THE DESIGN, PREPARATION AND EVALUATION OF *ARTEMISIA AFRA* AND PLACEBOS IN TEA BAG DOSAGE FORM SUITABLE FOR USE IN CLINICAL TRIALS

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A thesis submitted in partial fulfilment of the requirements for the degree of Magister Pharmaceuticiae in the School of Pharmacy, University of the Western Cape, Bellville, South Africa.

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KEY WORDS

Artemisia afra

Standardized plant material

Plant freeze-dried aqueous extract

Tea bag dosage form

Infusion from tea bag

Luteolin

HPLC

Herbal stability

Herbal placebo

Clinical trials

Summary

The design, preparation and evaluation of *Artemisia afra* and placebos in tea bag dosage form suitable for use in clinical trials.

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Artemisia afra, a popular South African traditional herbal medicine is commonly administered as a tea infusion of the leaves. However, clinical trials proving its safety and efficacy are lacking mainly due to the absence of good quality dosage forms and credible placebos for the plant.

The objectives of this study were to prepare a standardized preparation of the plant leaves and freeze-dried aqueous extract powder of the leaves, in a tea bag dosage form and to design and prepare credible placebos for these plant materials. It was hypothesised that the intra-batch variation in the plant flavonoid constituents would be lower in the standardized *A. afra* leaves and freeze-dried aqueous extract powders compared to the traditionally used non-standardized leaves, that the tea bag would be a pharmaceutically suitable dosage form for the traditionally used plant leaves, that the infusion profiles of luteolin (the flavonoid marker), from the tea bag would be similar to that of the loose leaves traditional form and that credible placebos for the plant materials devoid of pharmacological activity could be prepared.

To realise the objectives, different batches of plant materials were blended and an aqueous decoction and freeze-drying method used to prepare the standardized leaves and freeze-dried aqueous extract powder of the plant leaves, respectively. These plant materials were packed in 36cm^2 tea bags which were pharmaceutically evaluated using the European Pharmacopoeia criteria, subjected to stability testing and their infusion profiles determined using the British Pharmacopoeia dissolution apparatus I (basket) and a modification of the BP apparatus II (paddle) incorporating a holding cell, for the loose leaves and the tea bag preparations, respectively, and the infusion profiles compared using the f_1 and f_2 mathematical method. The *A. afra* leaves were exhaustively solvent

extracted and inert in-organic salts blended to prepare the placebos for the leaves and freeze-dried aqueous extract powder, respectively. Finally, the placebos were evaluated for lack of pharmacological activity using the isolated guinea pig tracheal muscle preparation.

The *A. afra* standardized dried leaves and freeze-dried aqueous extract powder, contained a total luteolin content of 2.065 ± 0.2347 and 13.870 ± 1.2460µg/mg, respectively, with an intra-batch variation (% R.S.D) of 11.36% and 6.70%, respectively, reduced from an initial %R.S.D of 21.24%, 30.00% and 16.77%, for the three separately collected plant batches used. The tea bag of the *A. afra* standardized dried leaves was stable under room temperature and humidity conditions for 6 months, while the freeze-dried aqueous extract tea bag was not and both tea bag preparations were unstable at conditions of 40˚C/ 75% RH. The f_1 and f_2 values for the infusion of luteolin from the leaves in tea bag compared to the loose leaves were 73.52% and 13.85%, respectively, indicating that the profiles were not similar. Finally, the placebo materials prepared closely resembled the respective plant materials and the placebo of the *A. afra* leaves possessed only slight muscle relaxant activity while the placebo for the extract powder was pharmacologically inert.

In summary, the results showed that the tea bag was a suitable dosage form for the *A. afra* standardized dried leaves, but not the freeze-dried aqueous extract powder and that the tea bag preparation did not have similar infusion profiles to the loose leaves, but could still be used in clinical trials provided that adjustments in the dose preparation and administration methods are made. Finally, credible placebos for the plant materials suitable for use in clinical trials were prepared.

DECLARATION

I declare that the thesis The design preparation and evaluation of *Artemisia afra* and placebos in tea bag dosage form suitable for use in clinical trials 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 complete references.

A. Dube June 2006

Signed UWC, Bellville.

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DEDICATION

I dedicate this master's thesis to my parents Mr and Mrs Dube for their love, support and encouragement that has got me where I am today.

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Chapter 1

Introduction

Artemisia afra is a popular traditional herbal medicine widely used in South Africa for the treatment of a variety of ailments (Roberts, 1990; Van Wyk & Gerike, 2000). Infusions of the plant leaves are commonly prepared as teas for treatments ranging from asthma, malaria to diabetes, and the steam from such infusions may also be inhaled for headaches and colds (Hutchings *et al*., 1996; Thring & Weitz, 2006).

Despite the popularity and widespread use of this herb, clinical trials demonstrating its safety and efficacy in humans are lacking. For traditional herbal medicines in general, several reasons have been given to account for this state of affairs and include the variability in the preparation methods and poor quality of the traditional dosage forms, the variation in the phytochemical composition of the plant materials used and the lack of credible placebos for use in the clinical trials (Wolsko *et al*., 2005). For example, in the *Artemisia afra* traditional preparations, it is commonly directed that a quarter cup or a double handful of the wet or dried leaves be infused as a tea (Roberts, 1990) and from this it may be anticipated that such non-specific directions to measurement may lead to variations in dose each time a treatment is prepared. In addition, the use of wet leaves in the dosage preparations may result in poor product quality, as the presence of moisture may encourage microbial growth. The *A. afra* plant materials used may also be subject to factors that may affect their phytochemical composition such as variations in the geographic source of the materials, time of harvest, drying processes and storage conditions and as a result, variations in the observed therapeutic effects may be expected (Graven *et al*., 1990; Zidorn *et al*., 2005). Collectively, the above issues, together with the fact that there is currently no placebo material for *A. afra* plant, makes it difficult to conduct good quality clinical trials on the herb.

To address some of the above issues and therefore enable clinical trials on the herb, it was proposed to standardize the plant leaves and prepare them in a tea bag dosage form. It was anticipated that such a preparation would be of suitable pharmacopoeial quality and

would have release profiles of the actives that were comparable to those observed in the traditional infusions (and therefore could be used in place of the traditional preparation in clinical trials). In addition, the process of standardization would reduce the inherent variability in the phytochemical constituents of the plant materials, guarantee reproducible clinical effects and therefore enable good quality safety and efficacy data for the herb to be generated. Furthermore, in order to obtain an even more uniform and constant material composition, a freeze-dried aqueous extract powder of the plant leaves was to be prepared in the tea bag dosage form, for administration as an instant tea, with the added advantage of rapid preparation for the patients (Wichtl, 1994). Finally, it was intended to design placebo materials which looked, smelt and tasted similar to the *A. afra* plant materials and were devoid of pharmacological activity.

Suitable analytical methods such as HPLC were used to characterise the quality of the plant materials and of the dosage preparations. Methods were designed for the preparation of the placebo materials and their pharmacological activity was evaluated using the isolated guinea pig tracheal muscle preparation.

Chapter 2

Literature Review

2.1 Introduction

In the present chapter, an overview of *Artemisia afra* plant is given and this includes its description, uses and traditional methods of preparation. The shortcomings of the traditional preparations shall also be highlighted and the proposed methods of addressing them discussed. The need for safety and efficacy data, including the general aspects of clinical trials and quality aspects, for herbal products shall also be discussed. Finally, a review of the methods used for dissolution profile comparisons shall be presented.

2.2 *Artemisia afra* – An important indigenous medicinal plant

2.2.1 Vernacular names

Artemisia afra Jacq. Ex Willd, is one of the oldest known and most widely used of medicinal plants in South Africa (Roberts, 1990; Watt & Breyer-Brandwijik, 1962). Several of the indigenous ethnic groups of South Africa have a long tradition of use of this plant for various ailments, and as such different names relate to it some of which are given below (Watt & Breyer-Brandwijik, 1962):

Xhosa : Umhlonyane
Zulu : Mhlonyane
Sotho : Lanyana
Tswana : Lengana
English : African Wormwood
Afrikaans : Wildeals

2.2.2 Botanical classification and morphology of *Artemisia afra*

Artemisia afra belongs to the;

Division : Magnoliphyta

Class : Magnoliopsida

Sub class : Asteridae

Order : Asterales

Family : Asteraceae

Genus : *Artemisia*

Species : *A. afra*

Fig. 2.1: ***Artemisia afra*** **plant.**

Artemisia afra is an erect growing, shrubby, woody, perennial plant growing up to 2 m tall with a leafy and hairy stem (Van Wyk *et al*., 1997). Its leaves grow up to 8 cm long and 4 cm wide. The leaf shape is narrowly ovate, bi-pinnatipartite, feathery and finely divided. The ultimate segments which are linear in shape with an acute tip and smooth or toothed margin, grow up to 10 mm long and 2 mm wide. It has a pectinated midrib with similar lobes, a smooth or glandular-punctated upper surface and a canescent (grayish) lower surface. The petiole is up to 2 cm long, dilated at the base with a pair of simple or divided leaf-like stipules. The inflorescence is subglobose with nodding, leafy, terminal, racemose panicles up to 40 cm long (Hilliard, 1977).

The plant is easily identifiable by its characteristic aromatic odour. It flowers between January and June, producing yellow coloured flowers. The fruits are about 1mm long, somewhat 3-angled and slightly curved with a silvery-white coating. In winter, the branches die back but rapidly regenerate from the base (Hilliard, 1977; Van Wyk *et al*., 1997).

2.2.3 Geographical distribution of *Artemisia afra*

Artemisia afra is found growing abundantly in Namibia, Zimbabwe, and in the mountain regions of Kenya, Tanzania and Uganda. It is also found as far up as Ethiopia, in the east

and south of tropical Africa and down to the eastern part of South Africa including Swaziland and Lesotho (Van Wyk *et al*., 1997; Hilliard, 1977).

In South Africa, the plant can be found growing in regions from Stellenbosch, the Western Cape, Aliwal North and the Graaff Reinet Mountains in the North. It is also widespread in Natal from the coast to the Drakensberg (Hilliard, 1977). Its geographical distribution in South Africa is shown in the Figure 2.2 below.

Figure 2.2: Geographical distribution of *Artemisia afra* in South Africa (SATMERG, 1999).

2.2.4 Traditional uses and dosage forms of *Artemisia afra*

Artemisia afra is a herb used for the treatment of several ailments (Van Wyk *et al*., 1997). It is a well-known treatment for coughs, colds, croup, whooping cough, colic, heartburn, flatulence and gout (Roberts, 1990; Thring & Weitz, 2006). It is also commonly used for the treatment of asthma, acute bronchitis, hay fever, bladder and kidney disorders, convulsions, diabetes, fever, headache, inflammation, rheumatism, stomach disorders and worms (Felhaber, 1997; Thring & Weitz, 2006). The herb is also used for the treatment of malaria (Watt & Breyer-Brandwijik, 1962).

In traditional Zulu medicinal use, leaf infusions are taken as teas or administered as enemas and the steam from the infusions is commonly inhaled for the treatment of

headaches and colds (Hutchings *et al*., 1996). Enemas made from ground plants suspended in water or milk are administered for constipation or intestinal worms in children. Decoctions are also taken as blood purifiers for acne, boils, measles and smallpox (Bryant, 1966). In a survey of medicinal plant use in the Bredasdorp/ Elim region of the Southern Overberg in the Western Cape Province of South Africa, *Artemisia afra* was found to be the plant with the greatest use value amongst the respondents. In other words, *Artemisia afra* was used the most for the treatment of various ailments more than any other traditional plant medicine among the people surveyed (Thring & Weitz, 2006).

The usual dosage preparation of the plant is in the form of a tea or a decoction (Roberts, 1990; Felhaber, 1997; Van Wyk *et al*., 1997). Commonly, a quarter cup of fresh leaves to one cup of boiling water is allowed to stand and steep for 10 minutes, then strained and sweetened with honey before drinking (Roberts, 1990). This preparation may be administered orally for the relief of most of the ailments previously mentioned. An infusion may also be prepared, by pouring 2 litres of boiling water over 1 cup of fresh leaves and stems and allowed to draw for an hour before being strained and this may be used as a bath for measles, wounds, bites and stings (Felhaber, 1997). A strong brew of the herb may be prepared using a half-cup of leaves to 1 1/2 or 2 cups of boiling water, allowed to draw for 10 minutes and then strained. This can be used as a mouthwash for gumboils, mouth ulcers or for the relief of earache (Bryant, 1966). Apart from these orally administered dosage forms, poultices, vapours for inhalation and enemas are prepared for the treatment of a variety of ailments (Roberts, 1990; Felhaber, 1997).

2.2.5 Shortcomings of the traditional dosage formulations

As stated above, the common method of dosage preparation is that of a decoction which involves the use of wet leaves immersed in boiling water to make a tea. The traditional method of preparation has several disadvantages attributed to it. Firstly, the use of wet leaves is undesirable. This is because the presence of moisture in the leaves may promote microbial growth and accelerate degradation of the product (McCutcheon, 2002). The

microbes may however be killed by use of boiling water during the decoction process, but in some circumstances the microbial load may still be outside the acceptable limits as set by the European Pharmacopoeia for herbal medicinal products (EP, 2002c).

Under the traditional method, the directions for dosage preparation are often obscure. Often the directions are stated as 'a double handful', 'cup of boiling water' or 'quarter cup of leaves' (Roberts, 1990). It is difficult to accurately quantify a double handful or measure a quarter cup consistently. Furthermore the size of 'cups' varies and this may lead to variations in the actual dose the patient receives.

It is well known that plants grown in different geographic locations may contain different compositions of the active principles (Zidorn *et al*., 2005; Gilani & Atta-ur-Rahman, 2005). The chemical constituents may vary depending on time of harvest, plant origins, drying processes, storage times and other factors (Liang *et al*., 2004; Yang *et al*., 2005). Phenolic compounds such as flavonoids in plants have been shown to increase proportionally with increases in altitude and this has been attributed mainly to their function as UV-B protective agents (Zidorn *et al*., 2005). Graven *et al* (1990) have reported significantly higher oil yields from *Artemisia afra* when the crop was harvested during anthesis and early seed set as opposed to earlier or later harvesting in the reproductive period. The aforementioned observations may be expected to affect the plant materials used in the traditional setting, resulting in variations in the phytochemical composition of the plant and consequently the amount of actives the patient receives in a given dose.

In the traditional method of preparation, there is no mention of the expected degree of comminution of the leaves used. In the extraction of constituents from plant matrices, i.e. in the decoction or infusion process, the degree of comminution (leaf size) is of crucial importance as the amount of constituents released into the tea per given time period increases as the degree of comminution increases and the highest yields are often obtained with smaller leaf sizes (Wichtl, 1994). In addition to the leaf size, the method of

preparation or drying/ storage of the plant material may affect the kinetics of release of the active principles due to changes in the internal leaf structure (Jaganyi & Price, 1999).

2.3 The need for safety and efficacy data

According to the World Health Organization (WHO), about 65-80% of the world population rely on herbal medicines for their primary health care needs (WHO, 1993). A marked growth in worldwide use of phytotherapies has occurred over the last 20 years and in Germany, it is estimated that about 80% of physicians prescribe herbs and sales of herbal medicines worldwide are in the region of hundreds of billions of dollars a year (Gilani & Atta-ur-Rahman, 2005). However, the boom in use and popularity has not been followed by an increase in supporting scientific data and insufficient safety and efficacy data exists for most plants to support their widespread use (Bombardelli, 2001; Calixto, 2000; Chang, 2000). This state of affairs has been attributed mainly to the lack of patent protection and the diversity and relatively small-scale of the industry involved, which has seen many of them unable to meet the financial demands of efficacy and safety studies (Mills, 1998). In addition, quality issues such as lack of plant material standardization, good quality dosage forms and thorough characterization of the traditional plant materials has also hampered clinical trial research or where conducted, has led to poor quality studies (Wolsko *et al*., 2005). Finally, the use of different dosages and variations in the duration of treatment (as administered in the traditional setting) makes comparison and analysis of the clinical data difficult (Kroes & Walker, 2004).

However, it is important that claims for safety and efficacy of herbal medicines be scientifically validated. Once the herb has been scientifically validated, it can gain the acceptance of conventional medicine (Calixto, 2000). Ispaghula, Garlic, Ginseng, Gingko, St. John's Wort and Saw Palmetto are a few examples of herbs that have gained popularity and approval by physicians as a result of scientific validation through clinical studies (Gilani & Atta-ur-Rahman, 2005). In addition, clinical studies help reveal the toxic effects, risks and inherent side effects of the plants for safer and more effective use of the medicines (Calixto, 2000).

Once scientifically validated, the herb may be approved by drug regulators and be marketed for public use. Although preliminary assessments of efficacy can be obtained through *in vitro* testing and experiments on animals, authorities licensing new medicines for public use require evidence of their effect on human beings and clinical trials, with minimum bias, are able to satisfy these requirements (Mills, 1998).

2.3.1 Clinical trials

A clinical trial is any systematic study of a medicinal product in human subjects whether in patients or in non patient volunteers in order to discover or verify the effects of and/ or identify any adverse reaction to the investigational product and/ or study their absorption, distribution, metabolism and excretion in order to ascertain the efficacy and safety of the product (EEC, 1997).

Clinical trials on herbal medicines may have one of two types of objectives. One is to evaluate the safety and efficacy that is claimed for a traditional herbal medicine and the other is to develop new herbal medicines or examine a new indication for an existing herbal medicine or change of dose formulation, or route of administration. In some cases, trials may be designed to test the clinical activity of a purified or semi purified compound derived from herbal medicines (EEC, 1997; WHO, 1998).

Clinical trials are generally divided into 4 phases. Phase I trials are carried out on a small number of healthy volunteers or patients suffering from the disease for which the medicine is intended. The main purpose of this type of trial is to observe tolerance to the medicine and therefore get an indication of the dose that may be used safely in subsequent studies (EEC, 1997; WHO, 1998). Phase II studies are conducted on a limited number of patients to determine clinical efficacy and further confirm safety. Such studies are usually randomized, double-blind, controlled studies, using for control groups either an existing alternative treatment or a placebo. The dosage schedules used in such studies are then used for more extensive clinical studies (EEC, 1997; WHO, 1998). Phase III studies are performed on larger patient groups usually situated at several study centres

using a randomized double blind design to validate the preliminary evidence of efficacy obtained in earlier studies. Normally such studies are conducted in conditions which mimic the anticipated or normal conditions of use as closely as possible (WHO, 1998). Finally, phase IV studies are performed after the dosage form is available for public use and the main purpose of such studies is to detect toxic events that may occur so rarely that they are not detected in earlier studies (WHO, 1998).

Clinical trials are required to conform to the standards of Good Clinical Practice (GCP), which essentially, is an international ethical and scientific quality standard for designing, conducting, recording and reporting trials. Compliance with this standard provides public assurance that the rights, safety and well-being of trial subjects are protected, consistent with the principles in the Declaration of Helsinki, and that the clinical trial data is credible (EEC, 1997).

2.3.1.1 Placebos

A placebo, also commonly referred to as a dummy pill, is in theory a pharmacologically inert substance. The placebo may be defined as any therapy or component of therapy that is deliberately used for its non-specific, psychological, or psychophysiological effect, or that is used for its presumed specific effect, but is without specific activity for the condition being treated (Emilien *et al*., 1998).

Historically, placebos began to be utilized in therapeutic research around the early nineteenth century in response to accusations that the efficacy observed had to do with just the appearance of a treatment, as opposed to any real effect from the therapy itself (Kaptchuck *et al*., 2001). As a result, placebos are now included in clinical research in order to function as a mechanism for controlling and ensuring that any observed improvement in the condition under investigation is due to the effects of the treatment alone. The requirements for a credible placebo are that factors such as the appearance, the volume or quantity, the number of intakes, preparation and route of administration be similar to the treatment under investigation (Emilien *et al*., 1998).

Herbal materials are known to present significant challenges in designing credible placebos. This is mainly due to the nature of plant materials with respect to their appearance, smell and taste, which is difficult to mimic using synthetic materials or chemicals (Kaptchuck *et al*., 2001). In literature, few herbal clinical trials employing the use of a placebo actually discuss the ingredients used in the formulation of the placebo and from the studies found, none were identified as using a placebo of the plant parts themselves, i.e. the leaves, bark, roots, etc. All studies found used extracts of the herbs which were formulated as tablets or capsules. One example is that of a placebo of a Chinese herbal preparation, that contained 78.2% calcium hydrogen phosphate, 19.6% soy fibre, 0.3% cosmetic brown, 0.5% cosmetic yellow, 0.01% edicol blue 0.09% identical liquorice dry flavour, and 0.03% bitter flavour (Bensoussan *et al*., 1998).

2.4 Teas

Tea was first processed as a beverage in China over 3000 years ago and today millions of cups of tea are consumed everyday around the world making tea one of the most popular beverages in the world (Peterson *et al*., 2004).

Teas vary by type (i.e. black, oolong, green and pu'er), variety (i.e. blended and unblended), and processing (i.e. conventional, cut, tear, and curl). Unblended teas are named by their country of origin (e.g. Assam, China, Darjeeling), while blended teas are named generically (Earl Gray, Irish Breakfast), rather than by the teas they contain (Peterson *et al*., 2004).

In brewing a tea, several factors are involved in influencing the characteristics of the final brew. Apart from the quality of the tea leaf itself, the quality of the water used is important in determining the brew (Jaganyi & Wheeler, 2003). Bottled or filtered water is normally preferred and this has to do mostly with the influence on the resultant flavour of the tea (Anonymous, 2006a). The temperature of the water used for the brew is also important and it is recommended that black teas be brewed with boiling water, oolongs with water just below boiling point and green teas with water around 80˚C. Again, the

temperature affects the flavour as well as the constituents extracted (Anonymous, 2006a). The other important factor in brewing teas is the steeping time. Steeping times depend on the type of leaf and its grade. Black teas are normally steeped from 3 - 7 minutes while oolongs are steeped from 1 ½ - 4 minutes and greens from 2 - 3 minutes. Steeping time affects the amount of constituent extracted, which ultimately affects the flavour of the tea. Longer steeping times normally result in bitter teas (Anonymous, 2006a).

Flavonoids are known to be extracted from teas during the tea making process. The resultant flavonoid content in a cup of tea may depend on one of the two factors, i.e. the characteristics of the tea leaf itself and the brewing characteristics, as described above (Peterson *et al*., 2005). Currently, there is much interest in the type and amount of flavonoids extracted from teas and herbal infusions. This is largely due to the reported health benefits of teas such as antioxidant, artherosclerotic and anti- carcinogenic benefits and flavonoids are believed to be mainly responsible for these actions (Wang & Helliwell, 2001; Atoui *et al*., 2005).

2.4.1 Instant teas

An instant tea is produced as a result of an exhaustive extraction of the tea leaves/herb using water or in some instances water/ ethanol mixtures. The resultant powders are then mixed with hot water for consumption as a tea (Wichtl, 1994). Generally, two types of instant teas are available resulting from differences in the manufacturing process. Spray-dried extracts are dry and hollow pellets produced as the extract is sprayed through a nozzle and forms fine droplets in a current of warm air. The result is a low-density highly soluble powder. However, these extracts are often hygroscopic with caking as a major problem (Wichtl, 1994). Tea granules are granular or cylindrical aggregates produced when the extract is sprayed onto a carrier (usually saccharose) and dried with heating. These granular extracts are often very soluble in water and slightly hygroscopic. However, they contain a small percentage of drug extract, as carrier and filler substances occupy a large proportion of the granule (Wichtl, 1994).

Generally, spray- dried instant teas are considered the ideal for a tea for medicinal purposes. They have the advantage of rapid preparation, which can be of convenience for the patients. They also have the added advantage of a uniform and constant composition, which is important in order to produce reproducible pharmacological effects (Wichtl, 1994; Bombardelli, 2001).

2.4.2 Tea bags

Millions of cups of tea are consumed everyday around the world and most of these are brewed using tea bags (Jaganyi & Ndlovu, 2001). Different shapes and sizes exist on the market, all with the aim of capturing the attention of the consumer. Apart from the traditional square and rectangular shapes, round and pyramidal shaped tea bags are also common (Jaganyi & Ndlovu, 2001). Tea bag papers used are commonly manufactured from nonwoven fibres usually based on cellulose from the seeds of cotton or stem fibres of hemp, jute or abaca trees (Anonymous, 2006b). These may be oxygen bleached or be processed unbleached to form the tea bag paper. The fibres may also be processed in combination with synthetic polymers to form papers that are heat sealable (Schoeller & Hoesch, 2005). High strength at the sealing joints, good cut-ability, high wet strength, good particle/dust retention and flavour infusion are some of the requirements for a good quality tea bag paper (Schoeller & Hoesch, 2005).

2.4.2.1 Infusion from tea bags

It has been reported in literature that when infused in water, the tea leaf swells by a factor of about 4.25 (Spiro & Price, 1985) and therefore, depending on the size of the tea bag, some hindrance occurs to the swelling of the tea leaf and this affects the rate of infusion of the constituents (Jaganyi & Mdletshe, 2000). In experiments conducted on the effect of tea bag size and shape on the rate of caffeine extraction from Ceylon orange pekoe tea (Jaganyi & Ndlovu, 2001), it was observed firstly, that the infusion of caffeine from loose tea was much faster than from tea in the tea bag. Secondly, significant increases in the rate of extraction were observed with increases in tea bag size. From a 16 to 36 cm^2 tea

bag area, an increase of 25% was observed and smaller increases in the rate of extraction were observed from tea bags larger than 36 cm^2 up to an area of 64 cm^2 (Jaganyi & Ndlovu, 2001).

The above observations have been attributed to increases in the space available for the swelling and movement of the tea leaf inside the tea bag. The theory behind this explanation assumes that when the tea leaf comes into contact with water, it swells first and then infusion follows. In the case of smaller sized tea bags, the tea leaves after swelling a compacted together. This makes penetration of water to the leaf and to the centre of the tea bag, more difficult. Moreover, the extracted constituents from the middle of the tea leaf to the bulk of the solution have to follow a more tortuous passage within the leaf particles; hence, a slower infusion process results (Spiro & Lam, 1995; Jaganyi & Ndlovu, 2001).

The hindrance to infusion can also be explained from the point of view of Nernst diffusion layers (Jaganyi & Ndlovu, 2001). A Nernst diffusion layer by definition is a fictitious layer above the true concentration profile or diffusion layer (IUPAC, 1997). This diffusion layer is present both inside and outside the tea bag membrane. The Nernst diffusion layer on the inside decreases with increase in the tea bag size because of an increase in free movement of the tea leaves and solution inside the bag. This explains the observed increase in rate of infusion with tea bag size. It also explains why the infusion is faster from loose leaves than from a tea bag as the Nernst diffusion layer in the former is zero (Spiro & Jaganyi, 2000). It also follows that, any motion which decreases the thickness of the inner and outer Nernst layers adjacent to the tea bag paper membrane, will increase the rate of tea brewing and this explains empirical findings among tea drinkers that stirring the brew around the tea bag, moving the tea bag up and down and jiggling the tea bag all speed up the brewing of the tea (Spiro & Jaganyi, 2000).
Other factors which affect the rate of tea brewing in a tea bag include the transfer of the constituents through the membrane, diffusion towards and away from the membrane plus through the swollen leaf, method of manufacture/preparation and storage of the tea leaves

and composition and temperature of the extracting medium (Spiro & Jaganyi, 2000; Jaganyi & Mdletshe, 2000).

2.4.2.2 Advantages of tea bags as a dosage form

Tea bags offer several advantages compared to the traditional tea preparation. Firstly, the fact that the correct amount (dose) is already accurately measured out offers both safety (from over- or under- dosing) and convenience in preparation for the patient. This advantage is more apparent in the case of mixed herbal teas, where the correct proportions of the herbs (with similar particle size) are already measured into the tea bag and therefore the patient does not have to scoop and measure each constituent herb as would be necessary with loose teas (Wichtl, 1994). Secondly, the considerable degree of comminution, which is required to pack into tea bags, also allows for a better extraction of the constituents compared to the uncut herb. The amount of constituents in the tea has been shown to increase as the degree of comminution increases and higher yields are often obtained using powdered drugs (Wichtl, 1994).

Tea bag paper material has been shown to retard the rate of infusion by about 29% when compared to loose tea (Jaganyi & Mdletshe, 2000) and this may offer an advantage if one wishes to control the amount of actives the patient receives within a given time period. However, in practice, because of the considerable degree of comminution these smaller leaves infuse faster inside the tea bags such that the overall infusion rate is increased over and above the larger-sized loose tea leaves (Jaganyi & Mdletshe, 2000).

Finally, tea bags also offer practical advantages. For example, the tea is easier to handle and simpler and less messy to dispose of as there is no residue left in the cup (Jaganyi & Mdletshe, 2000).

2.5 Phytochemical constituents of *Artemisia afra*

Literature sources contain some information on the phytochemical constituents of *Artemisia afra.* The essential oil of *A. afra* is known to contain the compounds α-pinene, γ-terpinene, camphene, ρ -cymene, 1.8-cineole, α-thujone, β- thujone, camphor, borneol, Artemisia ketone and sesquiterpene- 1-3 (Graven *et al*., 1990; Van Wyk *et al*., 1997; Piprek, 1982). In addition to these compounds found in the essential oil, other compounds have been detected within the leaves and these include the tannins, saponins, terpenoids of the eudesmadien and germacratien types, triterpenes α- and β- amyrin, the friedelin alkanes ceryl cerotinate and n- nonacosane as well as the coumarins and acetylenes (SATMERG, 1999; Van Wyk *et al*., 1997).

However, little is documented in the literature concerning the flavonoid constituents of *A. afra*. Investigations in our laboratory by Waithaka (2004), Komperlla (2005) and Mukinda (2006) on leaf infusions and aqueous extracts of the plant leaves have revealed the presence of the flavonoids luteolin, kaempferol, apigenin and quercetin.

2.6 The flavonoids

Flavonoids are a class of natural compounds present in all vascular plants. They occur in virtually all plant parts including the leaves, roots, wood, bark, pollen, flowers, berries and seeds (Markham, 1982). They are the pigments responsible for the hues and various colours observed in flowers and are also required for the normal growth, development and defense mechanisms in plants (Di Carlo *et al*., 1999; Harborne & Williams, 2000).

Flavonoids are biosynthesized via a combination of the shikimic acid and acylpolymalonate pathways. A cinnamic acid derivative synthesized from shikimic acid acts as the starting compound in a polyketide synthesis in which additional acetate residues are incorporated into the structure (Figure 2.3). This is followed by ring closure and through subsequent hydroxylations and reductions, plants are then able to form different classes of flavonoids (Markham, 1982; Di Carlo *et al*., 1999).

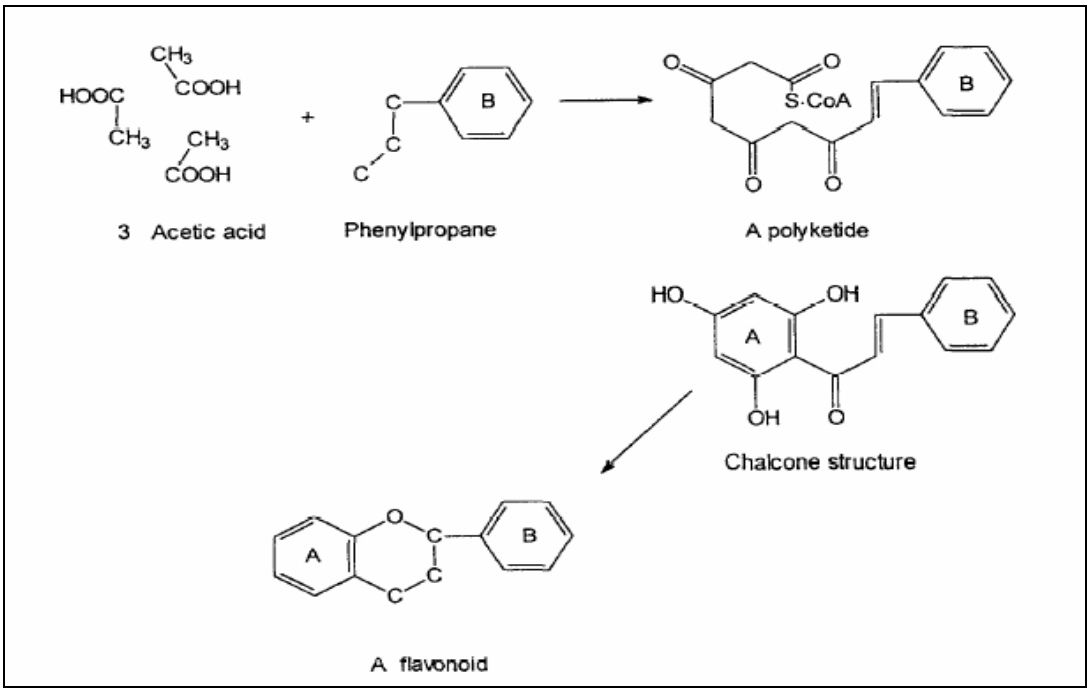

Figure 2.3: Schematic diagram showing the biosynthesis of the flavonoids (Di Carlo *et al.*, 1999).

Structurally, flavonoids consist of a fifteen-carbon atom basic nucleus (C_6-C_3- C_6 configuration) with several phenolic groups and, in some instances, attached sugars to form glycosides. They possess two benzene rings namely A and B, which are connected by an oxygen-containing heterocyclic ring C (Figure 2.4). Flavonoids are grouped according to the presence of different substituents on the rings and by the degree of ring saturation. 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 flavonones (e.g. hesperetin, naringenin) and flavones (e.g. luteolin, apigenin) (Markham, 1982; Heim *et al*., 2002).

Figure 2.4: The flavonoid nuclear structure (on the left) and the structure of luteolin (3′,4′,5,7-tetrahydroxyflavone) (on the right).

Flavonoids may occur as aglycones (consisting of a 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 as their methylated derivatives. Linking to various sugar residues increases the variation in structure and polymerization of the flavonoid. For example, quercetin alone is known to have over 179 glycosides and of the 2×10^3 different flavonoids already identified, up to 2×10^6 are thought to exist (Molnar-Perl & Fuzfai, 2005).

Flavonols and flavones are the most widely occurring flavonoids; of these luteolin (figure 2.4), quercetin, kaempferol, myricetin, chrysin and apigenin are widely distributed. The flavanones, flavanols, dihydroflavones and dihydrochalcones, are considered minor flavonoids because of their limited natural distribution (Di Carlo *et al*., 1999).

The starting step in the analysis of flavonoids, normally involves extraction or isolation of the flavonoids. Extraction with methanol or solid phase extractions are the commonly used methods. Optimum extracting conditions, however, vary and depend on the matrix to be isolated from e.g. plasma, urine or herbal material. Chromatographic techniques such as HPLC are the methods of choice in the separation, identification and quantitation of flavonoids. (Molnar-Perl & Fuzfai, 2005; de Rijke *et al*., 2006). In the analysis procedures, it is accepted practice to first acid hydrolyse the glycosides and then identify or quantify the released aglycones. This is mainly due to the large number of flavonoid conjugates present and the few numbers of commercially available reference compounds for them (Crozier *et al*., 1997). The hydrolysis procedure may also be used as a step to reduce the number of compounds to be determined, resulting in better resolution and improved characterization of the flavonoid constituents (Molnar-Perl & Fuzfai, 2005).

A wealth of information exists in the scientific literature concerning the pharmacological properties of flavonoids. Antioxidant, anti-mutagenic, anticancer, anti-hypertensive, anti-inflammatory, anti-diabetic, anti-allergy, anti-asthma, anti-ischaemic, anti-ulcer, antibacterial, antiviral and immune stimulating properties (Havsteen, 2002). Some of the

reported pharmacological actions of the flavonoids are similar to those reported for *A. afra*, which may give a lead as to the possible active compounds within the plant.

2.7 Quality considerations for herbal materials

Quality may be defined as the sum of variable characteristics that may significantly impact upon a product. For herbal medicines, such variable characteristics include the origins of the herb, botanical identity, purity, potency, stability and content of specified marker compounds (McCutcheon, 2002). Apart from these, issues of Good Agricultural Practices and Good Manufacturing Practices are also important and have a direct bearing on the final quality of the product (EMEA, 2001).

2.7.1 Standardization of herbal materials

Standardization refers to measures taken to ensure that there is a consistent quantity of a defined marker compound within a herbal material, as herbal materials are known to be highly variable in their make up (Gilani & Atta-ur-Rahman, 2005). Intrinsic factors (e.g. genetics) and extrinsic factors (e.g. growing, harvesting, storage and drying processes) may lead to variations in the chemical profiles of the herb (McCutcheon, 2002; Yang *et al*., 2005; Zidorn *et al*., 2006). In order to achieve reproducible biological data in terms of safety and efficacy, it is recommended that the herbal material be standardized to the active ingredients when they are known, or to specific markers when the actives are not yet known (Bombardelli, 2001).

Standardization is commonly achieved through blending different batches of the plant material (McCutcheon, 2002; Bombardelli, 2001). The assumption is that the content of the other constituents will also vary in proportion to the marker compound, and that if each batch contains the same amount of marker compound, other constituents will also be relatively consistent (Bombardelli, 2001). Alternatively, normalization may be employed. This is the process of adjusting the extraction ratio and/or adding fillers to achieve the targeted marker content. However, this is usually acceptable within narrow limits and

large adjustments are only permissible in cases where it has been established that the marker is responsible for the pharmacological activity (McCutcheon, 2002). Certain authorities consider this method similar to adulteration, particularly if this is not declared on the product label and consequently this method of standardization is not encouraged (McCutcheon, 2002).

2.7.2 Chemical fingerprints of herbal materials

Chemical fingerprints are commonly used to confirm the identity, authenticity and lot-lot consistency of a plant (Fan *et al*., 2006). Currently chromatographic and/or electrophoretic techniques are used to generate chromatographic patterns of plant materials (Springfield *et al*., 2005; Fan *et al*., 2006). Chemical fingerprints allow most of the phytochemical constituents of the herb to be determined and in this way, the full herbal plant can be considered as the active as opposed to the use of a single constituent (which may or may not be responsible for clinical efficacy). In this way, a form of chemical standardization can thus be applied as a quantitative ratio among the various constituents can be established, i.e. fixing a quantitative relationship between classes of compounds, the active principles or some characteristic compounds present in the plant (Bombardelli, 2001). It is generally accepted that materials with similar chromatographic fingerprints are likely to possess similar properties and as a result, the fingerprints allow the concept of phytoequivalence to be applied. This is the comparison of the chemical fingerprints for a material or product under question with that of a clinically proven reference product and is meant to guarantee patient safety as well as protection from adulterated products (Liang *et al*., 2004).

2.8 Quality assessment of herbal materials

Monographs exist for some of the commonly used and popular herbal medicines and the European Pharmacopoeia contains over 130 monographs to which the respective herbs must comply. However where monographs are not available, quality assessment is usually based on the results of test procedures performed on the plant material itself

(Springfield *et al*., 2005). Such tests include analysis of the starting material, tests on microbial quality or contaminants such as pesticides and fumigation agents. Quantitative determination of marker compounds with known activity is also assessed as a quality criterion (EMEA, 2001).

2.8.1 Quality assessment of herbal starting materials

An assessment of the quality of the starting material and excipients is required. Firstly, information on the site of collection, time of harvesting, stage of growth, drying and storage conditions should be documented (WHO, 2004) and in the case of herbal drugs with constituents with known activity, assays of their content using validated methods are required. This content must be stated as a range in order to ensure reproducibility (EMEA, 2001). Where the constituents are not known, suitable marker compounds may be selected and used (WHO, 2004; EMEA, 2001).

Generally, herbal materials must be tested for microbial contamination, pesticides and fumigation agents, toxic metals and other likely contaminants and adulterants. Acceptance criteria and limits exist but are diverse and there appears to be lack of consensus on these (WHO, 1998). For instance, the limits for some pesticides published in the *Pharmeuropa* 1993 are more restrictive than the WHO limits. In addition, the limits specified for microbial contamination in the European Pharmacopoeia 2002 are more restrictive than the WHO 1998 limits.

2.9 Quality assessment of herbal preparations

Guidelines require that the particulars of the characteristics, identification tests and purity tests for the product be established (EMEA, 2001). These may include details of tests on the performance of the dosage form such as dissolution or infusion. Chemical fingerprints can be used to trace the stability of the herbal preparation. Since the whole herbal drug or preparation may be considered to be the active, a determination of the stability of a single marker compound may not suffice; an analysis of the whole herbal material may be more

appropriate (EMEA, 2001). Heigl *et al* in a study to examine possible changes in the flavonoid pattern of common herbal drugs during long term and stress testing storage conditions, used HPLC fingerprint comparisons to demonstrate differences in stability of individual flavonoid components (Heigl *et al*., 2003). Such comparisons may allow determinations on substances present in the herbal preparations with respect to their stability and proportions, for quality purposes (Bombardelli, 2001).

2.9.1 Dissolution / infusion testing

In vitro drug release from dosage forms may be characterized as a quality control procedure as well as to establish *in- vitro in- vivo* correlations. Compendial methods such as the BP or USP apparatus I (basket), II (paddle) and III (reciprocating cylinder) may be used to evaluate the *in vitro* release characteristics of the dosage form.

Several factors are known to influence drug dissolution and include the effective surface area of the drug, dissolution coefficient of the drug, thickness of the diffusion layer, the saturation solubility of the drug, volume of the dissolution media and the amount of drug in the solution (Missaghi & Fassihi, 2005). Generally, the compendial methods of dissolution testing have been designed to test for dissolution from conventional pharmaceutical dosage forms and although a test for dissolution from medicated patches exists in the British Pharmacopoeia 2000, utilizing an extraction cell to hold the patch in place, by and large the methods are intended for tablets and capsule dosage forms.

In characterizing the infusion of tea bags, which are both floating and swelling matrices (a non conventional dosage form), the selection of an appropriate dissolution method requires special considerations in order to attain sensitive and reproducible dissolution data. Missaghi and Fassihi (2005) evaluated the effect of various hydrodynamic conditions on drug release from a floating and eroding matrix. The USP apparatus I, II, III and a modified apparatus II (paddle over mesh) were evaluated for similarity over various agitation speeds and the results from the determinations of these investigations showed that the paddle apparatus at 50 revolutions per minute (rpm) has similar

hydrodynamics to the paddle over a mesh at 50 rpm and to the basket apparatus at 100 rpm. These results may aid in the selection of an appropriate dissolution method and respective agitation rate, for similar non-conventional dosage forms such as tea bags.

2.9.2 Dissolution / Infusion profile comparison methods

Dissolution/infusion profile comparison methods have been developed in order to answer questions regarding the similarity in performance of pharmaceutical dosage forms. These comparisons also aid the development of *in- vitro in- vivo* correlations, which can help reduce costs, speed up product development and reduce the need to perform costly bioavailability/ bioequivalence studies. They also have applications in establishing final dissolution specifications for pharmaceutical dosage forms (O'Hara *et al*., 1998; Freitag, 2001).

A dissolution profile may be defined as the measured fraction (or percentage) of the labelled amount of drug that is released from a dosage unit at a number of predetermined time points when tested in a dissolution apparatus (O'Hara *et al*., 1998). The methods to compare dissolution profile data can be categorized as either, exploratory data analysis which includes graphical and numerical summaries of the data; mathematical methods that typically use a single number to describe the difference between the profiles and statistical and modelling methods which take both the variability and underlying correlation structure in the data into account in the comparison.

Exploratory data analysis methods as stated above, involve graphical and numerical analysis. The data may be illustrated graphically by plotting the mean dissolution profile data for each formulation with error bars extending to two standard errors at each dissolution time point. The dissolution profiles may be considered to differ significantly from each other if the error bars at each time point do not overlap (O'Hara *et al*., 1998). As a complement to the graphical summary, the data may be summarized numerically by presenting the mean and standard deviation at each time point. In addition, the difference between the mean profiles and a 95% confidence interval for the difference in the mean

profiles may be presented. If the 95% confidence interval does not contain zero, then the difference at that time point are considered significantly different at the 5% significance level (O'Hara *et al*., 1998). The exploratory method is not currently endorsed by the United States Food and Drug Administration (FDA) and another deficiency is that it is difficult to definitively conclude that profiles are different if the error bars overlap only at some time points and not at others, or if the 95% confidence interval for the difference in the profiles contains zero only at some time points. In addition, if several formulations are being compared, graphical and numerical illustrations may become too cluttered, with all the error bars and columns in the tables, making the data evaluation difficult (O'Hara *et al*., 1998).

Two mathematical comparison methods are described in the literature. The first described by Moore and Flanner and the second by Roscigno. Only the Moore and Flanner method shall be discussed here, as it is the most popular and accepted of the two. Moore and Flanner described two equations; a 'difference factor' f_1 (equation 2.1) and a 'similarity factor' f_2 (equation 2.2).

$$f_1 = 100\left\{\left[\sum_{k=1}^{p}|X_k - Y_k|\right] \Big/ \left[\sum_{k=1}^{p} X_k\right]\right\} \qquad \textbf{Eqtn: 2.1}$$

$$f_2 = 50\log_{10}\left(100\left[1 + \frac{1}{p}\sum_{k=1}^{p} w_k(X_k - Y_k)^2\right]^{\frac{1}{2}}\right) \qquad \textbf{Eqtn: 2.2}$$

Where n is the number of dissolution time point, R_t and T_t are the reference and test dissolution values at time t_t , respectively and w_t is an optional weighting factor (Freitag, 2001).

The f_1 equation is the sum of the absolute values of the vertical distances between the test and reference values. The f_1 equation is zero when the profiles are similar and increases proportionally as the difference between the profiles increases and values between 0 and 15 mean similarity between profiles (FDA, 1997). The f_2 equation is a logarithmic transformation of the average of the squared vertical distances between the test and

reference dissolution values at each time point. The f_2 value approaches 100 when the profiles are identical and values between 50 and 100 ensure similarity between the profiles (FDA, 1997).

This mathematical method is the most popular of the comparison methods as it is easy to compute and is accepted and recommended by the FDA. The main disadvantage is that it does not take into consideration the variability or correlation structure in the data. However, the FDA implies that the f_2 equation should only be used when the within-batch variation, in terms of coefficient of variation, is less than 15% (FDA, 1997). The f_1 and f_2 are also sensitive to the number of time points used and it has been criticized that the basis of the criteria for deciding the difference or similarity between the dissolution profiles is unclear. In other words, the difference between the dissolution profiles at which the difference is considered to be of practical importance or likely to affect *in vivo* performance is not clear (O'Hara *et al*., 1998; Saranadasa & Krishnamoorthy, 2005).

Statistical methods include the one-and two-way analysis of variance (ANOVA) methods, mixed effects model (multivariate methods), modelling-based methods and Chow and Ki's methods (O'Hara, 1998). Statistical methods take into consideration the variability and correlation structure of the data. However, they possess several disadvantages which limit their use. For example, ANOVA comparisons may only be statistically significant at some dissolution time points and not at others, thus making it difficult to conclude whether there is any difference. As a result, ANOVA methods are only recommended in the case of immediate release data at a single dissolution time point (O'Hara, 1998; Freitag, 2001). The other statistical methods also have similar pitfalls which limit their use, i.e. ambiguous in interpretation, tedious to perform, difficulty in implementation using standard statistical software, sometimes inefficient and a lack of regulatory endorsement. However, the multivariate method is recommended by the FDA when the within-batch variability has a coefficient of variation greater than 15%, but this method also suffers from some of the pitfalls mentioned above (FDA, 1997; O'Hara *et al*., 1998).

Chapter 3

Work Plan

3.1 Study objectives

The objectives of the study were:

- To prepare standardized dried leaves and freeze-dried aqueous extract powder of *A. afra* plant leaves and to characterise the physiochemical properties of the materials,
- To develop and validate an analytical method for the detection and quantitation of luteolin in the *A. afra* plant materials,
- To quantitate and determine the inter- and intra batch variation in the luteolin content of the plant materials,
- To prepare the standardized dried leaves and freeze-dried aqueous extract powder in tea bag dosage form and determine and compare the infusion profiles (using luteolin as a marker compound), of the tea bag dosage form and the loose leaves (traditional dosage form),
- To design and evaluate credible placebos for the dried leaves and the freeze-dried aqueous extract powder of *A. afra.*

3.2 Hypotheses

It was hypothesised that:

- The intra batch variation in the luteolin levels decreases in the order dried leaves > standardized dried leaves > freeze-dried aqueous extract,
- The tea bag dosage form is a suitable dosage form for the traditional plant leaves and will meet pharmacopoeial quality requirements,
- The infusion profile of luteolin from the tea bag dosage form will be similar to that of the loose leaves (traditional form) and therefore be suitable for use in clinical trials and,

- It is possible to design credible placebos for the *Artemisia afra* plant materials devoid of pharmacological activity.

3.3 Study approach

This study intended to enable clinical trials on the plant to be conducted by, firstly, standardizing the *A. afra* plant materials and preparing a freeze-dried aqueous extract powder of the plant leaves and secondly, by preparing the plant materials in a tea bag dosage form and determining the similarity of the infusion profiles of the tea bags and the loose leaves (in order to ensure interchangeability) and finally, by designing credible placebos for the plant materials devoid of pharmacological activity.

3.3.1 Why *Artemisia afra*?

Artemisia afra (Wilde als) has several uses attributed to it in literature. Several authors describe it as being one of the most widely used and popular plant medicines in South Africa. It is therefore important that such a popular medicinal plant be validated in terms of its safety and efficacy using clinical trials. In addition, its many uses, widespread distribution throughout Africa and several different methods of preparation and administration made the plant an ideal candidate to model as a plant standardized and developed into a suitable dosage form for evaluation in clinical trials following which, regulatory approval for marketing can be granted.

3.3.2 Why a tea bag dosage form?

Traditionally *A. afra* is administered in the form of a tea. This tea is commonly prepared by infusing the loose leaves in hot water (Roberts, 1990). Therefore, in order to mimic the traditional method of preparation, which would be necessary for the evaluation of the safety and efficacy of the plant as used traditionally, a dosage form which closely resembles the loose leaves, yet overcomes the disadvantages associated with the loose leaves was needed. Previous studies have attempted to manufacture tablet dosage forms of the plant leaves (Komperlla, 2005). These have by and large proved unsuitable due to

the hygroscopic nature of the extracts used to manufacture the tablets. In addition, these extracts may not contain all the constituents found within the leaves. Therefore, a tea bag containing the *A. afra* leaves would allow the complete plant leaves to be administered to the patient and closely resemble the way the plant is used traditionally.

3.3.3 Why luteolin?

It is generally recommended that marker compounds be used to identify and characterize the quality and stability of plant materials especially where the active ingredient is unknown (EMEA, 2005). In this study, the flavonoid luteolin was chosen as a marker compound for *Artemisia afra* leaves. This flavonoid has been found consistently in various batches of *A. afra* plant leaves, and was found to be stable in assay procedures applied (Waithaka, 2004). In addition, several literature sources describe the pharmacological effects of luteolin, e.g. anti-oxidant, anti-inflammatory, anti-allergic, anti-cancer and blood glucose modifying effects (Shimoi *et al,* 2004; Mino *et al*, 2004*)* and these effects, in relation to the traditional uses of *A. afra,* may suggest that this compound may play a role in the observed therapeutic effects of the herb. Also, much was known on the extraction, detection and quantitation of flavonoids in plant matrices, making the flavonoids and luteolin in particular, suitable marker compounds to characterise plant materials.

Chapter 4

Preparation and evaluation of *Artemisia afra* plant material and design of placebos

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4.1 Introduction

In this chapter, the methods used in the preparation of the standardized dried leaves and freeze-dried aqueous extract powder (herewith referred to as SDL and FDAE, respectively), of *Artemisia afra* leaves are presented. The development and validation of the HPLC assay used for evaluation of the plant materials is also described in detail. Finally, methods used in the design of the placebos as well as the results obtained are presented and discussed.

4.2 Equipment and materials

The following equipment were used;

-85˚C freezer (*Lozone CFC Freezer, Model U855360, New Brunswick Scientific, USA*), **balance** (*Scaltec SPB42, Model SPB71, Scaltec Instruments, Heiligenstadt, Germany*), **centrifuge** (*Labofuge 200, Germany),* **furnace** *(Naber Model L47T. Industrieofenbau 2804, Lilienthal/Bremen, West Germany)*, **freeze-drier** (*Virtis Freeze Mobile 72SL, The Virtis Company Gardner, New York, USA*), **light microscope** (*Nikon Monocular Model Sc, Japan*), **HPLC filter unit** (*Millipore Cameo 25 AS, DDA 02025So MSI: Micro separation Inc., USA*), **solvent filter paper** (*47mm filter membrane 0.45µm PVDF, Millipore, USA),* **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),* **laboratory blender** (*Waring Commercial Laboratory Blender 8010, Model 32BL79, New Hartford, Connecticut, USA),* **oven** (*Model Memmet 854 Schwabach, West Germany)*, **pH meter** (*Basic 20 Crison Instruments, S.A, Italy*), **spectrophotometer** *(DU 640 spectrophotometer, Beckman, USA),* **test sieve shaker** (*Endecott sieve shaker, E.F.L. 1MK11, Endecotts (test sieve) LTD, London, England*), **vortex machine** *(Vortex-2, G-*

560E, Scientific Industries, Inc. Bohemia, N.Y. 11716 USA), **water bath** (*Labcon, CDH 110, Maraisburg, South Africa*).

The HPLC system used consisted of an auto sampler (*Beckman Gold Module 507*), a programmable binary gradient pump (*Beckman Gold Module 126 series*), a diode array detector (DAD) (*Beckman Gold Module 168 series*) with a 32-Karat™-software package and a Synergy® Hydro- reverse phase column (*Phenomenex, USA*) having 4μm particle size and a column length of 250 x 4.60 mm.

The following materials were used;

Artemisia afra plant, quinine hydrochloride dihydrate Ph Eur (*Fluka Chemie GmbH, Germany*), acetonitrile (*Burdick & Jackson, M.I. USA),* ethyl acetate CP (*Saarchem, South Africa)*, luteolin, morin hydrate, hesperetin (*Sigma Aldrich, Germany),* sodium hydroxide (*UnivAR, Saarchem, South Africa)*, methanol AR, hydrochloric acid, potassium hydrogen phosphate (*Riedel-de Haen AG, Germany)*, lactose (*UniLab, Saarchem, South Africa)*, D.C brown (*AR Agenicies cc, South Africa)*, sodium starch glycollate (*UniLab, Saarchem, South Africa),* potato starch (*Saarchem, South Africa),* calcium chloride, microcrystalline cellulose, di-sodium hydrogen phosphate heptahydrate (*Merck, South Africa),* sodium saccharin (*Supelco. Bellefonte, USA)*, linalool (*Sigma-Aldrich Chemie GmbH, Germany),* silica gel (*BDH, South Africa),* dimethyl sulphoxide (*Sigma-Aldrich, Germany).*

4.3 Methods

4.3.1 Collection and preparation of the plant material

Whole *Artemisia afra* plants were collected from the Montagu district of the Western Cape Province of South Africa in February 2005. A sample of the collected plants was filed as a voucher specimen (Voucher No: 6735) in the herbarium of the Botany Department of the University of the Western Cape. This collected plant material was considered as batch 001. Additional plant material batches 002 and 003 were collected

from the same district in the months of March and April 2005, respectively. The freshly collected plant material was separated from earthy and other foreign material and the leaves (for use in the study) manually separated from the stems and flowers of the plant. The collected leaves were then weighed before being dried in an oven at a temperature of 30^{o}C for 3 days (Komperlla, 2005). After drying, the leaves were weighed again to obtain the final dry mass. Finally, the dried leaves were cut up into smaller fragment sizes using a laboratory blender and the resultant leaf fragments stored sealed in plastic bags in a cool dark place away from direct light.

4.3.2 Standardization of the *Artemisia afra* dried leaves

There are various ways of achieving standardization of plant materials. The most common and accepted method is blending different batches of plant material together (McCutcheon, 2002), and in this study, the 3 different batches of *A. afra* plant leaves were blended together. Equal portions of each plant batch were separately weighed and placed together in a large plastic bag before being mixed by shaking vigorously for 5 minutes. The resultant leaves blend was then stored in sealed plastic bags in a cool dry place, protected from direct light.

4.3.3 Preparation of the freeze-dried aqueous extract powder.

The extraction procedure used to prepare the extract, as far as possible, mimicked the methods described in the literature for the preparation of the traditional dosage. Commonly, a quarter cup quantity of *A. afra* leaves to a cup of boiling water is allowed to stand and seep for 10 minutes (Roberts, 1990). Therefore, the aqueous extract was prepared by adding boiling distilled water to the appropriate mass of standardized dried leaves (SDL) in 1:35 ratio of leaves to solvent (Komperlla, 2005) and the extraction mixture left to seep and draw for 10 min. After this period, the mixture was filtered using Whatman no. 1 filter paper and the filtrate frozen at -85^{o}C in a freezer. The frozen extract was then dried at -44^{o}C under vacuum over 3 days using a freeze drier. The resultant extract powders were then combined and weighed and the percentage yield calculated.

The FDAE powder was then placed in amber glass containers and these stored in desiccators until used (Komperlla, 2005).

4.3.4 Irradiation of the plant materials

There is a requirement for microbiological decontamination of materials of natural origin due to the high levels of microbes inherent in these materials. However, methods of decontamination are few and are often restricted. For example the use of ethylene oxide is forbidden, and the efficacy of some other methods has not been well established (WHO, 1998). Ionizing irradiation is a well established decontamination method which has been proved effective and safe, having been used for decontamination of spices and seasonings for several years (Razem & Katusin-Razem, 2002).

In this study, the SDL as well as the FDAE powder of *A. afra* were decontaminated using ionizing radiation from a Cobalt (Co) source at a level of 18 kGy. Pre- and post-irradiation microbial tests were conducted to check and confirm the effectiveness of the procedure in the decontamination of the plant materials.

4.3.5 Determination of the plant material organoleptic characteristics

Organoleptic characteristics refer to the appearance, colour, odour, and taste of a substance. An examination to determine these characteristics is normally the first step towards establishing the identity and degree of purity of materials (WHO, 1998; EMEA, 2005). Characterization of these properties is also a primary step in setting of specifications, as well as setting a standard to which a placebo should resemble if it is to be credible. In addition, changes in the stability of a material may be recognized by changes in the organoleptic properties. Sweeteners, flavorants and aroma chemicals may be added to mask objectionable odours and tastes present in a material (Zheng & Keeney, 2006).

For this study, the appearance, colour, odour and taste of the SDL and FDAE powder of *A. afra* were characterized using the human sensory evaluation (i.e. by eye, nose and tongue).

4.3.5.1 Determination of the plant material particle size and shape.

Particle size and shape have an effect on the physico-chemical properties of a dosage form. They play a great role in producing homogeneity of dosage forms and, in the case of tea bags, influence the rate of infusion of the soluble constituents. Smaller fragment sizes are known to infuse faster than larger sized ones (Wichtl, 1994; Jaganyi & Mdletshe, 2000) and this difference in the rate of infusion may affect the dose the patient receives for a given tea brewing time. The size and shape of the particles also has a direct impact on mixing and significant deviations in the particle size can result in the segregation of the mixture, with smaller fragments settling at the bottom and larger fragments at the top of the mixture (Fayed & Otten, 1984). Sieve and microscopic methods are the commonly used methods to determine particle size and shape and were the methods used in this study.

4.3.5.1.1 The sieve method

The degree of coarseness or fineness of a powder is classified according to the nominal aperture size expressed in micrometers of the mesh of the sieve through which the powder will pass (BP, 2000a). Using this criterion, powders are divided into 'coarse', 'moderately fine', 'fine', and 'very fine'. The degree of fineness of the powder is expressed as a weight-to-weight percentage of the powder passing through different size sieves. If a single sieve number is given, not less than 95% of the powder passes through the sieve of that number, unless otherwise indicated.

For the evaluation of the *A. afra* plant materials, sieves having numbers of 1400, 355, 180, 125 and 90 µm were assembled in a descending order, i.e. 1400 µm size sieve on top and 90 µm at the bottom. The assembled set of sieves was placed on a test sieve shaker

before 10 g of the SDL or FDAE powder was placed onto the top sieve, and the assembly shook for 30 minutes. Thereafter the powder collected on each of the sieves was weighed and the percentage (w/w) of each fraction determined.

4.3.5.1.2 Determination of the plant material particle shape

The microscopic method allows the determination of the shape of the particles and a Nikon light microscope was used to determine the particle shape of the plant materials used. A few milligrams of the SDL or FDAE powder were sprinkled onto a slide and viewed under the microscope. The particle shapes were observed and described as either irregular, round, cylindrical or rectangular.

4.3.5.2 Determination of the plant material colour

Colour can be used as a means of identifying a particular substance. Several Pharmacopoeias include the colour of the substance as part of the substances monograph. In this study, the colour of the *A. afra* SDL and FDAE powder as well as the colour of the hues produced when these materials are infused in hot water to produce teas was determined. This information was crucial for the design of the placebos which should be similar in appearance to the plant materials.

In determining the colour of the materials, 1 g of the material being examined was placed against a white background and its colour described. In determining the colour of the hues produced, 4 g of the SDL and 1g of the FDAE powder were infused in 200 ml of distilled water at a temperature of 80˚C, stirred for 2 minutes, and allowed to stand for 8 minutes. The resultant hue was then visually examined and described accordingly.

4.3.5.3 Determination of the plant material odour

Odour can be used as a quick method for identification of a substance. The presence or absence of odour in *A. afra* plant material was determined using the method prescribed in

the British Pharmacopoeia 2000. One gram of the SDL or the FDAE powder of *A. afra* was placed on a watch glass about 5 cm in diameter. The material was then allowed to stand for 15 minutes, smelt and the presence or absence of odour from the material noted (BP, 2000b).

4.3.5.4 Determination of the plant material bitterness value

The bitterness value is defined as the reciprocal of the dilution of a compound, liquid or extract that still has a bitter taste (EP, 2002a). The bitter properties of plant materials are determined by comparing the threshold bitter concentration of a preparation of the material with that of a dilute solution of quinine hydrochloride. The threshold bitter concentration is defined as the lowest concentration at which the material continues to provoke a bitter sensation 30 seconds after tasting it. The bitterness value is expressed in units equivalent to the bitterness of a solution containing 1g of quinine hydrochloride, the bitterness value of which is set at 200 000 (WHO, 1998). In the literature *A. afra* is described as having an extremely bitter taste (Van Wyk *et al*., 2000; Roberts, 1990). In order to aid effective design of the herbal placebos it was necessary to characterize the bitterness value of the plant materials.

For the bitterness value determinations, the SDL and FDAE of *A. afra* were dispersed in boiling distilled water at a concentration of 0.01 g/ml, stirred and allowed to stand for 10 minutes before being filtered using Whatman No. 1 filter paper. The solutions were allowed to cool before tasting. Serial dilutions of quinine hydrochloride and the plant materials, i.e. 0.00001 g/ml and 0.001, 0.0001, 0.00002, 0.00001 g/ml respectively, were then prepared as described in the European Pharmacopoeia 2002 (EP, 2002a).

A panel comprising of 6 tasters was set up. In order to correct for individual differences in tasting bitterness, a correction factor for each taster was established using quinine hydrochloride. The panel members then tasted serially diluted concentrations of the SDL and the FDAE solutions, starting with the most dilute concentration. The bitterness value

was determined as prescribed in the European Pharmacopoeia 2002 using the following equation:

$$\textbf{Bitterness Value} = \frac{\textbf{Y x } k}{\textbf{X x 0.1}} \qquad \textbf{Eqtn: 4.1}$$

Where; Y = Dilution factor of threshold bitter concentration

k = Correction factor for each panel member

X = Number of ml of threshold bitter concentration which when diluted to 10 ml still has a bitter taste.

4.3.6 Determination of the plant material ash values

Ash values can be considered as quality standards to indicate identity, purity or possible adulteration of a herbal material. The ash remaining following ignition of medicinal plant materials is determined by different methods which measure total ash, acid-insoluble ash and water-soluble ash. The total ash method is designed to measure the total amount of material remaining after ignition. This includes both "physiological ash", which is derived from the plant tissue itself, and "non-physiological" ash, which is the residue of the extraneous matter (e.g. sand and soil) adhering to the plant surface (WHO, 1998). Acid-insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth. Water-soluble ash is the difference in weight between the total ash and the residue remaining after treatment of the total ash with water (EMEA, 2005).

Total ash and acid-insoluble ash determinations were performed on the standardized dried leaf material and FDAE powder of *A. afra* according to the prescribed methods in the British Pharmacopoeia 2000. Water-soluble ash determination is a non-pharmacopoeial test and was performed by adding 25 ml of water to the crucible containing the total ash and boiling for 5 minutes. Thereafter, the insoluble matter was collected in a sintered-

glass crucible, washed with hot water, ignited in a crucible for 15 minutes at a temperature of 450°C and the remaining ash retrieved. The difference in weight (in mg) of this residue from the weight of the total ash was the water-soluble ash (WHO, 1998).

4.3.7 Determination of the plant material moisture content

The moisture content of the plant materials was determined using the method prescribed in the European Pharmacopoeia 2002 which involves the use of a drying oven. For this, 0.5g quantities of the SDL and the FDAE powder of *A. afra* were placed in a flat-bottomed dish about 50mm in diameter and dried in an oven for 3 hours at a temperature range of 100-105ºC (EP, 2002b). The moisture content was calculated as a mass percentage using the following formula:

$$\% \text{ Moisture} = \frac{\text{Initial weight (Wet mass)} - \text{Final weight (Dry mass)}}{\text{Initial Weight}} \times 100 \quad \text{Eqtn: 4.2}$$

4.3.8 Determination of microbial contamination of the materials

Medicinal plant materials normally carry a great number of bacteria and moulds, often originating from the soil. Bacterial and fungal contamination is of serious concern especially in terms of health risks (EMEA, 2005). Unacceptable levels of microbial contamination are often due to poor agricultural practices or improper drying and storage of the harvested plant material and typically result in degradation of the plant materials. In some cases microbial contamination may render the plant material toxic either by transforming benign chemicals in the plant into harmful substances, or through the microbes' production of toxic compounds (McCutcheon, 2002).

The European Pharmacopoeia provides guidance on which microbes are to be tested for as well as their minimum acceptable limits (EP, 2002c). Following this guidance, the total viable aerobic count, total yeasts and moulds, absence of *Escherichia coli*, *Salmonella* and enterobacteria were consequently tested for in the SDL and FDAE

powder of *A. afra*. In addition to this, tests not specified in the pharmacopoeia were also conducted. These were tests for absence of *Staphylococcus aureus* and *Pseudomonas aeruginosa.* Microbiological testing on the materials was conducted before and after irradiation of the materials, using standard microbial test methods as described in Appendix A7.

4.3.9 Development and validation of the HPLC assay

For the quantitation of luteolin in the plant materials, a reversed phase HPLC method using an internal standard was developed. The compounds hesperetin, p-coumaric acid, mefenamic acid, morin hydrate, cinnamic acid and salicylic acid were evaluated as possible internal standards. The requirements were that the compound should not be present in the *A. afra* plant materials, should absorb strongly at the luteolin absorption wavelength maximum (349 nm), be extractable in ethyl acetate, have similar physiochemical characteristics to luteolin, and have a chromatographic retention time that did not interfere with other peaks from the plant.

Different chromatographic conditions were evaluated for the separation of the luteolin peak from the internal standard peak and other possible interfering peaks. Three different types of reverse phase chromatographic columns (i.e. silica C-18 (250 x 4.6 mm, 5 µm), Luna ® C-18 (150 x 4.60 mm, 5 µm), Luna® C-18 (250 x 4.6 mm, 5 µm)), different mobile phase compositions and flow rates were evaluated and optimized. The mobile phases consisted of either methanol or acetonitrile, with a mixture of acetic acid or formic acid in water or a phosphate buffer at different concentrations to control the pH and to reduce peak tailing. To validate the assay, the linearity, recovery, limit of detection (LOD), limit of quantitation (LOQ) and intra- and inter-day precision, for the assay were determined.

In performing the validation, firstly, stock solutions of luteolin and morin hydrate (1mg/ml) were prepared in dimethyl sulphoxide (DMSO), wrapped with aluminium foil to protect from light and stored at -21˚C. Standard solutions were freshly prepared each

day by appropriate dilution of the stock solutions with the mobile phase (acetonitrile 30%, phosphate buffer 70% at pH 2). Concentrations of 2.5, 5, 10, 20, 50 and 100 µg/ml of luteolin and 100 µg/ml of morin were prepared.

For the preparation of the calibration curve, the stock solutions were diluted with mobile phase to produce the concentrations described above. To each concentration of luteolin, 50 µg of morin hydrate was added. 50 µl of each standard was injected onto the column and the peak height ratios between luteolin and morin plotted against the corresponding concentrations of the injected luteolin. The complete procedure was repeated over 3 consecutive days.

The limit of detection (LOD) was obtained by successively decreasing the concentration of luteolin as long as a signal to noise ratio of 3: 1 appeared. The limit of quantitation (LOQ) was defined as the lowest concentration where an accuracy better than 20 % was achieved (Ruckert *et al*., 2004).

Finally, for the determination of intra-day precision and accuracy, 6 replicates of the standards were analysed on the same day. The precision and the accuracy for the inter-day analysis were determined on 3 different days.

4.3.10 Quantitation of luteolin in the plant materials

The flavonoid luteolin was used as a marker compound for the *A. afra* plant materials. As part of the characterization of the plant materials, different batches of the plant leaves, the SDL and the FDAE were evaluated for the average content of total, free and glycosidic or conjugated luteolin using the validated HPLC assay. In addition, the intra- and inter-batch variation in the content of the different forms of luteolin in the various plant materials, were also determined.

A defined procedure was used for sampling from the various plant material batches. This entailed randomly taking 20g of the plant material under evaluation from the batch bulk

and spreading it on a tile 10 x 10cm. From here, 6 sampling positions were defined (as shown in Figure 4.1) and 250 mg samples were removed from each position.

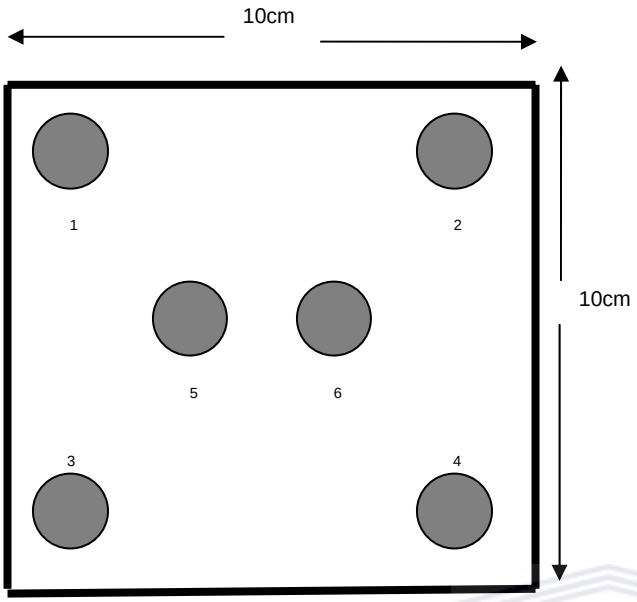


Figure 4.1: **Sampling positions used in the quantitation of luteolin in the *A. afra* plant materials.**

The sampled plant material was then prepared following the common traditional method for the preparation of the tea. The 250 mg sample was placed in 10 ml of hot (80˚C) distilled water (25 mg/ml), vortexed for 2 minutes and then allowed to stand for 8 minutes before being filtered using Whatman no. 1 filter paper. The filtrate was separated into two 1 ml portions for analysis of the total luteolin (hydrolysed) and free luteolin (unhydrolysed).

For the total luteolin assay, the 1 ml sample was transferred to a tube containing 4.8 ml of 2N HCl and an appropriate amount of internal standard. The mixture was vortexed for 30 seconds, before being left to 'hydrolyse' in a water bath at 80˚C for 40 minutes. Following this, the sample was allowed to cool, 5 ml of ethyl acetate added, vortexed for 2 minutes (to ensure the luteolin was extracted into the ethyl acetate layer) and then centrifuged at 3000 rpm for 10 minutes to separate the ethyl acetate from the aqueous

layer. Finally, the top layer of ethyl acetate was pipetted off into a clean tube and evaporated to dryness under a cool stream of nitrogen.

For unhydrolysed (free) luteolin assay, the 1 ml sample was transferred to a tube containing 5ml of ethyl acetate and internal standard. This was vortexed for 2 minutes before being centrifuged at 3000 rpm for 10 minutes. The top layer of ethyl acetate was then removed and the sample evaporated to dryness as described above.

To quantify the amount of luteolin in the extracted samples, the dry residues were reconstituted in 1ml of the mobile phase. 50 µl of the solution was then injected onto the column and isocratically eluted at a flow rate of 1 ml/min at room temperature with a run time of 30 minutes using a mobile phase of acetonitrile 30% and phosphate buffer 70% at pH 2). Detection was conducted at a wavelength of 349 nm and the luteolin in the samples quantitated from a standard curve based on the peak height ratio of luteolin and internal standard.

4.3.11 Design of the plant material placebos

Designing credible herbal placebos is known to present special challenges especially with regard to achieving and maintaining concealment. This is mainly due to the fact that herbal medicines are more elaborate in their composition compared to the simple chemical structures of drugs commonly encountered in pharmaceutical placebo design (Kaptchuck, 2001). In this study, the objective was to design placebos which looked, smelt and tasted similar to the *A. afra* SDL and FDAE.

4.3.11.1 Design of placebo for the standardized dried leaves

The SDL of *A. afra* were used to produce the placebo. Since the phytochemical constituents, in particular the flavonoids, were thought to be responsible for the observed therapeutic effects of the plant leaves, it was inferred that *A. afra* leaves without these phytochemical constituents or with very low levels of them, would be devoid of

pharmacological activity. Therefore, to produce the placebo of the dried leaves, the phytochemical constituents were to be extracted out of the *A. afra* leaves as much as possible.

A method utilising exhaustive solvent extraction with hydrolysis was developed to extract as much as possible of the flavonoids (as well as any other compounds soluble in the extracting solvents) within the leaves. In selecting appropriate solvents, flavonoid solubilities were taken into consideration. For example, for the extraction of water-soluble flavonoids, a more polar solvent was selected. The safety or hazardous potential of the solvents were also taken into consideration. Various solvents were evaluated for their effectiveness in extracting the constituents using the minimal number of extractions.

Based on the above considerations, a total of 15 extractions using a combination of either water, methanol/ water (50/50, v/v), methanol or ethyl acetate, (depending on the particular extraction step) including a hydrolysis and neutralisation step (to free up the sugar bound flavonoids for optimal extraction), were conducted. In all extractions a mass to solvent ratio of 1:20 was used. In conducting the extractions, the leaf and solvent mixture was stirred for 2 minutes then allowed to stand for 10 minutes before being filtered using Whatman no. 1 filter paper. The leaf to solvent ratio was kept constant in all extractions. From the supernatant, a 1 ml sample was retained for analysis using UV spectrophotometry and to the remaining residue of leaves, additional solvent was added. UV spectrophotometry was conducted at the luteolin absorbance maxima (349 nm) as well as the absorbance maxima for the plant material extract. The process was repeated using the next solvent in the procedure, while monitoring the UV absorbance of the extract, until the absorbance was acceptably low. Samples of the initial and final extracts were also analysed using the validated HPLC assay in order to obtain a chromatographic fingerprint of the material as well as obtain a measure of the extraction process through a comparison of the pre- and post extraction chromatograms.

4.3.11.2 Design of the placebo for the freeze-dried aqueous extract powder

There are few clinical trial studies in the literature, where placebos of herbal extracts have been employed (Pan *et al*., 2000). In many of these trials, extracts in capsules or tablets were used with the result that the patient could not see the extract itself. In making these placebos, elaborate formulas of inorganic salts were employed to mimic the extract as well as impart good flow properties to the powder to aid the manufacture of the tablets or capsules. One example is that of a placebo of a Chinese herbal preparation containing 78.2% calcium hydrogen phosphate, 19.6% soy fibre, 0.3% cosmetic brown, 0.5% cosmetic yellow, 0.01% edicol blue 0.09% identical liquorice dry flavour and 0.03% bitter flavour (Bensoussan *et al*., 1998).

In the design of the placebo of the *A. afra* FDAE, various selected inorganic salts were blended together. These salts were chosen based on their organoleptic properties relative to the organoleptic properties of the FDAE. In addition, in order to match the hue produced when the *A. afra* FDAE is dissolved in water, a colourant was included in the placebo formulation. The appropriate proportions of inorganic salts to be added were determined on a trial and error basis, where a formulation was evaluated against the aqueous extract and modified as needed. The various formulations and proportions of each salt tried are shown in Table 4.1 below.

Table 4.1: Various excipients and their proportions used in different formulations in the design of the placebo for the *A. afra* freeze-dried aqueous extract powder.

Excipient	Amount of various excipients in the various formulations (g)					
	1	**2**	**3**	**4**	**5**	**6**
Potassium dihydrogen phosphate	0.275	---	---	0.200	0.400	0.475
Microcrystalline cellulose	0.225	0.300	0.300	---	---	---
Sodium starch glycollate	---	0.100	---	0.100	---	---
Potato starch	---	---	0.130	0.130	0.050	0.03
Lactose	---	---	0.050	---	0.035	0.025
Calcium chloride	----	---	---	0.050	---	---
DC Brown	0.050	0.015	0.020	0.020	0.02	0.02
Di-sodium hydrogen phosphate heptahydrate	---	0.135	---	0.050	---	---
Total	0.55	0.55	0.55	0.55	0.55	0.55

4.3.11.3 Matching of odour between the placebos and the plant materials

A. afra is a highly aromatic plant. The dried leaves and infusions from the leaves (aqueous extract) also possess a characteristic odour. In order to make the odours similar between the placebos and the *A. afra* plant materials, an aroma chemical was employed to provide an aroma to the placebos as well as to spike the *A. afra* plant materials so that the plant material odours are matched.

An appropriate aroma chemical was selected from a range of commercially available synthetic aroma chemicals. In selecting the appropriate chemical, the fragrance characteristics, flavour properties, toxicity, fixative properties and substantivity (which refers to the ability of the perfume to last on a specific surface) of the chemical was taken into consideration. A chemical with a more natural or green fragrance with a high tenacity and substantivity was preferred in order to resemble the herbal smell of *A. afra*.

The aroma chemical selected to mask the odour of the *A. afra* plant materials was linalool (Figure 4.2). This chemical possesses a fruity, herbal woody, rosewood odour (BASF, 2006). Linalool is a substance widely used in functional and alcoholic perfumery. It is also frequently used in fruit imitations (peach, apricot, pineapple, grape, berry flavours etc), and for chocolate and spice complexes (BASF, 2006). It is accepted by the Council of Europe for use in foods as an artificial flavouring and is considered Generally Recognised as Safe (GRAS) by US Food and Drug Administration (OECD, 2004). Based on this, the aroma chemical was deemed suitable for inclusion in the formulations.

In order to determine the appropriate concentration of the aroma chemical sufficient to mask the smell of an *A. afra* tea solution, a modification of the European Pharmacopoeia method for the determination of bitterness of materials was used. Serial dilutions of the aroma chemical were prepared, i.e. 0.0042, 0.0048, 0.0052, 0.0056 and 0.0058 g/ml and mixed with equal proportions of a 20 mg/ml hot water extract of *A. afra*. Using a panel of four evaluators, the natural sense of smell was used to discriminate and evaluate the odours. The concentration of the aroma chemical at which the odour of the *A. afra* in the mixed solution could not be determined was taken to be the threshold masking concentration. This concentration capable of masking the characteristic odour of *A. afra* was therefore used in matching the smell properties of the materials.

In order to incorporate the aroma chemical into the materials, a method was devised whereby a silica gel adsorbent would adsorb the aroma chemical and be able to release the aroma when in solution. The aroma chemical was dissolved in 1ml of ethyl acetate and the solution added to 0.125 g of silica gel powder. The ethyl acetate was then evaporated off under a stream of nitrogen to leave a dry silica gel powder containing the aroma chemical. Appropriate amounts of the aroma-impregnated silica gel were then added to the *A. afra* SDL and FDAE, as well as the placebo materials.

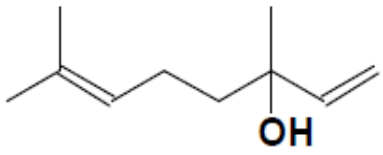


Figure 4.2: Chemical structure of linalool.

4.3.11.4 Matching of taste between the placebos and the plant materials

A. afra is an extremely bitter tasting plant (Roberts, 1990). Therefore the inclusion of a sweetening agent was considered in order to improve the patient acceptance of the plant medicine as well as to match the taste properties of the plant with that of the placebo (Zheng & Keeney, 2006). In the selection of an appropriate sweetener, the safety profile and classification of the sugar compound was taken into consideration. Sodium saccharin, a synthetic sweetener was preferred as opposed to a nutritive caloric natural sweetener such as glucose. This was to avoid possible confounding of results if the preparation was to be used in a trial such as a diabetes trial.

In order to determine the concentration of sodium saccharin required to mask the bitterness of the plant materials, a method was devised based on a modification of the European Pharmacopoeia bitterness value determination method. Serial dilutions of the chemical were prepared and mixed with equal proportions of a 20 mg/ml hot water extract of *A. afra*. Using a panel of 4 tasters, the natural sense of taste was used to establish the solution in which the bitter taste could no longer be tasted and the solution that was determined to be sweet. The amount of sodium saccharin in this solution was then adsorbed onto the silica gel adsorbent as described in section 4.3.11.3 above and incorporated into the *A. afra* and placebo materials.

4.4 Results and Discussion

4.4.1 Preparation of the plant materials

For this study, the leaves were removed from each of the 3 collected *A. afra* plant batches. On average 43.5% of the total *A. afra* plant weight consisted of the leaves. Drying of these collected leaves in the oven for 3 days at 30˚C in resulted in an average 57.36 ± 7.51% loss in weight. This result therefore indicates that approximately 50% of the mass of harvested *A. afra* leaves is in the form of moisture. This information may be useful to researchers in future studies on the plant, planning the amount of plant material required in order to attain a certain dry mass of leaves. The complete data for the collection and preparation are presented in Appendix A1.

To prepare the FDAE powder, a mass ratio of leaves to solvent of 1:35 was used. Several extractions of the SDL were conducted and a summary of the yields obtained for each extraction are presented in Appendix A3. The average yield for the FDAE powder obtained was 21.96 ± 1.97%. This value was similar to that obtained previously by other investigators, viz 27.88% and 19.9% by Komperlla (2005) and Mukinda (2006), respectively. Figure 4.3 shows an image of the SDL and the resultant FDAE powder.

Figure 4.3: **The standardized dried leaves of *A. afra* (left) and the freeze-dried aqueous extract powder of the *A. afra* standardized dried leaves (right).**

4.4.2 Organoleptic properties of the *A. afra* plant materials

The results of the organoleptic determinations on the *A. afra* SDL and FDAE powder are shown in Table 4. 2 below. The leaves were observed to be grey-green in colour, while the FDAE powder resulting from them was light brown in colour (see Figure 4.3). This difference in colour may point to a possible breakdown or change in the compounds present in the leaves occurring during the infusion process. When the FDAE was allowed to stand for 15 minutes, the extract became darker in colour and the powder clumped together. This observation was similar to that made by Komperlla (2005) and was attributed to the hygroscopic nature of the extract. The taste, odour and colour of the teas (hue) of the leaves and FDAE were however similar. The *A. afra* leaves and FDAE powder were also both bitter in taste. The bitterness of the leaves is described in the literature and in some traditional preparations sugar or honey is added to the preparations to mask the bitterness and improve the patient acceptance of the medicine (Van Wyk & Gerike, 2000; Roberts, 1990). The above information gathered during the organoleptic evaluation was used to set specifications for the plant materials and with these specifications, set standards for the placebo materials to meet.

Table 4.2: Organoleptic characteristics of the *A. afra* standardized dried leaves and freeze-dried aqueous extract powder.

Organoleptic characteristic	*A. afra* Standardized dried leaves	*A. afra* freeze-dried aqueous extract powder
Appearance	Particulate leaves, finely divided, hydrous, with a rough texture.	Brittle, free flowing, small particulate powder, which clumps together to form a solid mass on prolonged exposure to air.
Colour of material	Grey-green.	Light brown powder changing to dark brown on prolonged exposure to air.
Colour of hue produced	Light brown to golden brown hue.	Light brown to golden brown hue.
Odour	Characteristic odour, highly aromatic. Odour still present after 15mins on watch glass.	Characteristic odour, aromatic. Odour still present after 15mins on watch glass.
Taste	Extremely bitter.	Extremely bitter.

4.4.2.1 Particle size and shape of the *A. afra* plant materials

Under the microscope, the particles of the SDL and FDAE powder were both observed to be irregular in shape. The results of the particle size determinations on the materials are presented in Appendix A4. Based on the BP 2000 classification system, both the SDL and FDAE were classified as being coarse powders. The particles of the SDL passing through the 1400 µm but retained by the 355 µm sieves i.e. those in the particle size range 1400-355 µm were retained for use in this study, while the FDAE powder was used un-sieved.

4.4.2.2 Bitterness value of the *A. afra* plant materials

The results of the determination and calculation of the bitterness values are presented in Appendix A5. The bitterness value for both the *A. afra* SDL and FDAE was calculated to be 600 000. From this, it meant that the *A. afra* leaves and FDAE were 3 times more bitter than quinine hydrochloride which is known to be an extremely bitter tasting compound. To our knowledge this is the first estimate of the bitterness of *A. afra* leaves and FDAE powder, and this result can be added to the list of already known specifications for *A. afra* plant materials. The fact that the bitterness values for the dried leaves and the FDAE were similar, indicated that the compounds responsible for the bitterness were extracted from the leaves during the infusion process and were probably present in similar amounts in the two preparations. This therefore implied that in the formulation of the placebo materials, the placebo of the FDAE powder and dried leaves needed to be of similar taste. In addition, as a result of the bitterness, a sweetener chemical was required in order to mask the bitter taste of the materials and therefore improve the patient acceptance of the medicines.

4.4.3 Ash values of the *A. afra* plant materials

The complete data sets and results for the ash value determinations on the *A. afra* materials are presented in Appendix A6. The average percentage of total ash, acid

insoluble ash and water soluble ash for the SDL were 9.9832 ± 0.0617%, 0.9308 ± 0.0012% and 4.4052 ± 0.0012%, respectively. These results are comparable to the 8.558% and 1.341% previously reported by Komperlla (2005) for the total ash and acid-insoluble ash, respectively, of *A. afra* dried leaves.

The average percentage of total ash, acid insoluble ash and water-soluble ash for the FDAE was found to be 21.820 ± 0.0886%, 0.0094 ± 0.0793% and 16.5952 ± 0.6760%, respectively. These results were also comparable to those obtained by Komperlla (2005), viz. 17.436% and 0.027% for total ash and acid insoluble ash, respectively. To the best of our knowledge, values for water-soluble ash of *A. afra* have not previously been reported and the values above are the first such for *A. afra* plant leaves and FDAE. These values may be added to the current list of specifications for the plant materials.

Collectively, the ash values, in addition to the organoleptic characteristics results helped to confirm the identity of the *A. afra* plant materials used. It was noted that the *A. afra* materials used by the previous investigators mentioned above was collected from the same source as that used in this study. It may therefore be inferred that *A. afra* plants growing in similar conditions (similar climate and soil conditions) possess similar organoleptic and physiochemical properties.

4.4.4 Moisture content of the *A. afra* plant materials

The results of the moisture content determinations on the *A. afra* SDL and FDAE powder are shown in Tables 4.3 and 4.4, respectively.

Table 4.3: Residual moisture content of the *A. afra* standardized dried leaves

Sample No	Initial mass of leaves (g)	Final mass of leaves (g)	Loss on drying (g)	Percentage moisture content
1	0.5253	0.4700	0.0553	10.53
2	0.5191	0.4650	0.0541	10.42
3	0.5206	0.4722	0.0484	9.38
4	0.5213	0.4613	0.0600	11.51
5	0.5149	0.4558	0.0591	11.48
Ave	0.5202	0.4649	0.0554	**10.66**
S. D	0.0038	0.0066	0.0046	**0.88**

Table 4.4: Residual moisture content of the *A. afra* freeze-dried aqueous extract powder.

Sample No	Initial mass of aqueous extract (g)	Final mass of aqueous extract (g)	Loss on drying (g)	Percentage of moisture
1	0.5039	0.4609	0.0430	8.53
2	0.5071	0.4645	0.0426	8.40
3	0.5145	0.4695	0.0450	8.75
4	0.5081	0.4642	0.0439	8.64
5	0.5020	0.4577	0.0443	8.82
Ave	0.5071	0.4634	0.0438	**8.62**
S. D	0.0048	0.0044	0.0010	**0.17**

The average moisture content for the SDL and FDAE powder of *A. afra* were 10.66 ± 0.88% and 8.62 ± 0.17%, respectively. Moisture content determinations are necessary, particularly for materials known to be hygroscopic (EMEA, 2005). In the present study the moisture content determinations were conducted immediately following the drying of the plant materials and therefore were an indication the residual moisture present in the materials at that stage. The values obtained in this part of the study were therefore used as the moisture content specifications for the materials and were used in the later part of the study to determine moisture absorption by the materials upon storage.

4.4.5 Microbial contamination of the plant materials

Microbial contamination tests were conducted before and after irradiation of the plant materials. The tests conducted before irradiation included tests for total microbial activity, and for absence/presence of *Escherichia coli, Pseudomonas* and *Staphylococcus aureus*. All testing was conducted externally by a company contracted to do the testing.

The results of the microbial contamination tests are presented in Appendix A7. Before irradiation, considerable contamination was detected within the dried leaves, i.e. a total microbial activity of more than 10^6 and the presence of more than 10^4 and 300 colony forming units (CFU) per gram, of *Pseudomonas* and *E. coli* bacteria respectively. Based on the European Pharmacopoeia 2002 guidelines, which state that not more than 200 CFU/gram *E. coli* and not more than 10^7 bacteria should be present (EP, 2000c), the dried leaves did not comply with the specification on microbial contamination of herbals. The

presence of *E. coli* in the plant leaves may have reflected the quality of the growing and harvesting practices at the source of the material, as *E. coli* bacteria are not normally found as part of the flora and fauna of the soil (WHO, 1998). The tests on the FDAE powder before irradiation however indicated the presence of a reduced total microbial activity of 984 000 and the absence of all the other organisms previously detected in the dried leaves. This implies that hot water extractions, and presumably also the traditional decoction/infusion process, reduce the microbial load present in the plant materials.

After irradiation of the plant materials, no contamination was detected for the tests conducted (i.e. absence of total microbial activity, yeasts & moulds, *E. coli, Salmonella* and *Enterobacteriaceae)* demonstrating the efficacy and usefulness of the irradiation process. From these results, it was thus assured that the plant materials were suitable for use in the manufacture of the medicinal dosage forms.

4.4.6 Development and validation of the HPLC assay

A reversed-phase HPLC method, using morin as internal standard, was developed for the quantitation of luteolin in *A. afra* plant materials. The optimal conditions of separation and detection were achieved on a Synergy® Hydro - reverse phase column, packed with a 4 μm particle size resin and a column length of 250 x 4.60 mm using a mobile phase of acetonitrile (30%) and phosphate buffer 100mM (70%, pH 2) at a flow rate of 1 ml/min and UV detection at 349 nm.

The standard curve of peak height ratio versus concentration of luteolin was linear in the range of 2.5 - 100 μg/ml (i.e. 125 – 5000 ng luteolin on column) (Figures 4.4 and 4.5), and was described by the equation Y = 0.03407X + 0.008233 (where Y = peak height ratio, X = luteolin concentration in μg/ml) and had a regression correlation coefficient, r value of 0.9996. The concentration of internal standard used was 50 μg/ml and the HPLC injection volume was 50 μl.

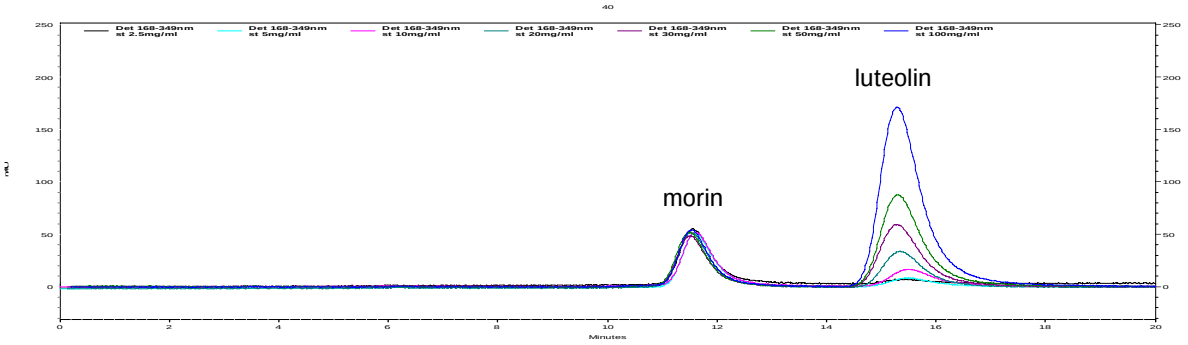
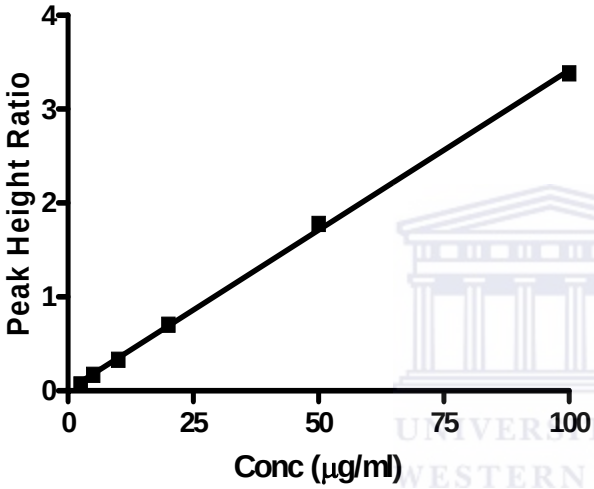


Figure 4.4: **Retention times and peak heights of morin and luteolin standards injected for generation of the standard curve. The average retention times for morin and luteolin were 11.90 ± 0.06 minutes and 15.86 ± 0.05 minutes, respectively.**



Best-fit values	
Slope	0.03407 ± 0.0004926
Y-intercept	0.008233 ± 0.02296
X-intercept	-0.2416
1/slope	29.35
95% Confidence Intervals	
Slope	0.03271 to 0.03544
Y-intercept when X=0;	0.05550 to 0.07196
X-intercept when Y=0;	-2.171 to 1.587
Goodness of Fit	
R	0.9996
Sy.x	0.04172

Figure 4.5: **The standard curve and linear regression line values, used in the quantitation of luteolin.**

The results of the accuracy and recovery of the assay are shown in table 4.5 and the inter- and intra- day precision results are presented in Table 4.6. The mean recovery of the method was 94.70 ± 8.62%. The concentration of 2.5 µg/ml had accuracy greater than 20.0% and was therefore considered to be the limit of quantitation (LOQ) for the assay (Ruckert *et al*., 2004). The LOD was found to be 50 ng on column, from an injected volume of 20 µl corresponding to a concentration of 2.5 µg/ml. The average intra- and inter-day precision were found to be 2.497 ± 0.6470% and 3.123 ± 1.068%, respectively. Collectively, these values indicated the good validity and reproducibility of the assay.

Table 4.5: Accuracy and recovery of the luteolin standard.

Given (µg/ml)	Found, mean ± S.D (µg/ml)	Accuracy (% deviation)	% Recovery
2.5	1.810 ± 0.196	-22.639	77.361
5.0	4.772 ± 0.177	-4.071	98.703
10.0	9.467 ± 0.169	-3.386	96.614
20.0	20.470 ±1.040	-1.100	99.853
50.0	48.110 ± 0.308	-3.779	96.221
100.0	99.010 ± 0.403	-0.540	99.460

Table 4.6: Intra-day and inter-day precision of the assay.

Given (µg/ml)	Intra-day		Inter-day	
	Precision (R.S.D., %)	Accuracy (% deviation)	Precision (R.S.D., %)	Accuracy (% deviation)
10.0	1.79	-3.386	4.06	-2.563
50.0	3.06	-3.779	3.35	+1.542
100.0	2.64	-0.540	1.96	-2.384

4.4.7 Quantitation of luteolin in the *A. afra* plant materials

The data sets and results of the quantitation of luteolin at each sampled position for each plant batch are presented in Appendix B. Figure 4.6 shows a chromatogram of luteolin and the internal standard morin within the plant matrix.

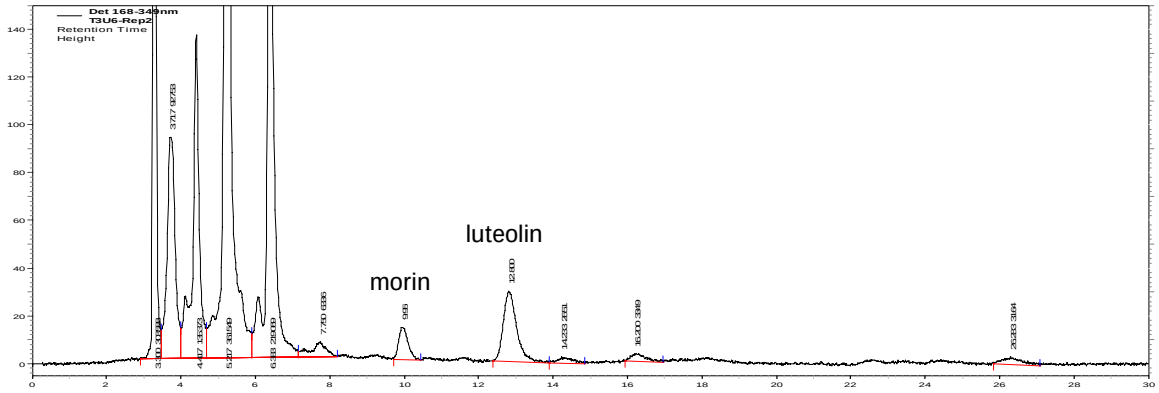


Figure 4.6: Chromatogram showing the retention of morin and luteolin within the plant matrix.

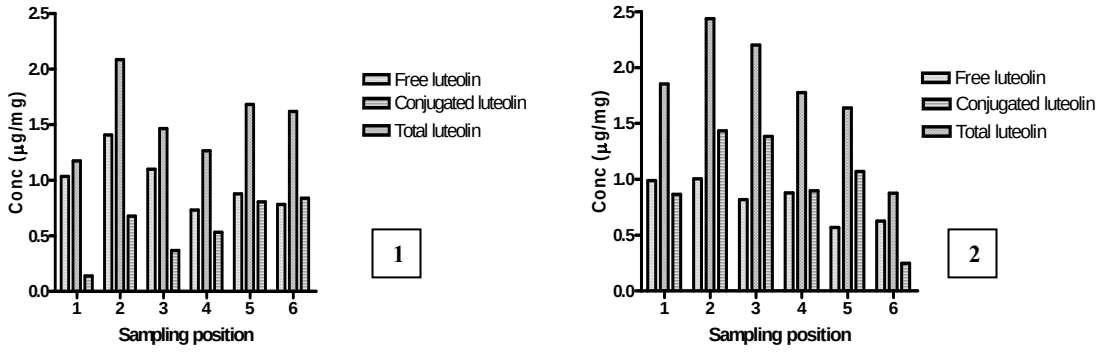
The *A. afra* leaves batches 001, 002 and 003 contained 0.9879 ± 0.2494, 0.8141 ± 0.1825 and 1.0980 ± 0.0991 µg/mg of free luteolin, respectively. The levels of conjugated/glycosidic luteolin were 0.5597 ± 0.2706, 0.9829 ± 0.4317 and 1.5780 ±

0.5743 µg/mg for batches 001, 002 and 003 respectively, and the levels of total luteolin content were 1.548 ± 0.3287, 1.7967 ± 0.5391 and 1.7350 ± 0.2909 µg/mg, respectively. From a one-way, non parametric analysis of variance (ANOVA) of the luteolin content for the different plant batches, the results showed that there was no significant inter-batch difference (p values; 0.2010, 0.0949 and 0.5471 for free, conjugated and total luteolin, respectively (alpha < 0.05)) between the luteolin content of the different plant batches i.e. the luteolin content present in each plant batch was similar. This similarity may be attributed to the similar method of preparation and storage of the plant materials and the fact that the plant materials were harvested from the same geographic location. The time of harvest was however, separated by a period of one month for each batch and from the results obtained, it can be concluded that this separation period had no effect on the levels of luteolin in the plant batches.

The SDL contained 0.8628 ± 0.0991, 1.203 ± 0.1862 and 2.065 ± 0.2347 µg/mg of free, conjugated and total luteolin, respectively. A one way ANOVA of this data and that obtained for batches 001, 002 and 003, showed that there was no significant difference in the content of free and total luteolin for the plant batches, i.e. p values were 0.1766 and 0.1399 for free and total luteolin, respectively (alpha < 0.05). This result is as expected, as the standardized batch was produced by blending together the 3 plant batches.

The FDAE powder contained 7.0880 ± 0.4751, 6.781 ± 1.152 and 13.870 ± 1.2460 µg/mg of free, conjugated and total luteolin respectively. These values obtained showed that the freeze-dried extract contained approximately 7 times more luteolin on a weight for weight basis than the *A. afra* SDL. This may be attributed to the extraction and freeze-drying process, whereby the constituents of the leaves are concentrated into a given volume of extracting solvent and then evaporated under vacuum. Therefore, dosage forms containing the FDAE powder should contain 7 times less material on a weight for weight basis compared to those containing the SDL. The amount of total luteolin found in the FDAE was comparable to the 6.965 ± 0.024 µg/mg determined in a previous investigation by Waithaka (2004).

There was however, considerable intra-batch variation in the luteolin levels of the plant materials as indicated by the percentage relative standard deviation for each batch. For instance, the % R.S.D for the total luteolin in the batches 001, 002 and 003 were 21.24, 30.00 and 16.77%, respectively. This intra-batch variation could be attributed to inconsistencies in the plant material make up, i.e. some batches contained a certain proportion of twigs and branches (as foreign matter), and also could have been due possibly to inconsistencies in the sample preparation, i.e. there could have been variation in the weighing or infusion of the leaves, across the batches. However, the % R.S.D for the total luteolin in the SDL was considerably less (% R.S.D = 11.36), and indicated a reduced variation within that batch attributable to the fact that this batch represented the 'mean' of the 3 batches having being produced by blending of batches. Similar trends were also observed for the conjugated and free luteolin content for each of the plant batches. Moreover, the intra-batch variation was much less (i.e. total luteolin % R.S.D = 6.70) for the FDAE powder compared to that for the SDL, and this may be attributed to the decoction process used to produce the extract, as the solution from which the latter resulted was homogeneous. It could therefore be concluded that standardization of the plant leaves reduced the inherent variation in the levels of luteolin and this variation was further reduced through preparation of a FDAE of the leaves. The low % R.S.D present for the FDAE and SDL, therefore showed that the FDAE powder was a more consistent material and that these materials were suitable for use in the manufacture of the medicinal dosage forms. To further highlight the inter and intra-batch variation in the luteolin content, the results obtained for the different plant material batches are also presented in bar graph form in Figure 4.7 below.



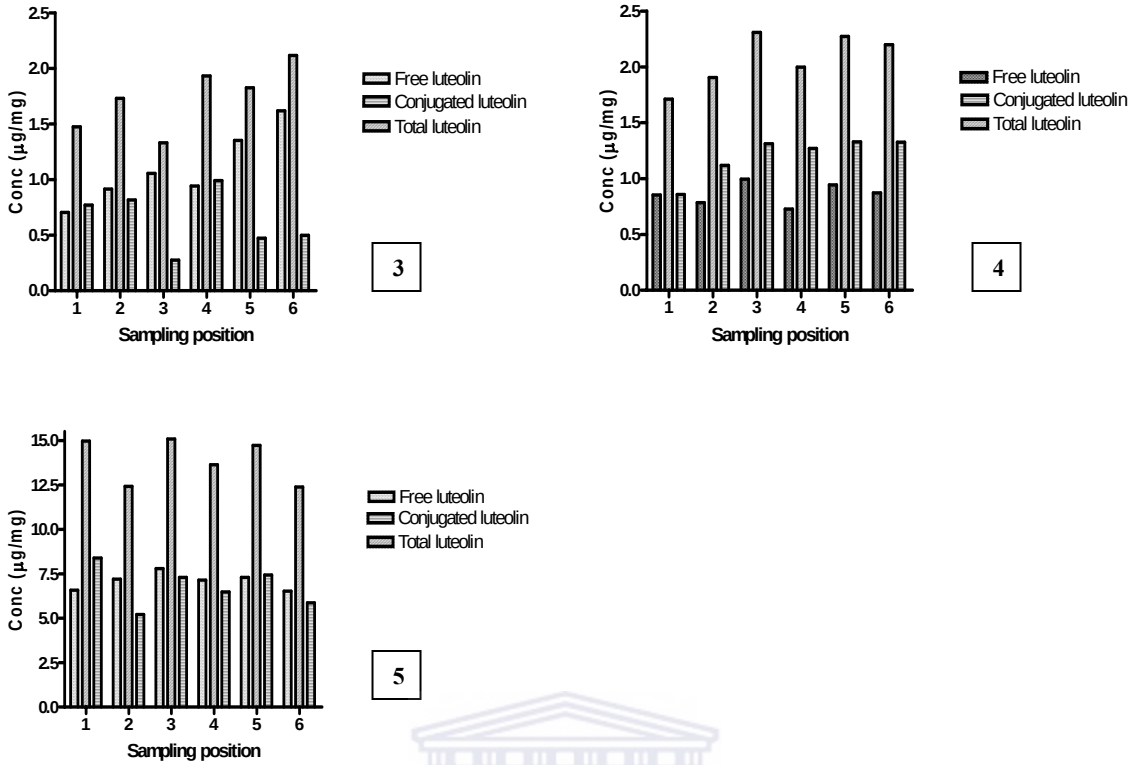


Figure 4.7: **Concentrations of the different forms of luteolin at various sampled positions in the *A. afra* plant materials. Graphs (1), (2), (3), (4) and (5), show the concentrations of luteolin in plant batches 001, 002, 003, the standardized leaves batch and the freeze-dried aqueous extract powder, respectively.**

4.4.8 Design of the placebos for the plant materials

4.4.8.1 Design of placebo for the *A. afra* standardized dried leaves

Using the 15 extractions, it was possible to reduce the absorbance of an aqueous solution extract of the leaves (at UV wavelength 220 nm) from an initial value of 4.8851 to a final value of 0.0854, i.e. a reduction of approximately 98% in absorbance was attained. The solvent extractions conducted and the resultant absorbances for a single batch is shown in Table 4.7 below. The process developed had an average recovery of 41%, i.e. from an initial mass of leaves of 10 g, 4.1 g of placebo leaves were obtained. This low recovery may be attributed to the several extraction steps involved, which may have contributed to the high loss in material.

Table 4.7: The series of solvent extractions employed to produce the placebo material of the *A. afra* leaves. The UV results presented are from a single manufactured 10 g batch.

Extraction step	Procedure/ 10g leaves	UV Absorbance	
		349nm	220nm
1	200ml cold water, stir for 2 minutes, allow to stand 10 minutes, then filter. To residue conduct step 2.	3.7798	4.1132
2	200ml hot water (80˚C), stir 2 minutes, allow to stand 10 minutes, then filter. To residue conduct step 3.	3.9441	4.2586
3	200ml hot water (80˚C), stir for 2 minutes, allow to stand 10 minutes, then filter. To residue conduct step 4.	3.8926	4.1577
4	200ml methanol/water (50/50 v/v), stir 2 minutes, allow to stand 10 minutes, then filter. To filter residue conduct step 5.	4.8851	4.9241
5	200ml methanol/water (50/50v/v) + 40ml 2N HCl (place in water bath for 120 minutes at 80˚C) then neutralise with 5N NaOH. Filter. To residue conduct step 6.	5.0400	5.2291
6	200ml methanol. Stir for 2 minutes, allow to stand for 10 minutes. Filter. To residue conduct step 7.	4.1889	4.2348
7	200ml methanol. Stir for 2 minutes, allow to stand for 10 minutes. Filter. To residue conduct step 8.	3.6138	3.8694
8	200ml methanol. Stir for 2 minutes, allow to stand for 10 minutes. Filter. To residue conduct step 9.	3.3656	3.7540
9	200ml methanol. Stir for 2 minutes, allow to stand for 10 minutes. Filter. To residue conduct step 10.	1.3457	1.5540
10	200ml methanol. Stir for 2 minutes, allow to stand for 10 minutes. Filter. To residue conduct step 11.	0.4295	0.5399
11	200ml ethyl acetate. Stir for 2 minutes, allow to stand for 10 minutes. Filter. To residue conduct step 12.	0.1923	0.2811
12	200ml ethyl acetate. Stir for 2 minutes, allow to stand for 10 minutes. Filter. To residue conduct step 13.	0.1326	0.2281
13	200ml ethyl acetate. Stir for 2 minutes, allow to stand for 10 minutes. Filter. To residue conduct step 14.	0.1192	0.1251
14	200ml ethyl acetate. Stir for 2 minutes, allow to stand for 10 minutes. Filter. To residue conduct step 15.	0.0976	0.0907
15	200ml ethyl acetate. Stir for 2 minutes, allow to stand for 10 minutes. Filter.	0.0707	0.0854

Figure 4.8 shows an image of the placebo of the dried leaves obtained after the repeated solvent extractions of *A. afra* leaves. The resultant placebo material was slightly darker than the original *A. afra* leaves (with a burnt appearance) and had a rougher texture than the *A. afra* leaves. This material also lacked the characteristic odour and bitter taste of the *A. afra* leaves.

The HPLC chromatographic fingerprints of the plant leaves before and after the solvent extractions are shown in Figures 4.9 and 4.10, respectively. As indicated by the reduced number of peaks, it is evident that most of the phytochemical constituents had been removed and therefore, the placebo produced may be expected to posses little pharmacological activity compared to the *A. afra* leaves. However, further evaluation in the form of pharmacological tests was warranted in order to prove the inertness of the material.

Figure 4.8: **Placebo leaves (on the left) produced by repeated solvent extractions of *A. afra* dried leaves (on the right).**

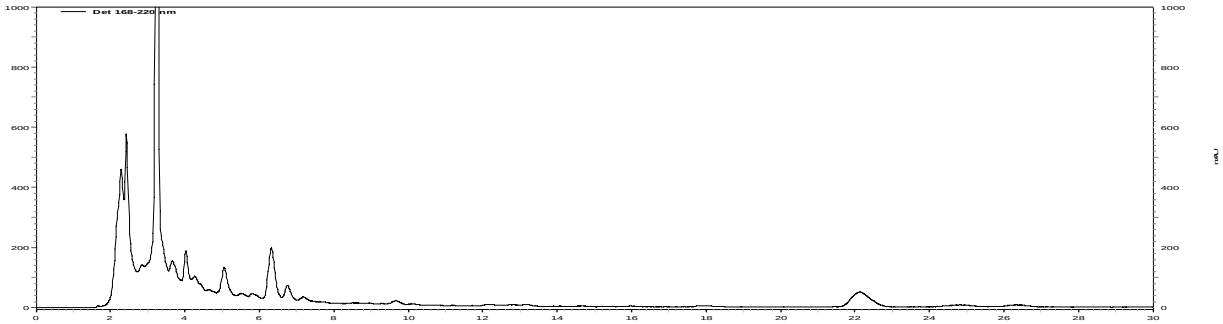


Figure 4.9: ***A. afra* standardized dried leaves chromatographic fingerprint after first solvent extraction.**

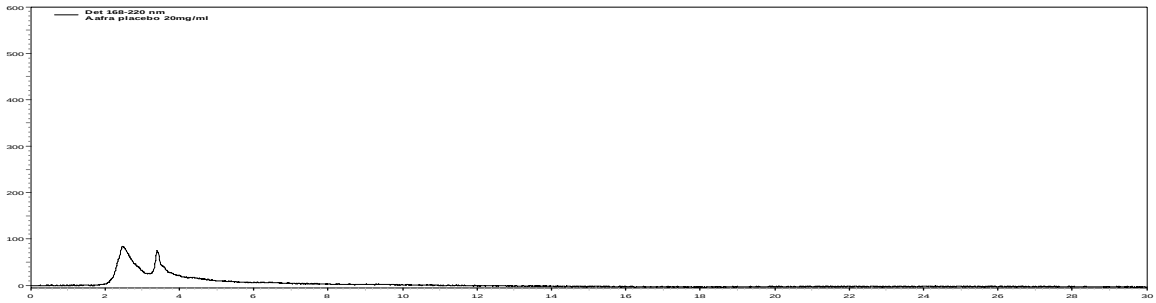


Figure 4.10: ***A. afra*** **leaves chromatographic fingerprint after0 final solvent extraction.**

4.4.8.2 Matching of odour of the placebo and plant materials

The amount of linalool capable of masking the *A. afra* smell was found to be 100 mg. This amount was adsorbed onto 0.125 g of silica gel as described in section 4.3.11.3 and included in the *A. afra* plant and placebo materials.

4.4.8.3 Matching of taste of the placebo and plant materials

0.15 g of sodium saccharin was found to mask the bitterness of the *A. afra* SDL and FDAE solutions. This amount was included in the preparations of the plant and placebo materials.

4.4.8.4 Design of placebo for the freeze-dried aqueous extract powder

The formula for the placebo for the FDAE is presented in Table 4.8 below. The placebo contained similar amounts of the aroma chemical and sweetener as the dried leaves preparations. The concentrations of linalool and sodium saccharin of 0.125 g and 0.15 g in the formulation constituted percentages of 0.063 and 0.075%, respectively. These levels are in line with the recommended safety levels of these chemicals. Linalool is generally approved for use as in fragrances up to a level of 1.5% and up to 500 mg/body weight/day of sodium saccharin may be ingested by humans without harm (OECD, 2004; SCF, 1997). 0.02 g of the synthetic colourant D.C. Brown was found to match the colour of hue produced by the *A. afra* plant materials. The chemicals included in the formulation (except for D.C. Brown) have been used in studies in the literature, (in similar amounts as

well as with other chemicals), to make placebo formulations which have been evaluated and considered pharmacologically inert (Bensoussan *et al*., 1998; Kaptchuck, 2000). Based on this, no further pharmacological evaluation for the FDAE placebo was warranted, and as such the extract was considered suitable for use in clinical trials.

Table 4.8: Excipients and quantities used in the formulation of the placebo for the *A. afra* freeze-dried aqueous extract powder.

Excipient	Quantity (g)	Use levels per 200 ml tea solution (%)
Potassium hydrogen phosphate	0.475	0.238
Lactose	0.025	0.013
Potato starch	0.03	0.015
D.C. Brown	0.02	0.010
Total	0.55	0.275
Sodium saccharin	0.15	0.075
Silica gel (100mg linalool)	0.125	0.063

4.5 Conclusions

The chapter addressed certain objectives of the study, viz. the preparation of the SDL and FDAE of *A. afra*, the characterization of the physicochemical properties of the plant materials, development and validation of an analytical method for use in the quantitation of luteolin in the materials, and the design of placebos for the plant materials.

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

- Approximately 50% of the mass of harvested *A. afra* leaves is due to moisture.
- Ionizing irradiation is an effective tool for the microbiological decontamination of the *A. afra* plant materials.
- Some new physicochemical specifications for the *A. afra* plant leaf materials were established, viz. water soluble ash values and bitterness values, which were 16.5952 ± 0.6760% and 16.5952 ± 0.6760%, for the dried leaves and freeze dried aqueous extract powder, respectively and the bitterness value of 600 000 for both materials.

- A suitable and reproducible assay method for the quantitation of luteolin in the *A. afra* plant materials using morin hydrate as internal standard was developed and validated.
- There is significant intra-batch variation but insignificant inter-batch variation in the luteolin levels of *A. afra* plant leaves collected from the same geographic location. A time of harvest, separated by a period of one month for each batch has no effect on the levels of luteolin in the plant batches.
- The FDAE powder contains approximately 7 times more total luteolin on a weight for weight basis than the SDL material.
- Standardization of the *A. afra* leaves reduces the inherent intra-batch variation in luteolin levels. This intra-batch variation is further reduced by preparation of a FDAE powder of the plant leaves.
- Placebo materials which resemble the SDL and FDAE powder can be produced using repeated solvent extractions and mixing of inorganic salts, respectively. Linalool, sodium saccharin and D.C brown can mask the odour, taste and match the colour of tea produced, respectively, of the *A. afra* plant materials.

In summary, the above results obtained showed that plant materials with reduced intra-batch variation in luteolin content and with acceptable pharmacopoieal microbial load had been produced, and were therefore suitable for the manufacture of the tea bag dosage form.

Chapter 5

The preparation and evaluation of tea-bags and placebos of *Artemisia afra*

5.1 Introduction

This chapter presents the materials and methods used in the preparation and evaluation of tea bags containing the standardized dried leaves (SDL), freeze-dried aqueous extract (FDAE) powder and placebos of the plant materials. The results obtained are also presented and discussed.

5.2 Equipment, materials and animals

In this part of the study, the following equipment were used;

balance (*Scaltec SPB42 Model SPB71, Scaltec Instruments, Heiligenstadt, Germany*), **centrifuge** (*Labofuge 200, Germany)*, **climatic chamber** *(Labcon, FSIE-H 20, Labdesign Engineering, South Africa)*, **dissolution test apparatus** (*Hanson Research, Northridge, CA, USA*), **HPLC filter uni**t (*Millipore Cameo 25 AS, DDA 02025So MSI: Micro separation Inc., USA*), **solvent filter paper** (*47mm filter membrane 0.45µm PVDF,Millipore, USA)*, **organ bath**, with **transducer** (custom made) and **recorder** (*Rikadenki R-03, Tokyo, Japan)*, **spectrophotometer** (*DU 640 spectrophotometer, Beckman, USA*), **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)*, **pH meter** (*Basic 20 Crison Instruments, S.A, Italy.*), **vortex** *(Vortex-2, G-560E, Scientific Industries, Inc. Bohemia, N.Y. 11716 USA)*, and a **water bath** (*Labcon, CDH 110 Maraisburg, South Africa*).

The HPLC system used consisted of an auto sampler (*Beckman Gold Module 507*), a programmable binary gradient pump (*Beckman Gold Module 126 series*), a diode array

detector (DAD) (*Beckman Gold Module 168 series*) with a 32-Karat^{TM}-software package and a Synergy® Hydro-reverse phase column (*Phenomenex, USA*) having 4 μm particle size and a column length of 250 x 4.60 mm.

The following materials were used;

Artemisia afra plant material, tea bag filter paper (*Dynapore 117/7/0, Schoeller & Hoesch, Germany),* morin hydrate, luteolin, isoprenaline, sodium pentobarbitone (*Sigma-Aldrich, Germany),* methacholine (*Fluka Chemie GmbH, Germany),* high density polyethylene (HDPE) storage container, silica gel (*BDH, South Africa),* sodium chloride (*UnivAR, Saarchem, South Africa)*, potassium hydrogen phosphate (*Riedel-de Haen, Germany)*, glucose, sodium hydrogen carbonate (*UniLab, Saarchem, South Africa),* calcium chloride, potassium chloride (*Merck, South Africa),* magnesium sulphate (*UnivAR, Saarchem, South Africa),* acetonitrile HPLC grade (*Burdick & Jackson, M.I. USA),* ethyl acetate CP (*Saarchem, South Africa)*, hydrochloric acid *(Kimix, South Africa).*

The following animals were used;

Male and female Dunkin Hartley guinea pigs of between 400 g and 700 g weight, obtained from the animal facility at the University of Cape Town, a few days before use. The animals were kept in an environmentally controlled animal room with temperature control and a 12 hr light darkness cycle. Ethics clearance was obtained for the use of the guinea pigs from the UWC Senate Ethics committee.

5.3 Methods

5.3.1 Preparation of the tea bags

The primary objective of the study was to prepare the SDL and FDAE powder of *A. afra* in a tea bag dosage form containing amounts of the materials equivalent to those used in the traditional infusions.

5.3.1.1 Determination of the amount of plant material for the tea bags

The most common traditional method of preparation directs that a quarter cup of leaves be infused in hot water, allowed to seep for 10 minutes and the resultant tea drunk (Roberts, 1990). Based on this, a 200 ml tared cup was loosely filled to the brim with dried *A. afra* leaves, the weight recorded and divided by 4 to obtain the amount equivalent to a quarter cup (i.e. dose of leaves required).

To determine the amount of FDAE powder to be incorporated in the tea bags, the total luteolin content present in the SDL was used. It was known (section 4.4.7), that the FDAE powder, on a weight-to-weight basis, contained approximately 7 times more total luteolin than the standardized *A. afra* leaves and therefore, the amount of FDAE incorporated into the tea bag was a seventh of that contained in the dried leaves tea bag.

5.3.1.2 Determination of the appropriate tea bag size

It has been reported in the literature, that various sizes of tea bags result in different rates of extraction of the soluble species (Jaganyi & Ndlovu, 2001). In fact, the rate of extraction increases with increases in tea bag size and in one study, viz. an investigation of the effect of tea bag size on the extraction of caffeine from Ceylon Orange Pekoe tea, the results showed that beyond a mass to surface area ratio of 1:9, only small increments in the rate of extraction were observed (Jaganyi & Ndlovu, 2001). This 'threshold ratio' of 1: 9 was therefore adopted for the manufacture of the *A. afra* plant material and placebo tea bags, i.e. the tea bags had a content mass to surface area ratio of 1:9. Tea bags containing the FDAE powder also had a content mass to surface area ratio of 1:9, although the material in these bags was only a seventh of the mass of the dried leaves used. This was done in order to increase the mass to surface area ratio beyond the threshold and therefore ensure an almost instant infusion and in addition, it was anticipated that depending upon the clinical trail design, the FDAE powder may be evaluated against the dried leaves and therefore the two preparations needed to be similar in size to facilitate clinical trial blinding.

5.3.1.3 Selection of the appropriate tea bag paper

In selecting the tea bag paper with the appropriate specifications, particle retention (pore size), wet strength and paper smell of the material were taken into consideration. Consequently, tea bag filter paper with high particle retention (and therefore suitable for containing the FDAE powder), high wet strength, absence of paper odour, made from oxygen-bleached raw materials and with heat sealable properties was selected (see Appendix C for the specifications). The oxygen-bleached tea bag paper was preferred in the hope that the bleaching process would have oxidised any pharmacologically active phytochemical constituents within the paper material and therefore the tea bag paper would be pharmacologically inert.

5.3.1.4 Preparation of the *Artemisia afra* and placebo tea bag dosage forms

Generally tea bags may be filled using an automated method, where the tea material flows through a hopper and is wrapped around by the tea bag paper and the latter sealed to enclose its contents. Typically, heated rods on either side of the tea bag paper will press together to seal the tea bag before it is cut into the desired shape (Anonymous, 2006a).

In this study, the tea bags of the *A. afra* plant materials were prepared and sealed manually. From the tea bag paper roll, strips 7 cm wide were cut, folded over, and cut to make squares 7 cm x 7 cm. Two of the three open sides of the square were then sealed closed, 0.5 cm into each side, using a hot iron. The appropriate amount of the sieved SDL of *A. afra* (particle size range of 0.35 - 1.40 mm) was then accurately weighed and transferred into the bag via the remaining open side and thereafter the latter side sealed, 0.5 cm into the side. This therefore meant that the available surface area for the plant material was 36 cm^2 (6 cm x 6 cm). The above procedure was repeated for the preparation of the tea bags containing the FDAE powder and the placebo materials.

5.3.2 Pharmaceutical evaluation of the tea bag dosage forms

Few quality control tests exist in the pharmacopoeia for the specific evaluation of tea bag dosage forms. Control tests are typically conducted on the herbal or tea material itself and include identity and microbiological evaluation tests. However, the test for uniformity of mass is specified in the European Pharmacopoeia 2002 to be conducted on herbal teas in sachets (EP, 2002d). The tea bag dosage forms of *A. afra* plant material were consequently evaluated on the latter in addition to other quality tests as described below.

5.3.2.1 Determination of uniformity of mass of the tea bag dosage forms

Twenty tea bags were randomly selected from each of the manufactured batches containing the *A. afra* SDL and FDAE powder. Each tea bag was weighed and then completely emptied of its contents and any remaining particles removed using a brush. To obtain the mass of the tea bag contents, the mass of the empty tea bag was subtracted from the initial mass of the tea bag (EP, 2002d) and from masses of the 20 tea bags, the average mass and deviation in the individual tea bags content was calculated.

5.3.2.2 Determination of the infusion profile of the *A. afra* dosage forms

Few studies are available in the literature on the release profiles of flavonoids in tea infusions. The majority of studies have focused on the influence of temperature, manufacturing method or composition of the extracting media on the infusion and equilibria of the tea infusions (Jaganyi & Mdletshe, 2000; Jaganyi & Wheeler, 2003; Spiro & Jaganyi, 2000).

To establish the infusion profiles of the *A. afra* dosage forms, the British Pharmacopoeia dissolution apparatus I (basket apparatus) at 100 revolutions per minute (rpm) and a modified apparatus II (paddle apparatus) at 50 rpm were used for the loose leaves and tea bag preparations, respectively. In the experiments, water, as the dissolution media, at a temperature of 80˚C was used (Jaganyi & Wheeler, 2003; Spiro & Lam, 1995), as it is the

temperature closest to that normally used in the preparation of teas (Anonymous, 2006a). At various times, samples from the infusions were collected and analysed for dissolved plant constituents.

The flavonoid luteolin was used as a marker compound to characterize the infusion profiles of the *A. afra* dosage forms and the validated HPLC assay described in section 4.4.6 was used to quantitate the amount of luteolin released over time. Infusion samples were acid hydrolysed yielding total luteolin (i.e. free aglycones plus the aglycones initially in glycosidic linkages) and unhydrolysed yielding free luteolin and the sugar moieties of luteolin, at each sampling time point and in addition, the amount of glycosidic luteolin present at each sample time point was calculated (i.e. total luteolin – free luteolin). Luteolin is known to be present in plants as luteolin-7- O-glucuronide, luteolin-5-O-glucoside, luteolin-3'- glucuronide, luteolin 3′-*O*- β - D -glucuronide and/or luteolin-3',4'-diglucuronide forms (Markham, 1982; Heitz *et al*., 2000) and these were the likely glycosidic forms of luteolin to be quantitated at each time point.

The samples collected from the infusion of the dosage forms were also analysed using a UV spectrophotometric assay, which provided a profile of the infusion of all the soluble plant constituents over time.

5.3.2.2.1 Determination of the infusion profile of the *A. afra* loose leaves

The British Pharmacopoeia dissolution apparatus I (basket method) was used for the infusion profile characterization of the loose leaves. The dissolution apparatus was fitted with a heater on the water bath capable of attaining and maintaining a temperature of 80˚C within a range of ±5˚C. One gram of the sieved SDL (particle size range 0.35-1.40 mm) was accurately weighed and loosely filled into the apparatus basket. The dissolution apparatus vessel containing 900 ml of de-aerated distilled water was weighed with its contents, allowed to equilibrate with the water bath temperature and once equilibrated, the basket containing the leaves lowered into the vessel and rotated at 100 rpm. Sampling

was conducted through a fixed glass rod inserted through the cover disc at a depth of 5 cm into the vessel, at a position 1 cm from the wall of the vessel.

Samples of 3 ml each were withdrawn from the vessel using a plastic syringe. 1 ml was transferred into a tube containing 5 ml of distilled water for UV spectrophotometric analysis, 1 ml into a tube for hydrolysed luteolin analysis using HPLC and the other 1 ml into a tube for unhydrolysed luteolin analysis using HPLC. Each sample withdrawn was immediately replaced with an equal volume of de-aerated distilled water. For UV and HPLC analysis, a total of 8 samples for each were withdrawn at the sampling time points: 0.0 (blank), 5.0 10.0, 30.0, 60.0, 120.0, 180.0 and 240.0 minutes. At the end of the run, the apparatus vessel was again weighed with its contents and the difference in weight taken as ΔV the volume lost through evaporation and sampling (assuming a specific gravity of 1 for the lost media). This was used to calculate the rate of loss for correction of the concentrations (Spiro & Lam, 1995).

5.3.2.2.2 Determination of the infusion profile of the *A. afra* tea bag dosage forms

The British Pharmacopoeia dissolution apparatus II (paddle method) was used for the infusion profile characterization of the *A. afra* tea bag dosage forms, i.e. tea bags containing the *A. afra* SDL and FDAE powder. However, because tea bags have a propensity to float in aqueous medium thus affecting the hydrodynamic conditions within the dissolution vessel, a holding cell was devised to hold the tea bag and maintain it in a fixed position within the vessel. The holding cell devised for this study incorporated a wire mesh cage and two stainless steel weight rods and was a modification of the double ring mesh assembly designed by Durig and Fassihi (2000) in investigating the dissolution of floating and sticking delivery systems and that used by Jaganyi and Mdletshe (2000), where stainless steel bolts were attached to the tea bag to prevent floating.

The holding cell devised and shown in Figure 5.1, was made from netting wire (wire specifications 900 x 13 x 0.71 mm), which was cut and folded to make a holding cell 7

cm x 7 cm, with a height of 1 cm. To ensure that the holding cell would not move during the infusion process, weights were added in the form of two stainless steel rods, on either side at the bottom of the holding cell. The rods had dimensions of 6.5 x 1 cm and the complete holding cell had a weight of 115 g. The shape of the dissolution vessel and the dimensions of the holding cell ensured that the holding cell would only go a certain distance within the vessel (i.e. 2.5 cm from the bottom of the vessel) and no further. The tea bag was placed inside the holding cell, such that it lay flat and the wire mesh did not hinder any swelling of the tea bag (see Figure 5.1).

In determining the infusion from the tea bags, once the media had been weighed and allowed to equilibrate to the water bath temperature (as described in section 5.3.2.2.1), the holding cell containing the tea bag was placed inside the vessel taking care to ensure that water did not splash out of the vessel during this process. The dissolution apparatus paddles were immediately lowered into the vessel up to a distance of 3.0 cm above the holding cell and rotated at a speed of 50 rpm. For UV and HPLC analysis, a total of 12 samples for each analysis method were withdrawn at the sampling time points: 0.0 (blank), 5.0 10.0, 30.0, 60.0, 120.0, 180.0, 240.0, 300.0, 420.0, 480.0 and 540.0 minutes. At the end of the run, the dissolution vessel was weighed with its contents and the difference in weight taken as ΔV, the volume lost through evaporation and sampling for use in calculation of the concentrations.

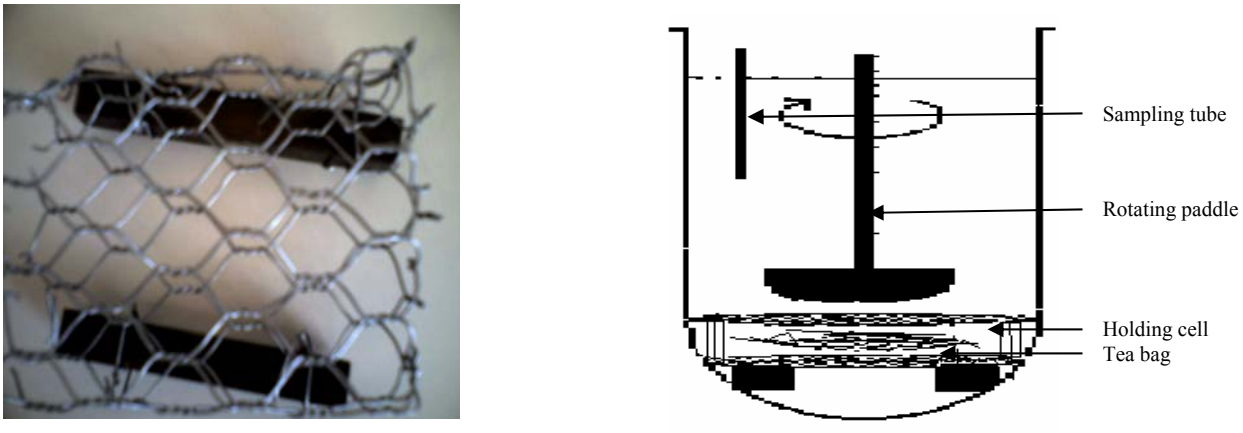


Figure 5.1: **The holding cell devised to contain the *A. afra* tea bags during the infusion process (on the left) and on the right, a schematic diagram of the modified dissolution apparatus II, used for the determination of the infusion profile of the tea bags containing the plant materials.**

For the determination of the infusion profile of the tea bags containing the FDAE, the same procedure as described above was used. However, in this case, the infusion samples for UV spectrophotometric analysis were withdrawn at the time points: 0 (blank), 1.0, 2.0, 3.0, 4.0, 5.0, 10.0 and 15.0 minutes. From the infusion profile generated using the spectrophotometric analysis, the time point at which 85% of the extract powder had infused was determined. This point was then used as a sampling time point for a single point dissolution assay for luteolin using HPLC (FDA, 1997) and total, free and conjugated luteolin quantified at this time point.

5.3.2.2.4 Sample preparation, analysis and comparison of the infusion profiles

Samples collected from the infusion experiments were hydrolysed and extracted using the procedure described in section 4.3.10 and assayed for total (hydrolysed) and free (unhydrolysed) luteolin using the validated HPLC assay. The quantity of total, free and the calculated conjugated luteolin concentrations at each time point were plotted against time to generate the infusion profile for the dosage forms.

For the UV spectrophotometric analysis, the 1 ml sample withdrawn was placed in a tube containing 5 ml of distilled water and immediately cooled, by placing in a cooler box, to prevent evaporation of the sample as well as any unknown degradation of the sample which could occur. The UV absorbance of the samples were measured at 349 nm (luteolin λ max) and at 220 nm, the λ max for the infusion samples, and the infusion profiles generated by plotting the absorbance measurements against time.

The infusion profiles of the *A. afra* loose leaves and the tea bag dosage forms generated from the UV spectroscopic and the HPLC quantitation of the different forms of luteolin were compared by calculating the similarity factor (f_2) and the difference factor (f_1) between the profiles.

5.3.2.3 Determination of the stability of the dosage forms upon storage

The tea bags containing the *A. afra* SDL and FDAE powder were subjected to real time and accelerated stability testing. Real time stability testing was conducted at normal room temperature and normal room humidity for a period of 6 months i.e. at Site A and accelerated stability testing conducted at 40˚C, 75% relative humidity for a period of 3 months i.e. at Site B. In addition, the effect of temperature alone on the stability of the FDAE tea bag preparation was evaluated. For this, tea bags containing the FDAE powder were placed in a secondary packaging comprising a high density polypropylene (HPDE) container containing a 5 g sachet of silica gel desiccant. These containers were placed under 2 different conditions of temperature, *viz.* room temperature and 40˚C, for a period of 3 months (i.e. at Site C and D respectively).

The stability of the tea bags upon storage were evaluated based on their organoleptic properties, i.e. appearance, odour, leakage of tea bag contents, moisture content of tea bag contents, infusion profile and chromatographic fingerprint of the tea bag contents. The latter was assessed using HPLC with diode array detection and the chromatographic fingerprints of the plant materials obtained, at 14 and 30-day intervals for the FDAE and dried leaves respectively, compared to determine the stability of the plant material as a whole. To obtain the chromatographic fingerprints, 25 mg samples were withdrawn from the preparations under storage, placed in 1ml of distilled water at 80˚C, vortexed for 2 minutes and left to stand for 8 minutes before being filtered using Whatman no. 1 filter paper. Fifty microliters of the filtrate was injected directly onto the HPLC column, under the conditions described in section 4.3.10. The chromatographic fingerprints obtained were overlaid onto each other in order to show any changes that may have occurred. The UV spectra of the HPLC assayed samples were also overlaid, and compared, at a wavelength and retention time corresponding to the peak with maximum absorbance. This comparison would show the purity and identity of the material throughout the period of the stability test, i.e. any degradation or change could be observed through the UV spectra (Springfield *et al*., 2005). Finally, the procedure for determining the organoleptic

properties, moisture content and infusion profile of the preparations was as described in section 4.3.5, 4.3.7 and 5.3.2.2, respectively.

5.3.3.4 Comparison of the *A. afra* tea bag dosage forms and their placebos

The organoleptic properties of the placebos of the *A. afra* leaves and FDAE powder were compared to that of the *A. afra* leaves and FDAE in tea bags, respectively. This was in terms of the overall appearance of the tea bags, colour and taste of the teas produced and odour of the product. For this evaluation, the natural senses of sight, taste and smell were used.

5.3.4 Pharmacological evaluation of the placebo for the plant leaves

In designing a placebo, it is essential that the placebo be evaluated for lack of pharmacological activity before it can be considered inert and suitable for use in a clinical trial. It therefore follows that the placebo should be evaluated against the 'active' for pharmacological activity, which the 'active' possesses. Bioassays utilizing either whole animals, isolated organs and tissues, blood and its components, tissue culture cells or subcellular cells can be used for evaluating the efficacy of the test material (WHO, 1993). In selecting an appropriate test system, consideration should be given to the sensitivity, reproducibility and general acceptance of the test system. Dose selection may be based on doses administered traditionally or clinically and results are often presented as dose-response relationships (WHO, 1993).

In this study, the muscle relaxant activity of the *A. afra* placebo and plant materials was evaluated using an isolated guinea pig tracheal muscle mounted in an organ bath system. This part of the study was ethically approved by the Ethics Committee of the UWC Senate Research Committee, as part of another more comprehensive project *viz.* 'The investigation of the mechanism for the respiratory smooth muscle relaxant effect of a flavonoid-containing aqueous extract of *Artemisia afra*' (M. Pharm research project of L. Manthata, School of Pharmacy, UWC), i.e. the animal tissue was isolated for use in the

afore-mentioned project and after an experimental run, was used for the present evaluation. The procedures used for the preparation of the test solutions and animal tissue to establish the activity of the *A. afra* plant material and placebos is described below.

5.3.5.1 Preparation of solutions used in the tracheal muscle experiments

For the evaluation of the muscle relaxant activity, similar concentrations of the placebo and *A. afra* leaves, i.e. 5, 10, 20, 50 and 100 mg/ml in distilled water, were prepared. For this, the appropriate mass of material was placed in a volume of hot (80˚C) distilled water, vortexed for 2 minutes, allowed to stand for 8 minutes and then filtered using Whatman no. 1 filter paper before being used. Stock solutions of 60 mM potassium chloride, 10^{-1}M methacholine and 1M isoprenaline for use in the experiments, were prepared in distilled water. Krebs Henseleit solution for sustaining the tissues was made by dissolving 35.05 g NaCl, 1.78 g KCl, 816.55 mg KH_2PO_4, 1.48 g $MgSO_4.7H_2O$, 10.50 g $NaHCO_3$, 1.84 g $CaCl_2.2H_2O$ in distilled water, and adding 9.90 g of glucose just before use.

5.3.5.2 Procedure for evaluation of the effect of the *A. afra* and placebo materials on the isolated guinea pig tracheal muscle

For this study, the tracheal muscle was isolated for mounting in the organ bath as follows; firstly, the guinea pig was weighed and anaesthetised with an intra-peritoneal injection of sodium pentobarbitone (190 mg/kg body weight). The trachea was then removed from the animal, placed in a petri-dish containing cold Krebs Henseleit solution, the excess tissue and fat trimmed off using a razor blade and the muscle opened by cutting longitudinally through the cartilage rings, diametrically opposite the trachealis muscle. The flat tissue was then pinned onto a corkboard (still immersed in the cold Krebs Henseleit solution) and cut into zig-zag strips by making transverse slits at equal intervals in the tissue (Uhlig, 1998). Finally, the strip was divided into 4 equal pieces, cotton threads inserted at each end and the strips mounted in the isolated organ bath system.

The isolated organ bath system used in this study, and shown in Figures 5.2 and 5.3, consisted of (i) a circulating water bath, (ii) 4 custom made water-jacketed, 15 ml glass organ baths each connected, with tubing and tap, to (iii) a reservoir containing Krebs-Henseleit solution, (iv) a force transducer and amplifier system and (v) a 4-pen strip chart recorder. The organ baths were filled with pre-warmed medium from the reservoir via glass tubing located in the water-jacketed glass systems, the flow into the bath being controlled via an inlet tap and flow out via a drain tap at the bottom of the bath. The contents of the bath and the glass perfusion lines were kept at 37ºC by pumping water through the glass jackets of the organ bath and glass systems using the circulating water bath. The contents in the organ bath were also continuously aerated with 5%CO_2 / 95%O_2 via an inlet at the bottom of the bath. The tissue was mounted in the bath by hooking one cotton thread to a hook at the bottom of the bath and the other end to the force transducer. The transducer converted the mechanical movements of the contracting/relaxing muscle into an electrical signal that was amplified and the result eventually recorded on a chart recorder.

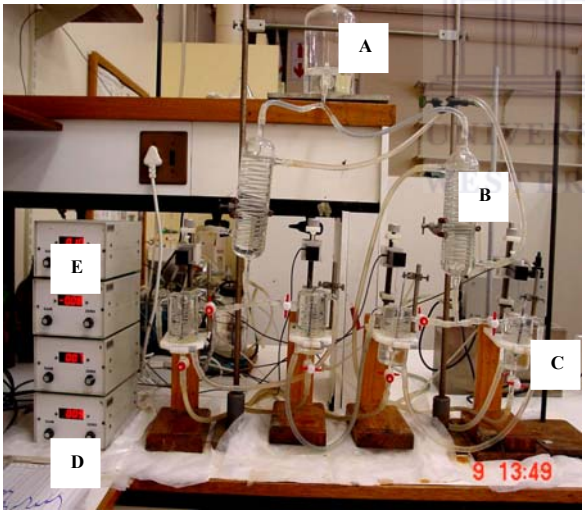


Figure 5.2 **The organ bath system used for evaluation of the activity of the plant materials. A is the bath reservoir containing Krebs Henseleit solution, B is one of the water-jacketed glass reservoirs C is the water bath temperature controller, D is the 4-pen strip chart recorder and E is the signal amplifiers.**

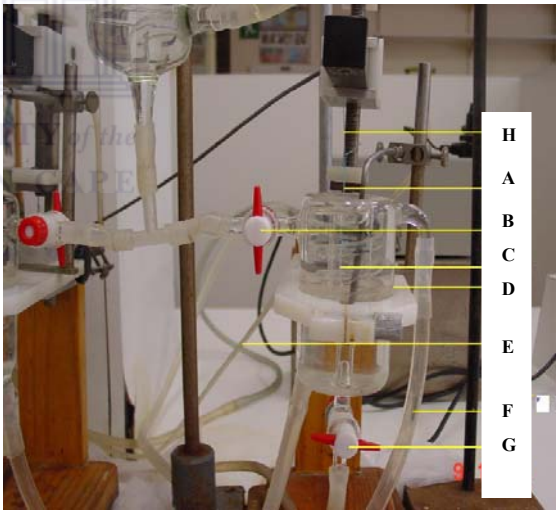


Figure 5.3: **A close up picture of a single organ bath. A shows cotton thread used to tie the tissue to the transducer, B is the Krebs' inlet tap, C shows the suspended tissue, D shows the bath, E shows the gas inlet, F, shows one of the pipes to the water-bath that transport warm water to maintain the temperature, G shows the Krebs' outlet tap and H shows the transducer.**

Once mounted in the organ bath the tissue was placed under a tension of 2 g and allowed to equilibrate for 1.5 to 2 hours, while being continuously irrigated and aerated with Krebs Henseleit solution at a temperature of 37 ºC (that was replaced at 15 min intervals) and a mixture of 95% O_2 and 5% CO_2. Thereafter, the tissue was depolarised with 2 doses of 0.1 ml potassium chloride (60 mM) injected into the bath and after each depolarising dose, and sufficient time allowed for the response, the tissue was washed and allowed time to recover before the next dose was given. Following this, the tissue was contracted using 0.1 ml methacholine (10^{-1}M), the contraction left to stabilise for 20 minutes and the response recorded on the strip chart recorder. The methacholine was then washed out and another methacholine dose administered, i.e. the process repeated in order to check the reproducibility of the tissue response. After the contractions had stabilized, cumulative 0.1ml doses of the placebo leaves solutions (i.e. 5, 10, 20, 50, and 100 mg/ml) were injected into the organ bath starting with the lowest concentration and allowing enough time for its effect to stabilise before the subsequent dose was injected. After the injection and stabilisation of the effect of the last dose (i.e. 0.1 ml of 100 mg/ml), 0.1ml of $1x10^{-1}$M isoprenaline was injected to obtain the maximal relaxation response. Throughout, the responses of the tissue to the injected solutions were continuously recorded on the chart recorder. The traced responses (i.e. the change from base-line or maximum contraction), were then measured and the relaxant effect of the injected solutions expressed as a percentage of the maximum relaxation induced by the isoprenaline. A log dose response curve of the percent relaxation *vs.* log dose was then plotted.

The same procedure was repeated for solutions of the *A. afra* plant leaves.

5.7 Results and discussion

5.7.1 Preparation of tea bags containing the *A. afra* plant material

The mass of a quarter cup of the *A. afra* dried leaves, as determined in section 5.3.1.1 was found to be 4 g and this was thus taken as the amount of material in one traditional dose and therefore the amount to be incorporated in one tea bag. Based on the ratio of the total luteolin content in the SDL to that in the FDAE powder, found in section 4.4.7, a seventh

of the *A. afra* leaves quantity i.e. 0.55 g, was taken as the amount of FDAE powder to be incorporated in the tea bags. These dose quantities used were in agreement with those obtained by previous investigators, *viz.* Mukinda (2006), in determining the amount of dried leaves that were equivalent to the traditional dosage preparation, found a quantity of 3.5 g, while Komperlla (2005) found that 0.60 g of the FDAE powder was equivalent to the traditional dried leaves preparation

Four grams of the SDL and 0.55 g of FDAE powder were thus, separately prepared in Dynapore 117/7/0 tea bag paper to produce tea bags having 36 cm^2 surface area. Images of the prepared tea bags containing the dried leaves and FDAE powder are shown in Figure 5.4. Although equivalent in luteolin content and tea bag surface area, the two products were clearly distinguishable from each other, with the *A. afra* leaves completely filling and extending the bag while the extract powder only occupied a small portion of the tea bag.

Figure 5.4: Prepared tea bag containing 4g of the standardized dried leaves of *A. afra* (on the left) and that containing 0.55g of the *A. afra* freeze-dried aqueous extract powder (on the right).

5.7.2 Uniformity of mass of the dosage forms

The uniformity of mass data for the manufactured tea bags containing the *A. afra* SDL and FDAE powder, is shown in Tables 5.1 and 5.2, respectively.

Table 5.1: Uniformity of mass of the prepared tea bags of the *A. afra* standardized dried leaves.

	Mass of tea bag contents (g)		Mass of tea bag contents (g)		Mass of tea bag contents (g)		Mass of tea bag contents (g)
1	4.0020	**6**	4.0004	**11**	3.9995	**16**	4.0008
2	4.0039	**7**	3.9995	**12**	4.0075	**17**	4.0021
3	4.0038	**8**	3.9992	**13**	4.0032	**18**	4.0206
4	4.0004	**9**	4.0002	**14**	4.0085	**19**	4.0023
5	4.0002	**10**	3.9992	**15**	3.9998	**20**	3.9997
Average tea bag content mass ± S.D = 4.003 ± 0.0013 % R.S.D = 0.12							

Table 5.2: Uniformity of mass of the prepared tea bags of the freeze-dried aqueous extract powder of *A. afra.*

	Mass of tea bag contents (g)		Mass of tea bag contents (g)		Mass of tea bag contents (g)		Mass of tea bag contents (g)
1	0.5564	**6**	0.5487	**11**	0.5533	**16**	0.5531
2	0.5679	**7**	0.5421	**12**	0.5604	**17**	0.5663
3	0.5609	**8**	0.5539	**13**	0.5645	**18**	0.5501
4	0.5574	**9**	0.5618	**14**	0.5681	**19**	0.5522
5	0.5645	**10**	0.5585	**15**	0.5544	**20**	0.5543
Average tea bag content mass ± S.D = 0.557 ± 0.0069 % R.S.D = 1.24							

The *A. afra* tea bags containing the SDL had an average content of 4.003 ± 0.0013 g and those containing the FDAE powder, an average of 0.557 ± 0.0069 g of material. The percent deviation in the content of the manufactured tea bags was well within the European Pharmacopoeia 2002 requirements (i.e. R.S.D of 0.12% and 1.24 %) for uniformity of mass of herbal teas in sachets. The latter requires a percent deviation of not more than 7.5% for an average mass more than 2.0 g and a percent deviation not more than 15% for a mass less than 1.5 g. Taken together with the results obtained in the identity and microbial tests conducted in Chapter 4, it was thus concluded that the manufactured tea bags complied with the European Pharmacopoeia 2002 requirements.

5.7.3 Infusion profile of the *A. afra* loose leaves

The infusion profiles of the *A. afra* loose leaves are presented below and Figure 5.5 shows the UV infusion profiles and the percentage of infused plant material released from the *A. afra* loose leaves. Figure 5.6 shows the infusion profiles of the luteolin release

(free, conjugated and total luteolin) and the percentage luteolin released from the *A. afra* loose leaves. The results shown were corrected for 4 g of plant material, since only 1 g of plant material, the maximum that could be loosely filled in the apparatus basket, was used. The results were also corrected to allow for evaporation and sampling losses.

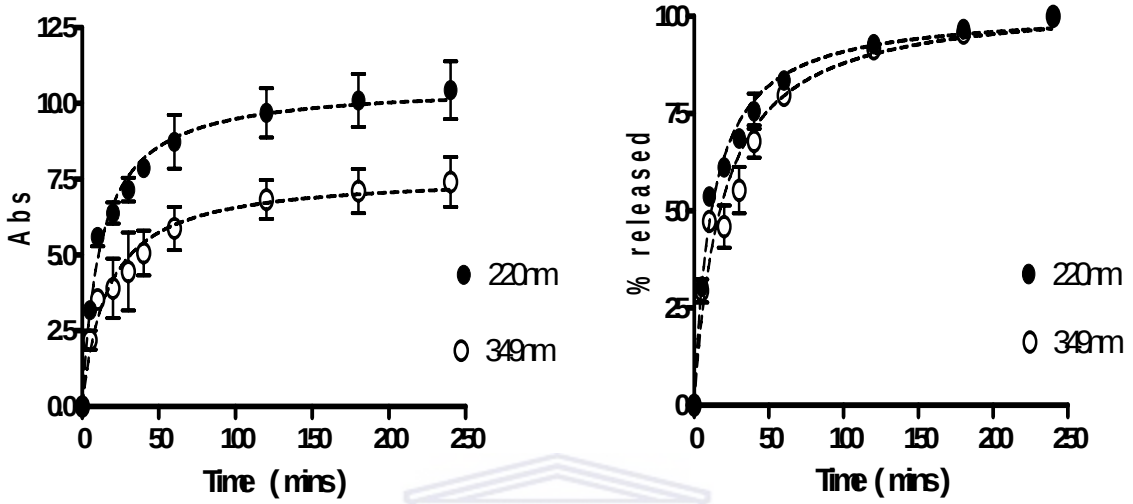


Figure 5.5: **The infusion profiles of the *A. afra* loose leaves (on the left) and the percentage release profile (on the right), determined using UV spectroscopic analysis at 220 (-●-) and 349 (-○-) nm.**

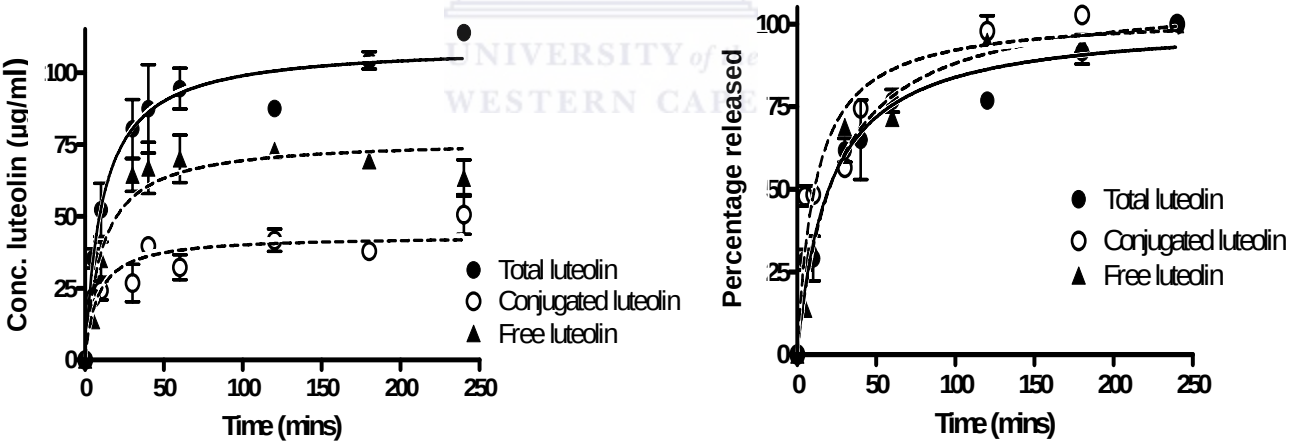


Figure 5.6: **The infusion profile of luteolin (total -●-, conjugated -○- and free -▲-) release (on the left) and the percentage release profile of luteolin (on the right) from the *A. afra* loose leaves.**

From the UV and HPLC generated infusion profiles, it was apparent that the profiles generated from the two methods were qualitatively similar, suggesting that luteolin was a suitable marker to use to characterise the infusion profiles of the *A. afra* leaves and

therefore the following discussion of the infusion results for this dosage form is based on the HPLC luteolin data only.

The infusion profiles obtained demonstrated a characteristic pattern of release, i.e. an initial burst release phase followed by a controlled release phase which, from the literature, is known to be typically associated with swelling (of the leaves). The initial burst phase normally accounts for 50% of the release and the swelling phase from 50% up to the point where 100% release is reached (Missaghi & Fassihi, 2005).

From the results obtained, insight into the amount and form of luteolin present in the traditional teas over the infusion period can be gained. Commonly, the traditional method of preparation allows the tea to stand and seep for 10 minutes before being consumed (Roberts, 1990). At this time point, based on the present results, an average of 35.01 ± 10.09 µg/ml of free luteolin (aglycone) and 24.20 ± 11.14 µg/ml of conjugated luteolin (glycosidic) were present in the tea and this corresponded to an average total luteolin concentration of 52.24 ± 16.04 µg/ml and an average percentage release of 31.35%, 47.39% and 34.61% of the free, conjugated and total luteolin, respectively. The final concentrations of free, conjugated and total luteolin present in the tea at steady state (i.e. after 100% release) were 63.30± 10.93 µg/ml, 47.35 ± 16.72 µg/ml, and 113.90 ± 11.18 µg/ml, respectively.

From the percentage released plots, it was evident that the different forms of luteolin were released into solution at different rates. The glycosidic or conjugated luteolin was released into solution faster than the aglycone form *viz.* 47.39% *vs.* 31.35% released in the first 10 minutes, respectively. This observation was expected as it is known that glycosylation renders flavonoids more water soluble (Markham, 1982). The conjugated luteolin was released into solution rapidly and reached an asymptote in concentration within 40 minutes, i.e. approximately 100% of the glycosidic luteolin content of the leaves was released within 40 minutes. On the other hand, the free luteolin aglycone was released at a slower rate and only reached an asymptote in concentration at approximately 180 minutes. A possible explanation for this could be that the conversion of the

conjugated luteolin to the aglycone (free) form, occurred in the tea solution, such that the concentration of conjugated luteolin did not increase further (an equilibrium existed between the released and converted conjugated luteolin), as the conjugated luteolin in solution was converted to free luteolin resulting in a gradual increase in free luteolin and no increase in conjugated luteolin.

The total luteolin content present in the *A. afra* SDL (section 4.4.5) was found to be 2.065 ± 0.235 µg/mg of plant material and this was expected to translate (under the infusion experiment volume conditions) to a total luteolin concentration of 9.18 µg/ml after a 10-minute period of infusion. However, instead an average concentration of 52.24 µg/ml (i.e. approximately 5 fold higher) was actually obtained after 10 minutes infusion. This could be attributed to differences in the experimental conditions, as in the quantitation determinations (section 4.3.10), the leaves were stirred for only 2 minutes before being allowed to stand and seep for 8 minutes, while in the infusion experiments (section 5.3.2.2.1), the leaves were continuously rotated at 100 rpm for 10 minutes and beyond. Under the latter conditions it could be expected that a greater amount of luteolin would infuse from the leaves and, therefore, the value for the total luteolin released after 250 mins (i.e. at steady state conditions) found in the infusion study may be the more correct quantity of total luteolin present in the standardised *A. afra* leaves.

In summary, the results obtained suggested, firstly that luteolin was a suitable marker for the characterization of the infusion of the *A. afra* loose leaves. Secondly, that the different forms of luteolin were released into solution at different rates, with conjugated luteolin being released at a faster rate than the aglycone form. Thirdly, that the method used to determine the amount of luteolin present in the plant materials may not give a correct estimation of the true luteolin content present. Finally, the amount of luteolin released over time in the traditional infusions has for the first time been characterised and the results obtained could be used to calculate the actual amounts of luteolin subjects would ingest when using *A. afra* teas brewed for different periods of time. Such information would be critical, when conducting a clinical trial to assess the efficacy of this traditional preparation.

5.7.4 Infusion profile of the tea bags containing the *A. afra* leaves

The UV infusion profile of the *A. afra* SDL in tea bags is shown in Figure 5.7 below, while Figure 5.8 shows the infusion profiles and percentage of luteolin released. All the results shown were corrected to allow for evaporation and sampling losses.

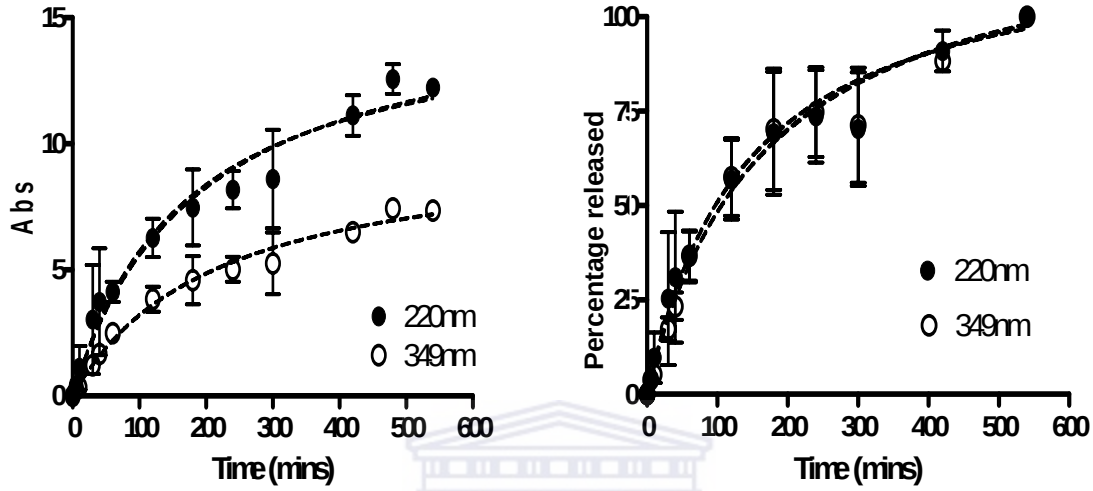


Figure 5.7: **The infusion profiles (on the left) and the percentage release profile (on the right) of the tea bags containing the *A. afra* leaves determined using UV spectroscopic analysis at 220 (-●-) and 349 (-○-) nm.**

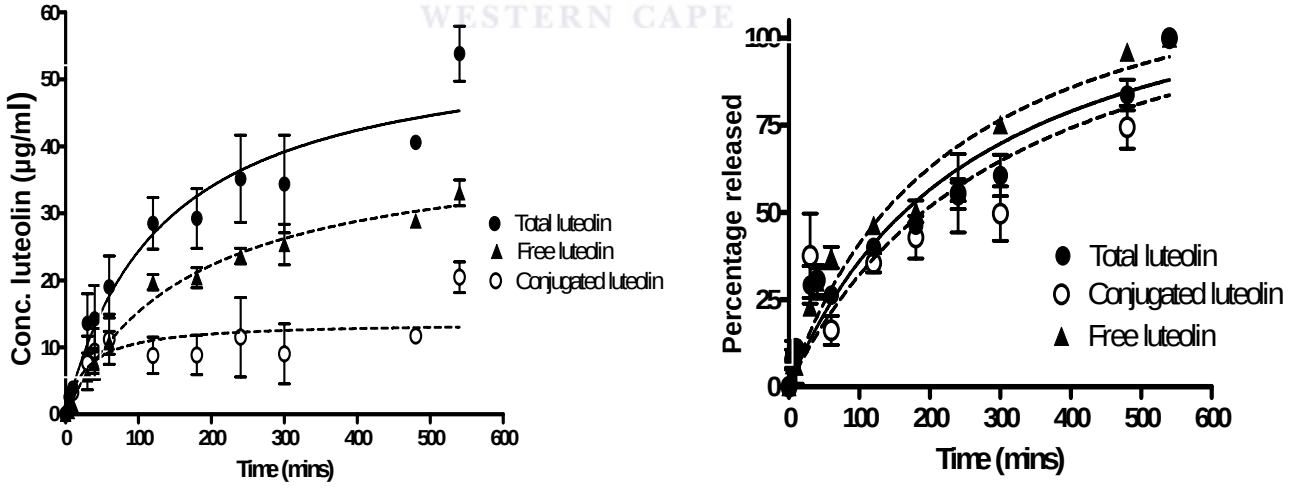


Figure 5.8: **The infusion profile of luteolin (total -●-, conjugated -○- and free -▲-) release (on the left) and the percentage release profile of luteolin (on the right) from the tea bags containing the *A. afra* leaves.**

As was the case for the loose leaves, the infusion profiles determined using the HPLC and UV methods were similar and the discussion below will again be based on the HPLC luteolin results only.

The infusion profiles for the *A. afra* leaves in the tea bag also demonstrated a characteristic pattern of release, i.e. an initial burst followed by a controlled release phase. However, in this case, the initial burst phase encompassed release up to approximately 35% of the total amount released at steady state and the swelling phase from 35% onwards. As a result of the short burst release phase and longer controlled release phase compared to that of the loose leaves, the rate of release in this case was expected to be slower. Using the same infusion time point as used for the loose leaves (of 10 minutes), an average of 1.66 ± 0.64 µg/ml of free luteolin and 2.71 ± 0.66 µg/ml, conjugated and 4.81 ± 0.55 µg/ml of total luteolin were present in the tea and at this time point, an average of only 8.88%, 10.90% and 10.08% of free, conjugated and total luteolin, respectively, were released. A comparison of these values with those obtained for the loose leaves at the same point, showed that the initial rate of infusion from the tea bags was approximately 3.63 times slower than that of the loose leaves. As the only difference between the two preparations was the tea bag membrane, the difference in the rate must have been due to this, i.e. the tea bag paper retarded the infusion of luteolin from the leaves by a factor of 3.63.

As was the case for the loose leaves, the glycosidic luteolin was released into solution faster than the aglycone form. Steady state conditions for luteolin release were reached after 540 minutes of brewing and this showed that a longer infusion time was required for the release of luteolin (and presumably the other phytochemical constituents of *A. afra* leaves) from the tea bags compared to that from the loose leaves. In fact, the infusion time to steady state was 3 times longer than that required for the loose leaves (i.e. 540 *vs.* 180 mins), clearly indicating the rate retarding effect of the tea bag paper.

Collectively, the results of the *A. afra* tea bag infusion study suggested that although the pattern of luteolin release was similar to that for the standardized leaves alone, the rate of

luteolin release was retarded (3 times slower) by the presence of the tea bag paper and it therefore might be worthwhile, in future studies, to investigate the effect of different types of tea bag papers on the rate of infusion of the plant leaves.

5.7.5 Infusion profile of the tea bags containing the freeze-dried aqueous extract of *A. afra*

Figure 5.9 shows the UV infusion profile of the FDAE powder of *A. afra* in tea bag dosage form and from the profile it can be observed that the powder was rapidly dissolving with an asymptote in concentration being reached within 10 minutes of infusion. Also, the rapid infusion was also quite evident, with 85% of the constituents being released within 5 minutes and 100 % released after 15 minutes of brewing.

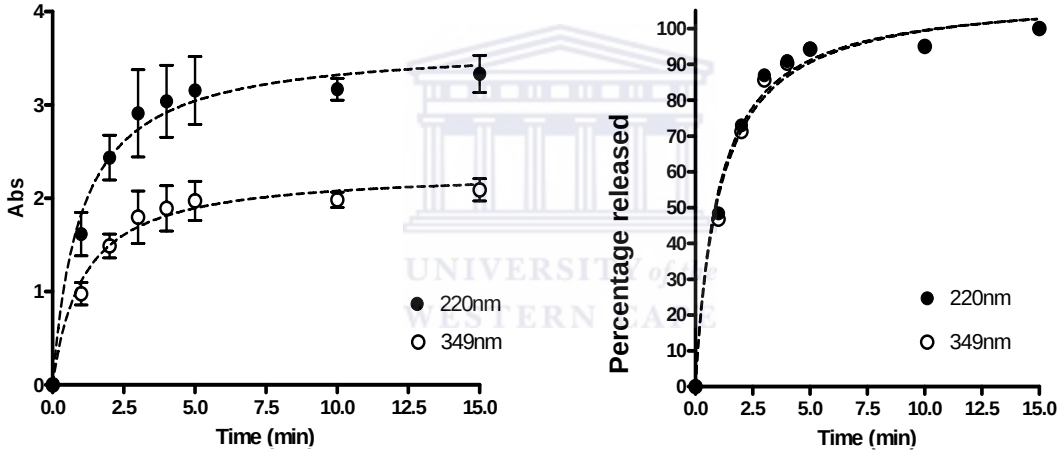


Figure 5.9: **The infusion profile (on the left) and percentage release profile (on the right) of the tea bags containing the *A. afra* freeze-dried aqueous extract determined using UV spectroscopy analysis at 220 (-●-) and at 349 (-○-) nm.**

At the infusion time point of 85% release, i.e. at 5 minutes, quantitation of the luteolin content using HPLC, found an average of 4.8510 ± 0.9048 µg/ml, 2.352 ± 0.4387 µg/ml and 2.499 ± 0.4661 µg/ml of total, free and conjugated luteolin, respectively, present in the tea solution. Based on the results of the determination of total luteolin content in the FDAE powder (section 4.4.7), a total luteolin content in solution after 100% release of 9.18 µg/ml was expected. Therefore, the result of the luteolin content present after 5 minutes showed that the tea bag containing the FDAE powder was capable of releasing 52.84% of the total luteolin content within a period of 5 minutes, thus demonstrating that

this tea bag was capable of releasing high amounts of luteolin within a much shorter period of time compared to the loose SDL and SDL in the tea bags. This finding was expected, as the FDAE was a finer particulate powder and highly soluble in water (as it was prepared using water). In addition, the fact that the powder was prepared from a 10-minute infusion of the dried leaves meant that the preparation contained the desired luteolin content (for a 10-minute brewing time) and this may be a distinct advantage of this preparation as in a clinical trial this tea bag could be allowed to infuse until 100% release is obtained, without one having to stop the infusion process as would be necessary with the tea bags or the loose leaves. Also, the rapid release of constituents within a short period of time compared to that found for the loose leaves and tea bags may make this preparation more suitable for use in clinical trials evaluating the loose leaves. Finally, the luteolin concentrations obtained at the 5-minute infusion time point served the purpose of setting a value against which the stability of the tea bags containing the extract powder could be evaluated.

In summary, the results showed that the contents of this dosage form were highly soluble and rapidly dissolving, with 85% of the constituents released within 15 minutes and as a result this dosage form could suitable for use in clinical trials evaluating the loose leaves.

5.7.6 Comparison of the infusion profiles of the *A. afra* loose leaves and tea bags containing the leaves and freeze-dried aqueous extract powder.

Table 5.3 gives a summary of the mean percentage release values obtained for the infusion of luteolin from the loose leaves and leaves in tea bag and in Table 5.4, a comparison of the infusion profiles based on the mean percentage release values is presented, i.e. the calculation of the f_1 (difference factor) and f_2 (similarity factor). In the calculation of the factors, the loose leaves were considered the reference product and the leaves in tea bag the test.

Table 5.3: Mean percentage release values (n = 6) of luteolin from the *A. afra* loose leaves and leaves in tea bag

Time (mins)	Infusion of the *A. afra* loose leaves (% ± S.D)			Infusion of *A. afra* leaves in tea bag (% ± S.D)		
	Free luteolin	Conjugated luteolin	Total luteolin	Free luteolin	Conjugated luteolin	Total luteolin
5	18.300±9.051	30.952±8.663	19.452±8.434	2.100±0.768	1.517± 1.070	1.774±0.861
10	31.346±10.574	47.388±8.331	34.610±8.871	8.881± 2.122	10.908±3.008	10.08±2.401
30	60.481± 7.395	73.904±5.960	60.061±6.754	15.150±4.643	11.230±6.844	12.500±5.357
60	78.145 ± 6.629	85.545±5.246	75.523±5.560	27.239±6.882	20.698±10.347	24.785±8.382
120	91.097 ± 8.078	92.650±6.059	86.343±6.176	46.249±7.949	36.588±13.510	40.844±9.819
180	96.375 ± 8.910	95.264±6.627	90.633±6.991	59.549±7.290	48.505±12.915	53.325±9.207
240	99.269 ± 9.372	96.636±6.916	92.958±7.882	68.887±6.685	57.285±11.931	62.281±8.434
480				91.367±9.895	80.113±17.092	84.667±12.417
540				94.635±11.166	83.639±19.719	88.014±14.033

Table 5.4: Comparison of the infusion profiles of luteolin from the *A. afra* leaves in tea bag (test) against that from the loose leaves (reference).

Form of luteolin	f_1 (%)	f_2 (%)
Free	65.51	18.41
Conjugated	78.20	9.48
Total	73.52	13.85

Dissolution or infusion f_1 values between 0 and 15 and f_2 values between 50 and 100 are regarded by the FDA as indicating similarity between profiles (FDA, 1997). From the values obtained in this study, i.e. f_1 more than 15 and f_2 less than 50, it was determined that the infusion profiles of the two preparations were not similar. This lack of similarity therefore meant that the tea bag dosage form did not have comparable release profiles to the loose leaves and therefore could not be used as a replacement for the loose leaves in clinical trials. However, although the infusion profiles were not similar, the tea bag dosage form could still be used in clinical trials if adjustments in the dose preparation and administration methods were done. For example, the final desired luteolin concentration in the tea could be achieved by allowing the tea bag to seep and draw 3 times longer than the loose leaves would be allowed, or alternatively, adjustments in the amount of tea

consumed could be done, e.g. the patient could consume 10 ml of the tea bag tea solution equivalent to consuming 1 ml of the loose leaves tea in order to achieve the desired doses. The above suggestions therefore meant that in this case, the infusion profiles needed not be similar in order for the tea bags to be used in place of the loose leaves in clinical trials.

By observation of the infusion profiles of the loose leaves and the FDAE powder in tea bag, it was determined that the profiles were also not similar based on the fact that the infusion of the extract tea bag was complete within 15 minutes, while that of the leaves required 240 minutes and moreover, due to the very different time points, it was not possible to calculate f_1 and f_2 values. However, the tea bag containing the FDAE powder, released a total luteolin content corresponding to 52.84% of the luteolin present in the tea bag within 15 minutes compared to the loose leaves and leaves in tea bag which only reached this level after approximately 30 minutes and 180 minutes of infusion, respectively. As a result of this rapid release, this dosage form could be a suitable replacement for the traditional dosage form for use in clinical trials even though the infusion profile is not similar to that of the loose leaves.

In summary, the results obtained showed that the infusion profiles for the tea bags containing the leaves and FDAE powder, were not similar to that of the loose leaves. However, they could still be used in clinical trials if adjustments in the preparation and administration methods were done.

5.7.7 Results of the stability studies conducted on the dosage forms

5.7.7.1 Organoleptic and chromatographic evaluation: Conditions at site A

The results obtained in the organoleptic evaluation of the tea bags subjected to the storage conditions at site A (room temperature and normal humidity) are presented in Appendix D1. The *A. afra* dried leaves in tea bag showed no changes in the appearance, odour, or leakage of the tea bag contents over the 6-month storage period. Figure 5.10 shows images of the tea bags at the beginning and at the end of the stability study at this site.

At the end of the stability study, the moisture content of the SDL in tea bag was 11.45 ± 1.02% from an initial average percentage of 10.13 ± 1.51% (see Table 5.5 below) and this showed that there were no significant increases in moisture in the leaves (p value 0.4145 at alpha < 0.05) and it could therefore be concluded that the SDL in tea bag do not absorb significant amounts of moisture under these test conditions

The HPLC chromatographic fingerprint analysis collected at 30-day periods, over the stability test period (see Appendix D2 for the overlaid chromatograms and UV spectra) also showed no changes occurring within the phytochemical constituents of the plant leaves as represented by the similar peaks. This lack of change and therefore similar identity of the plant materials was further validated by means of the UV spectra which were conducted at the peak with the maximum absorbance, which was at 220 nm and at an average retention time of 3.52 minutes. From this, it was observed that no changes in the UV spectral plots occurred indicating the similarity of the peaks throughout the stability period.

Tea bags of the FDAE powder stored under the conditions at site A, however, showed significant changes in the organoleptic properties after 7 days of storage, i.e. the extract became a dark brown, sticky paste, which leached out of the tea bag (see Figure 5.11), however, no changes in the odour of the material were observed. Changes were also observed in the chromatographic fingerprints, which showed that almost all the peaks initially present were no longer present after the 7-day period (see Appendix D2).

In summary, the results showed that the tea bags containing the SDL were stable under normal room temperature and humidity conditions, but that the tea bags of the FDAE were not stable under these conditions.

Table 5.5: Loss on drying of the *A. afra* dried leaves after 180 days stability testing at site A.

Sample No	Initial mass of leaves (g)	Final mass of leaves (g)	Loss on drying (g)	Percentage moisture content
1 2 3	0.5052 0.5021 0.5013	0.4466 0.4402 0.4494	0.0586 0.0619 0.0519	11.60 12.38 10.35
Ave	0.5029	0.4454	0.0575	**11.45**
S. D	0.0021	0.0047	0.0051	**1.023**

Figure 5.10: A tea bag of *A. afra* dried leaves at day 0 (on the left) and on the right, a tea bag of *A. afra* leaves at day 180, stored under conditions of normal room temperature and humidity.

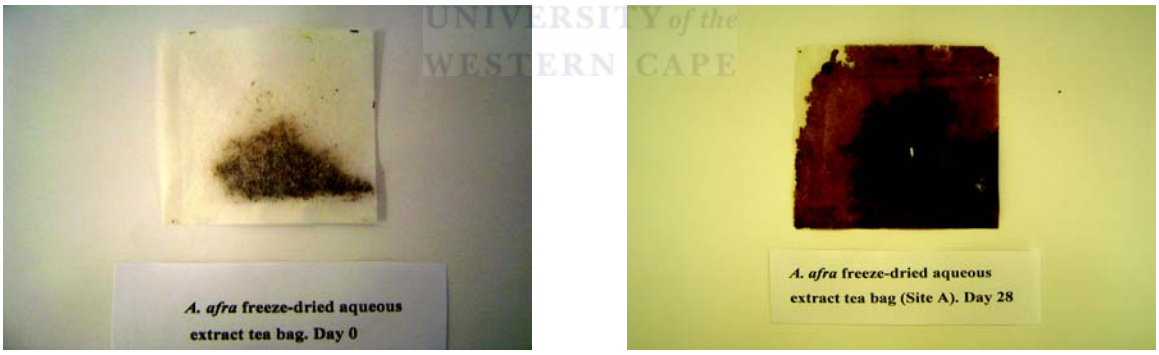


Figure 5.11: A tea bag containing the *A. afra* FDAE powder at day 0 (on the left) and on the right, a tea bag containing the FDAE powder at day 28, stored under conditions of normal room temperature and humidity.

5.7.7.2 Organoleptic and chromatographic evaluation: Conditions at site B

The tea bags of the dried leaves subjected to accelerated storage testing conditions of 40ºC /75% relative humidity for a period of 3 months, were found to show no noticeable changes in the physiochemical properties up to a period of 30 days. However, after 30

days, a green colouration on tea bag surface was noticed, and this colouration increased in intensity up to the end of the stability study. Figure 5.12 shows images of the tea bags at day zero and at the end of the stability study at this condition.

At the end of the storage period, the moisture content of the dried leaves from the stored tea bags had changed significantly from an initial loss on drying of 10.13 ± 1.51%, to 16.60 ± 1.01% after 90 days (p value 0.010 at alpha < 0.05) (see Table 5.6 below). The preparation thus absorbed significant amounts of moisture under the storage conditions and this may have been responsible for the green colouration observed on the tea bag paper as the high humidity wetted the leaves within the tea bag. Despite this change, the HPLC chromatographic fingerprint analysis and UV scan at 220 nm, showed no differences in the peaks, indicating that the tea bag leaf constituents did not change in composition over the test period.

Table 5.6: Loss on drying of the *A. afra* dried leaves after 90 days stability testing at site B.

Sample No	Initial mass of leaves (g)	Final mass of leaves (g)	Loss on drying (g)	Percentage moisture content
1 2 3	0.5019 0.4995 0.5028	0.4140 0.4157 0.4248	0.0879 0.0838 0.0780	17.51 16.78 15.51
Ave	0.5014	0.4182	0.0832	**16.60**
S. D	0.0017	0.0058	0.0050	**1.012**

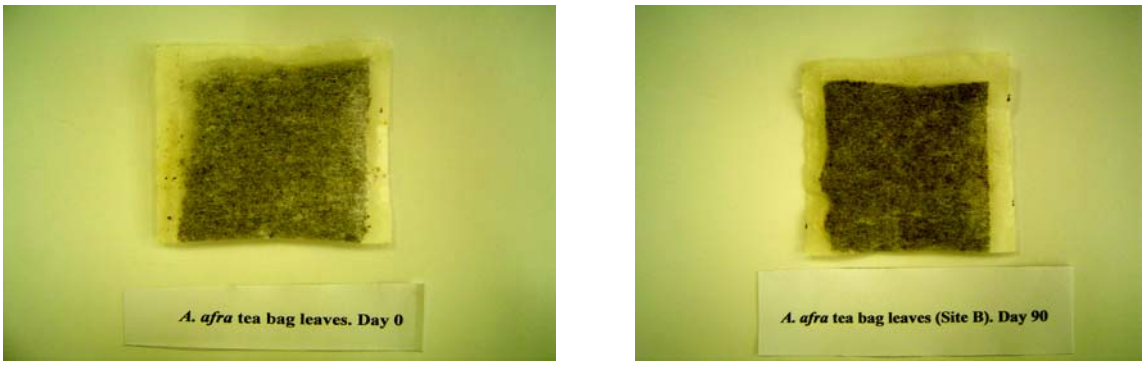


Figure 5.12: Shows, on the left, a tea bag containing the *A. afra* leaves at day 0 and on the right, a tea bag containing the *A. afra* leaves after 90 days storage at conditions of 40ºC /75% relative humidity. Note the green colouration on the tea bag surface after 90 days.

Nevertheless, the above results showed that the tea bags containing the SDL were not stable under the high temperature and humidity storage condition as shown by the greenish colouration on the tea bag surface.

5.7.7.3 Organoleptic and chromatographic evaluation: Conditions at site C and D

Tea bags containing the freeze-dried extract powder were also evaluated under conditions of room temperature or 40ºC and zero humidity, i.e. at site C and D, respectively. The results of the organoleptic evaluation of the tea bags stored under these conditions are presented in Appendix D1. No changes in organoleptic properties were observed from day 0 to day 28 at both storage conditions, however, after 28 days the powders were no longer free flowing and became hard solid masses within the tea bags, with those under the higher temperature condition harder than those at the lower temperature condition (as the former tended to crumble easily). Figure 5.13 shows images of the tea bags at the beginning and end of the storage under the two storage conditions.

The chromatographic fingerprint analysis of samples of the extract powder from the tea bags under the lower temperature condition, showed no changes over the entire test period (see Appendix D2). However, the fingerprint analysis of the samples from the tea bags at the higher temperature condition, showed a change after 60 days, i.e. peaks, which had previously been observed at 8 minutes and 23 minutes, were no longer present (see Appendix D2).

Figure 5.13: **A tea bag containing the *A. afra* FDAE at day 0 (on the left), in the middle, a tea bag containing the FDAE at day 90 stored under conditions at site C and on the right, a tea bag containing the FDAE powder after 90 days storage at the conditions at site D.**

From the results, it was clear that the temperature affected the stability of the FDAE powder in tea bag and from the data obtained the most suitable storage conditions for the tea bags containing the FDAE powder would appear to be at zero humidity and normal room temperature.

5.7.7.4 Infusion profile characteristics of the dried leaves tea bags stored under the conditions at site A and B.

Determinations of the percentage of plant material infused at a single time point for the preparations subjected to the real time and accelerated stability testing was conducted and the results obtained are presented below. Table 5.7 shows the percentage of the phytochemical constituents released after 180 minutes of infusion (determined from the UV spectroscopic analysis) of the tea bags containing the *A. afra* SDL stored at the storage sites A and B.

Table: 5.7 Percentage released by the tea bags of the *A. afra* dried leaves at 180 minutes from tea bags stored at storage sites A and B.

Storage condition site	Percentage released by the *A. afra* dried leaves tea bag at 180 minutes ± S. D		
	Day 0	Day 90	Day 180
A	53.7929 ± 5.5370%	53.8849 ± 5.3210%	57.6955± 6.520%
B	53.7929 ± 5.5370%	23.3427 ± 3.6160%	

From the comparison of the infusion of the tea bags under the conditions of normal room temperature and humidity, no significant differences in the percentage of phytochemical constituents infused at the 180 minute time point, for the duration of the stability study, was found (p value 0.9733 at alpha < 0.05). This suggested that the preparation remained stable and within specification under this storage condition.

However, there was a significant difference in the mean percentage material infused from the tea bags containing the SDL after 90 days of storage under the accelerated storage conditions (p value 0.0001 at alpha < 0.05). Initially, a mean value of 53.80% was

released within 180 minutes and after 90 days the mean value at this time point was found to be 23.34%, which represented a reduction of approximately 50%. This reduction in the rate and extent of the infusion suggested that this condition of high temperature and humidity, adversely affected the stability of the preparation over the time period evaluated. Taken together with the changes in organoleptic properties observed, i.e. that the leaves became wet (increased moisture content) and stuck together, it could be possible that the storage conditions created a more tortuous passage for the leaf constituents to infuse through (through sticking together of the leaves), as well as made it difficult for the media to wet the leaves and this could explain the observed reduction in the rate of infusion.

Collectively, the results obtained from the stability evaluations (i.e. the infusion profile analysis, chromatographic fingerprint analysis, loss on drying and organoleptic tests), suggested that only normal room temperature and humidity were suitable storage conditions for the tea bags containing the dried leaves of *A. afra*.

5.7.7.5 Infusion profile characteristics of the freeze-dried aqueous extract powder in tea bag stored under conditions at site C and D.

Determinations of the amount of luteolin infused after 5 minutes from the tea bags stored under conditions of zero humidity and normal room temperature or 40ºC for 3 months, i.e. at site C and D, respectively are presented in Table 5.8.

Table: 5.8 Luteolin content (µg/ml) infused after 5 minutes infusion of the tea bags containing the freeze-dried aqueous extract powder stored under conditions at site C and D.

Form of luteolin	Luteolin content (µg/ml) infused after 5 minutes from the tea bags stored under conditions at site C		Luteolin content (µg/ml) infused after 5 minutes from the tea bags stored under conditions at site D	
	Day 0	Day 90	Day 0	Day 90
Free	2.3520 ± 0.4387	2.8690 ± 0.2640	2.3520±0.4387	1.0250 ± 0.0567
Conjugated	2.4990 ± 0.4661	0.0225 ± 0.0061	2.4990±0.4661	0.0827 ± 0.1113
Total	4.8510 ± 0.9048	2.8915 ± 0.9048	4.8510±0.9048	1.1040 ± 0.0603

For the tea bags containing the FDAE powder subjected to storage under conditions of zero humidity and room temperature (site C), resulted in a significantly less (p value 0.0065 at alpha < 0.05) amount of luteolin released after 5 minutes of infusion. Initially, 4.8510 µg/ml of total luteolin was released and after 90 days storage, 2.8915 µg/ml was released representing an almost 50 % reduction. This difference may be attributed to the organoleptic observation that the FDAE became hard and crusty mass and this may have reduced the rate of dissolution by making it more difficult for the extract to dissolve in the solution. Furthermore, there was a significant decrease in the conjugated luteolin content observed (p value 0.0001 at alpha < 0.05) from the preparations stored at this site and from this it may be concluded that the FDAE in tea bags are not stable when stored under these conditions as a result of the decrease in the conjugated luteolin and the reduction in the rate of infusion.

The infusion of the tea bag preparations at under zero humidity and 40˚C (site D) also showed a significant decrease in the amount of luteolin infused after 5 minutes (p value 0.0006 at alpha < 0.05) and a reduction in the rate of infusion of approximately 4 times (4.8510 *vs.* 1.1040 µg/ml released after 5 minutes). Organoleptic evaluations at this site (section 5.7.7.3) revealed that the extract powder solidified and this could have been responsible for the observed reduction in the luteolin infused. Changes in conjugated luteolin content were also observed and the changes were similar to those that occurred

under stability site C and therefore it could be concluded that the FDAE powder in tea bag undergoes a reduction in the conjugated luteolin content when stored.

Collectively, the results obtained from the stability evaluations (i.e. the infusion profile analysis, chromatographic fingerprint analysis, loss on drying and organoleptic tests), on the FDAE powder in tea bag, suggested that the preparations were not stable under conditions of normal room temperature, 40ºC and zero humidity as a reduction in the rate of infusion and in the conjugated luteolin and as a result, these tea bags cannot be used in clinical trials as a replacement for the traditional loose leaves preparation.

5.7.8 Comparison of tea bags of the *A. afra* plant material and their placebos

The organoleptic properties of the placebos for the *A. afra* SDL and FDAE powder in tea bag were compared against the plant material tea bags and Tables 5.9 and 5.10 give a summary of the characteristics of the tea bags and placebos prepared.

The addition of the aroma chemical and sweetener to the placebo and *A. afra* plant materials resulted in preparations with similar aroma and taste. Also the colour of the teas produced by both the placebos and plant preparations were found to be similar (see Figure 5.15c). From an observation of the images (Figure 5.14 and 5.15a and b) it can be observed that there were no apparent differences between the tea bag containing the plant leaves and that containing the placebo for the leaves. On close inspection of the tea bags containing the placebo and the FDAE powder, it could be observed that the placebo preparation was slightly more reddish in colour compared to the tea bag containing the FDAE powder. However, the teas resulting from the powder preparations were of similar intensity, (crucial in the clinical trials where teas will be prepared) indicating that the FDAE placebo could still function as a credible placebo.

In summary, the placebo preparations were judged to match the *A. afra* plant materials (spiked with inert aroma and sweetener chemicals) and as such, the placebo preparations could be used in clinical trials evaluating the plant materials.

Table 5.9: Physicochemical comparison of tea bags of the *A.afra* leaves and placebo for the leaves

Parameter	*A.afra* leaves in tea bag	Placebo for *A.afra* leaves in tea bag
Description	7x7cm square grey tea bag containing material with rough texture.	7x7cm square grey tea bag containing material with rough texture.
Taste/flavour	Sweet, citrus	Sweet, citrus
Odour	Citrus, herbal woody, rosewood.	Citrus, herbal woody, rosewood.
Hue produced by tea	Reddish brown clear hue	Reddish brown clear hue

Table 5.10: Physicochemical comparison of tea bags containing the *A.afra* freeze-dried aqueous extract and extract placebo.

Parameter	*A. afra* FDAE in tea bag	Placebo of the *A. afra* FDAE in tea bag
Description	7x7cm square grey tea bag containing a brittle, free flowing, brown, particulate material.	7x7cm square grey tea bag containing a brittle, free flowing, reddish, particulate material.
Taste/flavour	Sweet, citrus	Sweet, citrus
Odour	Citrus, herbal woody, rosewood.	Citrus, herbal woody, rosewood.
Hue of tea produced	Reddish brown clear hue	Reddish brown clear hue

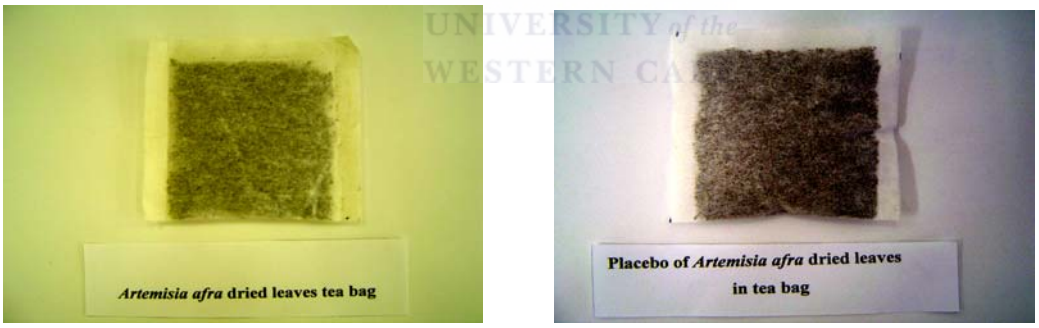


Figure 5.14: Shows the *A. afra* standardized dried leaves in tea bag on the left and the *A. afra* dried leaves placebo in tea bag on the right.

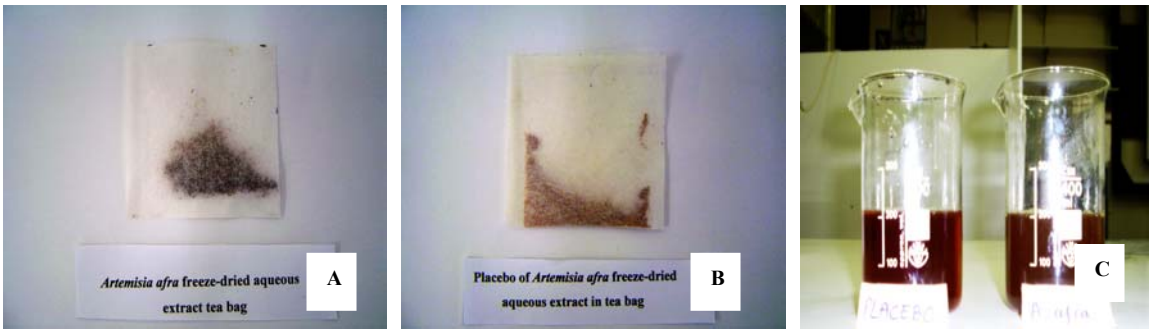


Figure 5.15: **(A) Shows the FDAE of *A. afra* in tea bag and (B) shows the placebo of the FDAE of *A. afra* in tea bag. (C) Shows the colours of the teas produced by *A. afra* placebo (on the left) and *A. afra* freeze-dried extract powder (on the right).**

5.7.9 Results of test for absence of activity of the placebo for the *A. afra* dried leaves.

The results of the pharmacological evaluation of the *A. afra* placebo and plant leaves are shown in tabular and graphic form in Table 5.11 and Figure 5.16, respectively.

Table 5.11: Percentage relaxation produced by cumulative concentrations of the *A. afra* standardized dried leaves and placebo of the leaves on the methacholine-induced contractions of the isolated guinea pig tracheal muscle.

	Conc. (mg/ml)	Cumulative conc. in bath (mg/ml)	Average % Relaxation ± SD	EC50	Max. relaxation ± SD
PLANT	5 10 20 50 100	0.0333 0.7000 0.8333 1.1666 1.8333	0 0 3.3710 ± 0.8720 3.7780 ± 0.3449 49.7760 ± 2.5100	236.18	49.776 ± 2.5100

	Conc. (mg/ml)	Cumulative conc. in bath (mg/ml)	Average % Relaxation ± SD	EC50	Max. relaxation ± SD
PLACEBO	5 10 20 50 100	0.0333 0.7000 0.8333 1.1666 1.8333	0 0 0.660 ± 1.143 1.9610 ± 1.698 3.9550 ± 0.9622	-	3.955 ± 0.9622

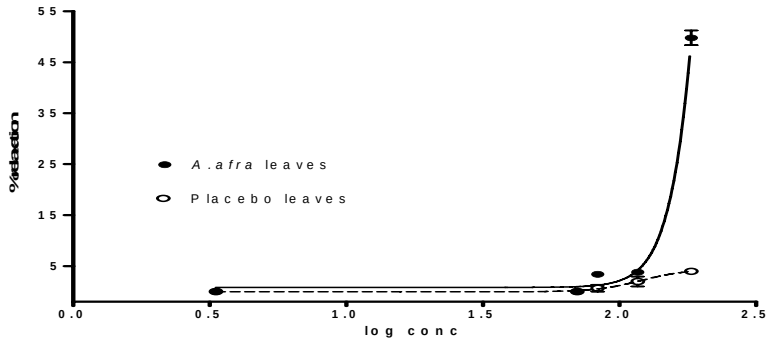


Figure 5.16: Percentage relaxation versus log concentration of the *A.afra* standardized dried leaves (-●-) and placebo (-○-) effect on the isolated guinea pig tracheal muscle.

Cumulative concentration of the *A. afra* plant material (up to 185 mg/ml or 1.833 mg/ml in the organ bath) injected onto the guinea pig trachea produced a maximal relaxation of 49.776 ± 2.51%, relative to the isoprenaline maximal relaxation, while the same cumulative doses of the placebo material produced a maximum relaxation of only 3.955 ± 0.962%. Generally, the traditional teas would be prepared as 4 g of leaves in 200 ml of hot water, which is equivalent to a 20 mg/ml solution and this concentration corresponded to 0.133 mg/ml in the organ bath. At this concentration the *A. afra* leaves were found to produce an average percentage relaxation of 0.804%, while the placebo had an average percentage relaxation of only 0.068%. Furthermore, depending on the volume of the tea the patient ingests, increased concentrations of the tea solution may be absorbed. For example, if a patient consumed 15 ml or a tablespoon of the tea, this would correspond to a concentration of approximately 1.833 mg/ml in the organ bath (assuming 100% absorption), which would result in a percentage relaxation of 46.206% and 3.921% from the plant material and placebo, respectively. The above results therefore showed that the plant material was active at the concentrations used traditionally, while the placebo was only slightly active at the same concentrations.

With the experimental design, it was difficult to conclude whether the slight relaxation seen with the placebo was due to the natural relaxation of the tissue or due to the actual effect of the placebo. Also, it was not possible to determine whether this slight effect would have any observable therapeutic effect in humans and therefore further studies of the effect of this preparation in humans are required. The relaxation seen from the *A. afra*

dried leaves was however apparent and validated the traditional use of *A. afra* leaves in the treatment of asthma and other bronchial conditions requiring bronchodilation.

5.8 Conclusions

Collectively, as for the results obtained in the preparation and evaluation of tea bags and placebos of *Artemisia afra*, the following conclusions could be drawn;

- The manufactured tea bags containing 4 g and 0.55 g of *A. afra* SDL and FDAE powder, respectively, were equivalent to the traditional dose and met the European Pharmacopoeia 2002 quality requirements.
- Luteolin is a suitable marker compound to use to characterise the infusion of the *A. afra* dosage forms.
- The method used to quantitate the luteolin content of the *A. afra* leaves does not give a correct estimation of the true luteolin content present in the material.
- The rate of infusion of luteolin from the manufactured tea bags is three times slower than that from the loose leaves.
- The tea bags containing the SDL and those containing the FDAE powder do not have comparable release profiles of luteolin to the loose leaves. However, they could still be used in clinical trials if adjustments in the tea preparation and administration methods are done.
- Tea bags containing the SDL are stable under conditions of normal room temperature and normal room humidity. However, tea bags containing the FDAE powder are not stable and as such this dosage form is not suitable for the FDAE powder.
- It is possible to design credible placebos for the *A. afra* SDL and FDAE powders. However, the placebo for the SDL possesses slight muscle relaxant activity.

In summary, the results suggested, firstly, that the tea bag was a suitable dosage form for the *A. afra* standardized dried leaves, but not for the freeze-dried aqueous extract powder and secondly, that the tea bags containing the standardized dried leaves do not have

comparable release profiles to the loose leaves traditional preparation and finally, that it is possible to prepare credible placebos for the plant material, which possess little pharmacological activity compared to the plant material.

Chapter 6

Conclusion

The primary objectives of the study were; to prepare standardized *A. afra* plant leaves and freeze-dried aqueous extract powder, to prepare these materials in a tea bag dosage form and compare the infusion profiles of the tea bag and the traditional loose leaves dosage forms and to design credible placebos for the *A. afra* leaves and freeze-dried aqueous extract powder in tea bags, suitable for use in clinical trials.

It was hypothesised that; the variation in the luteolin levels in standardized plant leaves and freeze-dried aqueous extract powder would be lower than that in the traditionally used non-standardized leaves, that a tea bag would be a suitable dosage form replacement for the traditional plant leaves and would meet pharmacopoieal quality requirements, that the infusion profiles of luteolin from the tea bag dosage form and traditional loose leaves will be similar and, finally, that credible placebos for the *A. afra* plant materials, devoid of pharmacological activity could be designed and prepared.

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

- Standardization of the *A. afra* leaves reduces the intra-batch variation in luteolin content present and this variation can be further reduced by preparation of a freeze-dried aqueous extract powder of the plant material.
- The tea bag is a suitable dosage form for standardized dried *A. afra* leaves, but not for the freeze-dried aqueous extract powder of the plant.
- The infusion profile of luteolin from tea bags containing standardized dried *A. afra* leaves is not similar to that of the loose leaves, however, the tea bags can still be used in clinical trials provided appropriate adjustments in the dose preparation and administration methods are made.
- Credible placebos for *A. afra* plant material tea bags which appear, smell and taste similar to the plant materials and possess little pharmacological activity and thus suitable for use in clinical trials were made.

In summary, *A. afra* plant materials with reduced intra-batch variation in luteolin content were prepared and manufactured in a tea bag dosage form, of which only the standardized dried leaves were stable upon storage. The release profiles of luteolin from the tea bag were not similar to that from the loose leaves, however, the tea bags could still be used in clinical trials if adjustments in the dose preparation and administration of the tea bag tea are made. Finally, credible placebos of the plant dosage form were prepared and can be used in clinical trials.

From the results, several areas for further research were noted. Firstly, there is a need to further evaluate the effect of the geographical source and period of harvest of the plant materials and different storage conditions, on the intra- and inter-batch luteolin content. Secondly, the effect of various leaf sizes, tea bag sizes and types of tea bag paper on the rate of infusion and infusion profiles of the plant tea bag dosage form requires further investigation. Finally, characterization of the phytochemical constituents still present in the placebo materials and further evidence of their pharmacological inertness is required. Notwithstanding the aforementioned further studies, the tea bags of the *A afra* standardized dried leaves and placebo materials can still be used in clinical trials with human subjects.

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APPENDICES

APPENDIX A1

Artemisia afra collected from the Montagu district of the Western Cape, was analysed to determine the proportion of the plant that constituted the leaves, flowers and stems/branches.

Table showing results of the quantitative determination of percentage of leaves, flowers and stems which constitute *Artemisia afra* plant.

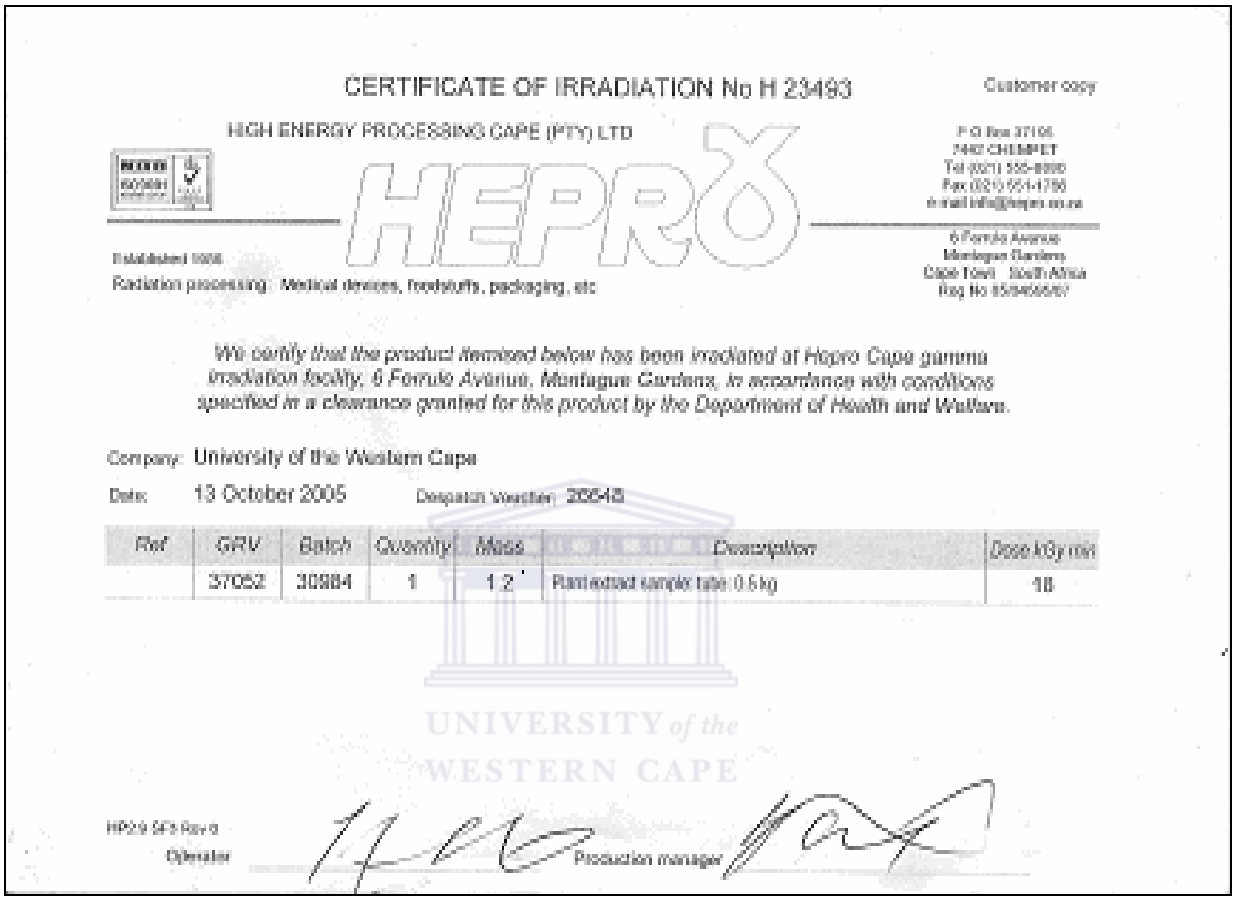
	Batch 001 (%)	Batch 002 (%)	Batch 003 (%)	% Average ± S.D
Leaves	43.47	48.54	41.57	44.53 ± 3.60
Flowers	30.14	29.58	32.16	30.63 ± 1.36
Stems/branches	23.90	25.67	24.29	24.62 ± 0.93

Table showing the average loss on drying of the *A. afra* leaves after drying in an oven for 3 days at 30˚C.

	Batch 001	Batch 002	Batch 003
Initial mass (g)	352.51	2787.11	6128.20
Final mass (g)	202.20	1305.35	2613.00
Loss on drying (g)	150.31	1481.76	3515.20
% Loss on drying	42.64	53.16	57.36
% Average ± S.D	51.05 ± 7.58		

APPENDIX A2

Figure showing the certificate of irradiation for the *Artemisia afra* dried leaves and freeze-dried aqueous extract powder.



CERTIFICATE OF IRRADIATION No H 23493

Customer copy

HIGH ENERGY PROCESSING CAPE (PTY) LTD

HEPRO

P O Box 37166
7462 CHEMPET
Tel (021) 555-6000
Fax (021) 551-1755
e-mail info@hepro.co.za

6 Ferula Avenue
Montague Gardens
Cape Town South Africa
Reg No 85/04655/07

Established 1998

Radiation processing: Medical devices, foodstuffs, packaging, etc

We certify that the product itemised below has been irradiated at Hepro Cape gamma irradiation facility, 6 Ferula Avenue, Montague Gardens, in accordance with conditions specified in a clearance granted for this product by the Department of Health and Welfare.

Company: University of the Western Cape

Date: 13 October 2005 Despatch Voucher: 25548

Ref	GRV	Batch	Quantity	Mass	Description	Dose kGy min
	37052	30984	1	1.2	Plant extract sample tote 0.6 kg	18

HP2.9.04.5 Rev 0

Operator

Production manager

APPENDIX A3

Table of the extractions conducted and resultant yields obtained in the preparation of the freeze-dried aqueous extracts of *Artemisia afra.*

Sample No	Volume of aqueous extract freeze dried (ml)	Mass of freeze dried extract obtained (g)	Yield of freeze-dried aqueous extract (%)
1	300	2.20	21.28
2	350	2.76	22.87
3	380	2.70	20.61
4	350	2.30	19.10
5	200	1.60	23.05
6	450	3.20	20.62
7	380	2.70	24.87
8	300	2.60	25.13
9	300	2.30	21.52
10	300	2.25	20.58
Average ± SD			**21.96 ± 1.97**

APPENDIX A4

Particle size classification of the *A. afra* plant materials

Table of the results of the particle size classification of the *A. afra* dried leaves.

Sieve Number	Av. Weight passing through (g)	Av. % w/w passing through	Particle size classification (BP 2000)
1400	9.80	96.41	
355	2.00	19.71	
180	0.37	3.64	**Coarse powder**
125	0.23	2.32	
90	0.17	1.66	

Table of the results of the particle size classification of the *A. afra* freeze-dried aqueous extract.

Sieve Number	Av. Weight passing through (g)	Av. % w/w passing through	Particle size classification (BP 2000)
1400	9.63	95.73	
355	5.13	50.99	
180	2.83	28.14	**Coarse powder**
125	1.93	19.20	
90	0.93	9.28	

APPENDIX A5

Method and results obtained for the determination of bitterness value of *Artemisia* afra dried leaves and freeze-dried extract powder (EP 2002a).

1. Determination on *A. afra* standardized dried leaves stock solution 0.01 g/ml

- Correction factor using Quinine Hydrochloride 0.01 mg/ml (Bitterness value 200 000)

	Q1(0.042mg)	Q2 (0.044mg)	Q3 (0.046mg)	k (n/5.00)
Taster 1	B	B		0.72
Taster 2	B	B		0.72
Taster 3	B	B		0.72
Taster 4	B	B		0.72
Taster 5	B	B		0.72

- The threshold bitter concentration for tasting quinine is at 0.042mg. The number of ml of stock solution in this concentration was 3.6. Therefore the correction factor k, (which is calculated as this number of ml divided by 5 was 0.72 for all tasters)

	C4 (DF 100 000)	C3A (DF 50 000)	C3 (DF 10 000)
Taster 1	B		
Taster 2	B		
Taster 3	B		
Taster 4	B		
Taster 5	B		

- The solution with the threshold bitter concentration for the tasters is solution is C4, with dilution factor 100 000 (Y).
- Upon tasting 1.2 ml (X) of C4 + 8.8 ml of water, the solution was found to be bitter by all tasters.

- Bitterness value for tasters;
  (Y x k) / (X x 0.1)

  $$\frac{= 100\,000 \times 0.72}{1.2 \times 0.1} = \mathbf{600\,000.}$$

Where Y is the dilution factor for the threshold bitter concentration.
X is the volume of bitter concentration judged to be bitter after addition of water.
k is the correction factor for each taster.

2. Determination of *A.afra* freeze-dried extract stock solution 0.01g/ml

	C4 (DF 100 000)	C3A (DF 50 000)	C3 (DF 10 000)
Taster 1	B		
Taster 2	B		
Taster 3	B		
Taster 4	B		
Taster 5	B		

The threshold bitter concentration is C4.

- After tasting 1.2 ml of C4 + 8.8 ml of water, the solution was found to be bitter by all tasters.

- Bitterness value for all tasters = (Y x k) / (X x 0.1)
  = 600 000

APPENDIX A6

Results of the ash value determinations on the *A. afra* plant materials

Table showing the data and results of the total ash and acid-insoluble ash value determinations for the *Artemisia afra* dried leaves.

Sample No	Mass of the dried leaves powder (g)	Mass of carbon free ash (g)	Percentage of Total ash	Mass of Acid-insoluble ash (g)	Percentage of Acid-insoluble ash
1	2.0006	0.1986	9.9270	0.0145	0.7248
2	2.0017	0.1991	9.9465	0.0192	0.9592
3	2.0003	0.1994	9.9551	0.0194	0.9685
4	2.0009	0.2014	10.065	0.0213	1.0645
5	2.0021	0.1986	9.9196	0.0156	0.7792
6	2.0022	0.2020	10.088	0.0221	1.1038
7	2.0001	0.1983	9.9145	0.0178	0.8899
8	2.0036	0.2009	10.0270	0.0191	0.9533
9	2.0006	0.1998	9.9870	0.0183	0.9147
10	2.0001	0.2000	9.9995	0.0190	0.9499
Ave	2.001	0.1998	**9.9831**	0.0186	**0.9308**
SD	0.0115	0.0127	**0.0617**	0.0230	**0.0012**

Table showing the data and results of the total ash and water-soluble ash value determinations for the *Artemisia afra* dried leaves.

Sample No	Mass of the dried leaves powder (g)	Mass of carbon free ash (g)	Percentage of Total ash	Mass of Water-soluble ash (g)	Percentage of Water-soluble ash
1	2.0005	0.2013	10.039	0.0874	4.3689
2	2.0050	0.2023	10.089	0.0876	4.3691
3	2.0035	0.1977	9.8677	0.0941	4.6968
4	2.0094	0.2015	10.027	0.0901	4.4839
5	2.0015	0.2018	10.082	0.0887	4.4317
6	2.0027	0.1988	9.9266	0.0850	4.2443
7	2.0025	0.1969	9.8327	0.0866	4.3246
8	2.0001	0.1987	9.9345	0.0888	4.4338
9	2.0028	0.1986	9.9161	0.0876	4.3739
10	2.0045	0.1996	9.9576	0.0867	4.3253
Ave	2.003	0.1997	**9.9675**	0.0886	**4.4052**
SD	0.0267	0.0188	**0.0886**	0.0248	**0.0012**

Table showing the data and results of the total ash and acid-insoluble ash value determinations on the *Artemisia afra* freeze-dried extract powder.

Sample No	Mass of aqueous extract (g)	Mass of carbon free ash (g)	Percentage of Total ash	Mass of Acid-insoluble ash (g)	Percentage of Acid-insoluble ash
1	2.0044	0.4350	21.7023	0.0002	0.00998
2	2.0013	0.4357	21.7708	0.0014	0.06995
3	2.0016	0.4406	22.0124	0.0037	0.1849
4	2.0008	0.4469	22.3361	0.0034	0.1699
5	2.0006	0.4366	21.8235	0.0007	0.0349
6	2.0011	0.4461	22.2927	-	-
7	2.0012	0.4514	22.5565	-	-
8	2.0014	0.4231	21.4020	-	-
9	2.0001	0.4198	20.9889	-	-
10	2.0009	0.42571	21.2759	-	-
Ave	2.001	0.4361	**21.820**	0.0019	**0.0094**
SD	0.0116	0.0106	**0.0886**	0.0016	**0.0793**

Table showing the data and results of the total ash and water-soluble ash value determinations on the *Artemisia afra* freeze dried extract powder.

Sample No	Mass of aqueous extract (g)	Mass of carbon free ash (g)	Percentage of Total ash	Mass of Water-soluble ash (g)	Percentage of Water-soluble ash
1	2.0044	0.4350	21.7023	-	-
2	2.0013	0.4357	21.7708	-	-
3	2.0016	0.4406	22.0124	-	-
4	2.0008	0.4469	22.3361	-	-
5	2.0006	0.4366	21.8235	-	-
6	2.0011	0.4461	22.2927	0.108	16.8957
7	2.0012	0.4514	22.5565	0.0987	17.6244
8	2.0014	0.4231	21.4020	0.0970	16.2936
9	2.0001	0.4198	20.98895	0.0947	16.2542
10	2.0009	0.42571	21.2759	0.1074	15.9083
Ave	2.001	0.4361	**21.820**	0.1012	**16.5952**
SD	0.01159	0.01062	**0.08862**	0.0061	**0.6760**

APPENDIX A7

The microbiological testing methods used and the results obtained in the determination of the microbial contamination on the *A. afra* standardized dried leaves and freeze-dried aqueous extract powders before and after irradiation of the plant materials.

Mr Admire Dube
University of the Western Cape
Department of Pharmacology
School of Pharmacy
Private Bag X17
Bellville
7535

19 October 2005

MICROBIOLOGICAL TESTING METHODS

The method numbers reflected on the Micro Report are our inhouse method numbers. These methods have been validated inhouse and approved by the SANAS (South African National Accreditation Systems). Herewith a list of our inhouse methods, the relevant standard reference methods if possible and a brief description.

SWJM 35 : Enumeration of Total Microbial Activity (TMA) *same as Total viable Plate Counts*
This method is based on the ISO method 4833. Plate count agar is used with incubation at 30°C for 48 ± 4 hours.

SWJM 49 : Enumeration of *Enterobacteriaceae*
The ISO method no. 5552 is followed. Violet Red Bile Glucose agar (VRBGA) is used with incubation at 37 °C for 24 ± 2 hours.

SWJM 50 : Enumeration of Yeasts & Moulds
This method is based on the ISO method 7954. Potato Dextrose agar (PDA) is used with incubation at 25 °C for 5 days.

SWJM 45 : Enumeration of *Escherichia coli*
Samples are plated out onto Violet Red Bile Lactose agar with MUG supplement (VRB – MUG) and incubated at 37 °C for 24 ± 2 hours. Plates are viewed under UV light to facilitate bacterial counts. Samples are also inoculated into tubes of E.coli broth. Broth cultures of samples are incubated at 44 ± 0.5 °C for 24 ± 2 hours. Tubes are then examined for Gas and Indole production.

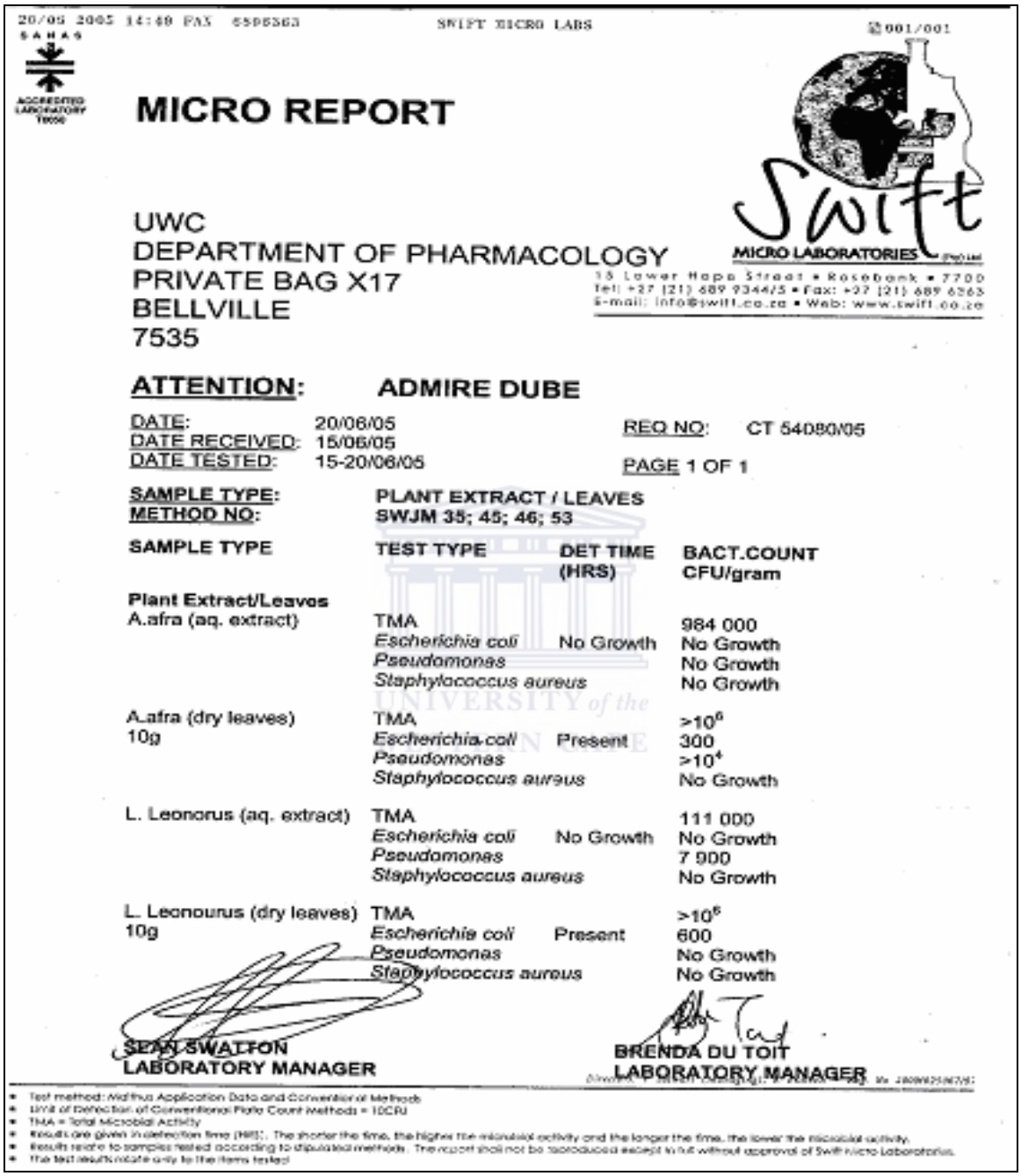
SWJM 42 : Detection of *Salmonellae*
This is an AOAC approved method, utilizing Malthus technology. Samples are pre- enriched in a broth at 37°C for 18 – 24 hours. From here tubes of Salmonella selective broth are inoculated and incubated at 37 °C for a maximum period of 30 hours. Presumptive positive cultures are confirmed by means of agglutination.

Feel free to contact me should you require any further information in this regard.

Kind regards

Deidrè Williams
Quality Co-ordinator
Swift Micro Laboratories

Figure showing the results of microbial contamination tests on the *A. afra* standardized dried leaves and freeze-dried aqueous extract powders before irradiation of the plant materials.



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SAHAS ACCREDITED LABORATORY T0056

MICRO REPORT

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UWC
DEPARTMENT OF PHARMACOLOGY
PRIVATE BAG X17
BELLVILLE
7535

ATTENTION: ADMIRE DUBE

DATE: 20/06/05
DATE RECEIVED: 15/06/05
DATE TESTED: 15-20/06/05

REQ NO: CT 54080/05

PAGE 1 OF 1

SAMPLE TYPE: PLANT EXTRACT / LEAVES
METHOD NO: SWJM 35; 45; 46; 53

SAMPLE TYPE	TEST TYPE	DET TIME (HRS)	BACT.COUNT CFU/gram
Plant Extract/Leaves			
A.afra (aq. extract)	TMA		984 000
	Escherichia coli	No Growth	No Growth
	Pseudomonas		No Growth
	Staphylococcus aureus		No Growth
A.afra (dry leaves) 10g	TMA		$>10^6$
	Escherichia coli	Present	300
	Pseudomonas		$>10^4$
	Staphylococcus aureus		No Growth
L. Leonorus (aq. extract)	TMA		111 000
	Escherichia coli	No Growth	No Growth
	Pseudomonas		7 900
	Staphylococcus aureus		No Growth
L. Leonourus (dry leaves) 10g	TMA		$>10^6$
	Escherichia coli	Present	600
	Pseudomonas		No Growth
	Staphylococcus aureus		No Growth

SEAN SWATTON
LABORATORY MANAGER

BRENDA DU TOIT
LABORATORY MANAGER

- Test method: Malthus Application Data and Conversion of Methods
- Limit of Detection of Conventional Plate Count Methods = 10CFU
- TMA = Total Microbial Activity
- Results are given in detection time (HRS). The shorter the time, the higher the microbial activity and the longer the time, the lower the microbial activity.
- Results relate to samples tested according to stipulated methods. This report shall not be reproduced except in full without approval of Swift Micro Laboratories.
- The test results relate only to the items tested

Figure showing the results of microbial contamination tests on the *A. afra* standardized dried leaves and freeze-dried aqueous extract powders after irradiation of the plant materials.

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MICRO REPORT

UWC
DEPARTMENT OF PHARMACOLOGY
PRIVATE BAG X17
BELLVILLE
7535

ATTENTION: **ADMIRE DUBE**

DATE: 18/10/05
DATE RECEIVED: 13/10/05
DATE TESTED: 13-18/10/05

REQ NO: CT 58457/05
PAGE 1 OF 1

SAMPLE TYPE: **PLANT MATERIAL**
METHOD NO: **SWJM 35; 42; 45; 49; 50**

SAMPLE TYPE	TEST TYPE	DET TIME (HRS)	BACT.COUNT CFU/gram
Plant Material			
No.1	TMA		No Growth
	Yeast & Mould		No Growth
	Enterobacteriaceae		No Growth
No.2	TMA		No Growth
	Yeast & Mould		No Growth
	Enterobacteriaceae		No Growth
No.3	TMA		No Growth
	Yeast & Mould		No Growth
	Enterobacteriaceae		No Growth
No.4	TMA		No Growth
	Escherichia coli	No Growth	No Growth
	Yeast & Mould		No Growth
	Salmonella		Absent/25g
	Enterobacteriaceae		No Growth

SEAN SWATTON
LABORATORY MANAGER

BRENDA DU TOIT
LABORATORY MANAGER

- Test method: Malthus Application Data and Conventional Methods
- Limit of Detection of Conventional Plate Count Methods = 10CFU
- TMA = Total Microbial Activity
- Results are given in detection time (HRS). The shorter the time, the higher the microbial activity and the longer the time, the fewer the microbial activity.
- Results relate to samples tested according to stipulated methods. The report shall not be reproduced except in full without approval of Swift Micro Laboratories.
- The test results relate only to the items tested

APPENDIX B

The data and results of the determinations of the luteolin content and variation in the plant material batches.

Table showing the data and results of the determinations of the free luteolin content and variation in the plant material batches.

Concentration of free luteolin in plant batches (µg/mg)					
Sampling Position	**Plant Batch 001**	**Plant Batch 002**	**Plant Batch 003**	**Plant Batch Standardized leaves**	**Freeze dried aqueous extract**
1	1.0339	0.9882	0.7040	0.8533	6.5789
2	1.4071	1.0052	0.9145	0.7854	7.2001
3	1.0976	0.8175	1.0557	0.9970	7.7892
4	0.7313	0.8784	0.9407	0.7267	7.1435
5	0.8765	0.5680	1.3524	0.9428	7.2922
6	0.7808	0.6269	1.6180	0.8719	6.5220
Average	**0.9879**	**0.8141**	**1.0980**	**0.8628**	**7.0880**
S.D	**0.2494**	**0.1825**	**0.3318**	**0.0991**	**0.4751**
% R.S.D	**25.25**	**22.42**	**30.23**	**11.48**	**6.70**

Table showing the data and results of the determinations of the conjugated/ glycosidic luteolin content and variation in the plant material batches.

Concentration of conjugated luteolin in plant batches (µg/mg)					
Sampling Position	**Plant Batch 001**	**Plant Batch 002**	**Plant Batch 003**	**Plant Batch Standardized leaves**	**Freeze dried aqueous extract**
1	0.1393	0.8649	0.7714	0.8585	8.3880
2	0.6781	1.4333	0.8168	1.1186	5.2185
3	0.3666	1.3835	0.2745	1.3119	7.2968
4	0.5316	0.8975	0.9909	1.2706	6.4871
5	0.8052	1.0698	0.4736	1.3291	7.4305
6	0.8372	0.2483	0.4977	1.3265	5.8680
Average	**0.5597**	**0.9829**	**0.6375**	**1.203**	**6.781**
S.D	**0.2706**	**0.4317**	**0.2658**	**0.1862**	**1.152**
% R.S.D	**48.35**	**43.92**	**41.69**	**15.49**	**16.98**

Table showing the data and results of the determinations of the total luteolin content and variation in the plant material batches.

Concentration of total luteolin in plant batches (µg/mg)					
Sampling Position	**Plant Batch 001**	**Plant Batch 002**	**Plant Batch 003**	**Plant Batch Standardized leaves**	**Freeze dried aqueous extract**
1	1.1732	1.8531	1.4754	1.7118	14.9669
2	2.0852	2.4385	1.7313	1.9040	12.4186
3	1.4642	2.2010	1.3302	2.3089	15.0860
4	1.2629	1.7759	1.9316	1.9973	13.6306
5	1.6817	1.6378	1.8260	2.2719	14.7227
6	1.6180	0.8752	2.1157	2.1984	12.3900
Average	**1.548**	**1.7967**	**1.735**	**2.065**	**13.87**
S.D	**0.3287**	**0.5391**	**0.2909**	**0.2347**	**1.246**
% R.S.D	**21.24**	**30.00**	**16.77**	**11.36**	**8.98**

APPENDIX C

The specifications for the Dynapore 117/7/0 tea bag paper used for manufacture of the *A. afra* plant material tea bags.

Parameter	Specification
Grammage	16.75g/m^2
Thickness	64.40pm
Apparent density	0.260g/cm^3
Tensile force MD	12.90N/15mm
Tensile force CD	3.80N/15mm
Wet tensile force CD	1.28N/15mm
Sand fall out	8.67%
Air permeability	1.561 Akustron (1/ms^2)
Heat-seal CD	1.34N/15mm

APPENDIX D1

The results of the organoleptic evaluation conducted on the tea bags of the *A. afra* leaves subjected to different storage conditions.

The results of the organoleptic evaluation conducted on the tea bags of the *A. afra* leaves subjected to stability testing at sites A and B. Changes occurring are highlighted in italics.

Condition & Sample Days	Organoleptic characteristic		
	Appearance	**Odour**	**Leakage**
Site A			
0	Grey square tea bag with material of rough texture.	Odour characteristic of *A. afra*	No leakage
30	Grey square tea bag with material of rough texture	Odour characteristic of *A. afra*	No leakage
60	Grey square tea bag with material of rough texture	Odour characteristic of *A. afra*	No leakage
90	Grey square tea bag with material of rough texture	Odour characteristic of *A. afra*	No leakage
120	Grey square tea bag with material of rough texture	Odour characteristic of *A. afra*	No leakage
180	Grey square tea bag with material of rough texture	Odour characteristic of *A. afra*	No leakage
Site B			
0	Grey square tea bag with material of rough texture.	Odour characteristic of *A. afra*	No leakage
30	*Grey square tea bag with material of soft texture.*	Odour characteristic of *A. afra*	No leakage
60	*Green/grey square tea bag with material of soft texture.*	Odour characteristic of *A. afra*	Green colouration on tea bag paper
90	*Green/grey square tea bag with material of soft texture.*	Odour characteristic of *A. afra*	*Green colouration on tea bag paper*

Table showing the results of the organoleptic evaluation conducted on the tea bags of the freeze-dried aqueous extract subjected to stability testing at sites at sites A, C and D. Changes occurring are highlighted in italics.

Site & Sample Days	Parameter		
	Appearance	**Odour**	**Leakage**
Site A			
0	Grey square tea bag with dark, free flowing powder material.	Odour characteristic of *A. afra*	No leakage
7	Dark brown stained tea bag with sticky material inside.	Odour characteristic of *A. afra*	*Leakage of contents out of the tea bag*
Site C			
0	Grey square tea bag with dark, free flowing powder material.	Odour characteristic of *A. afra*	No leakage
14	Grey square tea bag with dark, free flowing powder material.	Odour characteristic of *A. afra*	No leakage
28	Grey square tea bag with dark, free flowing powder material.	*Faint odour of A. afra*	No leakage
42	*Grey square tea bag with dark, hard crusted powder.*	*Faint odour of A. afra*	No leakage
60	*Grey square tea bag with dark, hard crusted powder.*	*Faint odour of A. afra*	No leakage
74	*Grey square tea bag with dark, hard crusted powder.*	*Faint odour of A. afra*	No leakage
88	*Grey square tea bag with dark, hard crusted powder.*	*Faint odour of A. afra*	No leakage
Site D			
0	Grey square tea bag with dark, free flowing powder material.	Odour characteristic of *A. afra*	No leakage
14	Grey square tea bag with dark, free flowing powder material.	*Faint odour of A. afra*	No leakage
28	*Grey square tea bag with dark, hard crusted powder.*	*Faint odour of A. afra*	No leakage
42	*Grey square tea bag with dark, hard crusted powder.*	*Faint odour of A. afra*	No leakage
60	*Grey square tea bag with dark, hard crusted powder.*	*Faint odour of A. afra*	No leakage
74	*Grey square tea bag with dark, hard crusted powder.*	*Faint odour of A. afra*	No leakage
88	*Grey square tea bag with dark, hard crusted powder.*	*Faint odour of A. afra*	No leakage

APPENDIX D2

Chromatographic fingerprint and UV spectral overlays of the samples from the *A. afra* plant materials in tea bag after storage at the various conditions.

Figure showing the chromatographic overlays of the *A. afra* dried leaves (in tea bag) over a 180 day stability testing period at site A.

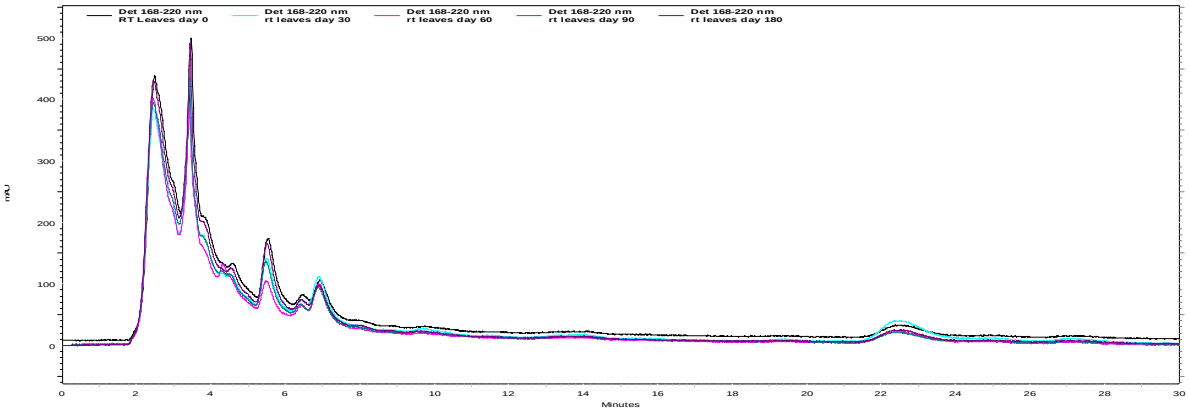


Figure showing the UV spectra overlay of *A. afra* dried leaves at day 0 and day 180 at site A. (λmax 220nm, retention time 3.52 minutes).

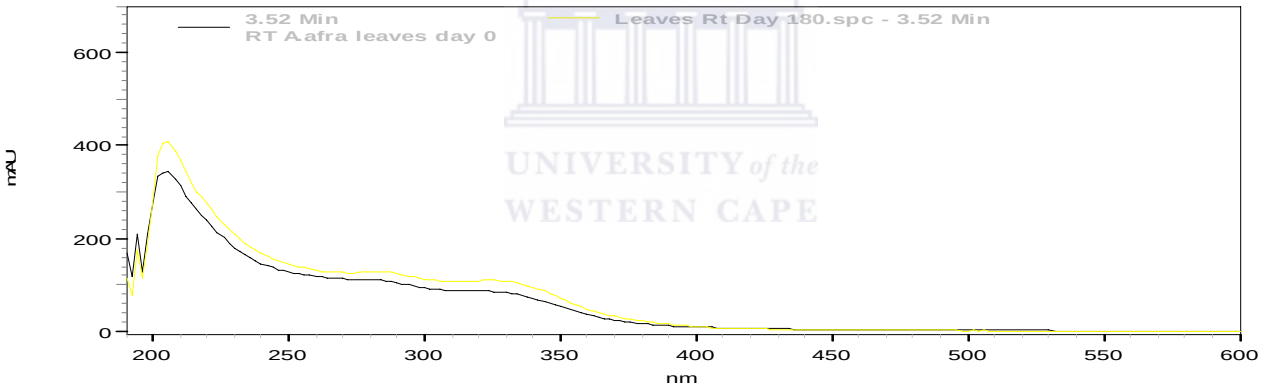


Figure showing the chromatographic overlay of *A. afra* freeze-dried aqueous extract in tea bag subjected to stability testing at site A.

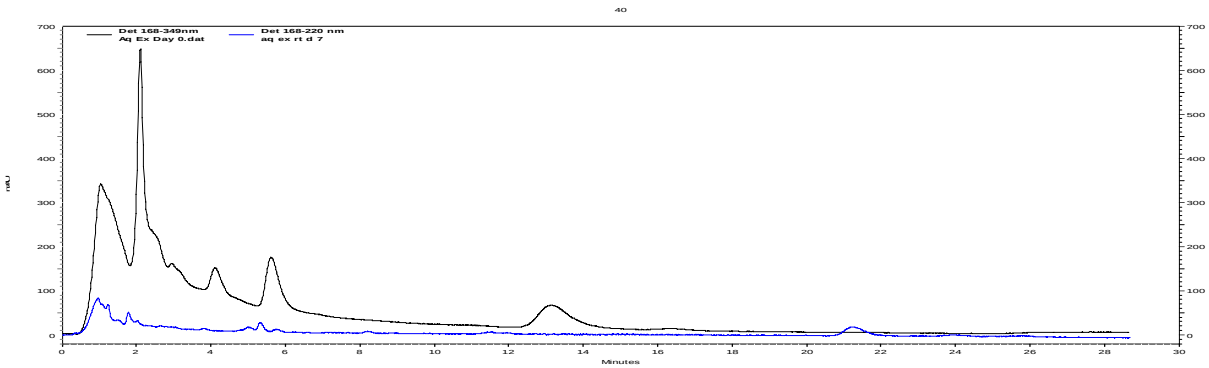


Figure showing the UV spectra overlay of *A. afra* freeze-dried aqueous extract in tea bag at the beginning and end of stability testing at site A. (λmax 220nm, retention time 3.52 minutes).

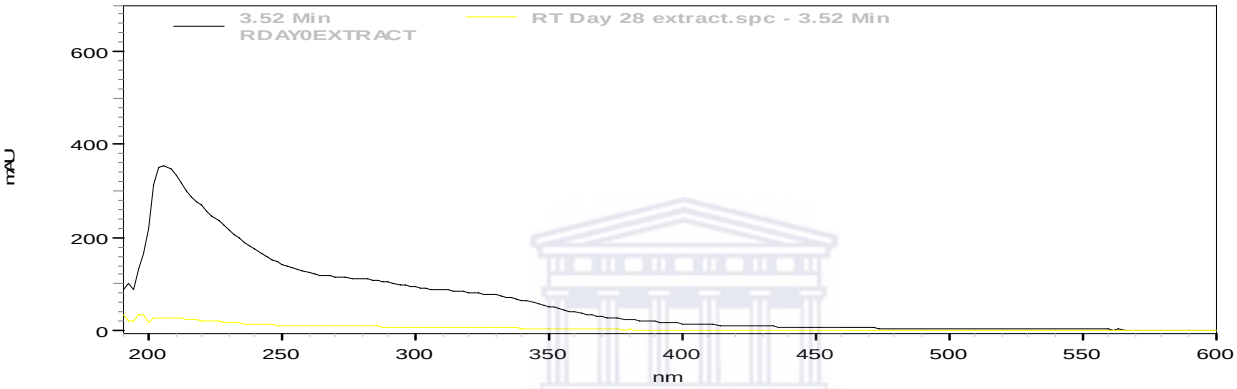


Figure showing the chromatographic overlays of the *A. afra* dried leaves (in tea bag) subjected to stability testing at site B.

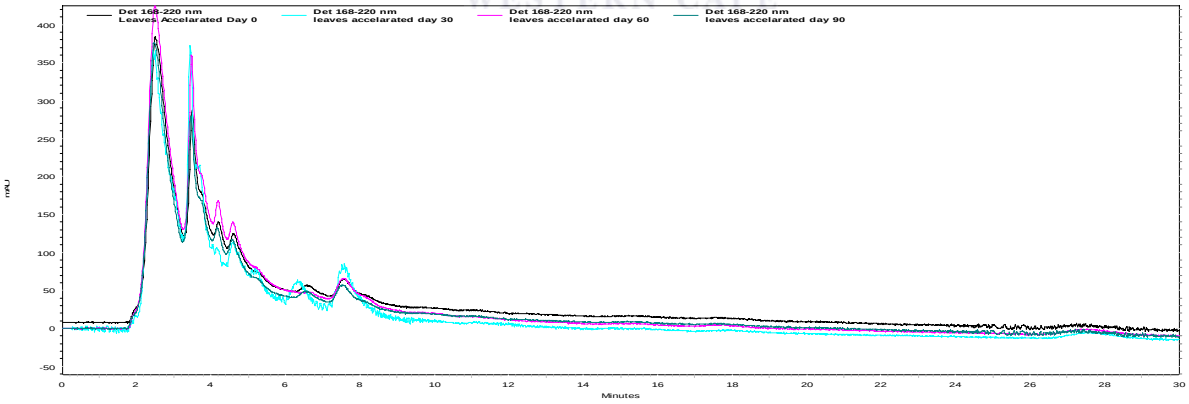


Figure showing the UV spectra overlay of the *A. afra* dried leaves in tea bag subjected to stability testing at site B. (λmax 220nm, retention time 3.52 minutes).

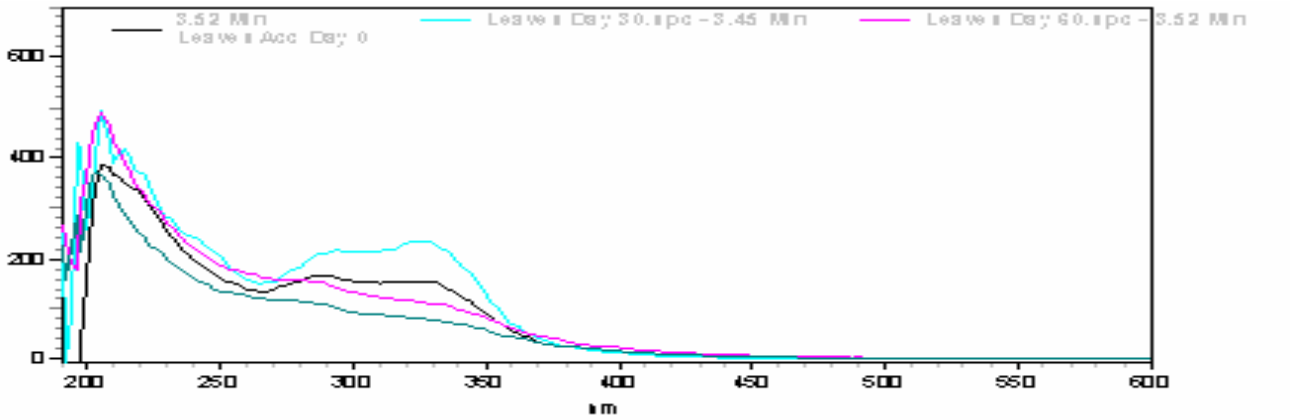


Figure showing the chromatographic overlays of the *A. afra* dried leaves (in tea bag) subjected to stability testing at site C.

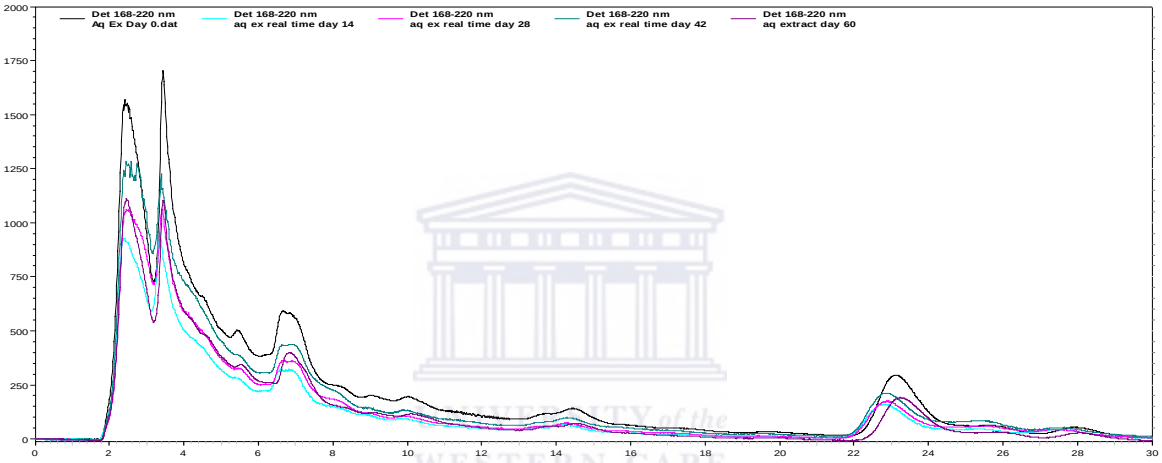


Figure showing the UV spectra overlay of the *A. afra* dried leaves in tea bag subjected to stability testing at site C. (λmax 220nm, retention time 3.52 minutes).

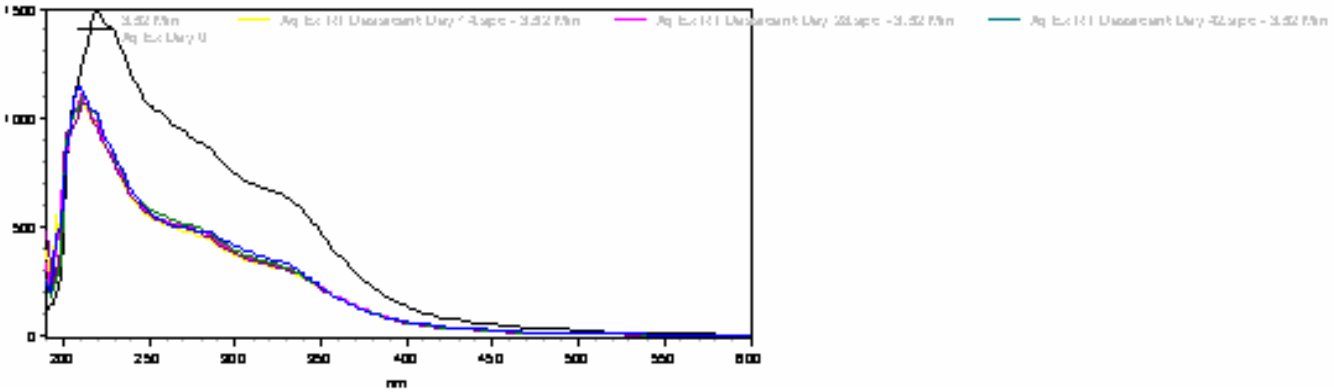


Figure showing the chromatographic overlays of the *A. afra* freeze-dried aqueous extract (in tea bag) subjected to stability testing at site D.

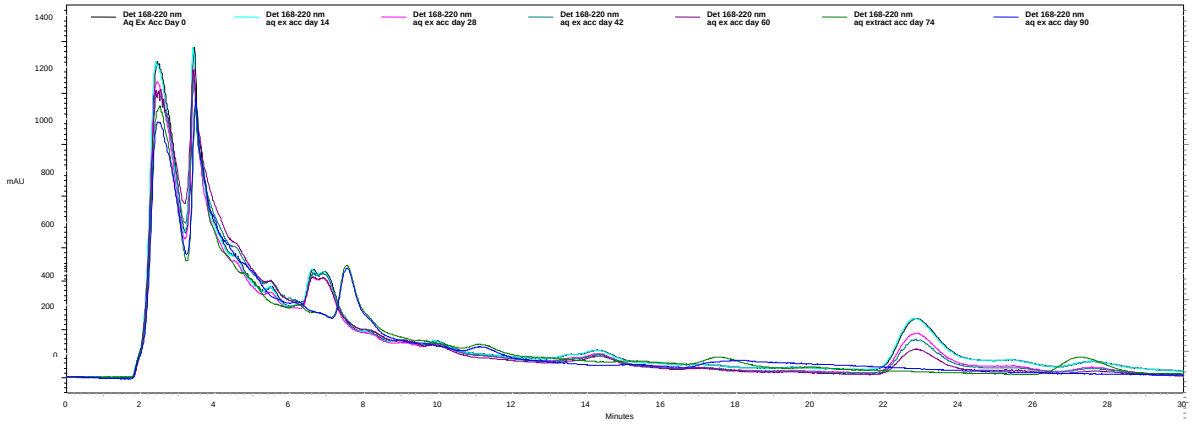
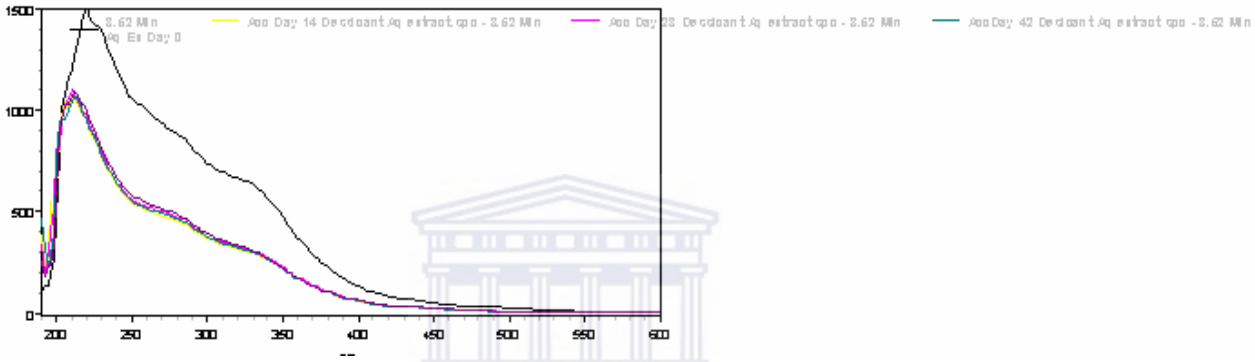


Figure showing the UV spectra overlay of the *A. afra* dried leaves in tea bag subjected to stability testing at site D. (λmax 220nm, retention time 3.52 minutes).



APPENDIX E

The pen recordings (data) from the pharmacological evaluation of the placebo material using the isolated guinea pig trachea model. Cumulative concentrations of the placebo and the *A. afra* plant materials were injected onto the perfused trachea. The 100% tissue relaxation was obtained using 0.1ml of $1x10^{-2}$M isoprenaline. The 'p' and 'l', markings on the charts represent the injected concentrations of the placebo and *A. afra* leaves solutions respectively.

Figure showing pen chart recordings from recorder A.

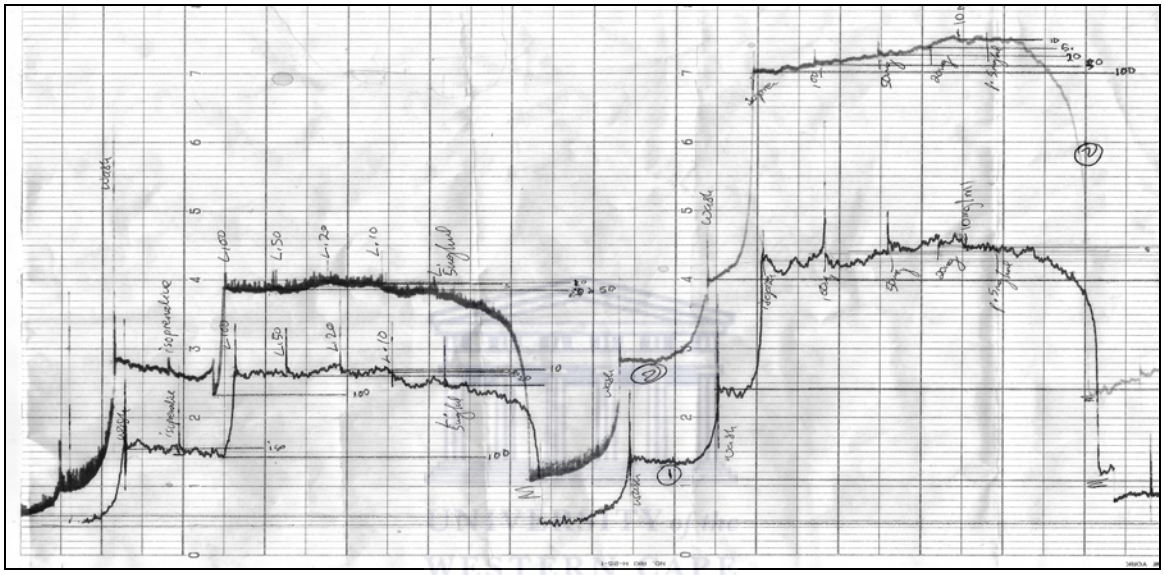
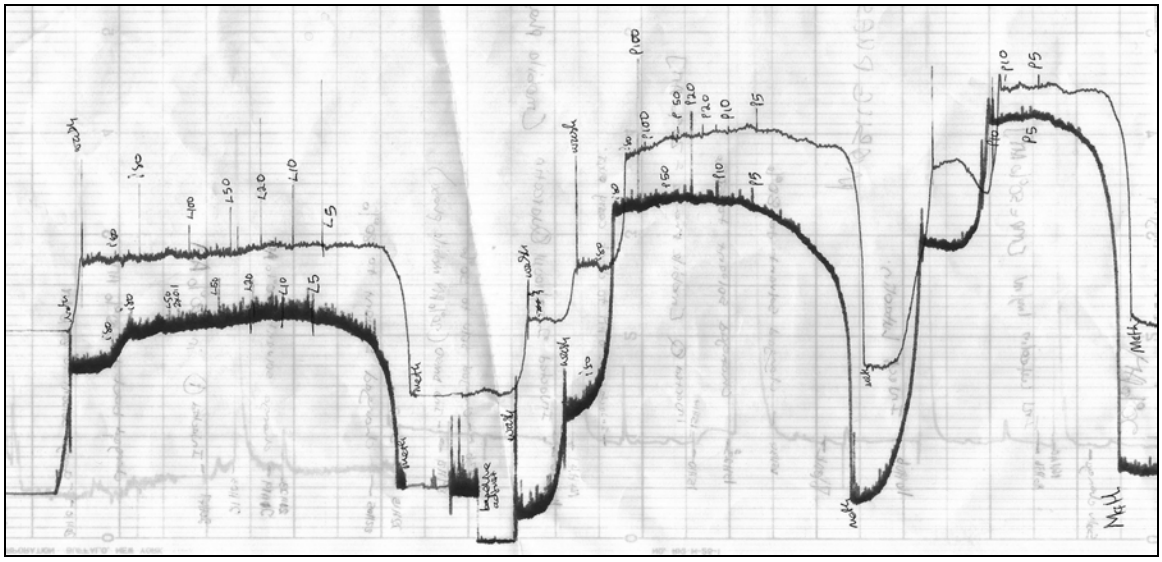


Figure showing pen chart recordings from recorder B.



Vitae

The author was born in Harare, Zimbabwe on the 1st of March in 1980. His primary and high school education was done in Harare and in July 2003 he obtained a Bachelor of Pharmacy (Hons) degree from the University of Zimbabwe. After completing his pharmacy internship and working briefly in the area of medicines regulation in Zimbabwe, the author enrolled for a M. Pharm. degree at the University of the Western Cape, South Africa, in February 2005.