Investigation of the *in vitro* bioavailability of luteolin from modified preparations of *Artemisia afra*

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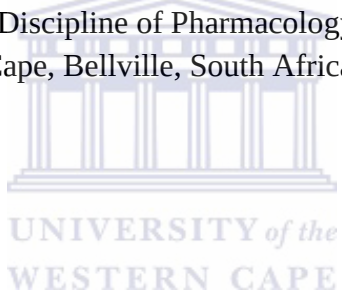
A thesis submitted in fulfilment of the requirements for the degree of *Magister Scientiae* (Pharmaceutical Sciences) in the Discipline of Pharmacology at the University of the Western Cape, Bellville, South Africa.



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Key words

Artemisia afra

Flavonoid

Luteolin

Permeability

Freeze dried

Alginate

Polymethylmethacrylate

Single pass intestinal perfusion

HPLC

Summary

Artemisia afra (*A. afra*) is traditionally used for a variety of ailments and contain flavonoids e.g. luteolin which may contribute to some of its activity. It is generally administered as a tea or decoction, and such liquid dosage forms present challenges as far as long term storage and stability are concerned, as well as sub-optimal oral bioavailability of actives they contain. Freeze dried aqueous extracts (FDAE) can alleviate such problems but may be hygroscopic and unstable. The use of modified forms of FDAE can counter the problem of hygroscopicity (e.g. use of alginate) and alleviate the issue of sub-optimal bioavailability of plant actives (e.g. polymethylmethacrylate).

The objectives of this study, were to: (1) prepare the freeze dried aqueous extract (FDAE) and modified forms, which include alginate-extract beads (alginate-FDAE) and polymethylmethacrylate coated alginate matrix beads of herbal extract (PMMA-alginate-FDAE) of the FDAE of *A. afra*, (2) determine and compare the pharmaceutical characteristics of the above mentioned preparations of *A. afra*,(3) quantify and compare the total flavonoid and specifically luteolin levels of the different forms of *A. afra*,(4) evaluate and compare the release characteristics of FDAE of *A. afra* from the alginate-FDAE and PMMA-alginate-FDAE beads in gastrointestinal fluids and (5) determine the intestinal permeability of luteolin contained in selected modified *Artemisia afra* extract preparations. It was hypothesized that making the alginate beads and the polymethylmethacrylate coated alginate beads would make the FDAE less hygroscopic with a lower moisture content, that the rate of release of luteolin from *A. afra* FDAE into gastrointestinal fluids would be faster than from the modified forms, and that the effective gastrointestinal permeability of luteolin in the alginate-FDAE and PMMA-alginate-FDAE beads of *A. afra* is equal to that in FDAE.

To realize these objectives, the FDAE was prepared by freeze drying the aqueous extract of the *A. afra* dried leaves, alginate-FDAE prepared by dispersing FDAE into 4% sodium alginate solution, then adding the resulting stock solution into a 2% calcium chloride solution and drying resulting beads and PMMA-alginate-FDAE prepared by a modified water-in-oil-in-water emulsion solvent evaporation method using water as an internal aqueous phase. Using

pharmacopoeial methods and methods adapted from other workers the organoleptic and pharmaceutical characteristics were determined to compare the pharmaceutical quality of these preparations of *A. afra*. To identify and determine the levels of luteolin in the plant preparations, a validated HPLC assay was developed. Finally, the *in situ* perfused rat intestine model was used to determine the *in vitro* bioavailability, i.e. gastrointestinal permeability, of luteolin from solutions containing luteolin in pure form, FDAE, alginate-FDAE and PMMA-alginate-FDAE.

The *A. afra* forms were obtained in moderate to good yields and FDAE was brown and hygroscopic in nature, the alginate beads dark brown free flowing and spherical in shape and the PMMA-alginate beads light brown in colour with rough edges. The *A. afra* plant forms on average contained 0.185 ± 0.24, 0.067 ± 0.014, 0.012 ± 0.071 µg/mg of free luteolin (n=3) in FDAE, alginate-FDAE and PMMA-alginate-FDAE respectively and 0.235 ± 0.026, 0.079 ± 0.093, 0.058 ± 0.082 µg/mg of total luteolin (n=3) in FDAE, alginate-FDAE and PMMA-alginate-FDAE respectively.

The P_{lumen} values for intestinal uptake of luteolin were significantly higher from solutions of *A. afra* forms than the pure luteolin solution (i.e. P_{lumen} values in the range of 0.02 - 0.035 cm/s for all plant forms *vs* P_{lumen} values in the range of 0.010 - 0.014 cm/s for pure luteolin, t-test $p = 0.0252$). The permeability of luteolin in FDAE appeared to be slighter greater than that of the modified forms (P_{lumen} values >0.03 cm/s for FDAE and P_{lumen} values <0.03 cm/s for both modified forms).

In summary, the results showed that, the modified *A. afra* forms; alginate-FDAE and PMMA-alginate-FDAE were of acceptable pharmaceutical quality with luteolin better taken up in the plant forms than in its pure form. The *A. afra* forms prepared had similar rates of uptake (permeability) of free and total luteolin with the rates being highest for the FDAE. Collectively, these results indicate that alginate-FDAE and PMMA-alginate-FDAE bead forms should be suitable for use in a solid dosage form (e.g. tablet or capsule) of *A. afra*.

Declaration

I declare that the thesis **Investigation of the *in vitro* bioavailability of luteolin from modified preparations of *Artemisia afra*** 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.

Anjong Nkengla
December, 2014

Signed: UWC, Bellville

Dedication

I dedicate this master's thesis to my dear parents Awa Joseph Nkengla and Felicitas Mengwi Nkengla and my uncle Dr Nkengla John Muluh for their unending love, support and constant encouragement.

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Chapter 1

Introduction

Artemisia afra (*A. afra*) also known as "Wilde als" or "wormwood" (Roberts, 1990) is one of the oldest and best known traditional herbal medicines used in South Africa for the treatment of a variety of ailments (Watt and Breyer-Brandwijk, 1962, Van Wyk and Gerike, 2000). The list of uses includes treatment of coughs, fever, asthma, malaria and diabetes. *A. afra* is commonly administered as a tea infusion of its leaves and the steam from such infusions may also be inhaled for the treatment of headaches and colds (Thring and Weitz, 2006, Hutchings et al., 1996b).

The usual dosage preparation of the plant is in the form of a tea or decoction (Roberts, 1990; Van Wyk et al., 1997) which involves immersing wet leaves in boiling water. The traditional method of preparation has several disadvantages attributed to it. Firstly, the preparations are often left in a pot and heated up daily before use leading to a very dark colour of the liquid aqueous extract. The dark appearance of the tea is most likely due to more constituents being extracted while the water remains in prolonged contact with the plant material. This may result in the alteration of many active constituents (especially due to decomposition during the boiling phase) and difficulties in ensuring quality control of the herbal ingredients of the final preparation. Also, the directions for dosage preparation are often very inaccurate in that quantities are measured as "a double handful", "cup of boiling water" or "quarter cup of leaves" (Roberts, 1990). This may cause a lot of variability in different preparations due to the inaccuracy in consistently quantifying or measuring either a quarter cup or double handful. Moreover, this may lead to variations in dose each time a treatment is prepared which may in turn alter the bioavailability and the pharmacokinetics of the active constituents. One solution to address these problems was to prepare a freeze-dried aqueous extract powder of the plant which will provide a more uniform and constant composition material that is also more amenable for preparation into a solid dosage form e.g. tablet or capsule.

However, scientific literature and past experience have shown that freeze-dried aqueous preparations are also problematic. In particular, they tend to be very sticky and hygroscopic and are prone to moisture sorption when exposed to an environment with even moderate humidity. The hygroscopic material becomes physically changed with an increase in volume and changes in particle size and shape (Egieyeh, 2011). The active compounds in these preparations typically have less than satisfactory pharmacokinetic characteristics which include rapid absorption rates, quicker metabolism and shorter half-lives. These problems may however also be remedied for instance by using appropriate excipients to modify the hygroscopic nature of the extract and enhance the *in vivo* delivery (i.e. bioavailability) and pharmacokinetics of active constituents in the active herbal ingredient (AHI) extract. The International Pharmaceutical Excipient Council (IPEC) defines an excipient as any substance other than the active drug that is included in the manufacturing process. Excipients are commonly used to improve the less than satisfactory characteristics e.g. pharmaceutical (i.e. excessive hygroscopicity) or pharmacokinetic (i.e. low bioavailability) of active ingredients. Excipients such as alginate and dextran have been used to reduce hygroscopicity. For example, Egieyeh showed a 35-75% significant reduction in hygroscopicity when a freeze-dried aqueous extract of *Artemisia afra* was incorporated into a matrix of sodium alginate (Egieyeh, 2011). Also, Tong *et al.,* in another experiment incorporated dextran into dried aqueous extract of *Radix ophiopogonis* and observed a reduction of hygroscopicity in the extract (Tong et al., 2008). Excipients can also be used to prepare controlled or modified release preparations. Tapas *et al*., (2011) used polymethylmethacrylate coated alginate matrix microcapsules to prepare a controlled release dosage form of diclofenac sodium which was found to have prolonged release in the intestine with improved absorption. This was done using sodium alginate (SAL) as a matrix material in a modified water-in-oil-in-water emulsion solvent evaporation method. It would be interesting to explore whether alginate-extract beads (alginate-FDAE) and polymethylmethacrylate coated alginate matrix beads (PMMA-alginate-FDAE) could reduce the hygroscopicity of the *A. afra* freeze dried aqueous extract (FDAE), and enhance the bioavailability (permeability and absorption) of the actives contained in the extract (AHI).

Scientifically, it can be assumed that the activities of medicinal plants are due to the active ingredients they contain. In most cases, however, the active ingredient(s) actually responsible for the therapeutic activities of plants are not known. Very little is known about the chemical

constituents responsible for the activity of *Artemisia afra*. However, several studies have revealed that *Artemisia afra* contains flavonoids e.g. quercetin, acacetin, kaempferol and luteolin amongst other potential active chemicals which may play a role in the beneficial activities of this plant (Van Wyk et al., 2000, Waithaka, 2004). Selected phytochemicals such as flavonoids may thus be appropriate markers to use to monitor quality aspects such as pharmaceutical quality and bioavailability of different *A. afra* plant preparations. Few studies have, however, addressed the ability of flavonoids to reach proposed *in vivo* sites of action. These limited number of studies conducted in humans and rats have indicated very poor and variable intestinal absorption of phenolic compounds (Walgren et al., 1998). In plants, flavonoids usually exist as glycosides and aglycones. The former is absorbed to a very limited extent (Scalbert and Williamson, 2000), are cleaved by gut bacteria (Day et al., 1999) and are usually hydrolysed (by intestinal enzymes and/or colonic micro flora) to the corresponding aglycone molecule (Manach et al., 2004; Pong et al., 2005) while the latter is absorbed freely across the intestinal mucosa by passive diffusion and conjugated in the mucosa and liver by phase II enzymes (Crespy et al, 1999). The mechanism by which flavonoids, whether as aglycone or glycosides are taken up into the cells of the gastrointestinal tract (GIT) has also not yet been fully resolved. Muganga found that the bioavailability of luteolin from aqueous extract of *A. afra* in the vervet monkey was significantly better than in its pure form (Muganga, 2004). It is thus speculated that either the plant extract might contain the luteolin in a better bioavailable glycoside form and/or might contain other compounds, which may beneficially influence the absorption characteristics of the flavonoid luteolin. Furthermore, an *in vitro* study should allow an insightful and more controlled approach to investigate the gastrointestinal permeability (i.e. absorption) of the flavonoid luteolin in the *A. afra* forms mentioned above before an *in vivo* study is conducted.

Given the above arguments, the objectives of this study were to: (1) prepare the freeze dried aqueous extract (FDAE) and modified forms, viz. alginate-extract beads (alginate-FDAE) and polymethylmethacrylate coated alginate matrix beads (PMMA-alginate-FDAE) of the freeze dried aqueous extract of *Artemisia afra*, (2) determine and compare the pharmaceutical characteristics of the above mentioned preparations of *A. Afra*, (3) quantify and compare the total flavonoid and specifically luteolin levels of the different forms of *A. afra*,(4) evaluate and compare the release characteristics of FDAE of *A. afra* from the alginate-FDAE and PMMA-

alginate-FDAE beads in gastrointestinal fluids and (5) determine the intestinal permeability of luteolin in the *A. afra* plant forms.

The literature reviewed in this study is covered in chapter 2; the plan of work in chapter 3; preparation and evaluation of different preparations of *Artemisia afra*in chapter 4; investigation of the *in vitro*bioavailability (permeability) of luteolin from modified *A. afra* forms in chapter 5 and finally, the overall conclusion and recommendations are given in chapter 6.

Chapter 2

Literature review

2.1 Introduction

In this chapter, an overview of *Artemisia afra* (*A. afra*) is given and this includes its botany, pharmacological effects, traditional uses and dosage form. In addition, this chapter also discusses: the shortcomings of the traditional preparation and methods of addressing them, the rationale for the preparation of modified forms of *A. afra*, an overview of flavonoids with focus on their chemistry and classification, and includes a brief description of the flavonoid luteolin with focus on its biological activities and pharmacokinetics as well as the rational for the use of the single pass intestinal perfusion (SPIP) method for investigation of the permeability of luteolin contained in the *A. afra* plant preparations.

2.2 What is *Artemisia afra*?

2.2.1 Vernacular names

Artemisia afra is one of the oldest and best known traditional herbal medicines used in South Africa for the treatment of a variety of ailments (Watt and Breyer-Brandwijk, 1962, Van Wyk and Gerike, 2000). Locally, a variety of names relate to this plant such as “African wormwood” in English, “Umhlonyane” in Xhosa, “Mhlonyane” in Zulu, “Lanyana” in Sotho, “Lengana” in Tswana and “Wilde als” in Afrikaans (Burits et al., 2001, Watt and Breyer-Brandwijk, 1962).

2.2.2 Taxonomy and morphology (plant description)

Artemisia afra belongs to the:

Kingdom: *Planta*

Family: *Asteraceae*

Genus: *Artemisia*

Species: *A. afra*

Artemisia afra is a medium sized, erect growing multi-stemmed woody perennial shrub, which grows up to 2 m in height with a leafy, hairy-ridged stem (Van Wyk et al., 1997, Van der Walt, 2004). Its soft leaves are finely-divided (like a fern), silver-grey due to the presence of fine hairs and grow up to 8 cm long and 4 cm wide (Figure 2.1).

Figure 2.1: *Artemisia afra* in its natural habitat at Montague gardens, South Africa.

2.2.3 Geographical distribution

A. afra is a very common species and easily recognized medicinal plant in Southern Africa. In South Africa, it usually grows in rocky mountainous areas along forest margins and stream sides with its natural distribution extending from the Northern and Eastern Transvaal to the Western Cape. It is also wide spread in Kwazulu-Natal from the coast to the Drakensberg (Hilliard, 1977). Its geographical distribution in South Africa is shown in figure 2.2.

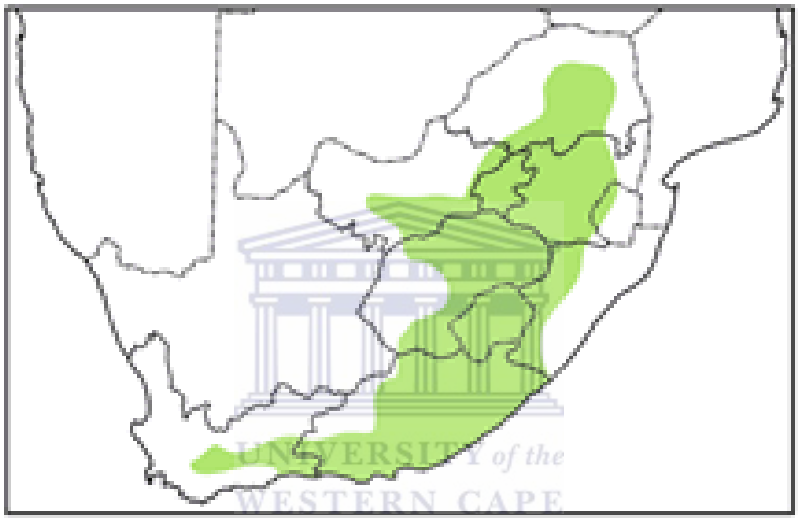

Figure 2.2: Geographical distribution of *A. afra* in South Africa (Adapted from SATMERG Pharmacopoeia, Monographs Project, 1999).

2.2.4 Uses and Pharmacological effects

A. afra is exceptionally and widely used in many parts of the world either alone or in combination with other plants as herbal remedies for a variety of ailments ranging from relatively minute discomforts like simple headache to neurological disorders like epilepsy. In South Africa, its name "African wormwood" is derived from its ability to eradicate worms but it is also used in the treatment of numerous ailments including colds, coughs, heartburn, flatulence, bladder and kidney disorders, convulsions, diabetes, headache, inflammation, rheumatism and stomach disorders (Felhaber, 1997, Thring and Weitz, 2006). The infusions or decoctions are applied as a lotion to bathe haemorrhoids and for ear ache as well as for treatment of diabetes in the

Eastern Cape Province of South Africa (Erasto et al., 2005, Mahop and Mayet, 2007). In addition to these, it is also commonly used for the treatment of acute bronchitis, hay fever and, asthma as it is said to clear the respiratory and bronchial passages (Graven et al., 1990, Watt and Breyer-Brandwijk, 1962, Hutchings et al., 1996b, Van Wyk and Wink, 2004).

A. afra is known to have strong antihistaminic, narcotic and analgesic activities (Van Wyk et al., 1997, Bruneton, 1995). Recent studies have demonstrated that steam distilled *A. afra* oil possesses antimicrobial properties (Graven et al., 1992). However, there is limited data on pharmacological investigations done on *A. afra*.

2.2.5 Phytochemical constituents of *Artemisia afra*

Artemisia afra contains many phenolic compounds which are dominated by flavonoids, a group of phytochemicals well known for their many biological activities (Harborne, 1973). However little is documented in the literature concerning the flavonoid constituents of *A. afra*. Although terpinoids, coumarins, acetylenes, scopoletin as well as triacontane and umbelliferone have been shown to be present in *A. afra*, their contribution to the biological activities of the plant is not yet known (Iwu, 1993, Van Wyk and Gerike, 2000).

The flavonoids that have been isolated include luteolin, apigenin, quercetin, chrysosplenetin and a tetra-hydroxymethoxyflavone (Tang et al., 2000). Several studies done at the School of Pharmacy of the University of the Western Cape though unpublished, revealed the presence of the flavonoid luteolin, kaempferol, apigenin and quercetin leaf infusions and aqueous extracts of plant leaves (Waithaka, 2004, Komperlla, 2005, Mukinda, 2006)

The essential oils of *A. afra* are known to contain mainly 1, 8-cineol, α-thujone, camphor, borneol, Artemisia ketone and sequiterpene -1-3 (Hutchings et al., 1996a{Hutchings, 1996 #319, Graven et al., 1990, Piprek et al., 1982). In addition, tannins, saponins, terpinoids, coumarins and acetylenes have been detected within the leaves of *A. afra* (SATMERG, 1999, Hutchings et al., 1996a, Van Wyk et al., 1997).

2.2.6 Dosage form of *Artemisia afra*

The usual dosage preparation of the plant is in the form of an infusion or decoction which involves the use of fresh leaves immersed in hot or boiling water and allowing it to seep to make a tea and as Wilde Als brandy, an alcoholic preparation. The leaf infusions are taken as teas or administered as enemas and the steam from the infusions arising from the *Artemisia afra* leaves is commonly inhaled for the treatment of headaches and colds (Hutchings et al., 1996). Enemas are made by suspending the ground *A. afra* plant in hot water or milk and the mixture used for constipation or intestinal worms in children. Decoctions are also taken as blood purifiers for acne, boils, measles and small pox (Bryant, 1996). The infusions or decoctions are made with variable quantities of fresh leaves and various forms of infusions are employed for treatment of diseases by different tribes (Roberts, 1990). However, despite the extensive traditional dosage forms (e.g. teas and enemas) and use of this plant, there is little or no data describing the effectiveness of these various dosage forms and the methods of use.

2.2.7 Shortcomings of the traditional dosage form preparation

The aforementioned traditional dosage forms of *A. afra* have several disadvantages arising from poor stability during storage and inconsistent directions for preparation and administration.

Firstly, the use of wet leaves in the preparation of teas is inappropriate. This is because moisture present in the leaves may promote bacterial growth which may lead to deterioration of the product (McCutcheon, 2002). Secondly, directions for dosage preparations are often generally obscure for example; the use of inexact measures such as a quarter cup, a handful, tumblerful and calabashful in the directions to prepare and use the traditional dosage form makes the instructions vague and incorrect. This leads to lack of mass and content uniformity as it is difficult to accurately quantify a handful or measure a quarter cup consistently (Sofowora, 1982, Williamson et al., 1996). Thus, it may be anticipated that such variability in the preparation may lead to variations in dose each time a treatment is prepared thus altering the bioavailability and the pharmacokinetics of the active constituents. Thirdly, *A. afra* infusions or teas/decoctions are often smelly and awful tasting for oral use. Sweeteners e.g. honey and sugar is then added to these teas or infusions to make them more palatable and to improve patient acceptability.

However, these additives in turn act as good media for the growth of microorganisms like bacteria, mould and fungi (Burlage et al., 1963). Lastly, intake of tea decoctions can differ significantly depending on the method of preparation of the tea. The size of the leaves and the extraction surface seems to be important in relation to flavonoid content (Aherne and O'Brien, 2002).

In the traditional method of preparation, there is no mention of the expected degree of comminution of the *A. afra* leaves. Particle size largely explains the differences seen in flavonoid yield between tea prepared from tea bags and loose leaves. Tea produced from tea bags has higher flavonoid levels compared with tea prepared from loose tea leaves (Hertog et al., 1993).

2.2.8 Lack of bioavailability data on the *Artemisia afra* plant

There is no commercially available preparation of *Artemisia afra* that is registered with the Medicines Control Council of South Africa and there exists very limited data about their bioavailability and bioequivalence. Thus there is no pharmacokinetic basis for the recommended dosage regimens of the *Artemisia afra*preparations due to the absence of pharmacokinetic data that usually results from bioavailability studies. Pharmacokinetics involves processes like absorption, distribution, metabolism and elimination and these processes have a crucial impact on the safety and efficacy of a medicine. The above mentioned pharmacokinetic processes are usually quantitatively described by means of various pharmacokinetic parameters such as volume of distribution, elimination or absorption half-lives or rate constants, and rates of excretion and metabolism. These parameters are obtained through pharmacokinetic studies and are important to support the labelling of drugs as well as for establishing dosage regimens (Shargel and Yu, 1999). For a new medicine, the pharmacokinetic parameters are usually determined in animal studies before the medicine is tested in humans (Spilker, 1991). This therefore implies that the proposed therapeutic effectiveness of *A. afra* is backed by very little or no evidence. The bioavailability and bioequivalence studies are usually conducted to quantitatively describe the absorption characteristics of a compound and to quantitatively compare the absorption of compounds respectively. The design of such studies is crucial to

obtain valid results. In this thesis, the focus is on investigating the permeability of luteolin in *A. afra* across the intestine as an indication of bioavailability.

2.3 Need for modified forms of *Artemisia afra* plant

As discussed in section 2.2.7 the traditional dosage form (i.e. tea, infusion, steam inhalation) of *Artemisia afra* has a number of disadvantages and are also not suitable for the evaluation of the herbal extract in clinical trials. Various studies conducted at the School of Pharmacy, University of the Western Cape, have investigated the production of freeze dried aqueous extract of *A. afra* and its formulation into various dosage forms (e.g. tea bags, tablets and capsules) (Dube, 2006, Egieyeh, 2009). These attempts were aimed at achieving a consistent dosage form of *A. afra* with high quality that will be suitable for clinical trials. However, the major challenge encountered with these dosage forms thus far prepared was the hygroscopicity of the freeze dried aqueous extract (FDAE) of *A. afra*. The hygroscopic nature of the FDAE of *A. afra* and other herbal extracts has been a major challenge for many researchers and manufacturers in the herbal drug and nutraceutical industries. Attempts to formulate these herbal extracts into an acceptable standardised dosage form that can be used for clinical trials have been very daunting (Tong et al., 2005). Hygroscopic and/or deliquescent substances, by definition, are prone to moisture sorption when exposed to environments with even moderate humidity. The sorption of moisture by drugs and herbals can create significant problems. Moisture can affect the physical, chemical and mechanical properties of a solid drug/herbal substance (Stubberud et al., 1996). There is therefore a general, and in the case of *Artemisia afra*, a specific need for a pharmaceutically acceptable non hygroscopic solid herbal formulation. In an attempt to achieve this, excipients were added to the prepared FDAE to further modify the form. The International Pharmaceutical Excipient Council (IPEC) defines an excipient as any substance other than the active drug that is included in the manufacturing process. Excipients are commonly used to improve the less than satisfactory characteristics e.g. pharmaceutical (i.e. excessive hygroscopicity) or pharmacokinetic (i.e. low bioavailability) of active ingredients. Excipients can also be used to prepare controlled or modified release preparations. These controlled preparations are usually multi-particle drug delivery systems suitable for achieving controlled or delayed release of oral formulations (Dey, 2008). Furthermore, they maintain adequate therapeutic plasma level of drug

avoiding peak-and-valley effect and thereby prolong the release of drug over extended period of time (Deasy and Dekker, 1984, Welling and Dobrinska, 1987). Tapas *et al.,* used polymethylmethacrylate coated alginate matrix microcapsules to prepare a controlled release dosage form of diclofenac sodium which was found to have prolonged release in the intestine with improved absorption (Tapas et al., 2011).The release of drug from the matrix microcapsules was less than that from the non-matrix microcapsules. While the non-matrix microcapsule prepared released 100% drug in 12 h, the matrix microcapsules released only 76.06% drug in 12 h. For this study, the freeze dried aqueous extract of *A. afra* was prepared and was further modified by adding alginate to the extract to prepare alginate-FDAE beads, and polymethylmethacrylate (PMMA) to prepare PMMA-alginate-FDAE beads.

2.3.1 Freeze-dried aqueous extract

Freeze drying the frozen aqueous extract allows water to be eliminated by sublimation. This operation allows drying to be conducted under moderate temperatures thus resulting in a high quality product. The freeze dried aqueous extract also has maximum antioxidant capacity since drying is done at moderate temperatures leading to reduced chemical degradation and less exposure to air during drying.

As aqueous solutions, freeze drying will stabilize the extract in a solid powder form that can be repackaged, formed into tablets, encapsulated, or used for further processing. Freeze drying preserves the chemical integrity of the extract ensuring high potency and effectiveness. In a stable, powder form, scientific studies on the extracts can be performed using known, repeatable quantities. Unfortunately, the solid particles formed from freeze drying of most aqueous extract of herbal medicines are hygroscopic. This leads to poor processing properties, poor stability of manufactured dosage forms and facilitation of microbial contamination (Schiller, 2002), thus the modification into less hygroscopic forms by incorporating excipients such as alginate, dextran and polymethylmethacrylate.

2.3.2 Alginates

Alginate is a natural biopolymer composed of randomly distributed units of two sugars which are urinates, 1, 4-linked β-D-mannuronic and α-L-guluronic acid residues (George and Abraham, 2006). Alginate, a hydrogel, is used as an excipient for the formulation of beads because of its ability to absorb 200-300 times its own weight of water (Rowe et al., 2009). Biopolymers like alginate have been shown to improve the gastrointestinal uptake of poorly absorbable drugs like insulin by entrapment of the protein to provide degradative protection in the gastrointestinal tract and facilitate transport into the systemic circulation (Sarmento et al., 2007). In addition, the encapsulation process using alginate hydrogels in the formation of the calcium alginate beads is done under extremely mild conditions and uses non-toxic reactants (Song et al., 2012). Alginate was chosen because as part of controlled release technology, it has advantages such as lower consumption of the active agent, reduced frequency of administration, and minimized side effects of drugs. Calcium alginate beads formed are spherical and are expected to improve the flowability of the freeze dried aqueous extract of *Artemisia afra*. Figure 2.3 shows the structures of mannuronic and guluronic acid residues and the linkage between these residues in alginate in the presence of calcium ions.

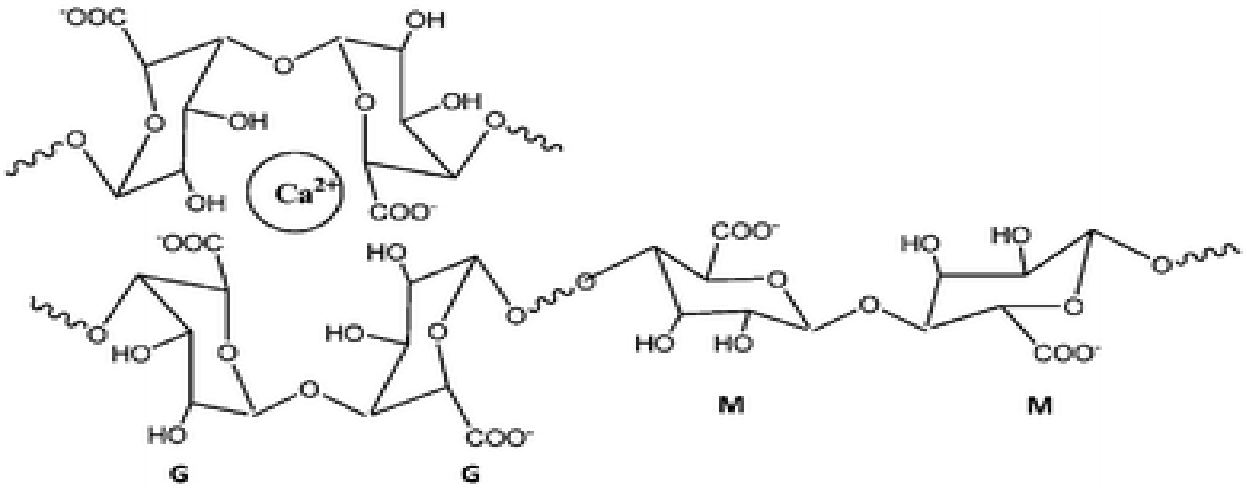

Figure 2.3: Chemical structure of alginate and its schematic gelation mechanism in the presence of calcium ions. Shown is a polymer chain of 2 guluronic acid (G) monomers and 2 mannuronic acid (M) monomers, with (1–4) linkages (George and Abraham, 2006).

2.3.3 Polymethylmethacrylate

Polymethylmethacrylate can be used as a coating polymer to produce a multiunit controlled release dosage form, which maintains adequate therapeutic plasma level of drug and prolongs the release of drug over extended period of time (Tapas et al., 2011). One of the objectives of this study was to develop PMMA coated alginate matrix microcapsules by $W_1/O/W_2$ emulsion solvent method and to compare its release profile with the other preparations mentioned above. Figure 2.4 shows the chemical structure of polymethylmethacrylate.

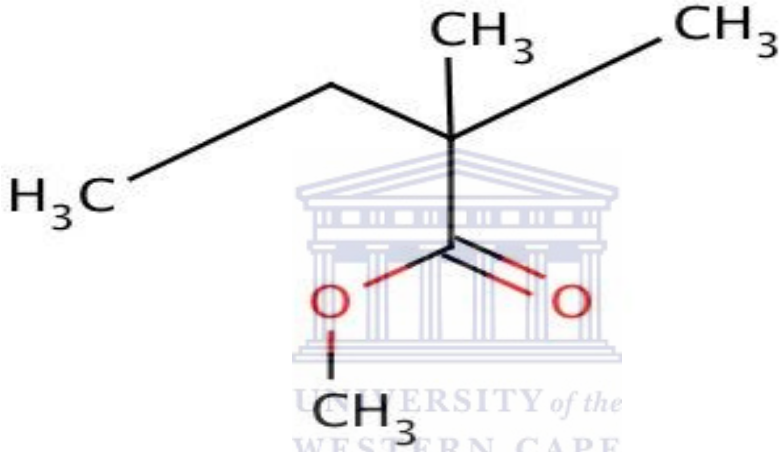

Figure 2.4: Chemical structure of Poly (methyl methacrylate).

2.4 Use of single pass intestinal permeability (SPIP) method to investigate permeability and absorption of modified forms of *Artemisia afra*

Despite tremendous innovations in drug delivery methods in recent decades, the oral route of drug administration still remains the most preferred route for most drug entities. The oral route of administration of drugs is preferred by virtue of the fact that it is convenient, inexpensive and offers high patient compliance as compared to alternate routes (Balimane et al., 2000). However, compounds intended for oral administration must have adequate aqueous solubility and intestinal permeability in order to achieve therapeutic concentrations. Poor permeability and/or absorption makes a molecule unsuitable for further development, and there has been great interest recently

in finding ways to avoid the situation of having a potent, yet impermeable molecule. Thus, prediction of drug absorption and permeability is therefore very important for the design of an oral preparation. For several decades, human *in vivo* studies using earlier intestinal techniques have been applied to investigate absorption, permeability and gastric emptying. Although human *in vivo* studies will be the most desirable, they are difficult, expensive and time consuming to conduct. As a result, a number of *in vitro* and *in situ* experimental methods have so far been utilised to estimate gastrointestinal absorption of drugs and the mechanism of absorption.

Animal tissue-based methods like the everted gut technique, isolated membrane vesicles and the *in vitro* transport across intestinal segment (side-by-side Ussing chamber) have been extensively used to explore the mechanism of absorption of nutrients from the intestine (Wilson and Wiseman, 1954, Ussing and Zerahn, 1951). Animal intestinal tissues are made up of essentially the same kind of endothelial cells as human tissue and have thus been extensively used because it is extremely difficult to obtain viable human tissues for permeability studies on a regular basis. However, the viability of the excised tissues is difficult to maintain since the tissues are devoid of direct blood and nerve supply and need constant oxygenation. Other disadvantages of these methods include rapid loss of viability of the tissues during the experiment and changes in morphology and functionality of transporter proteins during the process of surgery and mounting of tissues. Cell-based methods like caco-2 cells and TC-7 clone have been the most extensively characterized and useful cell model in the field of drug permeability and absorption (Artursson et al., 1994, Rubas et al., 1996). The predictability and utility of this model to rank order a large number of compounds in terms of absorption potential have been demonstrated many times by many investigators (Artursson and Karlsson, 1991, Chong et al., 1997). However, this method has a shortcoming in that it is very difficult to compare the absolute permeability coefficient value of individual compounds, particularly with compounds that primarily permeate via the paracellular route. Among these methods, the *in situ* method, single-pass intestinal perfusion (SPIP) approach is one of the most widely used classic technique employed in the study of intestinal absorption compounds, which provides experimental conditions closer to what is faced after oral administration (Crouthamel and Sarapu, 1983, Sutton et al., 2001). The biggest advantage of this technique is the presence of an intact blood and nerve supply. In addition, the technique has lower sensitivity to pH variations because of a preserved microclimate above epithelial cells (Hogerle and Winne, 1983). The technique also provides the unique advantages

of experimental control (compound concentration and intestinal perfusion rate) and the ability to study regional differences; and factors that may influence the intestinal absorption of the compound.

In situ perfusion of intestinal segments of rodents (rats or rabbits) is frequently used to study the permeability and absorption kinetics of drugs and it involves perfusion of drug solution prepared in physiological buffer through isolated cannulated intestinal segments. Permeability is assessed based on the disappearance of drug (difference between inlet and outlet concentrations) from intestinal lumen. Since water absorption and secretion during the perfusion may cause errors in the calculated net effective permeability values (P_{eff}), a non-absorbable marker is required to correct water reflux (Sutton et al., 2001). For this reason, phenol red is co-perfused with drug compounds.Phenol red was first introduced as a non-absorbable marker by Gorham in 1923 (Gorham, 1923).

Previous studies have shown that the extent of absorption in humans can be predicted using the single pass intestinal perfusion in rat (Salphati et al., 2001). However, the single pass intestinal perfusion method is limited in that the method relies on the disappearance of compound from the luminal side as an indication for absorption. Moreover the rate of absorption of drug into the systemic circulation is not always represented by the rate of decrease of concentration in the perfusate. Uhing and Kimura also demonstrated that the surgical manipulation of the intestine combined with anaesthesia caused a significant change in the blood flow to the intestine and had a remarkable effect on absorption rate (Uhing and Kimura, 1995). In this study, the single pass intestinal perfusion was employed to investigate the permeability and absorption of flavonoids from the different modified preparations of *Artemisia afra*. The limitations mentioned above affected this study in that the concentration of luteolin indicated by the effluent perfusate samples was not an exact representative of the concentration of luteolin absorbed into the systemic circulation. In addition the incisions made on the intestine were seen to reduce the blood flow to the intestine thus affecting the concentration of luteolin absorbed into systemic circulation.

2.4.1 Criteria for selection of animal model for permeability study

Interspecies differences in gastrointestinal (GI) transit time, size and shape of intestinal villi, gastric pH, lymphatic uptake and drug metabolism occurring in the gut are among the physiological variables that can contribute to differences in absorption (Rogge and Taft, 2005) and consequently, in the bioavailability of a drug material. Much of the interspecies' diversity in GI anatomy and function reflects differences in primary sources of dietary constituents (Rogge and Taft, 2005). In light of these interrelationships, the basis for selection of an animal model for bioavailability studies should be on the closeness of evolutionary and dietary similarities to man.

2.5 Flavonoids

Flavonoids are a class of polyphenolic compounds that occur naturally in the plant kingdom. They occur ubiquitously in virtually all plant parts including the leaves, roots, wood, bark, pollen, flowers, berries and seeds (Markham, 1982). They are common dietary components of vegetables, fruits, wine and tea. They are the pigments responsible for the various colours observed in flowers and are also required for the normal growth, development and defence mechanisms in plants (Di Carlo et al., 1999, Harborne and Williams, 2000).

2.5.1 Chemistry and classification

Flavonoids are a group of low-molecular weight phenolic substances formed from the combination of derivatives synthesized from phenylalanine and acetic acid. All flavonoids have a common biosynthesis origin namely, a chalcone resulting in condensation of a "triacetate'' with cinnamic acid, and cyclization forming the central pyran ring (Bruneton, 1999). The structure of flavonoids is based on the flavonoid nucleus (Figure 2.5), which consists of 3 phenolic rings referred to as A, B, and C rings (Kuhnau, 1976).

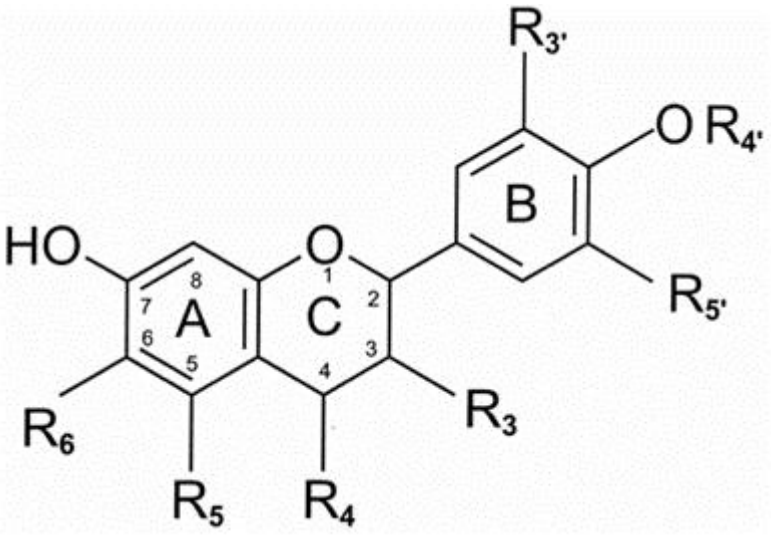

Figure 2.5: General structural formula of flavonoids.

The flavonoids are divided into various classes according to the position of multiple combinations of hydroxyl groups, sugars, oxygen, and methyl groups attached to the basic structure. Variations in the heterocyclic ring C give rise to flavonols, flavones, catechins, flavanones, anthocyadins and isoflavonoids (Figure 2.6) (Di Carlo et al., 1999, Markham, 1982). In addition, the basic structure of flavonoids allows a multitude of substitution patterns in the benzene ring A and B within each class of flavonoids: phenolic hydroxyls, O-sugars, methoxy groups, sulphates and glucuronides. Flavonoid *per se* are compounds in which the benzenoid substitution is at the 2-position while compounds with substitution at 3-position are termed isoflavonoids. Flavonoids may also be aglycones (i.e. consisting of benzene ring condensed with a six-member ring, which possesses a phenyl ring at the 2 position), glycosides (that carry one or more sugar residues on the ring) or methylated derivatives.

Flavonoids containing a pyran ring i.e. a hydroxyl group in position C3 of the C ring are classified as 3-hydroxyflavonoids (i.e. the flavonols, anthocyanidins and catechins) and those lacking a hydroxyl group in position C3 as 3-desoxyflavonoids i.e. the flavanones (e.g. hesperetin, naringenin) and flavones (e.g. luteolin, apigenin) (Markham, 1982, Heim et al., 2002). In this study, luteolin is the main flavonoid of interest and is further discussed below.

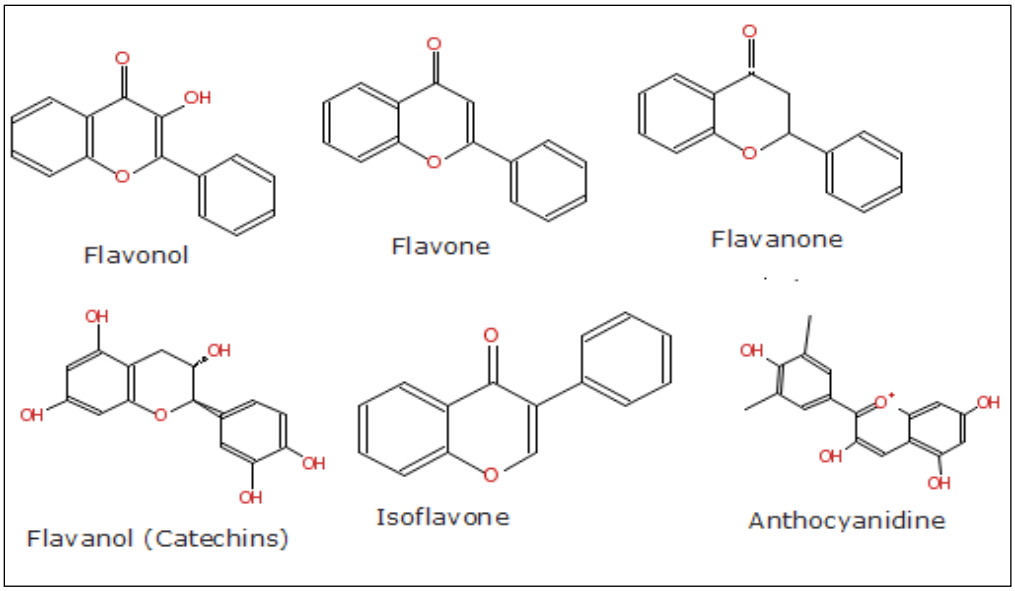

Figure 2.6: Structures of the main classes of flavonoids.

2.5.2 Metabolism and bioavailability of flavonoids

The metabolism and bioavailability of flavonoids is likely to be of crucial importance to their ability to help protect human health against disease (Setchell et al., 2002). Deglycosylation of flavonoid glycosides has been proposed as the first stage of metabolism. Day *et al*. used human small intestine and liver cell-free extracts to see whether there is glucosidase activity toward flavonoid glycosides. Some but not all flavonoid glycosides were hydrolysed by the small intestine and liver extracts (Day et al., 1998). After absorption, flavonoids are bound to albumin and transported to the liver via the portal vein (Manach et al., 1996). The liver seems to be the chief organ involved in flavonoid metabolism; nevertheless, it must be noted that other tissues, especially intestinal mucosa or kidneys must not be ruled out (Hackett, 1986). The liver also synthesizes conjugated derivatives, by coupling with a sulphate or a glucoronic acid molecule. The conjugated flavonoid derivatives are excreted in urine and in bile, the major route depending on the species. When excreted in bile, they are poured into the duodenum and metabolized by bacteria in the large intestine, which results in the production of fragmentation products and/or the hydrolysis of glucorono- or sulpho-conjugated and the release of flavonoid metabolites.

These may be reabsorbed and undergo an enterohepatic cycling, thus non negligible concentrations of flavonoid metabolites may be present in the blood plasma.

Flavonoid metabolism in humans has been reported to also depend on the participation of intestinal microflora (Kuhnau, 1976, Bokkenheuser et al., 1987, Griffiths, 1982). Flavonoids that are not absorbed in the small intestine can be metabolized by colonic microflora into aglycones and phenolic acids. These in turn may be absorbed from the colon (Manach et al., 1998, Hollman et al., 1995, Macdonald et al., 1983). Flavonoid metabolism produces a series of phenolic compounds that have been identified as aromatic acids. They form by detachment of the A ring from the residual flavonoid molecule and the opening of the heterocyclic C ring.

2.6 Luteolin

Luteolin is a yellow microcrystalline substance which is an active compound in *Reseda luteola L*. (Hoppe, 1975) found by Chevreul in 1833. In 1896, Pekin confirmed the presence of luteolin in weld. Paris later isolated, characterized and identified luteolin glycoside in 1955 (Cristea et al., 2003). It is sparingly soluble in water, but soluble in alkali.

Luteolin is one of the most common flavonoids present in edible plants and in plants used in traditional medicine to treat a wide variety of ailments. It is most often found in leaves, barks, clover blossom, rag weed and pollen.

Luteolin is present as an aglycone (molecule without any sugars bound to it) in some plants such as perilla seeds and as glycosides (molecule with one or several sugars bound to it) in celery, green pepper, perilla leave, chamomile tea, etc. (Shimoi et al., 1998, Shimoi et al., 2001). Most glycosides of luteolin are O-glycosides, i.e., the sugar moieties are bound to the aglycone through one or more four free hydroxyl (OH) groups (Figure 2.7). These glycosides usually have sugar moieties at position 5, 7, 3’ and 4’, as luteolin is 5, 7, 3’, 4’- tetrahydroxyflavone (Lopez-Lazaro, 2009).

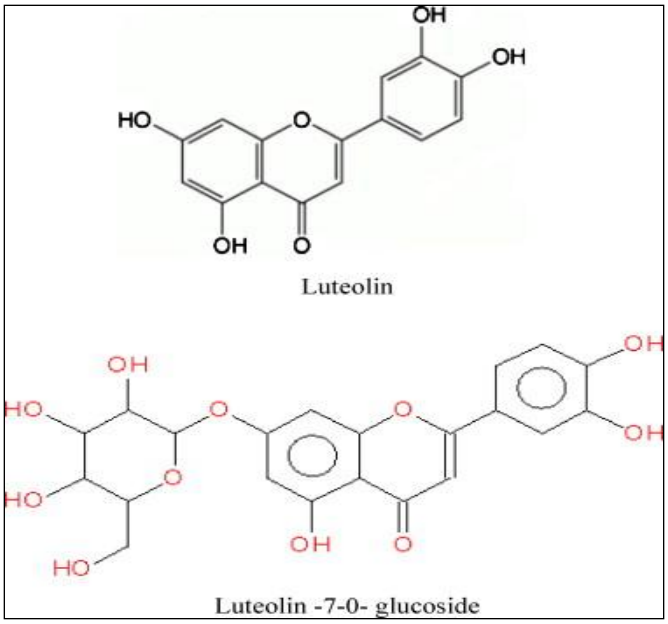

Figure 2.7: The structure of Luteolin and Luteolin-7-0-glucoside.

2.6.1 Biological activities of luteolin

Luteolin may be a valuable available emerging pharmacological agent due to its many beneficial properties. It has been reported that luteolin has anti-inflammatory and antispasmodic activity (Hoffmann, 1995), antioxidant activity (Areias et al., 2001), anti-allergic and, anti-cancer activities and immune-modulatory properties to suppress hyperactive immune systems (Shimoi et al., 1998). Due to its anti-allergic, anti-inflammatory and smooth muscle relaxing properties, luteolin has potential in the prevention and treatment of many respiratory disorders including asthmatic conditions and chronic bronchitis (Wang, 2000). Luteolin has been demonstrated to be an emerging anti-cancer agent that completely inhibits the catalytic activity of eukaryotic DNA topoisomerase I. (Chowdhury et al., 2002, Galvez et al., 2003). Luteolin is an important dietary cancer preventive agent (Cai et al., 1997, Elangovan et al., 1994, Ubeda et al., 1995) and shows strong antiproliferative activity against different human cancer cell lines (Ramanathan et al., 1993, Fotsis et al., 1997). Luteolin is also known to be an effective smooth muscle relaxant and protects the heart lipids against doxorubicin-induced lipid peroxidation (Sanchez de Rojas et al., 1996, Sadzuka et al., 1997). Luteolin also aids in prevention of the neovascular disease of the eye by inhibiting corneal angiogenesis *in vivo* (Joussen et al., 2000).

Based on this profile of action, luteolin, thus, appears to be one of the potential pharmacological constituents that could contribute to the medicinal values of many plants. However, very limited information is available on the absorption and bioavailability of luteolin in human or in experimental models.

2.6.2 Absorption and metabolism of luteolin

Very few studies have been done demonstrating the ability of flavonoids to reach proposed sites of action *in vivo*. The limited number of studies in rats and humans has indicated very poor and variable intestinal absorption of phenolic compounds (Walgren et al., 1998). It is generally assumed that flavonoids are absorbed as their aglycones after hydrolysis of the glycosides along the digestive tract (Walle, 2004). However controversies exist concerning which form of flavonoid is actually absorbed i.e. glycoside, aglycone or both forms. Glycosides are absorbed to a very limited extent (Scalbert and Williamson, 2000) are cleaved by gut bacteria (Day et al., 2000) and are usually hydrolysed (by intestinal enzymes and/or colonic micro flora) to the corresponding aglycone molecule (Manach et al., 2004, Pong et al., 2005). Aglycones on the other hand are absorbed freely across the intestinal mucosa by passive diffusion and conjugated in the mucosa and liver by phase II enzymes (Crespy et al., 1999). Kuhnau, 1976, reported that flavonoids present in foods cannot be absorbed from the intestine because they are bound to sugars as glycosides and further explained that only free flavonoids without a sugar molecule (aglycones) are considered to be able to pass through the gut wall (Kuhnau, 1976). In contrast, Hollman and his team (1999) suggest that for quercetin glycosides, the absorption of the intact glycosides is much better than the pure aglycones (Hollman and Katan, 1999). In addition, the mechanism through which flavonoids are absorbed in the GIT whether as aglycones or glycosides has not yet been fully resolved. It is however generally assumed that the aglycones of the flavonoids are absorbed mainly by passive diffusion (Pong et al., 2005).

The metabolism of flavonoids after absorption may have a considerable effect on their biological properties (Nielsen et al., 1997, Erlund et al., 2002) and bioavailability. The liver seems to be the chief organ involved in flavonoid metabolism (Hackett, 1986). Other important sites of flavonoid metabolism include the gastrointestinal lumen, cells of the intestinal wall (Erlund et al., 2002). Day *et al*., showed that the enzymes lactase–phloridzin hydrolase and ß-glycosidase were able to

cleave flavonoid glycosides in the small intestine although it wasn't yet well characterized (Day et al., 1998, Day et al., 2000). There is also evidence suggesting that flavonoids can be metabolized prior and during absorption and be subject to first-pass metabolism (Scalbert and Williamson, 2000, Shimoi et al., 1998, Erlund et al., 2002).

Spencer *et al*., investigated the absorption of luteolin aglycone and luteolin 7-O-ß-glucoside through the perfusion of the jejunum and ileum in an isolated rat intestine model. They found at least six different glucoronides in the plasma after treatment with ß-glucoronides (Spencer et al., 1999). The results suggested that luteolin was mono-glucoronidated at different sites and could also possibly be di- or tri- glucoronidated. Shimoi *et al* investigated the absorption of luteolin and luteolin 7-0-ß-glucoside in rats by High-Performance Liquid Chromatography (HPLC) using rat everted small intestine. The absorption analysis demonstrated that luteolin was converted to glucoronides during passing via the intestinal mucosa and that luteolin 7-0-ß-glucoside was absorbed after hydrolysis to luteolin. They confirmed that free luteolin, its conjugates and methylated conjugates were present in rat plasma after its oral administration in pure form and that some luteolin could possibly escape the intestinal conjugation and the hepatic sulfation/methylation (Shimoi et al., 1998).

Very few studies have however been done on the pharmacokinetics of luteolin contained in plant materials. Wittemer *et al*., investigated the bioavailability of luteolin glycosides contained in Artichoke leaf extracts in humans. It was found that no free luteolin or luteolin-7-O-glucoside was present in plasma or urine (Wittemer et al., 2005). Muganga found that the bioavailability of luteolin from aqueous extract of *Artemisia afra* in the vervet monkey was significantly better than in its pure form (aglycone) (Muganga, 2004). Thus there is a need to know in which of the modified forms luteolin will be better absorbed from the plant.

2.6.3 Analytical methods suitable for luteolin analysis in plants and use in bioavailability studies

Several methods are used to separate, identify and quantitate flavonoids in plant material. Several steps are involved in these methods which include separation, purification and identification of the different constituents that may be present in the plants. For both quantitative

and qualitative analysis, a very important step is proper preparation of the sample, which differs depending on:

1) The physical state of the sample (fluid or solid).

2) The concentration of the compound in the sample (flavonoids in biological samples are usually at a fairly low level, so it may be necessary to enrich the sample by extraction).

3) The type of matrix containing flavonoids (particular components of a matrix may interfere with the analyte, making the analysis difficult).

4) The chemical nature of the analysed compound. Flavonoids in physiological human materials and in plant samples are most frequently in the form of conjugates: in food samples, they mainly occur as glycosides, whereas in the material isolated from animals and humans, the most forms are glucoronic or sulfonic derivatives.

Many solvents can be used to extract the flavonoids found in plants, depending on the form of the flavonoid (aglycone or glycoside). To extract flavonoids in aglycone form, non-polar solvents such as ether, hexane and chloroform are usually used (Markham, 1982). Glycosides or flavonoids possessing a number of un-substituted hydroxyl groups are polar and generally soluble in solvents such as ethanol, methanol, dimethyl sulfoxide and water.

Several chemical tests and calorimetric methods can be used to identify individual flavonoids or flavonoid classes. One of such methods is thin layer chromatography (TLC) which is a very useful and frequently applied technique (Markham, 1982). However, most flavonoids are not visible on TLC plates and are therefore viewed under ultraviolet light at wavelengths 254 and 366 nm after the spots have been exposed to ammonia vapours. The TLC technique has a very low sensitivity and a low limit of detection of 5-10 µg flavonoid on the plate (Wagner et al., 1984). Therefore, high performance liquid chromatography (HPLC) which is a sensitive and rapid technique is increasingly utilised for the separation, identification and quantification of flavonoids in plant extracts (Springfield et al., 2005). Indeed, HPLC and mass spectrometry are modern techniques used for identification and quantitative analysis of flavonoids (Harborne and Williams, 2000) and several studies have indicated the successful use of HPLC for the

identification and quantification of flavonoids (Cristea et al., 2003, Wittemer and Veit, 2003, Valentao et al., 1999).

Reversed-phase HPLC (RP-HPLC) combined with different detectors is also a commonly used analytical method for separation of flavonoids (Escarpa and Gonzalez, 1998, Brolis et al., 1998). RP-HPLC is also being used because flavonoids are usually very soluble in polar solvents. Several steps are involved in the analyses of flavonoids. The first step is the acid hydrolysis of the aqueous plant material followed by organic phase extraction to separate the free aglycones from the other flavonoids and the use of HPLC to isolate individual compounds (Tura and Robards, 2002). In developing the HPLC assay for analyses, selection of suitable solvents, columns and wavelengths of maximum absorption are taken into consideration. In HPLC assays, various proportions of solvents such as water-methanol, water-methanol-acetic acid or formic acid and water-acetonitrile have been reported for the elution of flavonoids such as luteolin and hesperetin (Markham, 1982). The most popular HPLC stationary phase used both generally and specifically for analyses of phenolics is octadecylsilane (ODS, C18, RP-18) (Majors, 2001). The final step in the analysis of flavonoids is the measurement of the detector response, development of the calibration curve and validation of the assay. Suitable HPLC assays can thus be developed and used to quantify flavonoids such as luteolin in plant material and can also be used to compare and investigate the bioavailability (cell membrane permeability) of luteolin from different preparations of the *A. afra* plant extract (i.e. FDAE *vs*alginate-FDAE *vs* PMMA-alginate-FDAE).

Chapter 3

Plan of work

3.1 Introduction

This chapter outlines the specific objectives, hypotheses set, and the study approach used for the investigation of the *in vitro* bioavailability (i.e. gastrointestinal permeability) of luteolin from modified forms of *A. afra*.

3.2 Study objectives

The specific objectives of this study were:

1. To prepare the freeze dried aqueous extract (FDAE) and its modified forms, alginate – extract beads (alginate-FDAE) and polymethylmethacrylate coated alginate matrix beads of herbal extract (PMMA-alginate-FDAE) of the freeze-dried aqueous extract of *A. afra*,
2. To determine and compare the pharmaceutical characteristics of the above mentioned preparations of *A. afra*,
3. To quantify and compare the total flavonoid (and specifically luteolin levels) of the different forms of *A. afra*,
4. To evaluate and compare the release characteristics of FDAE of *Artemisia afra* from the alginate-FDAE and PMMA-alginate-FDAE beads in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) and,
5. To determine the intestinal permeability of luteolin contained in the selected in selected modified *A. afra* extractpreparations.

3.3 Hypotheses of the study

The following hypotheses were postulated;

1) **Hypothesis 1**: making the alginate beads and the polymethylmethacrylate coated alginate matrix beads of the FDAE of *A. afra* makes the FDAE less hygroscopic with a lower moisture content i.e.

   $H_{FDAE} > H_{alginate\text{-}FDAE} > H_{PMMA\text{-}alginate\text{-}FDAE}$

   and

   $MC_{FDAE} > MC_{alginate\text{-}FDAE} > MC_{PMMA\text{-}alginate\text{-}FDAE}$

   Where H = hygroscopicity and MC = moisture content

2) **Hypothesis 2**: the rate of release of luteolin from *A. afra* FDAE into gastrointestinal fluids is faster than the release from the alginate-FDAE and PMMA-alginate-FDAE beads and the rate of release of luteolin from *A. afra* alginate-FDAE faster than that from *A. afra* polymethylmethacrylate coated alginate matrix beads i.e.

   $RR_{FDAE} > RR_{alginate\text{-}FDAE} > RR_{PMMA\text{-}alginate\text{-}FDAE}$

   Where RR = rate of release

3) **Hypothesis 3**: the effective gastrointestinal permeability (P_{eff}) of luteolin in the alginate-FDAE and PMMA-alginate-FDAE beads of *A. afra* FDAE is equal to that in FDAE i.e.

   $P_{eff}^{lut}{}_{FDAE} = P_{eff}^{lut}{}_{modified\ extract}$

3.4 Study Approach

To realize the above objectives, the following were done. Firstly the modified preparations of *Artemisia afra* were prepared and pharmaceutically characterised. Next, an HPLC assay for luteolin in plant samples was developed and the level of luteolin determined in the different preparations of *Artemisia afra*. Finally, this assay was used to determine the *in vitro* bioavailability i.e. gastrointestinal permeability, of luteolin in the *A. afra* forms using the *in-situ* perfused rat intestine model.

3.4.1 Rationale for the modified preparations of *A. afra* freeze dried aqueous extract

In traditional practise, *A. afra* is mainly used as an infusion or decoction of the loose leaves in hot water and these forms have a number of disadvantages as highlighted in chapter 2. Past researchers investigated the formulation of freeze driedaqueous extract of *A. afra* into a dosage form in an attempt to produce a more uniform dosage form. However, the major challenge encountered in this dosage form was the problem of hygroscopicity. Thus in this study, the FDAE was modified by the incorporation of excipients in an attempt to counter hygroscopicity (e.g. use of alginate) and alleviate issues of sub-optimal bioavailability of plant actives (e.g. polymethylmethacrylate).

Alginate, a hydrogel, was used as an excipient in the formation of the beads because of its ability to absorb 200-300 times its own weight of water (Rowe, 2009). In addition, the beads are formedunder extremely mild environment from sodium alginate by ionic gelation, a process which takes advantage of the ability of sodium alginates to form solid gels by reaction with divalent cations such as Ca^{2+} (George and Abraham, 2006). The calcium alginate is expected to provide a barrier to diffusion of moisture into the herbal extract thus reduce the hygroscopicity of freeze dried aqueous extract of *Artemisia afra*. Polymethylmethacrylate was used as a coating polymer to reduce hygroscopicity andproduce a multiunit controlled release, which maintains adequate therapeutic plasma level of drug and prolongs the release of drug over extended period of time. PMMA-alginate-FDAE and alginate-FDAE beads are spherical and are expected to improve the flowability of the freeze dried aqueous extract of *Artemisia afra*.

3.4.2 Rationale for the selection of the characteristics to evaluate the forms of *A. afra* prepared

To establish the pharmaceutical quality of these *A. afra* plant preparations certain organoleptic and pharmaceutical characteristics were chosen to be determined and compared viz, colour, taste, odour, texture, particle size, particle shape, moisture content (hygroscopicity), aqueous solubility, entrapment efficiency of FDAE in modified forms and total flavonoid content of *A. afra* forms. These parameters affect the physico-chemical properties, bulk properties, product performance, processability, stability, bioavailability, appearance of end product and play a great role in producing homogeneity of dosage forms, thus their evaluation is of paramount importance.Organoleptic properties were determined using natural senses while the pharmaceutical properties were determined using pharmacopoeial methods and those standard methods adapted from other workers.

3.4.3 Rationale for the focus on luteolin

Flavonoids form one of the major constituents of medicinal plants. In recent years, flavonoids have attracted increasing attention due to their various beneficial pharmacological effects. Luteolin was selected for this investigation firstly because it has been found as a major flavonoid constituent in the crude extracts of *A. afra* (Waithaka, 2004). Secondly, luteolin has been shown to have *in vitro* pharmacological activities as mentioned in the previous chapter that can be associated with the uses and *in vivo* effects of *A. afra* and therefore should be a reliable pharmacological marker agent whose pharmacokinetic characteristics will have a major impact on the therapeutic effect of this plant. In addition, flavonoid levels in the aqueous extracts of the plant could be easily quantified by HPLC assay because it is a sensitive, economic and rapid technique. Lastly, luteolin is found in *A. afra* in both aglycone and glycoside forms and would thus be an ideal marker compound to establish the effect the different preparations may have on the absorption and permeability of these different flavonoid forms.

3.4.4 Rationale for the use of single-pass intestinal perfusionmodel

The *in situ* method, single-pass intestinal perfusion (SPIP) approach provides experimental conditions closer to what is faced after oral administration (Crouthamel and Sarapu, 1983, Sutton et al., 2001). The biggest advantage of this technique is the presence of an intact blood and nerve supply. In addition, the technique has lower sensitivity to pH variations because of a preserved microclimate above epithelial cells (Hogerle and Winne, 1983).On the other hand, the rat perfusion model was considered suitable for this study because,

1. Laboratory rats have been shown to be similar to humans in many of their biochemical responses to drugs, including the absorption, distribution, metabolism and excretion of drugs. Thus, human biological, behavioural and many other characteristics can be replicated in rats. Moreover, if interpreted with due care, the extrapolation of the biological results obtained with these animals to humans may be considered valid.
2. These experimental animal species are small, easily maintained and can, with practice, be easily handled in addition to the fact that they can be housed in large numbers in a relatively small area.
3. Rats are relatively inexpensive and can be bought in large quantities from commercial producers that breed rodents specifically for research. The rodents are also generally mild tempered and docile, making them easy to handle although some types of rats can be more difficult to restrain than others.

Chapter 4

Preparation and evaluation of different preparations of *Artemisia afra*

4.1 Introduction

In this chapter, the equipment, materials and methods used for the preparation of the freeze dried aqueous extract (FDAE) of *A. afra* and the modified forms of the FDAE of *A. afra* i.e. the alginate-FDAE beads and the PMMA-alginate-FDAE beads, are discussed. The organoleptic and pharmaceutical properties of *A. afra* preparations are also presented. The development and validation of the HPLC assay used for quantitation of luteolin in *A. afra* preparations is also described in detail. Finally, the results obtained are presented and discussed.

4.2 Equipment, Chemicals and materials

4.2.1 Equipment

Analytical balance (*Mettler®, model PE 6000*), a scientific balance (*Ohaus®, model GA 110*), a freeze- dryer (*Virtis ®, Freeze mobile model 125L*), -85°C freezer (*Lozone CFC Freezer, Model U855360, New Brunswick Scientific, USA*), a water bath (*Labcon®, model CDH 110 Maraisburg, SA*), filtration system (SUPELCO) connected to vacuum pump (*Medi-Pump Model 1132-2, Thomas Industries, Inc., USA*), filter paper (*Whatman No. 41 & Whatman No.1, Whatman, England*), oven (*Model Memmet 854 Schwabach, West Germany*), a centrifuge (*Multex MSE®*, England), desiccators, a microplate reader (*VERSAmax, Molecular Device, USA*), a vortex mixer (Vortex-2, G-560E, Scientific Industries, Inc. Bohemia, N.Y. 11716 USA), test sieve shaker (*Endecott sieve shaker, E.F.L. 1MK11, Endecotts (test sieve) LTD, London, England*), a pH meter (*Basic 20 Crison Instruments, S.A, Italy*), micropipettes, an HPLC system (Agilent model 1200, Chemetrics) consisting of an auto sampler and a diode array detector, all used with a C18 reverse phase column (Phenomenex,USA) having 4µm particle size and a

column length of 250 x 4.60 mm, HPLC filter unit (*Millipore Cameo 25 AS, DDA 02025So MSI: Micro separation Inc., USA*).

4.2.2 Chemicals

Acetonitrile and methanol, were purchased from Burdick & Jackson (USA), ethanol and aluminium chloride from Merck (Darmstadt, Germany), formic acid and sodium acetate from BDH Chemicals Ltd (England), hydrochloric acid from Kimix (USA), monobasic potassium phosphate, distilled water (prepared with Millipore filtration system) and ethyl acetate from Saarchem, (South Africa), calcium chloride from EMD Biosciences (Germany), sodium hydroxide and sodium alginate from Sigma Aldrich (Germany), polymethylmethacrylate, calcium chloride, dichloromethane, Tween 80, Span 80, luteolin, morin, pepsin and pancreatin were purchased from 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 (Groenvlei farm, South Africa), placed in plastic bags and stored in a cool place at room temperature away from sunlight until use.

4.3 Methods

4.3.1 Preparation of the freeze-dried aqueous extract

The study plant material was extracted in a way that closely mimicked the method traditional herbal practitioners have, as described in the literature, used over generations, to extract their plant medicines. Usually, a quarter cup quantity of *A. afra* leaves and a cup of boiling water is allowed to stand and steep for 10 minutes (Roberts, 1990). However, in this study, the dried leaves were suspended in distilled water (100 g of dried leaves in 3.5 L of distilled water) and the mixture boiled for 30 minutes. Three batches were prepared. After boiling, the decoction obtained was left to cool before it was filtered using Whatman no. 1 filter paper and the filtrate

transferred to a round bottom flask and frozen at -85 °C in a freezer. The frozen extract was then dried under vacuum over 3 days using the Virtis™ mobile freeze-dryer. The resultant extract powders from the different batches were then 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 and stored at room temperature in desiccators until used.

4.3.2 Preparation of alginate beads (alginate-FDAE) of aqueous extract of *Artemisia afra*

The stock solution was prepared by dispersing 2 g of FDAE into appropriate volumes of sodium alginate solution (4% w/w). The calcium-alginate beads were prepared by drop wise addition of 10 ml of the stock solution into 20 ml of $CaCl_2$ solution (5% w/w) through a stainless steel needle with a diameter of 0.7 mm (Song et al., 2012). In this process, the distance between the edge of the needle and the surface of the $CaCl_2$ solution was maintained at 5 cm and the syringe plunger was operated manually. Finally the resulting calcium-alginate-FDAE beads were allowed to cure in the medium for 10 min, then separated from the solution by filtration, rinsed with deionised water, and dried in a vacuum drying oven at 60 °C until a constant weight was obtained. A schematic representation of the general production process is shown in figure 4.1.

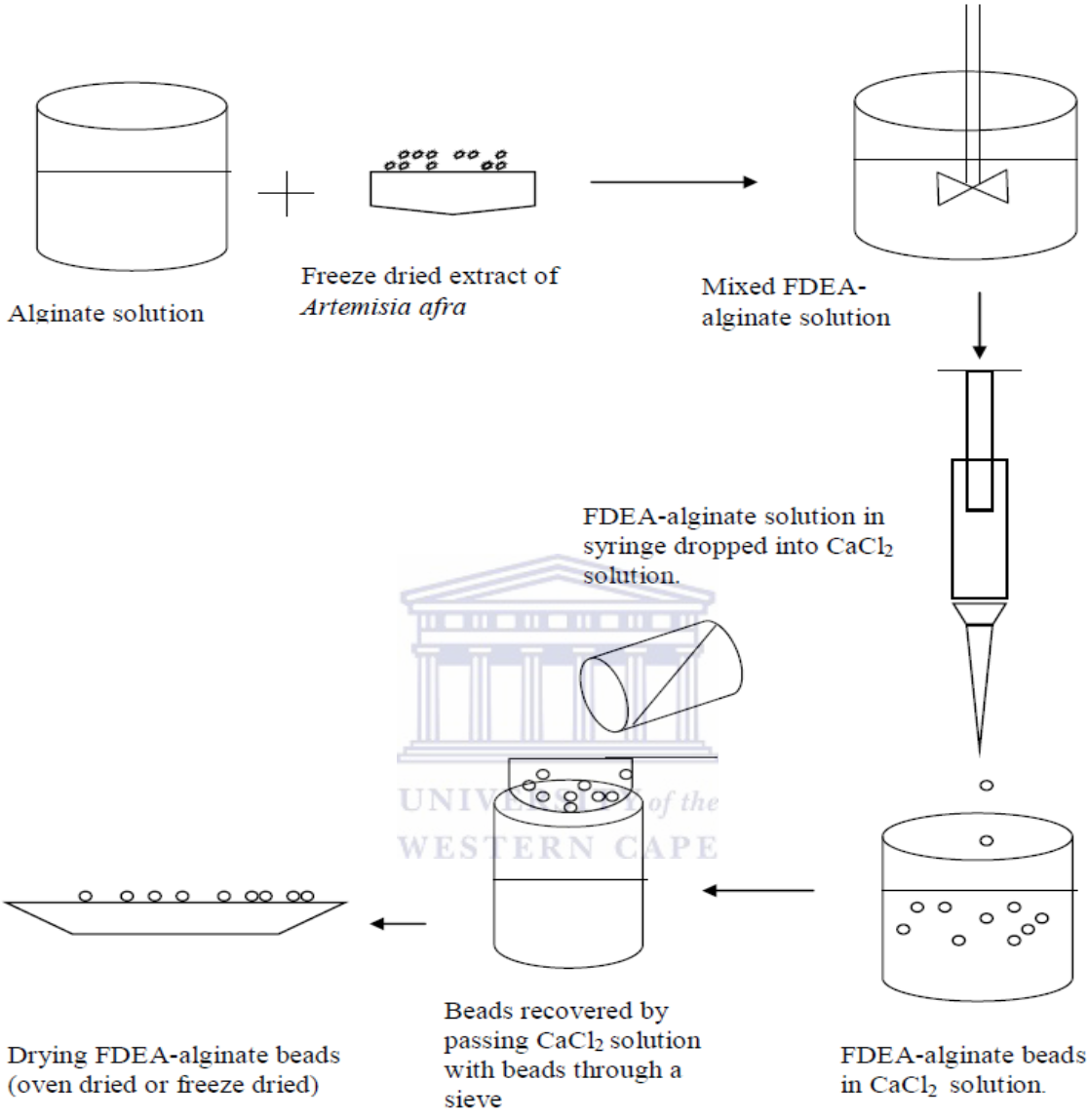

Figure 4.1: A schematic representation of the production of alginate-FDAE beads (adapted from Egieyeh, 2011 unpublished data).

4.3.3 Preparation of polymethylmethacrylate coated alginate extract (PMMA-alginate-FDAE) of *A. afra*

PMMA-alginate-FDAE was prepared by a modified water-in-oil-in-water ($W_1/O/W_2$) emulsion solvent evaporation method using water as internal aqueous phase(Tapas et al., 2011). First, sodium alginate (0.5 - 2.5% w/v) was dissolved in 3 ml water at 30 -35 °C by stirring with a magnetic stirrer for 20 minutes. Then 200 mg of FDAE was added and the solution stirred for a further 20 min. The resulting mixture was drawn up in a 20 ml syringe and then added through a 16 gauge needle to 20 ml solution of PMMA (4% w/v) in dichloromethane containing 1% v/v Span 80 and emulsified at 4000 rpm for 2 min in a homogenizer. The resulting W_1/O primary emulsion was then added through a 16 gauge needle into 100 ml of water containing 1.25% v/v tween 80 and 2% w/v $CaCl_2$ and emulsified at 850 rpm to form a $W_1/O/W_2$ emulsion. Stirring was continued for 1.5 hours with a mechanical stirrer to evaporate off the organic solvent. The resultant microcapsules were separated by decantation, washed thrice with water and then vacuum dried at 60 °C for 8 hours. The microcapsules were stored in a vacuum desiccator until used (Tapas et al., 2011).

4.3.4 Determination of organoleptic and pharmaceutical properties of *A. afra* preparations

In this study, certain organoleptic and pharmaceutical characteristics were chosen to be determined and compared viz, colour, taste, odour, texture, particle size, particle shape, moisture content, aqueous solubility, entrapment efficiency of FDAE in modified forms, total flavonoid content of *A. afra* forms and release of flavonoids (FDAE) from modified *A. afra* forms. Recognizable changes in the organoleptic properties of a material may be an indication of changes in stability of the material. Unpleasant odours, tastes and smell of a material can be masked by addition of sweeteners, flavourants and aroma chemicals (Zheng and Keeney, 2006).

4.3.4.1 Determination of colour and odour of *A. afra* preparations

The colour of the plant material preparations was determined by weighing and placing 100 mg of each untreated plant material preparation and against a white background. The colour observed against the white background was described.

The British Pharmacopoeia 2000(BP., 2000b)method was used to determine the presence or absence of odour in the plant material preparations. For this, 100 mg of each plant material preparation was weighed and placed on a watch glass about 10 cm in diameter. The material was allowed to stand for 15 minutes and then smelled to determine the presence or absence of any odour.

4.3.4.2 Determination of particle size and shape of *A. afra* preparations

Particle size and shape can affect the physico-chemical properties of a dosage form, bulk properties, product performance, processability, stability, bioavailability and appearance of the end product. These parameters also play a significant role in the homogeneity of dosage forms. The sieve and microscopic methods are the commonly used methods for determining particle size and shape. The method of the British Pharmacopoeia (BP., 2000b) was used in this study to determine the particle size grading of the *A. afra* preparations.

Briefly sieves No's 335, 180, 125 and 90 were used for the *A. afra* dried leaves and the FDAE while sieves No's 1700, 1400, 1000 and 500 were used for the alginate and the PMMA-alginate beads of *A. afra.* The sieves were arranged in order of decreasing aperture size with a receiving pan closely fitted at the bottom. The assembled sets of sieves were mounted on a test sieve shaker (Figure 4.2). An accurately weighed amount (10 g) of the plant material preparation was placed onto the top-most sieve and the assembly shook for 20 minutes until sifting was practically complete. The retained powder on each sieve was collected, weighed and calculated as a percentage (w/w) of total feed. The degree of fineness of each feed was made according to the BP 2000 specifications. In this way, the plant materials could be described as being "coarse", "moderately fine", "fine' or "very fine".

The shape of the different plant material preparations was determined by microscopic examination. For this, 5 mg of the FDAE, alginate-FDAE beads or PMMA-alginate-FDAE beads were sprinkled onto a microscopic slide and viewed under a light microscope "Nikkon Abbe 1.25" after which they were micrographed for their particle shapes.

Figure 4.2: Mechanical test sieve shaker.

4.3.4.3 Determination of moisture content of *A. afra* preparations

When a substance is known to be hygroscopic, it is very critical and important to determine its moisture content (EMEA, 1999). The method prescribed in the European Pharmacopoeia 4th edition 2002 (EP., 2002b)was used for the determination of the moisture content of the plant material preparations. This method was used to monitor the change in weight in plant material preparation resulting from any moisture loss. In this method, 0.3 g quantities each of the FDAE, alginate beads and PMMA- alginate beads of *A. afra* were separately weighed in a flat bottom dish, dried in an oven for 3 hours at a temperature of 100 °C, allowed to cool for 10 minutes in a desiccator over anhydrous silica gel and the final weight recorded (EP., 2002b). The moisture content was calculated as a mass percentage using the following formulas;

Moisture weight= Initial weight (Before drying) – Final weight (After drying) -----Eqn. 4.1

Moisture content = (Moisture weight/ Initial weight) X 100% ----------Eqn. 4.2

4.3.4.4 Determination of aqueous soluble fraction of *A. afra* preparations

Solubility is an important property of a drug that determines the dissolution rate of the active ingredient. An active ingredient must be in the solution of gastric fluids, before it can be absorbed into the systemic circulation. Poor solubility and dissolution are undesirable properties of the active ingredient in oral solid dosage forms. In the case of the *A. Afra* preparations, the yield of extract obtained with distill water depends on the water-soluble matter present in the powder.

In this study, the aqueous soluble fraction of the plant material preparations was determined at room temperature as follows: First, 0.2 g of plant material was accurately weighed in a 5 ml eppendorf tube, mixed with 2 ml of distilled water and vortexed for 1 minute and the mixture then kept at room temperature for 8 hours with additional vortexing after every hour. At the end of the extraction, the mixture was filtered under vacuum, the residue collected on a previously weighed filter paper, the residue plus filter paper oven dried at 35 °C for 4 hours and then left in open air for an hour to equilibrate at room temperature. The joint weight of dried filter paper and residue was noted and the weight of the filter paper subtracted to obtain the weight of the residue. The solubility or water extractable matter was then determined by subtracting the weight of the residue from the total plant material weight and expressing it as a percentage i.e.

$$\text{Solubility} = \frac{\text{(Weight of initial plant material – Weight of dried residue)}}{\text{Volume of solvent}} \text{ X 100\%- - - Eqn. 4.3}$$

From the results obtained and the descriptive phrases provided in the BP 2000 (Table 4.1) the solubility of each plant material was then derived.

Table 4.1: Common descriptive phrases of solubility and the corresponding quantitative solubility ranges as per BP 2000.

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

4.3.4.5 Evaluation of entrapment efficiency of FDAE in alginate beads and PMMA-alginate beads

The alginate-FDAE and PMMA-alginate-FDAE beads were prepared as explained in sections 4.3.2 and 4.3.3, respectively. Three samples (100 mg) each of the alginate and PMMA-alginate beads were weighed in 15 ml test tubes, 10 ml of simulated intestinal fluid (phosphate buffer solution pH6.8, without enzyme) added, and the mixture warmed at 37 °C and stirred overnight using a magnetic stirrer. Thereafter the mixture was allowed to settle for 30 minutes and 3 ml aliquots of the supernatant containing the extract drawn into a 5 ml syringe, filtered through a 0.45 µm filter (with the first 0.5 ml filtrate discarded) and filtrate collected in an eppendorf tube. From each filtrate 200 µl was transferred to a microplate well and its absorbance measured at 349 nm using a microplate reader. A calibration curve of the 349 nm absorbance of solutions containing various amounts of dissolved FDAE in distilled water *versus* concentration was then established and used to determine the amount of FDAE released from the beads.

Finally, the amount of extract in the beads was compared to the expected amount and the entrapment efficiency calculated as:

$$\%EE = (A_D/A_E) * 100 \text{ ------- Eqn. 4.5}$$

Where EE = entrapment efficiency,

A_D=the determined amount of extract per gram of beads and,

A_E=the expected amount of extract per gram of beads.

This was done for triplicate samples and the average %EE of FDAE for alginate-FDAE *versus* PMMA-alginate-FDAE beads compared.

4.3.4.6 Determination of total flavonoid content of *Artemisia afra* preparations

The total flavonoid content of the preparations was estimated using the aluminium chloride colorimetric method (Lamien-Meda et al., 2008, Hoerudin, 2004) with some modifications. Luteolin was used as the reference and a standard curve of known concentrations of luteolin was generated. A stock solution of luteolin was prepared by dissolving 2 mg of luteolin in 2 ml of 50% ethanol in water (v/v). Standard working solutions of concentrations 2.5, 20, 50, 100, 200, and 250 µg/ml were made up by diluting aliquots of the stock solution and diluting with 50% ethanol to achieve the above mentioned concentrations.

To a 15 ml test tube, 0.5 ml of each standard solution, 1.5 ml of 95% ethanol (v/v), 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water were added and the mixture incubated at room temperature for 30 minutes. Thereafter, 200 µl of the reaction mixture was transferred to a 96 well plate and its absorbance determined at 349 nm using a microplate reader. The absorbance *versus* luteolin concentration data was inserted GraphPad™ Prism, the standard curve established and the latter used for the quantification of the total flavonoids in the *A. afra* samples. For the blank, the 10% aluminium chloride was substituted with an equivalent volume of distilled water.

For the determination of the total flavonoid content in the *A. afra* preparations, 2.5 mg of each plant preparation were weighed in a 5 ml screw capped Kimax test tube, 0.5 ml of distilled water

added and the mixture vortexed for 3 minutes to dissolve and provide the unhydrolysed samples. For hydrolysed samples, 1 ml of 2M hydrochloric acid was added to the solution, heated in a water bath at 80 °C for 40 minutes, allowed to cool and 1ml of 2M sodium hydroxide solution added to neutralize the solution. Then the solutions were reacted with aluminium chloride as described above and the flavonoid concentration in each hydrolysed and unhydrolysed plant test sample determined from, the luteolin standard curve generated with GraphPad™ Prism. Finally, the average total flavonoid levels in 3 replicates of each *A. afra* preparation was determined and compared.

4.3.4.7 Evaluation of the release characteristics of modified*Artemisia afra*preparations

The release study was performed on 200 mg of each of the *Artemisia afra* preparations in Simulated Gastric Fluid (HCl solution, pH 1.2, without enzyme) and Simulated Intestinal Fluid (phosphate buffer solution pH 6.8, without enzyme), prepared according to the United States Pharmacopoeia 29 (USP) (http://www.pharmacopeia.cn/v29240).

Briefly, 20 ml volume of SIF or SGF was placed in a 50 ml beaker, covered and heated to 37 °C in a water bath while the temperature was monitored with a thermometer. When a constant temperature had been achieved, 200 mg samples (alginate-FDAE beads and PMMA-alginate-FDAE beads) were added into the fluid and the mixture stirred with a magnetic bar. Samples of the fluid (1 ml) were withdrawn at 5, 10, 20, 30, 40, 50, 70 and 90 minutes and replaced with same volume of fresh fluid in order to maintain constant volume. The withdrawn samples were centrifuged, the supernatant collected and 200 µl of the supernatant placed in a 96 well plate. The FDAE content was determined by reading the absorbance of triplicate samples of each herbal preparation at 349 nm. To determine the amount of FDAE released, a calibration curve of the 349 nm absorbance values obtained for various concentrations (50-300 µg/ml) of FDAE in simulated intestinal fluid was established and used to quantitate the amount of FDAE in the samples obtained for the 2 other preparations. From the amount released at each time point, the cumulative amount of FDAE extract released *versus* time was generated, analysed by GraphPad™ Prism and the percentage release of FDAE at 30 minutes (T_{30}) determined and compared for the alginate-FDAE and PMMA-alginate-FDAE beads in the different media.

To determine the mechanism of release of the extract (FDAE) from FDAE-alginate and PMMA-alginate FDAE beads,a simple relationship which describes drug release from a simple swellable polymeric matrix derived by Peppas *et al*., was used. This relationship is given by the Korsmeyer-Peppas model which is given by:

$$F = Kt^n \quad \text{----------Eqn. 4.6}$$

where F = the percentage fraction of extract released at time t,

K = the release rate constant incorporating the structural and geometric characteristics of the drug dosage form,

n = the release exponent indicative of the drug release mechanism.

The n value is used to characterize differential release and the value of n characterizes the release mechanism of drug.

4.3.5 Development and validation of HPLC method

Luteolin was selected as a marker for the comparison of level of active compound in the different preparations of *A. afra*. The luteolin levels in FDAE, alginate-FDAE beads and PMMA-alginate-FDAE beads were determined by HPLC using a reverse phase method. Separation was effected on a Phenomenex Luna C-18 column with 4 µm particle size and a column length of 250 x 4.60 mm i.d; 5u. The mobile phase consisted of 0.1% formic acid aqueous solution (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The elution gradient was: 0-22 min 15%B; 22-27 min 100%B; 27-33 min 15%B. The solvents were filtered and degassed by membrane filtration prior to use. The column temperature was kept at 25 °C, eluent pumped at flow rate of 0.8 ml/min, 20 µl samples injected and the luteolin peaks detected at 350 nm. As internal standard morin was used, mainly because it has similar physicochemical properties to luteolin, is extracted in ethyl acetate and absorbs at the same wavelength as luteolin i.e. 350 nm.

To validate the assay, the following parameters were determined: linearity, precision and accuracy, lowest limit of detection and lowest limit of quantification.

In performing the validation, stock solutions of luteolin and morin were prepared in 50 % methanol water and stored in a refrigerator at -80 °C until use. Standard solutions were prepared by diluting the luteolin stock solution in 50% methanol water to a concentration range of 4.3 – 26 µg/ml and internal standard morin to obtain a concentration of 100 µg/ml. For the standard curve 100 µl of morin was added to each luteolin standard solution and the ratios of luteolin: morin peak area *versus* luteolin concentration plotted. GraphPad™ Prism was used for the plotting of the standard curve and determination of curve linearity (R^2) and concentration in the unknownsamples. The precision of the analytical method was determined by assaying 3 replicates of the low, medium and high concentration standard solutions on three consecutive days and on each occasion the average, standard deviation and % relative standard deviation (RSD) calculated and compared. The accuracy of the method was determined from the mean concentrations obtained for the replicates and the percentage difference. The limit of detection (LOD) and quantitation (LOQ) for luteolin was determined using the response (peak area) with LOD being determined as the analyte concentration giving a response 3 times that of the noise level i.e. signal to noise ratio 3:1 and with mean baseline noise of 0.4 mAU and the LOQ as the lowest concentration where an accuracy better than 20% was achieved (Ruckert et al., 2004). Lastly the percentage bias was determined as the difference between the concentration measured and the prepared concentrations expressed as a percentage of the prepared concentration.

4.3.6 Quantification of luteolin in the *Artemisia afra* preparations

For the quantification, the HPLC assay was used and the FDAE, alginate-FDAE and PMMA-alginate-FDAE were assayed in two ways, the acid-hydrolysed and unhydrolysed methods. The latter provides the level of free flavonoid and the former the level of total flavonoid.

First, hydrolysed and unhydrolysed sample solutions of the *A. afra* plant preparations were prepared. For the unhydrolysed samples, 75 mg of each plant preparation was placed in labelled 15 ml conical-shaped centrifuge tubes, 100 µl of morin (IS, 100 µg/ml) and 3 ml of aqueous methanol (50%) added and the solution vortex mixed for 1 minute. For the hydrolysed plant sample solution, 3 ml of acidic aqueous methanol (MeOH: 2NHCL 50:50) was added, the mixture heated in a water bath at 80 °C for 40 minutes and then cooled. To both hydrolysed and

unhydrolysed plant sample solutions, 5 ml ethyl acetate was added, the mixture vortex mixed for 3 minutes and centrifuged for 15 minutes at 3500 rpm. Using a pipette, the ethyl acetate supernatant was transferred to a clean test tube and evaporated to dryness under a gentle stream of nitrogen gas. The residue was reconstituted in 1 ml of mobile phase solution (water: acetonitrile 50: 50), the mixture transferred to HPLC vials and 20 µl aliquots injected unto the HPLC column. From the chromatograms the peak areas for luteolin and internal standard peaks were obtained.

To identify luteolin in the plant samples, the UV spectra and retention times obtained from the chromatograms of the plant samples were compared to that obtained with the pure luteolin standard solutions. Finally, the height of the luteolin peak was noted, the luteolin: internal standard peak area ratio calculated and the levels of free or total luteolin in each sample determined from the standard curve regression equation. Three replicates of each preparation were done and average values for the 3 plant materials compared using student's t-test.

4.4 Results and discussion

4.4.1 Preparation of plant materials

The characteristics of the freeze dried aqueous extract (FDAE), alginate-FDAE and PMMA-alginate-FDAE beads of *A. afra* prepared as described in sections 4.3.1, 4.3.2 and 4.3.4 are summarized in table 4.2. Three batches of the FDAE were prepared and the particle size of the leaves, the temperature of the solvent and the time duration for the extraction were constant for all extractions. The average yield for the freeze dried aqueous extract was 21.8% and this was similar to that obtained by previous investigators e.g. 19.9% by Komperlla (2005) and 21.96% by Dube (2006).

The yields for the modified forms were slightly lower at 16.5% and 12.6% for alginate-FDAE beads and PMMA-alginate-FDAE beads respectively (Table 4.2). In the preparation of the FDAE, 2 g of dried leaves was calculated to be equivalent to 8 g of dried leaves used to produce one 150 ml cup of the traditional infusion. For the modified forms, 4 g of FDAE was used to prepare the alginate-FDAE and PMMA-alginate-FDAE beads. The increase in quantity of FDAE used to prepare the modified forms was to compensate for any subsequent leaching that may occur from the beads into the calcium chloride solution and dichloromethane for the alginate-FDAE beads and PMMA-alginate-FDAE respectively. The small difference in yields seems to reflect the FDAE entrapment efficiency in these modified forms (Table 4.2).

4.4.2 Organoleptic and pharmaceutical properties of the FDAE, alginate-FDAE beads and PMMA-alginate-FDAE beads of *Artemisiaafra*

A summary of the organoleptic and pharmaceutical properties of the *A. afra* forms is shown in table 4.2 and pictures of the FDAE, alginate-FDAE and PMMA-alginate-FDAE are given in figures 4.3, 4.4 and 4.5 respectively.

Table 4.2: Organoleptic and pharmaceutical properties of *Artemisia afra* forms.

Test	FDAE	Alginate-FDAE	PMMA-alginate-FDAE
Yield (%)	21.733 ±1.850	16.543 ± 0.039	12.613 ± 0.044
Physical appearance	Brittle, hygroscopic	Small free flowing spherical beads	Small free flowing spherical beads
Colour	Light brown	Dark brown	Brown
Odour	Aromatic	Pungent	Pungent
Taste	Bitter	Bitter	Bitter
Particle size	Coarse	Not gradable	Not gradable
Moisture content (%)	10.68 ± 0.727	6.759 ± 0.151	8.034 ± 0.279
Water soluble fraction (%)	83.858 ± 1.753	7.494 ± 0.492	0.939 ± 0.440
Entrapment efficiency(%)	N/A	42.812 ± 0.979	37.603 ± 4.599
Total flavonoid content in hydrolysed samples (µg LE/mg of extract)	19.905	12.133	3.676
Total flavonoid content in un-hydrolysed samples (µg LE/mg of extract)	24.533	11.257	2.609

Where LE = luteolin equivalent.

Figure 4.3: Photomicrograph of FDAE (4X).

Figure 4.4: Photomicrograph of alginate-FDAE beads (4X).

Figure 4.5: Photomicrograph of PMMA-Alginate-FDAE beads (4X).

The freeze dried aqueous extract of the *Artemisia afra* leaves were initially light brown in colour but rapidly changed to a dark brown colour because of its hygroscopicity. The alginate-FDAE beads were dark brown in colour unlike the PMMA-alginate-FDAE which was brown in colour. The dripping techniques used in the production of the beads produced spherical droplets which upon falling down into the calcium chloride solution resulted in spherical gel particles due to the ionic interactions between the alginate and the Ca^{2+} ions (Finotelli et al., 2010). The FDAE, alginate-FDAE and PMMA-alginate-FDAE had unpleasant odours and, taste and presence of such characteristics normally will result in poor patient acceptability of dosage form. However, such negative characteristics can be masked with excipients when the extracts are incorporated into a tablet or capsule form.

4.4.2.1 Particle size and shape of *Artemisia afra* preparations

Particle size and shape are crucial parameters worth considering in the manufacture of dosage forms in that they influence dissolution and bioavailability. Under the microscope, the FDAE particles appeared to be irregularly shaped while the alginate-FDAE and the PMMA-alginate-FDAE beads appeared to have a fairly regular spherical shape (Figures 4.4, 4.5 and 4.6). The particle size distribution of the various preparations of *A. afra* is given in table 4.2, figure 4.6 and Appendix 3. According to the BP 2000 classification system, *A. afra* dried leaf powder and the freeze dried aqueous extract powder were both classified as coarse, with the FDAE appearing to be much finer than the *A. afra* dried leaves. The BP 2000 classification was also used to classify the size distribution of the modified forms. On this basis, the alginate-FDAE beads and the PMMA-alginate-FDAE which were seen to be non-uniform in particle size (due to the variation in the sizes of the beads obtained) were thus classified as not gradable (Table 4.2). The ranges in particle size seen with the modified forms may have an impact in dosage form preparation such as causing inconsistent weight and content of dosage form. An option to prevent this problem is the removal of all the bigger particles by additional sieving. Figure 4.6 shows the particle size range of the different *A. afra* plant preparations.

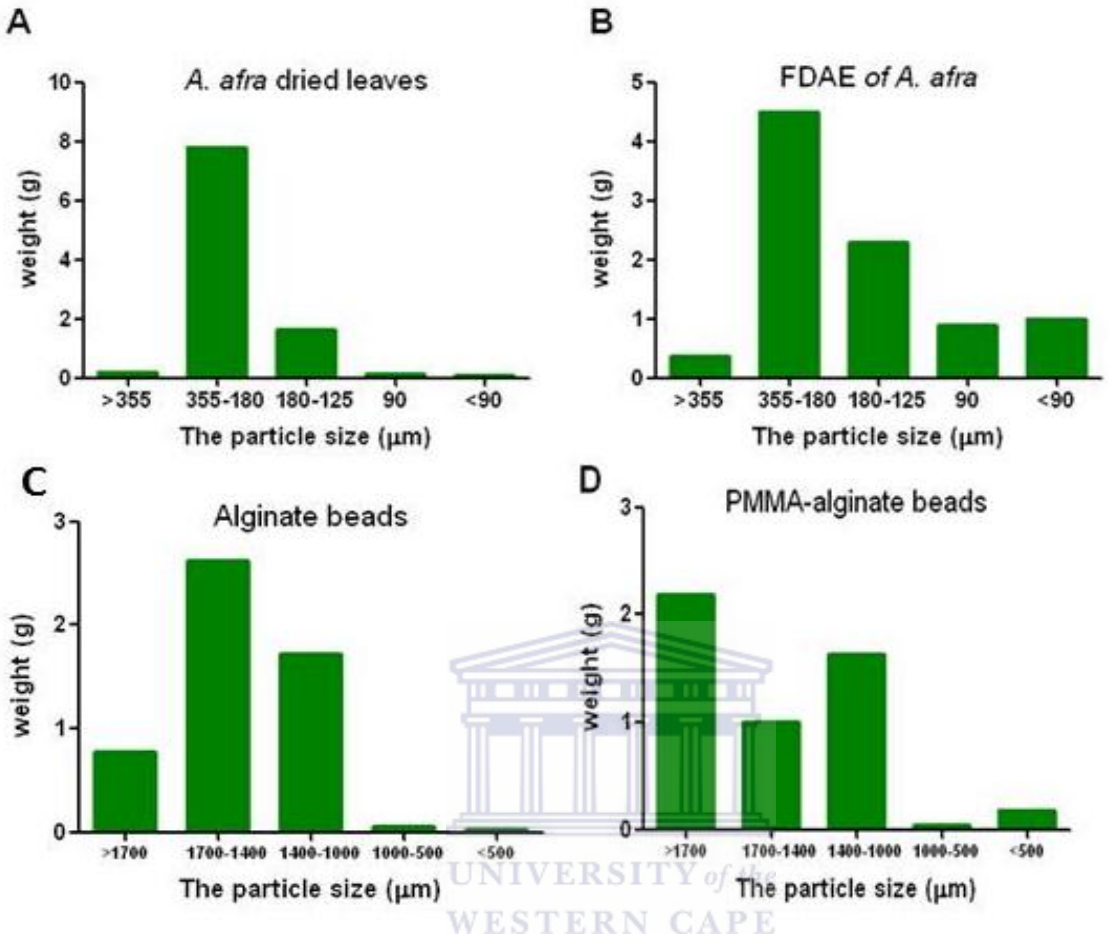

Figure 4.6: Particle size of A) *A. afra* dried leaves, B) FDAE of *A. afra*, C) Alginate beads and D) PMMA-alginate beads measured using the sieve method.

4.4.3 Moisture content of *Artemisia afra* preparations

The percentage moisture content data for the FDAE, alginate beads and PMMA-alginate beads of *A. afra* is given in Appendix 4 and summarised in table 4.2. The average moisture content for FDAE, alginate-FDAE beads and PMMA-alginate-FDAE beads were 10.68 ± 0.73%, 6.76 ± 0.15%, 8.03 ± 0.27% respectively. The FDAE is known to be hygroscopic, thereby explaining its higher moisture content compared to that of the other preparations. The moisture content of the modified preparations of the FDAE is low and this is an indication that they are less hygroscopic than the FDAE. The low moisture content observed in the modified forms could be due to the excipients added to the FDAE and the methods of preparation used to modify the FDAE into

beads. In addition, at room temperature, the physical appearance of the FDAE changed from a light brown powder to a sticky dark brown gum thus indicating that its moisture content increases with increasing storage hours. Unlike the FDAE, the physical appearance of the modified forms is stable on storage without any visible physical changes suggesting that they were reasonably non-hygroscopic. A full investigation on their hygroscopicity was however not done in this study.

4.4.4 Aqueous soluble fraction of *Artemisia afra* preparations

The results of the experiments to determine the aqueous solubilities obtained at room temperature for the FDAE, alginate-FDAE beads and PMMA-alginate-FDAE beads of *A. afra* are given in Appendix 5 and summarised in table 4.2. For oral solid dosage forms, aqueous solubility is a crucial factor influencing the bioavailability of drugs and the solubility of compounds may be described as per the description given in table 4.3.

Table 4.3: Common descriptive phrases of solubility and the corresponding quantitative solubility ranges as per BP (2000).

Description	Approximate Weight Of Solvent (G) Necessary To Dissolve 1 G Of Solute
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1000
Very slightly soluble	1000 to 10,000
Practically insoluble	More than 10, 000

On average, 25.8 mg of the FDAE of *A. afra* was soluble in 1 ml of distilled water (Appendix 5.1) thus indicating that 1000 mg of FDAE powder required approximately 38.7 ml of distilled water to dissolve and that the FDAE powder could be categorized as being sparingly soluble. For the alginate-FDAE beads of *A. afra*, 1000 mg of the beads required approximately 441 ml of distilled water to dissolve and therefore it could be classified as being slightly soluble in water. Lastly, for the PMMA-alginate-FDAE beads, 1000 mg of the beads required approximately 3529 ml of distilled water to dissolve classifying it as being very slightly soluble. As expected the 2 modified forms were less soluble in water than the FDAE, but even the aqueous extract of FDAE can only be classified as being sparingly soluble in water at room temperature. This suggests the traditional tea infusion method of preparing the FDAE (as was used in this study) resulted in a preparation containing additional solutes (and possibly active compounds) that are not fully soluble in water at room temperature. The method used to prepare extracts used to establish or evaluate the pharmacological effects of *A. afra* aqueous preparation can thus possibly be an important factor to keep in mind.

4.4.5 Entrapment efficiency of FDAE in modified *Artemisia afra* preparations

Entrapment efficiency is the ratio of the determined drug content in the formulation to the loaded amount (experimental drug content). The average entrapment efficiency of alginate-FDAE beads and PMMA-alginate-FDAE beads was 42.812% and 37.603% respectively (Tables 4.2 and Appendix 6). At present, there is very little information on the entrapment efficiency of herbal material in alginate or polymethylmethacrylate preparations but EE% of 51.7% to 68.6% was found for curcumin in alginate beads (Song, 2012 #106).The loading efficiency of water soluble drugs in alginate beads is much lower than that of water insoluble drugs (Aslani and Kennedy, 1996) and this is due to leakage of drugs from alginate beads having large gel porosity (Liu et al., 1997). The low values obtained in this study may thus be the result of leaching of the water soluble FDAE extract from the beads into the calcium chloride solution. The drug entrapment efficiency is important in bead drug delivery systems because the actual amount of drug in the beads is what will be available for absorption (Ito et al., 2010). In all, the entrapment efficiency of the alginate-FDAE and PMMA-alginate-FDAE bead formulation was fair but may be improved.

4.4.6 Determination of total flavonoid content in the *Artemisia afra* preparations

This experiment was done to determine the total flavonoid content (TFC) of the plant material using the aluminium chloride method. Luteolin was the standard used to establish the calibration curve (Figure 4.7), which was linear from 20 to 250 µg/ml (r^2 = 0.9934). The total flavonoid content of the plant materials were expressed as luteolin equivalent (LE) per mg of extract and are shown in appendix 7and figure 4.8.

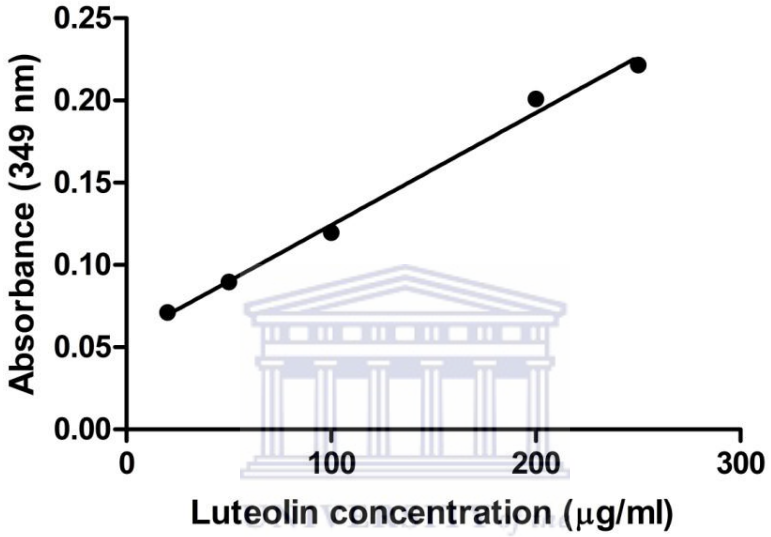

Figure 4.7: Standard curve of luteolin concentration *versus* absorbance at 349 nm used for the quantification of the total flavonoid content in the different *A. afra* preparations (r^2 = 0.9934; slope = 0.0007 ± 0.0559).

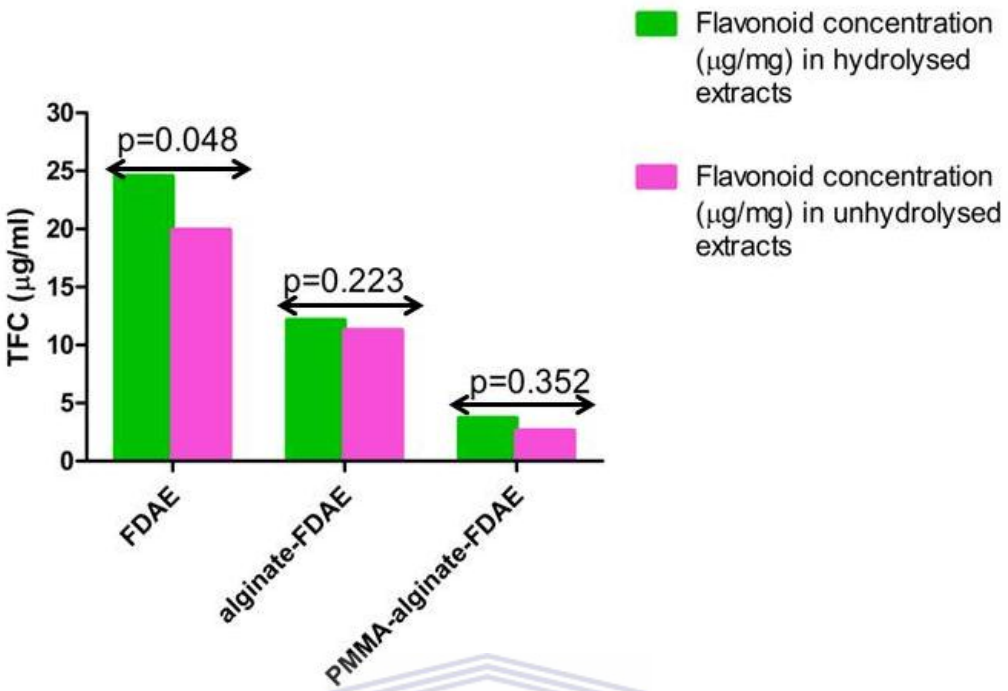

Figure 4.8: The total flavonoid content (µg LE/mg) of the hydrolysed and unhydrolysed extracts of *A. afra* plant preparations. The P values are for the t-test of TFC in hydrolysed vs. unhydrolysed plant preparations.

Flavonoids are one of the most widespread and diverse group of natural phenolic compounds. The flavonoids in the *A. afra* plant combined with aluminium to form a complex flavonoid-aluminium that could be measured. Using the standard curve generated for luteolin ($r^2 = 0.9934$), the total flavonoid content of the various *A. afra* preparations (µg of LE/mg of *A. afra* plant preparation) was found to vary from 3.67 µg/mg in hydrolysed PMMA-alginate-FDAE beads, 12.133 µg/mg of hydrolysed alginate-FDAE beads to 24.5 µg/mg in hydrolysed FDAE.

When the FDAE of *A. afra* plant was subjected to acid hydrolysis, the level of TFC was significantly increased compared to the level in the unhydrolysed FDAE (t-test $p_{FDAE} = 0.0482$). However, when the modified preparations were subjected to hydrolysis the levels of TFC in both preparations were relatively similar as compared to the unhydrolysed preparations (i.e. t-test $p_{alg.beads} = 0.223$ and $p_{pmma\text{-}alg.} = 0.352$). Compared to the FDAE, the 2 modified products thus contained very little acid hydrolysable (i.e. conjugated) flavonoids. Plant preparations normally contain several different flavonoids. The increase in total flavonoid content observed after acid-hydrolysis can be explained by the fact that glycosides (and other conjugates) present in the plant preparations, broke down to produce flavonoid aglycones that were able to form complexes with

aluminium chloride by binding at the *hydroxy-oxo- and ortho-hydroxy-oxo*-groups (Smirnova and Pervykh, 1998). The FDAE thus had a fair proportion of its flavonoid content in this glycoside (or conjugate) form but not the modified preparations. The latter contained more of its flavonoids in aglycone form.

Many investigators determined the total flavonoid content in foods and plants using the aluminium chloride method used in this study. Meda *et al*., (2004) were able to determine the TFC in Burkina Faso honey using this method and they reported that the method was only specific for flavanols and flavones and thus did not represent the real total flavonoid content. Thus in this study, a high performance liquid chromatography method was used to quantify luteolin in the *A. afra* plant preparations.

The % entrapment efficiencies of 42.8% and 38.6% for alginate-FDAE and PMMA-alginate-FDAE are reflected in the total flavonoid content of these forms. From the TFC (µg of luteolin/mg of bead) values, it can be calculated that to formulate a 250 mg capsule containing alginate-FDAE or PMMA-alginate-FDAE, each will contain approximately 3 mg and 0.9 mg of luteolin respectively. These values of active herbal ingredient (AHI) seem insufficient and impractical to be formulated into a herbal medicine dosage form expected to produce a therapeutic response. Thus the loading of active herbal ingredient (sufficient to produce a therapeutic effect) into the plant product is of great importance and suggests that modified forms should be prepared in a manner in which they contain a higher load of active ingredient per mg of material.

4.4.7 Release of FDAE from modified *Artemisia afra* preparations in gastrointestinal fluids

Multi-unit controlled release dosage forms pass via the gut in a manner that avoids the gastric emptying and different transit rates (Breimer, 1980) and release drugs more uniformly in a predictable manner (Follonier and Doelkar, 1992). Consequently, much research efforts has been concentrated on the development of micro particles using natural polymers as they are derived from natural sources, do not require organic solvent during manufacture, is readily available and qualified for a number of chemical modifications (Vyas and Khar, 2002). One objective of this

study was to use alginate and polymethylmethacrylate to produce beads containing the freeze dried aqueous extract as active herbal ingredient that will be released slower and more uniformly when taken orally.

Consequently, the *in vitro* release of FDAE from alginate-FDAE and PMMA-alginate-FDAE beads in simulated gastric fluid and simulated intestinal fluid was investigated and the release characteristics of the FDAE from the modified preparations obtained is shown in figure 4.9.

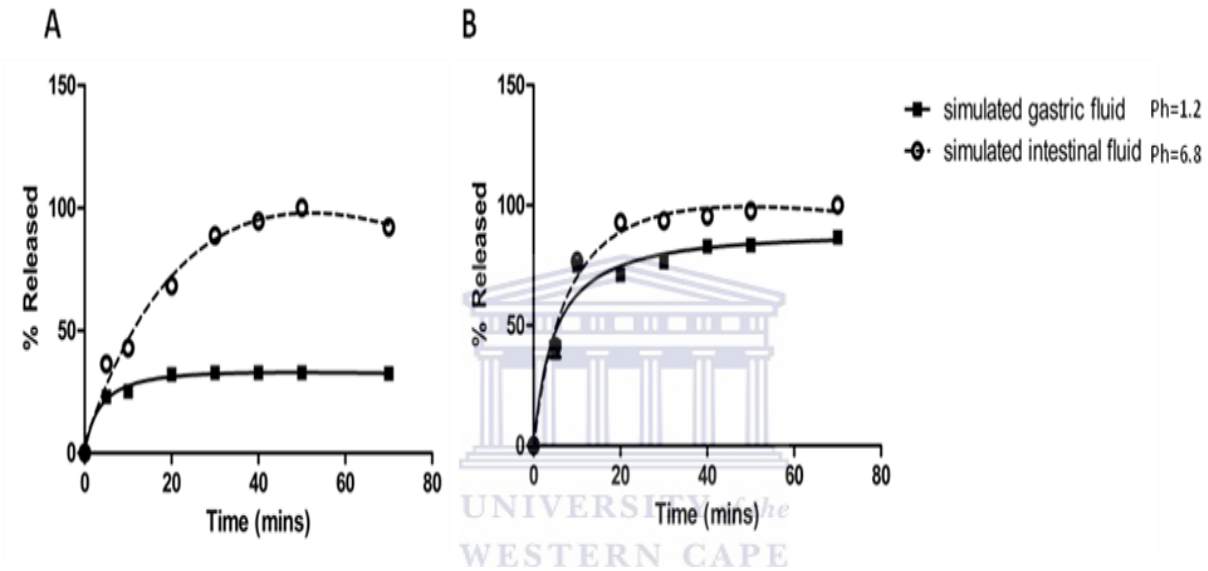

Figure 4.9: The percentage release profiles of FDAE from (A) alginate-FDAE beads and (B) PMMA-alginate-FDAE beads in SIF and SGF determined using UV at 349 nm.

At 30 minutes at pH 1.2, alginate-FDAE released 32.780% of FDAE and PMMA-alginate-FDAE released 76.297% of FDAE (Table 4.5) whileat 30 minutes at pH 6.8, alginate-FDAE released 88.718% of FDAE and PMMA-alginate-FDAE released 93.518% of FDAE (Table 4.5).

For both the alginate-FDAE and PMMA-alginate-FDAE rapid release of FDAE occurs at pH 6.8 whereas only 32.780% of FDAE is released from alginate-FDAE and 76.297% of FDAE released from PMMA-alginate-FDAE atpH 1.2 respectively. The faster and more extensive FDAE release in SIF could possibly be due to the fact that the beads swelled and eventually burst giving rise to the increase in the release of extract. Similar rapid release of water soluble drugs from alginate beads at higher pH have been reported (El-Kamel et al., 2003) and this has been

considered as a major problem in sustaining drug release in SIF. Previous data has shown that alginate beads swell less at pH 1.2 thus slow penetration of SGF into the almost unswelled beads. The slow penetration together with complex drug release mechanisms involving displacement of drug from alginate by the counter ions present in the medium and subsequent diffusion of the free drug out of the beads were responsible for the slow release of the drug from alginate beads in SGF (Halder et al., 2005). This therefore suggests that in the stomach (at low pH), minimum release of FDAE (active herbal ingredient) from the beads will be achieved while in the intestines at higher pH maximum release of FDAE will be effected from the beads.

The alginate-FDAE and PMMA-alginate-FDAE beads alone do not seem suitable as oral controlled release dosage forms. Some studies have shown that the incorporation of resinate to the alginate beads appeared to extend drug release up to 6 hours although the major portion (90%) of the drug was released in 1.5 hours (Ray et al., 2008), thus the resin alginate beads provided marginal improvement in prolonging drug release in SIF. In conclusion, a pH responsive release profile displayed by the alginate-FDAE and PMMA-alginate-FDAE beads was observed, with faster release observed at a higher pH in SIF (6.8) compared to SGF (1.2) (Figure 4.9). The release can however be extended over a longer period by the incorporation of resinate to the beads containing the active herbal ingredient to obtain a more controlled release.

To ascertain the release mechanism of the extract from FDAE-alginate and PMMA-alginate FDAE beads in SIF and SGF (i.e. obtain the n value describing the release mechanism as given in table 4.4), the data obtained from the *in vitro* release experiment was fitted to the Korsmeyer and Peppas model (Korsmeyer et al., 1983, Hayashi et al., 2005). The values of n obtained (for alginate-FDAE and PMMA-alginate-FDAE beads) were less than 0.5 in both SGF and SIF as shown in table 4.5. This indicated that the release from both modified forms was predominantly by classical Fickian diffusion. Thus the n values of > 0.5 obtained had no relevant significance in the release pattern observed for alginate-FDAE and PMMA-alginate-FDAE in both SIF and SGF. For this study, it was observed that the addition of alginate and polymethylmethacrylate, reduced the swelling and erosion of beads thus facilitating drug release by Fickian diffusion.

Table 4.4: Release exponent and equivalent release mechanism.

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

Table 4.5: Parameters of release and goodness of fit to Korsmeyer- Peppas model.

	***A. afra* preparation**	**Release rate (T_{30}) %**	**Korsmeyer-Peppas model**	
			Release exponent, n (Mean ± SD) n = 2	**Regression coefficient, r^2**
SIF	Alginate-FDAE beads	88.718	0.367 ± 0.026	0.94125
	PMMA-alginate-FDAE beads	93.518	0.232 ± 0.019	0.9327
SGF	Alginate-FDAE beads	32.780	0.136 ± 0.039	0.9648
	PMMA-alginate- FDAE beads	76.297	0.202 ± 0.019	0.9362

4.4.8 Development and validation of the HPLC assay

A reverse phase HPLC method using morin as an internal standard was developed for the quantitation of luteolin in different preparations of *A. afra.*

Under the HPLC conditions described in section 4.3.5, good symmetrical peaks with retention times of 13.87 ± 0.05 and14.31 ± 0.03 min were found for morin and luteolin respectively (Figures 4.10 and 4.11). The standard curve of peak height ratio *versus* luteolin concentration was linear (r^2= 0.9917) over the concentration range of 4.3 to 26 µg/ml (Figure 4.12) and, described by the equation Y = 0.1354X ± 0.2076 (where Y= peak height ratio, X = luteolin concentration in µg/ml). The salient parameters for the validated assay are shown in table 4.6.

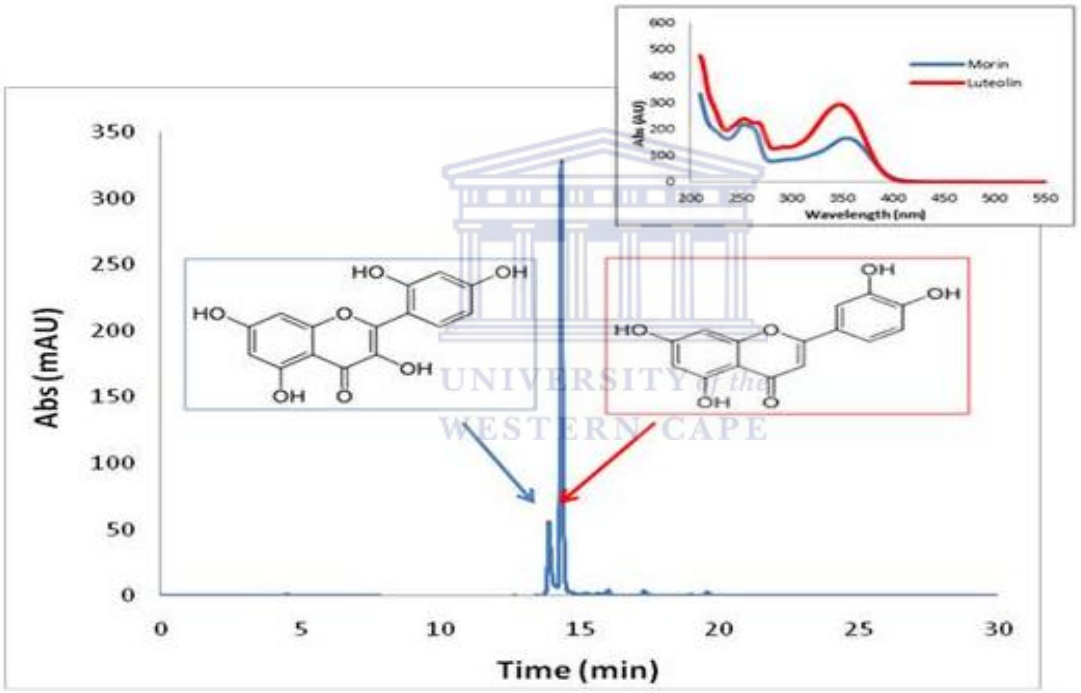

Figure 4.10: HPLC chromatogram for the luteolin reference standard and morin (internal standard) at 370 nm. Insert: UV/V is spectrum for the reference compound at 14.3 min (indicated in red on chromatogram).

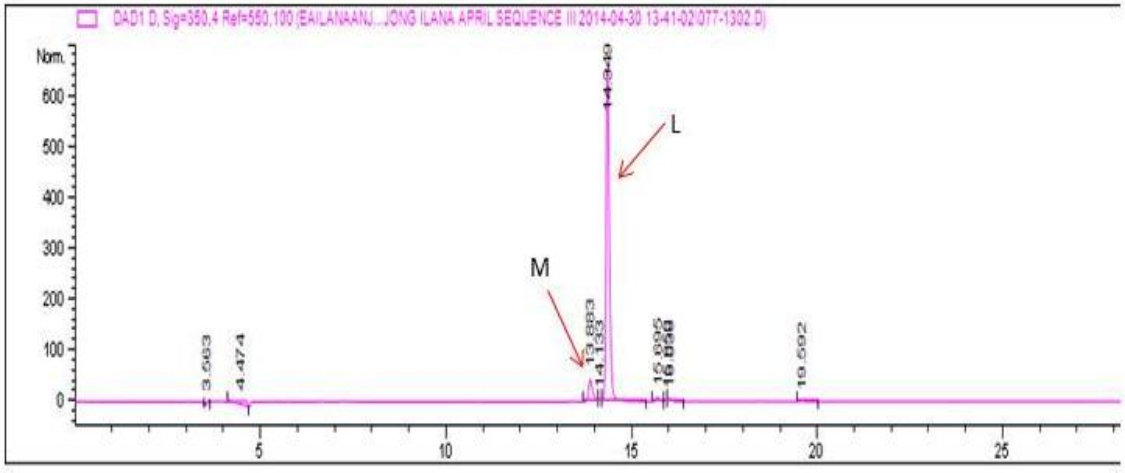

Figure 4.11: Representative HPLC chromatogram of morin (peak M) at retention time 13.9 and luteolin (peak L) at retention time 14.3.

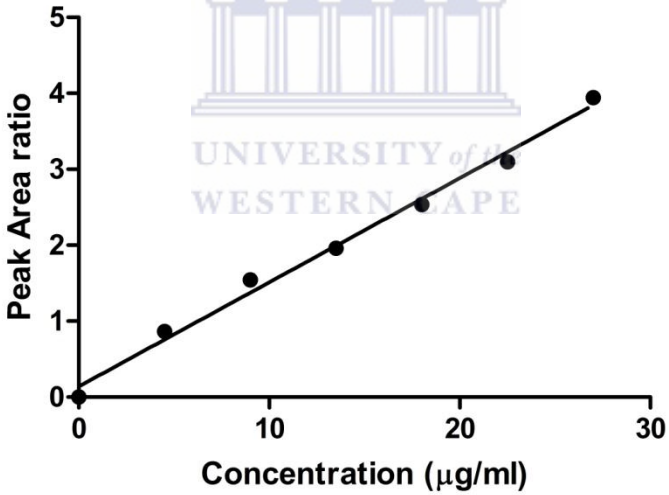

Figure 4.12: Luteolin standard curve used in the quantitation of luteolin in *Artemisia afra* plant preparations by HPLC assay. Linear regression equation: Y = 0.1354X ± 0.2076; r^2= 0.9917.

Table 4.6: Assay validation parameters for luteolin quantification in *Artemisia afra* preparations

Validation parameters	Luteolin Assay
Retention time of luteolin (min)	14.3
Linearity-Regression of coefficient (R^2)	0.9917
Inter-assay precision (RSD in 3 days) (%)	4.895
LOD(ng on column)	22.4
LOQ (µg/ml)	4.3

The results of the inter-day and intra-day accuracy and precision of the assay are shown in appendix 8.1. The limit of detection was found to be 22.4 ng on column, from an injected volum of 20 µl with a corresponding concentration of 1.3 µg/ml (Appendix 8.2) . The concentration of 4.3 µg/ml had accuracy greater than 20% and was therefore considered to be the limit of quantitation (LOQ) for the assay. The average inter-day precision was found to be 4.895 ± 1.421%. In this study, the absorbance of luteolin was read at 350 nm which differed slightly from the 349 nm maximum wavelength for luteolin recommended by Markham (Markham, 1982). Overall, these values indicated good validity and reproducibility of the assay.

4.4.8.1 Identification and level of luteolin in *Artemisia afra* preparations

The above described validated HPLC assay was used to identify and quantitate the levels of luteolin in the unhydrolysed and hydrolysed *A. afra* plant preparations. The hydrolysed and unhydrolysed samples of plant extracts were analysed and representative chromatograms obtained for each plant are given in figures 4.13 to 4.18.

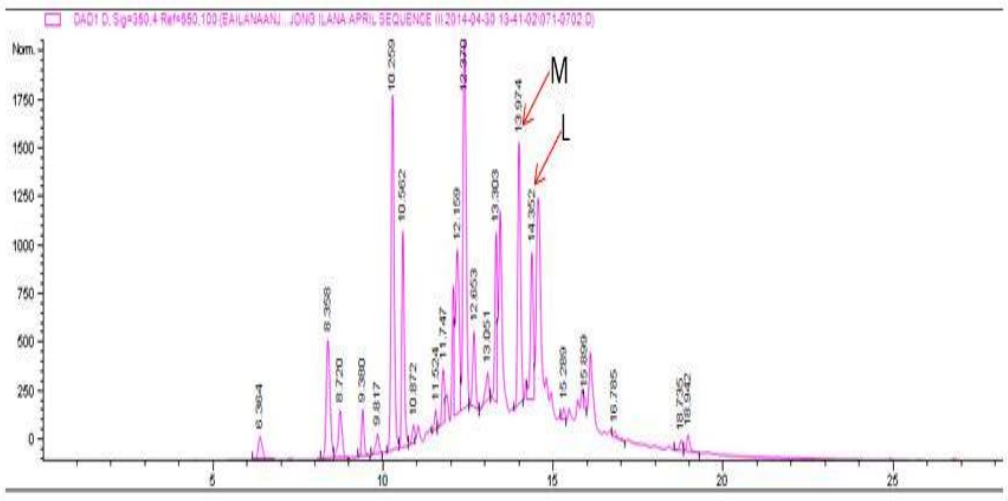

Figure 4.13: Representative HPLC chromatogram of unhydrolysed FDAE of *A. afra*. The retention time of morin (peak M) is 13.9 and luteolin (peak L) is 14.3.

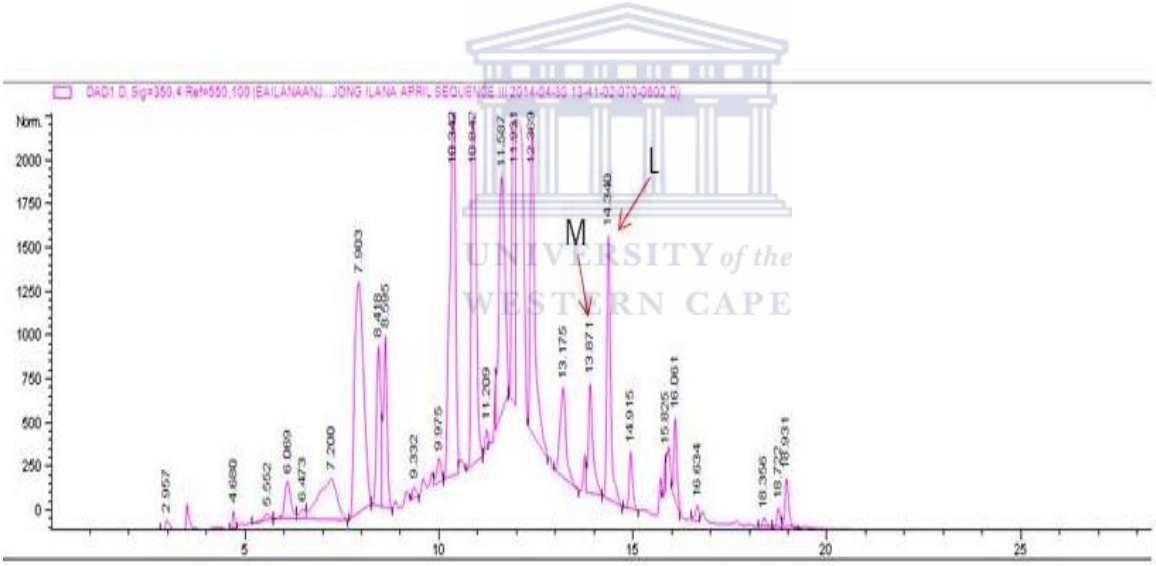

Figure 4.14: Representative HPLC chromatogram of hydrolysed FDAE of *A. afra*. The retention time of morin (peak M) is 13.9 and luteolin (peak L) is 14.3.

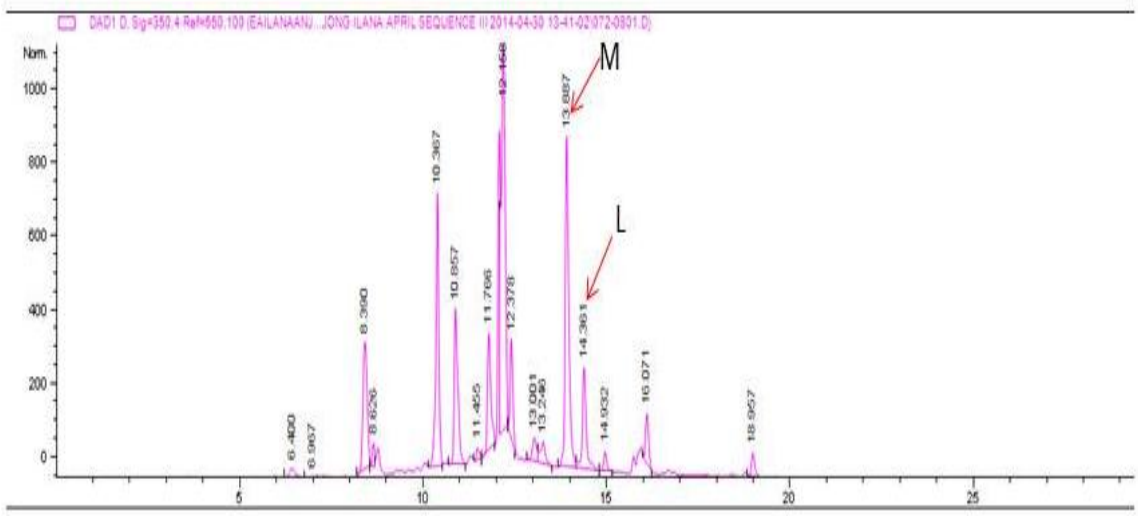

Figure 4.15: Representative HPLC chromatogram of unhydrolysed PMMA-alginate-FDAE of *A. afra*. The retention time of morin (peak M) is 13.9 and luteolin (peak L) is 14.3.

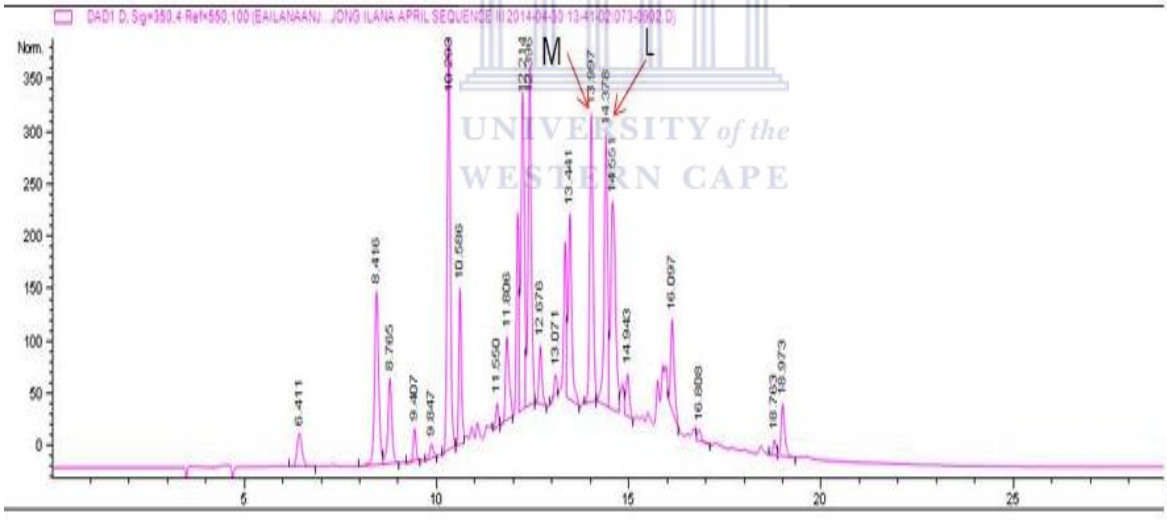

Figure 4.16: Representative HPLC chromatogram of hydrolysed PMMA-alginate-FDAE of *A. afra*. The retention time of morin (peak M) is 13.9 and luteolin (peak L) is 14.3

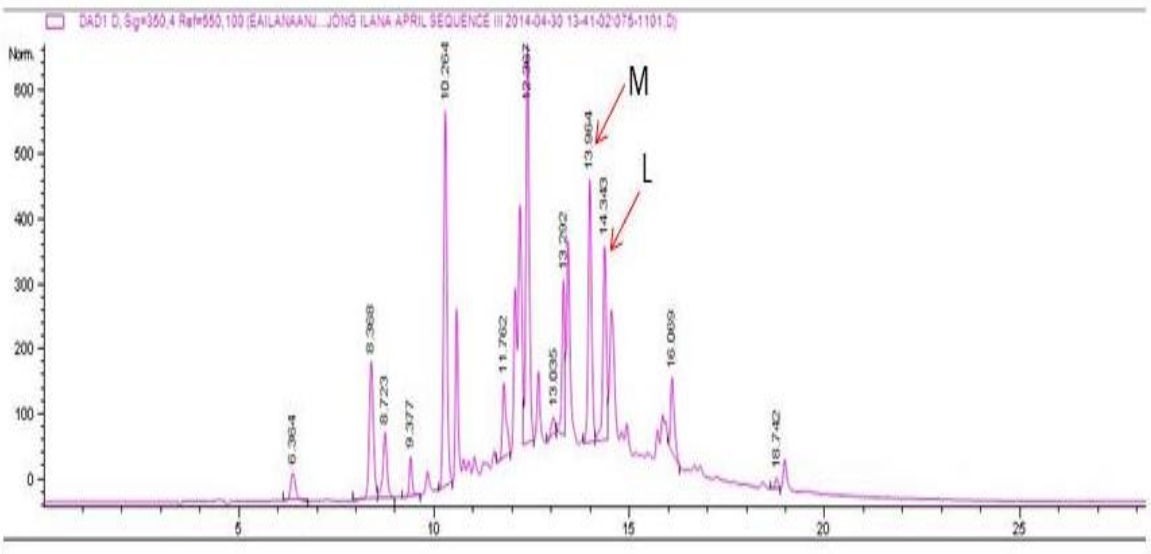

Figure 4.17: Representative HPLC chromatogram of unhydrolysed alginate-FDAE of *A. afra*. The retention time of morin (peak M) is 13.9 and luteolin (peak L) is 14.3.

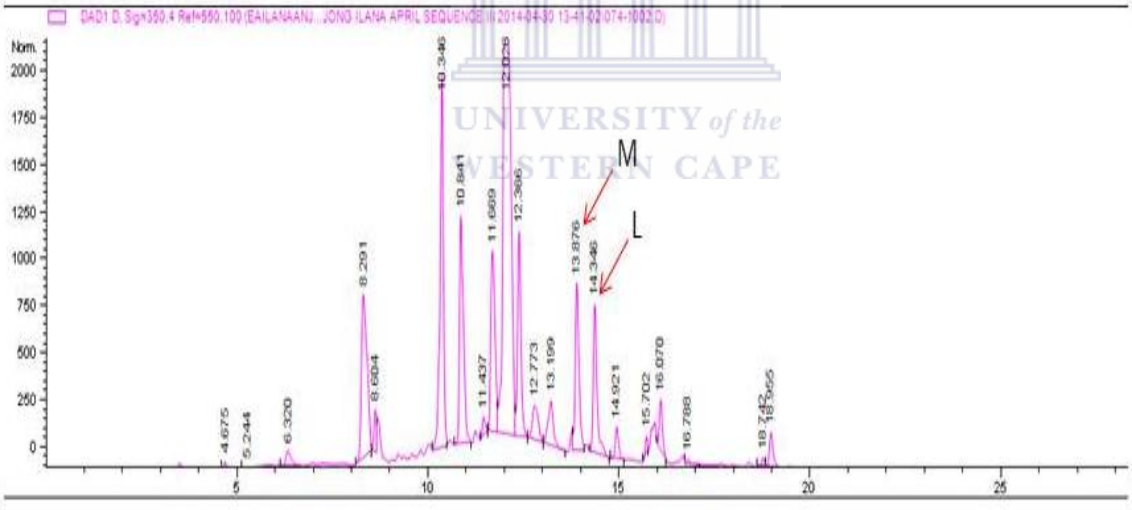

Figure 4.18: Representative HPLC chromatogram of hydrolysed PMMA-alginate-FDAE of *A. afra*. The retention time of morin (peak M) is 13.9 and luteolin (peak L) is 14.3.

Table 4.7: Level of luteolin in the *Artemisia afra* plant preparations

Plant samples	Luteolin concentration (µg/mg of plant extract) Mean ± SD (n=3)		Conjugated luteolin (%)
	Free luteolin	Total luteolin	
FDAE	0.185 ± 0.24	0.235 ± 0.026	21
Alginate-FDAE	0.067 ± 0.014	0.079 ± 0.093	15
PMMA-alginate-FDAE	0.012 ± 0.0710	0.058 ± 0.082	79.3

The FDAE, alginate-FDAE and PMMA-alginate-FDAE beads contained 0.185 ± 0.24, 0.012 ± 0.071 and 0.067 ± 0.014 µg free luteolin/mg, and 0.235 ± 0.026, 0.079 ± 0.093 and 0.058 ±0.082 µg total luteolin/mg, respectively (Table 4.7). As expected, the FDAE contained three to four times more luteolin than the modified forms. The alginate-FDAE contained more luteolin than the PMMA-alginate-FDAE but not significantly so (t-test p = 0.216), indicating that the former had more aglycones than the later. After the acid- hydrolysis, the levels of luteolin were increased in all the *A. afra* preparations of (Table 4.7).When the PMMA-alginate-FDAE was subjected to acid hydrolysis, the level of total luteolin was significantly increased compared to the free luteolin level (t-test p= 0.0412). This suggested that PMMA-alginate-FDAE had a substantial greater percentage of glycosides (79.3%) than FDAE and alginate-FDAE (21% and 15%), meaning that although it has a lower load of luteolin it has more of the conjugated form. Compared to the PMMA-alginate-FDAE, the 2 other products thus contained very little acid hydrolysable (i.e. conjugated) flavonoids (Table 4.7). These differences in luteolin concentration were clearly associated with the methods of preparation for the various forms of *A. afra*. For the FDAE, extraction and freeze drying was involved whereby the constituents of the leaves were concentrated into a given small dry mass having a relatively high concentration of luteolin. For the modified forms, that same FDAE was dispersed in calcium chloride solution or dichloromethane and mixed (i.e. diluted) with alginate or polymethylmethacrylate and subjected to emulsification to form the alginate-FDAE and PMMA-alginate-FDAE beads respectively. In these processes, deterioration of flavonoids and/or complex formation between flavonoids and

the many constituents present in the plant extract and/or added excipients very likely occurred reducing the levels of detectable luteolin, as seen in the results obtained.

The data therefore suggests that for the modified forms to contain luteolin in a more detectable form, they should be prepared in a manner in which they contain a higher load of active ingredient per mg of material.

4.5 Conclusion

The chapter addressed certain objectives of the study, viz, the preparation of the freeze dried aqueous extract (FDAE) and its modified forms, comparison of the organoleptic and pharmaceutical characteristics viztotal flavonoid (specifically luteolin levels) of the different forms of *A. afra* and evaluationof the release characteristics of FDAE of *Artemisia afra*from the alginate-FDAE and PMMA-alginate-FDAE beads in gastrointestinal fluids.

From the results obtained the following conclusions could be drawn;

Moderate to good yields of 21.8%, 16.5% and 12.6% was obtained for FDAE, alginate-FDAE and PMMA-alginate-FDAE beads respectively. The FDAE powder was coarse and irregularly shaped while the modified forms were not gradable and were both spherical in shape. The modification of FDAE into alginate-FDAE and PMMA-alginate-FDAE beads reduced porosity and this may lead to improved flowability when preparing dosage forms. The FDAE was confirmed to be hygroscopic while the modified forms less so. Modification to alginate-FDAE and PMMA-alginate-FDAE thus appeared to solve the problem of hygroscopicity.

It was possible to load moderate amounts (37 - 42%) of FDAE into the bead forms suggesting that a reasonable amount of FDAE can be delivered via these forms. In addition the alginate-FDAE and PMMA-alginate-FDAE showed similar FDAE release patterns of faster release at intestinal fluid than gastric fluid. Both are thus expected to predominantly release the actives in the intestine. The EE% of the modified forms related to the total flavonoid content (TFC) in that alginate-FDAE contained more luteolin per mg of material than PMMA-alginate-FDAE.

Finally, the FDAE contained higher levels of total and free luteolin than the modified forms, with relatively low values of total and free luteolin observed in the modified forms. However, the PMMA-alginate-FDAE showed a greater percentage of conjugated luteolin than the other forms. These differences in luteolin concentration could be attributed to the methods used in preparing the various forms of *A. afra*.

Chapter 5

Determination of the *in vitro*intestinal permeability of luteolin from *Artemisia afra* preparations

5.1 Introduction

The work reported in this chapter aimed at investigating the absorption and gastrointestinal permeability of luteolin from the different preparations of *A. afra* prepared as discussed in chapter 4. To address this goal, the single-pass intestinal perfusion method was used and this method along with the other accompanying procedures used and the results obtained are presented and discussed.

5.2 Materials and methods

5.2.1 Reagents and equipment

Luteolin was purchased from Sigma Aldrich (St Louis, USA), sodium pentobarbitone 6% from Kryon Lab (Pty) Ltd (South Africa) and phenol red from Merck (Germany). Acetonitrile, formic acid and methanol were HPLC grade and obtained from Merck (Germany). Double distilled waterproduced with Millipore filtration system was used during the entire HPLC procedure. Surgical equipments were supplied by Lasec (Pty) Ltd, South Africa. All other materials and reagents were either of analytical grade or of the highest purity and used as received.

5.2.2 Animals

Healthy male rats, weighing 250-350 g were purchased from the Animal Unit at the University of Stellenbosch, South Africa and were housed at room temperature in a well-ventilated animal room with free access to food and water. The lighting in the animal room was regulated to reproduce a 12-hr cycle of day and night conditions. Before the intestinal perfusion experiment, all animals were placed in cages and fasted for 12 hours with free access to tap water. For the experiment, the rats were anaesthetized with an intraperitoneal injection of pentobarbitone solution (40 mg/kg body weight), restrained in a supine position and then placed on a 37 ˚C

heating pad under a heating lamp during surgery throughout the *in situ* intestinal perfusion. The protocol for this study was approved by the University of the Western Cape Senate Research Ethics Committee (Registration no. 12/7/4, Appendix 1).

5.2.3 Surgical procedures

The surgical protocol used in the study was similar to that described elsewhere (Dang et al., 2012, Singhal et al., 1998, Cummins et al., 2003, Zhang et al., 2006), with minor modifications.

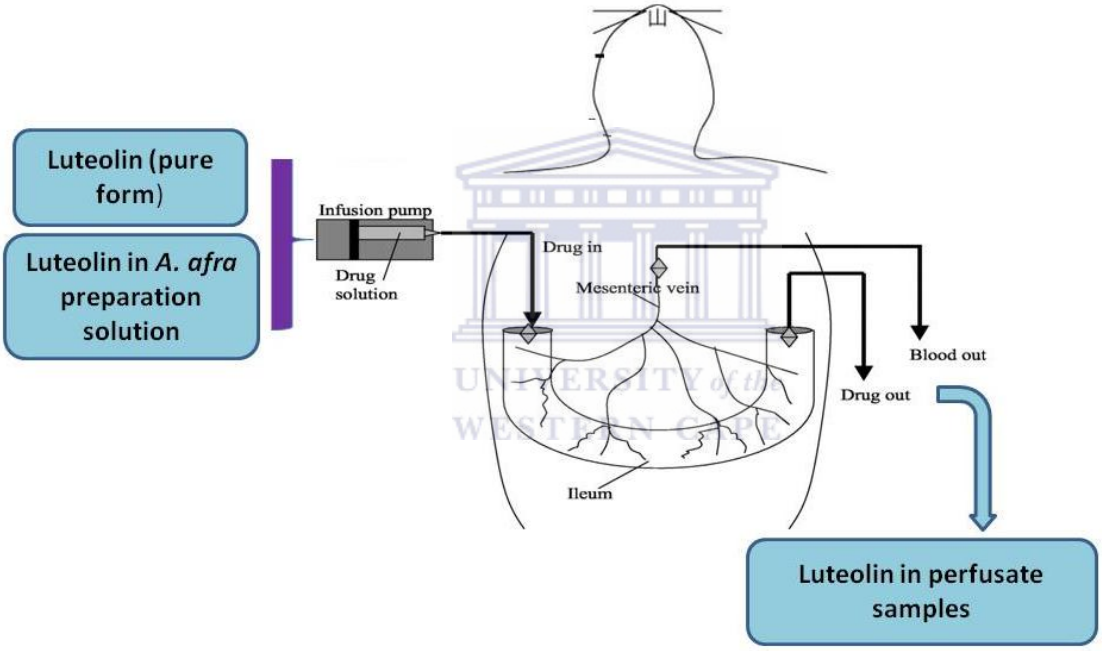

Figure 5.1: Illustration of single pass rat intestine perfusion model set up (modified from Cummins et al., 2003).Note: In the experiments for the data reported, only the ileum was cannulated.

A 5-cm-long midline incision into the abdomen was performed on the anaesthetized animal, the intestines located and the ileal segment carefully isolated for perfusion. Care was taken to handle the small intestine gently in order to maintain an intact blood supply. Using surgical scissors, incisions were made at both ends of the isolated intestinal segment. Glass cannulas (2 cm long, 4 mm o.d. (outside diameter)) connected to tubing (6mm o.d.) were inserted at both ends (at inlet

cannula to infuse and at outlet cannula to collect perfusion fluids). At the inlet side, syringe filled with pre-warmed saline was connected to gently flush the intestine so that intestinal content exited at outlet cannula. A syringe pump was then connected to the inlet cannula and pre-warmed normal saline (blank perfusate vehicle) infused into the segment at rate of 0.25 ml/min to test the free flow through the segment at the outlet. The isolated intestine was then reinserted into the abdominal cavity, the cut abdominal wall partially stitched closed and the closed abdominal area covered with moist pad of surgical gauze. The isolated intestine model was now ready for perfusion. The experimental set up was performed as illustrated in figures 5.1 and 5.2.

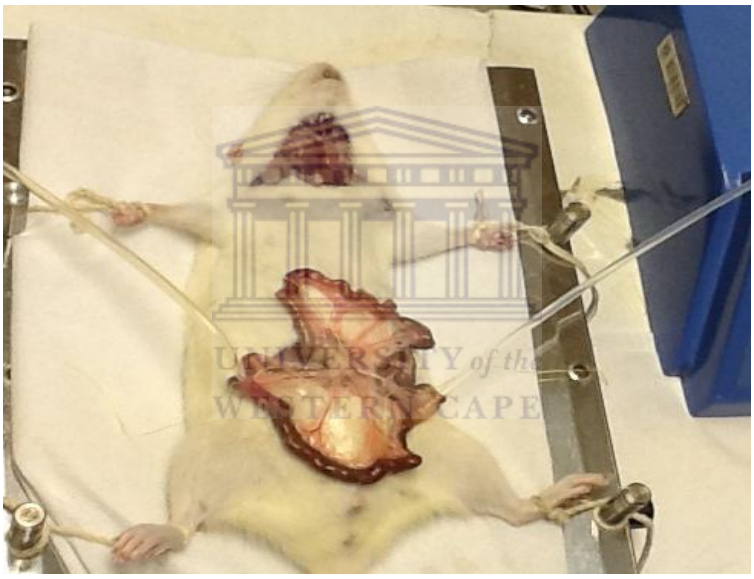

Figure 5.2: Cannulated intestinal segment of Wistar rat prior to reinsertion into abdominal cavity.

5.2.4 Single-pass intestinal perfusion

The perfusion vehicle was normal saline. All perfusion solutions contained 20 µg/ml phenol red to act as a non-absorbable marker for measuring water flux and to correct for changes in the water flux across the intestine (Sutton et al., 2001). Solutions containing 20 µg/ml of luteolin in pure form, and 5 µg/ml of luteolin in FDAE, 2.5µg/ml of luteolin in alginate-FDAE and 2.3 µg/ml of luteolin in PMMA-alginate-FDAE in saline were infused into the rat ileum. The *in*

*situ*intestinal infusions were initiated by infusing the test solution at 1 ml/min for 2 min to ensure filling the segment rapidly , followed by perfusion at 0.25 ml/min for the remainder of the experiment (i.e. 50-60 min) (Mukinda, 2010). Samples of the intestinal outflow were continuously collected into pre-weighed vials over 10 min intervals for up to 60 min. The collected perfusate samples were kept on ice until the end of the study where after they wereimmediately frozen and stored at -80 °C until analysis. At the end of the experiment, the length of the segment of cannulated intestine from the end of the inlet cannula to the beginning of the exit cannula was measured without stretching it, and then the animal was sacrificed by performing bilateral thoracotomy.

5.2.5 Quantitation of phenol red for water flux measurement

To correct for volume changes due to changes in the water flux across the intestine, phenol red was added to all perfusion solutions at a concentration of 20 µg/ml (Sutton et al., 2001). The quantitation of phenol red in the perfusate samples was performed using high performance liquid chromatography (HPLC) and an external phenol red calibration curve obtained for standard solutions of 1 and 50µg/ml phenol red in normal saline (linear regression R^2 =0.9978).

5.2.6 HPLC assay of luteolin in plant sample perfusate

For the assay of luteolin in the plant perfusatesamples, the stored frozen samples were thawed, and then extracted, either as such or after acid hydrolysis, into ethyl acetate (which is then evaporated to dryness and the residues used) for the quantitation of the free and total luteolin levels in the samples. The validated assay reported in section 4.4.8 was used.

All samples were analysed by HPLC (Agilent HPLC system-1200 series). The optimum separation of the test compounds (i.e. luteolin and phenol red) was achieved with a mobile phase composed of 0.1% formic acid aqueous solution (solvent A) and 0.1% formic acid in acetonitrile (solvent B), flowing through a Phenomenex Luna column (5 µm, 4.6 x 250 mm) at a flow rate of 0.8 ml/min and at 25 °C with UV detection at 350 nm. The elution gradient was: 0-22 min 15%B; 22-27 min 100%B; 27-33 min 15%B. The volume of samples injected was 20 µl for all perfusate samples and the run time was 33 min.

5.2.7 Data analysis

The permeabilities of luteolin and luteolin in *A. afra* plant preparations i.e. FDAE, alginate-FDAE beads and PMMA-alginate-FDAE beads across the rat intestine were calculated based on the disappearance of the parent compound (luteolin) from the lumen (P_{lumen}) using equation 1 (Singhal et al., 1998; Cummins et al., 2003).

$$\mathbf{P_{lumen}=-Q/2\pi rLxIn(C_{lumen}/C_0)}(1)$$

Where, r = radius of the intestinal lumen (0.3cm)

L = length of the segment (cm),

Q = flow rate of treatment through the intestine (0.25ml/min),

C_0 = concentration of the test compound at the start of the perfusion (in syringe, µg/ml),

C_{lumen} = steady-state concentration of the test compound exiting the lumen (µg/ml).

The concentrations of luteolin in the perfusate were corrected for changes in the water flux at each time interval using equation (2):

$$\mathbf{Conc_{corrected} = Conc_{measured} \times [phenol\ red]_{in}/[phenol\ red]_{out}}(2)$$

Graphs of total or free luteolin concentration (µg/ml) were plotted against time. Student t-test was used to compare differences and data presented as mean ± SD. Statistical significance was set at $p \leq 0.05$.

5.3 Results and discussion

5.3.1 Absorption of luteolin from *Artemisia afra* preparations in the rat single-pass intestinal perfusion model

To assess the effect alginate and PMMA-alginate modifications has on the absorption of luteolin (total and free) contained in solutions of pure luteolin, and *A. afra*preparations i.e.(FDAE, alginate-FDAE, PMMA-alginate-FDAE), solutions were individually perfused through a segment of the rat intestine in the absence of gut contents. The HPLC validated assay developed in section 4.4.8 was used for the simultaneous quantitation of luteolin and phenol red in the *in situ* perfusate samples. Before analysis, the latter were either un-hydrolysed or acid-hydrolysed to quantitate the free and total luteolin levels in the samples.

Under the HPLC conditions described in section 5.2.5, good symmetrical peaks with retention times of 11.52 ± 0.06 min and 13.81 ± 0.03 min was found for phenol red and luteolin respectively (Figure 5.3 and Appendix 9).

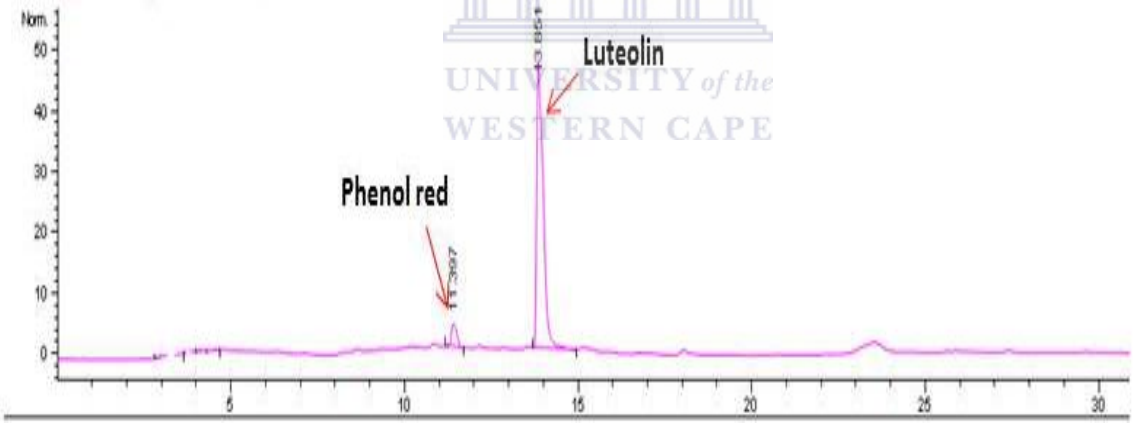

Figure 5.3: A representative HPLC chromatogram of *in situ* perfusion samples containing luteolin and phenol red at 350 nm. The retention time of luteolin is 13.8 while that for phenol red is 11.4.

Profile of total and free luteolin content of perfusate samples collected at different time points is shown in figures 5.4 and 5.5 and tables 5.1 and 5.2.

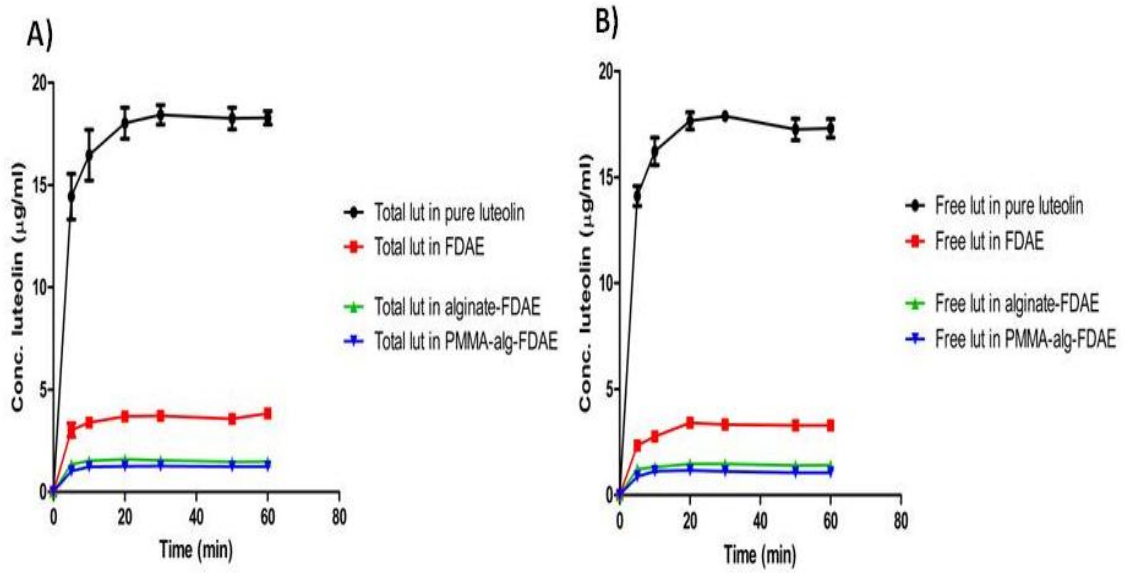

Figure 5.4: Profiles of A) total luteolin content and B) free luteolin content in effluent perfusate samples as a function of time.The rat intestine segment was perfused with saline solutions containing 20 µg/ml pure luteolin, FDAE containing 5 µg/ml of luteolin, alginate-FDAE containing 2.5 µg/ml of luteolin and PMMA-alginate-FDAE containing 2.3 µg/ml of luteolin. Luteolin content is expressed as µg/ml and the curves represent mean values of three datasets i.e. n =3.

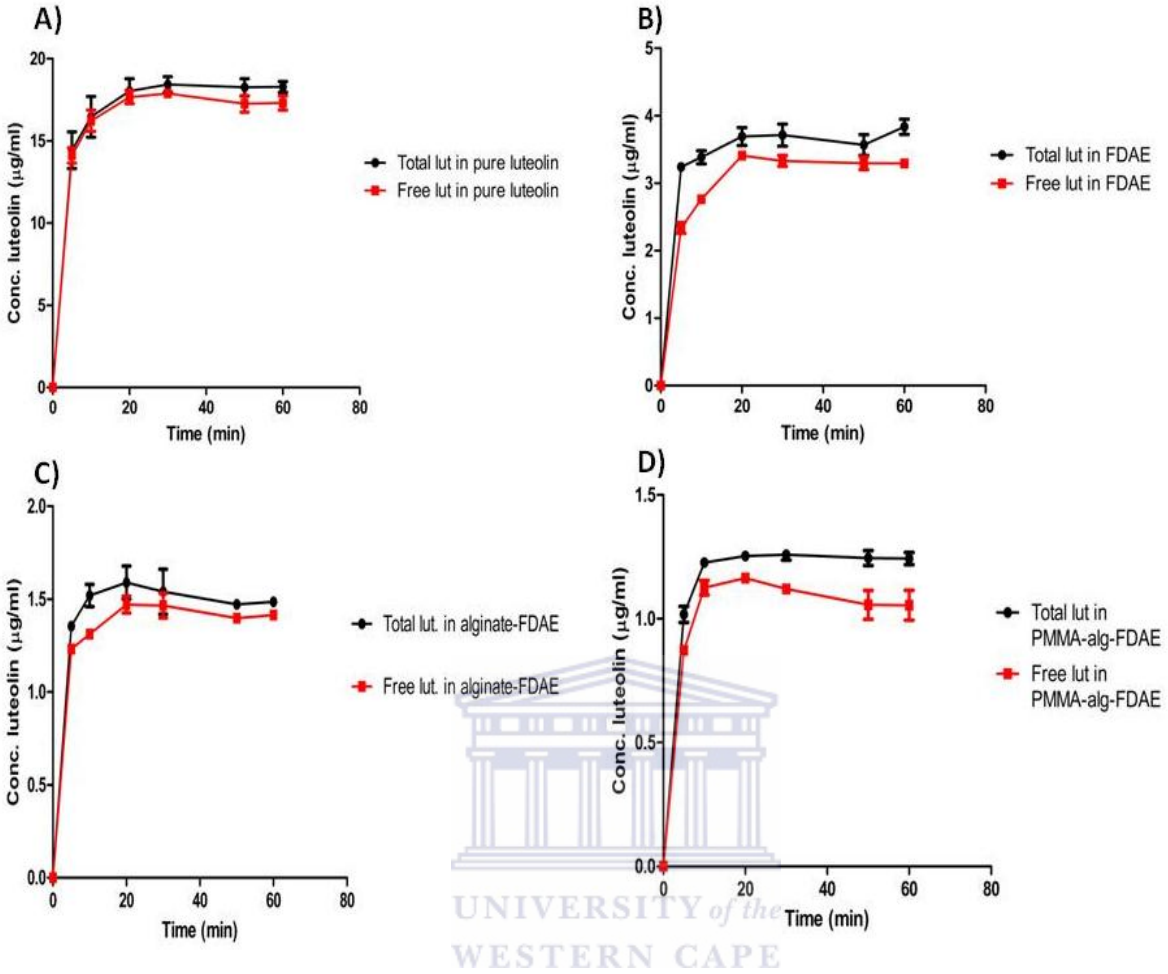

Figure 5.5: Profiles of free and total luteolin concentrations versus time following perfusion with A) pure luteolin B) FDAE C) alginate-FDAE D) PMMA-alginate-PMMA. The rat intestine segment was perfused with saline solutions containing 20 µg/ml pure luteolin, FDAE containing 5 µg/ml of luteolin, alginate-FDAE containing 2.5 µg/ml of luteolin and PMMA-alginate-FDAE containing 2.3 µg/ml of luteolin. Luteolin content is expressed as µg/ml and the curves represent mean values of three datasets i.e. n =3.

Table 5.1: Total luteolin content in rat intestinal perfusate at various time intervals during perfusions with pure luteolin and *A. afra* forms.

Time interval (min)	Total luteolin perfusate concentrations (Mean ± SD (µg/ml)) after perfusions with			
	Luteolin (pure) (*C_{in} = 20 µg/ml)	FDAE (*C_{in} = 5 µg/ml)	alginate-FDAE (*C_{in} = 2.5 µg/ml)	PMMA-alginate-FDAE (*C_{in} = 2.3 µg/ml)
0-5	15.55 ± 0.13	3.223 ± 0.07	1.329 ± 0.04	1.05 ± 0.17
5-10	17.70 ± 0.03	3.481 ± 0.05	1.579 ± 0.17	1.234 ± 0.18
10-20	18.78 ± 0.02	3.824 ± 0.32	1.678 ± 0.14	1.247 ± 0.36
20-30	18.91 ± 0.06	3.875 ± 0.30	1.501 ± 0.26	1.2370 ± 0.11
40-50	18.76 ± 0.04	3.721 ± 0.28	1.464 ± 0.20	1.215 ± 0.22
50-60	18.61 ± 0.07	3.946 ± 0.15	1.460 ± 0.05	1.218 ± 0.16
R_{10-60}	18.05 ± 0.05	3.678 ± 0.20	1.501 ± 0.14	1.200 ± 0.20

Total luteolin content as mean ± SD (n =3). Each value represents the total luteolin content of perfusate at various time points. R_{10-60} = average of the steady-state data (samples obtained between 10 and 60 min).

Table 5.2: Free luteolin content in rat intestinal perfusate at various time intervals during perfusions with pure luteolin and *A. afra* forms.

Time interval (min)	Free luteolin perfusate concentrations (Mean ± SD (µg/ml)) after perfusions with			
	Luteolin (pure) (*C_{in} = 20 µg/ml)	FDAE (*C_{in} = 5 µg/ml)	alginate-FDAE (*C_{in} = 2.5 µg/ml)	PMMA-alginate-FDAE (*C_{in} = 2.3 µg/ml)
0-5	14.981 ± 0.13	2.417 ± 0.29	1.213 ± 0.38	0.89 ± 0.33
5-10	16.997 ± 0.08	2.738 ± 0.24	1.320 ± 0.38	1.095 ± 0.35
10-20	18.589 ± 0.18	3.456 ± 0.27	1.425 ± 0.44	1.175 ± 0.42
20-30	18.908 ± 0.11	3.410 ± 0.22	1.398 ± 0.30	1.140 ± 0.35
40-50	18.789 ± 0.14	3.360 ± 0.15	1.415 ± 0.21	1.114 ± 0.31
50-60	17.958 ± 0.12	3.392 ± 0.25	1.409 ± 0.26	1.115 ± 0.22
R_{10-60}	17.647± 0.13	3.127 ± 0.24	1.363 ± 0.33	1.088 ± 0.33

Free luteolin content as mean ± SD (n =3). Each value represents the free luteolin content of perfusate at various time points. R_{10-60} = average of the steady-state data (samples obtained between 10 and 60 min).

From the results obtained, insight into the amounts and form of luteolin present in the perfusate samples over the perfusion period can be gained. Generally, it was observed that the

disappearance of luteolin from each plant preparation was relatively steady after 10 min perfusion. The luteolin content disappearing from pure solution at steady state was relatively low (˜12%), as can be seen from the total and free luteolin concentrations in perfusate samples at steady state (Tables 5.1 and 5.2) when compared to that of the *A. afra* forms.This indicated that not much was disappearing from pure luteolin, indicating poor uptake (permeability) than the plant forms.

Table 5.3: Total luteolin content uptake at various time intervals from perfusions of pure luteolin and *A. afra* forms.

Time interval (min)	Effective permeability (Peff) values of total luteolin (cm/s)			
	Luteolin (pure)	FDAE	alginate-FDAE	PMMA-alginate-FDAE
0-5	0.0106	0.0343	0.0250	0.0305
5-10	0.0110	0.0335	0.0309	0.0298
10-20	0.0115	0.0327	0.0301	0.0280
20-30	0.0123	0.0324	0.0296	0.0279
40-50	0.0131	0.0289	0.0325	0.0318
50-60	0.0147	0.0289	0.02980	0.0260
R_{10-60}	0.0122	0.0318	0.0294	0.029

Table 5.4: Free luteolin content uptake at various time intervals from perfusions of pure luteolin and *A. afra* forms.

Time interval (min)	Effective permeability (Peff) values of free luteolin (cm/s)			
	Luteolin (pure)	FDAE	alginate-FDAE	PMMA-alginate-FDAE
0-5	0.0103	0.0335	0.0323	0.0257
5-10	0.0140	0.0331	0.0309	0.0262
10-20	0.0110	0.0317	0.0291	0.0276
20-30	0.0113	0.0312	0.0295	0.0276
40-50	0.0121	0.0285	0.0317	0.0290
50-60	0.0131	0.0281	0.0295	0.0307
R_{10-60}	0.0119	0.0310	0.0299	0.0278

Table 5.5: Statistical parameters of total and free luteolin uptake from perfusions of pure luteolin and *A. afra* preparations.

Sample	Luteolin uptake	p value, (T-test result for total *vs* free luteolin uptake in *A. afra* preparations)
Luteolin *vs* FDAE	total	0.0022, S
	free	0.0022, S
FDAE *vs* alginate- FDAE	total	0.5887, NS
	free	0.8182, NS
FDAE *vs* PMMA-alginate FDAE	total	0.0649, NS
	free	0.0260, S
alginate- FDAE*vs* PMMA-alginate-FDAE	total	0.1481, NS
	free	0.0200, S

The above-mentioned findings were supported by the permeability coefficient (P_{lumen}) data shown in figure 5.6 and tables 5.3 and 5.4. The P_{lumen} values were calculated based on the luminal disappearance of total and free luteolin using the Eqn. 1 at the steady-state period. The intestinal effective permeability represents a direct measurement of the local absorption rate and reflects the transport velocity across the epithelial barrier, expressed as centimeter per second (Lennernas, 1998). The P_{lumen} values for intestinal uptake of total and free luteolin were higher from solutions of *A. afra* forms than the pure luteolin solution (i.e. P_{lumen} values in the range of 0.02 - 0.035 cm/s for all plant forms *vs* P_{lumen}values in the range of 0.010 - 0.014 cm/s for pure luteolin, t-test p = 0.0252) confirming that the plant matrix form seemed to improve the intestinal disappearance of both the glycoside and aglycone forms of luteolin (Tables 5.3 and 5.4). This could be as a result of the uptake of luteolin glucoside and other luteolin derivatives in the

unhydrolysed form as opposed to the uptake of luteolin aglycone only in the hydrolysed form.These findings, thus confirm the suggestion made by Mukinda in his study, stipulating that plant matrix appeared to improve the intestinal disappearance of both the aglycone and glycoside forms of luteolin (Mukinda et al., 2010). It had been previously found in vervet monkeys that luteolin in the aqueous extract of *A. afra* had a significantly higher oral bioavailability than that of luteolin in a pure solution (Muganga, 2004).

In addition, Mukinda *et al*., showed that luteolin in *A. afra* aqueous extract, regardless of its form (i.e. whether aglycone or glucoside), was better taken up and more efficiently metabolized than its aglycone and glucoside forms administered as pure solutions (Mukinda et al., 2010). This suggested that the plant matrix had an interesting effect on the uptake and subsequently metabolism of luteolin from *A. afra*.

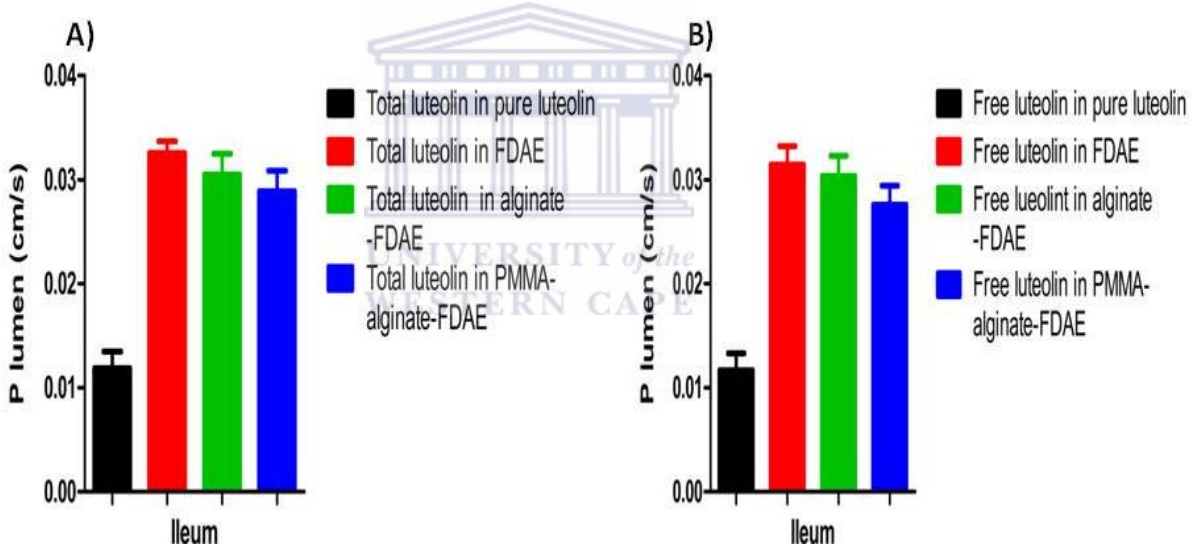

Figure 5.6: **Permeability coefficients of A) total luteolin and B) free luteolin in pure luteolin and *A. afra* preparations across rat ileum. The rat intestine segment was perfused with 20 µg/ml pure luteolin, FDAE containing 5 µg/ml of luteolin, alginate-FDAE containing 2.5 µg/ml of luteolin and PMMA-alginate-FDAE containing 2.3 µg/ml of luteolin.P_{lumen} was calculated using Eq. 1, at the steady-state (i.e. between 10 and 60 min). The histograms represent the mean values of three datasetsi.e. n=3.**

The permeability of total and free luteolin in the FDAE appeared to be greater than that of the modified forms (P_{lumen}>0.03 cm/s for FDAE and P_{lumen}<0.03 cm/s for both modified forms), although the difference in uptake of luteolin (free and total) between FDAE and alginate-FDAE was statistically not significant (Table 5.5) whereas the difference in uptake of free luteolin between FDAE and PMMA-alginate-FDAE was statistically significant (p = 0.0260). The alginate-FDAE displayed greater uptake of luteolin (free and total) than the PMMA-alginate-FDAE. The difference in uptake of total luteolin in these modified forms was statistically not significant (p = 0.0649) whereas that for free luteolin was statistically significant (p = 0.0260) (Table 5.5).

Despite the fact that many studies have been done investigating the absorption of luteolin in either, the pure form or the plant form (Shimoi et al., 1998; Spencer et al., 1999), no studies have been conducted investigating the absorption of luteolin from different modified forms of the aqueous plant extract. Very little is known about the extent to which flavonoids are absorbed and the mechanisms of absorption involved. According to Chen *et al*, poor bioavailability of flavonoids results from their poor intrinsic permeation and transporter-mediated efflux (Chen et al., 2008). Liu *et al*, in their study, concluded that the low bioavailability of flavonoids is as a result of extensive phase II metabolism in the intestine (Liu and Hu, 2002). Song *et al* compared the permeability values among high, medium and low concentrations of metformin in the duodenal segment. A concentration-dependent change was observed, exhibiting a decrease in permeability at the highest drug concentration. The result suggested that intestinal absorption of metformin was via passive and transcellular mechanisms involving an active saturable process (Song et al., 2006). This could mean that the absorption sites in the ileum were saturated and hence the less of the pure luteolin was absorbed or disappeared, although it had a higher concentration (20 µg/ml) as compared to the amount of luteolin present in the plant forms (i.e. 5µg/ml of luteolin in FDAE, 2.5 µg/ml of luteolin in alginate-FDAE and 2.3 µg/ml of luteolin in PMMA-alginate-FDAE). Such a deduction will be in accordance with the results found in this study in which the amount of free and total luteolin disappearing frompure luteolin solution is lower than that of the*A. afra* plant forms as seen in figures 5.4 and 5.5. However, due to the very small amounts of luteolin identified and quantified in the *in situ* perfusate plant samples, special care should be paid to the loading of active herbal ingredient (AHI) into the plant preparation

when preparing plant dosage form formulations in order to maintain the effective blood concentration of AHI.

With the experimental design, it was difficult to accurately compare the permeabilities of luteolin in pure form and *A. afra* plant forms due to the lack of mass balance done for all forms. This made it difficult to specifically ascertain which modified form showed better luteolin uptake. The mass of luteolin in the FDAE and beads was incomparable to the mass of luteolin (pure form) used in this study as seen from the luteolin concentrations in the perfusion solutions. To mitigate this problem, it was thus recommended that actives in plant forms be extracted and mass balance done for all forms to be investigated for future purposes. In addition it was not possible to determine whether the amount of luteolin will have any observable therapeutic effect in human. Thus further studies on the effect of these *A. afra* preparations in humans are required.

5.4 Conclusion

The results obtained from this study clearly suggested that luteolin present in the *A. afra* plant forms is absorbed into the circulation, although the exact mechanisms of absorption remain a subject of argument. The disappearance of luteolin was improved in the *A. afra* plant forms when compared to the pure luteolin solution with the FDAE showing slightly greater disappearance of luteolin that the modified forms.The total and free luteolin content in the perfusate samples of the alginate-FDAE and PMMA-alginate-FDAE beads indicated similar luteolin content disappearing from these beads. Collectively, these results indicate that the modified forms of the FDAE prepared i.e. alginate-FDAE and PMMA-alginate-FDAE beads should thus be suitable for use in the preparation of a solid dosage form e.g. tablet or capsule for *Artemisia afra*.

Chapter 6

Conclusions and Recommendations

The objectives of this study were; to prepare the freeze dried aqueous extract (FDAE) and its modified forms (alginate-FDAE and PMMA-Alginate-FDAE),to determine and compare the pharmaceutical characteristics of the various forms, to evaluate and compare the release characteristics of FDAE from the alginate-FDAE and PMMA-alginate-FDAE beads in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), and to determine the intestinal permeability of luteolin contained in the modified *Artemisia afra* extractpreparations.

It was hypothesized that; making the modified forms of the FDAE of *A. afra* would make the FDAE less hygroscopic with a lower moisture content, the rate of release of luteolin from alginate-FDAE would be faster than that from PMMA-alginate-FDAE beads and finally that the effective gastrointestinal permeability (P_{eff}) of luteolin in the alginate-FDAE and PMMA-alginate-FDAE beads of *A. afra* FDAE would be equal to that in the FDAE.

From the results obtained the following conclusions could be drawn;

1. The yield of freeze dried aqueous material obtained from the aqueous extract of *A. afra* dried leaves was consistent with that obtained from other investigators.The modified preparations of *A. afra* also had organoleptic features, viz, colour, taste and odour which were identifiable and very distinctive. Unlike the FDAE whose colour changed from light brown to dark brown on storage in a desiccator at room temperature, the physical appearance of the modified forms (alginate-FDAE beads and PMMA-alginate-FDAE beads) was stable on storage without any visible physical changes.
2. The moisture content of the modified forms of *A. afra*; alginate-FDAE beads and PMMA-alginate-FDAE beads were relatively low as compared to the FDAE. This result thus indicated improvement in counteracting the hygroscopicity of the FDAE and therefore improved storage over longer periods of time.
3. A pH responsive release profile displayed by the alginate-FDAE and PMMA-alginate-FDAE beads was observed, with faster release observed at a higher pH in SIF (6.8) compared to SGF (1.2) thus indicating slow release of flavonoids from the beads in the stomach and faster release in the intestine.

4. The FDAE contained higher levels of the marker luteolin than the modified forms which increased in all forms after hydrolysis. Relatively very small amounts of the marker compound luteolin were identified and quantified in the modified forms with PMMA-alginate-FDAE having a higher percentage of conjugates than the two other forms.
5. Luteolin was better taken up in the plant forms than in its pure form. The *A. afra* forms prepared had similar rates of uptake (permeability) of free and total luteolin with the rates being highest for the FDAE.

In summary, the modified forms which include alginate-FDAE and PMMA-alginate-FDAE beads should be suitable for use in a solid dosage form (e.g. tablet or capsule) of *A. afra*.

From the results, several areas for further research were noted. Firstly, there is a need to improve the entrapment efficiency of FDAE in alginate-FDAE and PMMA-alginate-FDAE beads. This will lead to an increase in the percentage of the FDAE (containing the actives) embedded in the beads. Secondly suitable excipients (e.g. Eudragit™) that can impact a non-hygroscopic nature unto the herbal extract and in turn improve bioavailability of actives should be investigated to determine if the bioavailability of the *A. afra* flavonoids can be improved by preparing modified forms with comparable flavonoid chemical content (i.e. same Active Herbal Constituents (AHC) as the aqueous extract) but improved *in vitro* availability (i.e. dissolution) and intestinal uptake (i.e. trans-intestinal stability and permeability) characteristics. Lastly, further studies are required to indicate whether the permeability of luteolin as determined by the single pass intestinal perfused model also occur *in vivo*. A good marker which can be easily detected and quantified and a method of quantification for *A. afra* is needed. In addition to this, a study needs to be conducted with this marker on different batches of the various *A. afra* forms to give a broad and clear understanding of the absorption of luteolin in these forms and to further ascertain which of the forms is better.

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Appendices

Appendix 1

Copy of Research Approval by Ethics Committee of the UWC Senate

UNIVERSITY of the
WESTERN CAPE

OFFICE OF THE DEAN
DEPARTMENT OF RESEARCH DEVELOPMENT

27 March 2013

To Whom It May Concern

I hereby certify that the Senate Research Committee of the University of the Western Cape, at its meeting held on 17 August 2012, approved the methodology and ethics of the following research project by: Prof J Syce (School of Pharmacy)

Research Project:	The investigation of the in vitro bioavailability of flavonoids (*lutecolin*) from various preparations of *Artemisia afra extracts*.
Registration no:	12/7/4

Any amendments, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.

The Committee must be informed of any serious adverse event and/or termination of the study.

Ms Patricia Josias
Research Ethics Committee Officer
University of the Western Cape

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Appendix 2

Table showing the resultant yields obtained in the preparation of the freeze dried aqueous extract of *Artemisia afra*.

Batch No	Weight of the A. afra plant leaves (g)	Volume of the water (L)	Yield of the freeze dried aqueous extract (g)
1	100	3.5	19.9
2	100	3.5	21.6
3	100	3.5	23.5
Ave			**21.733**
Std Dev			**1.850**

Appendix 3

Particle size analysis of *Artemisia afra* preparations

Artemisia afra plant preparation weight (g)	Sieve No.	Weight of powder passing through sieve (g)	Weight of powder passed through as % of feed	Fineness grading (BP 2000)
***A. afra* dried leaves**	**355**	**2.2**	**21.8**	**Coarse powder**
	180	**0.47**	**4.7**	
	125	**0.33**	**3.3**	
	90	**0.24**	**1.2**	
FDAE of *A. Afra*	**355**	**5.9**	**59**	**Coarse powder**
	180	**2.87**	**28.7**	
	125	**1.92**	**19.2**	
	90	**0.89**	**8.9**	
Alginate beads of *A. Afra*	**1700**	**7.82**	**78.2**	**Not gradable**
	1400	**6.82**	**68.2**	
	1000	**5.19**	**51.9**	
	500	**5.15**	**51.5**	
PMMA-alginate beads of *A. Afra*	**1700**	**9.22**	**92.2**	**Not gradable**
	1400	**6.30**	**63**	
	1000	**4.58**	**45.8**	
	500	**4.52**	**45.2**	

Appendix 4

Moisture content of *Artemisia afra* preparations

Appendix 4.1: Table showing results of moisture content of FDAE of *A. afra*

Sample No	Initial weight of plant material(g)	Final weight of dried plant material (g)	Difference (g)	Percentage of moisture
1	0.3176	0.2861	0.0315	9.92
2	0.3069	0.2739	0.033	10.75
3	0.3087	0.2736	0.0351	11.37
Ave	**0.3110**	**0.2779**	**0.0332**	**10.68**
Std Dev	**0.0057**	**0.0071**	**0.0018**	**0.7275**

Appendix 4.2: Table showing results of moisture content of alginate-FDAE beads of *A. afra*

Sample No	Initial weight of plant material(g)	Final weight of dried plant material (g)	Difference (g)	Percentage of moisture
1	0.3992	0.3724	0.0268	6.713
2	0.3724	0.3466	0.0258	6.928
3	0.3873	0.3616	0.0257	6.6357
Ave	**0.3863**	**0.3602**	**0.0261**	**6.7591**
Std Dev	**0.0134**	**0.0129**	**0.0006**	**0.1514**

Appendix 4.3: Table showing results of moisture content of PMMA-alginate-FDAE beads of *A. afra*

Sample No	Initial weight of plant material(g)	Final weight of dried plant material (g)	Difference (g)	Percentage of moisture
1	0.3339	0.3081	0.0258	7.7268
2	0.3071	0.2817	0.0254	8.2709
3	0.3221	0.296	0.0261	8.1030
Ave	**0.3210**	**0.2952**	**0.0257**	**8.0336**
Std Dev	**0.0134**	**0.0132**	**0.0003**	**0.2786**

Appendix 5

Aqueous soluble fractionof preparations of *A. afra*

Appendix 5.1: Table showing the aqueous soluble fractionof FDAE of *A. afra*

Sample No	Initial weight of plant material(mg)	Weight of the residue on filter paper(mg)	Weight of the soluble portion in the plant material (mg)	Percentage of soluble material
1	30.6	5.4	25.2	82.353
2	30.2	5.1	25.1	83.113
3	29.2	3.8	25.4	86.986
4	32.5	5.3	27.2	83.692
5	33.5	5.9	27.6	82.388
6	28.6	4.4	24.2	84.615
Ave	**30.767**	**4.983**	**25.783**	**83.858**
SD	**±1.896**	**±0.757**	**±1.324**	**±1.753**

Appendix 5.2: Table showing the aqueous soluble fractionof alginate-FDAE beads of *A. afra*

Sample No	Initial weight of plant material(mg)	Weight of the residue on filter paper(mg)	Weight of the soluble portion in the plant material (mg)	Percentage of soluble material
1	30.3	28.1	2.2	7.261
2	29.7	27.4	2.3	7.744
3	30.7	28.3	2.4	7.818
4	31.2	28.7	2.5	8.013
5	30.1	28.1	2	6.645
6	29.4	27.2	2.2	7.494
Ave	**30.233**	**27.967**	**2.267**	**7.494**
SD	**±0.656**	**±0.565**	**±0.175**	**±0.492**

Appendix 5.3: Table showing the aqueous soluble fractionof PMMA-alginate-FDAE beads of *A. afra*

Sample No	Initial weight of plant material(mg)	Weight of the residue on filter paper(mg)	Weight of the soluble portion in the plant material (mg)	Percentage of soluble material
1	30.1	29.7	0.4	1.329
2	30.4	30.2	0.2	0.658
3	29.7	29.3	0.4	1.347
4	29.6	29.5	0.1	0.338
5	30.7	30.3	0.4	1.303
6	30.3	30.1	0.2	0.660
Ave	**30.133**	**29.85**	**0.2833**	**0.939**
SD	**±0.423**	**±0.408**	**±0.133**	**±0.440**

Appendix 6

Entrapment efficiency of the FDAE in the alginate-FDAE beads and PMMA-alginate-FDAE beads of *A. afra*

Appendix 6.1: Table showing entrapment efficiency of the alginate-FDAE beads

	Expected weight of extract per weight of beads	Determined weight of extract per weight of beads	Entrapment efficiency (%)
Sample 1	0.3552	0.156	43.919
Sample 2	0.3552	0.149	42.061
Sample 3	0.3552	0.151	42.455
Mean			42.812
Standard deviation			0.979

Appendix 6.2: Table showing entrapment efficiency of the PMMA-alginate-FDAE beads

	Expected weight of extract per weight of beads	Determined weight of extract per weight of beads	Entrapment efficiency (%)
Sample 1	0.3365	0.122	36.107
Sample 2	0.3365	0.114	33.938
Sample 3	0.3365	0.144	42.764
Mean			37.603
Standard deviation			4.599

Appendix 7

Total flavonoid content

Appendix 7.1: The absorbance readings at 349 nm of the hydrolysed and unhydrolysed preparations of *A. afra*

Plant Sample	hydrolysis	Absorbance reading at 349nm in AUFs (Mean ± SD, n = 3)
FDAE	No	0.0907 ± 0.0012
	Yes	0.0988 ± 0.003
Alginate- FDAE beads	No	0.0756 ± 0.0013
	Yes	0.0771 ± 0.001
PMMA-alginate-FDAE beads	No	0.060 ± 0.001
	Yes	0.0623 ± 0.003

Appendix 7.2: The total flavonoid content (µg of luteolin equivalents (LE) per 2.5 mg) of unhydrolysed and acid-hydrolysed plant material

Plant Sample	Hydrolysis	Equivalent luteolin conc. in µg per 2.5 mg of plant sample (Mean ± SD, n = 3)
FDAE	No	49.762 ± 1.786
	Yes	61.333 ± 5.167
Alginate- FDAE beads	No	28.143 ± 1.789
	Yes	30.333 ± 1.929
PMMA-alginate FDAE beads	No	6.524 ± 1.668
	Yes	9.190 ± 3.786

Appendix 7.3: Comparison of the total flavonoid content expressed in µg of luteolin equivalents (LE) per mg for the unhydrolysed and acid-hydrolysed plant material

Plant Sample	Hydrolysis	TFC (µg of LE/mg of extract)	p value, (T-test result for unhydrolysed *vs* hydrolysed)
FDAE	No	19.905	0.0482; S
	Yes	24.533	
Alginate- FDAE beads	No	11.257	0.2232; NS
	Yes	12.133	
PMMA-alginate FDAE beads	No	2.609	0.3522; NS
	Yes	3.676	

Appendix 8

Appendix 8.1: Inter and intra day precision of HPLC asay for luteolin. Luteolin concentration is 4.3 µg/ml

Assay day	Measured luteolin concentration in sample (µg/ml)				RSD(%)
	Time 1	Time 2	Time 3	Mean ± SD	
1	4.8591	4.8130	4.8716	4.848 ± 0.03	0.637
2	4.7955	5.068	5.1105	4.991 ± 0.18	3.427
3	4.8586	4.8409	4.8426	4.847 ± 0.01	0.201
Inter-Day assay precision (RSD in 3 days)				4.895 ± 0.045	1.421

Appendix 8.2: LOD and LOQ data for luteolin over the 4.5 – 27.0 µg/ml range at 350 nm

Prepared concentration (µg/mL)	Mean peak area (mAUFs)	Concentration found (µg/mL)	RSD (%)	Bias[&](%)	LOD[#]	LOQ
8.7	775.59	8.47	0.25	-2.33	Accept	Accept
4.3	425.82	3.91	0.44	-9.74	Accept	Accept
2.17	269.15	1.87	5.10	-13.62	Accept	Reject
1.30	211.77	1.12	7.47	-13.49	Accept	Reject
0.43	-*	-*	-*	-*	Reject	Reject

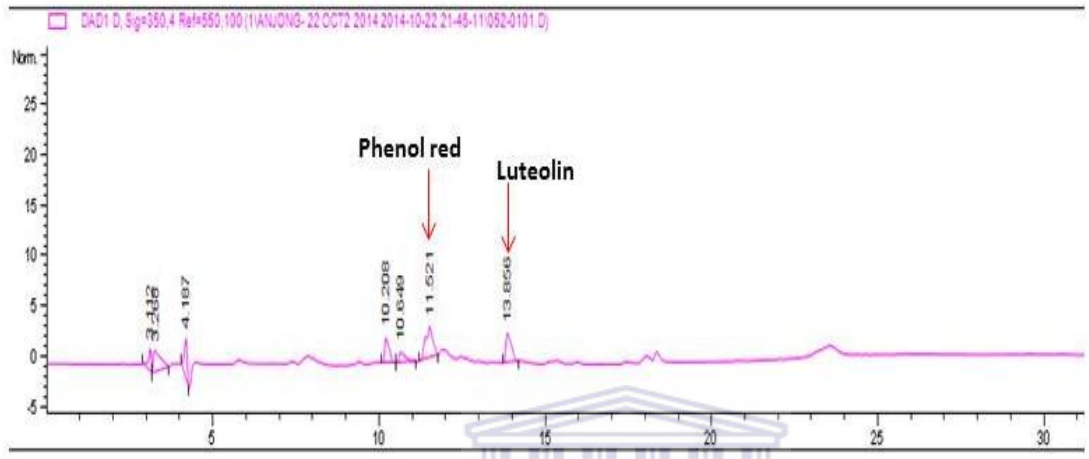
[&]Bias: The difference between the mean concentration measured and the prepared concentrations as a percentage of the prepared concentration.

[#]LOD: Determined using an analyte response 3 times that of the noise (signal-to-noise ratio of 3:1) with the mean baseline noise = 0.4 a.u., n=3.

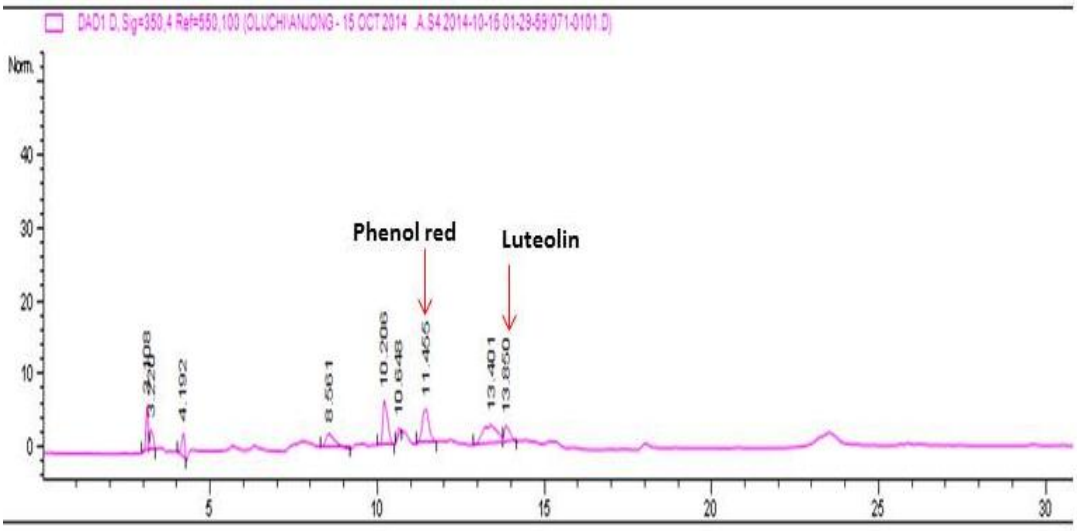
*Concentration could not be determined since the peak area was less than the regression equation intercept.

Appendix 9

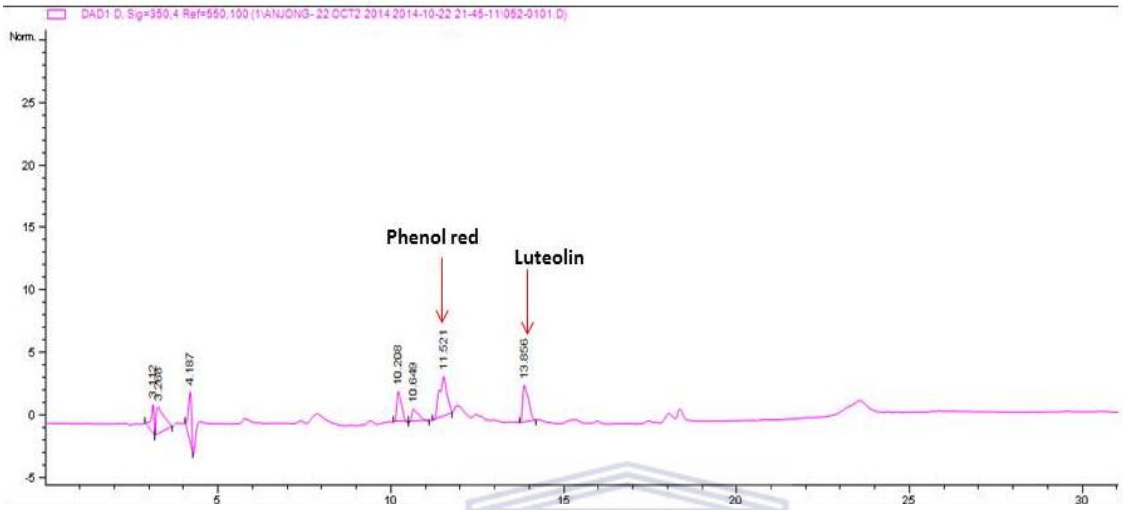
Appendix 9.1: A representative HPLC chromatogram of unhydrolysed PMMA-alginate-FDAE *in situ* perfusate sample at 30 min containing luteolin and phenol red. The retention time of luteolin is 13.8 while that for phenol red is 11.5. The chromatographic conditions are described in section 5.2.6.



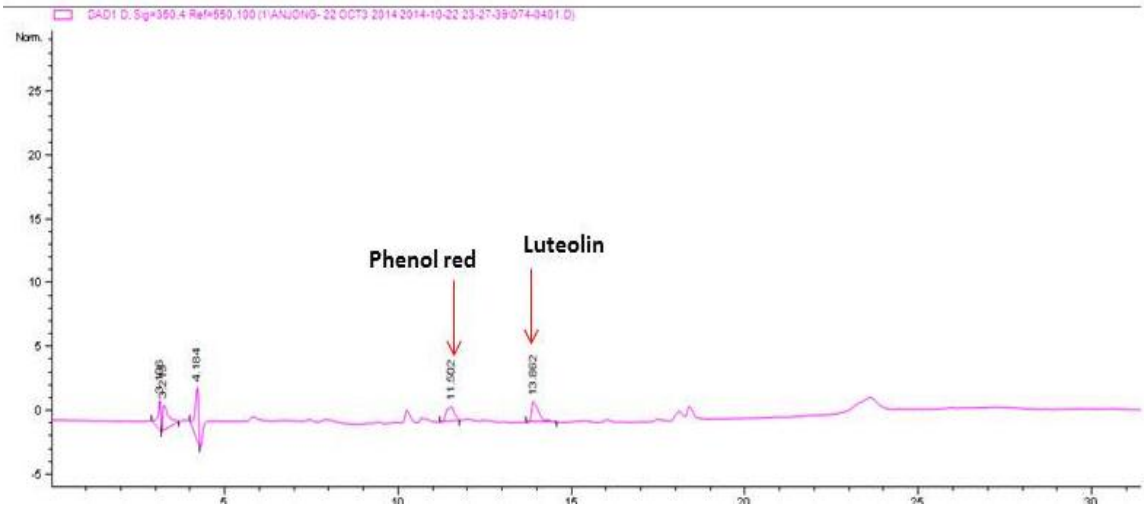
Appendix 9.2: A representative HPLC chromatogram of hydrolysed PMMA-alginate FDAE *in situ* perfusate sample at 30 min containing luteolin and phenol red. The retention time of luteolin is 13.8 while that for phenol red is 11.4. The chromatographic conditions are described in section 5.2.6.



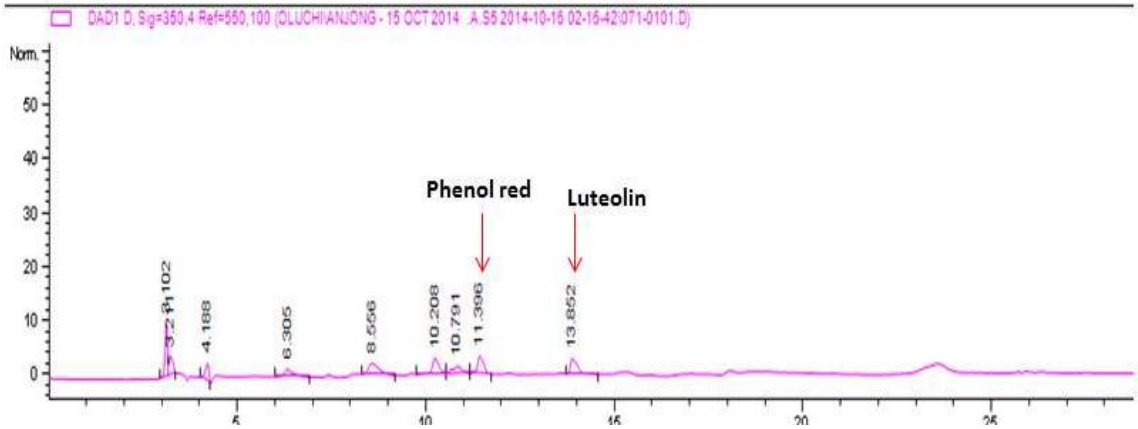
Appendix 9.3: A representative HPLC chromatogram of unhydrolysed alginate-FDAE *in situ* perfusate sample at 30 min containing luteolin and phenol red. The retention time of luteolin is 13.8 while that for phenol red is 11.5. The chromatographic conditions are described in section 5.2.6.



Appendix 9.4: A representative HPLC chromatogram of hydrolysed alginate-FDAE *in situ* perfusate sample at 30 min containing luteolin and phenol red. The retention time of luteolin is 13.8 while that for phenol red is 11.5. The chromatographic conditions are described in section 5.2.6.



Appendix 9.5: A representative HPLC chromatogram of unhydrolysed FDAE *in situ* perfusate sample at 30 min containing luteolin and phenol red. The retention time of luteolin is 13.8 while that for phenol red is 11.5. The chromatographic conditions are described in section 5.2.6.



Appendix 9.6: A representative HPLC chromatogram of hydrolysed FDAE *in situ* perfusate sample at 30 min containing luteolin and phenol red. The retention time of luteolin is 13.8 while that for phenol red is 11.5. The chromatographic conditions are described in section 5.2.6.