THE FORMULATION, MANUFACTURE AND EVALUATION OF CAPSULES CONTAINING FREEZE-DRIED AQUEOUS EXTRACTS OF *LEONOTIS LEONORUS* OR *MENTHA LONGIFOLIA*.

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SUMMARY

THE FORMULATION, MANUFACTURE AND EVALUATION OF CAPSULES CONTAINING FREEZE-DRIED AQUEOUS EXTRACTS OF *LEONOTIS LEONORUS* OR *MENTHA LONGIFOLIA.*

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Leonotis leonorus and *Mentha longifolia* are two herbs commonly used in South Africa, mostly in oral liquid dosage forms. Several disadvantages are associated with these traditional dosage forms which can perhaps be remedied by using an appropriate oral solid dosage form, provided the actual plant material in the latter still resemble, as closely as possible, the traditionally used material and provide products of suitable pharmaceutical quality.

The objectives of this study were to prepare and evaluate the pharmaceutical suitability of the freeze-dried aqueous extracts of *L. leonorus* and *M. longifolia* as plant raw material for the capsule dosage of these two therapies and to formulate and manufacture capsules of *L. leonorus* and *M. longifolia* aqueous extract that would contain amounts of the plant materials equivalent to that found in their traditional liquid dosage forms, and have immediate release characteristics and suitable stability.

To realize these objectives the decoction of the plants were made and freeze-dried, and each dried extracts evaluated, in a pre-formulation study, for its organoleptic and physicochemical (e.g. particle size and shape, powder

density, flowability, solubility, etc) properties and levels of flavonoid marker compounds. For the latter a validated HPLC assay was used. Based on the pre-formulation study results the formulation of the capsules (i.e. excipients, type and size of capsule, capsule filling method, etc to use) was decided and the capsules manufactured. Thereafter the manufactured capsules were pharmaceutically evaluated for weight and content uniformity, moisture content, microbial contamination, dissolution profile and stability using conventional pharmaceutical methods.

The freeze-dried aqueous extracts of *L. leonorus* and *M. longifolia* produced were moderately fine powders with irregular particle shapes, were sparingly soluble in water, but highly wettable, had good flow properties, on average contained 4.89 ± 0.25% and 8.40 ± 0.14% moisture for *L. leonorus* and *M. longifolia,* respectively, had microbial contamination counts well within the specifications and were suitable plant raw materials for hard capsule manufacture. The capsules produced were physically elegant and acceptable, on average contained 165.3 ± 1.629 µg luteolin and 382.1 ± 10.77 µg apigenin (n=6) in the *L. leonorus* and *M. longifolia* capsules, respectively, and met the pharmacopoeia specifications for content and weight uniformity (The average deviation in weight were 1.19±1.78% and 1.58±1.24%, respectively), microbial contamination levels and rapid dissolution. The capsules were however very unstable even when stored, outside a container, at room temperature and humidity conditions, had practically no shelf-life (t_{90} = 3.67 weeks and 5.39 for *L. leonorus* and *M. longifolia* capsules, respectively), and significantly different dissolution profiles (f_2 = 25.06 and 44.61 for *L. leonorus* and *M. longifolia* capsules, respectively), after 12 weeks storage.

Collectively, the results showed that the aqueous extracts *L. leonorus* and *M. longifolia* were suitable as raw materials of the plants as far as manufacture of

capsules were concerned, but that the stability and shelf-life of these extract containing capsules were unacceptable. As such these capsules were thus not adequate replacements for the traditional decoction forms of these two commonly used medicinal plants.

DECLARATION

I declare that the thesis "*The formulation, manufacture and evaluation of capsules containing freeze-dried aqueous extracts of Leonotis leonorus or Mentha longifolia"* 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.

Name : HaiQiu Ma

Signature: ……..

Date: ……

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DEDICATION

I dedicate this master's thesis to my mother *HanYing Song* and my father *XinMin Ma* for their love and support.

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CHAPTER 1

INTRODUCTION

Traditional medicine(TM) refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being (World Health Organization, 2003). According to WHO, as many as 80% of the world's people rely for their primary health care on traditional medicine, most of which are remedies made from plants. In South Africa, most people also associate traditional medicine with herbs (Richter, 2003).

Leonotis leonorus and *Mentha longifolia* are two herbs commonly used in South Africa. *Leonotis leonorus* is one of the oldest cultivated South African medicinal plants, having been grown in Holland in 1663. The Zulu make an infusion of the aboveground parts of this plant for coughs and colds. In the case of *Mentha longifolia*, the colonialists prized this plant as an antispasmodic and calmative and used an infusion of it to treat flatulent colic, indigestion and colds (Dyson, 1988).

Plant medicines are generally considered to be safer and less damaging to the human body than synthetic drugs. Furthermore, there is a current upsurge of interest in plants that is further supported by the fact that many important drugs in use today were derived from plants or starting molecules of plant origin: digoxin / digitoxin, the vinca alkaloids, reserpine and tubocurarine are some important examples (Williamson et al, 1996).

In most cases, as is the case with *L. leonorus* and *M. longifolia,* liquid dosage forms (e.g. decoction, infusion, teas, etc) of plant remedies are used. The

traditional liquid dosage form, however have several disadvantages. Firstly they are not easy to keep free of microbial contamination. Secondly, the traditional measurements and directions for their use are not exact (e.g. take half a cupful, two teaspoonful, etc). Such inaccurate directions will influence the uniformity of the dosing in the individual users. Thirdly, long-term storage of the liquid dosage forms may be problematic as the active principles in them may be quite unstable over time. Fourthly, liquid dosage forms are not convenient to prepare. Most of these problems may be remedied by using an appropriate solid dosage form, e.g. tablets or capsules, of the plant material. To our knowledge such tablet or capsule solid dosage forms have not yet been produced for *L. leonorus* and *M. longifolia.*

The actual plant material used to make the solid dosage form must however still, as closely as possible, resemble the traditionally used material while, at the same time, be amenable to easy formulation into the solid dosage form. Ground, dried plant material seldom have the appropriate pharmaceutical properties e.g. uniform particle size, adequate flow characteristics, etc, that allows the easy incorporation of such plant material into a solid dosage form of good quality. To circumvent the afore-mentioned problems associated with ground dried plant material one might want to prepare and use the freeze-dried aqueous extract of the plant and use that for the preparation of the solid dosage form (e.g. a directly compressed tablet or capsule). If the prepared dosage forms are also rapidly disintegrating and releasing then they may also quite accurately mimic the traditional dosage forms and be suitable to be used in place of the liquids.

Previous studies with other plants e.g. *Artemisia afra* (Komperlla, 2004), *Dodonaea angustifolia* (Kayitare, 2001) have however indicated that the freeze-dried aqueous extracts of plant materials are frequently very hygroscopic and not easily amenable for tabletting. No such information is

however available for the freeze-dried aqueous extracts of *L. leonorus* and *M. longifolia,* but it was anticipated that these would also be hygroscopic.

The use of the hard gelatin capsule dosage form may perhaps be an alternative to overcome the problem, which the hygroscopicity of the extracts may pose. The capsule shell is an excellent barrier to air, and it also has some other advantages such as being easy to swallow and tasteless, may allow rapid release, flexibility of formulation, short manufacturing steps, etc. The preparation of a capsule containing freeze-dried aqueous extracts of *L. leonorus* and *M. longifolia* could thus lead to products of acceptable pharmaceutical quality. To test this hypothesis (i.e. to test for the presence of the quality of the capsules) appropriate marker constituents in the plant material could be monitored.

Several articles had revealed that flavonoids are present in *L. leonorus* and *M. longifolia* and these phytochemicals may thus be appropriate markers to use to assess the quality control aspects, such as content uniformity and the stability testing, of *L. leonorus* and *M. longifolia* capsule dosage form. Furthermore, the flavonoid marker compounds could quite easily be quantitated using a HPLC analytical method.

Given the above arguments, the objective of this study consequently were (1) to prepare and evaluate the pharmaceutical suitability of the freeze-dried aqueous extracts of *L. leonorus* and *M. longifolia* as plant raw material for the capsule dosage form of these two phytotherapies and (2) to formulate and manufacture capsules of *L. leonorus* and *M. longifolia* that would contain an amount of plant material equivalent to that found in its traditional liquid dosage forms and would have immediate release characteristics and appropriate stability.

CHAPTER 2

LITERATURE REVIEW

2.1 Traditional medicine.

According to the World Health Organization, traditional medicine refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being (WHO, 2003). Examples of traditional medicine include traditional Chinese medicine, Ayurveda (Dubey et al, 2004) and traditional African medicine or traditional African medical systems (Okpako, 1999). In industrialized countries, adaptations of traditional medicine are termed “Complementary” or “Alternative” medicine (CAM).

One of the definitions given for ‘African Traditional Medicine’ by the WHO Center for Health Development is the following:

> The sum total of all knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental, or societal imbalance, and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing (WHO).

Over one-third of the population in developing countries lack access to essential medicines and it is claimed that in Africa, up to 80% of the population uses traditional medicine (TM) for primary health care. The provision of safe and effective TM/CAM therapies could thus become a critical tool to increase access to health care (WHO, 2003). It is therefore not surprising that, in Africa,

traditional healers and remedies made from plants play an important role in the health of millions of people (Overview on Medicinal Plants and Traditional Medicine in Africa).

2.1.1 Traditional medicinal plants.

A medicinal plant is any plant that provides health-promoting characteristics, temporary relief or symptomatic problems or has curative properties (http://davesgarden.com).

Without plants, most medicines would not exist. Over 40% of medicines now prescribed in the U.S. contain chemicals derived from plants. Indeed a comprehensive search of plants known for their medicinal chemicals would be an enormous task, especially since of the estimated 250,000 plant species on earth, only 2% have been thoroughly screened for chemicals having potential for medicinal use (http://www.nps.gov). Furthermore, most developing countries are endowed with vast resources of medicinal and aromatic plants, much of which are still under-explored.

Man in his environment has used these plants over the millennia for human welfare. Even today, a large proportion of people in developing countries still living in rural areas, continue to depend on these plants. They are precluded from the luxury of access to modern therapy, mainly for economic reasons.

Africa has a long and impressive list of medicinal plants that are used based on local knowledge. For instance *Securidaca Longepedunculata* is a tropical plant found almost everywhere in Africa. (http://www.conserveafrica.org.uk).

All over, including in Africa, it is claimed that the use of traditional plant medicines offer many advantages.

2.1.2 Advantages and disadvantages of traditional plant medicine.

Traditional plant medicine has many advantages, which can be exploited. Medicinal plants are potential sources of new drugs, sources of cheap starting materials for synthesis of known drugs or a cheap source of known drugs. It is claimed that the human body better accepts drugs derived from natural sources than substances invented in the laboratory (Muhizi, 2002). Furthermore, plant medicines generally have fewer side effects when compared with synthetic products.

Things always exist dialectically. Therefore, traditional plant medicines also have some disadvantages. Firstly, plants as biologic systems have inherent potential variability in their chemistry and resulting biologic activity. Secondly, plants from different geographic areas also have biologic diversity (Fabricant and Farnsworth, 2001). In addition, for most traditional medicines, the specific constituent that causes a therapeutic effect is not known. Many constituents exist in the plant, and it is likely that they work together to produce the desired therapeutic effect. However, what the exact combination of active ingredients is often the crucial problem for traditional plant medicine development.

Leonotis leonorus and *Mentha longifolia* are two popular traditional plant medicines used in South Africa.

2.1.3 *Leonotis leonorus* and traditional use.

Vernacular names

English - Wild dagga, red dagga, wild hemp, lion's ear/tail, minaret flower

Afrikaans – Kilpdagga, wildedagga

Sotho – Lebake, levake

Xhosa - Umfinoafinoane

Zulu – Imunyane, umunyane

(Dyson, 1988)

Figure 2-1. *Leonotis leonorus*

Leonotis leonorus grows wild in all the Cape Provinces, Kwaulu-Natal and Northern and Eastern Transvaal. It is one of the oldest cultivated South African plants, having been grown in Holland in 1663 (Dyson, 1988).

The plant is mainly used, orally, in the form of an aqueous decoction, and per rectum as a topical application. Medicinally it is traditionally used, internally, for the treatment of cough, cold, influenza, chest infections, diabetes, hypertension, eczema, epilepsy, delayed menstruation, intestinal worms, constipation, spider bites and scorpion stings and as an antidote for snakebite, and externally, for the relief of hemorrhoids, eczema, skin rashes and boils (http://www.sahealthinfo.org).

The following is the general dosage preparation and directions for use of *Leonotis leonorus*:

1 tablespoonful of chipped dried herb (approx. 10,0 g) added to 3 cupfuls (approx 500 ml) of boiling water, boil for 10 minutes; allow cooling overnight, strain and use the clear liquid for both internal and external use. If fresh material is used, 3-4 young twigs (leaf and stem) are boiled with one liter of water. For internal use, adults must take half a cupful (approx. 90 ml); elderly patients a quarter of a cupful (approx. 45 ml); children 6-12 yrs of age, a

quarter of a cupful (approx. 45 ml) and children 2-6 yrs, two teaspoonfuls (approx. 8 ml) to be taken two to three times daily. For external use, the decoction may be applied to the affected area using a clean cloth (http://www.sahealthinfo.org).

2.1.4 *Mentha longifolia* and traditional use.

Vernacular names

English – Spearmint, wild mint

Afrikaans – Kruisement

Sotho – Kwena, kwena-ya-thaba

Xhosa – Inxina, inzinziniba

Zulu – Ufuthanelamhlanga

(Dyson, 1988)

Figure 2-2. *Mentha longifolia*

In South Africa, *Mentha longifolia* grows commonly along streams in the Transvaal district; also in the Cape Provinces, Orange Free State, Swaziland, KwaZulu-Natal and Lesotho (Dyson, 1988).

It is traditionally used for a variety of purposes with various dosage directions and dosage forms. The early colonists prized this plant as antispasmodic and calmative and used an infusion to treat flatulent colic, indigestion and colds. More commonly it is used as follows. A quarter of a cup of fresh leaves is added to one cup of boiling water, allowed to stand for three minutes and then drunk. Alternatively, a fresh leaf can be chewed to provide relief from heartburn and colic, aid digestion and relieve flatulence. Externally, mint can be applied to glandular and other swellings. Furthermore the fresh leaves rubbed onto pillows and blankets, make an excellent mosquito repellent. Finally, the Xhosa (indigenous tribe in South Africa) of Kokstad, Butterworth, and Cederville, districts of South Africa, have also been reported to make a tea from fresh or

dried leaves (Dyson, 1988).

The dosage forms in which *Leonotis leonorus* and *Mentha longifolia* are used can, as for many of the traditionally used plant medicines, be quite varied.

2.1.5 Traditional dosage forms.

Traditional medicines are dispensed in dosage forms such as liquids (e.g. infusions, decoctions, elixirs and tinctures), semi-solids (e.g. pastes, creams and ointments), solids (e.g. whole or powdered plant parts, pills and tablets), and gases (e.g. incense, fumigants and inhalants). Most of these preparations are given orally or applied externally on the affected parts of the body (http://banglapedia.search.com.bd). Liquid dosage forms are the most popular dosage form used.

Infusions are typically used for delicate herbs, leaves and fresh tender plants. Preparing an infusion is much like making a cup of tea. Water is brought just to a boil and then poured over an herb (or combination of herbs). The ratio of herb to water can vary depending on the remedy, the plant, and whether cut herb or powdered herb is used. Generally using 1 teaspoon (approx 3 g) of powdered herb or 2 teaspoons (approx 6 g) of more bulky cut herb in a 6-8 ounce (170ml – 230ml) cup of water is sufficient (Taylor, 2004). Infusions are best prepared as needed and taken the same day (Dharmananda).

For decoctions, instead of just steeping it in hot water, the plant material is boiled for a longer period of time to soften the hard woody material so that it releases its active constituents (Taylor, 2004). Preparing decoctions of herbs is a relatively easy but inefficient method of getting out active ingredients. The amount of ingredients to be extracted is relatively large because of the high dose of total material. Decoctions are generally inconvenient and unpleasant

herbal preparations that are used only when the situation might require. An hour or so is required to cook up the tea. The inefficiency of decoction preparation is made worse when careful attention is not paid to the different cooking times that might be appropriate (Dharmananda).

The use of tinctures and alcoholic extracts are other, somewhat popular, means of delivering Chinese herb formulas (Dharmananda). A tincture is an alcohol and water extract which is used when plants have active chemicals that are not very soluble in water, and/or when a larger quantity is prepared for convenience and wanted for longer term storage. The compliance for certain patients (e.g. children, women, and those on restricted diets) using tinctures is usually very limited (Taylor, 2004).

Macerations. This method of preparation is certainly the easiest. The fresh or dried plant material is simply covered in cool water and soaked overnight. The herb is strained out and the liquid is drunk. Normally this is used for very tender plants and/or fresh plants, or those with delicate chemicals that might be harmed by heating or which might be degraded in strong alcohol (Taylor, 2004).

In general, the traditional dosage forms have some disadvantages. Firstly, they are not easy to keep free of microbial contamination. Secondly, the large volumes of preparation required may not be comfortable for patients. Thirdly, the traditional dose measures of plant medicines used are not exact (e.g. half a cupful, two teaspoonful, etc). Such inaccurate dosage directions will affect the uniformity of the dosing in the individual users. Most of these problems may however be remedied by using an appropriate solid or other dosage forms.

2.1.6 Options of alternate dosage forms available and their advantages and disadvantages.

For many plant medicines there are already some solid dosage forms (i.e. powders, granules, tablets, capsules) available that are used instead of the traditional liquid dosage forms. The term ‘powder’, when used to describe a dosage form describes a formulation in which a drug powder has been mixed with other powdered excipients to produce the final product. The function of the added excipients depends upon the intended use of the product. Coloring, flavoring and sweetening agents may, for example, be added to powders for oral use (Summers, 2002).

Granules, which are used as a solid dosage form, consist of powder particles that have been aggregated to form a larger particle, which is usually 2-4 mm in diameter. This is much larger than granules prepared as an intermediate for tablet manufacture (Summers, 2002).

‘Powders’ and ‘Granules’ as solid preparations are chemically more stable than liquid preparations. They are convenient forms in which drugs are dispensed in large doses. Orally, administered powders and granules of soluble medicaments have a faster dissolution rate than tablets or capsules. But they also have some disadvantages. Firstly, bulk powders or granules are far less convenient for the patient to carry than a small container of tablets or capsule, and are as inconvenient as liquid preparations, such as mixtures. Secondly, the masking of unpleasant tastes may be a problem with this type of preparation. Thirdly, bulk powders or granules are not suitable for administering potent drugs used in low doses. Finally, the use of powders and granules are not a suitable method for the administration of drugs, which are inactivated in, or cause damage to, the stomach (Summers, 2002).

Tablets are defined as ‘solid preparations each containing a single dose of one or more active ingredients and obtained by compressing uniform volumes of

particles'. The tablet is one of the most popular oral dosage forms for several reasons. Firstly, when compared to liquid dosage forms, tablets have general advantages in terms of the chemical and physical stability of the dosage form. Secondly, the preparation procedure for tablets leads to accurate dose amounts of the drug in each tablet. Thirdly, tablets are convenient to handle and can be prepared in a way that is versatile with respect to their use and to the delivery of the drug. Fourthly, tablets can be mass-produced with robust and quality-controlled production procedures providing elegant preparations of consistent quality and, in relative terms, low price (Alderborn, 2002).

It seems to be a good choice for *L. leonorus* and *M. longifolia* to be formulated as tablets of their dried aqueous extracts, but previous studies with other plants e.g. *Artemisia afra* (Komperlla, 2004), *Dodonaea angustifolia* (Kayitare, 2001) have however indicated that the freeze-dried aqueous extracts of plant materials are frequently very hygroscopic and not easily amenable for tabletting. Capsules, another popular dosage form, may however be another option.

Herbal products are typically divided into either liquid or solid dosage forms. Most liquid dosage forms are produced from fluid extracts, and most solid dosage forms are produced from solid extracts. An 'extract' is defined as a concentrated preparation of a liquid, powder or viscous substance, ordinarily prepared from dried plant parts (the crude drug) by using an appropriate solvent (https://www.cedrugstorenews.com).

Generally, solid extracts are made by two methods: evaporation (dried decoction) and sublimation (freeze-dried extract). The dried decoctions are produced by making large batches of either single herbs or traditional herb formula mixtures as decoctions (in large tanks), and then draining the liquid

from the dregs (Dharmananda). The liquid is evaporated (using heat and vacuum) to form syrup. The syrup is then put into a spray-drier along with a powder carrier (usually starch or the dried, powdered, herb dregs), and the remaining water is evaporated, leaving a dry powder (Dharmananda). However, the process of removing the water and spray drying the granules can lead to some losses as the water is evaporated off, by heating, and the syrup is then slowly heat-dried to a sold condition. In this process, some of the ingredients may also breakdown. The end product is thus not necessarily one that is much better than the decoction.

Freeze-drying of the liquid extract will result in the freeze-dried extract. In this process the initial liquid solution or suspension is frozen, the pressure above the frozen state is reduced and the water removed by sublimation. Thus a liquid-to-vapor transition takes place as summarized in see figure 2-3 (Aulton, 2002).

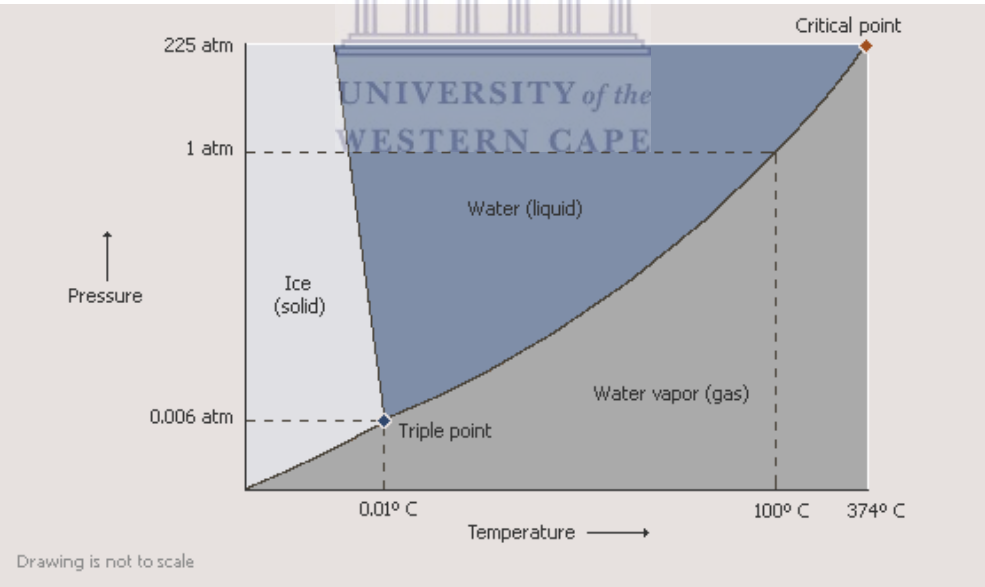


Figure 2-3 *Phase diagram for water (http://encarta.msn.com).*

Freeze drying has certain special advantages. Firstly, the drying takes place at very low temperatures, so that enzyme action is inhibited and chemical decomposition, particularly hydrolysis, is minimized. Secondly, the solution is

frozen such that the final dry product is a network of solid material occupying the same volume as the original solution. Thus, the product is light and porous. Thirdly, the porous form of the product gives ready solubility. Fourthly, there is no concentration of the solution prior to drying. Hence, salts do not concentrate and denature proteins, as occurs with other drying methods. Finally, since the process take place under high vacuum there is little contact with air, and oxidation is minimized (Aulton, 2002). The above characteristics reveal that freeze-drying is a good method for making plant material extracts. But the porosity, ready solubility and complete dryness may yield a hygroscopic product and this may be the big challenge in the formulation of solid dosage forms of herbs.

No term can describe the hygroscopicity definitively. This is because there is both a kinetic and a thermodynamic component in the term (Carstensen, 1980). A hygroscopic material will pick up moisture from the atmosphere and the term *hygroscopic* is relative. Ammonium chloride, for instance, would be hygroscopic if stored in an atmosphere above 79.5% relative humidity (RH) but not in atmospheres below 79.5% RH at 20℃ (Carstensen, 1977).

2.2 The capsule dosage form.

The word ‘capsule’ in the English language is derived from the Latin word ‘capsula’, which means a small box or container (Jones, 2004). In more recent times, capsule has been used primarily to describe a solid oral dosage form, which consists of a container, usually made of gelatin, filled with a medicinal substance. There are many forms of capsules and they can be divided into two main categories, which in current English usage are described by the adjectives ‘hard’ and ‘soft’. The ‘hard capsule’ consists of two separate parts, each a semi-closed cylinder in shape. One part, the ‘cap’, has a slightly larger

diameter than the other, which is called the 'body' and is longer (figure 2-4). The cap fits closely over the body to form a sealed unit (Jones, 2004).

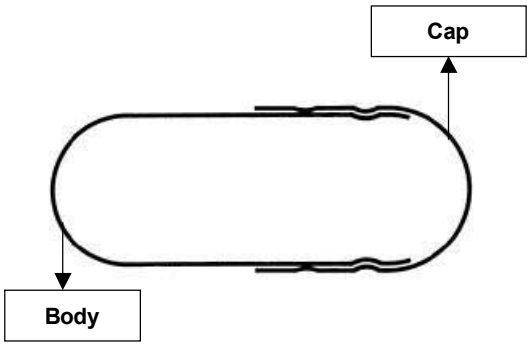


Figure 2-4. *Self-lock capsule*

The gelatin capsule was invented early in the 19th century as a result of the need to mask the obnoxious taste of many of the medicinal substances, which were in vogue at that time. The actual inventor of the gelatin capsule appears to have been Mr F. A. B. Mothes in 1834 (Jones, 2004). One procedure introduced to overcome Mothes's patent resulted in the production of a new type of capsule, the hard two-piece, which was the forerunner to the modern hard two-piece capsule. Another Parisian pharmacist, J. C. Lehuby, who was granted a patent on 20th October 1846, invented the hard two-piece capsule (Jones, 2004).

Gelatin is the commercial protein derived from the native protein collagen, which is present in animal skin and bone, and the term 'gelatin' originates from the Latin 'gelatus', meaning stiff or frozen. Gelatin has all the properties required to meet the technical needs of the pharmaceutical capsule industry. These include solubility, solution viscosity and thermally reversible gelation properties in aqueous solution. It produces strong, clear, flexible, high-gloss films, which dissolve readily under the conditions existing in the stomach. Furthermore, current scientific evidence indicates that gelatin is a safe raw material (Jones, 2004).

Hard gelatine capsules can be filled with a large variety of materials of different physicochemical properties (i.e. dry solids, semisolids, non-aqueous liquids, etc) (Jones, 2004), while soft gelatin capsule are generally used to contain liquid and semisolid materials.

Specialist capsules have been made to meet the demands of certain applications, e.g. gastro-resistant capsules, modified-release capsules, self-locking capsules, capsules for liquid filling, capsules for administration to animals and capsules used for certain clinical trials. For current applications there are certain design features that all hard capsules must possess, viz. a feature to hold the empty capsule shells together, a self-locking feature, an air venting system and a feature to allow accurate rejoining after filling (Jones, 2004).

Capsules offer many advantages. 1. Capsules, because of their elongated shape, are easy to swallow, which is one reason for the number of capsule-shaped tablets manufactured today. 2. Flexibility of formulation is another advantage of the capsule dosage form. However the biggest formulation advantage of capsules is that there is less need for additional excipients. 3. Since capsules are tasteless, they effectively mask any unpleasant taste or odor of their contents. 4. They offer rapid release characteristics, due to the rapid dissolution rate of the capsules. 5. The use of hard capsules is also a common feature in clinical trials, as the filling of tablets or even capsules themselves will blind the dosage forms studied (Podczeck, 2004). 6. The manufacture of capsules as illustrated in figure 2-5 also involves a much shorter process compared to that for other modern dosage forms (e.g. tablet). 7. Controlled release can be achieved using capsules. Dry powder mixtures, granules, pellets and tablets can be filled into hard capsules. Moreover combination of two or three types (i.e. dry powder mixtures, tablets or pellets) also can be put into capsules.

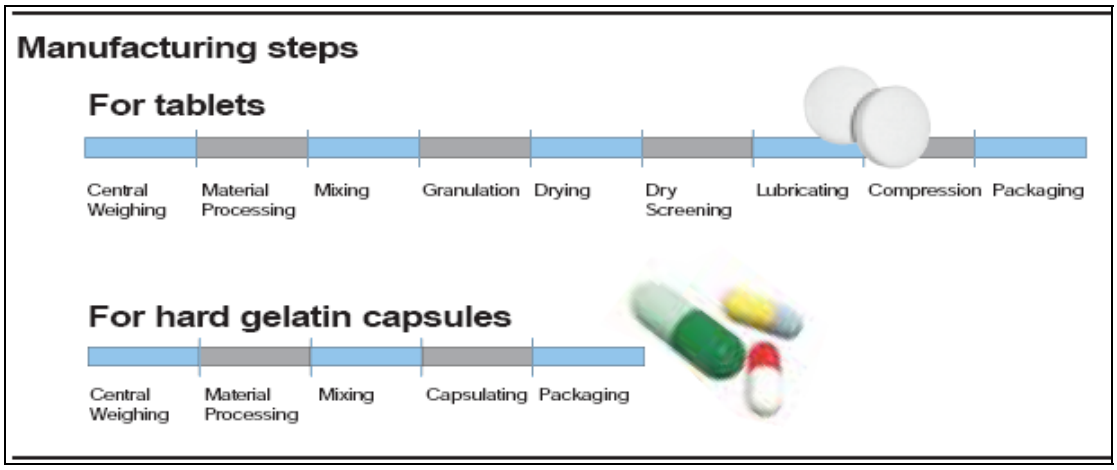


Figure 2-5. *Comparison of manufacturing process of tablets and hard gelatin capsules (www.capsugel.com)*

Some or all of the above-mentioned advantages of capsules can also be help, when used as dosage form for pant medicines, help improve the quality of herbal preparations.

2.3 Quality evaluation of herbal materials

Just like conventional drugs the quality of herbal medicines must, for safety and efficacy reasons, be strictly controlled. Criteria/specifications for such quality assessments of herbals are however less well defined. The European Medicines Evaluation Agency (EMEA, 1999) offers suggestions. Tests and assay for herbal materials and finished products such as total ash, ash Insoluble in hydrochloric acid, particle size, water content, microbial limits, dissolution/disintegration, hardness/friability, uniformity of dosage units are generally recommended.

For most herbs, the specific ingredient that causes a therapeutic effect is not known. Herbal medicines are complex mixtures, which usually comprise at least 50 or so chemical constituents, although most contain many more

(Barnes, 2002). So, reference material is important for the evaluation of the quality of a herbal medicine. A reference standard, or reference material, is a substance prepared for use as the standard in an assay, identification, or purity test. In the case of herbal substances, the reference standard may be a sample of the plant or a chemically defined substance (e.g. a known active constituent, a marker substance or a known impurity) (EMEA, 1999). The reference standard has a quality appropriate to its use. The composition of reference standards intended for use in assays should be adequately controlled and the authenticity and/or purity of a standard should be determined by validated procedures. In the case of herbal materials where the constituents responsible for the therapeutic activity are unknown, appropriate marker substances may be used. Furthermore, appropriate statistical analysis should be applied, when necessary, to the quantitative quality data obtained (EMEA, 1999).

For many herbal products the marker compounds suitable for use to assess herbal product quality have not been established, but one should of course start by selecting one or other of the constituents of the plant medicine.

2.4 Phytochemical constituents of *Leonotis leonorus* and *Mentha longifolia* useful for quality control.

The chemical constituents in plants can be classified as indicated in the following scheme:

Plant → **Primary metabolism**

Primary metabolites:

Proteins, Amino acids, Carbohydrates, Lipids, etc.

↓

Secondary metabolism

Secondary metabolites:

Glycosides, alkaloid, terpenes, **Phytochromes**,etc.

↓

Flavonoid, Chlorophyll, Lutein, Carotene, etc.

↓

Flavonols, Flavanones, Catechins, Anthocyanidins, Isoflavones, **Flavones**, etc.

↓

Apigenin, Luteolin, etc**.**

General scheme of phytochemical constituents of plants. (http://www.100md.com)

The sum of the processes by which a particular substance is handled (as by assimilation and incorporation, or by detoxification and excretion) in the living body is called metabolism (http://www.amfar.org). It is distinguished between primary and secondary metabolism. The primary metabolism contains all pathways necessary to keep the cell alive. In the secondary metabolism, compounds are produced and broken down that are essential for the whole organism (http://www.biologie.uni-hamburg.de).

The following phytochemical constituents have thus far been identified for *L. leonorus* and *M. longifolia*.

For *L. leonorus:* tannins, quinones, saponins, alkaloids and triterpene steroids were detected in preliminary tests, while iridoids were not detected

(Laonigro et al, 1979). Flavonoids are also present in *L. leonorus* (Ascensao and Marques, 1997).

For *M. longifolia*, tannins, saponins and flavonoids were detected, but not alkaloids. Common flavonoids e.g. acacetin, hesperidin, as well as luteolin and apigenin glucuronides have been reported for European populations of the plant (Bourweig and Pohl, 1973).

CHAPTER 3

PLAN OF WORK

3.1 Objective.

The first objective of this study was to prepare and evaluate freeze-dried aqueous extracts of *L. leonorus* and *M. longifolia* as suitable plant raw materials for capsule dosage forms of these two phytotherapies. The second objective was to formulate and manufacture capsule dosage forms of *L. leonorus* and *M. longifolia* aqueous extracts that contained amounts of plant materials equivalent to that found in their traditional liquid dosage forms and capsules that would meet conventional pharmaceutical standards for immediate release dosage forms.

3.2 Hypothesis

It was hypothesized that freeze-dried aqueous extracts of *L. leonorus* and *M. longifolia* would have suitable characteristics (especially not be hygroscopic) for use as plant raw material for capsules. And secondly, that immediate release capsules (i.e. > 75% dissolution of plant material in 45 mins) containing an amount of plant material equivalent to that found in traditional liquid dosage forms of *L. leonorus* and *M. longifolia* can be manufactured.

3.3 Study produces:

To achieve the above objectives, the following were done.

1) Preparation of *L. leonorus* and *M. longifolia* freeze-dried aqueous extracts.

2) Determination of the flavonoid content of *L. Leonorus* and *M. longifolia* by HPLC.

3) Pre-formulation study of *L. leonorus* and *M. longifolia* freeze-dried aqueous extracts.

4) Formulation, manufacture and evaluation of *L. leonorus* and *M. longifolia* capsules.

3.3.1 Preparation of *L. leonorus* and *M. longifolia* freeze-dried aqueous extracts.

Freeze-dried aqueous extracts of *L. leonorus* and *M. longifolia* were the major drug substances used in the study because it was believed that the aqueous extract of the leaves of the plant would closely resemble the liquid dosage solution form of the plants with the advantages that freeze-drying would produce a convenient form of the plant material. Consequently, the leaves of *L. leonorus* and *M. longifolia* were to be collected, washed, dried and a decoction prepared by using distilled water as the solvent. Finally, the decoctions were to be frozen in a dry ice – acetone bath and freeze-dried by freeze-dryer to afford an acceptable solid form of the plant material.

3.3.2 Determination of the flavonoid content of *L. Leonorus* and *M. longifolia* by HPLC.

To evaluate the pharmaceutical properties of the final *L. leonorus* and *M. longifolia* capsules a suitable assay for the flavonoid markers in the plant materials were needed. Consequently an HPLC assay was to be developed that could be used to first, determine the presence and relative levels of flavonoids present in the plant extracts and decoctions, and then to use for the

assessment of the pharmaceutical properties of the capsules – see 3.3.4 Flavonoids were chosen a appropriate markers because of their known abundance in these plants and the HPLC assay because of its many advantages e.g. selectivity, sensitivity.

3.3.3 Preformulation study of *L. leonorus* and *M. longifolia* freeze-dried aqueous extracts.

To assess the suitability of the freeze-dried aqueous extract for formulation into a capsule, a preformulation study that checked the physicochemical properties of the *L. leonorus* and *M. longifolia* extract powders was required. The results of the preformulation study would provide the information needed for the rational formulation and manufacture of the capsules. In the pre-formulation study the organoleptic properties, particle size and shape, powder density, angle of repose, moisture content and solubility were to be determined using the appropriate techniques.

3.3.4 Formulation, manufacture and evaluation of *L. leonorus* and *M. longifolia* capsules.

Based on the pre-formulation results, formulation of the capsules could be decided (i.e. which and how much excipients should be used; the type and the size of the capsule shell; the capsules filling method, etc). The final step in this study would consequently then be to evaluate the filled capsules, by assessing their weight and content uniformity, moisture content; microbial contamination, dissolution profile and stability. For these tests the levels of suitable flavonoid markers found in the extracts were to be monitored using an HPLC assay – see 3.3.2 above.

CHAPTER 4

PREPARATION AND PRE-FORMULATION STUDY OF *LEONOTIS LEONORUS* AND *MENTHA LONGIFOLIA* FREEZE-DRIED AQUEOUS EXTRACTS.

4.1 Introduction

Prior to the development of dosage forms, it is essential that certain fundamental physical and chemical properties of the drug molecule and other derived properties of the drug powder are determined. This information dictates many of the subsequent events and approaches in formulation development. This first learning phase is known as pre-formulation (Wells, 2002). Pre-formulation is an important step in the development of a new drug. It influences the safety, effectiveness, controllability, stability and compliance of the drug (Cui, 1980). Generally, pre-formulation includes the study of the following:

1. The physicochemical properties of manufactured batches of the API (active pharmaceutical ingredient), and an assessment of their relevance to the final formulation,
2. The chemical and physical stability of the API,
3. The impurity profiles of the API, including the typical content of synthetic products and degradation products, and
4. The chemical compatibility of the active with potential excipients.

These studies give clues as to how to achieve the desired performance of the finished product (Walters, 2003).

The majority of drug substances in use today occur as solid materials. Most of

them are pure chemical compounds of either crystalline or amorphous constitution. Some are powdered animal or vegetable drugs (Ansel, 1981). For this study, *L. leonorus* and *M. longifolia* freeze-dried aqueous extract powder were prepared and analyzed in the pre-formulation study. In total nine assessments were performed in the pre-formulation study and they are described below. In addition, the equipment and materials and procedures used for the preparation of the plant raw materials are also described.

4.2 Equipments and materials

The following equipments were used in the preparation of the plant extracts and the pre-formulation study:

Oven *(Memmert 854 Schwabach, West Germany*); Disintegrator (*Dynamics Corporation Of America, USA*); Balance 1 (*Wirsam Scientific &Precision Equipment (PTY) Ltd. Model GA 110, West Germany*); Balance 2 (*Mettler Pe 6000 Mettler Instrumente Ag Ch-8606 Greifensee- Zurich, Switzerland*); Hot Plate (*Type Rct13 Kika-Werke Gmbh&Co.Kg D-79219 Staufen, Germany*); -85 ℃ Freezer (*Lozone Cfc Freezer Model U855360, New Brunswick Scientific, USA*); Freeze-Dryer (*Model Virtis Freeze Mobile 72sl, The Virtis Company Gardner, New York, USA*); Light Microscope (*Nikon Abbe 1.25 Japan*); Stage Micrometer (*Graticules Ltd, Tonibridge Kent, England*); Eyepiece Micrometer (*Olympus, Japan*); Filtration System (*Supelco*); Watch Glass: (*8cm Diameter*) Vacuum Pump (*Medi-Pump Model 1132-2, Thomas Industries, Inc., USA*); Sieve Shaker (*Endecott Sieve Shaker, E.F.L. 1mk11, Endecotts (Test Sieve) Ltd, London, England*); Sieves (*Incorporating Madison Test Sieves (Pty) Ltd. Republic Of South Africa*); Water Bath (*Cph110, Manufactured By Labdesign Engineering Pty Ltd*.); Mechanical Tapping Device (*Chadwell Heath Essex, England*); Buchner Funnel (*127c-2a, Haldenwanger Berlin*);

Spectrophotometer (*DU 640 spectrophotometer, Beckman, USA*); microscope (*Olympus, dp2, Tokyo Japan*) with a camera (*u-CMAD-2*) and a lamp (*BX50*).

The following materials were used in the preparation of the plant extracts and the pre-formulation study:

Acetone (*Cp. Kimix.*); *Carbon Dioxide (CO_2)*; *Leonotis Leonorus* Leaves (*Cogmanskloof in Montagu in the Western Cape Province of South Africa*); *Mentha Longifolia* Leaves (*Kirstenbosch Garden Western Cape Province of South Africa*);

4.3 Methods

4.3.1 Collection of leaves and preparation of freeze-dried aqueous extracts of *Leonotis leonorus* and *Mentha longifolia*

The fresh leaves of *L. leonorus* were collected from Cogmanskloof in Montagu in the Western Cape Province of South Africa on 7th March 2005. A sample of the collected plants was deposited as a specimen (voucher No: 6736) in the herbarium, Botany Department, University of the Western Cape. The fresh leaves could not be kept for a long period in the air, so the leaves were washed using water and dried in the oven at 60℃ (for approximately 3 days) until they retained a constant weight. Because water constitutes the bulk of most plant material, dried herbs tend to contain a higher concentration of non-volatile bioactive compounds and one would need to consume larger amounts of fresh herbs to obtain the same quantity of active components present in the dried plant. Because we were also interested in the non-volatile actives, the use of dried *L. leonorus* and *M. longifolia* leaves instead of the fresh leaves were, in addition to being easier to store, etc, considered more appropriate.

The fresh leaves of *M. longifolia* were collected from Kirstenbosch Garden Western Cape Province of South Africa on 21st March 2004. The sample of the collected plants was filed as a specimen (voucher No: 6635) in the herbarium, Botany Department, University of the Western Cape. These leaves were dried in the same way as that used for *L. leonorus*.

To prepare the aqueous extracts the following procedure was used. For *Leonotis leonorus*, 1 tablespoonful of chipped dried herb (10g) were added to 3 cupfuls (500ml) of boiling water, boiled for 10 minutes, and allowed to cool overnight as per traditional procedure (http://www.iamshaman.com). The mixture was filtered on a Buchner funnel using filter paper (Whatman number 1), the extract frozen in a dry ice – acetone bath and then freeze-dried under vacuum over 2 days using the Savant Freeze Drying System. Once the material was completely dried, it was collected, placed in a moisture-free package, sealed and kept in a desiccator until use.

For the *M. longifolia,* a quarter of a cup of dried leaves (2g) was added to one cup of boiling water (150ml) and the mixture allowed to stand until cool as per the traditional procedure (Dyson, 1988). The mixture was then filtered, freeze-dried, and the powder collected and stored as described for *L. leonorus*.

4.3.2 Determination of the organoleptic properties of the plant extracts.

Usually it is difficult to measure organoleptic properties since there are no standard laboratory tests for this and requires personnel that are well experienced with the process. In this study the following organoleptic properties of the plant materials were assessed: physical appearance, odour and taste. For these samples of *M. longifolia* and *L. leonorus* extracts were inspected and assessed using the natural senses (e.g. eyes, nose, mouth).

4.3.3 Determination of the solubility of the plant extracts.

All drugs, by whatever route they are administered, must exhibit at least limited aqueous solubility for therapeutic efficiency. The transfer of molecules or ions from a solid state into solution is known as dissolution (Aulton, 2002). The extent to which the dissolution proceeds under a given set of experimental conditions is referred to as the solubility of the solute in the solvent. Thus, the solubility of a substance is the amount of it that passes into solution when equilibrium is established between the solution and excess (undissolved) substance. The solution that is obtained under the latter conditions is said to be saturated. (Aulton, 2002) Solubility is an important factor for drug absorption. It is described by the *Noyes-Whitney* equation (Eqn. 4-1):

$$\mathbf{dC / dt = DA (C_s - C) / h.} \quad \text{(Eqn. 4-1)}$$

Where $\mathbf{dC / dt}$ *is the rate of dissolution of the drug particles,* $\mathbf{D}$ *is the diffusion coefficient of the drug in solution in the gastrointestinal (GIT) fluids,* $\mathbf{A}$ *is the surface area of the particle in contact with the GIT fluids.* $\mathbf{C_s}$ *is the saturation solubility of the drug in solution in the diffusion layer.* $\mathbf{C}$ *is the concentration of the drug in solution in the bulk of the gastrointestinal fluids.* $\mathbf{h}$ *is the thickness of the diffusion layer around each drug particle.*

The equilibrium solubility of the freeze-dried aqueous extracts of *L. leonorus* and *M. longifolia* was determined as follows.

A saturated solution obtained by stirring excess extract powder (pass through 180 sieve) solute with distilled water for 3 hours at the required temperature

(25℃, 37℃, 60℃) by using water bath until equilibrium has been attained. From the start of stirring samples are withdrawn every 30 minutes. Membrane filters (0.45 μm) were used for filter samples. Absorbance of the sample was measured at an appropriate maximum wavelength (280 nm for *L. leonorus*; 285 nm for *M. longifolia*) using UV Spectrophotometer. The AUFS (absorbance) reading should increase until one gets to a maximum when equilibrium is reached. This indicates the time required for equilibration. Finally, *Time-Absorbance* curves were withdrawn at 25℃, 37℃ and 60℃. The amount of solute contained in the sample of saturated solution was determined by Gravimetric analysis. The solubility was obtained by the following equation (Eqn. 4-2):

Solubility = (weight of initial powder – weight of dried residue) / volume of solvent ×100%

(Eqn. 4-2)

4.3.4 Determination of extract particle size and shape.

The dimensions of particulate solids are important in achieving optimum production of efficacious medicines. For some herbal substances intended for use in herbal teas or solid herbal medicinal products, particle size can have a significant effect on dissolution rates, bioavailability, and/or stability (EMEA, 1999). Particle size influences the production of formulated medicine as solid dosage forms. Both tablets and capsules are produced using equipment that controls the mass of drug and other particles by volumetric filling (Staniforth, 2002). Size measurements also provide an important in vitro indicator of the bioavailability of a given formulation, allowing formulators to predict the dynamics of drug release for dosage forms (Burgess, 2004). When medicine is administered, the dosage form should release the drug into solution at the

optimum rate. This depends on several factors, one of which will be the surface area of drug as predicted from the *Noyes-Whitney equation* (Eqn. 4-1) (Staniforth, 2002). The surface area could be increased by reducing the particle size of material.

$$\mathbf{dm / dt = D A (Cs – C) / h} \quad \text{(Eqn. 4-1)}$$

A *is the surface area of the particle in contact with the gastrointestinal fluids.*

One of the most fundamental and easy methods for determining particle size is a sieving method. This method involves passing the material being sized through openings of a particular standard size in sieves. This is also the method described in British Pharmacopoeia (British Pharmacopoeia 2000[1]), and the method used in the present study where the degree of fineness of powders is determined by sieving (see Figure 4-1). The sieve receiver and the sieves of number 90, 125, 180, 355 were arranged in a descending order on the sieve shaker. And then 10 g of *L. leonorus* or *M. longifolia* extract was poured in the top sieve. Finally, the cover was put on the top, and the shaker was started. The process of shaking took 30 minutes. Thereafter the powder collected on each of the sieves was weighed and the percentage (w/w) of each fraction determined.

Figure 4-1. *Sieves shaker and sieves*

Particle shape refers to the outline of a particle. It can influence the production of medicines formulated as solid dosage forms. Both tablets and capsules are produced using equipment that controls the mass of drug and other particles by volumetric filling. Therefore, any interference with the uniformity of fill volumes may alter the mass of drug incorporated into the tablet or capsule and hence reduce the content uniformity of the medicine. Normally the particle shapes are irregular. So, the particles are usually described using the following terms: spherical, cubical, platy, pyramidal, flaky, granular, rod -like, needle-like, blocky, fibrous, sponge, etc. With the exception of spherical and cubical particle shapes, the other particle shapes are difficult to describe accurately (Cui, 1980). In order to describe the particle shape quantificationally, the term degree of sphericity (θ) can be used (*Cui, 1980*). The equation for the degree of sphericity is as follows:

Degree of sphericity $\theta = D_a / D_p$ (Eqn. 4-3)

θ: *Degree of sphericity*

D_a *: Projected area diameter*
D_p *: Projected perimeter diameter*
*(** D_a and D_p see Figure 4-2.)

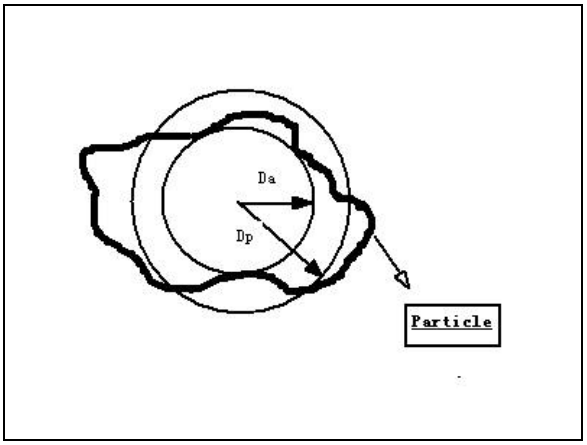
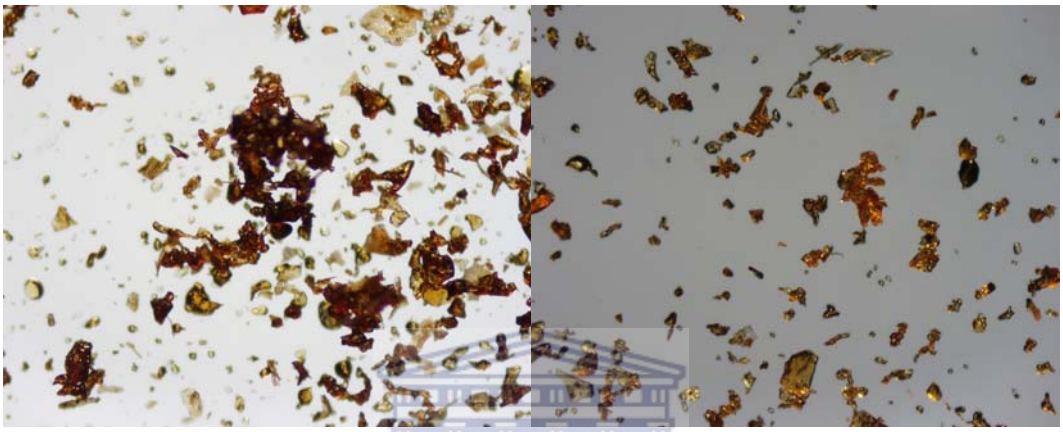


***Figure 4-2**. D_a : Projected area diameter and D_p : Projected perimeter diameter.*

In this study, the extract powder of *L. leonorus* or *M. longifolia* was left on a slide and a microscope was used to observe the particle shape (see Figure 4-3). The D_a and D_p were measured by using micrometer. And the Degree of sphericity (θ) was calculated by using Eqn. 4-3.



M. longifolia *L. leonorus*

***Figure 4-3**. The particle shape of M. longifolia and L. leonorus freeze-dried aqueous extract powders.*

Recently, the use of microscopy and image analysis is increasingly being recognized as the most reliable technique to characterize particle shape and particle size and volume distribution. Industry experts, especially pharmaceutical experts, are now looking to characterize particle shape in addition to particle size, to gain a better understanding of how shape can affect the various properties of a product (http://www.clemex.com).

4.3.5 Determination of the density of the extracts.

A simple test has been developed to evaluate the flow ability of a powder by comparing the poured density (bulk density) and taped density of a powder and the rate at which it packed down. A useful empirical guide is given by

Carr's compressibility index (Eqn. 4-4) ('compressibility' is a misnomer, as compression is not involved) (Wells, 2002).

Carr's index (%) = (Tapped density – Poured density) / Tapped density

(Eqn. 4-4)

Bulk density can be determined by measuring the volume of a known mass of powder, which has been passed through a screen, (in order to break up agglomerates) into a graduated cylinder. The *tapped density* measurement is achieved by mechanically tapping a measuring cylinder containing a powder sample. After observing the initial volume, the cylinder is mechanically tapped, and volume readings are taken until little further volume change is observed (British Pharmacopoeia 2000[2]). The tapping machine used in the present study shown in Figure 4-4.

***Figure 4-4**. Equipment for density test*

In study the density of *L. leonorus* or *M. longifolia* aqueous extract powder was determined as follows*:*

Sufficient *L. leonorus* or *M. longifolia* aqueous extract powder (that has passed

thru 180 sieve) was poured into the tared cylinder on apparatus up to a volume between 8-10ml before compacting. The cylinder was then weighed and the weight of extract recorded. Thereafter the cylinder was secured in its holder and the reading of unsettled apparent volume, V_0, was taken to the nearest millilitre. The machine was switched on, the powder in the cylinder tapped for approximately 1250 times and the final volume V_{1250}, again taken to the nearest millilitre. The bulk and tapped densities were then calculated using the following equations (Eqn. 4-5 and 4-6).

Bulk density (poured density): m / V_0, in g per ml

Bulk density = weight of the powder / bulk volume

(Eqn. 4-5)

Tapped density: m / V_{1250}, in g per ml.

Tapped density = weight of the powder / tapped volume

(Eqn. 4-6)

4.3.6 Determination of the flowability of the plant extracts

Figure 4-5. *Apparatus for measuring angle of repose.*

The angle of repose **(θ)** is another important parameter that can be used to describe the flowability of a powder (Wells, 2002). In the present study a

special apparatus was used for the test (Figure 4-5). The apparatus consisted of a glass cylinder kept in the centre of the plate, a plate with scale and a ruler for measuring the height of powder mound.

To determine the angle of repose, the glass cylinder was filled with 10 g of plant extract (pass 180 sieve), the cylinder smoothly lifted allowing the powder to flow out at the bottom unto the plate leaving a conical mound. The height and radius of the mound was measured and angle of repose then calculated using the following equation (Eqn. 4-7):

$$\tan\theta = h / r \quad \text{(Eqn. 4-7)}$$

θ*: Angle of repose*

h *: height of the conical mound*
r *: radius of the conical mound*

4.3.7 Determination of the ash residues of the plant extracts

The total ash and acid-insoluble ash was determined for each extract. 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. 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 (World Health Organization, 1998).

For the testing of *L. leonorus* and *M. longifolia* freeze-dried aqueous extracts, the British Pharmacopoeia methods (British Pharmacopoeia 2000[3,4]) were followed:

For the total ash testing, 2 g of *L. leonorus* or *M. longifolia* freeze-dried aqueous extract powder was heated in a tared crucible at a furnace (temperature 450℃) until free from carbon, cool and weigh. If a carbon-free ash cannot be obtained in this way, exhaust the charred mass with hot water, collect the residue on an ashless filter paper, incinerate the residue and filter paper, add the filtrate, evaporate to dryness and ignite at a temperature 450℃. Calculate the percentage of ash with reference to the air-dried drug.

For the acid-insoluble ash testing, the total ash of *L. leonorus* or *M. longifolia* freeze-dried aqueous extract was boiled for 5 minutes with 25 ml of 2M hydrochloric acid, collect the insoluble matter on an ashless filter paper (Whatman number 41), wash with hot water and ignite at temperature 450℃. Calculate the percentage of acid-insoluble ash with reference to the air-dried drug.

4.3.8 Determination of the moisture content of extracts.

The loss on drying test is important when the herbal substances are known to be hygroscopic (EMEA, 1999). An excess of water in medicinal plant materials will encourage microbial growth, the presence of fungi or insects, and deterioration following hydrolysis (World Health Organization, 1998). Furthermore, water determination occupies an extremely important position among today's analytical methods in industry. In modern pharmaceutical technology, the water content provides information concerning the shelf life and the quality of the drugs. The most likely cause of drug instability is hydrolysis, and water plays a dominant role in hydrolysis reaction (Wells, 2002).

There are numerous methods for water content analysis (e.g. oven method, infrared drying method, microwave drying method, titration methods, chemical extraction of water, refraction method, and electrolytic method, etc.). The oven drying method was the original method used and is still a method that is used in the majority of fields (e.g. food industry, pharmaceutical industry, etc).

For this study the European Pharmacopoeia (European Pharmacopoeia 4th edition 2002[2]) method described below was used. About 0.50 g of the *L. Leonorus* or *M. longifolia* extract was finely powdered and rapidly weighed in a flat-bottomed dish. The extract was then dried in an oven at 100-105℃ for 3 hours, allowed to cool (approximately 10 minutes) in a desiccator over anhydrous silica gel, weighed and the weight recorded. The moisture content as determined by this gravimetric method was then calculated using the following equations. (Eqn. 4-8 and 4-9):

Moisture weight = Initial weight (before drying) – Final weight (after drying) (Eqn. 4-8)

Moisture content = (Moisture weight / Initial weight) 100% (Eqn. 4-9)

4.3.9 Determination of the microbial contamination of the extracts

Before the microbial testing, the freeze-dried aqueous extracts were irradiated to kill the microorganism in the extracts. Gamma irradiation is generally used as a sterility quality assurance step in manufacturing processes. For instance, gamma irradiation is internationally used for sterilizing spices and herbs (ICGFI).

In this study the extract was packed in sealed brown glass containers sent to

the radiation facility (Hepro (Cape) Pty Ltd in Montague Gardens in Western Cape Province, South Africa) where it was irradiated at the level of 18 KGY The product remained intact in its packaging and was never opened at the radiation facility and in this way the integrity of the packaging was maintained, the product never exposed directly to the Cobalt 60 and only to the energy emitted by the cobalt 60. This means that products were also not rendered radioactive.

After irradiation, the freeze-dried aqueous extracts were taken for the following microbial tests to be done at Swift Micro Laboratories, in 15 Lower Hope Street, Rosebank, Cape Town, South Africa: Total microbial activity; Escherichia coli; Yeast & Mould; Salmonella and Enterobacteriaceae. For total viable count, Enterobacteriaceae and Yeast & Mould testing the ISO method 4833, 5552 and 7954 was followed; for Escherichia coli testing, samples are plated out onto Violet Red Bile Lactose agar with MUG supplement and incubated at 37 ℃ for 24±2 hours, plates are viewed under UV light to facilitate bacterial counts; for Salmonella testing, an AOAC method, utilizing Malthus technology, was used (See Appendix 43: Microbial contamination testing methods).

4.3.10 Determination of the wet ability of the plant extracts

The wet ability is an important parameter that influences the solubility and disintegration properties of some solid dosage forms (Cui, 1980). Wetting studies usually involve the measurement of contact angles (see Figure 4-4) as the primary data. The lower the contact angle the greater the wetting. Contact angles below 90° indicate that the liquid wets the solid spontaneously. Contact angles above 90° indicate that the liquid does not wet the solid. To produce a quantitative measure of wetting, i.e. to obtain the contact angle, two approaches are generally used: goniometric and tensiometric.

In this study, goniometry was used. The goniometric approach to contact angles involves placing a piece of glass a thin layer of *L. leonorus* or *M. longifolia* extract powder homogeneously and placing a drop of water on a flat solid surface and analyzing the shape of the drop formed. The half-circle protractor was used to measure the contact angle (Figure 4-4).

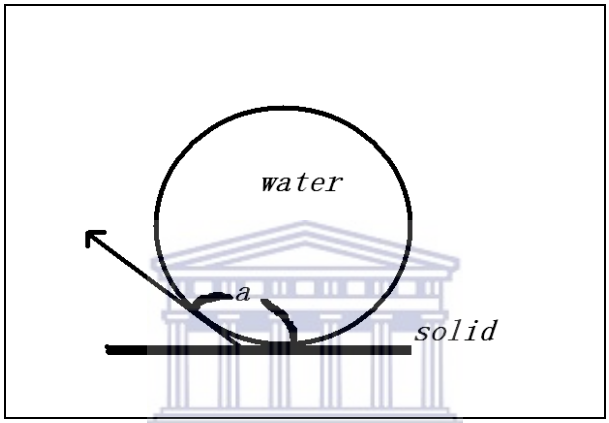


Figure 4-6. *The contact angle between solid and liquid (**a** is the contact angle).*

4.4 Results and discussion

A summary of the pre-formulation testing results is shown in Table 4-1.

Table 4-1 *the summary of pre-formulation testing results on L. leonorus and M. longifolia.*

Testing	*L. leonorus*	*M. longifolia*
Yield of freeze-dried aqueous extracts (%)	**18.42±0.9432**	**12.42±1.281**
The solubility of extracts (g/ml)	**0.0242±0.0032**	**0.0301±0.0025**
Degree of sphericity	**0.6050±0.1173**	**0.6913±0.1119**
Particle size	**Moderately fine**	**Moderately fine**
Carr's index (%)	**13.24±1.015**	**7.193±1.805**
Angle of repose (°)	**34.83±1.063**	**38.52±5.332**
Total ash (%)	**17.2±0.460**	**20.98±0.257**
Acid-insoluble ash (%)	**1.41±0.190**	**2.28±0.377**
The moisture content (%)	**4.89 ± 0.2354**	**8.398±0.1357**

Microbial contamination		
TMA	**No Growth**	**No Growth**
Escherichia coli	**No Growth**	**No Growth**
Yeast & Mould	**No Growth**	**No Growth**
Salmonella	**Absent/25g**	**Absent/25g**
Enterobacteriaceae	**No Growth**	**No Growth**
The contact angle (°)	**6.75±1.581**	**6.5±1.512**

4.4.1 Yield of freeze-dried aqueous extract of *L. leonorus* and *M. longifolia*.

The yields of freeze-dried aqueous extract obtained from *L. leonorus* and *M. longifolia* using the method described in section 4.3.1 are summarized in table 4-1 and appendices 1 and 2. On average 18.42±0.9432% of aqueous extract was obtained from *L. leonorus* and 12.42±1.281% from *M. longifolia* crude leaves. Based on these yields, 562 mg of *L. leonorus* or 248 mg of *M. longifolia* were the doses of aqueous extract that were equivalent to the traditional dose. These results show a low level of yield compared with *Artemisia afra* (28.4± 1.25%) (Komperlla, 2004) and *Dodonaea angustifolia* (25.07 ± 3.032%) (Kayitare, 2001).

4.4.2 The organoleptic properties of the freeze-dried aqueous extract of *L. leonorus* and *M. longifolia*.

The picture of the freeze-dried aqueous extracts (Figure 4-7) and a summary of the organoleptic properties (Table 4-2) of the freeze-dried aqueous extract of *L. leonorus* and *M. longifolia* are given.

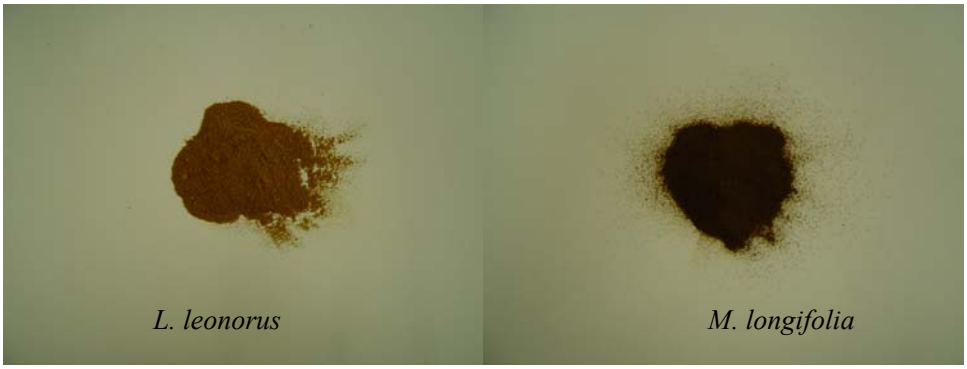


Figure 4-7. *Freeze-dried aqueous extract of L. leonorus and M. longifolia.*

Table 4-2 *The organoleptic properties of the freeze-dried aqueous extract of L. leonorus and M. longifolia:*

Properties:	*L. leonorus*	*M. longifolia*
Physical appearance	**Brittle, free-flowing, small particulate powder.**	**Brittle, free-flowing, small particulate powder.**
Color	**Brown, darker than ground leave powder.**	**Light brown, darker than ground leave powder.**
Odour	**Characteristic odour.**	**Like mint aromatic odour.**
Taste	**Bitter.**	**Bitter and cool.**

The bitter taste and unpleasant odors normally result in poor patient acceptance of dosage forms. Hopefully these negative characteristics still present in the extracts can be masked when incorporated in capsule form.

4.4.3 The solubility of *L. leonorus and M. longifolia* extracts.

For oral solid dosage forms aqueous solubility is a crucial factor influencing the bioavailability of drugs. The solubility of compounds may be described as per the description given in table 4-3.

Table 4-3. *Descriptive solubilities.*

Description	Approximate Weight Of Solvent (G) Necessary To Dissolve 1 G Of Solute.
Very soluble	**Less than 1**
Freely soluble	**From 1 to 10**
Soluble	**From 10 to 30**

Sparingly soluble	**From 30 to 100**
Slightly soluble	**From 100 to 1000**
Very slightly soluble	**From 1000 to 10,000**
Practically insoluble	**More than 10,000**

This table indicates the meanings of the terms used in statements of approximate solubilities. (Aulton, 2002)

The results obtained in the solubility testing of the aqueous extracts of *L. leonorus* and *M. longifolia* are summarized in table 4-1 and appendixes 3. At room temperature 1g of *L. leonorus* extract powder dissolved in 41.82±5.153 ml of water and 1g of *M. longifolia* extract powder dissolved in 33.43±2.466 ml of water. Both extracts could thus be described as being sparingly soluble. The solubility of *L. leonorus* and *M. longifolia* freeze-dried aqueous extract showed similar solubility to the *Artemisia afra* freeze-dried aqueous extract i.e. 1g of *Artemisia afra* extract powder can dissolved in 40 ml of water (Komperlla, 2004).

The effect of dissolution time and temperature on the solubility of extracts was also checked and the results of that study are given in appendix 4. The time allowed for dissolution and temperature did not apparently influence the solubility of *L. leonorus* and *M. longifolia* extracts.

4.4.4 The size and shape of particles of *L. leonorus* and *M. longifolia* extracts

Particle size and shape are crucial parameter. They are important for the manufacture of the dosage forms, influence dissolution and bioavailability. Particles can be classified under 4 different classes in Table 4-4.

Table 4-4*. British Pharmacopoeia 2000 Appendix XVII A. Particle Size of Powders*

Coarse powder	**Not less than 95% by weight passes through a number 1400 sieve and not more than 40% by weight passes through a number 355 sieve.**
Moderately fine powder	**Not less than 95% by weight passes a number 355 sieve and not more than 40% by weight passes through a number 180 sieve.**
Fine powder	**Not less than 95% by weight passes a number 180 sieve and not more than 40% by weight passes through a number 125 sieve.**
Very fine powder	**Not less than 95% of the powder by weight passes a number 125 sieve and not more than 40% by weight passes through a number 90 sieve.**

** The above terms are used in the description of powders.*

The results of the particle size study are given in Tables 4-5 and Figure 4-8, the particle shape dimensions are given in appendix 5.

Table 4-5. *Particle size of L. leonorus and M. longifolia freeze-dried extract powders.*

Sieve:	*L. leonorus* Extract (g)	*M. longifolia* Extract (g)
1400	**0.000**	**0.000**
355	**0.144**	**2.0055**
180	**2.329**	**2.1880**
125	**2.258**	**0.8746**
90	**2.289**	**0.9853**
Pass through 90 sieve	**2.980**	**2.6317**

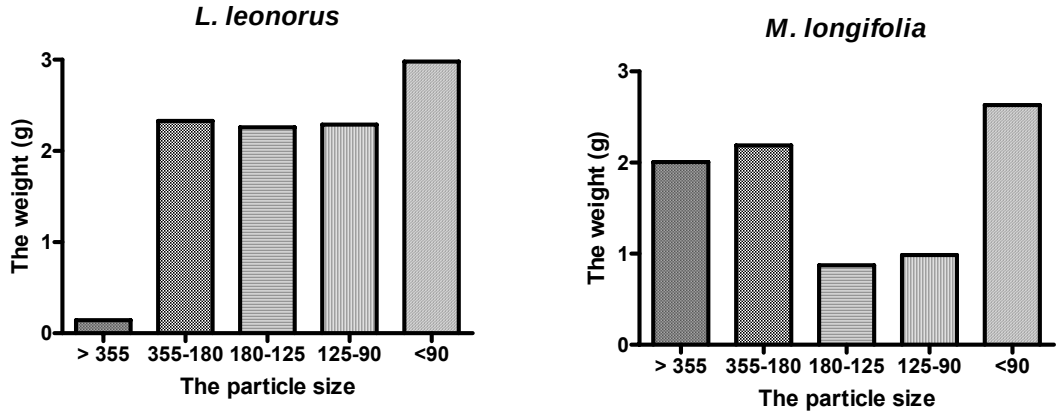


Figure 4-8. *Particle size of L. leonorus and M. longifolia freeze-dried extract powders.*

According to the above results, the *L. leonorus* and *M. longifolia* freeze-dried aqueous extract powders were both categorized as moderately fine powders

based on the British Pharmacopoeia (British Pharmacopoeia 2000[1]) standard. Their particle shapes were irregular. The degree of sphericity (θ) of *L. leonorus* freeze-dried aqueous extract powder was 0.6050±0.1173 and *M. longifolia* freeze-dried aqueous extract powder was 0.6913±0.1119 (see appendix 5). Generally, if θ approaches 1, the particle is close to sphericity. The results obtained showed that the *L. leonorus* and *M. longifolia* freeze-dried aqueous extract powders possessed appropriate flowability for the manufacture of the capsule dosage form, and the 'moderately fine powder' classification implied that the powder possessed a relatively bigger surface area and could be easily dissolved by water. The same particle size result was also found in a study on the *Artemisia afra* freeze-dried aqueous extract powder (Komperlla, 2004).

4.4.5 The densities of the freeze-dried aqueous extract powders.

According to Carr, powders can be described under 6 classifications as shown in Table 4-6.

***Table 4-6**. Carr's index as an indication of powder flow (Wells, 2002)*

Carr's index (%)	Type of flow
5 -15	**Excellent**
12 - 16	**Good**
18 - 21	**Fair to passable**
23 - 35	**Poor**
33 - 38	**Very poor**
>40	**Extremely poor**

The results of the determinations for the extracts are summarized in Table 4-1 and appendices 6 and 7. The Carr's index of Compressibility for *M. longifolia* extract is 7.193±1.805% for *L. leonorus* is 13.24±1.015%. The density study results shows that the extract of *L. leonorus* and *M. longifolia* freeze-dried aqueous extract powders can all be categorized as having excellent or good

flow properties. And these results show a better flowability compared to the other plant extracts viz. 18.233±1.7785% for the *Artemisia afra* freeze-dried aqueous extract powder (Komperlla, 2004)

4.4.6 The flowability of the freeze-dried aqueous extract powder.

There is usually an empirical relationship between angle of repose (**θ)** and the ability of the powder to flow as expressed in the table 4-7.

Table 4-7. *Angle of repose as an indication of powder flow properties (Wells. 2002).*

Angle of repose (degrees)	Type of flow
<20	**Excellent**
20-30	**Good**
30-34	**Passable**
>40	**Very poor**

The results of the determinations on the powders are given in appendix 8. The *L. leonorus* and *M. longifolia* freeze-dried aqueous extract powders had angles of repose of 34.83±1.063°and 38.52±5.332°, respectively. Both therefore had passable flow properties. The above results are similar to those obtained in a previous study on the *Artemisia afra* freeze-dried aqueous extract of 37.736± 3.0564°(Komperlla, 2004).This implicated that the *L. leonorus* and *M. longifolia* freeze-dried aqueous extract powders possessed appropriate flowability for the manufacture of capsule dosage form.

4.4.7 The ash values for the extract powder powders.

The total ash and acid insoluble ash values for the freeze-dried aqueous extract powders of *L. leonorus* and *M. longifolia* are given in appendices 9 and 10. For *L. leonorus* extract the total and acid insoluble ash were 17.2±0.460%

and 1.41±0.190% respectively. For *M. longifolia* was 20.98±0.257% and 2.28± 0.377%. This ash testing was the first ash testing for *L. leonorus* and *M. longifolia* aqueous extracts. And this result could be used for future reference.

4.4.8 The moisture content of the extracts.

The percentage of moisture content of *L. leonorus* and *M. longifolia* extracts are given in appendices 11 and 12. The results were 4.89±0.2354% and 8.398 ±0.1357% respectively. The *L. leonorus* freeze-dried aqueous extract showed comparable moisture content to the *Artemisia afra* freeze-dried aqueous extract powder of 5.000±0.5429% (Komperlla, 2004). Moisture content is an important parameter for capsule dosage form and it is also important for herbal medicines, which are hygroscopic. Moisture content cannot represent the hygroscopic property of the powder, but it can serve as a reference for quality control (e.g. stability study).

4.4.9 The microbial quality of the extracts.

The results of the determination of the microbial contamination of the extracts are summarized in appendices 13 and 14. The limitation criteria of microbial contamination were in compliance with European Pharmacopoeia (European Pharmacopoeia 4th edition[1]) as follow:

CATEGORY 4: Herbal remedies consisting solely of one or more vegetable drugs (whole, reduced or powdered)

B. Herbal remedies to which boiling water is not added before use

(a) Total viable aerobic count. Not more than 10^5 aerobic bacteria and not more than 10^4 fungi per gram or per milliliter.

(b) Not more than 10^3 enterobacteria and certain other Gram-negative bacteria per

gram or per milliliter.

(c) Absence of Escherichia coli (1 g or 1 ml).

(d) Absence of Salmonella (10 g or 10 ml).

The microbial contamination testing results on the extracts were all within the limitation.

4.4.10 The wet ability of the extract powders.

The contact angle of *L. leonorus* and *M. longifolia* extract powders were 6.75± 1.581° and 6.5±1.512°. both of them <90°(see appendix 15), and this implied that they all could be wetted spontaneously. There is currently no contact angle testing results for other plant extracts can be compared to the best of our knowledge.

4.5 Conclusions.

This part of the study was intended to evaluate the physical-chemical properties of the *L. leonorus* and *M. longifolia* aqueous extracts powder. The results obtained provided important information for the formulation design.

The yields of freeze-dried aqueous extract obtained from *L. leonorus* and *M. longifolia* was 18.42±0.9432% and 12.42±1.281% respectively. It shows a lower level of yield compared with other plant extracts. The solubility testing revealed that the *L. leonorus* and *M. longifolia* aqueous extract powders were sparingly soluble in water. Particle size and shape determinations showed that the *L. leonorus* and *M. longifolia* aqueous extracts powder were moderately fine powders with irregular particle shapes. While the density studies and

angle of repose results revealed that the *L. leonorus* and *M. longifolia* aqueous extract powders had good flow properties. The moisture content tests showed that the *L. leonorus* aqueous extract contained comparable moisture to other plant extract, but *M. longifolia* aqueous extract contained higher level of moisture. The microbial quality tests revealed that *L. leonorus* and *M. longifolia* aqueous extracts powders were safe to be used as pharmaceutical raw materials. Finally, the wet ability testing showed that *L. leonorus* and *M. longifolia* aqueous extract powders were highly wettable.

In general, *L. leonorus* and *M. longifolia* aqueous extract powders were thus suitable plant raw materials for hard capsule manufacture.

CHAPTER 5

FORMULATION, MANUFACTURE AND EVALUATION OF *LEONOTIS LEONORUS* AND *MENTHA LONGIFOLIA* CAPSULES

5.1 Introduction.

In this chapter, the equipment and materials and procedures used in the formulation, manufacture and evaluation of the capsules containing freeze-dried aqueous extracts of *Leonotis leonorus* and *Mentha longifolia* are presented.

5.2 Equipment and materials

The following equipment was used in the formulation, manufacture and the evaluation of the capsules:

Spectrophotometer (*DU 640 spectrophotometer, Beckman, USA*); dissolution tester (*VK 700 Vankel, Optolabor, South Africa*); millipore Filter Unit (*Cameo 25 AS, DDA 02025So MSI: Micro Separation Inc., USA*); vortex-2 mixer (*G-560E, Manufactured By Scientific Industries, Inc. Bohemia, N.Y. 11716 USA*); centrifuge (*Labofuge 200, Germany*); pH meter (*Basic 20 Crison Instruments, S.A.*); capsule filler (*S. L. Sanderson and Co. Canada*); micropipette (*Gilson Medical Electronics (France) S.A*); Oven *(Memmert 854 Schwabach, West Germany);* HPLC system consisting of an auto sampler (*Beckman Gold Module 507*), a programmable binary gradient pump (*Beckman Gold Module 126 series*), a diode array detector (*Beckman Gold Module 168 series*) with a 32-Karat™-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; balance (*Model GA 110, Wirsam Scientific & Precision Equipment (PTY) Ltd., West Germany*); "0" Size Capsule filler (*S.L. Sanderson and Co. 173 Sandy Springs Lane Berry Creek, CA 95916*);

The following materials were used in the formulation, manufacture and the evaluation of the capsules:

Freeze-dried aqueous extract of *Mentha longifolia* and freeze-dried aqueous extract of *Leonotis leonorus (see chpt 4);* hard gelatin capsules (*Size 0; Colour: clear, A White Chemist, Cape Town*); potassium dihydrogen phosphate (KH_2PO_4) (*Holpro Analytics (PTY) LTD. Johannesburg*); potassium chloride (KCl) and ethyl acetate (*Analar grade, Saarchem Merck Chemicals (PTY)*

LTD),South Africa); hydrochloric acid (HCl) (*Analar grade) and me*thanol (*AR) (KIMIX Chemicals & Laboratory Suppliers*); acetonitrile (*HPLC Grade, Allied Signal Inc., USA*);. apigenin ($C_{15}H_{10}O_5$ *Fluka, Germany*); and luteolin ($C_{15}H_{10}O_6$) and dimethyl sulfoxide (*DMSO) (Sigma-Aldrich Co., USA).*

5.3 Methods.

5.3.1 Determination of the amount of freeze-dried aqueous extract per capsule.

In order to formulate and manufacture capsules of *L. leonorus* and *M. longifolia* that would contain an amount of active ingredient equivalent to that found in the traditional liquid dosage forms, the amount of freeze-dried aqueous extract to be used in the *L. leonorus* or *M. longifolia* capsules was decided as described below.

Firstly, decoctions of *L. leonorus* and *M. longifolia* were prepared according to the method given in the literature (see 4.3.1). The decoctions were frozen and then freeze-dried as described in 4.3.1. The masses of freeze-dried aqueous extracts derived from the decoctions were recorded and the yield obtained calculated. Secondly, the active ingredient markers (i.e. flavonoids) in the decoctions and the freeze-dried aqueous extracts derived from those decoctions were separately quantified, using an HPLC assay, and the levels obtained compared. If the marker level in the decoctions and their freeze-dried aqueous extracts was not the same, then the amount of extract to be used was adjusted accordingly.

5.3.2 Formulation and manufacture of the *L. leonorus* and *M. longifolia* capsules.

In this section, the selection of the capsule size, the filling machine, the filling method and the exicipients are described.

Selection of the capsule size: The volume of material that was to be filled into the capsule determined the size of the capsule that was needed. Generally, capsules of sizes “0” to “4” were readily available in the market and the relationship between the capsule size and related body volume are shown in Table 5-1. For pharmaceutical products it is unusual to use a size larger than “0” because of the difficulty in swallowing larger size capsules, whilst size “5” is rarely used due to difficulties in the automatic filling process (Cole, 1987).

Table 5-1 *Capsule size and body fill volumes:*

Capsule size	Body volume (ml)
0	**0.67**
1	**0.48**
2	**0.37**
3	**0.28**
4	**0.20**

Selection of the filling method. It was also necessary to take into account the type of the filling machine that was available and how each type of product is handled (Jones, 2002). Generally, there are two kinds of filling machines, viz. manually operated and automatic filling machines. For this study manual filling equipment i.e. the plate method using the Feton hand operated capsule filler (Figure 5-1) was used for the manufacture of the *L. leonorus* and *M. longifolia* capsules.

Extract | Filled capsule

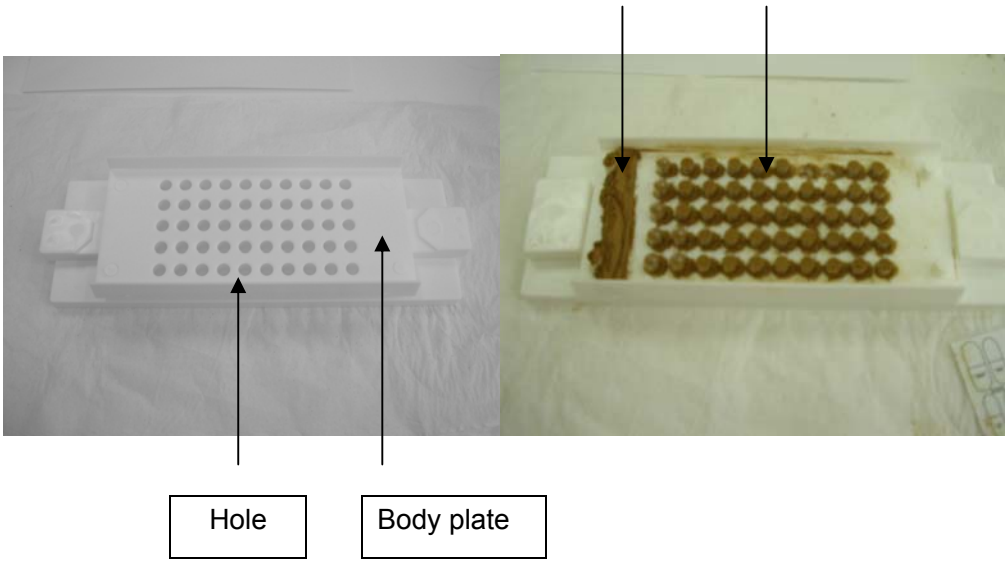


Figure 5-1 *Filling machine and the filling of capsules.*

The plate method was the original method used for filling hard shell capsules. It developed from the hand filling of single capsules procedure used in the middle of the 19th century to its present form and is today still widely used in pharmacies and development laboratories (Cole, 1987). The equipment used for this study consisted of sets of plastic plates, which had predrilled holes to take 50 capsules of size "0". Empty "0" size capsules were placed into the holes by hand with the bodies of the capsule fitting snugly into the plate. The caps of the capsules were then removed, powder placed onto the surface of the body plate (plate containing the capsule bodies) and spread with a spatula so that it flowed into the empty capsule bodies. Because the uniformity of the fill weight was very dependent upon the good flow properties of the powder, the capsule bodies were filled using alternating gentle shaking of the plate, to remove any air pockets, and the spreading of more material into the capsule body. Once filled to the brim, the cap of each capsule was repositioned over the material filled body of the capsule and the two capsule parts rejoined using gentle pressure (Jones, 2002). The quantity of the powder that could be poured into the capsule was more or less established by a trial and error method i.e. alternating shaking with filling until the desired result (i.e. end

weight) was obtained.

Selection of the excipients. The majority of materials poured into capsules are formulated as powders that are typically mixtures of the active ingredient(s) together with a combination of different types of excipients (Jones, 1995). This is so because placing powders into hard capsules often lead to improved bioavailability owing to a higher porosity of the filled mass and the requirements for powder flow is less stringent for filling capsules than for tabletting (Podczeck, 2004).

The last step of formulation of was to choose the excipients. Normally, there are three types of excipients used in powder filled capsules i.e. diluents, glidants and lubricants.

The *diluent,* also known as the filler, is used to increase the bulk volume of the powder. It is also chosen for its plug forming properties and the most frequently used examples are: lactose, starch and microcrystalline cellulose. Diluents are classically defined as inert materials added to a mixture to increase its bulk to a more manageable quantity. Although they are chemically relatively inert, they do play a role in the release of the active substances. In fact, the diluent should be carefully chosen taking the solubility of the active compound into consideration. If a soluble diluent such as lactose is added to a poorly or insoluble compound it will make the powder mass more hydrophilic, enabling it to break up more readily on capsule shell disintegration. The converse is also true: actives that are readily soluble are best mixed with insoluble diluents such as starch or microcrystalline cellulose, because they help the powder mass to break up without interfering with their solubility in the medium (Jones, 2002).

Based on the results of the pre-formulation study, the freeze-dried aqueous extracts of *L. leonorus* and *M. longifolia* were both categorized as being sparingly soluble and hygroscopic. Powders, which tend to adsorb moisture (i.e. are hygroscopic), should not be mixed with excipients such as cellulose or starch that contain larger amounts of water. Instead moisture-regulating excipients such as mannitol or anhydrous lactose can be included (Podczeck, 2004). On the other hand diluents are also not necessary if the dose of the drug is high.

Glidants are materials that reduce inter-particulate friction, so that there is improved flowability of the powder. Examples of glidants include colloidal and anhydrous silica. The concentration of glidant used is important; quantities above 1% tend to decrease the flow rate of the mix while about 0.1% is usually adequate (Cole, 1987).

Lubricants are materials that reduce powder-to-metal adhesion and one example is magnesium stearate. Lubricant use is however very much depended on the type of the machine to be used, e.g. lubricants are typically not needed when simple capsule filling equipment is used.

All the above-mentioned issues, as well as the amount of extract that could be filled into one capsule (see Table 5-1), were carefully considered to arrive at the final formulation for the capsules to be made in this study. Eventually, it was decided that the freeze-dried aqueous extract powders of *L. leonorus* or *M. longifolia* should be the only materials filled into the capsules and based on the extraction yields (see 5.4.1), 365 mg of *L. leonorus* freeze-dried aqueous extract and 314 mg of *M. longifolia* freeze-dried aqueous extract was to be placed in separate size “0” capsules using the capsule filling method described above.

5.3.3 Determination of uniformity of weight and the amount of material in the capsules.

For the determination of the uniformity of weight, the British Pharmacopoeia (British Pharmacopoeia 2000[7]) method was used. Twenty of the *L. leonorus* or *M. longifolia* capsules prepared as described above were taken at random, their contents individually weighed and the average weight (mass) of the content determined. Not more than two of the individual weights (masses) had to deviate from the average weight (mass) by more than 7.5% and none of the deviates by more than twice that percentage.

The amount of powder actually filled into the capsules was also compared with the desired quantity and the difference (in percentage) between the desired and actual quantity calculated. According to the formulation, 365mg of *L. leonorus* freeze-dried aqueous extract and 314mg of *M. longifolia* freeze-dried aqueous extract was to be filled in one capsule each. Twenty capsules were thus randomly chosen, their contents weighed, the percentage difference between this and the desired weight calculated and averaged for the 20 capsules to assess the accuracy of the filling process.

5.3.4 Quantification of the level of flavonoid markers in the plant material in the capsules.

Quantifying the active ingredients in the capsule is important for dosage form quality control and for the plant medicine analysis, the referenced standard (i.e. the marker) and assay method to be used should be decided before the quantification study. In this study, an HPLC method was used.

5.3.4.1 Selection of suitable marker compound(s)

There are usually several ingredients in plant material. To quantify the amount of active material in the capsule, a specific constituent(s) should be chosen as reference material(s).

Flavonoids are virtually ubiquitous in green plants and as such are likely to be encountered in any work involving plant extracts (Markham, 1982). So it was anticipated that flavonoids would be ideal reference material or markers to use for quality control of *L. leonorus* and *M. longifolia* dosage forms.

Luteolin, apigenin, acacetin, diosmetin, hesperetin and eriodictyol are flavonoids that have been found in *M. longifolia* (Bourweig and Pohl, 1973). Very little is however known about the flavonoids that are present in *L. leonorus,* although the results of one study have revealed that flavonoids are also present in this plant (Ascensao and Marques, 1997).

In this study, several flavonoids i.e. luteolin, rutin, apigenin and quercetin were therefore evaluated as candidate markers for the quality control analysis of the *L. leonorus* and *M. longifolia* capsules. Solutions of these pure flavonoids and the *L. leonorus* and *M. longifolia* freeze-dried aqueous extracts were prepared and assayed using the HPLC method described below (see 5.3.4.2). From these assays chromatograms such as those shown in appendices 18 to 27 were obtained and the compounds identified from their retention times and their peak characteristics (shape and size) evaluated. Eventually, luteolin (Figure 5-2) and apigenin (Figure 5-3) was selected as markers for *L. leonorus* and *M. longifolia,* respectively. Representative chromatograms of these compounds are showed in Appendix 18, 19, 20, 23, 24 and 25. Although luteolin and apigenin were not the only flavonoids detected in the plant materials, they were present in appreciable quantities in the solutions of the respective plant extracts, gave easily identified, well separated

chromatographic peaks with good peak shape and reasonable retention times. The purity of the luteolin and apigenin peaks in *L. leonorus* and *M. longifolia* chromatograms were also confirmed by UV-Diode Array detection (see Appendix 21, 26).

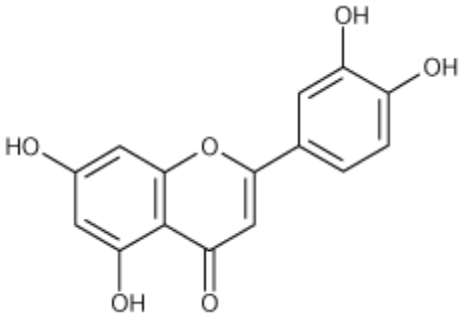


Figure 5-2 *Luteolin ($C_{15}H_{10}O_6$)*

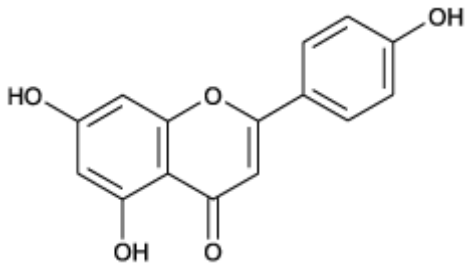


Figure 5-3 *Apigenin ($C_{15}H_{10}O_5$)*

5.3.4.2 HPLC assay for flavonoids in plant materials.

The sample preparation. In this study, the freeze-dried aqueous extracts of *L. leonorus* and *M. longifolia* prepared as described in section see 4.3.1 were used and had to be prepared into samples suitable for HPLC analysis. Generally sample preparation in HPLC assays comprises the removal of any interfering components of the sample material (e.g. plant) matrix, and particulates from the analyte solution, pre-concentration of the analytes and steps to enhance the sensitivity of the assay. Further, the plant samples can be prepared using the hydrolyzed or un-hydrolyzed method, with the latter providing the free flavonoid level and the former method the total flavonoid levels (Markham.1982). The actual methods used to assay for the flavonoids in the two plants were as follows.

To quantitate the free flavonoid levels (i.e. the un-hydrolyzed method), 25 mg of *L. leonorus* or *M. longifolia* extract were weighed, placed in a screw-capped Kimax tube and dissolved in 1 ml of distilled water, 5 ml ethyl acetate added to the extract solutions and the mixture vortex-mixed for 1 minute and centrifuged

for 10 minutes at 3000 rpm. The top layer of ethyl acetate was removed to another clean tube, evaporated to dryness under a gentle stream of nitrogen and the residue reconstituted in 1 ml of mobile phase (for luteolin) or methanol (for apigenin) before injection onto the HPLC.

To quantitate the total (free plus conjugated) flavonoid levels (i.e. the hydrolyzed method), 25 mg of *L. leonorus* or *M. longifolia* were weighed, placed in screw-capped tube and dissolved in 1 ml of distilled water. Then 3 ml of 2M hydrochloric acid were added to the solution and the mixture heated at 80℃ for 40 min in a water bath to hydrolyse the sample. Thereafter the solutions were allowed to cool, 5 ml ethyl acetate added, and the mixture vortex-mixed for 1 minute and centrifuged for 10 minutes at 3000 rpm. The top layer of ethyl acetate was removed, evaporated to dryness under a gentle stream of nitrogen and the residue reconstituted in 1ml of mobile phase (for luteolin) or methanol (for apigenin) before injection on the HPLC.

To quantify the level of the flavonoids in the plant material contained in the capsules, the hydrolyzed method was used, but for the stability testing (5.3.8), both the hydrolyzed and un-hydrolyzed methods were used.

The standard curve. As indicated earlier, luteolin and apigenin were selected as markers for the HPLC analysis of the *L. leonorus* and *M. longifolia*, respectively. Pure luteolin and apigenin were prepared as 1 mg/ml stock solutions (dissolved in DMSO) and from these, standard solutions of different concentrations (i.e. 5 µg/ml, 10 µg/ml, 15 µg/ml, 25 µg/ml, 25 µg/ml and 50 µg/ml) of luteolin (dissolved in water) or apigenin (dissolved in methanol) were prepared. One milliliter samples of the standard solutions were extracted as described above, and eventually 50µl of the reconstituted solutions injected onto HPLC and the markers eluted under the following conditions: mobile

phase consisting of 30% acetonitrile and 70% KH_2PO_4 buffer (pH 1.0); isocratic elution; flow rate of 1ml per minute; C18 reverse phase column (Phenomenex, 4μm particle size and 250 x 4.60 mm); spectrophotometric detector wavelength of was 349 nm(λ); and HPLC system consisting of auto sampler (Beckman Gold Module 507), programmable binary gradient pump (Beckman Gold Module 126 series), diode array detector (Beckman Gold Module 168 series) 32-Karat™-software package.

To identify the luteolin or apigenin, the retention times of the peaks representing the marker compounds were noted and to quantitate the level of the marker compound the peak height measured in the computer-stored chromatograms was used. Finally, a graph of the concentrations *vs*. peak height was plotted and the curve analysed by least squares linear regression method using the Graphpad Prism 4 program (Graphpad Prism 4, 2003). To determine the sensitivity (quantitation limit), precision and linear concentration range of the method, tubes containing several appropriate concentrations of the two markers were prepared and analyzed as described above.

Quantification of the flavonoid in the plant material in the capsules. After the extraction and HPLC elution of samples of the plant materials, the chromatograms of *L. leonorus* and *M. longifolia* samples were acquired and the peak representing luteolin or apigenin identified by comparing the retention times. The peak height was then measured from the HPLC computer generated chromatogram and the quantity of luteolin or apigenin in the capsule determined by correlating the peak heights with the corresponding concentrations in the concentrations *vs*. peak height standard curves. Samples of the plant material were also spiked with known amounts of marker compounds, assayed and the percent recovery calculated to further validate the assay.

5.3.5 Determination of moisture content of *L. leonorus* and *M. longifolia* capsules.

For this study, the shell of the capsules was removed and the moisture level of the contents of the capsules determined using the European Pharmacopoeia (European Pharmacopoeia 4th edition 2002[2]) method described earlier under section 4.3.8. entitled “Determination of the moisture content of extracts”.

5.3.6 Determination of the microbial contamination of *L. leonorus* and *M. longifolia* capsules.

The same microbial testing methods described in section 4.3.9 - Microbial quality test - was used. Again the contents of the capsules were assessed and the European Pharmacopoeia criteria/specifications on microbial contamination were used to assess the acceptability of the level of microbial contamination of the capsules (European Pharmacopoeia 4th edition 2002[1]).

5.3.7 Determination of the dissolution profile of *L. leonorus* and *M. longifolia* capsules.

The dissolution test measures the rate at which a drug is released into solution from a dosage form and is used as an indication of the bioavailability of a pharmaceutical product and of product quality. In order to simplify testing procedures the major pharmacopoeias have chosen to standardize on three apparatuses: the revolving basket, the paddle and the flow through cell (Jones, 2004). In this study the basket method (Figure 5-4) was used. Further, the quantitation of the amount of plant material dissolved was measured based on UV absorbance measured at 280 nm for *L. leonorus* and 285 nm for *M. longifolia*, the wavelengths for maximum UV absorption of solutions of the *L. leonorus* and *M. longifolia* freeze-dried aqueous extracts determined from the

spectrophotograms obtained over 190 to 500 nm. The dissolution samples could not be analyzed using the HPLC assay described above, because the maximum concentration possible in the dissolution medium (calculated to be 0.184 μg/ml of luteolin for *L. leonorus* and 0.425 μg/ml of apigenin for *M. longifolia)* was too low to be detected.

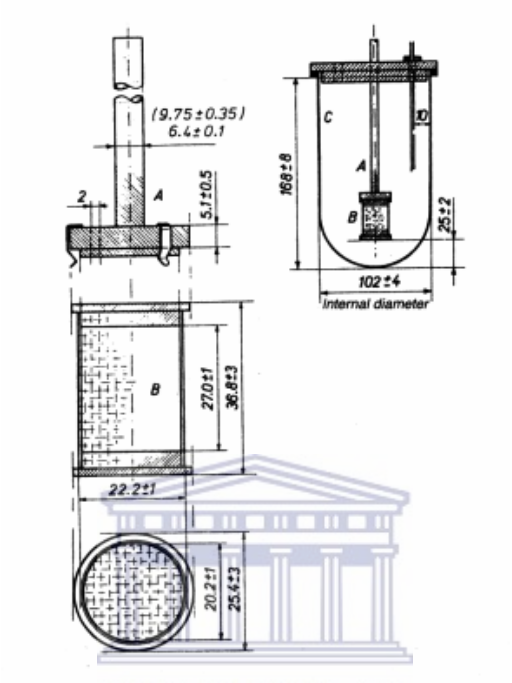


Figure 5-4 *Basket apparatus (Dimensions in mm) Appendix XII D. Dissolution Test for Tablets and Capsules (Dissolution Test for Solid Dosage Forms) (from British Pharmacopoeia).*

Consequently, for the dissolution study, 900 ml of pH 1.2 hydrochloric acid buffer was degassed, introduced into the vessel of the apparatus, warmed to 37±0.5°Cin the water bath. One capsule was placed in a dry basket, the basket was lowered into position and the apparatus were operated immediately at the rotation speed 100 rpm. At various time points, viz. at 5, 10, 15, 25, 35, 45 and 60 minutes after start, 3 ml samples of the medium was withdrawn from a point halfway between the surface of the dissolution medium and the top of the rotating basket and not less than 10 mm from the wall of the vessel. Each time the withdrawn medium was immediately replaced by 3 ml of pH 1.2 hydrochloric acid buffer introduced into the vessel. The samples were filtered

and the UV absorbance of the solution was determined at the wavelengths mentioned earlier and using the solution of one of the empty capsule shell dissolved in the 900 ml volume of dissolution medium as a blank reference solution. From the standard curve of known concentrations of plant material *versus* UV absorbance, the amounts of dissolved plant material in the dissolution media were determined, the percent material dissolved at each time point calculated and the percent dissolved over time profiles for each plant capsule derived (appendixes 38 and 39). For each plant product the dissolution profile of 6 capsules were determined and for each capsule not less than 70% of the stated amount had to dissolve within 45 min in order for the product to meet the specification for immediate release (British Pharmacopoeia 2000[6]). If one of the capsules did not meet this requirement a further six had to be tested individually and all had to comply.

5.3.8 Determination of stability of *L. leonorus* and *M. longifolia* capsules.

Degradation of active drug is the major and most important cause of the drug product stability changes. Drug degradation occurs by four main processes: hydrolysis, oxidation, photolysis, and trace metal catalysis. The most likely cause of drug instability is hydrolysis. Water plays a dominant role and in many cases it is implicated passively as a solvent vector between two reacting species in solution (Wells, 2002). Thus storage of the product in atmospheres of high humidity will typically accelerate decompositions that result from hydrolysis. Also, an increase in temperature causes an increase in the rate of chemical reaction (Pugh, 2002). Further, the information on the stability of a product should include, as appropriate, the results from physical, chemical, and biological tests, including those related to particular attributes of the dosage form. Thus information on the dissolution rate and changes in organoleptic properties for solid oral dosage forms; the level of active ingredient and moisture content for plant medicine, etc. are useful product

stability indicating information.

For this study, the capsules were stored without packaging under two conditions: (A) 40℃±2℃ / 75% ± 5% relative humidity (RH) and (B) room temperature and room RH i.e. 25℃±2℃ / 40% ± 5(RH) (EMEA, 2003). Condition A was as attained by using a climate chamber, while condition B referred to the general customary environment. The manufactured *L. leonorus* and *M. longifolia* capsules (section 5.3.2) were stored under the afore-mentioned conditions and every two weeks samples of capsules were taken from each site and assessed for organoleptic properties (i.e. gross physical nature, color and odor of the powder content and overall size, shape and appearance of the capsule) and the level of flavonoid markers. At the end of 12 weeks the moisture content of the capsules and the dissolution of the capsules were again tested. The organoleptic properties and the moisture level of the content of the test capsules were compared with that of the content of two other batches of capsules wrapped in plastic packaging and stored in the same environmental conditions A and B. The flavonoid levels were determined by HPLC assay as described above (section 5.3.4.2) and the levels obtained for the different storage conditions and various times and at end of storage compared. For the dissolution profile of *L. leonorus* and *M. longifolia* capsules the UV assay method described above (section 5.3.7) was used and the profiles obtained at the start and after 12 weeks compared by determining the similarity factor (f_2). The similarity factor (f_2) (Eqn. 5-1) is a logarithmic reciprocal square root transformation of the sum of squared error and is a measurement of the similarity in the percent (%) dissolution between the two curves (FDA, 1997).

$$f_2 = 50 \log \{[1+(1/n)\sum_{t=1}^{n} (R_t - T_t)^2]^{-0.5} 100\} \quad \text{(Eqn. 5-1)}$$

5.4 Results and discussions.

5.4.1 The amount of freeze-dried aqueous plant extract per capsule.

A summary of the results pertaining to the traditional doses, yields of aqueous extracts and dose equivalence obtained for the *L. leonorus* and *M. longifolia* plant materials are presented in table 5-2. The 90 ml decoction dose of *L. leonorus* yielded 561.70±35.45 mg of aqueous extract (n= 6). From the 2 g in 150 ml *M. longifolia* in water mixture effectively only 117 ml of decoction could be isolated and this yielded 248±25.63 mg of freeze-dried aqueous extract.

Table 5-2 *The traditional doses, yields of aqueous extracts and dose equivalence for the L leonorus and M. longifolia plant materials*

Plant	Traditional dose	*Amount of Aqueous Extract (mg) ± SD (n=6)	**Marker level in decoction dose (x) and Aqueous extract (y) (µg) ± SD (n=6)	***Equivalent amount of aqueous extract (mg) ± SD (n=6)
L. leonorus	**10,0 g dried herb added to 500 ml of boiling water, boil for 10 minutes, allow to cool overnight, and use 90 ml of decoction for**	**561.7 ± 35.45**	**X = 355 ± 18.37 (3.94 ± 0.021 µg /ml)** **Y = 273 ± 7.98**	**730 ± 46.06**

	internal use.		**(0.486 ± 0.0039 µg /mg)**	
M. longifolia	**Use 2 g of dried leaves to 150 ml boiling water let it stand for three minutes and then drink. (117 ml of decoction)**	**248.3 ± 25.63**	**X = 396 ± 6.22 (3.38 ± 0.041 µg /ml)** **Y = 313 ± 5.53 (1.261 ± 0.082 µg/mg)**	**314 ± 32.42**

* *Amount (mg) of aqueous extract derived from the traditional dose (i.e. 90 or 117 ml of the decoction)*

** *Level of luteolin or apigenin in 90 or 117 ml decoction dose (mg and µg/ml) and aqueous extracts (mg and µg/mg) of L. leonorus or M. longifolia, respectively.*

*** *Equivalence between traditional and aqueous extract based on flavonoid levels i.e. this amount of aqueous extract has the same amount of luteolin contained in the volume of decoction (i.e. 90 or 117ml) traditionally consumed.*

When the level of flavonoid markers in the traditional decoctions and aqueous extracts were however compared, the levels in the decoction were significantly ($p_{luteolin} = 0.002$; $p_{apigenin} < 0.0001$, t-test) higher than that in the aqueous extract. Some marker material was obviously lost in the freeze-drying process. This may have been due to degradation and/or the evaporation during the vacuum process. Why or how the loss occurred is unclear at this stage, but had to be taken into account when deciding the dose of extract to use. Based on the marker levels the 90 ml traditional decoction dose of *L. leonorus* and the 117 ml of *M. longifolia* were calculated to be equivalent to 730±46.06 and 314± 32.42 mg of aqueous extracts, respectively. Consequently the amount of aqueous extract per capsule was set at 314 mg for *M. longifolia* and 365 mg (i.e. half of 730 mg) for the *L. leonorus.* It was clear that 730 mg of *L. leonorus* extract would not fit into one size '0' capsule, but quite easily into two such capsules.

The above results thus indicated that the flavonoid marker levels in traditional decoctions of herbal medicines and the freeze-dried aqueous extract derived from such decoctions may differ significantly and this had to, and could be, taken into account when formulating the equivalent capsule dosage form. The appropriateness of this strategy and whether the same results would be found with other marker compounds obviously needs further investigation.

5.4.2 Evaluation of the manufactured *L. leonorus* and *M. longifolia* capsules

Several factors had to be considered in the formulation and manufacture of the capsules. Largely, the bulk density of the contents governed the size of the capsule shell that could be used (Cole, 1987). The results of the pre-formulation study (section 4.4.5) indicated that the bulk densities of *L. leonorus* and *M. longifolia* freeze-dried aqueous extracts were 0.5063±0.01349 g/ml and 0.4556±0.02703 g/ml, respectively and, from the results in section 5.4.1 and table 5-2, that 730 mg and 314 mg, respectively, were the single doses of each that was required. Given this information, it was decided for practical reasons to use size “0” capsules for both preparations and, to split the actual bulk of one dose of *L. leonorus* freeze-dried aqueous extract (i.e. 730mg), into two capsules. Capsules containing 365mg *L. leonorus* and 314mg *M. longifolia* were thus manufactured using the plate method (see 5.3.2) and pictures of the final products produced are shown in Figure 5-5. By and large the products had a highly acceptable pharmaceutical appearance and the results of the evaluation of various aspects of these capsules are given below.

Figure 5-5 *Capsules of the aqueous extracts of L. leonorus and M. longifolia*

5.4.2.1. Uniformity of weight and content of capsules.

The results of the uniformity of weight and content of the *L. leonorus* capsules and *M. longifolia* capsules are given in appendices 16 and 17. The average deviation in weight from average for *L. leonorus* and the *M. longifolia* capsules were 1.19±1.78% and 1.58±1.24%, respectively and the average total content per capsule 101.6±1.43% and 102.8±2.14%, respectively. According to the British Pharmacopoeia (British Pharmacopoeia, 2000[5,7]), the limit on the acceptable deviation in weight from average for capsules is ±7.5% and the limits on the amount of content in the capsules 90% to 110%. The afore-mentioned results thus indicated that both the *L. leonorus* and *M. longifolia* capsules met the British Pharmacopoeia specifications.

5.4.2.2. Level of flavonoid markers in the capsule plant material

The levels of luteolin or apigenin in the capsuled plant material were determined using a sensitive validated HPLC assay method. Representative chromatograms of the markers in standard solutions and the plant materials are given in appendices 18 to 27, the representative standard curves for luteolin and apigenin in figures 5-6 and 5-7 and a summary of the assay

validation results in table 5-3. The assays were reproducible, sensitive and robust enough for use in this study.

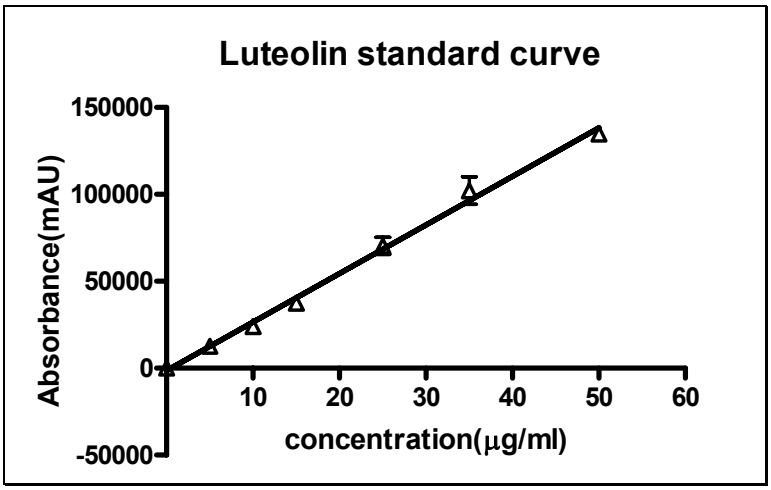


***Figure 5-6**. Luteolin standard curve. Linear regression equation: Absorbance(mAU) = -1445 + 2796* Conc (μg/ml), r^2=0.9911 (n=6)*

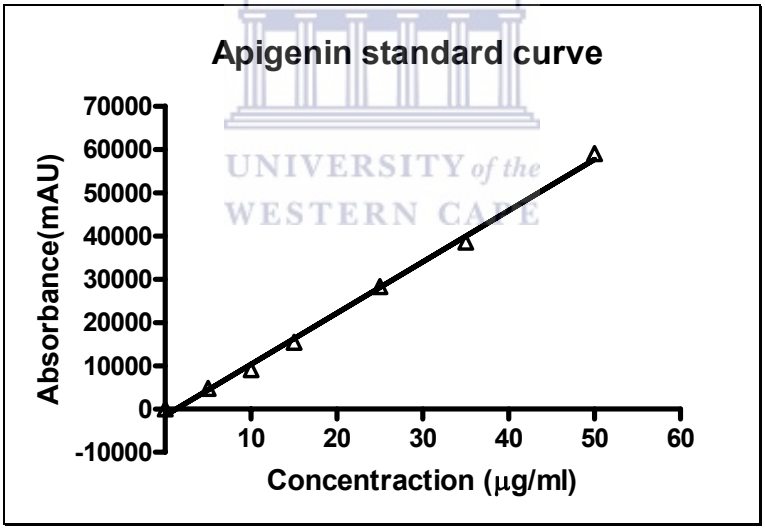


***Figure 5-7.** Apigenin standard curve. Linear regression equation: Absorbance(mAU) = -1515 + 1185* conc (μg/ml); r^2=0.9966 (n=6)*

***Table 5-3** Validation parameters of the HPLC assays for luteolin and apigenin.*

Validation Parameter	Luteolin Assay	Apigenin Assay
Linearity – Regression coefficient (r^2)	0.9911	0.9966
Quantitation limit (ng on column)	5	6
Inter-assay precision (RSD in 3 days) (%)	5.78	2.49

Using this HPLC assay the amounts of luteolin and apigenin per *L. leonorus* or

M. longifolia capsule were determined and the results obtained are given in Appendices 29 and 32. The average amount of luteolin per *L. leonorus* capsule was 165.3±1.629 µg with %RSD of 0.99% while the average amount of apigenin per *M. longifolia* capsule was 382.1±10.77 µg with %RSD equal to 2.82%. To confirm the accuracy of the assay for the markers in the capsules recovery studies were also done (i.e. capsule plant material spiked with known amounts of markers and then assayed) and the results obtained from these studies are given in appendices 30 and 33. The average percent recoveries and RSD for luteolin in *L. leonorus* and apigenin in *M. longifolia* were 97.24 and 3.3% and 83.40 and 9.16%, respectively. While the accuracy of the assay for apigenin in the *M. longifolia* was lower, and the content uniformity based on the determination of marker levels in only 6 capsules, the capsules still complied with the British Pharmacopoeia (British Pharmacopoeia 2000[8]) specification i.e. based on marker levels the capsules still met the content uniformity specification (85-115%). Collectively, these results suggested that the capsules produced met all the content uniformity specifications.

5.4.2.3 Moisture level of the content of *L. leonorus* and *M. longifolia* capsules.

After the capsules were filled the moisture level of its contents were again (within 5 days) tested just to ascertain if there had been changes in moisture level during the manufacturing procedure. The results of these tests are given in appendices 34*A* and 35*A* and indicated that the moisture level of the contents of the *L. leonorus* and *M. longifolia* capsules were 7.15±0.38% and 8.76±0.45%, respectively. When analysed in the pre-formulation study, the moisture content for the *L. leonorus* and *M. longifolia* extracts (given in Appendix 11 and 12) were however 4.89±0.23% and 8.39±0.13%, respectively.

There thus appeared to have been a slight increase in the moisture level of the *L. leonorus* material after encapsulation. This suggested that this extract absorbed some moisture during the filling procedure, presumably because it was more hygroscopic than the *M. longifolia* extract. Since the moisture absorbed may speed up degradation, the humidity conditions during the manufacture of the capsules can thus be a crucial factor and these capsules should preferably be manufactured under more tightly controlled humidity conditions.

5.4.2.4 Microbial contamination of *L. leonorus* and *M. longifolia in* capsules.

The microbial contamination test results for the *L. leonorus* and *M. longifolia* capsule contents are given in appendices 36 and 37 and showed that even though the capsule material was not irradiated (as was the case with the aqueous extract tested in the pre-formulation study) all the preparations continued to meet the European Pharmacopoeia limit on microbial contamination (see 4.4.9) (European Pharmacopoeia 4th edition[1]). This specification was in fact even more stringent than that recommended by WHO (WHO, 2005). So, while herbal products often have high levels of microbial contamination e.g. in case of crude ginseng radix (Nakajima, et al, 2005) or general herbal preparations (Adeleye et al, 2005; Aschwanden, 2001). The results from the present study do suggest that the preparation and use of the aqueous extract of these plant materials in capsule form bring and keep microbial contamination levels to acceptable levels.

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5.4.2.5 Dissolution profile of *L. leonorus* and *M. longifolia* capsules.

The results of the dissolution studies on the *L. leonorus* and *M. longifolia* capsules are summarised in Figures 5-8 and 5-9 and showed that >70% of the *L. leonorus* or *M. longifolia* capsule contents dissolved in the dissolution medium within 45 minutes. These results are within the specification set in the British Pharmacopoeia (British Pharmacopoeia 2000[6]) and indicated that both the *L. leonorus* and *M. longifolia* capsules were immediate release solid oral dosage forms with good *in vitro* bioavailability.

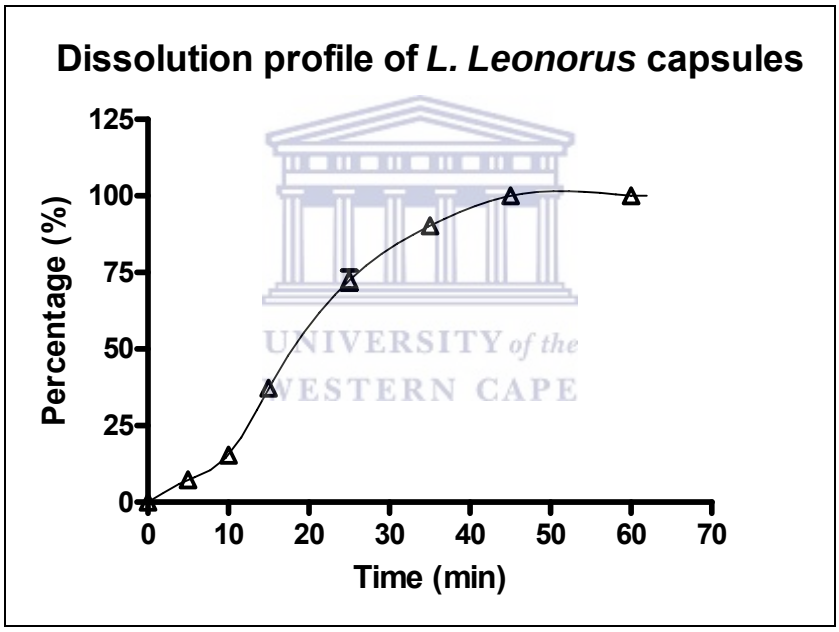

Figure 5-8. *Dissolution profile of L. leonorus capsules. Dissolution conditions: basket method, 900ml pH 1.2 hydrochloric acid buffer; 37±0.5℃; samples quantitated by UV assay.*

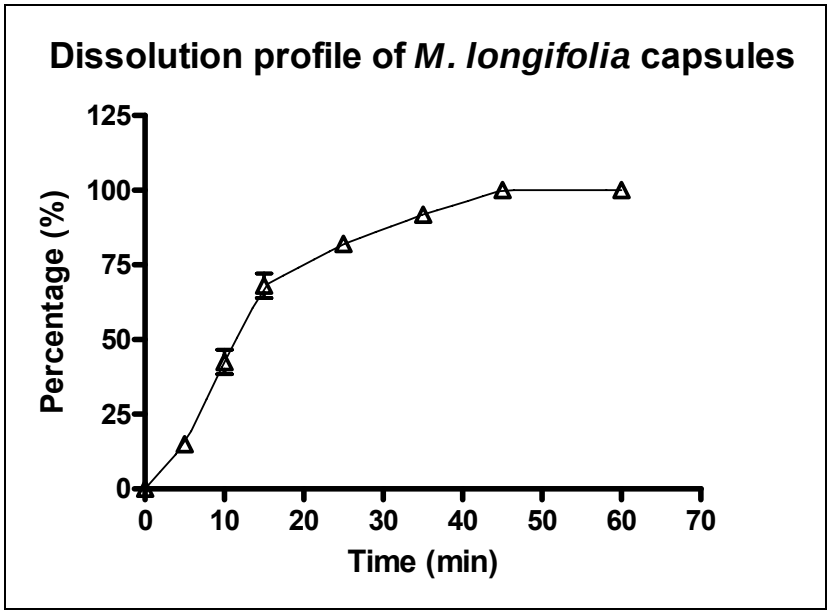

Figure 5-9*. Dissolution profile of M. longifolia capsules. Dissolution conditions: basket method, 900ml pH 1.2 hydrochloric acid buffer; 37±0.5℃; samples quantified by UV assay.*

5.5.2.6 Stability of *L. leonorus* and *M. longifolia* capsules.

A summary of the changes in the organoleptic properties that occurred during storage of the *L. leonorus* and *M. longifolia* capsules at 40±2℃ / 75%±5% relative humidity (RH) and at room temperature and relative humidity (RH) over 12 weeks period are given in Appendices 40 and 41. For the study of the organoleptic properties four batches of capsules were stored under the different conditions (see Table 5-4).

Table 5-4 *The storage conditions for the stability study of the L. leonorus or M. longifolia capsules.*

Batch #	Temperature℃	Relative humidity (RH)	Container
1	*Room temperature	**Room relative humidity	Outside container
2	Room temperature	Room relative humidity	In container
3	40±2 ℃	75%±5% (RH)	In container

4	40±2 ℃	75%±5% (RH)	Outside container

**** 25℃±2℃ ** 40% ± 5%(RH)***

The *L. leonorus* or *M. longifolia* capsules that were not in a plastic container and stored at high temperature (40±2 ℃) and relative humidity (75%±5% RH) underwent drastic changes in physical integrity within 2 days (i.e. the capsule shell broke and the capsule content liquefied). So, this batch of capsules could not be further evaluated for their organoleptic properties, dissolution profiles and moisture content.

For the *L. leonorus* or *M. longifolia* capsules stored at room temperature and room humidity and outside the container (batch 1 in Table 5-4), the size and shape of the capsules were maintained but very obvious changes occurred in most of the organoleptic properties (i.e. gross nature, colour and odours) of the capsule content in the first 2 weeks of storage. The *L. leonorus* powder first became hard and crispy, then hard and rubbery and eventually slightly sticky, changed in colour from brown to deep brown and quickly lost most of its strong odour. Similar changes occurred in the *M. longifolia* material – the powder became hard and crispy losing its crispiness towards the end of the storage period, changed in colour from light brown to deep brown and also quickly lost most of its strong distinctive odour. Clearly, when these capsules were stored at room temperature and relative humidity unprotected from light (i.e. outside a plastic container) significant deterioration occurred in the plant materials.

When the *L. leonorus* or *M. longifolia* capsules were stored in the plastic container, whether at room temperature and relative humidity or high temperature and relative humidity i.e. 25±2℃ / 40%±5% RH and 40±2℃ / 75%±5% RH (batches 2 and 3 in Table 5-4), the organoleptic properties of the plant material remained relatively unchanged during the 12 weeks storage.

Some agglomeration of the powder (i.e. clumping) occurred towards the latter stages of the storage period in the case of *L. leonorus* (appendix 40) stored at room conditions, while, interestingly, no such clumping was obvious in the *M. longifolia* material even after 12 weeks storage. Perhaps this was due to the *L. leonorus* extract being more hygroscopic than *M. longifolia* material. When both sets of capsules were stored under the higher humidity condition the clumping of the powders occurred sooner, for both after 6 weeks storage.

Collectively, the above results suggested that the capsules of *L. leonorus* and *M. longifolia* freeze-dried extract were not very stable upon storage and that the hygroscopic nature or moisture content of the extracts may possible contribute to this state of affairs.

When the moisture levels of the *L. leonorus* and *M. longifolia* capsule contents were determined the results depicted in figures 5-10 and 5-11 were obtained.

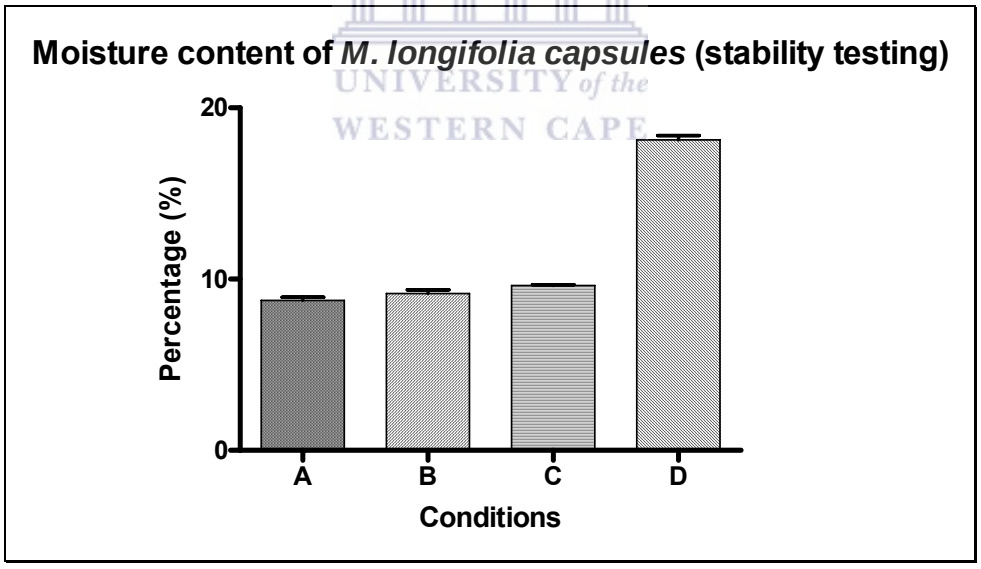

Figure 5-10*. Moisture content of M. longifolia capsules. (A) Before storage; (B) After 12 weeks in plastic container at room temperature and relative humidity; (C) After 12 weeks in plastic container at 40±2℃ and 75% ± 5% RH; and (D) After 12 weeks outside the container and at room temperature and relative humidity.*

For *M. longifolia* there was no change in the moisture levels, irrespective of the

temperature and humidity storage conditions, as long as the capsules were stored inside the plastic container. For the *M. longifolia* capsules stored at room temperature and humidity but outside the container, there was a significant ($p<0.0001$, t-test) increase in the moisture content of the plant material (see Figure 5-10 D). When stored at the higher temp and humidity conditions outside the container the plant material actually liquefied. Clearly, the *M. longifolia* extract in the capsules required protection against moisture. Furthermore, because there was no significant difference ($p=0.1204$, t-test) in the moisture level of the capsule contents stored in the container, whether at room or higher temperature conditions (see B and C in figure 5-10), strongly suggesting that moisture/humidity rather than temperature was the significant factor driving the organoleptic degradation of the stored *M. longifolia* capsules.

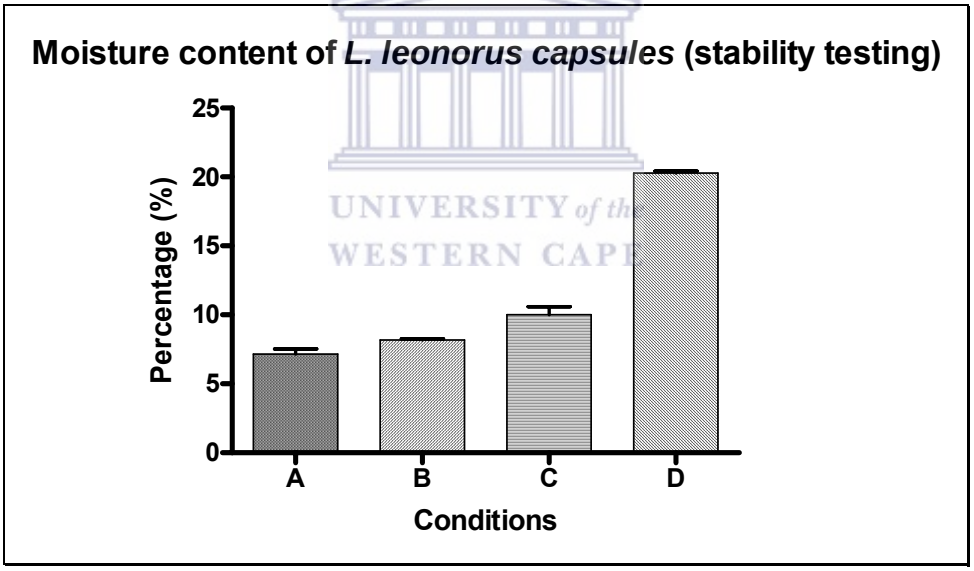

Figure 5-11. *Moisture content of L. leonorus capsules. (A) Before storage; (B) After 12 weeks in plastic container at room temperature and relative humidity; (C) After 12 weeks in plastic container at 40±2℃ and 75% ± 5% RH; and (D) After 12 weeks outside the container and at room temperature and relative humidity. * Significantly different from A before storage*

Similar to the case of the *M. longifolia* capsules there was also a significant increase ($p<0.0001$, t-test) in the moisture content of the plant material in *L.*

Leonorus capsules stored outside the container under room conditions (Figure 5-11 D). In this case however there were also significant increases (P=0.0058, t-test) in the moisture content for the capsules stored in the containers under high temperature and RH *vs.* room temperature and RH conditions (Figure 5-11 B *vs.* C) as well as that stored under high temperature and RH *vs.* before storage ($p<0.0001$, t-test) (Figure 5-11 A *vs.* C). In this case temperature might thus also, along with moisture, be a significant contributory factor driving the degradation. Alternatively, it might just be that the higher hygroscopic nature of the *L. leonorus* caused this particular set of results.

Taken together, the results strongly suggested that storage in plastic containers significantly lowered the moisture content of the *L. leonorus* and *M. longifolia* capsule contents and that the humidity of the environment and/or hygroscopicity of the plant material have a greater influence than temperature on the stability of *L. leonorus* or *M. longifolia* capsules. It must however also be noted that, the effect that the exposure to natural light might also have were not assessed in the present study.

Apart from the capsules containing the *L. leonorus* and *M. longifolia* aqueous extract not being particularly stable upon storage (i.e. will not have a long effective pharmaceutical shelf-life) it was also important to ascertain how the capsules would perform as far as releasing their contents was concerned. A comparison of the dissolution profiles of the *L. leonorus* or *M. longifolia* capsules tested at the beginning and end of the storage period obtained in this study are shown in Figures 5-12 and 5-13.

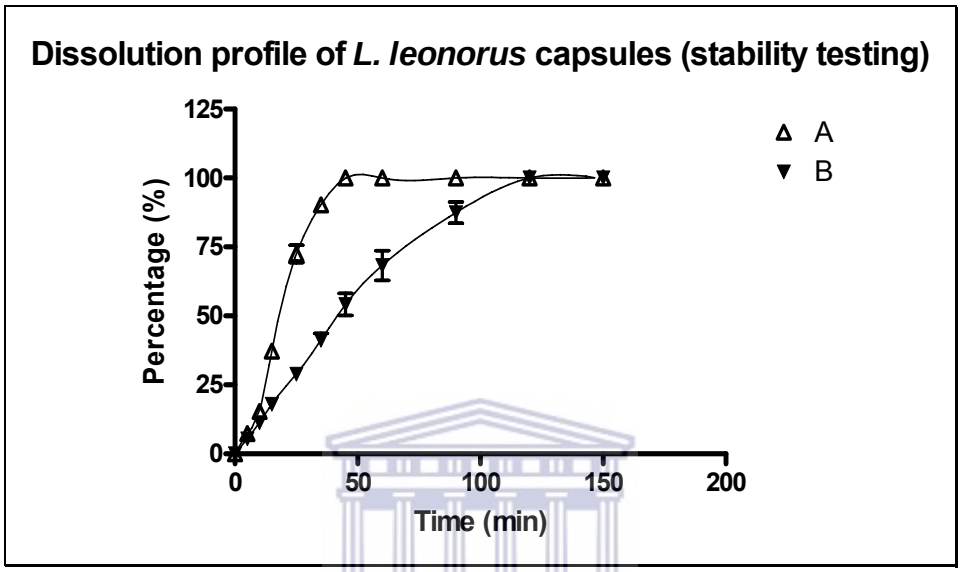

Figure 5-12. *Dissolution profile of L. leonorus capsules. (A) Dissolution profile before storage; (B) Dissolution profile after 12 weeks storage outside the container at room temperature and relative humidity. Dissolution conditions: basket method, 900ml pH 1.2 hydrochloric acid buffer; 37±0.5℃; samples quantitated by UV assay.*

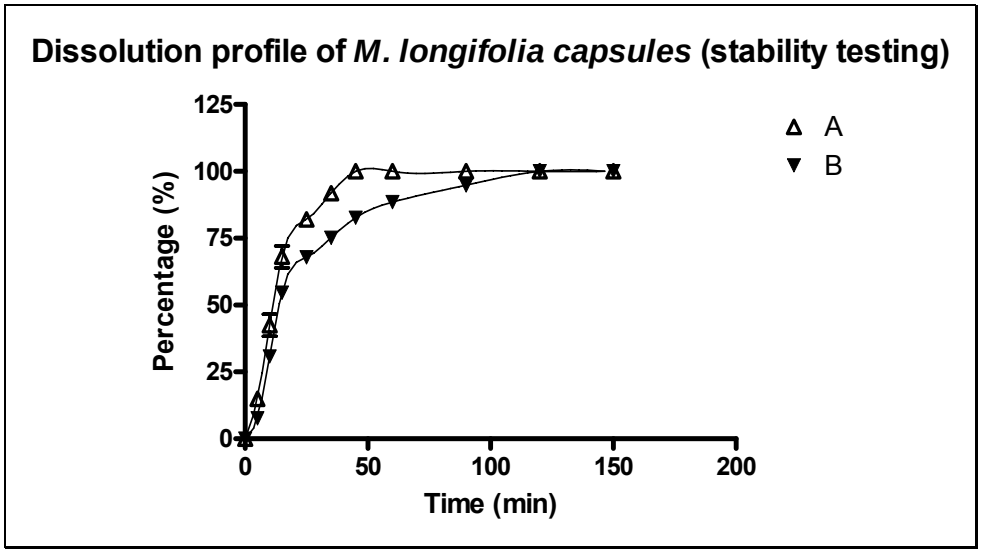

Figure 5-13. *Dissolution profile of M. longifolia capsules. (A) Dissolution profile before storage;*

(B) Dissolution profile after 12 weeks storage outside the container at room temperature and relative humidity. Dissolution conditions: basket method, 900ml pH 1.2 hydrochloric acid buffer; 37±0.5℃; samples quantitated by UV assay.

After 12 weeks storage at room temperature and relative humidity outside a container, the *M. longifolia* but not *L. Leonorus* capsules were still within the British Pharmacopoeia single point specification for a rapid release oral dosage form, i.e. >70% of the capsule contents dissolved in the dissolution medium within 45 minutes (British Pharmacopoeia, 2000[6]). However, the dissolution characteristics of both capsules had changed significantly upon storage, more so for the *L. leonorus* capsules (Figure 5-12) for which a similarity or f_2 value of 25.06 was obtained than the *M. longifolia* capsules (Figure 5-13) for which the f_2 value was 44.61. The dissolution profiles are normally considered similar when the f_2 values are at least above 50 and closer to 100 (MCC, 2005). The results obtained in the present study thus indicated that there were substantial changes in the dissolution profiles of *L. leonorus* and *M. longifolia* capsules after 12 weeks storage, in the open, under conditions of normal room temperature and humidity. One would thus also anticipate differences in the bioavailability of these stored capsules.

Finally, the results of the experiments assessing the effect that 12 week storage of the *L. leonorus* and *M. longifolia* capsules under room temperature and humidity and high temperature-high humidity (i.e. 40±2℃/ 75%±5%RH) conditions might have on their levels of luteolin and apigenin, respectively, are given in Figures 5-14, 5-15, 5-16 and 5-17. From these results the shelf-lives of the capsules and hence appropriate expiry dates can be determined. Normally, shelf-life (t_{90}) is defined as the time required for a drug to decompose to 90% of its initial concentration at a specific temperature, that is, when 10% decomposition has occurred (Álvarez-Lueje. 2005).

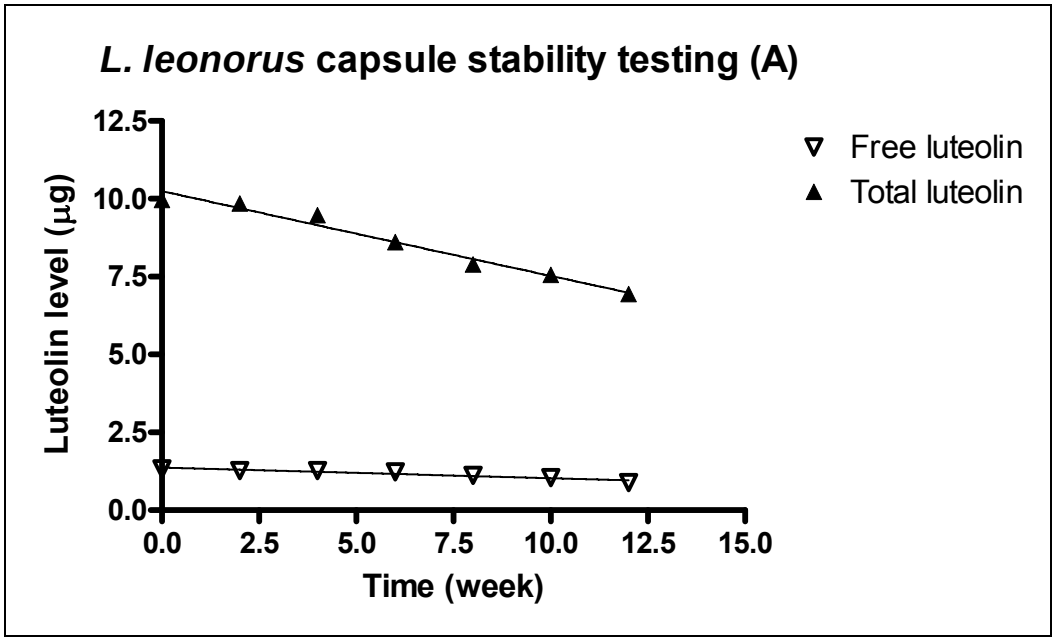

***Figure 5-14**. The luteolin level in L. leonorus capsules during 12 weeks storage outside container at room temperature and room relative humidity.*

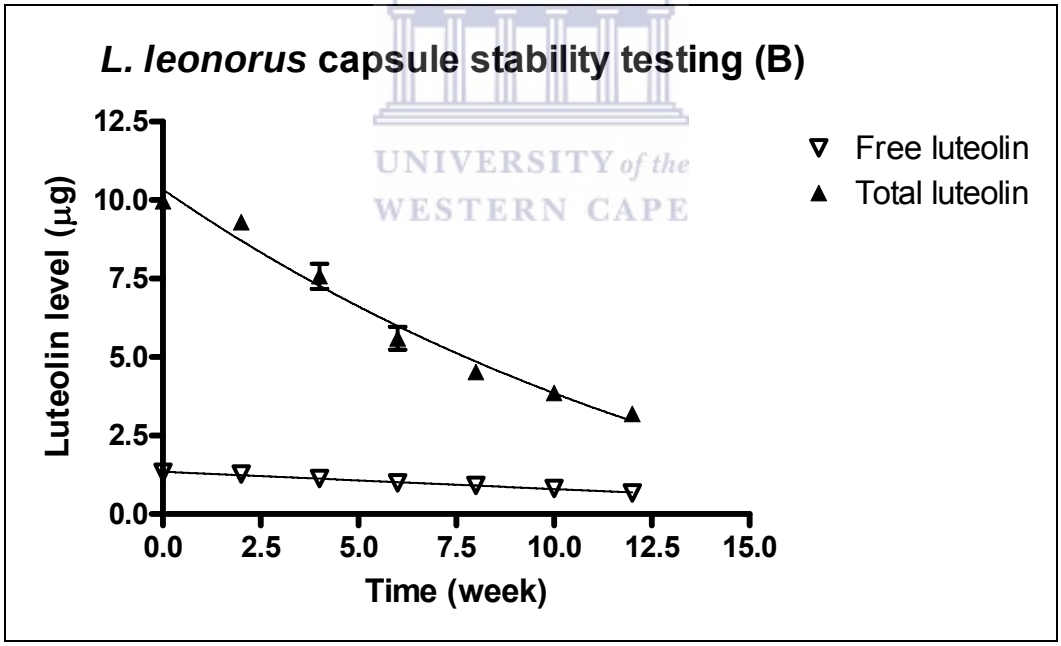

***Figure 5-15**. The luteolin level in L. leonorus capsules during 12 weeks storage outside container under 40±2℃, 75%±5%RH conditions.*

In the *L. leonorus* capsules stored outside a container at room temperature and humidity conditions there was a steady average drop in the total luteolin

content of 0.2719 μg/week over the 12 weeks with the level of total luteolin content after 12 weeks being only 67% of the initial level. Significantly, the level had already dropped to 90% of the initial level within 3.67 weeks (i.e. t_{90} < 4 weeks). Since shelf-life (i.e. the expiry date) is normally based on the t_{90} value this meant that the *L. leonorus* capsules essentially had no practical shelf-life when stored outside a container even under room conditions. The situation is even more drastic when the results of the capsules stored under the accelerated stability testing conditions (i.e. higher temperature and humidity – Figure 5-15) are considered, viz. – approx. average rate of decrease of 0.6114 μg/week and t_{90} = 1.63 weeks). The luteolin marker results thus reflected the instability results found based on the analysis of the organoleptic properties of the capsules, strongly suggesting that the luteolin was indeed an appropriate marker to measure or monitor the stability of the *L. leonorus* capsules.

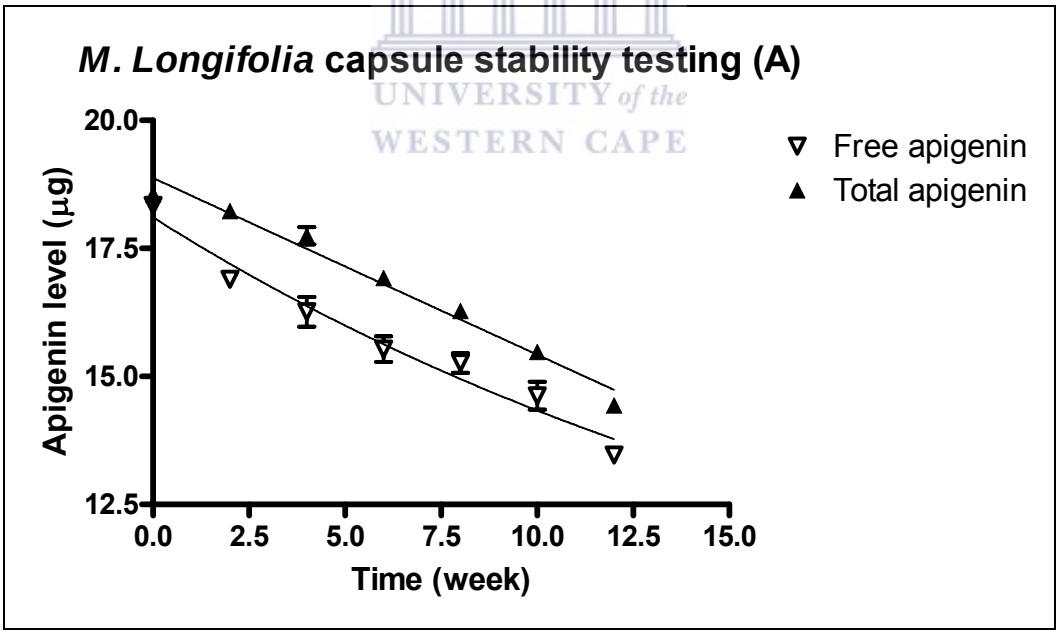

***Figure 5-16**. The apigenin level in M. longifolia capsules during 12 weeks storage outside a* container at r*oom temperature and room relative humidity.*

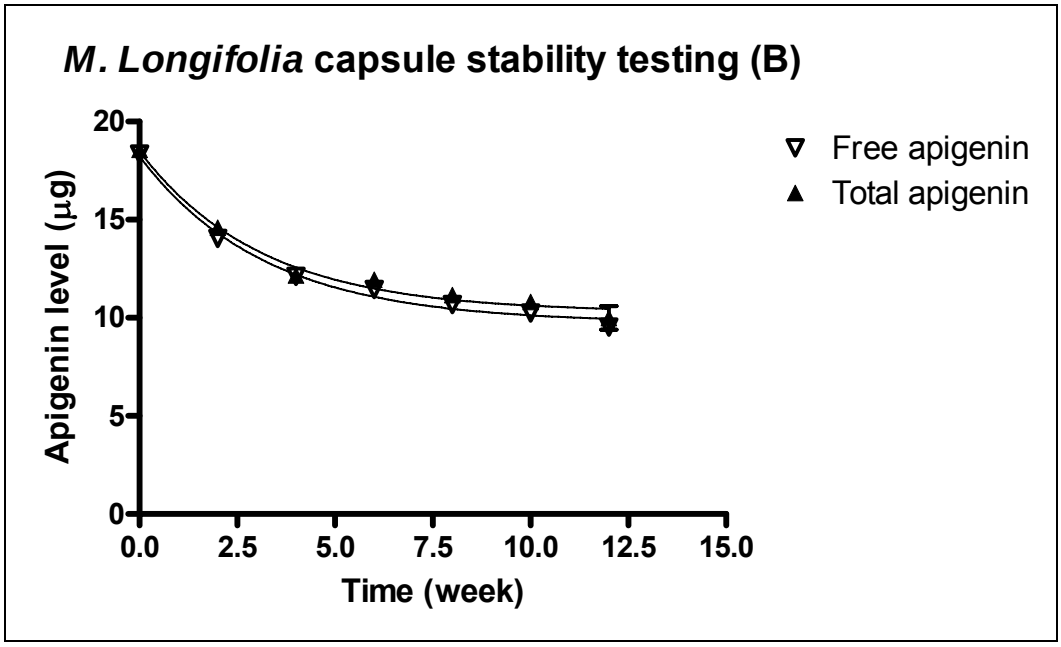

***Figure 5-17**. The apigenin level in M. longifolia capsules during 12 weeks storage outside a container at 40±2℃, 75%±5%RH*

A similar picture emerged for the apigenin in the *M. longifolia* capsules. Under room temperature and humidity conditions (Figure 5-16) the total and free apigenin levels in the capsule content decreased at average rates of 0.3450 and 0.3396 μg/week, respectively resulting in levels of 14.44 and 13.46 μg and 77.84 and 73.39 % of the initial values, respectively at 12 weeks. In this case the shelf-life or t_{90} were approx. 5.39 weeks, again not really of any practical use.

Even more drastic were the results obtained after storage under the accelerated stability testing conditions (Figure 5-17). In this case the total and free apigenin levels showed a fast drop for the first 4 weeks (average rates of decrease of 1.60 and 1.56 μg/week) followed by a slower steady drop for the subsequent 8 weeks (average rates of decrease of 0.55 and 0.64 μg/week, respectively), resulting in levels of 53.85 and 51.91% of the initial values of total and free apigenin, respectively at 12 weeks. The estimated shelf-life i.e. t_{90} was approx.1.16 weeks. Again, the apigenin marker results also appeared

to reflect the instability results found during the analysis of the organoleptic properties of *M. longifolia* capsules and suggesting that as for the luteolin with *L. leonorus,* the apigenin also was an appropriate marker to use to monitor the stability of the *M. longifolia* capsules.

Collectively, the stability testing results obtained indicated that the manufactured capsules essentially had no practical shelf-lives, had changed profoundly different dissolution profiles after storage, implicated the humidity of the environment and the hygroscopic nature of the plant extract materials as major drivers for the instability of the capsules and finally confirmed that the markers luteolin and apigenin could effectively be used in stability studies of the *L. leonorus* and *M. longifolia* capsules, respectively. Although the active constituents of the two plant medicines are not known, the possibility is strong that the active constituents might show similar instability trends.

5.6 Conclusions.

The primary purpose of this chapter was to report and discuss the manufacture, and evaluation of hard gelatin capsules containing *L. leonorus* and *M. longifolia* plant material that would meet the conventional pharmaceutical standards and might make them equivalent and suitable replacements for the traditional liquid dosage forms of these two traditional plant medicines.

From the results obtained the following conclusions could be drawn:

1. Although there were significant differences in the actual levels, by comparing the levels of flavonoid markers found in the traditional decoctions and the freeze-dried aqueous extracts of *M. longifolia* and *L. leonorus* it was however possible to calculate doses of the extracts in capsule form that should approximate the traditional doses. The

appropriateness of this strategy and whether the same results would be found if other marker compounds are used obviously needs further investigation.

2. Elegant capsules uniform in content and weight could be manufactured by using the plate filling method and the use of the freeze-dried aqueous extract of the plant materials in capsule form appeared to bring and keep microbial contamination levels to acceptable levels. Moreover, the manufactured capsules met the British Pharmacopoeia dissolution specification for immediate release solid oral dosage forms and had good *in vitro* bioavailability.

3. *L. leonorus* extract absorbed some moisture during the capsule filling procedure, presumably because it was more hygroscopic than the *M. longifolia* extract. Since the moisture absorbed may speed up degradation, the humidity conditions during the manufacture of the capsules could thus be a crucial factor and these capsules should preferably be manufactured under more tightly controlled humidity conditions i.e. in conditions of < 40% relative humidity (RH).

4. Finally, the stability testing results obtained collectively indicated that the manufactured capsules essentially had no practical shelf-lives, had profoundly different dissolution profiles after storage, implicated the humidity of the environment and the hygroscopic nature of the plant extract materials as major drivers for the instability of the capsules and confirmed that the markers luteolin and apigenin could effectively be used in stability studies of the *L. leonorus* and *M. longifolia* capsules, respectively. Although the active constituents of the two plant medicines are not known, the possibility was also high that the active constituents might show similar instability trends.

Overall, capsules containing freeze-dried aqueous extract powder of *L. leonorus* and *M. longifolia* at doses equivalent to that found in their traditional liquid dosage forms and meeting most of the conventional pharmaceutical standards for immediate release dosage forms were successfully manufactured. However the manufactured capsules had essentially no practical shelf-lives and profoundly different dissolution profiles after storage. Although offering some advantages, these capsules were thus not yet suitable replacements for the traditional liquid dosage forms.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

The primary objectives of this study were to prepare and evaluate a freeze-dried aqueous extract of *L. leonorus* and *M. longifolia* as suitable raw material for capsule dosage form and to formulate and manufacture capsules of *L. leonorus* and *M. longifolia* containing the active ingredient in amounts equivalent to that found in their traditional decoction dosage forms and capsules that would meet conventional pharmaceutical standards for immediate release dosage forms.

It was hypothesized that, the freeze-dried aqueous extracts of *L. leonorus* and *M. longifolia* would have suitable characteristics (esp. not be hygroscopic) for use as raw material for capsules, and that the capsule dosage forms of *L. leonorus* and *M. longifolia* that contain an amount of plant material extract equivalent to that found in its traditional liquid dosage forms would have comparable and acceptable dissolution rates.

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

1. Freeze-dried aqueous extract powders of *L. leonorus* and *M. longifolia* have good flowability, irregular particle size and shape, are sparingly soluble with high wet ability, on average contained 4.89 ± 0.25% and 8.40 ± 0.14% moisture for *L. leonorus* and *M. longifolia,* respectively, had microbial contamination counts well within the specifications and were suitable plant raw materials for incorporation in hard capsule dosage form.

2. Although the actual levels may differ significantly, comparison of the levels of the flavonoid markers luteolin and apigenin found in the traditional decoctions and the freeze-dried aqueous extracts of *M. longifolia* and *L. leonorus*, respectively, provided a convenient method to calculate doses of the extracts in the capsule dosage form that would approximate the traditional doses. The appropriateness of this strategy and whether the same results would be found if other marker compounds are used however needs further investigation.

3. Elegant capsules that were uniform in content and weight, on average contained 165.3 ± 1.629 µg luteolin and 382.1 ± 10.77 µg apigenin in the *L. leonorus* and *M. longifolia* capsules, respectively, and had microbial contamination levels within the European Pharmacopoeia specifications could be manufactured by using the plate filling method and the freeze-dried aqueous extracts of the plants. Moreover, the manufactured capsules met the British Pharmacopoeia dissolution specification for immediate release solid oral dosage forms and had good *in vitro* bioavailability.

4. Because the freeze-dried extract of *L. leonorus* extract absorbed some moisture during the capsule filling procedure, presumably because it was hygroscopic (and more so than the *M. longifolia* extract) and since the moisture absorbed may speed up degradation, the humidity conditions during the manufacture of the capsules could be a crucial factor and these capsules should preferably be manufactured under more tightly controlled humidity conditions i.e. in conditions of < 40% relative humidity (RH).

5. Unfortunately, capsules produced using the freeze-dried aqueous extract of *L. leonorus* and *M. longifolia* are very unstable, have no practical shelf-lives and have profoundly different dissolution profiles after storage, with the

humidity of the environment and the hygroscopic nature of the plant extract materials being implicated as major drivers for the instability of the capsules.

6. The flavonoid luteolin for *L. leonorus* and apigenin for *M. longifolia*, both assayed by HPLC method were useful markers to monitor the stability of capsules of the two plants and, although the active constituents of the two plant medicines are not known, to indicate the high possibility that the active constituents in the aqueous extracts and capsules of these plants might also be unstable.

Collectively, the results showed that the aqueous extracts *L. leonorus* and *M. longifolia* were suitable as raw materials of the plants as far as manufacture of capsules were concerned, but that the stability and shelf-life of these extract containing capsules were unacceptable. As such these capsules were thus not adequate replacements for the traditional decoction forms of these two commonly used medicinal plants.

RECOMMENDATIONS:

The hygroscopicity of the *L. leonorus* and *M. longifolia* freeze-dried aqueous extract capsules was probably the most important factor contributing to the less than satisfactory stability results found in this study. To overcome this problem the following may be recommended:

1. Use different types of solvent(s) to prepare the extract powder e.g. methanol or methanol-water extract. This may, of course, make the extract less similar to the traditional decoction forms of the plant.

2. Control the manufacturing environment especially the humidity level.

Generally, pharmaceutical air conditioning is usually set below 50% RH, but very hygroscopic products, particularly moisture sensitive ones as was the case in this study, are best manufactured and stored at humidity levels below 40% RH.

3. Use a different capsule shell e.g. vegetable capsule. Because hard gelatin capsules are made of 86% bovine and/or pigs skin and 14% purified water and vegetable capsules are made of 92% methylcellulose and 8% purified water, the latter may be better to use for the two plant extracts prepared in this study.

4. Use another type of dosage forms e.g. controlled-release capsule or tablets, etc. Again, however this option may lead to substantial dissimilarity between the new dosage form and the traditional decoction form.

5. Use a different type of container e.g. glass bottles, foil blisters and dessicant to store the products.

Several of these options are viable and worth further investigation.

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APPENDICES

Appendix 1: *Yield of freeze-dried aqueous extract of L. leonorus.*

Sample	Weight of the dry leaves (g)	Volume of the water (mL)	Yield of freeze dried aqueous extract		Amount of aqueous extract in single 90 ml dose (g) (i.e. decoction)
			(g)	**%**	
1	**10**	**500**	**1.80**	**18.0**	**0.53**
2	**10**	**500**	**1.96**	**19.6**	**0.59**
3	**10**	**500**	**1.71**	**17.1**	**0.51**
4	**10**	**500**	**1.78**	**17.8**	**0.60**
5	**10**	**500**	**1.88**	**18.8**	**0.56**
6	**10**	**500**	**1.92**	**19.2**	**0.58**
Average & SD			**1.842 ± 0.0943**	**18.42 ± 0.9432**	**0.5617±0.0355**

Appendix 2: *Yield of freeze-dried aqueous extract of M. longifolia.*

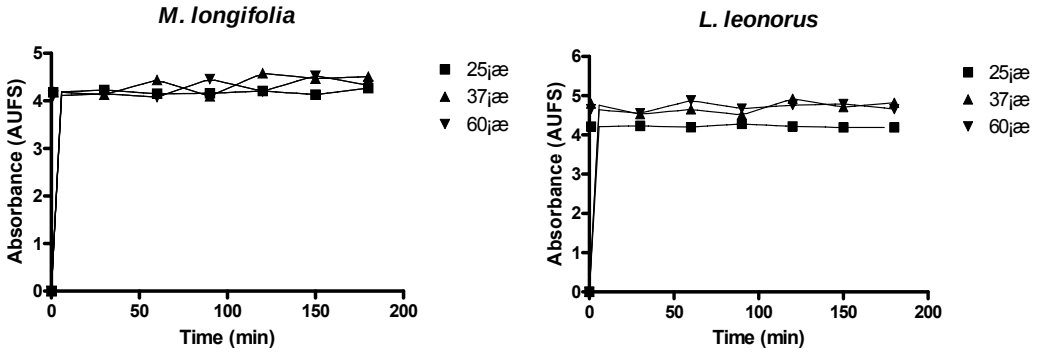
Sample	Weight of the dry leaves (g)	Volume of the water (mL)	Yield of freeze dried aqueous extract	
			(g)	**%**
1	**2**	**150**	**0.24**	**12.0**
2	**2**	**150**	**0.23**	**11.5**
3	**2**	**150**	**0.22**	**11.0**
4	**2**	**150**	**0.28**	**14.0**
5	**2**	**150**	**0.28**	**14.0**
6	**2**	**150**	**0.24**	**12.0**
Average & SD			**0.2483±0.0256**	**12.42±1.281**

Appendix 3*: The solubility of L. leonorus and M. longifolia freeze-dried aqueous extracts at room temperature.*

Sample	***L. leonorus***			***M. longifolia***		
	Initial (g)	**Residue (g)**	**Solubility (g/ml)**	**Initial (g)**	**Residue (g)**	**Solubility (g/ml)**
1	**0.0397**	**0.0100**	**0.0297**	**0.0370**	**0.0019**	**0.0351**
2	**0.0336**	**0.0094**	**0.0242**	**0.0315**	**0.0019**	**0.0296**
3	**0.0337**	**0.0109**	**0.0228**	**0.0342**	**0.0050**	**0.0292**

4	**0.0376**	**0.0150**	**0.0226**	**0.0305**	**0.0019**	**0.0286**
5	**0.0388**	**0.0132**	**0.0256**	**0.0326**	**0.0033**	**0.0293**
6	**0.0354**	**0.0149**	**0.0205**	**0.0441**	**0.0155**	**0.0286**
Average & SD			**0.0242±0.0032**			**0.0301±0.0025**

Appendix 4: *Effect of dissolution time and temperature on solubility of L. leonorus and M. longifolia freeze-dried aqueous extracts.*



Appendix 5: *Particle shape dimensions of L. leonorus and M. longifolia.*

Sample	*L. leonorus*			*M. longifolia*		
	Da (10^{-2}mm)	Dp (10^{-2}mm)	θ	Da (10^{-2}mm)	Dp (10^{-2}mm)	θ
1	**4**	**5.5**	**0.67**	**5**	**7**	**0.71**
2	**3**	**6.5**	**0.43**	**4**	**6**	**0.67**
3	**2.5**	**5**	**0.6**	**3**	**6**	**0.5**
4	**2.5**	**3.5**	**0.86**	**3**	**3.5**	**0.86**
5	**5**	**8**	**0.63**	**5**	**9**	**0.56**
6	**4**	**6.5**	**0.57**	**4.5**	**7**	**0.64**
7	**4**	**9**	**0.5**	**6**	**9**	**0.67**
8	**2**	**3**	**0.57**	**7**	**8**	**0.88**
9	**1.5**	**2**	**0.75**	**4**	**8**	**0.5**
10	**5**	**7.5**	**0.63**	**5**	**8**	**0.625**
11	**4.5**	**7**	**0.71**	**8**	**11**	**0.72**
12	**7**	**16**	**0.41**	**8**	**9**	**0.89**
13	**6**	**11**	**0.58**	**5**	**6.5**	**0.77**
14	**5**	**11**	**0.55**	**5.5**	**8**	**0.69**
15	**8**	**10**	**0.73**	**3**	**4**	**0.75**
16	**4**	**7**	**0.5**	**6**	**8**	**0.75**
17	**9**	**11**	**0.75**	**2.5**	**3.5**	**0.71**
18	**5**	**10**	**0.45**	**4**	**5.5**	**0.73**
19	**6**	**12**	**0.58**	**7**	**11**	**0.64**
20	**4**	**8**	**0.63**	**5**	**9**	**0.56**

AVE & SD			**0.6050± 0.1173**			**0.6913± 0.1119**

Da = Projected area diameter

Dp = Projected perimeter diameter

θ = Degree of sphericity

Appendix 6: *Density parameters for the freeze-dried aqueous extract of L. leonorus.*

Sample No	Weight of the dried aqueous extract (g)	Loose volume (ml)	Loose density (g/ml)	Packed volume (ml)	Packed density (g/ml)	Index of Compressibility (%)
1	**4.4732**	**8.8**	**0.5083**	**7.6**	**0.5886**	**13.64**
2	**5.0231**	**9.9**	**0.5074**	**8.7**	**0.5774**	**12.12**
3	**4.8687**	**9.2**	**0.5292**	**8.1**	**0.6011**	**11.96**
4	**4.5007**	**8.9**	**0.5057**	**7.6**	**0.5922**	**14.61**
5	**4.8429**	**9.7**	**0.4993**	**8.4**	**0.5765**	**13.39**
6	**4.6371**	**9.5**	**0.4881**	**8.2**	**0.5655**	**13.69**
AVE & SD			**0.5063± 0.01349**		**0.5836± 0.01282**	**13.24±1.015**

Appendix 7: *Density parameters for the freeze-dried aqueous extract of M. longifolia.*

Sample No	Weight of the dried aqueous extract (g)	Loose volume (ml)	Loose density (g/ml)	Packed volume (ml)	Packed density (g/ml)	Index of Compressibility (%)
1	**4.1312**	**9.0**	**0.4590**	**8.6**	**0.4804**	**4.45**
2	**3.7728**	**8.8**	**0.4287**	**8.1**	**0.4658**	**7.96**
3	**4.1163**	**9.6**	**0.4288**	**8.8**	**0.4678**	**8.34**
4	**3.7199**	**8.3**	**0.4482**	**7.6**	**0.4895**	**8.44**
5	**4.3595**	**9.3**	**0.4688**	**8.5**	**0.5129**	**8.60**
6	**4.6502**	**9.3**	**0.5000**	**8.8**	**0.5284**	**5.37**
AVE & SD			**0.4556± 0.02703**		**0.4908± 0.02516**	**7.193±1.805**

Appendix 8: *Angle of repose of L. leonorus and M. longifolia extracts.*

Samples	***L. leonorus***			***M. longifolia***		
	Height (cm)	Radius (cm)	Angle of repose (°)	Height (cm)	Radius (cm)	Angle of repose (°)
1	**2.2**	**3.05**	**35.80**	**2.3**	**3.60**	**32.57**
2	**2.1**	**2.90**	**35.91**	**3.0**	**3.25**	**42.71**
3	**2.2**	**2.95**	**36.71**	**2.6**	**3.65**	**35.46**
4	**2.0**	**2.95**	**34.14**	**3.2**	**3.50**	**42.44**
5	**2.0**	**2.85**	**35.06**	**2.6**	**3.30**	**38.23**
6	**2.0**	**2.90**	**34.59**	**2.0**	**3.85**	**27.45**
7	**2.1**	**3.05**	**34.55**	**2.8**	**3.55**	**38.26**
8	**2.0**	**3.05**	**33.25**	**3.1**	**3.20**	**44.09**
9	**2.0**	**2.90**	**34.59**	**2.9**	**3.25**	**41.74**
10	**2.0**	**3.00**	**33.69**	**3.0**	**3.30**	**42.27**
AVE & SD			**34.83±1.063**			**38.52±5.332**

Appendix 9*: Total ash and acid insoluble ash values for the freeze-dried aqueous extract of L. leonorus.*

Sample	Weight of the dried aqueous extract (g)	Weight of carbon free ash (g)	Percentage of total ash (%)	Weight of acid-insoluble ash (g)	Percentage of acid-insoluble ash (%)
1	**2.0035**	**0.3575**	**17.84**	**0.0332**	**1.66**
2	**2.0174**	**0.3426**	**16.98**	**0.0308**	**1.53**
3	**2.0445**	**0.3474**	**16.99**	**0.0286**	**1.40**
4	**2.0021**	**0.3518**	**16.66**	**0.0272**	**1.29**
5	**2.0192**	**0.3520**	**17.43**	**0.0239**	**1.18**
AVE & SD			**17.2±0.460**		**1.41±0.190**

Appendix 10: *Total ash and acid insoluble ash values for the freeze-dried aqueous extract of M. longifolia.*

Sample	Weight of the dried aqueous extract (g)	Weight of carbon free ash (g)	Percentage of total ash (%)	Weight of acid-insoluble ash (g)	Percentage of acid-insoluble ash (%)
1	**2.0300**	**0.4281**	**21.09**	**0.0450**	**2.22**
2	**2.0341**	**0.4232**	**20.81**	**0.0424**	**2.08**
3	**2.0069**	**0.4141**	**20.63**	**0.0410**	**2.04**
4	**2.0198**	**0.4254**	**21.06**	**0.0432**	**2.14**
5	**2.0527**	**0.4371**	**21.29**	**0.0605**	**2.95**
AVE & SD			**20.98±0.257**		**2.28±0.377**

Appendix 11: *The moisture content of the L. leonorus freeze-dried aqueous extract.*

Sample No	Initial weight of aqueous extract (g)	Final weight of aqueous extract (g)	Difference (g)	Percentage of moisture (%)
1	**0.3690**	**0.3503**	**0.0187**	**5.07**
2	**0.3715**	**0.3526**	**0.0189**	**5.09**
3	**0.3595**	**0.3428**	**0.0167**	**4.65**
4	**0.3701**	**0.3525**	**0.0176**	**4.76**
5	**0.3661**	**0.3472**	**0.0189**	**5.16**
6	**0.3573**	**0.3407**	**0.0166**	**4.65**
AVE & SD				**4.89±0.2354**

Appendix 12: *The moisture content of the M. longifolia freeze-dried aqueous extract.*

Sample No	Initial weight of aqueous extract (g)	Final weight of aqueous extract (g)	Difference (g)	Percentage of moisture (%)
1	**0.5054**	**0.4624**	**0.0430**	**8.51**
2	**0.7324**	**0.6698**	**0.0626**	**8.55**

3	**0.6031**	**0.5532**	**0.0499**	**8.27**
4	**0.5044**	**0.4625**	**0.0419**	**8.31**
5	**0.5465**	**0.5014**	**0.0451**	**8.25**
6	**0.6245**	**0.5714**	**0.0531**	**8.50**
AVE & SD				**8.398±0.1357**

Appendix 13: *The results of the microbial contamination testing of L. leonorus freeze-dried aqueous extract.*

SAMPLE TYPE	TEST TYPE	DETERMINATION TIME 24±2(HRS)	BACT.COUNT CFU/gram
Leonotis leonorus	**TMA**		**No Growth**
	Escherichia coli	**No Growth**	**No Growth**
	Yeast & Mould		**No Growth**
	Salmonella		**Absent/25g**
	Enterobacteriaceae		**No Growth**

TMA = Total microbial activity

Appendix 14: *The results of the microbial contamination testing of M. longifolia freeze-dried aqueous extract.*

SAMPLE TYPE	TEST TYPE	DETERMINATION TIME 24±2(HRS)	BACT.COUNT CFU/gram
Mentha longifolia	**TMA**		**No Growth**
	Escherichia coli	**No Growth**	**No Growth**
	Yeast & Mould		**No Growth**
	Salmonella		**Absent/25g**
	Enterobacteriaceae		**No Growth**

TMA = Total microbial activity

Appendix 15: *The contact angle of L. leonorus and M. longifolia freeze-dried aqueous extracts.*

Sample	*L. leonorus (°)*	*M. longifolia (°)*
1	**7**	**7**

2	7	5
3	8	5
4	7	8
5	7	6
6	9	9
7	5	5
8	4	7
AVE & SD	6.75±1.581	6.5±1.512

Appendix 16: *Content uniformity test results for L. leonorus capsules.*

	Weight of empty capsule (g)	Weight of filled capsule (g)	Weight of extract(g)	* Deviation in weight from average (%)	**Amount of content of capsules (%).
1	0.0926	0.4537	0.3611	2.48%	99%
2	0.0966	0.4637	0.3671	0.86%	101%
3	0.1024	0.4731	0.3707	0.11%	102%
4	0.0927	0.4641	0.3714	0.30%	102%
5	0.0976	0.4575	0.3599	2.81%	99%
6	0.0879	0.4571	0.3692	0.30%	101%
7	0.0954	0.4687	0.3733	0.81%	102%
8	0.0977	0.4732	0.3755	1.40%	103%
9	0.0995	0.4751	0.3756	1.43%	103%
10	0.0938	0.4573	0.3635	1.84%	100%
11	0.0943	0.4706	0.3763	1.62%	103%
12	0.0953	0.4670	0.3717	0.38%	102%
13	0.0947	0.4615	0.3668	0.95%	100%
14	0.0958	0.4756	0.3798	2.57%	104%
15	0.0958	0.4642	0.3684	0.51%	101%
16	0.0931	0.4581	0.3650	1.43%	100%
17	0.0940	0.4685	0.3745	1.13%	103%
18	0.0915	0.4648	0.3733	0.81%	102%
19	0.0961	0.4715	0.3754	1.38%	103%
20	0.1022	0.4697	0.3675	0.76%	101%
AVE & SD	0.0954	0.4657	0.3703	1.194	101.6

	±0.0034	±0.0066	±0.0054	±1.778%	±1.432%

* = [Weight of extract (g) - Average weight of extract (g)] / Average * 100%

** = Weight of extract (g) / 0.365 (g) * 100%

Appendix 17*: Content uniformity test results for M. longifolia capsules.*

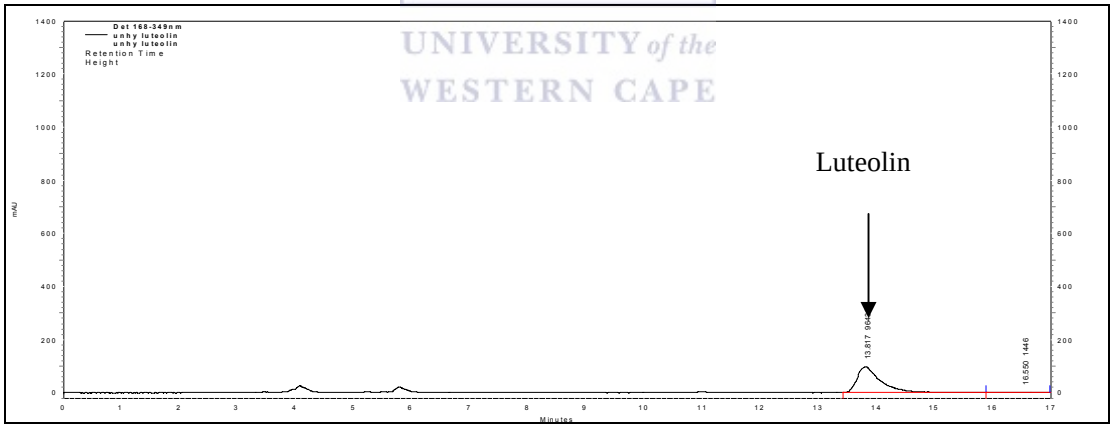
	Weight of empty capsule (g)	Weight of filled capsule (g)	Weight of extract(g)	*Deviation in weight from average (%)	**Amount of content Of capsules (%).
1	**0.0971**	**0.4223**	**0.3252**	**0.59%**	**103%**
2	**0.0951**	**0.4123**	**0.3172**	**1.89%**	**101%**
3	**0.0986**	**0.4167**	**0.3181**	**1.61%**	**101%**
4	**0.0989**	**0.4230**	**0.3241**	**0.25%**	**103%**
5	**0.0911**	**0.4225**	**0.3314**	**2.51%**	**105%**
6	**0.0986**	**0.4220**	**0.3234**	**0.03%**	**103%**
7	**0.0935**	**0.4204**	**0.3269**	**1.11%**	**104%**
8	**0.0941**	**0.4157**	**0.3216**	**0.53%**	**102%**
9	**0.0984**	**0.4135**	**0.3151**	**2.54%**	**100%**
10	**0.0891**	**0.4117**	**0.3226**	**0.22%**	**103%**
11	**0.0960**	**0.4223**	**0.3263**	**0.93%**	**104%**
12	**0.0971**	**0.4314**	**0.3343**	**3.40%**	**106%**
13	**0.0927**	**0.4181**	**0.3254**	**0.65%**	**103%**
14	**0.0977**	**0.4097**	**0.3120**	**3.50%**	**99%**
15	**0.0896**	**0.4162**	**0.3266**	**1.02%**	**104%**
16	**0.0948**	**0.4248**	**0.3300**	**2.07%**	**105%**
17	**0.0977**	**0.4264**	**0.3287**	**1.67%**	**105%**
18	**0.1023**	**0.4231**	**0.3208**	**0.77%**	**102%**
19	**0.0987**	**0.4274**	**0.3287**	**1.67%**	**105%**
20	**0.1004**	**0.4086**	**0.3082**	**4.67%**	**98%**

AVE & SD	**0.0961 ±0.0035**	**0.4194 ±0.0062**	**0.3233 ±0.0066**	**1.582 ±1.241%**	**102.8 ±2.142%**

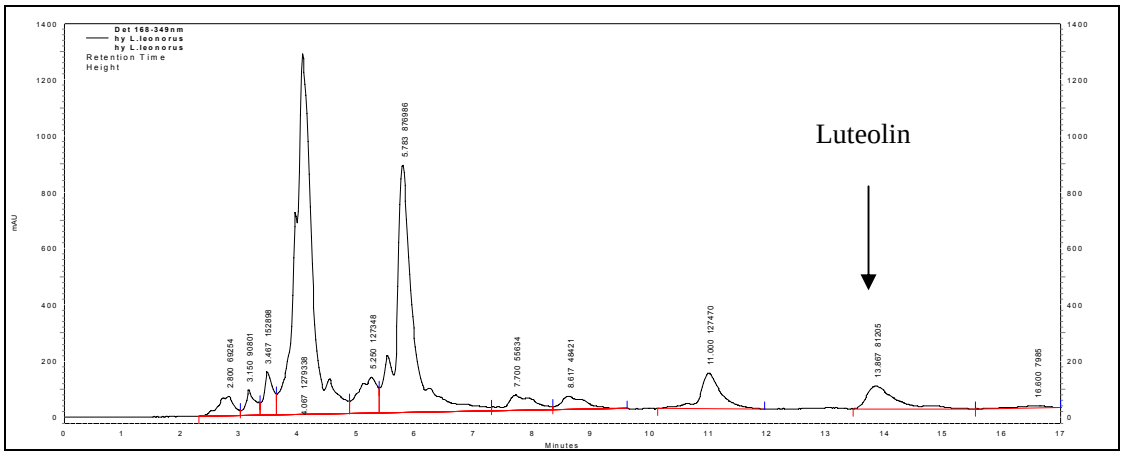
* = [Weight of extract (g) - Average weight of extract (g)] / Average * 100%

** = Weight of extract (g) / 0.314 (g) * 100%

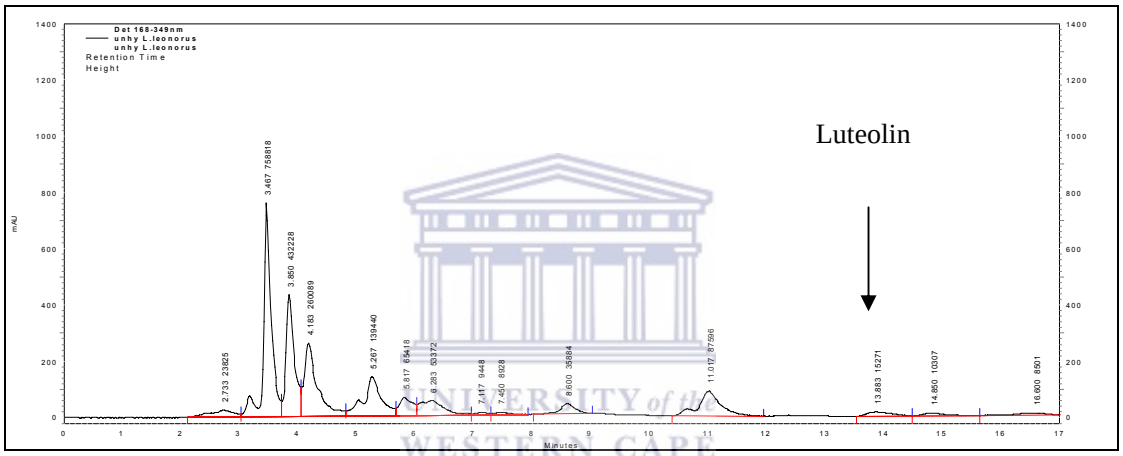
Appendix 18: *Chromatogram of luteolin extracted from aqueous solution. (The retention time = 14 min; Mobile phase: isocratic 70% acetonitrile and 30% KH_2PO_4 buffer; flow rate 1ml/min).*



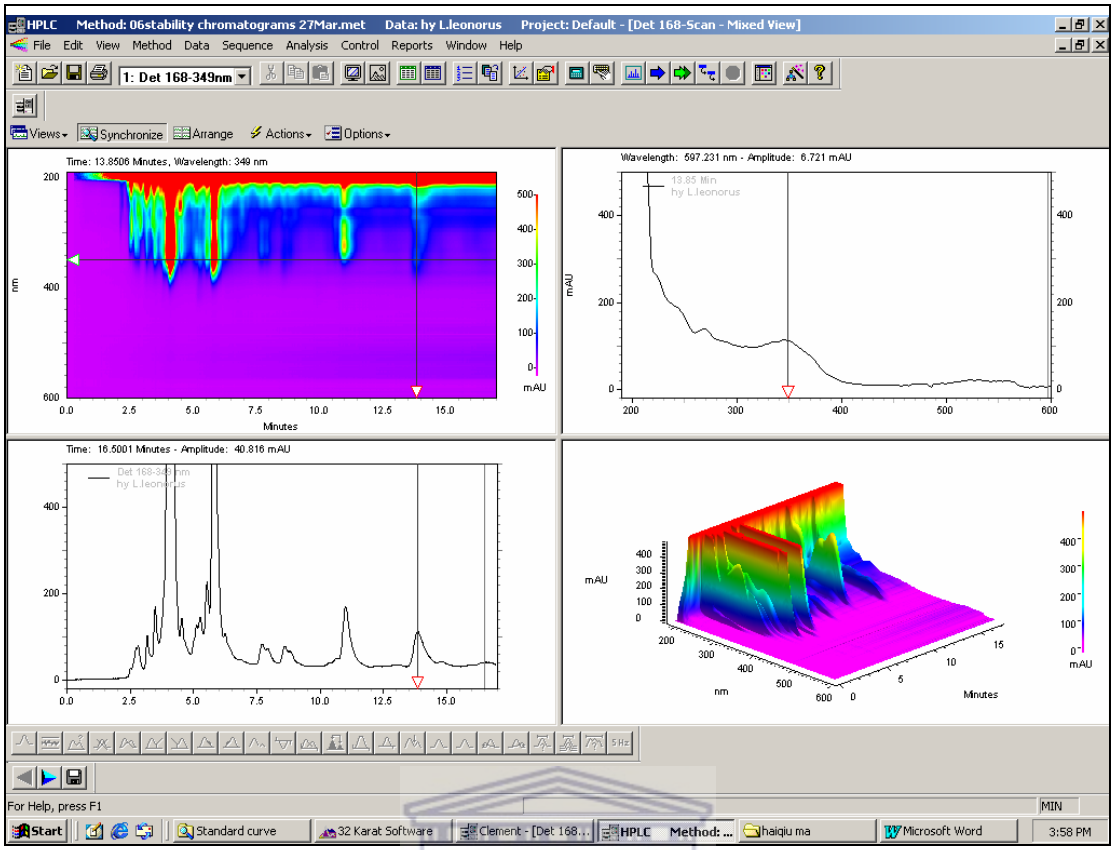
Appendix 19: *Chromatogram of luteolin in extracted hydrolysed L. leonorus. (The retention time = 14 min; Mobile phase: isocratic 70% acetonitrile and 30% KH_2PO_4 buffer; flow rate 1ml/min).*



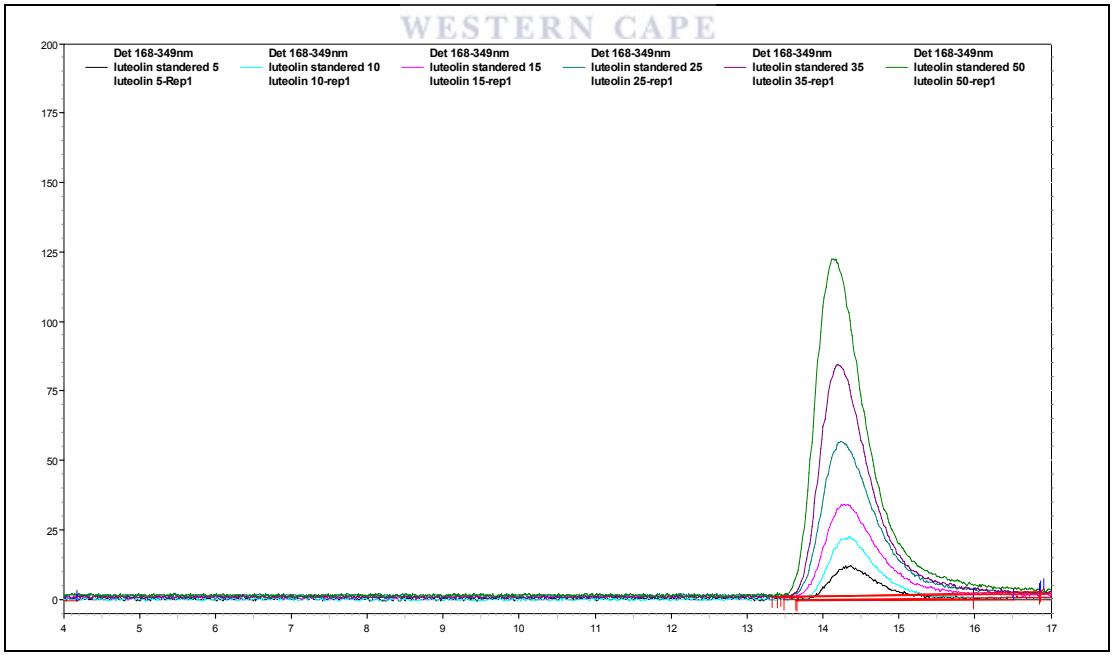
Appendix 20: *Chromatogram of luteolin in extracted unhydrolysed L. leonorus (The retention time = 14 min; Mobile phase: isocratic 70% acetonitrile and 30% KH_2PO_4 buffer; flow rate 1ml/min).*



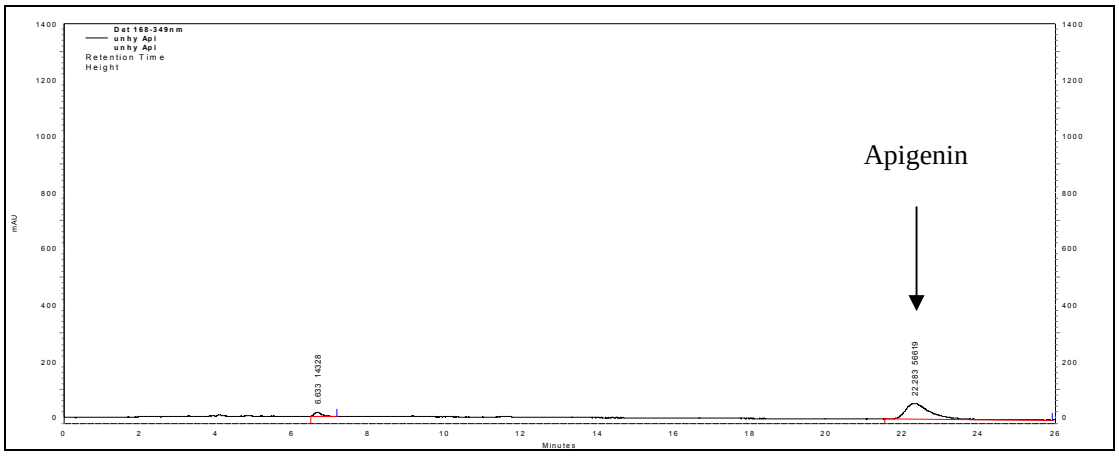
Appendix 21: *3D (diode array) chromatogram of hydrolysed extract of L. leonorus. (The retention time = 14 min; Mobile phase: isocratic 70% acetonitrile and 30% KH_2PO_4 buffer; flow rate 1 ml/min).*



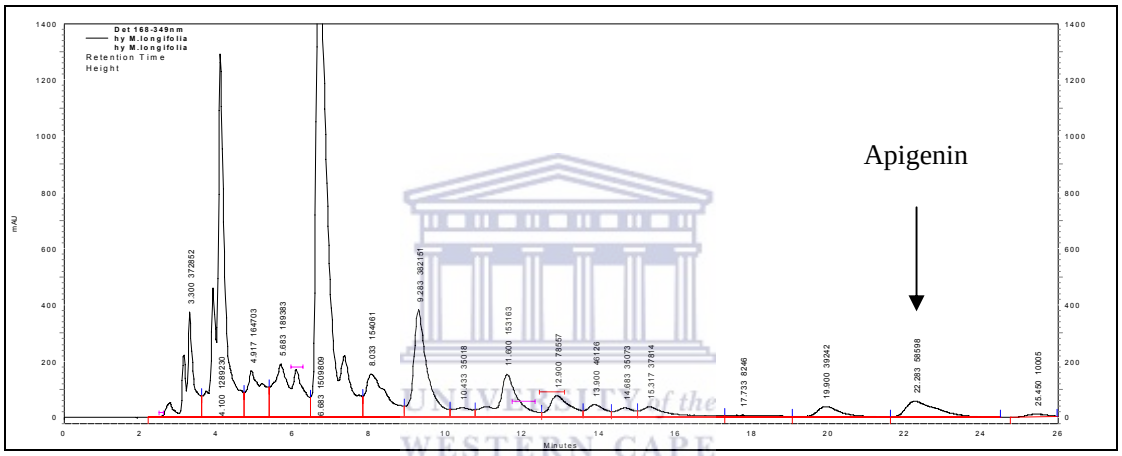
Appendix 22: *Chromatogram of different concentrations of luteolin extracted from water. (The retention time = 14 min; mobile phase: isocratic 70% acetonitrile and 30% KH_2PO_4 buffer; flow rate 1 ml/min).*



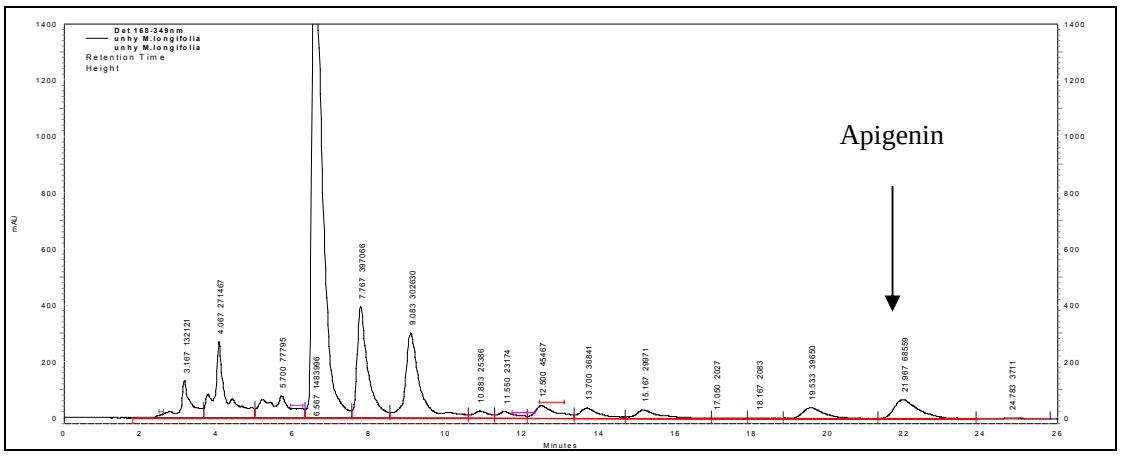
Appendix 23: *Chromatogram of apigenin extracted from methanol. (The retention time = 22 min; Mobile phase: isocratic 70% acetonitrile and 30% KH_2PO_4 buffer; flow rate 1ml/min).*



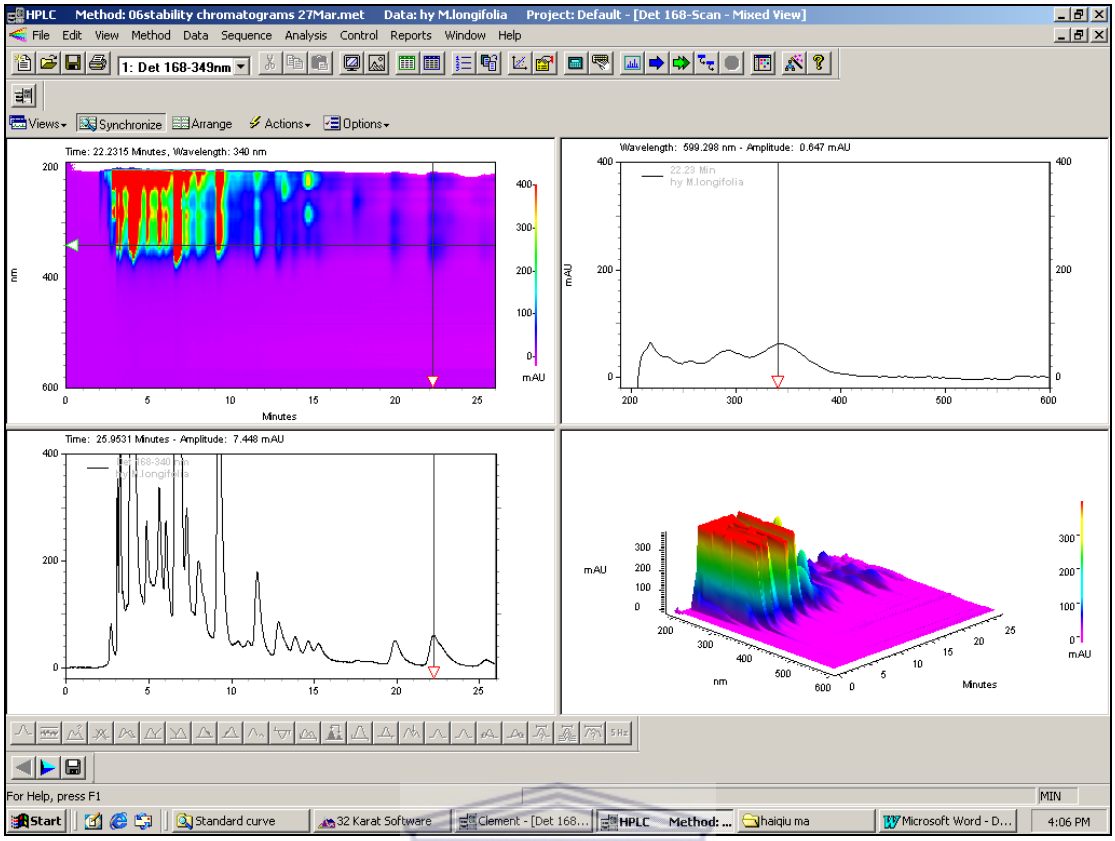
Appendix 24: *Chromatogram of apigenin in extracted hydrolysed M. longifolia (The retention time = 22 min; Mobile phase: isocratic 70% acetonitrile and 30% KH_2PO_4 buffer; flow rate 1ml/min).*



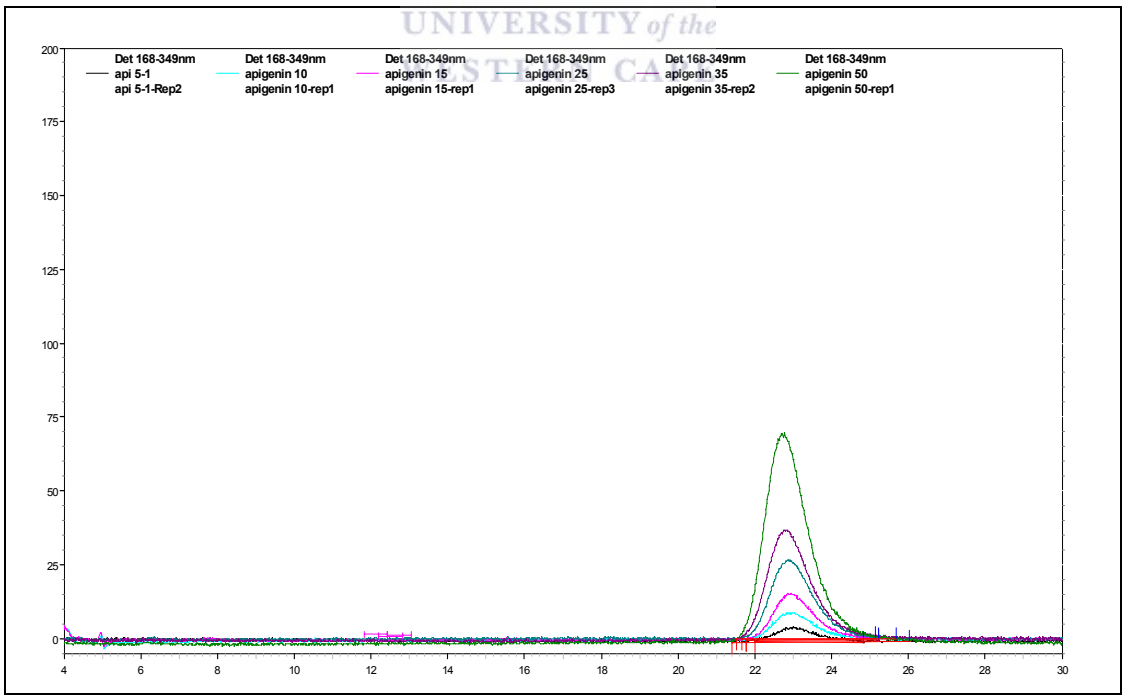
Appendix 25: *Chromatogram of apigenin in extracted unhydrolysed M. longifolia (The retention time = 22 min; Mobile phase: isocratic 70% acetonitrile and 30% KH_2PO_4 buffer; flow rate 1ml/min).*



Appendix 26: *3D (diode array) chromatogram of hydrolysed extract of M. longifolia. (The retention time = 22 min; Mobile phase: isocratic 70% acetonitrile and 30% KH_2PO_4 buffer; flow rate 1 ml/min).*



Appendix 27: *Chromatogram of different concentrations of apigenin extracted from methanol. (The retention time = 22 min; Mobile phase: isocratic 70% acetonitrile and 30% KH_2PO_4 buffer; flow rate 1 ml/min).*



Appendix 28: *Inter- and intraday precision of HPLC assay for luteolin. Luteolin concentration is 10µg /ml; inject volume: 50µl.*

Assay day	Measured luteolin concentration in sample ((µg /ml)				RSD (%)
	Time 1	**Time 2**	Time 3	AVE ± SD	
1 day	**9.24**	**9.20**	**8.86**	**9.10 ± 0.208**	**2.29**
2 day	**10.16**	**10.02**	**10.30**	**10.16 ± 0.140**	**1.38**
3 day	**10.10**	**9.80**	**9.98**	**9.96 ± 0.151**	**1.52**
Inter-day assay precision (RSD in 3 days)				**9.74 ± 0.509**	**5.23**

Appendix 29: *The amount of luteolin in L. leonorus capsules.*

Capsule	1	2	3	4	5	6	AVE & SD (n = 6)	RSD%
Luteolin (µg)	**162.72**	**163.57**	**165.28**	**166.76**	**166.62**	**166.85**	**165.3 ± 1.629**	**0.99%**

Appendix 30: *Recovery of luteolin from L. leonorus capsule content (n = 6) as a percent of the labeled amount.*

Capsule	The amount of *L. leonorus* in extract from capsule (mg)	* Assayed amount of luteolin in extract form capsule (µg)	Amount of luteolin added to the sample (µg)	**Assayed amount of luteolin (µg)	Recovery (%)
1	**50.7**	**22.6029**	**20**	**40.9970**	**91.97**
2	**44.9**	**20.1219**	**20**	**40.1045**	**99.91**
3	**44.7**	**20.2409**	**20**	**39.1502**	**94.55**
4	**32**	**14.6200**	**20**	**34.8985**	**101.39**
5	**39**	**17.8037**	**20**	**37.1539**	**96.75**
6	**38.6**	**17.6453**	**20**	**37.4197**	**98.87**
AVE & SD					**97.24 ± 3.529**
RSD					**3.63%**

* *Amount of luteolin in extract form capsule as determined by HPLC assay.*

** *Amount of luteolin in extract form capsule as determined by HPLC assay, after addition of luteolin.*

Appendix 31: *Inter- and intraday precision of HPLC assay for apigenin. Apigenin concentration is (5µg/ml), inject volume: 50µl.*

Assay day	Measured apigenin concentration in sample (µg /ml)				RSD (%)
	Time 1	Time 2	Time 3	AVE & SD	
1 day	**5.24**	**5.39**	**5.27**	**5.30±0.079**	**1.50**
2 day	**5.49**	**5.18**	**5.28**	**5.32±0.158**	**2.98**
3 day	**5.21**	**5.22**	**4.82**	**5.08±0.228**	**4.49**
Inter-day assay precision (RSD in 3 days)				**5.23±0.183**	**3.50**

Appendix 32: *The amount of apigenin in M. longifolia capsules:*

Capsule	1	2	3	4	5	6	AVE & SD	RSD
Apigenin (µg)	**363.91**	**388.01**	**379.07**	**378.53**	**394.23**	**388.82**	**382.1 ± 10.77**	**2.82%**

Appendix 33: *Recovery of apigenin from M. longifolia capsule content (n = 6) as a percent of the labeled amount.*

Capsule	The amount of *M. longifolia* in extract from capsule (mg)	* Assayed amount of apigenin in extract form capsule (µg)	The amount of apigenin add to the sample (µg)	**Assayed amount of apigenin (µg)	Recovery (%)
1	**24**	**27.8144**	**20**	**44.0759**	**81.31**
2	**26**	**32.1282**	**20**	**47.4388**	**76.55**
3	**23.3**	**28.1283**	**20**	**45.7131**	**87.92**
4	**29.6**	**35.6827**	**20**	**52.2667**	**82.92**
5	**27.5**	**34.5267**	**20**	**49.6549**	**75.64**
6	**24.5**	**30.3375**	**20**	**49.5436**	**96.03**
AVE & SD					**83.40 ±7.638**
RSD					**9.16%**

* *Amount of apigenin in extract form capsule as determined by HPLC assay.*

** *Amount of apigenin in extract form capsule as determined by HPLC assay, after addition of apigenin.*

Appendix 34A: *Moisture content of L. leonorus sampled from filled capsules before storage.*

Sample No	Initial weight of aqueous extract (g)	Final weight of aqueous extract (g)	Difference (g)	Percentage of moisture (%)
1	**0.5462**	**0.5066**	**0.0396**	**7.25**
2	**0.5625**	**0.5255**	**0.0370**	**6.58**
3	**0.5457**	**0.5086**	**0.0371**	**6.80**
4	**0.5393**	**0.4987**	**0.0406**	**7.53**
5	**0.5268**	**0.4885**	**0.0383**	**7.27**
6	**0.5800**	**0.5365**	**0.0435**	**7.50**
AVE & SD				**7.155 ± 0.3843**

Appendix 34B: *Moisture content of L. leonorus sampled from filled capsules stored in container at room temperature and humidity for 12 weeks.*

Sample No	Initial weight of aqueous extract (g)	Final weight of aqueous extract (g)	Difference (g)	Percentage of moisture (%)
1	**0.3514**	**0.3139**	**0.0375**	**8.08**
2	**0.374**	**0.3383**	**0.0357**	**8.20**
3	**0.3462**	**0.3122**	**0.034**	**8.25**
AVE & SD				**8.177±0.0874**

Appendix 34C: *Moisture content of L. leonorus sampled from filled capsules stored in container at 40±2℃ and 75% ± 5%RH for 12 weeks.*

Sample No	Initial weight of aqueous extract (g)	Final weight of aqueous extract (g)	Difference (g)	Percentage of moisture (%)
1	**0.3638**	**0.3344**	**0.0294**	**10.67**
2	**0.3632**	**0.3334**	**0.0298**	**9.55**
3	**0.3468**	**0.3181**	**0.0287**	**9.82**
AVE & SD				**10.01±0.5845**

Appendix 34D: *Moisture content of L. leonorus sampled from filled capsules stored outside container at room temperature and humidity for 12 weeks.*

Sample No	Initial weight of aqueous extract (g)	Final weight of aqueous extract (g)	Difference (g)	Percentage of moisture (%)
1	**0.3854**	**0.3069**	**0.0785**	**20.42**

2	**0.388**	**0.3098**	**0.0782**	**20.15**
3	**0.3758**	**0.2996**	**0.0762**	**20.28**
AVE & SD				**20.28±0.1350**

Appendix 35A: *Moisture content of M. longifolia sampled from filled capsules before storage.*

Sample No	Initial weight of aqueous extract (g)	Final weight of aqueous extract (g)	Difference (g)	Percentage of moisture (%)
1	**0.3085**	**0.2806**	**0.0279**	**9.04**
2	**0.2974**	**0.2716**	**0.0258**	**8.67**
3	**0.3119**	**0.2855**	**0.0264**	**8.46**
4	**0.3150**	**0.2860**	**0.0290**	**9.21**
5	**0.3184**	**0.2893**	**0.0291**	**9.14**
6	**0.3004**	**0.2762**	**0.0242**	**8.06**
AVE & SD				**8.76±0.4502**

Appendix 35B: *Moisture content of M. longifolia sampled from filled capsules stored in container at room temperature and humidity for 12 weeks.*

Sample No	Initial weight of aqueous extract (g)	Final weight of aqueous extract (g)	Difference (g)	Percentage of moisture (%)
1	**0.3288**	**0.2971**	**0.0317**	**8.72**
2	**0.3119**	**0.2822**	**0.0297**	**9.52**
3	**0.303**	**0.2736**	**0.0294**	**9.21**
AVE & SD				**9.150±0.4034**

Appendix 35C: *Moisture content of M. longifolia sampled from filled capsules stored in container at 40±2℃ and 75% ± 5%RH for 12 weeks.*

Sample No	Initial weight of aqueous extract (g)	Final weight of aqueous extract (g)	Difference (g)	Percentage of moisture (%)
1	**0.305**	**0.2784**	**0.0266**	**9.64**
2	**0.3045**	**0.2755**	**0.029**	**9.52**
3	**0.3094**	**0.2809**	**0.0285**	**9.70**
AVE & SD				**9.620±0.0916**

Appendix 35D: *Moisture content of M. longifolia sampled from filled capsules stored outside container at room temperature and humidity for 12 weeks.*

Sample No	Initial weight of aqueous extract (g)	Final weight of aqueous extract (g)	Difference (g)	Percentage of moisture (%)
1	**0.3206**	**0.2609**	**0.0597**	**18.62**
2	**0.3324**	**0.2727**	**0.0597**	**17.96**
3	**0.3156**	**0.2594**	**0.0562**	**17.81**
AVE & SD				**18.13±0.4309**

Appendix 36: *Microbial test results for the L. leonorus capsule content.*

SAMPLE TYPE	TEST TYPE	DETERMINATION TIME 24±2(HRS)	BACTERIAL COUNT CFU/gram
Leonotis leonorus	**TMA**		**30**
	Escherichia coli	**No Growth**	**No Growth**
	Yeast & mould		**No Growth**
	Salmonella		**Absent/20g**
	Enterobacteriaceae		**No Growth**

TMA = Total microbial activity

Appendix 37: *Microbial test results for M. longifolia capsule content.*

SAMPLE TYPE	TEST TYPE	DETERMINATION TIME 24±2(HRS)	BACTERIAL COUNT CFU/gram
Mentha longifolia	**TMA**		**30 (mould)**
	Escherichia coli	**No Growth**	**No Growth**
	Yeast & Mould		**30 mould**
	Salmonella		**Absent/20g**
	Enterobacteriaceae		**No Growth**

TMA = Total microbial activity

Appendix 38: *Results of dissolution study of L. leonorus capsules. Each capsule was dissolved in 900ml pH 1.2 hydrochloric acid buffer, basket method, 100 rpm (BP 2000 Appendix XII D Method; samples taken at the times indicated and assayed for UV absorbance at 285 nm).*

Time (min)	Percentage dissolved from capsule #						% Dissolved
	1	2	3	4	5	6	AVE ± SD
5	**5.02**	**3.55**	**10.06**	**7.08**	**6.86**	**11.45**	**7.33±2.97**
10	**17.82**	**8.06**	**23.14**	**11**	**15.22**	**16.64**	**15.31±5.30**
15	**41.92**	**27.42**	**40.62**	**33.62**	**38.41**	**41.3**	**37.21±5.66**
25	**65.63**	**66.93**	**73.83**	**79.62**	**65.26**	**83.49**	**72.46±7.79**
35	**88.64**	**88.39**	**92.08**	**90.1**	**91.21**	**90.97**	**90.23±1.47**
45	**100**	**100**	**100**	**100**	**100**	**100**	**100.00**
60	**100**	**100**	**100**	**100**	**100**	**100**	**100.00**

Appendix 39: *Results of dissolution study of M. longifolia capsules. Each capsule was dissolved in 900ml pH 1.2 hydrochloric acid buffer, basket method, 100 rpm (BP 2000 Appendix XII D Method; samples taken at the times indicated and assayed by UV absorbance at 280 nm).*

Time (min)	Percentage dissolved from capsule #						% Dissolved
	1	2	3	4	5	6	AVE
5	**12.65**	**8.21**	**17.26**	**22.62**	**18.01**	**10.67**	**14.903±5.340**
10	**47.12**	**40.85**	**24.23**	**52.93**	**48.97**	**40.8**	**42.483±10.113**

15	**77.21**	**67.78**	**48.55**	**73.58**	**72.47**	**68.67**	**68.042±10.146**
25	**85.86**	**79.51**	**81.26**	**82.76**	**82.3**	**80.24**	**81.988±2.254**
35	**95.71**	**90.12**	**90.19**	**92.28**	**90.89**	**91.84**	**91.838±2.086**
45	**100**	**100**	**100**	**100**	**100**	**100**	**100.000±0.000**
60	**100**	**100**	**100**	**100**	**100**	**100**	**100.000±0.000**

Appendix 40: *The stability results of L. leonorus capsule organoleptic properties after 12 weeks storage.*

(Batch 1) ***Room temperature and relative humidity (Outside container)***				
Week	Size, shape of capsule	Gross nature of powder in capsule	Color of powder (Seen through capsule)	Odor of powder
0	**Regular "0" size & shape**	**Powder**	**Brown**	**Strong**
2	**No change**	**Hard and crisp**	**Deep brown**	**Faint**
4	**No change**	**Hard but not crisp**	**Deep brown**	**Faint**
6	**No change**	**Hard like rubber**	**Deep brown**	**Faint**
8	**No change**	**Hard like rubber**	**Deep brown**	**Faint**
10	**No change**	**Slightly sticky**	**Deep brown**	**Faint**
12	**No change**	**Slightly sticky**	**Deep brown**	**Faint**
(Batch 2) ***Room temperature and relative humidity (in container)***				
Week	Size, shape of capsule	Gross nature of powder in capsule	Color of powder (Seen through capsule)	Odor of powder
0	**Regular "0" size & shape**	**Powder**	**Brown**	**Strong**

2	**No change**	**Powder**	**Brown**	**Strong**
4	**No change**	**Powder**	**Brown**	**Strong**
6	**No change**	**Powder**	**Brown**	**Strong**
8	**No change**	**Powder**	**Brown**	**Strong**
10	**No change**	**Agglomeration**	**Brown**	**Strong**
12	**No change**	**Agglomeration**	**Brown**	**Strong**
(Batch 3)	***40±2 ℃, 75%±5% relative humidity (in container)***			
Week	Size, shape of capsule	Gross nature of powder in capsule	Color of powder (Seen through capsule)	Odor of powder
0	**Regular "0" size & shape**	**Powder**	**Brown**	**Strong**
2	**No change**	**Powder**	**Brown**	**Strong**
4	**No change**	**Powder**	**Brown**	**Strong**
6	**No change**	**Agglomeration**	**Brown**	**Strong**
8	**No change**	**Agglomeration**	**Brown**	**Strong**
10	**No change**	**Agglomeration**	**Brown**	**Strong**
12	**No change**	**Agglomeration**	**Brown**	**Strong**

Appendix 41: *The stability results of M. longifolia capsule organoleptic properties after 12 weeks storage.*

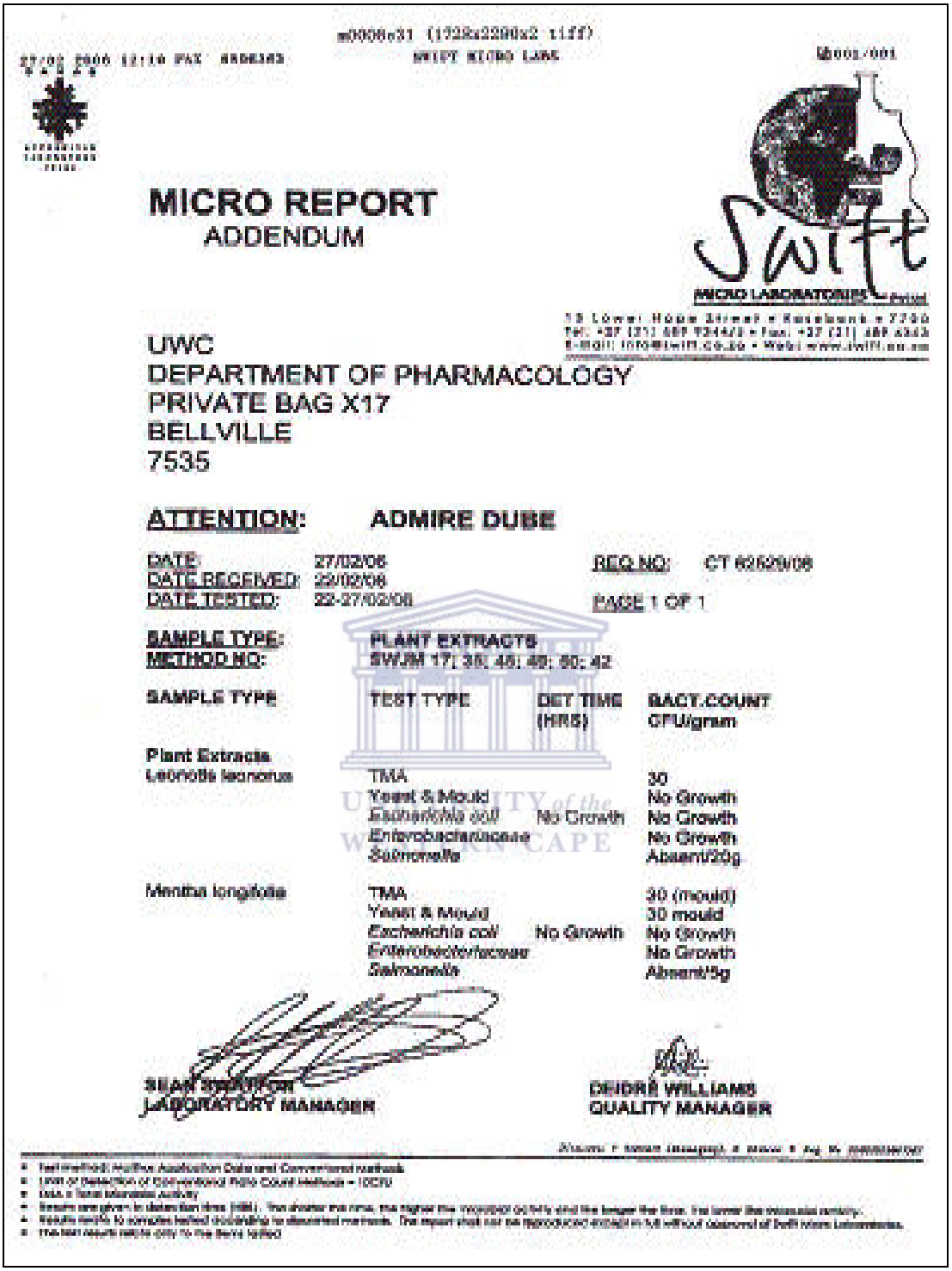
(Batch 1)	***Room temperature and relative humidity (Outside container)***			
Week	Size, shape of capsule	Gross nature of powder in capsule	Color of powder (Seen through capsule)	Odor of powder
0	**Regular "0" size & shape**	**Powder**	**Light brown**	**Strong**
2	**No change**	**Hard and crisp**	**Deep brown**	**Faint**
4	**No change**	**Hard and crisp**	**Deep brown**	**Faint**
6	**No change**	**Hard and crisp**	**Deep brown**	**Faint**
8	**No change**	**Hard and crisp**	**Deep brown**	**Faint**
10	**No change**	**Hard but not crisp**	**Deep brown**	**Faint**
12	**No change**	**Hard but not crisp**	**Deep brown**	**Faint**
(Batch 2)	***Room temperature and relative humidity (in container)***			
Week	Size, shape of capsule	Gross nature of powder in capsule	Color of powder (Seen through capsule)	Odor of powder

0	**Regular "0" size & shape**	**Powder**	**Light brown**	**Strong**
2	**No change**	**Powder**	**Light brown**	**Strong**
4	**No change**	**Powder**	**Light brown**	**Strong**
6	**No change**	**Powder**	**Light brown**	**Strong**
8	**No change**	**Powder**	**Light brown**	**Strong**
10	**No change**	**Powder**	**Light brown**	**Strong**
12	**No change**	**Powder**	**Light brown**	**Strong**

(Batch 3) ***40±2 ℃, 75%±5% relative humidity (in container)***

Week	Size, shape of capsule	Gross nature of powder in capsule	Color of powder (Seen through capsule)	Odor of powder
0	**Regular "0" size & shape**	**Powder**	**Light brown**	**Strong**
2	**No change**	**Powder**	**Light brown**	**Strong**
4	**No change**	**Powder**	**Light brown**	**Strong**
6	**No change**	**Agglomeration**	**Light brown**	**Strong**
8	**No change**	**Agglomeration**	**Light brown**	**Strong**
10	**No change**	**Agglomeration**	**Light brown**	**Strong**
12	**No change**	**Agglomeration**	**Light brown**	**Strong**

***Appendix 42**: Microbial contamination results:*



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SWIFT MICRO LAB

27/02 2006 12:10 FAX 8806383 001/001

MICRO REPORT
ADDENDUM

Swift
MICRO LABORATORIES (Pty) Ltd

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E-Mail: info@swift.co.za • Web: www.swift.co.za

UWC
DEPARTMENT OF PHARMACOLOGY
PRIVATE BAG X17
BELLVILLE
7535

ATTENTION: ADMIRE DUBE

DATE: 27/02/06
DATE RECEIVED: 22/02/06
DATE TESTED: 22-27/02/06

REQ NO: CT 62629/06

PAGE 1 OF 1

SAMPLE TYPE: PLANT EXTRACTS
METHOD NO: SWJM 17; 36; 45; 49; 50; 42

SAMPLE TYPE	TEST TYPE	DET TIME (HRS)	BACT.COUNT CFU/gram
Plant Extracts			
Leonotis leonorus	TMA		30
	Yeast & Mould		No Growth
	Escherichia coli	No Growth	No Growth
	Enterobacteriaceae		No Growth
	Salmonella		Absent/20g
Mentha longifolia	TMA		30 (mould)
	Yeast & Mould		30 mould
	Escherichia coli	No Growth	No Growth
	Enterobacteriaceae		No Growth
	Salmonella		Absent/5g

SEAN [illegible]
LABORATORY MANAGER

DEIDRÉ WILLIAMS
QUALITY MANAGER

***Appendix 43**: Microbial contamination testing methods.*

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.

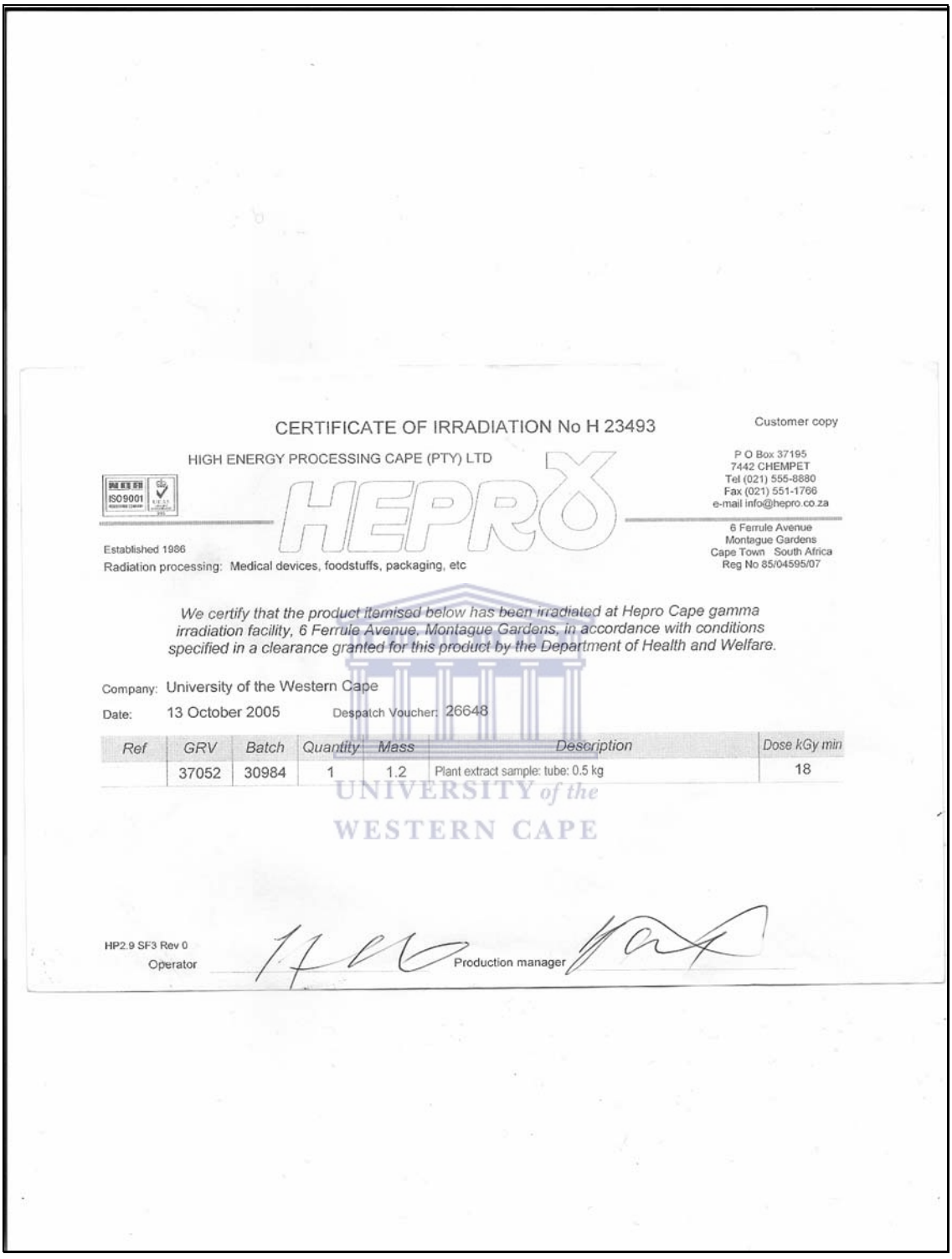
SWJM 42 : Detection of *Salmonellae*
This is an AOAC approved method, utilizing Malthus technology. Samples are pre- enriched in a broth at 37°C for 18 – 24 hours. From here tubes of Salmonella selective broth are inoculated and incubated at 37 °C for a maximum period of 30 hours. Presumptive positive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

***Appendix 44**: Irradiation certificate.*



CERTIFICATE OF IRRADIATION No H 23493

Customer copy

HIGH ENERGY PROCESSING CAPE (PTY) LTD

HEPRO

P O Box 37195
7442 CHEMPET
Tel (021) 555-8880
Fax (021) 551-1766
e-mail info@hepro.co.za

6 Ferrule Avenue
Montague Gardens
Cape Town South Africa
Reg No 85/04595/07

Established 1986

Radiation processing: Medical devices, foodstuffs, packaging, etc

We certify that the product itemised below has been irradiated at Hepro Cape gamma irradiation facility, 6 Ferrule 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: 26648

Ref	GRV	Batch	Quantity	Mass	Description	Dose kGy min
	37052	30984	1	1.2	Plant extract sample: tube: 0.5 kg	18

HP2.9 SF3 Rev 0

Operator Production manager

Appendix 45*: Introduction to the Author.*

NAME: HaiQiu Ma
BIRTH DAY: 10/04/1977 born in Shan Xi, China.
NATIONALITY: China.
EDUCATIONAL BACKGROUND:

1994---1997, Pharmaceutics, Certificate, **Tian Jin Pharmacy School, Tian Jin, CHINA.**

1997---2000, Pharmacy, National Diploma, **Tianjin Medical University, Tian Jin, CHINA.**

2004---2004, Pharmaceutical Sciences, B-Tech, **Tshwane University of Technology, Pretoria, SOUTH AFRICA.**

2005---2006, Pharmaceutical Sciences, M.Pharm, **University of the Western Cape, Cape Town, SOUTH AFRICA.**