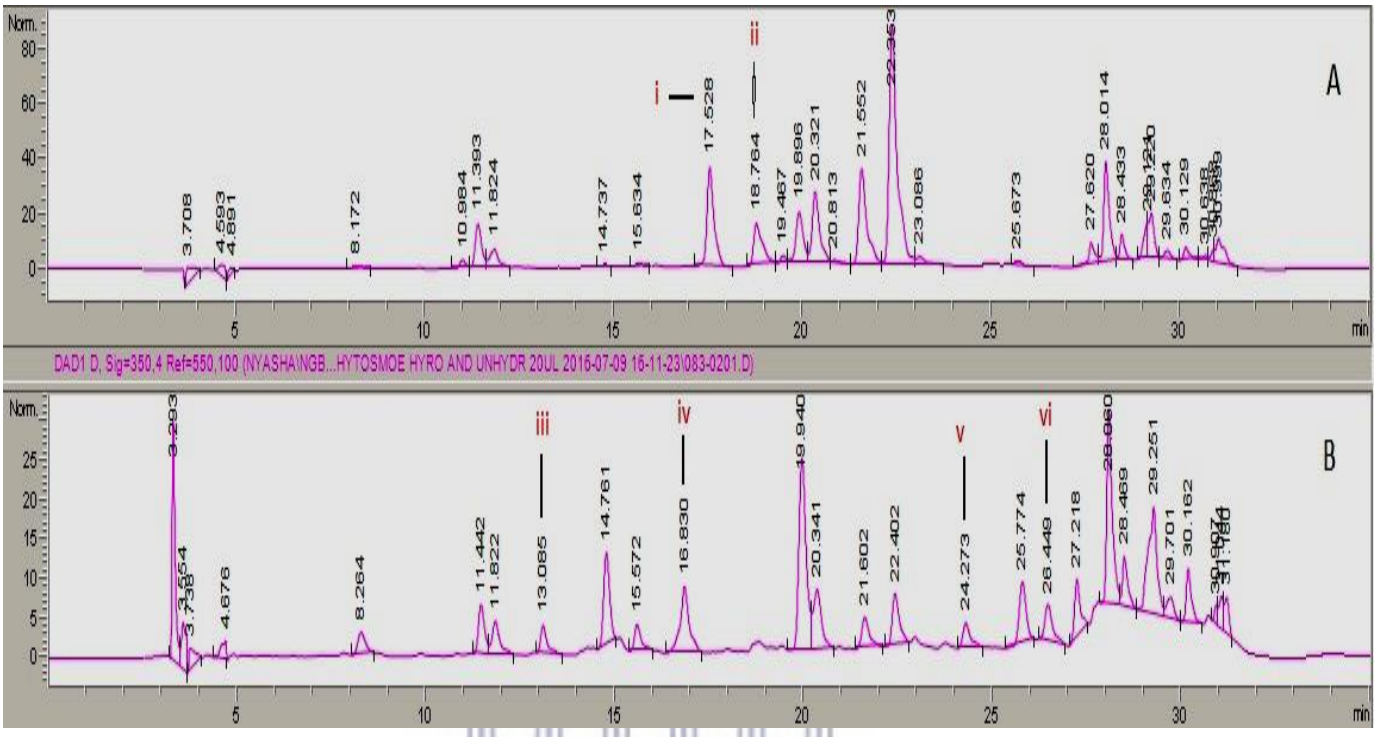

Figure 4.10: Representative chromatograms of unhydrolysed (A) and hydrolysed (B) phytoconstituents present in the phytosome complex at 350 nm. Luteolin detectable at ≈ 28.5 minutes

Interestingly, the chromatograms of the hydrolysed phytosome phytoconstituents showed no increase in the luteolin peak area, implying that complexing between PC and luteolin conjugates was either very low or non-existent. However, comparison of the phytosome chromatograms (Figure 4.10 A and B) revealed emergence of new peaks e.g. at Rt 13.085 (iii), 16.830 (iv), 24.402 (v) and 26.449 (vi) minutes (Figure 4.10B) in the hydrolysed phytosome chromatogram and disappearance of peaks e.g. 17.528 (i) and 18.764 (ii) minutes (Figure 4.10A), previously present in unhydrolysed phytosome chromatogram. This may be evidence of the presence of conjugates (e.g. glycosides) which are acid hydrolysed (resulting in the disappearance of peaks or decrease in peak areas) to the aglycone form which may result in either emergence of a new peak or increase in existing peak area, thus confirming complexation of PC and the conjugates. In order to identify the compounds corresponding to the disappeared peaks, the UV spectra were analysed and the results obtained are shown below.

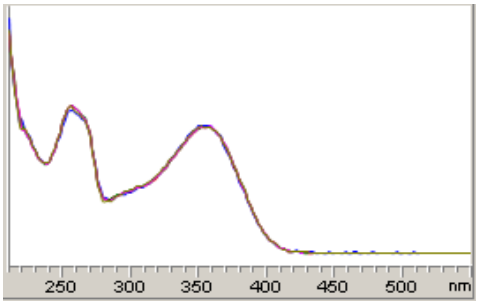

Figure 4.11: UV spectrum of peak at 17.528 minutes measured at 350 nm

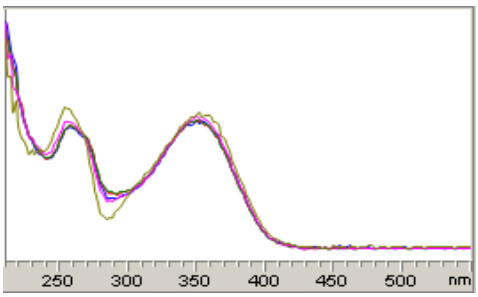

Figure 4.12: UV spectrum of peak at 18.764 minutes measured at 350 nm

Both spectra strongly suggested that the compounds were flavonoid glycosides (maximum absorbance at ≈ 350 nm) due to the disappearance of the peaks upon acid hydrolysis.

All in all, the phytosomes proved to be suitable drug carriers for at least the flavonoid phytoconstituents of *A. afra* FDAE as most of the phytoconstituents complexed with the phospholipids.

4.4.2.2 Experimental design

A full 2^3 factorial design incorporating one process (stirring speed) and two formulation variables (i.e. organic solvent and FDAE: PC mass ratio) taken at two levels, was used to design experimental batches to investigate the factors that affected CFE during the preparation of *A. afra* phytosomes. Selection of the factors (variables) and levels that could affect CFE were based on literature reports where varying molar ratios of drug to PC and different types of organic solvents were used. Stirring speed was also investigated since the chosen preparation method was largely based on stirring and stirring speed was expected to influence CFE. Results obtained from the experimental design analysis are shown in Table 4.10.

When the data obtained was subjected to mathematical modelling and fitted into a two factor interaction polynomial model and the polynomial equation relating Y, the response CFE (%) to the transformed factors, the following equation (model) (Equation 4.11) was obtained.

$$\mathbf{Y = 46.02 + 6.16\ X_1 - 5.57\ X_2 - 2.86\ X_3 - 1.92\ X_1X_3 - 2.92\ X_2X_3} \qquad \textbf{Eqn. 4.11}$$

The main effects (X_1, X_2 and X_3) represented the average result of changing one factor at a time from its low to high value, while the interaction terms (X_1X_2, X_2X_3, and X_1X_3) showed how the response changed when two factors were simultaneously changed. This polynomial equation was then used to draw conclusions after considering the magnitude of the coefficient, the mathematical sign carried (i.e., positive or negative) and the p-value of the factor. Results of the mathematical modelling exercise are shown in Table 4.11.

Table 4.11: Results of ANOVA analysis of factorial model

Source	Sum of squares	df	Mean squares	F value	P value Prob > F
Model	1428.31	5	285.66	3.10	***0.0076***
A – PC ratio	606.39	1	606.39	12.95	***0.0049***
B – Stirring speed	496.18	1	496.18	10.59	***0.0086***
C – organic solvent	130.53	1	130.53	2.79	0.1260
AC	58.91	1	58.91	1.26	0.2883
BC	136.1	1	136.31	2.91	0.1188
Lack of fit	4.49	2	2.25	0.039	***0.9622***

The fitted model was considered adequate if it showed statistical significance ($p < 0.05$) and the lack-of-fit was not significant ($p > 0.05$). The data analysed showed a fairly good correlation with the model ($p = 0.0076$, $R^2 = 0.7531$ and lack of fit $p = 0.9622$). Although the R^2 value was only 0.7531, it was deemed suitable for the purpose of the model which was to investigate factors affecting CFE (Statease, 2016). However, R^2 values close to 1 are important for purposes of optimising formulations.

The data (Table 4.11) showed that there were no significant interaction effects (i.e. X_1X_3, p = 0.2883 and X_2X_3, p = 0.1188), but factors X_1 and X_2 were found to significantly affect CFE. Moreover, from Equation 4.11, X_1 and X_2 had a positive and negative sign, respectively, implying that an increase in PC content resulted in a corresponding increase in CFE while a decrease in stirring speed increased the CFE. On the other hand, the X_3 factor had no significant effect on CFE, implying that irrespective of the organic solvent (i.e. tetrahydrofuran or dichloromethane) used, there were no noteworthy effects on the CFE. The relationship of factors X_1 and X_2 with CFE is further illustrated in Figures 4.13 and 4.14. As depicted by the response surfaces (Figures 4.13 and 4.14), increasing the PC amount resulted in an increase in CFE irrespective of type of organic solvent used.

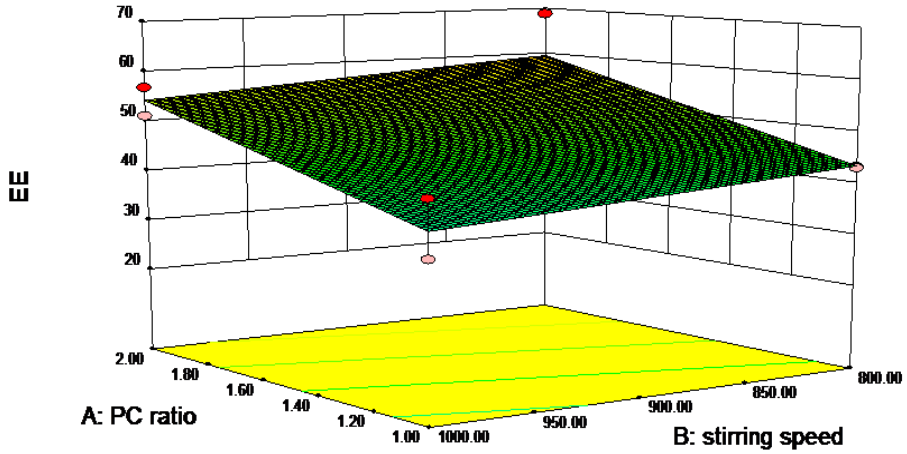

Figure 4.13: 3D representation of the effect of FDAE: PC ratio and stirring speed on CFE using dichloromethane

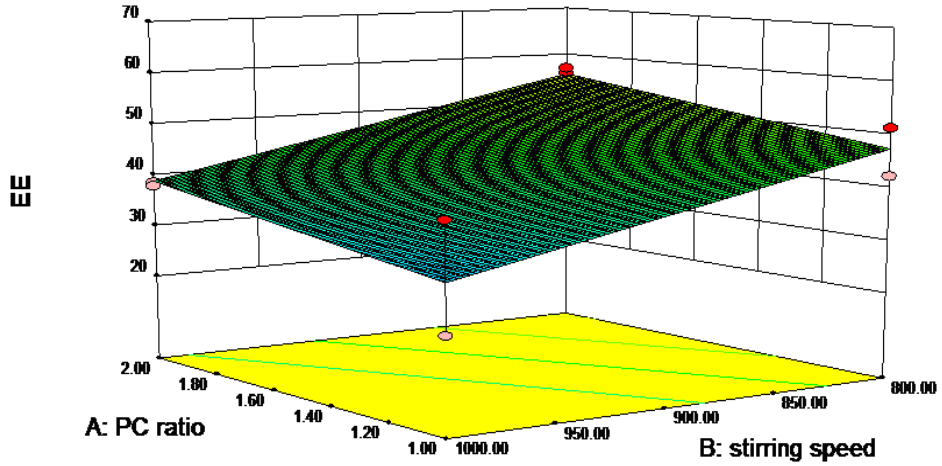

Figure 4.14: 3D representation of the effect of FDAE: PC ratio and stirring speed on CFE using tetrahydrofuran

This was most likely effected by an increase in the number of PC binding sites available for interaction with the polar phytoconstituents of the extract. In this present study, where mass ratios were used, due to the complexity of the phytoconstituents, a further increase in the amount of PC may also have resulted in increase of the CFE. It is generally assumed that one polyphenol molecule complexes with one PC molecule (Kidd, 2009), therefore an increase in the amount of PC would have resulted in a subsequent improvement in the CFE, though, this would have been within limits of saturation. Indeed, saturation of choline molecules results in a decrease in the CFE as any additional phytoconstituents (i.e. in excess of choline molecules) will remain unbound hence the need to use experimental designs to optimise the preparation variables, particularly the PC quantity.

One unique aspect of this drug delivery study was the complexation of a mixture of phytoconstituents with PC. The application of phytosome technology has generally thus far been with pure polyphenols and PC and the use of the stoichiometric ratio of 1:1. This ratio has been considered most suitable for formulating phytosomes (Khan *et al.*, 2013). However, higher ratios of 1 : 3 have also yielded high encapsulation efficiencies of up to 94% (Pathan & Bhandari, 2011; Yue *et al.*, 2010). In our unique scenario, to improve CFE, more PC interaction sites would therefore be required to interact with the numerous phytoconstituents as competition for interaction sites is high.

The stirring speed (X_2) also significantly affected the CFE ($p = 0.0086$). An increase in stirring speed decreased the CFE irrespective of the type of organic solvent used (Figures 4.13 and 4.14). High stirring speeds reduce the contact time for the reactions to take place, in this case the hydrogen bond formation and or Van der Waals interactions between the choline head and the polar compounds. In addition, at high speeds (1000 rpm) it was noted that by the end of the 60 minutes of stirring most of the organic solvent had evaporated. The rate at which a solvent evaporates has been shown to affect CFE, where rapid evaporation rates result in low CFE and low speeds increase CFE (Yeo & Park, 2004). This study therefore supported this finding.

In summary, based on the results from the experimental design, it was noted that in the preparation of *A. afra* phytosomes, the amount of PC and stirring speed significantly affected the CFE whereas the type of organic solvent used had no significant effects on the CFE.

4.4.2.3 Particle size, particle size distribution and colloidal stability of *A. afra* phytosomes

Apart from an acceptable CFE, particle characterisation (i.e. determination of particle size, particle size distribution and colloidal stability) is a crucial aspect in drug product development and quality control of dosage forms. Results of the three characteristics for the *A. afra* phytosomes are shown in Table 4.12.

Table 4.12: Particle size, polydispersity index (PDI) and zeta potential of *A. afra* phytosomes

Particle size (nm)	Particle size distribution (PDI)	Zeta potential (mV)
487 ± 35	0.201 ± 0.076	-54.6 ± 0.2

Firstly, the particle size of the phytosomes was in the nano range, thus increasing the surface area available for dissolution to occur, since, as per Noye-Whitney equation, the rate of dissolution is directly proportional to the surface area available for dissolution (Aulton & Taylor, 2013). In addition, the small size would also enable the phytosomes to penetrate into, and permeate through the physiological barriers (Saoji *et al.*, 2016).

Generally, PDI values of > 0.4 indicate extremely polydisperse systems which are undesirable, while mono-disperse systems ideally possess a PDI of 0 (Nobbmann, 2014). The phytosomes were moderately polydisperse (PDI = 0.1 – 0.4) and thus no significant effects on the dissolution, content uniformity, stability and bioavailability of the phytosome were expected. Broadly, polydisperse particles negatively affect the aforementioned parameters. With respect to dissolution, erratic drug release profiles are likely to occur as the polydisperse particles go into solution at contrasting rates. Similarly, content uniformity is also negatively affected as inconsistent amounts of the drug(s) will be present in the individual dosage forms. The phytosomes therefore had an acceptable PDI which was unlikely to adversely affect the dissolution and content uniformity.

Zeta potential is a measure of the magnitude of the electrostatic or charge repulsion/attraction between particles, and is one of the fundamental parameters known to affect particle stability. The measurement brings detailed insight into the causes of dispersion, aggregation or

flocculation, and can be applied to improve the formulation of dispersions, emulsions and suspensions. The phytosomes had a zeta potential of -54.6 ± 0.2 mV, and a zeta potential greater than 30 mV or less than -30 mV indicates a stable colloidal system (Aulton & Taylor, 2013). From the result obtained it could thus be concluded that the phytosomes formed a very stable colloidal system.

Overall, the prepared phytosomes were of nanoparticle size and formed a moderately polydispersed but very stable colloidal system.

4.4.2.4 Confirmation of phospholipid – phytoconstituent interaction

The FTIR analytical technique was used to investigate spectroscopic characteristics which reflect the interactions between PC and the phytoconstituents bound in the complex and therefore confirm formation of the complex. The spectra result of the FTIR analyses of PC, phytosomes, physical mixture of the FDAE and PC, and FDAE are shown in Figure 4.15.

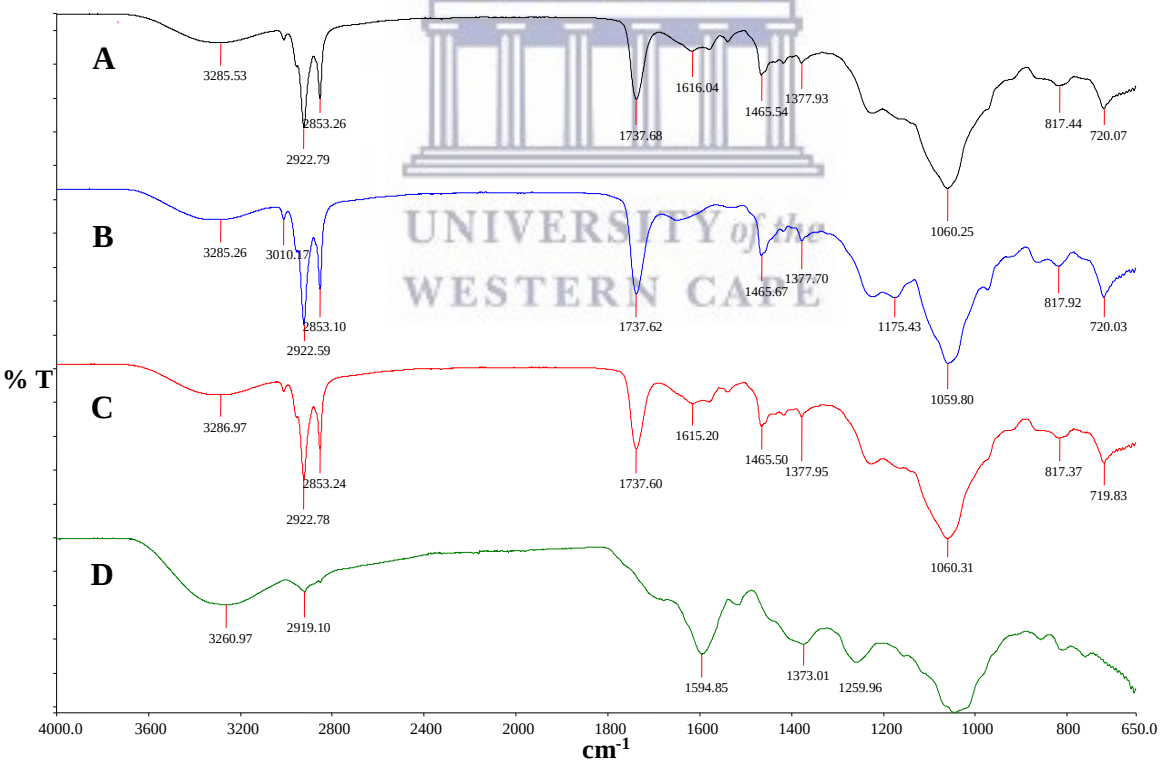

Figure 4.15: FTIR spectra of phospholipids (A), phytosomes (B), physical mixture (C) and FDAE (D)

The FTIR spectrum of the phospholipids (A) contained the characteristic C-H stretching signal present in the long fatty acid chain at 2922 and 2853 cm^{-1}, a C=C stretching band at 3010 cm^{-1} representing the internal fatty acid alkene, the C=O stretching band in the fatty acid ester at 1737 cm^{-1} and, a P=O stretching band at 1236 cm^{-1} and a P-O-C stretching band 1060 cm^{-1} for the choline (Saoji *et al.*, 2016).

For the FDAE, a broad peak at 3260 cm^{-1}, representing an –OH group substituted on an aromatic ring, common with flavonoids was present (Khan *et al.*, 2014). In addition a weak C-H stretching signal at 2919 cm^{-1} relating to the short aliphatic chains typically found in some flavonoids and glycosides was present as well as an aromatic ring stretch C=C-C, exhibited at 1594 cm^{-1}, which related to the presence of aromatic constituents such as flavonoids, tannins and saponins (Sikarwar *et al.*, 2008).

Finally, the FTIR spectrum of the complex was quite different, particularly in the fingerprint region (i.e. 1600 to 650 cm^{-1}), from that of the phospholipids, physical mixture and FDAE. Compared to the phospholipid spectrum, there was a slight shift of the P-O-C absorption band from 1060.25 to 1059.80 cm^{-1}, disappearance of a peak at 1616.04 cm^{-1} and a change in shape of the spectra around 970 cm^{-1} thus confirming interaction of the polar head of PC and the phytoconstituents.

The FTIR thus provided evidence of the formation of phytosomes and also confirmed that the formation was through interactions between the phospholipid polar head and the phytoconstituents.

4.4.2.5 Particle shape and surface morphology

The shape and surface morphology of the phytosomes was analysed using the SEM and the results data of this analysis are depicted in Figure 4.16. The phytosomes appeared spherical with a smooth surface and were generally all of the same size (mono-dispersed). The spherical shape was advantageous as it increased the surface area available for dissolution and, improved both drug release properties and flow properties of the phytosomal powder. Based on the dynamic light scattering method, the particle size was approximately 487 nm, while the SEM image indicated a much smaller size of about 70 nm. This discrepancy may have been due to the micelles that form when the phytosomes were in the aqueous media causing the larger size in contrast to the SEM image for which the dry powder form of the

phytosomes was used and, the zeta potential value of -54.6 mV eliminated any chances that the discrepancy could have been due to aggregation of the particles.

Generally, SEM images of phytosomes from other studies have shown them to either be rod shaped (Das & Kalita, 2014) or irregularly shaped (Arora, Sharma & Kaur, 2013; Habbu *et al.*, 2013; Sabzichi *et al.*, 2014), i.e. different to that found in the present study. This may have been due to the preparation techniques used which, in most cases, were either reflux or solvent evaporation methods. The stirring or homogenisation based preparation techniques, as used in the present study, generally yield spherical shapes.

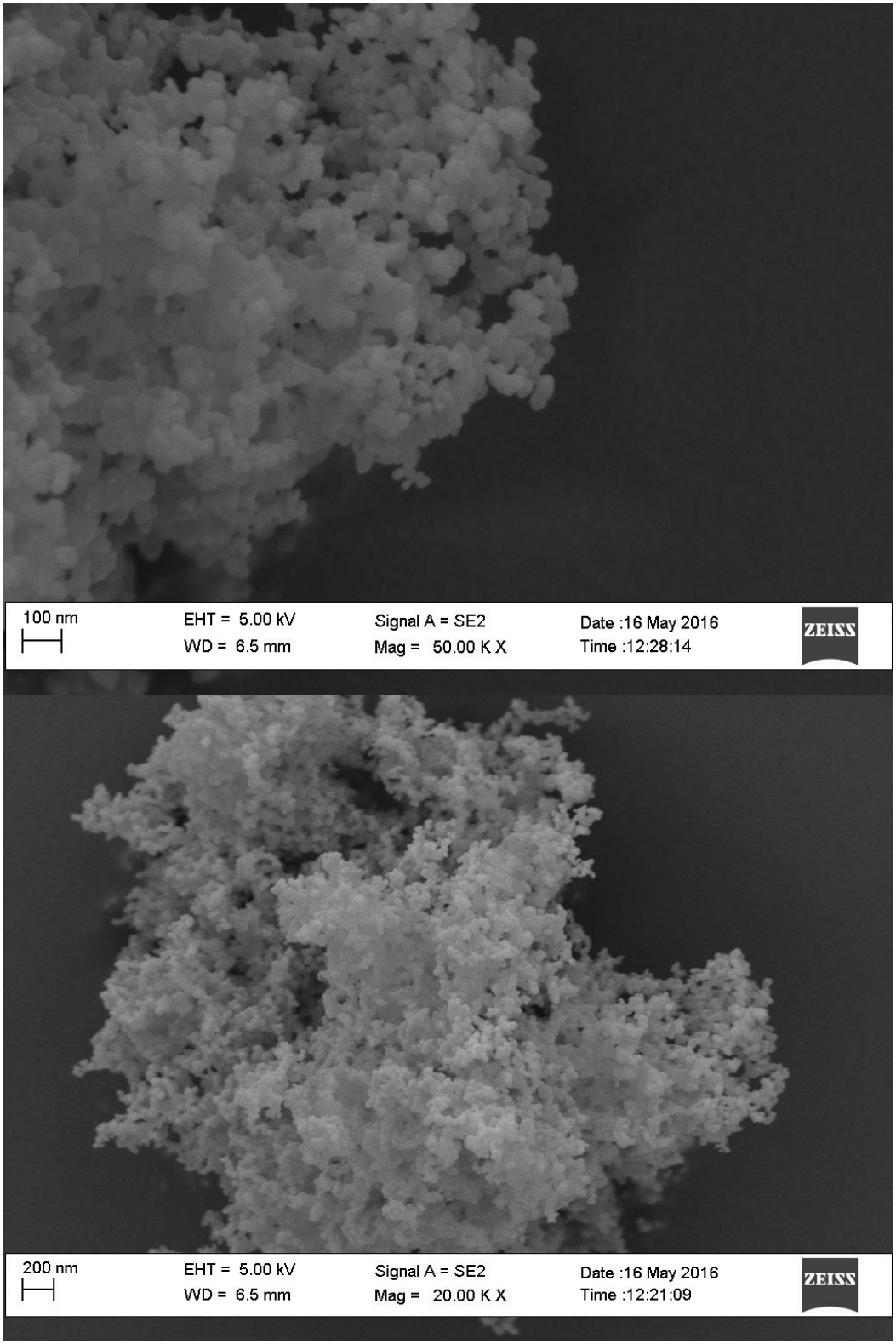

Figure 4.16: Scanning election microscope images of *A. afra* phytosomes

Overall, the phytosome shape and surface morphology were spherical and smooth, respectively, characteristics which were both ideal for pharmaceutical powders.

4.4.2.6 Apparent solubility of the FDAE and phytosomes

The apparent solubility of the FDAE and phytosomes, using luteolin as a marker compound, was determined in water and octanol at 25ºC and the results obtained are shown in Table 4.13.

The results indicated that complexation of phytoconstituents with PC significantly ($p < 0.0001$) decreased the water solubility of the phytoconstituents and significantly ($p = 0.001$) improved their lipid solubility. Several other authors have also noted the improved lipophilicity associated with phytosomes (Khan *et al.*, 2014; Zhao *et al.*, 2013). Phosphatidylcholine is an amphipathic molecule, having a positively charged head group and two neutrally charged tail groups, that renders it miscible in both water and lipid environments. By complexation of phytoconstituents with PC to make the phytosomes, the phytoconstituents come to share some of the PC's versatile solubility properties as evidenced by the results in Table 4.13. In this interaction the polar PC head is responsible for the water solubility while the tail groups are responsible for lipid solubility.

Table 4.13: Apparent solubility of luteolin in the FDAE and *A. afra* phytosomes in water and n-octanol at 25ºC

	Apparent solubility (µg/ml) *	
	Water	**n-octanol**
FDAE	13.62 ± 0.54	0.27 ± 0.18
***A. afra* phospholipid complex**	0.23 ± 0.003	1.35 ± 0.004

*n = 3

Extrapolating from the FTIR results, which showed that the phytoconstituents interacted with the polar head of the PC, the diminished water solubility of the phytosomes could be explained by the decreased availability of PC polar head molecules to form hydrogen bonds (an important determinant of water solubility) with water molecules. The ostensibly improved lipophilicity could thus be attributed to the enwrapping of the bound phytoconstituents by the two aliphatic tails to produce a lipophilic envelope which allows the complex to dissolve in low polarity solvents (Bombardelli 1989).

In summary, lipophilicity is an important determinant in the prediction of the absorption of substances and therefore from the apparent solubility findings of this study it can be deduced that phytosomes significantly improved the lipophilicity of the *A. afra* phytoconstituents and therefore, potentially also improve their absorption.

4.5 Conclusion

The objectives of this study were to prepare and characterise *A. afra* FDAE and phytosomes thereof. From the results obtained the following conclusions could be drawn;

Moderate yields of the FDAE were obtained and the extract was well characterised, showing good aqueous solubility, acceptable moisture content and a high flavonoid content. Also the extract contained moderate levels of luteolin, mostly in the conjugate form. However, the FDAE was hygroscopic and poorly lipid soluble. The HPLC method developed for the assay of luteolin proved to be sufficiently accurate and reproducible for the quantitative analysis of luteolin in the *A. afra* FDAE and phytosomes. Furthermore, the mechanical dispersion method was suitable for the preparation of *A. afra* phytosomes and yielded phytosomes with a good CFE and with suitable pharmaceutical physicochemical characteristics - smooth, spherical, mono-dispersed, nano-sized particles which had good colloidal stability and improved lipid solubility. However, the CFE was significantly affected by the phosphatidylcholine amount and stirring speed.

In conclusion, phytosome technology can be successfully implemented on the FDAE of *A. afra* to produce a phytosome delivery product having acceptable pharmaceutical physiochemical characteristics and improved lipophilicity suitable for drug delivery.

Chapter 5

Evaluation of drug release properties of *Artemisia afra* preparations: FDAE and phytosomes

5.1 Introduction

One of the objectives of this study was to compare the dissolution profiles of *A. afra* FDAE *versus* the phytosomes, in simulated gastric and/ intestinal fluid, using luteolin as the marker compound. In this chapter, the materials, equipment, methodology and procedures used to determine the dissolution of *A. afra* FDAE and phytosomes are presented. Included among the procedures explained is the comparison of dissolution profiles and evaluation of the luteolin release mechanisms and kinetics of the aforementioned preparations.

5.2 Equipment and materials

5.2.1 Equipment

The following equipment was used;

Square hot plate magnetic stirrer *(Dragonlab, Model MS7-H550 Pro, Dragon Laboratories, China),* **vacuum pump** *(Buchi, Model V-500, BuchiLarbotechnik AG, Switzerland),* **Whatman No. 1 paper filter, 5 ml syringes, nylon syringe filters** *(25 mm diameter, 0.45 µm pore size),* **micropipettes, HPLC filter unit** (*Millipore Cameo 25 AS, DDA 02025So MSI: Micro separation Inc., USA),* **membrane filters** *(Durapore, 0.45 µm HV, Millipore),* **semi automated dissolution tester with fraction collector, micro glass filters** *(Sotax AT7-Smart, Switzerland),* **pH meter** *(Basic 20,Lasec, South Africa)*

The **HPLC system** (*Agilent, Model 1200 series, Chemetrics*) used consisted of a **vacuum degasser** *(Agilent 1200 series, Model G1322A, Germany),* **autosampler** (*Agilent 1200 series, Model G1329A, Germany),* **thermostatted column compartment** *(Agilent 1200 series, Model G1316A, Germany),* **a quaternary pump** (*Agilent 1200 series, Model G1311A, Germany*), **a diode array detector (DAD)** (*Agilent 1200 series, Model G1315B, Germany),* a **PC with Chemstation® software** (*Agilent, OpenLAB CDS Chemstation Edition LC and CE version A.01.04(033))* and a **reverse phase column** *(Luna® 5 µm, C18, 250 x 4.60 mm, Phenomenex, United States of America).*

5.2.2 Materials

Phytosomes and FDAE of *A. afra* (prepared from previous chapter)

5.2.3 Chemicals

The following chemicals were used;

Acetonitrile *(HPLC grade)***, methanol** *(HPLC grade)***, sodium nitrate, aluminium chloride** *(Merck, Darmstadt, Germany)*, **formic acid** *(BDH Chemicals Ltd, England)*, **hydrochloric acid 32%** $^{w}/_{v}$ *(Kimix, USA)*, **sodium hydroxide pellets, anhydrous dibasic sodium phosphate, disodium hydrogen orthophosphate** *(Merck, SA)*; **Triton X-100** *(Sigma Aldrich, USA)*; **distilled water** *(Millipore, Milford, MA, USA)*.

5.3 Methods

5.3.1 Determination of the dissolution profile of the *A. afra* FDAE

The USP basket apparatus method was used to determine the dissolution of the *A. afra* phytoconstituents (using marker compounds, luteolin and TFC) from the FDAE in 0.1 N hydrochloric acid (pH 1.2).

Briefly, approximately 1 000 mg of the FDAE was weighed in each of the six baskets to ensure content weight uniformity in compliance with the USP guidelines. Standard procedures were used to prepare the dissolution media (Appendix 6) and the final pH was confirmed using a calibrated pH meter. For the dissolution tests, each vessel contained approximately 1 000 mg of extract in 450 ml of 0.1 N hydrochloric acid at 37 ± 0.5ºC and stirred at 100 rpm. At predetermined time intervals (5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes), 5 ml of sample was automatically withdrawn from each vessel through an in-line glass filter and replaced with an equal volume of the same pre-warmed (37 ± 0.5ºC) medium in order to maintain sink conditions. Aliquots were then taken from each 5 ml sample and used to determine the concentrations of free and total luteolin, and TFC dissolved at the various time points.

To determine the free luteolin dissolved at each time point, 20 µl was injected into the HPLC system analysed using the validated HPLC method reported in section 4.3.1.2.5. In the determination of the total (hydrolysed) luteolin dissolved at the various time points (5, 15, 30, 60 and 120 minutes), 1 ml of the dissolution sample was hydrolysed using the method described in section 4.3.1.2.6 and analysed also using the previously mentioned validated

HPLC method. Finally, to determine the TFC dissolved at 5, 15, 30, 60 and 120 minutes, 1 ml of the dissolution sample was analysed using the method described in section 4.3.1.2.4. The amount and percentage of free and total luteolin, and TFC dissolved was calculated from the peak area response of the HPLC chromatograms, using the highest peak response obtained from each vessel as the 100% value. The data was plotted as percentage dissolved ± standard deviation *versus* dissolution time using GraphPad Prism 6.

Lastly, to compare the dissolution profiles of free and total luteolin, and TFC from the FDAE, the f_2 and ANOVA methods were used. The f_2 values were calculated according to Equation 5.1 using the DDSolve® software.

$$f_2 = 50 \log \left(\frac{100}{\sqrt{\left[1 + \frac{1}{n} \sum_{t=1}^{n} W_t \ (R_t - T_t)^2\right]}} \right) \qquad \textbf{Eqn 5.1}$$

Where, the parameters, **Rt** and **Tt** are the mean percent dissolved at each time point for the reference and test products, respectively, and **n** is the number of observations /dissolution sample times being considered for the computation and $\mathbf{W_t}$ is an optional weight factor.

Comparison by ANOVA was determined using the DDSolve® software, where it compared corresponding time points and significance level was set at $p < 0.05$.

5.3.2 Determination of the dissolution profile of the *A. afra* phytosomes

The methodology was based on the physiological and physicochemical properties controlling drug absorption of phytosomes. Similar to the FDAE dissolution test, the USP basket apparatus was used in the dissolution test of the phytosomes.

The dissolution test was designed in a manner that mimicked the passage of the phytosomes through the gastrointestinal tract and the conditions thereof and, taking into consideration the lipophilicity of the phytosomes, USP delayed release method A was implemented. Briefly, the method consisted of an acid stage in 0.1 N hydrochloric acid (pH 1.2), that mimicked the stomach environment and a buffer stage (pH 6.8). The buffer stage was modified to mimic the small intestines and bile salts (which are an integral part of the absorption of lipids), and

contained a surfactant (Löbenberg & Amidon, 2000), 4% Triton X-100 phosphate buffer solution at pH 6.8. The surfactant was selected on the basis of FDA approval and its use in dissolution studies of other drugs such as ergocalciferol, alectinib, and carbozantinib (U.S. Food and Drug Administration, 2016). The concentration of 4% $^{v}/_{v}$ (4.28% $^{w}/_{v}$) was used as it was above the critical micelle concentration (0.0155% $^{w}/_{v}$).

For the dissolution procedure, briefly, approximately 1 000 mg of phytosomes were weighed into each of the six baskets, vessels were filled with 450 ml of 0.1 N hydrochloric acid and, allowed to equilibrate to 37 ± 0.5^{o}C, the phytosome loaded baskets placed in dissolution medium and rotated at 100 rpm. After 2 hours of operation, a 10 ml aliquot was automatically withdrawn through an in-line glass filter and stored at 4^{o}C for analysis. Immediately after withdrawing the aliquot and with the apparatus operating, 150 ml pre-warmed buffer (16% Triton X-100) was added and pH adjusted to 6.8 ± 0.05 within 5 minutes using 2 N hydrochloric acid or 2 M NaOH. Further 10 ml aliquots were then withdrawn at 2.5, 3.0, 4.0, 6.0, 8.0, 10.0 and 12.0 hours post start of the dissolution test. After each sample withdrawal an equivalent volume of fresh pre-warmed dissolution medium was added to maintain sink conditions. The replacement medium for the buffer stage was a mix of the acid and buffer media, and surfactant so as to mimic the initial buffer stage conditions to which the buffer was added to the acid stage. The pH of the replacement medium was adjusted to pH 6.8.

From each of the collected dissolution samples, 5 ml was withdrawn and placed in a 10 ml test tube, 3 ml ethyl acetate added, the mixture vortexed for 60 seconds and centrifuged at 5 000 rpm for 5 minutes. Thereafter, the ethyl acetate layer was pipetted into a separate 10 ml test tube, blown dry under a gentle stream of nitrogen and 100 µl of methanol added to the residue. The tube was gently swirled until the residue was uniformly mixed and finally 20 µl injected into the HPLC system for luteolin quantification using the validated HPLC method reported in 4.3.1.2.5.

The amount and percentage of luteolin dissolved was calculated from the peak area responses of the HPLC chromatograms, using the highest peak response obtained from each vessel as the 100% value. Data was plotted as percentage dissolved ± standard deviation *versus* dissolution time and Q-release were determined using GraphPad Prism 6.

5.3.3 Comparison of dissolution profiles of *A. afra* FDAE *versus* phytosomes

To compare the dissolution profiles of luteolin from the FDAE and phytosomes, two methods were employed. These were the ratio of percent dissolved (Q-values) and ratio of mean dissolution time (MDT) methods, both being model independent methods. Firstly, The Q-values were determined from the dissolution profiles by simply deriving the time taken for 75% of the luteolin to be released into solution (Q_{75}) and for this GraphPad Prism 6 was used. Secondly, the MDTs were calculated according to Equation 5.2, using the DDSolve® software.

$$MDT = \frac{\sum_{i=1}^{n} t_{mid}\,\Delta M}{\sum_{i=1}^{n} \Delta M} \qquad \textbf{Eqn 5.2}$$

where, ***i*** is the dissolution sample number (e.g., i = 1 for 5-min data, i= 2 for 10-min data, etc.), ***n*** is the number of dissolution sample times, t_{mid} is the time at the midpoint between ***i*** and ***i*-1**, and ΔM is the additional amount of drug dissolved between ***i*** and ***i*-1**.

Finally, the Q-values and MDTs were compared by ANOVA and significance level was set at $p < 0.05$.

5.3.3.1 Determination of the luteolin release mechanism from *A. afra* FDAE and phytosomes

The luteolin dissolution profiles of FDAE and phytosomes were also compared by means of release mechanisms and kinetics. For this, Weibull, Probit, Logistic, Makoid-Bakanar, Peppas-Sahlin and the Korsmeyer-Peppas models, and analysis using DDSolve® software (Zhang *et al.*, 2010), were employed and the best fit model selected on the basis of the lowest AIC.

5.4 Results and discussion

5.4.1 Dissolution of luteolin and TFC from *A. afra* FDAE

Initially, 1 000 mg of extract was immersed in 900 ml of dissolution medium but due to the low content of free luteolin in the plant material (< 0.5 mg/g), as well as to improve the

sensitivity of the method by increasing marker compound concentration, the dissolution medium was reduced to 450 ml, maintained at 37 ± 0.5ºC. The dissolution profile of free luteolin obtained is shown in Figure 5.1.

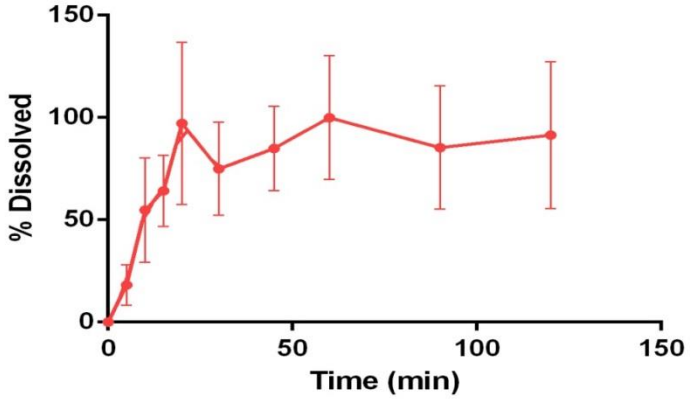

Figure 5.1: Dissolution profile of luteolin aglycone from *A. afra* FDAE at pH 1.2. Data is presented as mean ± SD (n = 6)

Although there are no pharmacopoeia specifications for the dissolution of *A. afra* preparations, the acceptance criteria set was based on the BP 2013, viz. that at least 75% of the marker compound be released within 45 minutes for immediate release dosage forms. Therefore, from the dissolution profiles, the percentage of luteolin dissolved at 45 minutes was determined in order to classify the release as immediate ($Q_{75} \leq 45$ minutes) or otherwise.

The Q_{75} value for free luteolin from the *A. afra* FDAE was less than 20 minutes (Table 5.1). This was illustrative of an immediate release profile as the time was much shorter than the 45-minute specification for immediate release classification. This was expected as the extract was aqueous based. Furthermore, the partition coefficient and apparent solubility values (sections 4.4.1.8 and 4.4.2.6, respectively) showed that the extract was hydrophilic hence also the burst release dissolution profile obtained. The results were similar to those obtained by Max (2007), in which more than 75% of the extract was released within 30 minutes (Max, 2007). In addition, the same immediate release characteristic was obtained by Komperlla (2004) where more than 70% of the tabletted extract was dissolved within 45 minutes (Komperlla, 2004).

The release profile of free luteolin from the extract was rather irregular with wide variations (mean RSD = 35%). This behaviour may be attributed to the different forms of luteolin present in the extract i.e. free and conjugated and their stability in the acidic medium. The

conjugate forms of luteolin may act as a reservoir that releases luteolin upon hydrolysis hence the fluctuating luteolin levels. On the contrary, this irregular trend was not evident in the total luteolin release profile (Figure 5.2).

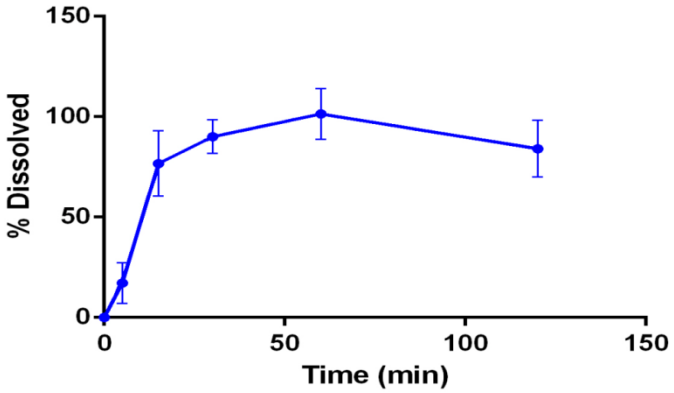

Figure 5.2: Dissolution profile of total (hydrolysed) luteolin from *A. afra* FDAE at pH 1.2. Data is presented as mean ± SD (n=6)

Similar to the free luteolin dissolution profile, the total luteolin release was also immediate, with 75% of the total luteolin being released within 20 minutes (Table 5.1). However, the total luteolin profile was more consistent than that for the free luteolin. This may have been due to a fewer number of sampling time points in the data profile such that inconsistencies may have been omitted. Also, because the sample was hydrolysed, it was less likely to be prone to fluctuations arising from conjugate forms of luteolin. After the 60-minute sampling point the dissolution profile also showed a gradual decrease in total luteolin released and one probable explanation for this may be the instability of luteolin in these acid conditions.

The TFC release profile was also assessed and it revealed a rapid release profile similar to the aforementioned profiles, with 75% of the total flavonoids being released in 15 minutes (Table 5.1).

Table 5.1: Classification of *A. afra* FDAE by dissolution rate

	Q = 75% (minutes)	**Dissolution characteristic**
Free luteolin	16	Immediate release
Total luteolin	15	Immediate release
TFC	13	Immediate release

The profile was rather consistent with a slight decrease in total flavonoids released post the 60-minute sampling point. Interestingly, this decrease was also the case for the total luteolin release profile, though that decrease was more evident in the TFC profile thus further supporting the instability of flavonoids under acidic gastric conditions.

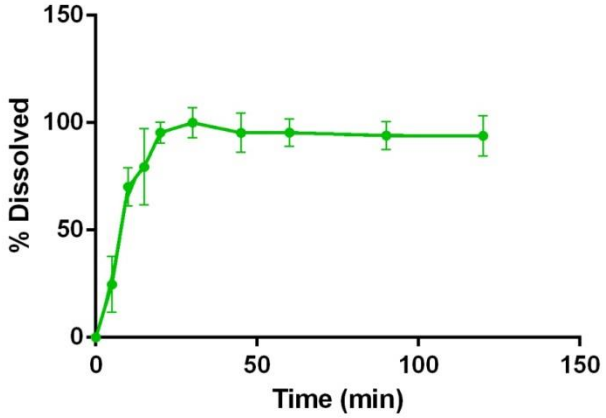

Figure 5.3: Dissolution profiles of total flavonoid content (TFC) from *A. afra* FDAE at pH 1.2. Data is presented as mean ± SD (n =6)

The superimposed free luteolin, total luteolin and TFC profiles (Figure 5.4), showed that the release of all the marker compounds appeared almost identical.

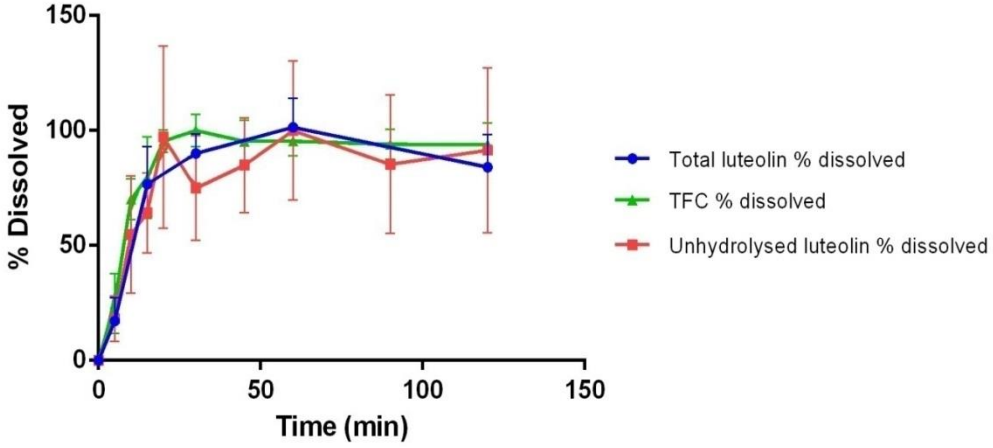

Figure 5.4: Dissolution profiles of free and total luteolin, and TFC from *A. afra* FDAE at pH 1.2. Data presented as mean ± SD (n=6)

Nevertheless, to quantitatively assess the similarity or dissimilarity of the dissolution profiles, the similarity factor (f2) and univariate ANOVA analysis were employed to compare the profiles. A summary of the comparison results is shown in Table 5.2 while the detailed ANOVA results are shown in Appendix 4.

Table 5.2: Comparison of dissolution profiles of *A. afra* using different marker compounds

	Similarity factor[+] (f_2)	ANOVA (p-value)
Free *versus* total luteolin	53	Not significant
Free luteolin *versus* TFC	N/A	0.0484*
Total luteolin *versus* TFC	N/A	0.0272*

[+] Bootstrapping similarity factor

*Significant difference was only at the 30-minute time point

The dissolution profiles of free and total luteolin were similar ($f_2 > 50$) and from the ANOVA results, all corresponding time points were not significantly different ($p > 0.05$) from each other. The expectation was that the total luteolin dissolution might be faster than the free luteolin because the luteolin conjugates are known to be more water soluble than the aglycone forms (Evans, 2009), but both were very rapid, i.e. > 80% released at 20 minutes.

The f_2 could not be used to compare the TFC dissolution profile with the free and total luteolin profiles as they are different chemical entities hence ANOVA was used. The TFC dissolution profile was generally similar to those of free and total luteolin with only significantly different 30-minute time points. Based on these results, either of the marker compounds was suitable for use as a quality control marker for the dissolution of the FDAE of *A. afra* as the dissolution profiles of the marker compounds were all similar, with no significant differences.

It is important to highlight that the immediate (burst) release of the phytoconstituents from the plant matrix may be undesirable therapeutically (Costa *et al.*, 2011). Due to the low partition coefficient and n-octanol apparent solubility, the rapid phytoconstituent release is not favourable for the FDAE of *A. afra* as ideal plasma concentrations may be not be attained due to inadequate time for absorption and their low lipophilicity. In addition, the rapid release may lead to dose dumping which in turn may result in the body being unable to absorb all the *A. afra* active constituents thus affecting the extracts' efficacy due to inability to maintain ideal plasma concentrations necessary for the desired therapeutic outcome. Lastly, such rapid release profiles may also lead to toxicity due to the large dose that is released over a short

period of time, hence the second objective of the study, viz. to modify the release profile of the *A. afra* phytoconstituents.

All in all, the *A. afra* FDAE exhibited a very rapid release profile and this was true for all the flavonoid marker compounds, i.e. free and total luteolin and TFC, measured.

5.4.2 Dissolution of *A. afra* phytosomes

From the previous chapter, the phytosomal preparation was shown to significantly improve the lipophilicity of the *A. afra* phytoconstituents and this physicochemical characteristic was likely to have an effect on the dissolution of the phytoconstituents. It was therefore hypothesised that there would be a decrease in the dissolution rate of the phytosomes in comparison to that of the FDAE. This section therefore looked into the effect the phytosomal dosage form had on the dissolution profile of the *A. afra* phytoconstituents.

In view of the physicochemical characteristics of the phytosomes, it was noted that they possessed poor flow properties hence no capsule filling was done and the phytosomes were filled directly into the dissolution baskets.

Also, due to the lack of complexation between luteolin conjugates and the phospholipids (see section 4.4.2.1), the dissolution of the total (hydrolysed) luteolin could not be measured. Similarly, the dissolution profile of the TFC also could not be measured, possibly due to the presence of Triton X-100 which may have interfered with the determination or, alternatively, due to low concentrations of the flavonoids in the dissolution vessel. However, the dissolution profile obtained for the free luteolin from the phytosomes could be measured and is shown in Figure 5.5.

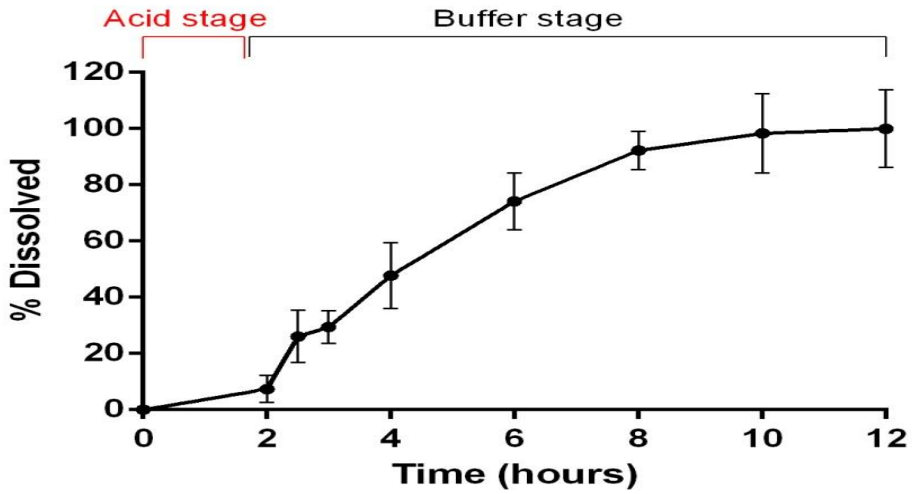

Figure 5.5: Dissolution profile of free luteolin from *A. afra* phytosomes. Red section represents the acid stage and black section represents buffer stage. Data is presented as mean ± SD (n= 6)

The dissolution profile of free luteolin from the phytosomes showed a delayed and extended release profile. The USP acceptance criterion for a delayed release is not more than 10% released by the 2-hour sampling point (USP 36, 2004). In this study, 8.6% had been released by the 2-hour mark thus the luteolin release from the *A. afra* phytosomes fitted into the delayed release criterion. The 2-hour sampling point also served to check for the presence or absence of dose dumping, which in this case was absent. Post the 2-hour mark and in the pH 6.8 (or simulated intestinal medium), there was a sudden release of luteolin which then steadied after the 2.5-hour sampling point until it slowed and plateaued around the 8-hour mark.

All in all, only the dissolution of free luteolin could be measured from the *A. afra* phytosomes and it showed a typical delayed and extended release profile with no dose dumping being evident.

5.4.3 Comparison of luteolin dissolution profiles of *A. afra* FDAE and phytosomes

The luteolin release from the phytosomes was consistent in contrast to that of free luteolin from the FDAE. This may have been due to complexation (between phospholipids and phytoconstituents) which controlled the amount of phytoconstituents that went into solution or possibly due to the absence of luteolin glycosides which would act as an 'extra' source of luteolin thereby resulting in fluctuations as those observed in Figure 5.1.

The Q_{75} and mean luteolin dissolution time (MDT) of the phytosomes were 366 and 269 minutes, respectively, as shown in Table 5.3. These Q_{75} and MDT values for the phytosomes were significantly ($p < 0.05$) different from those obtained for the FDAE, thus proving that the release of luteolin from the FDAE had been successfully modified.

Table 5.3: Comparison of Q_{75} values and MDTs of luteolin release from *A. afra* FDAE and phytosomes

	Q = 75% (minutes)	Dissolution characteristic	MDT (minutes)
FDAE	16	Immediate release	18.9
Phytosome	366	Delayed release	269.4
	$p < 0.05$		**$p < 0.01$**

Complexation of active constituents(s), e.g. with cyclodextrin, is a technique that has been widely used to alter their physicochemical and physiological properties (Jackson, Young & Pant, 2000). In this present study, the alteration of the dissolution profile of the phytoconstituents was hinged upon the decreasing water solubility (increasing lipophilicity) by complexation of phytoconstituents with phospholipids, thus from the comparison above, the dissolution rate of the phytoconstituents was successfully decreased.

Interestingly, the phytosome complex possibly imparted a gastro protective effect on the phytoconstituents of *A. afra*, thus conferring acid stability to the constituents (Singh *et al.*, 2011a). This is of importance as the majority of the flavonoids in *A. afra* are in the conjugate form, which under gastric acidic conditions may be hydrolysed to the aglycone form (Manach *et al.*, 2004). Glycosides have been associated with pharmacological actions hence their protection from hydrolysis is of great importance.

In addition to the delayed release which imparted improved gastric acid stability to the phytoconstituents, the extended release during the buffer stage was also beneficial. Extended release mechanisms are advantageous in that they reduce dosing frequency and quantity, improve absorption and bioavailability. These are all aspects that the FDAE lacked and therefore the phytosome drug delivery system addressed most of the flaws of the FDAE in addition to the primary goal of modifying the release of the phytoconstituents.

Phytosomes have been used as a mechanism to improve the aqueous solubility of poorly soluble drugs in many studies (Pathan & Bhandari, 2011; Arora, Sharma & Kaur, 2013; Habbu *et al.*, 2013; Zhao *et al.*, 2013; Das & Kalita, 2014; Khan *et al.*, 2014; Singh *et al.*, 2014), but in this present study the contrary was performed, i.e. phytosome technology was used to decrease aqueous solubility (decrease dissolution rate). In a similar study, that had the same objective of reducing the dissolution rate of an active ingredient, a controlled release pattern was shown by the gallic acid complex (which showed continuous release up to 93% of gallic acid) at the end of 24 hours in comparison to free gallic acid (which showed 81.9% burst release in just 0.5 hours) (Singh *et al.*, 2011b). The results of the aforementioned study therefore support the findings of this present study.

In summary, the use of the phytosome technique was effective in retarding the dissolution rate of the hydrophilic phytoconstituents of *A. afra* and therefore successfully modified the release of the phytoconstituents.

5.4.3.1 Kinetics of luteolin release from *A. afra* FDAE and phytosomes

It was however also important to further discern the kinetics and mechanism of release involved in the dissolution of the luteolin from the phytosomes and FDAE, so as understand the underlying processes involved. To elucidate the mechanisms behind the release of the aglycone marker compound, luteolin, from the *A. afra* preparations, the dissolution data obtained were fitted to mathematical models. This section describes the results obtained from such mathematical modelling of the marker compound release from the two preparations.

Generally, the free and total luteolin, and TFC dissolution data for the FDAE of *A. afra* best fitted the Peppas-Sahlin model (Figure 5.6 and Appendix 5). This was shown by the lower AIC values compared to that for the other models, which implied better model suitability. The AIC values and model parameters obtained are shown in Appendix 5.

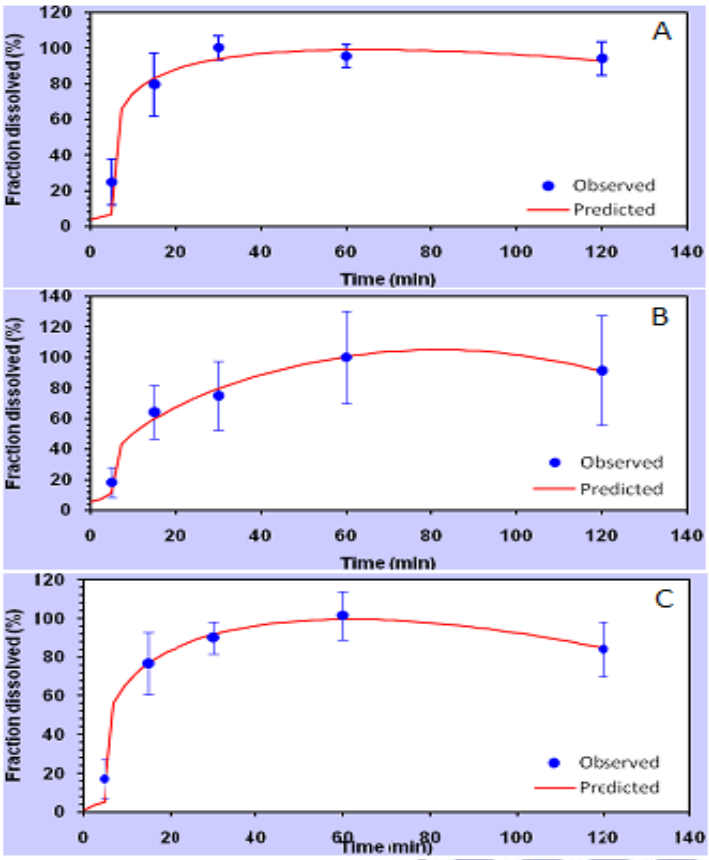

Figure 5.6: Model (Peppas-Sahlin 1 with Tlag) of best fit for *in vitro* release of TFC (A), free luteolin (B) and total luteolin (C) from *A. afra* FDAE at pH 1.2. Results are mean ± SD (n =6).

Peppas-Sahlin models describe release occurring *via* two mechanisms, namely, Fickian diffusion and matrix relaxation/erosion and their contribution is denoted by the k_1 and k_2 constants, respectively. The k_1 values for free and total luteolin, and TFC were 49.413, 54.706 and 69.717 respectively, whereas the k_2 values were all negative. This therefore indicated that the release mechanism of the marker compounds was exclusively by Fickian diffusion.

Similar to this present study, an investigation of the release mechanism of flavonoids (sutherlandins) from different *Sutherlandin frutescens* plant materials, also reported that some of that data fitted the Peppas-Sahlin model, but in contrast to findings of the present study, their k_1 and k_2 constants showed that the release mechanism was due a combination of both Fickian diffusion and matrix relaxation/erosion (Mbamalu, 2015).

In order to establish the suitability of the marker compounds for their use in dissolution tests of the *A. afra* extract, the Fickian constants were compared, i.e. the $k_1^{\text{free luteolin}}$

versus $k_1^{total\ luteolin}$, $k_1^{free\ luteolin}$ *versus* k_1^{TFC} and $k_1^{total\ luteolin}$ *versus* k_1^{TFC}. No significant differences ($p > 0.05$) were observed implying that use of either of the marker compounds gave a good representation of the dissolution of the phytoconstituents of the extract. This was also confirmed by the results (Table 5.2) obtained through statistical (ANOVA) and model independent (f_2) comparisons where no significant differences in the dissolution profiles of the marker compounds (free and total luteolin and TFC) under gastric conditions (pH 1.2) were found.

The luteolin dissolution data of the phytosomes fitted well ($R^2_{adj} > 0.9300$) into several models *viz.* Probit, Logistic, Makoid-Bakanar with Tlag, Korsmeyer-Peppas with Tlag and Weibull models (Appendix 5). Of these, the Weibull model gave the best fit as it had the lowest AIC value (Appendix 5). Generally, the Weibull model has been found to fit most dissolution data curves (Costa & Lobo, 2001) and the data of the present study was no exception (Figure 5.7).

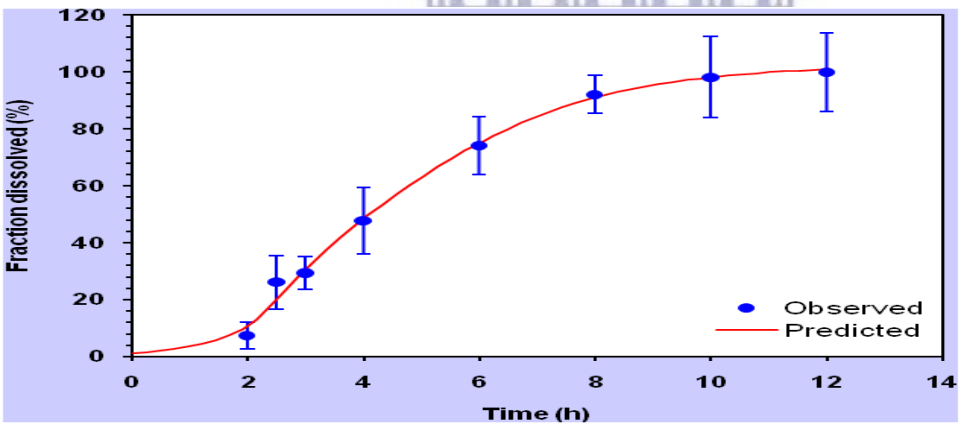

Figure 5.7: Model of best fit (Weibull_4) for *in vitro* release of luteolin from *A. afra* phytosomes. Results are Mean ± SD. (n = 6)

With a Weibull function, β, of 2.188 the shape of the luteolin dissolution curve was sigmoid and indicated that the release of luteolin from the phytosomes was by a complex mechanism. Overall, the rate of release did not change monotonically but rather initially increased nonlinearly up to an inflection point and thereafter decreased asymptotically. To further elucidate the mechanism of release, the data was also fitted to the Korsmeyer-Peppas model ($R^2_{adj} = 0.9750$) and yielded an *n* value of 1.367 (Appendix 5). This *n* value indicated a super case II transport mechanism which is a relaxational release similar to the complex release mechanism suggested by the obtained Weibull function. The Korsmeyer-Peppas model data

therefore also verified that the Weibull function, β may be accurately used to describe the release mechanisms of luteolin from *A. afra* phytosomes (Papadopoulou *et al.*, 2006).

Finally, comparison of the release mechanisms of luteolin from the FDAE and phytosomes revealed a clear difference as release from the former was by Fickian diffusion but from the latter, by a complex mechanism. It therefore further confirmed that the phytosome complexation technique modifies the release of the phytoconstituents not only in terms of dissolution time but also in terms of release mechanism and kinetics.

5.5 Conclusion

The overall objective of the chapter was to test the hypothesis that phytosomes of *A. afra* could modify the release profile of the phytoconstituents by decreasing their dissolution rate. From the results obtained the following conclusions could be drawn.

Free and total luteolin and TFC were suitable for use as quality control markers for the dissolution of the FDAE of *A. afra*. The dissolution of phytoconstituents of *A. afra*, using luteolin as the marker compound, was significantly modified from a very rapid immediate release in the FDAE form to a delayed and extended release profile in the phytosomal dosage form. Also, in terms of release mechanism, free and total luteolin, and TFC were all released from the FDAE through Fickian diffusion but luteolin was released from the phytosomal complex through a complex mechanism.

In summary, phytosome complexation technique can be successfully used to decrease the dissolution rate and modify the release mechanism of phytoconstituents of *A. afra*.

Chapter 6

Conclusions and recommendations

The overall aim of this study was to modify the *in vitro* release of *Artemisia afra* phytoconstituents in simulated gastrointestinal conditions using the phytosome drug delivery technique. The specific objectives were to prepare and characterise *A. afra* phytosomes, identify factors influencing the efficiency of complex formation between the *A. afra* phytoconstituents and phospholipids and, ultimately evaluate the phytosome dissolution profile in comparison to the *A. afra* freeze dried aqueous extract dissolution profile.

It was hypothesised that the phytosome technique could be successfully effectuated on the freeze dried aqueous extract of *Artemisia afra* to produce a phytosome drug delivery system having acceptable pharmaceutical physicochemical properties and, that the dissolution rate of phytoconstituents from such phytosomes would be lower than that of the freeze dried aqueous extract.

From the results obtained the following major conclusions could be drawn;

1. The mechanical dispersion method was suitable for the preparation of *A. afra* phytosomes and produced phytosomes with good complex formation (CFE) and with suitable pharmaceutical physicochemical characteristics - smooth, spherical, mono-dispersed, nano-sized particles which had good colloidal stability and improved lipid solubility.
2. In the preparation of the *A. afra* phytosomes, the CFE was significantly affected by the phosphatidylcholine amount and stirring speed, where it directly varied with the former and inversely varied with the latter. However, the type of organic solvent used had no significant effect of the CFE.
3. The dissolution of phytoconstituents of *A. afra*, using luteolin as the marker compound, was significantly modified from a very rapid immediate release in the FDAE form to a delayed and extended release profile in the phytosomal dosage form. Also, in terms of release mechanism, luteolin was released from the FDAE through Fickian diffusion in contrast to the release of luteolin from the phytosomal complex which was by a complex mechanism.

In summary, the phytosomal drug delivery technique is suitable for use in the delivery of *Artemisia afra* phytoconstituents and the technique is capable of modifying the phytoconstituent release profile so as to allay the challenges associated with immediate release profiles.

From the results, further areas of research were noted. Firstly, the *A. afra* phytosomes were sticky and non free flowing thus posing challenges in the manufacturing and handling processes such as capsule filling and hence there is need to formulate them with other excipients such as silicone dioxide, microcrystalline cellulose and magnesium stearate to improve the flow properties. Secondly, the complex formation efficiency was nowhere near 100% and some phytoconstituents such as luteolin glycosides, did not complex with the phospholipids. It is therefore necessary to optimise the preparation technique so as to improve the complex formation efficiency and ensure all phytoconstituents complex with the phospholipids. Thirdly, there is need to conduct *in vivo* studies on the absorption of the *Artemisia afra* phytosomal complex to verify if the intestinal permeability and bioavailability does improve as a result of the increased lipophilicity of the phytoconstituents. Finally, the pharmacological activity of the *Artemisia afra* phytosomes should also be evaluated to ensure the desired pharmacological activity is maintained or enhanced with the phytosome delivery system in contrast to the FDAE.

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Appendices

Appendix 1: Extractions conducted and resultant yields obtained in the preparation of the freeze-dried aqueous extracts of *Artemisia afra.*

Mass of leaves (g)	Volume of water (ml)	Mass of FDAE (g)	Yield of extract (%)
20.057	400	5.593	27.89
11.500	230	3.210	27.91
100.890	2000	27.08	26.84
102.400	2100	29.9	29.20
25.000	500	5.546	22.18
10.000	200	2.293	22.93
Average yield (%) ± SD (n=6)		**26.16 ± 2.90**	

Appendix 2: Extractable matter and solubility of *A. afra* FDAE

		HCL (pH 1.2)		Phosphate buffer (pH 6.8)		Water	
Mass of FDAE (mg)		217.8	199.0	207.4	203.5	205.3	200.0
Volume of solvent (ml)		5	5	5	5	5	5
Mass of filter paper + residue (mg)		834	816	811	822	868	1234
Mass of filter paper (mg)		801	801	807	819	836	1220
Mass of residue (mg)		33	15	4	3	32	14
Extractable matter (mg/g)		848.5	844.8	980.7	985.3	844.1	930
Mean ± SD (n=2)		**846.6 ± 2.6**		**983.0 ± 3.2**		**888.0 ± 62**	
Solubility	**mg/ml**	37.0	36.8	40.7	40.1	34.7	37.2
	Mean ± SD (n=2)	**36.9 ± 0.1**		**40.4 ± 0.2**		**35.9 ± 1.8**	
	Parts	27.1	27.2	24.6	24.9	28.9	26.9
	Mean ± SD (n=2)	**27.1 ± 0.1**		**24.8 ± 0.3**		**27.9 ± 1.4**	

Appendix 3: Residual moisture content of the *A. afra* freeze-dried aqueous extract powder

Sample	Mass of FDAE (g)	Moisture content (%)
1	0.153	2.63
2	0.179	2.25
3	0.206	2.44
4	0.208	2.40
5	0.152	2.65
Average ± SD (n=5)		**2.47 ± 0.15**

Appendix 4: Univariate ANOVA results for comparison of dissolution profiles of TFC and, free and total luteolin from FDAE of *A. afra*

	p – value		
Time (minutes)	**Free *versus* total luteolin**	**Free luteolin *versus* TFC**	**Total luteolin *versus* TFC**
5	0.8681	0.3517	0.2897
15	0.2243	0.1618	0.7856
30	0.1564	**0.0272**	**0.0484**
60	0.9182	0.7225	0.3252
120	0.6540	0.8696	0.1882

Appendix 5: Fitting of *A. afra* FDAE and phytosomes dissolution data for mathematical modelling. Best fit values are highlighted.

		FDAE			
Mathematical Model	**Parameter**	**Free luteolin**	**Total luteolin**	**TFC**	**Phytosome**
Probit_2	**A**	-3.687	-4.802	-4.369	-2.564
	B	4.071	5.517	5.144	4.129
	Fmax	97.817	92.072	99.977	111.065
	AIC	35.1920	32.1749	27.2665	48.8327
Weibull_3	**B**	1.454	2.422	2.103	2.377
	Fmax	96.437	91.691	97.938	100.944
	AIC	34.8151	32.4451	25.8831	49.3717
Weibull_4	**B**	1.112	1.286	2.407	2.188
	Ti	-2.936	-1.499	-6.964	-0.493
	Fmax	123.656	99.196	97.092	102.797
	AIC	29.7699	29.3640	27.1701	47.7687
Logistic 3	**K**	0.366	0.516	0.510	0.889
	Γ	12.539	9.359	9.200	4.256
	Fmax	94.512	91.926	97.332	99.654
	AIC	34.8135	32.3540	25.4667	49.6077
Peppas-Sahlin 1 with Tlag	**k1**	49.413	54.706	69.717	-604.597
	k2	-21.669	-11.213	-16.990	617.251
	M	0.621	0.371	0.327	0.387
	Tlag	-0.446	4.060	3.638	1.163
	AIC	28.6513	24.3334	24.2186	51.8195
Peppas-Sahlin 2 with Tlag	**k1**	22.434	27.953	27.769	41.921
	k2	-1.284	-1.883	-1.802	-2.753
	Tlag	3.612	4.471	3.424	2.135
	AIC	33.8283	34.9292	35.6259	50.5413
First order with Fmax	**k1**	0.075	0.083	0.095	0.067
	Fmax	98.143	95.375	99.433	216.613
	AIC	35.0982	37.7048	35.6470	57.6354
Makoid-Bakanar with Tlag	**kMB**	23.116	43.798	53.146	19.977
	N	0.997	0.472	0.276	2.152
	K	0.012	0.008	0.005	0.183

	Tlag	-3.124	2.828	3.258		0.354
	AIC	29.5818	24.2481	26.9513		48.0548
Korsmeyer-Peppas	**kKP**	X	X	X		5.099
	N	X	X	X		1.637
	R^2	X	X	X		0.9762
	R^2_{adj}	X	X	X		0.9750
	AIC					100.2955

Appendix 6: Preparation procedures for dissolution media

Dissolution media	Preparation procedure
0.1 N HCL pH 1.2	9.6 ml of 32% $^{w}/_{v}$ hydrochloric acid added to 500 ml of distilled water and then filled to 1 000 ml with distilled water. pH adjusted using either 2 N hydrochloric acid or 2 M NaOH.
Phosphate buffer pH 6.8	Sodium dihydrogen phosphate dihydrate (7.8 g) and sodium dihydrogen phosphate-12-hydrate (17.9 g) dissolved in 500 ml of distilled water and then filled to 1 000 ml with distilled water. pH adjusted using either 2 N hydrochloric acid or 2 M NaOH.