Pharmaceutical Evaluation of Phela Capsules Used as Traditional Medicine

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SUMMARY

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Phela is a traditional herbal medicine comprising four plants, which together are claimed to have immune boosting properties. The Indigenous Knowledge Systems (IKS) program of the South African Medical Research Council (SAMRC) focuses on investigating such claims. The purpose of this study was to establish (from literature) a series of criteria and specifications that are appropriate to establish the pharmaceutical quality of plant-containing capsules such as Phela and to assess the pharmaceutical quality of Phela, in capsule form, and its suitability for use in clinical trials. Thus, the organoleptic features and physical and chemical properties of the individual plant powders and the content uniformity, release characteristics and shelf life of the capsules were determined.

First, the current literature on the quality control of herbal provided by the British Pharmacopeia (BP), European Medicine Agency (EMEA) and World Health Organisation (WHO) were reviewed and a list of quality control parameters, methods and specifications that could be used to assess the pharmaceutical quality of the Phela plant materials and capsules were drawn up. Then, the freshly prepared powders of the four individual plant materials and the final mixture were assessed for their organoleptic features, ash value, flowability, moisture content, extractable matter and microbial contamination using standard pharmacopoeia methods. Gelatine hard capsules of the 4 combined plant materials were manufactured at the SAMRC's IKS Lead Programme under GMP conditions. The *in vitro* dissolution of the capsules was determined using the USP basket method and release of the plant drug monitored by

UV spectroscopy. The capsule was subjected to accelerated stability testing for estimation of a shelf life. An HPLC assay and fingerprint method was employed for this purpose.

Overall the Phela capsule contents had a uniformly light brown colour, bitter taste and characteristic medicinal odour which could be masked by a capsule dosage form. Particle size and shape determinations showed that the four plant powders and the mixture were not gradable. The four crude plant powders (RM, PT, CG, S) and the Phela mixture had similar flow properties (i.e. angles of repose of 39.233±3.85°, 39.41±1.85°, 35.91±3.24°, 38.16±4.59° and 37.92±1.28°, respectively) and acceptable moisture content levels (i.e. 9.28±0.31 %, 8.58±0.43 %, 9.31±0.06 %, 10.29±0.53 % and 9.77±0.08 % respectively) which was unchanged in the final mixture. The water soluble or extractable matter was very high for plant PT (77.99±5.82 %) and low for plant S (32.79±2.87 %). The heavy metal, pesticides and microbial levels were within acceptable WHO standards.

The manufacture of the capsules did not constitute any problems and the manufactured capsules had a high mass content uniformity (0.42 ±0.01 mg) and complied with BP standards in that the masses of not more than two of the individual capsules deviated from the average weight (mass) by more than 7.5 %. The capsules released 50.39 % of its active ingredients within 45 minutes. HPLC fingerprinting and pattern recognition analysis indicated that there were 5 compounds in the Phela capsules that decreased by 22 % and 70 % of their original levels after 12 and 24 weeks of storage, respectively, under elevated temperature 40 ^{0}C and 70 % relative

humidity conditions. The 5 compounds had an average shelf-life (i.e. t_{90}) of 6.1 weeks under these accelerated test conditions.

In conclusion, the results obtained firstly indicated that the BP, EMEA and WHO were in fairly good agreement on the criteria and specifications that can be used to assesses the pharmaceutical quality of a traditional plant medicine such as Phela. Secondly, the Phela plant powders were found to have acceptable pharmaceutical properties that did not complicate or adversely affected the capsule manufacture. Thirdly, the Phela capsules produced were generally of acceptable pharmacopoeial standard. Fourthly, HPLC fingerprinting and pattern recognition analysis proved useful to examine the chemical stability of selected marker compounds of Phela and indicated that the capsules had no practical shelf life under elevated temperature and humid conditions. Overall, the Phela capsules should thus be suitable for use in a short time clinical trial, but for use in a long period trial the long term stability of the Phela capsules under ambient conditions must still be confirmed.

DECLARATION

I declare that the thesis "*The pharmaceutical evaluation of Phela capsules used as traditional medicine"* is my own work and 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.

Brian J. Sehume **December 2010**

Signed: …….. **UWC, Bellville.**

Date: ……..

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DEDICATION

I dedicate this master's thesis to my mother (Lulu) and my father, not forgetting my big brother Jeffery Mathethe Sehume for their love and support.

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Chapter 1

Introduction

Herbs used in traditional medicine are nowadays frequently manufactured as conventional pharmaceutical dosage forms i.e. tablets, capsules and solutions, but these are generally not properly evaluated for their pharmaceutical properties. The Indigenous Knowledge Systems unit (IKS) of the South African Medical Research Council (SAMRC) works in partnership with Traditional Healers (TH's) to manufacture pharmaceutical dosage forms of traditional medicines for the TH's. Little is however known about the pharmaceutical quality of these traditional preparations, the characteristics of the plant raw material and how it will react with other excipients or adulterants, etc.

In a traditional setting, liquid dosage forms of herbal medicines are generally preferred and the general preparation of such plant-containing material does not seem to follow generally accepted pharmaceutical manufacturing procedures, nor are the final preparations likely to meet conventional pharmaceutical or suitable quality standards. In fact, the afore-mentioned traditional liquid dosage forms may suffer from several disadvantages. For example, they may be prone to physical, phytochemical and microbiological instability (Wichtl, 1994) the preparations are often bitter in taste and unpalatable resulting in poor patient compliance, the unit doses may produce storage difficulties, etc. In most cases, adequate standards for traditional herbal dosage forms have not been optimally formulated and the pharmaceutical quality of the traditional herbal medicines not adequately evaluated by standard scientific methods. The formulation, manufacture and evaluation of capsules of plant material should be equally or even more complicated.

The formulation, manufacture and evaluation of pharmaceutical herbal dosage forms are complex procedures and mainly so because of the nature of the herbal ingredients contained in such preparations. “Firstly, the herbal ingredients are complex mixtures of different secondary metabolites that can vary considerably depending on environmental and genetic factors. Secondly, the constituents responsible for the plants’ claimed therapeutic effects are frequently unknown or only partly explained which precludes the level of pharmaceutical control that can routinely be achieved with synthetic drugs and conventional pharmaceutical preparations. These complex positions of quality aspects of herbal drugs are further complicated by the use of combinations of herbal ingredients as is common in traditional practice” (Mukherjee, 2002).

The increasing demand for herbal remedies (which represent a substantial proportion of the global drug market) both in the developing and developed countries, has inevitably led to the requirement that the quality and purity of the herbal raw materials and finished products also must be stringently maintained. For instance in Germany, most herbal drugs, even mixtures of herbal drugs, are registered as conventional drugs. This means that they meet the same stringent criteria of quality, efficacy, and safety as synthetic drugs (Wagner, 1999). In Germany, both medical practitioners and patients alike have continuously harnessed the use of traditional medicines. Moreover, that country’s pharmaceutical industry specialises in and relies on herbal drugs and has developed, and supported projects aimed to optimise the quality of herbal drugs through standardisation.

The World Health Organisation (WHO), the Food and Drug Administration (FDA) and European Medicines Agency (EMEA) have set basic criteria for the evaluation of the quality of herbal medicines for scientific organisations and manufacturers. All procedures should be in accordance with Good Manufacturing Practice. (Annex I, WHO, 2000). In addition a quality measure for phytopharmaceuticals in the form of a "monograph" or "master file," is needed to define the individual quality criteria and specifications for every phytopharmaceutical (Bauer, 1998).

As indicated earlier on, the SAMRC's IKS unit works in partnership with TH's on herbal medicines based on the history of use. "This is done by providing indigenous communities with the support resources and tools to understand external influences on their environment and adapt to changes, ensuring the preservation and continuation of their practices" (SAMRC's IKS website). Thus SAMRC's IKS unit manufactures capsules of herbal medicines for TH's (SAMRC's IKS). Phela is one such capsule product that is presently being produced by SAMRC's IKS unit. This unit has opted to make capsules of the Phela herbal medicine because the use of hard gelatine capsule dosage form could perhaps overcome some of the problems associated with the liquid traditional dosage forms. The capsule is a small cylindrical soluble container enclosing a dose of medicine. The capsule shell is an excellent barrier to air, and it also has some advantages such as being easy to swallow and tasteless, and may allow rapid release, flexibility of formulation and a short manufacturing process (Haiqui Ma, 2006).

However the vegetable gelatine capsule herbal product designated Phela that IKS unit is presently making has not yet been optimally evaluated for its pharmaceutical

properties and quality. For instance**,** the actual plant material used has not been evaluated for optimal formulation into a solid dosage form preparation**.** Ground, dried plant material seldom have the appropriate pharmaceutical properties e.g. uniform particle size, adequate flow characteristics, mass uniformity, taste, etc, that allows easy incorporation of such plant material into solid dosage forms of good quality. In addition, the finished capsules need to be evaluated for mass and content uniformity, appearance, dissolution and stability.

Consequently, the objectives of this study were to:

a) Establish (from literature) a series of criteria and specifications that are appropriate to establish the pharmaceutical quality of plant-containing capsules such as Phela,

b) Assess the pharmaceutical quality of the finished Phela capsules,

c) Assess the pharmaceutical quality of the "formulated" Phela raw material, and

d) Provide a certificate of analysis for the finished capsules.

The realisation of the afore-mentioned objectives would allow the determination whether the Phela capsules made by SAMRC's IKS unit is of acceptable pharmaceutical quality and suitable for use in a clinical trial.

Chapter 2

Introduction (Background)

In this chapter an overview is presented on the traditional medicine Phela, pharmaceutical issues concerning herbal medicine and the pharmaceutical evaluation of herbal dosage forms.

2.1. What is Phela?

Phela is a crude botanical product constituting four plants collected in different regions of South Africa and combined in a specific ratio to make herbal traditional medicine. However, due to legal requirements (Intellectual Property Rights) individual plant names cannot be given in full in this dissertation. For the purposes of this study, the four constituent of Phela will thus be referred to as plants PT, CG, RM and S.

Plant PT is a perennial plant of the family *Agavaceae* which is endemic to Mexico and grows in forests or grasslands. The scales of these bulbs are reputed as antispasmodic and Plant PT is particularly known as a traditional aid for the treatment of malaria. Plant CG is a shrub which belongs to the family *Verbenaceae*, and grows in tropical Africa and Asia. Growing in dense bushveld, rocky hillsides and forest verges, the leaves are used in traditional medicine to help treat coughs, intestinal worms and convulsions. Plant RM is also a member of *Verbenaceae* family and is native to tropical East Africa. Ethiopians traditionally used it as treatment for pneumonia, malaria, gonorrhoea and urine infections (Desissa, 2000).

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Plant S belongs to the family *Fabaceae*, and varies from a semi-woody annual herb in warm temperate areas to a woody annual shrub or sometimes a short-lived perennial shrub found in frost-free areas. This plant is indigenous to Brazil and used traditionally to treat fevers, menstrual problems, tuberculosis, anaemia, liver complaints, and as a general tonic. The leaves are also used for gonorrhoea, fever, urinary tract disorders, oedema, and menstrual problems. In Panama, the tea of the leaf is used for stomach colic, while the crushed leaves are often used as filling for a poultice due to its anti-inflammatory properties.

The natural Phela product is under investigation for its potential health protection or disease inhibiting properties by the Indigenous Knowledge Systems (IKS) Unit of the South African Medical Research Council (SAMRC). Very little is known about the safety, efficacy, toxicity, formulation and pharmaceutical quality of Phela. The absence of quality control requirements for potency and purity of herbal preparations is a major concern due to the variation in chemical content and composition between samples and some may not even contain the assumed active contents (Patnala, 2008). Irrespective of its use, it is a requirement to assess for safety, efficacy and pharmaceutical quality if used by the people.

2.1.1. Traditional claims of Phela

Phela has been used for decades in patients with wasting conditions and for increasing energy. The medicine has been reported to have benefits for immune compromised individuals due to its immune stimulant effects and for the condition traditionally called “muyaka” (personal communication with traditional healer on MRC file). The

disease “muyaka” is characterised by the following symptoms in bed-ridden patients: severe chest problems with coughing, coated or pimply tongue, high temperature and fevers that resulted in shivers and headaches, severe weight and appetite loss, vomiting and diarrhoea, stiffness, oral ulcers and slow painful death. These observational and anecdotal findings have been reported by medical doctors, through patient testimonies and by traditional practitioners, who independently, also reported beneficial effects gained by patients using the traditional medicine (on SAMRC’ IKS files).

The South African Medical Research Council (SAMRC), through the Indigenous Knowledge Systems (IKS) program is actively involved in traditional medicine research. Their purpose is developing new forms of therapy from traditional herbal medication, which are efficacious and safe to use. The claims made by traditional healers about the therapeutic effect of herbs for certain diseases make it necessary to explore the potential of these medicines. Phela is one such herbal product that is being evaluated by the IKS unit but if the claims for efficacy, safety and quality of this traditional medicines is to be scientifically assessed an adequate and consistent dosage form is required.

2.1.2. Traditional dosage forms of Phela

The traditional method of preparation for Phela is by infusion and decoction. Such preparations are administered orally (from SAMRC’ IKS files). These liquid preparations are the most popular forms used. In general, infusions are typically made of delicate herbs, leaves and fresh tender plants and prepared by boiling water which is poured over the herbs or a combination of herbs (Ma, 2006). The ratio of herb to

water can vary depending on the remedy, the plant, and whether cut herb or powdered herb is being used. Generally, using 1 teaspoon (3 grams) of powdered herb or 2 teaspoons (6 grams) of more bulky cut herb in a 6 to 8 ounce (170 ml to 230 ml) cup of water is sufficient (Taylor, 2004).

Despite being relatively easy, preparing decoctions of herbs is not the most efficient method of extracting active ingredients. Quite a substantial amount of ingredients would have to be extracted to obtain the highest possible amount of extracted material. During the decocting process, the plant material is boiled for as long as it takes to soften the hard woody material for active ingredients be released (Taylor, 2004). Most of these problems may however be remedied by using an appropriate solid or alternative dosage forms.

2.1.3. Need for an alternative dosage form for Phela

Traditional dosage forms have some disadvantages. They have poor stability during storage, they are not immediately available for use and are susceptible to microbial contamination. The preparation of traditional medicine is time consuming and the product usually deteriorates when heated, has an inconsistent mass and has poor content uniformity. While, by subtle variation in method of preparation and formulation, these medicines can easily be made to suit the palatability of the patients and to elicit the required potency of the drug, such variation in preparation may however lead to inconsistencies and non-reproducibility in the quality and efficacy of such individualized medicines. To solve some of the afore-mentioned disadvantages several types of dosage forms for herbal preparations are under development at the

MRC i.e. the traditional herbal liquid preparation, standardised dried plant powder extracts and capsules or tablets containing dried plant powder.

The use of extracts allows one to achieve authenticity, assay and chemical constituent analysis (Torey, *et al,* 2010). Extracts are made by immersing large quantities of plant material into water and thereafter removing the water by freeze drying. Authenticity relates to proving that the material is true i.e. it corresponds with the right identity (Yadav, *et al,* 2008). Purity pertains to evaluating that there are no adulterants present in the plant material (Yadav, *et al,* 2008). The assay part of standardisation is the assessment of the chemical and biological profiling (Yadav, *et al,* 2008). Safety is assessed through clinical use. Extracts from plant material can be qualitatively and quantitatively assessed for authenticity.

The capsule or tablet dosage form contains the dry plant powder or plant extract powder that replaces 2-3 teaspoonfuls of herbs as the herbal dosage form. Apart from being smaller, capsules and tablet are also easier to carry and easy to swallow, better to produce, pack and store. However, all capsules and tablets must meet certain pharmaceutical standards, which are mostly based on physical appearance of the dosage form and the consistency of its chemical components. Whatever dosage a form is used to replace the traditional form should be of suitable quality is crucial.

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2.1.4. Specific aspects of the quality of Phela

Quality assurance of botanicals and herbal preparations is the prerequisite of clinical trials (Sahoo, *et al*, 2010). Therefore before any dosage form of Phela produced can be used in a clinical trial, the pharmaceutical quality of such a dosage form must be assured. This includes assuring the quality of each component plant materials of Phela as well as the final dosage form (e.g. capsule),

The basic prerequisites for quality control of each component include authentication of the herbal ingredient, inter/intra species variation in plants, environmental factors, plant parts used and the contamination of herbal ingredients. The authentication and cultivation is done according to Good Laboratory Practice (GLP) and Good Agricultural Practice (GAP) guidelines respectively. These guidelines ensure that the plant raw material fulfils highest quality standards.

To ensure quality, the individual tests for identity such as morphological identification (macroscopic and microscopic identification), chemical analysis such as Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), capillary electrophoresis, HPLC coupled with Mass Spectra (LC/MS), protein analysis and the use of molecular markers are conducted (Sahoo, *et al*, 2010). In addition, all the plant drugs are to be tested for microbiological contaminations, the content of heavy metals, pesticides and aflotoxins or other mycotoxins. These measurements ensure that only plant drugs of a high quality are used, which is a precondition for the production of standardized herbal preparations is complying with the set quality requirements (Kroll, *et al*, 2006). The quality of the herbal starting material shows more and more fluctuations due to varied geographical locations where the indigenous

plants grow, the different vernacular names they have and the great deal of substitution found in commercial markets (Yadav, *et al,* 2008).

The quality of the final dosage form (e.g. capsule or tablet) of Phela must also be assured, but for this one expects to be able to use the standardized methods and specifications generally applied for the pharmaceutical assurance of such dosage forms (see later).

2.2. Pharmaceutical issues concerning herbal medicines

The issues that are specific to quality control of herbal medicines are: (1) herbal drugs are mixtures of several constituents and are more difficult to characterize, (2) the active principle(s) are not always known and may be a mixture of compounds from different classes of flavonoids, (3) selective analytical methods may not yet exist, (4) reference compounds may not be commercially available, (5) harvesting, drying, and storage conditions have an influence on the raw material, (6) processing of extracts influences the chemical constituents e.g. solvent polarity and mode of extraction can alter the concentrations of constituents and instability of constituents may influence composition of the extract (Bauer, 1998).

Natural products have traditionally been the basis of most of the drugs in use, but these days we are witnessing an increase in herbal remedy usage throughout the western world, raising doubts about the safety of these rapidly emerging products. The dilemma facing most regulatory authorities is that consumers consider these products as either traditional medicines or natural food supplements (Rousseaux, *et al*, 2003). They do not necessarily concern themselves with product regulation. All

countries have laws concerning food, drugs and cosmetics, but the legislation seldom clearly define the regulation of alternative remedies. Complementary/alternative medicine (CAM) is an umbrella term for a collection of different approaches to diagnosis and treatment ((Barnes, 2003). In most countries alternative remedies are regulated as foods, provided that no medicinal claim has been made against the label (Rousseaux, *et al*, 2003).

The pharmaceutical quality requirement of many complementary medicines is a cause for concern (Barnes, 2003). Manufacturers of licensed complementary medicines, are required to demonstrate to the Medicines Control Agency (MCA) that their products meet standards for pharmaceutical quality (as well as safety and efficacy), i.e. that they manufacture in accordance with the principles of GMP (Barnes, 2003). However, manufacturers of unlicensed products are not required to do this and therefore there is no guarantee that such products meet standards for pharmaceutical quality (Barnes, 2003).

Existing herbal ingredients of complementary medicine encompass complex mixtures of secondary metabolites that can vary considerably depending on environmental and genetic factors which will make it a difficult to determine the expiry date or shelf-life. Even when the chemical composition of a plant extract is known, the pharmacologically active moiety (ies) may not be known (Boullata, *et al,* 2000). The evaluation of herbal dosage forms should be established on the source of the raw material, quality, purity and the herbal dosage form.

2.3. Evaluation of the quality of herbal dosage forms

The important basic factors in the evaluation of herbal dosage forms includes the criteria and specification for the manufactured and finished products, the quality control of the starting (raw) plant material of herbal products and quality control guidelines for herbal dosage forms in assessing the safety, efficacy of a herbal medicine and dissolution are discussed.

2.3.1. The criteria and specification for the quality control of herbal dosage forms

Acceptance criteria and specification are the foremost important features of the quality control for herbal dosage forms. Acceptance criterion is one of the key features of the quality control for manufactured and finished herbal products. An acceptance criterion establishes specifications, i.e. qualitative and quantitative characteristics, with test procedures and acceptance limits for the medicinal product during its intended shelf life (EMEA, 2006a). For herbal medicinal products, the quality control criteria used is mainly found in different pharmacopoeia like British pharmacopoeia (1999), European Pharmacopoeia (1997), etc. For example, the procedures and specifications concerning the microscopic characteristics, chemical identification tests, total ash and hydrochloric acid insoluble ash levels, particle size, inorganic impurities and heavy metals limit tests, microbial contamination limit tests, mycotoxins presence tests and pesticide residue tests can all (clearly) be obtained from the pharmacopoeias and used to evaluate the quality of the crude herbal drugs.

Specification is defined as a list of tests, references to analytical and biological procedures, and appropriate acceptance criteria, which are numerical limits, ranges, or

other criteria for the tests described. It establishes the set of criteria to which an (1) herbal substance (2) herbal preparation and (3) herbal medicinal product should conform to be considered acceptable for its intended use (EMEA, 2001). The setting of specifications for 1, 2 and 3 is part of an overall quality control strategy which includes control of raw materials and add-ons, in-process testing, process evaluation/validation, stability testing and testing for consistency of batches. When combined in total, these elements provide assurance that the appropriate quality of the product will be maintained (EMEA, 2006). Familiarity with the scientific and technical literature regarding the specifications of chemical substances for pharmaceutical use is of primary importance, particularly for those working in Quality Assurance (QA) /Quality Control (QC), production and formulation development.The differences in pharmacopoeial specifications among regions usually exist. A harmonized global specification is possible if the procedures and acceptance criteria defined are acceptable to regulatory authorities in all regions. In case there is no available pharmacopoeial monograph, raw material manufacturers must establish approved specification and all other scientific data according to the technical guidelines, general monographs and good manufacturing practice (GMP) requirements. To ensure whether the plant material meets the required quality criteria or specifications it is essential that the raw plant materials be subjected to quality control tests.

2.3.2. The quality of the starting (raw) plant material of herbal products

The quality requirements for raw material extends from the time the crude drug is collected and distributed, through the stages of processing and manufacture into an herbal medicinal product, and on to the final product reaching the consumer. Raw

material can be defined as starting material or any intermediate which will be used for further processing (WHO, 2010). The source and quality of raw materials, good agricultural practices (GAP) and manufacturing processes are certainly essential steps for the quality control of herbal medicines and play a pivotal role in sustaining the quality and stability of herbal preparations (EMEA, 2002; WHO, 1998; WHO, 2000). Before finished pharmaceutical dosage forms are produced, the identity, purity and quality of raw materials (as per specifications for impurities and other related substances present) must be established by means of suitable testing methods (Sapna, *et al,* 2007*).* The raw material must be tested for microbial contamination, pesticide and fumigation agents, toxic metals, and other likely contaminants and adulterants.

The control of botanicals must be carefully planned in order to obtain plant materials which are both suitable as well as safe for use. This includes the botanical source, plant parts used and its state (i.e. whole, reduced, powdered, fresh, or dry). An assessment of the quality of the starting material and excipients are required. The information on the site of collection, time of harvesting, stage of growth, drying and storage conditions should be documented (WHO, 2004). In the case of herbal drugs with constituents with known activity, assays of their content using validated methods are required. This content must be stated as a range in order to ensure reproducibility (EMEA, 2001).

2.3.3. Quality control guidelines for the assessment of herbal dosage forms

The quality control guidelines of raw material of herbal products and herbal dosage forms are done according to description set by the BP or USP monographs. The BP and USP provide useful guidelines on quality control assessment for herbal medicine

and their preparations are standardized, regulated and quality is controlled according to guidelines described in the official compendiums. These guidelines for the assessment of herbal medicines are intended to facilitate the work of regulatory authorities, scientific bodies and industry in the development, assessment and registration of herbal products (WHO, 1991). The herbal product monographs listed in the BP and USP are useful checkpoints for quality evaluation on herbal medicines and herbal medicine preparations such as qualitative and quantitative assessment of the active substance, description of the method of preparation of the herbal medicinal, stability and dissolution test.

2.3.4. Dissolution

Dissolution testing is a method for evaluating physiological availability that depends upon having the drug in a dissolved state (Saccone, *et al*, 2004). Dissolution test measures the portion (%) of the Active Pharmaceutical Ingredient (API) that has been released from tablets/capsules and has dissolved in the dissolution medium during controlled testing conditions within a defined period. It is one of the most important and useful in-vitro tests for assuring product quality and batch to batch consistency (Kanfer, 2009). *In vitro* dissolution often aids in guiding the section of prototype formulation and often helps to determine optimum amounts of ingredients needed to achieve requisite drug release profiles. Dissolution also provides information on the impact of changes in composition, process or site of manufacturing which can help in identify potential problems of *in vivo* release and bioavailability absorption following administration.

Dissolution of drugs from solid dosage forms is a key parameter in assessing the product quality and uniformity at the formulation stage and throughout the shelf-life of the product (He, *et al,* 2004). The significance of a dissolution test is the fact that a drug should be in solution form to ensure that it is absorbed and available to systemic circulation (He, *et al*, 2004). In order for a drug to have its desired effect after oral administration, it must be soluble and diffuse through the gut wall into the body. The first step in that process is the disintegration of the dosage form followed by dissolution of the active ingredient. Dissolution of a pure substance follows the Noyes Whitney Equation:

$$\mathbf{dc/dt = kS\ (Cs- Ct)} \qquad \text{equation} \qquad 2.4$$

Where dc/dt is the rate of dissolution, k is the dissolution rate constant, S is the surface area of the dissolving solid, Cs is the saturation concentration of the drug in the diffusion layer and Ct is the concentration of the drug in dissolution media (or the bulk).

There is a scientific framework for classifying drug substances based on their aqueous solubility and intestinal permeability. The Biopharmaceutics Classification System **(**BCS) was originally developed for chemically defined synthetic drug substances, but it may also help with Herbal Medicinal Products (HMPs) (Blume, *et al,* 2000). When combined with the dissolution of the drug product, the BCS takes into account three major factors that govern the rate and extent of drug absorption from immediate-release solid oral dosage forms: dissolution, solubility, and intestinal permeability.

According to the BCS, drug substances are classified as follows:

Class 1: High Solubility = High Permeability; Class 2: Low Solubility = High Permeability; Class 3: High Solubility = Low Permeability; and Class 4: Low Solubility = Low Permeability. In addition, solid oral dosage forms are categorized as having rapid or slow dissolutions. Within this framework, when certain criteria are met, the BCS can be used as a drug development tool to help funders justify requests for biowaivers.

Unlike orthodox medicines, specific guidelines for dissolution testing of complementary/alternative have not been developed (Nair, *et al*, 2008). Compendia methods for botanicals products, such as the BP or USP, apparatus I (basket), II (paddle) and III (reciprocating cylinder) and disintegration test may be used to evaluate the *in vitro* release characteristics of the dosage form. Generally, dissolution testing of solid oral dosage forms is carried out by the basket (USP apparatus 1) method under mild agitation (100 rpm with the basket) in aqueous buffer in the pH range 1.2 to 6.8. The data obtained could be presented as percentage of dissolved material as function of time and pH.

2.4. Characterisation of the quality of the starting plant materials of Phela

Herbal drug preparations are diverse in character and vary from simple, comminute plant material to extracts. Based on recent scientific data, a comprehensive specification must be developed for every herbal drug preparation.

2.4.1. Characteristics of relevance in the evaluation of Phela raw materials

The test parameters which are discussed below should be recommended for the quality control of herbal drug preparation relevant to Phela:

2.4.1.1. Organoleptic features

Organoleptic refers to any sensory properties of a product involving taste, colour, odour and feel or texture. Organoleptic testing involves inspection through visual examination, feeling and smelling of products.

Colour is a vital means of identification for many pharmaceuticals, capsules and is usually important for consumer acceptance (Odeku, 2005). The colour of the product must be uniform within a single tablet, from tablet to tablet and from lot to lot (Sekharan, *et al,* 2010). Non-uniformity of colouring not only lack visual appeal but also could be associated by the consumer with non-uniformity of content and poor product quality (Sekharan, *et al,* 2010). Non-uniformity of colour is referred to as mottling. The eye cannot differentiate small differences in colour nor can it precisely define colour and efforts have been made to quantitate colour evaluations.

Odour may also be important for consumer acceptance of tablets and can provide an indication of the quality of tablets as the presence of an odour in a batch of tablets could indicate a stability problem, such as the characteristic odour of acetic acid in degrading aspirin tablets. However, the presence of an odour may be characteristic of the drug (e.g. vitamins), added ingredients (e.g. flavouring agent) or the dosage form (e.g. film-coated tablets).

Taste is also important for consumer acceptance of certain tablets (e.g. chewable tablets) and many companies utilize taste panels to judge the preference of different flavours and flavour levels in the development of a product. Taste preference is however subjective and the control of taste in the production of chewable tablets is usually based on the presence or absence of a specified taste.

2.4.1.2. Microscopic and macroscopic properties of powders

Sieving is one of the fundamental methods for the classification of powders, and it is the method of choice for determining the size distribution of coarse powders (Brittain, 2002). Microscopic examination of the raw drug substance provides an indication of particle size and particle size range of the drug substance, as well as its structure (Allen, 2008). During some processing procedures, the solid drug powders must flow freely and not become entangled or agglomerated. Spherical and oval-shaped powders flow more easily than needle-shaped powders and may facilitate processing.

Particle size, and particle size distribution affects certain physical and chemical properties of drug substances, such as drug dissolution rate, bioavailability, content uniformity, taste, texture, color, and stability (Allen, 2008). They both play significant roles in flowability and other properties, such as bulk density, angle of repose, and compressibility of bulk solids (Ganesan, *et al,* 2008). Flow characteristics and sedimentation rates (suspensions) are also important factors related to particle size. It is essential to establish, as early as possible in the formulation process, how the particle size of the drug substance may affect formulation and product efficacy (Allen, 2008).

Even a small change in particle size can cause significant alterations in the resulting flowability. Particle size influences the production of formulated medicine as solid dosage forms. Reduction in particle size often tends to decrease the flowability of a given granular material due to the increased surface area per unit mass (Fitzpatrick, 2004a and Fitzpatrick, 2004b).

Quality control of herbal drugs has traditionally been based on appearance. Today, microscopic evaluation is crucial in the initial identification of herbs, as well as in identifying small fragments of crude or powdered herbs, and for detecting foreign matter and adulterants. A primary visual evaluation, which seldom needs more than a simple magnifying lens, can be used to ensure that the plant is of the required species, and that the right part of the plant is being used. Sometimes microscopic analysis is needed to determine the correct species or verify that the correct part of the species is present.

2.4.1.3. Water content

It is important to perform a verification test when the herbal drug preparation (especially extracts) are known to be (water-loving) hygroscopic. One characteristic of hygroscopic powders is that they continuously change their physicochemical properties when exposed to relative humidity and temperature conditions that either favour the absorption or loss of moisture (Teunou, 1999). The moisture acquired due to the hygroscopicity of the powder may lead to the degradation of the powder when enzymes like glycosidase are activated. Water impurities generally include minerals, viruses, bacteria and other organic material.

2.4.1.4. Aqueous solubility

The solubility of a compound depends upon the physical and chemical properties of the solute and the solvent. Solubility is sensitive to changes in temperature according to Le Chatelier's Principle. Solubility will increase with decreasing size of solute particle (or droplet) because of the additional surface energy. This effect is generally small unless particles become very small, typically smaller than 1 μm. The effect of the particle size on solubility constant can be quantified as follows:

$$\log({}^{*}K_A) = \log({}^{*}K_{A\to 0}) + \frac{2\gamma A_m}{3\ln(10)RT} \qquad \text{equation 2.1}$$

where ${}^{*}K_A$ is the solubility constant for the solute particles with the molar surface area A, ${}^{*}K_{A\to 0}$is the solubility constant for substance with molar surface area tending to zero (i.e., when the particles are large), γ is the surface tension of the solute particle in the solvent, A_m is the molar surface area of the solute (in m^2/mol), R is the gas constant and T is the absolute temperature.

The extraction of any crude drug with a particular solvent yields a solution containing different phyto-constituents. The use of a single solvent sample can be the means of providing preliminary information on the quality of a particular drug sample. This method determines the amount of active constituents extracted with solvents from a given amount of medicinal plant material. It is employed for materials for which there is still no suitable chemical or biological assay exists (WHO, 1998).

2.4.1.5. Microbial contamination

Microbial contamination is associated with algae, bacteria and fungi (Kneifel, *et al*, 2002). Inevitably, this microbiological background depends on several environmental factors and exerts an important impact on the overall quality of herbal products and preparations (Kneifel, *et al*, 2002). Risk assessment of the microbial load of medicinal plants has therefore become an important subject in the establishment of modern Hazard Analysis and Critical Control Point schemes. Microbial contamination of medicinal plant parts could be the result of inappropriate harvesting and cleaning of raw plant material, unhygienic processing of plants and incorrect transport mechanisms.

Plants intended for use in botanical dietary supplements should be cultivated using Good Agricultural Practice (GAP). This approach provides quality assurance by helping to prevent microbial, heavy metal, herbicide, and pesticide contamination and by excluding weeds and insects. If wild plant specimens are collected or plant material is purchased from suppliers without GAP assurance, they should be assayed for levels of pesticides, herbicides, heavy metals, and microbes (van Breemen, *et al*, 2007).

2.4.1.6. Level of heavy metal and pesticides

A common misperception is that natural substances cannot be present in toxic concentrations in a variety of herbal preparations and dietary supplements (Ernest, 1998). The study entitled “Heavy metal hazards of Nigerian Herbal Remedies” revealed that high levels of iron, nickel, cadmium, copper, lead, selenium, and zinc would cause adverse health effects when regularly taken as recommended (Obi, *et al*,

2006). Failure to establish the true cause of exposure means that the patient continues to take the metal-containing medication. Thus, the screening of traditional remedies for efficacy and safety has been recommended to protect the general public. Heavy metals are known contaminants or adulterants of many traditional remedies (Obi, *et al*, 2006). The WHO is not yet in a position to recommend limits for contaminants since these are too diverse and there remains a lack of consensus on the matter (WHO, 2005). The risks are particularly high when the therapeutic preparation contains numerous plants, like Phela capsules.

The need for inclusion of tests and acceptance criteria for inorganic impurities should be studied during the development and based on knowledge of relevant plant species, their cultivation and manufacturing processes. Acceptance criteria will ultimately depend on safety considerations. Where justified, procedures and acceptance criteria for sulphated ash or residue, on ignition, should follow pharmacopoeial precedents; other inorganic impurities may be determined by other appropriate procedures, e.g., atomic absorption spectroscopy.

The medicinal plants material can be contaminated with heavy metals and pesticides which can be attributed to many causes including environmental pollution and traces of pesticides (Street, 2008). As these components even in trace amounts can be dangerous, they have to be removed from the medicinal products. Limit test for these materials have been prescribed by the WHO (WHO, 2007). They have also established the maximum residue limit (MRL) for biocides in the medicinal plant cultivation. The MRL is calculated after safety test in human beings, which indicate

toxicologically accepted levels according to the reliable assay available. The MRL is calculated using the following formula.

$$\textbf{MRL} = \frac{\textbf{ADI x W}}{\textbf{MDI x [100 x (safety factor)]}} \qquad \text{equation 2.2}$$

Where MRL = Maximum Residue Limits (mg/kg)

ADI = Acceptable Daily Intake (mg compounds/ kg body weight)

W = Body weight (kg)

MDI = Mean daily intake of drug

When the herbal crude drug is used to prepare extracts, tinctures or other phytopharmaceutical formulations in which the manipulation may influence the pesticides concentration of the final product, the MRL is calculated as:

$$\textbf{MRL} = \frac{\textbf{ADI x W x E}}{\textbf{MDI x [100 x (safety factor)]}} \qquad \text{equation 2.3}$$

Where E = the extraction coefficient of the pesticide, which depend on the method of preparation and needs to be experimentally determined.

The WHO (2007) on guidelines for assessing quality of herbal medicines with reference to contaminants and residues were used to assess the acceptability level of heavy metals in herbal medicines.

2.5. Analytical techniques for quality control of herbal capsules

Analytical methods such as photometric analysis, Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) can be used to establish the constant composition of Phela herbal preparations. In cases where active ingredients are unknown or have become too complex, the quality of plant extracts can be assessed by means of a chromatographic fingerprint.

The approach for the mainstream pharmaceutical industry has been to identify novel single-entity drugs for any given disease or condition. Once a candidate drug is established, a harmonized, standard quality–control measure is applied. The quality of single-entity pharmaceuticals is based on the correct chemical identity, purity and consistency.

The chemical identity of pure starting material is established by physicochemical characteristics based on melting point; ultraviolet-visible (UV-Vis); infrared (IR); mass spectral (MS); nuclear magnetic resonance (NMR); spectra; and X-ray data. Analytical techniques such as high-performance thin-layer chromatography (HPTLC); high-performance liquid chromatography (HPLC); gas-chromatography (GC); and capillary electrophoresis (CE) reveal the purity or impurity profiles and establish consistency (Govindaraghavan, 2008).

Purity is closely linked with the safe use of drugs as it deals with factors such ash values, contaminants (e.g. foreign matter in the form of other herbs), and heavy metals. However, due to the application of improved analytical methods, modern purity evaluation also includes microbial contamination, aflotoxins, radio activity and

pesticide residue testing. In this study HPLC assay can then be used to measure the flavonoid content of the capsule dosage form.

2.6. Flavonoids used as chemical markers for quality control.

Phela is rich in chemicals (such as flavonoids) that can be used effectively as markers in the analytical procedures for the content uniformity and dissolution tests of Phela capsules. These flavonoids may contribute to the biological activity of and could be appropriate phytochemicals to monitor quality control studies of Phela herbal dosage forms. These chemical compounds should be used as markers and the quantification by HPLC analysis is a viable method to help evaluate the herbal capsules. HPLC and mass spectrometry are some of the modern techniques used as tools for identification and quantitative analysis of flavonoids (Harborne, 2000).

Attempts to adopt a similar concept (as explained above) for quality control purposes of herbal medicine raise immediate difficulties. Firstly, herbal medicines are complex mixture of diverse constituents, therefore purity or impurity profiles cannot be easily established as can be done with single-entity drugs. Secondly, the quality of herbal medicine has to be evaluated with an understanding of the complexity of the raw materials which it contains. Strict quality control guidelines are not available to help assess complex interactions, such as synergism and antagonism among constituent mixtures (Govindaraghavan, 2008).

Chapter 3

Plan of work

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

3.1. Objectives

The overall aim was to determine whether capsules of Phela made for traditional use were of suitable quality to use in clinical trials.

The specific objectives of the study were to:

a) Establish (from literature) a series of criteria and specifications that are appropriate to establish the pharmaceutical quality of plant-containing capsules such as Phela,

b) Assess the pharmaceutical characteristics of the finished Phela capsules,

c) Assess the pharmaceutical characteristics of the "formulated" Phela raw material, and

d) Provide a certificate of analysis for the finished capsules.

3.2. Hypotheses

It was hypothesized that,

Firstly, the capsule dosage form of Phela plant material would meet most of the general pharmacopoeial quality specifications,

Secondly, plant material contents of Phela capsules would be rapidly dissolving and meet the BP specification of 75 % of the extractable plant material dissolving in the acidic solution at pH 1.2 within 45 minutes, and

Thirdly, the Phela plant materials contain flavonoids-like compounds that will be susceptible to degradation and may be suitable as marker compounds to monitor the stability Phela capsules during storage.

3.3. Study approach

To realize the above objectives the following were done:

3.3.1. Establishment of a list criteria and specifications for pharmaceutical quality of plant material containing capsules

Information provided by pharmacopoeia and regulatory agencies e.g. (BP, EMEA, WHO, etc) via internet was to be searched and assessed for criteria that may be used to establish the pharmaceutical quality of Phela. The information provided by the BP comprises of a series of monographs, each detailing mandatory standards for active substances, excipients and formulated preparation, together with supporting general notices, appendices (test methods, reagents, etc.) and reference spectra (BP, 2009). The United State Pharmacopeia (USP), nor the Japanese Pharmacopeia (JP) or the German Pharmacopeia (GP) were not used because most of the information provided in the BP would be exactly the same. Most of the information provided by EMEA relates to the conclusions reached by the European Medicines Agency's scientific committees following their procedures for evaluating the quality, safety and efficacy of medicines (EMEA, 2010). The WHO was chosen because of worldwide recognition for regulation of quality control of herbal medicines and the work of

WHO in supporting the preparation of model guidelines in this field, have been helpful in strengthening recognition of their role in health care (Zhang, 1998).

3.3.2. Assessment of the pharmaceutical quality of the finished Phela capsules

Because the Phela capsules had already been manufactured by the time this present study commenced, the first step in the evaluation of the pharmaceutical quality of Phela was to focus on the manufactured Phela capsules. For this, the set of tests, methods and specifications arrived at in part 3.3.1 was to be used. The capsules were assessed specifically for their organoleptic characteristics, uniformity of weight, moisture content, dissolution profile and stability using pharmacopoeias methods.

3.3.3. Assessment of the pharmaceutical quality of the "formulated" Phela raw materials

Because the Phela capsules contain a mixture of 4 different plant raw materials, each of which will affect the quality of the final Phela product, the pharmaceutical quality of each of the raw material powders and the mixture was also evaluated. Specifically, the physicochemical properties of the individual and the final formulated Phela raw material was assessed using the methods and specifications suggested by the guidelines discussed under 3.3.1. From the results the rational and appropriateness of the formulation and manufacture of these capsules could be assessed. In this study, the generally used or suggested characteristics i.e. organoleptic features, flow properties, ash values, moisture content, contaminant (microbial, heavy metal, pesticide) levels, particle size, HPLC fingerprint, aqueous solubility and stability were determined using pharmacopoeia methods.

3.3.4. Determination of stability profile of Phela capsules

The final step in this study was to determine the stability profile of the manufactured Phela capsules. For clinical trials, the capsule dosage form of Phela should remain stable over a suitably long period and this can be determined by monitoring the level and integrity of selected chemical constituents of the herbal preparation over time. In this study, HPLC chromatographic fingerprints of the Phela material were generated and analysed. As marker compounds for the stability assessment flavonoids were chosen because of their known abundance in these plants (Treutter, 2006), their known ability to cause pharmacological effects relevant to Phela (Ratty, *et al*, 1988) and their chemistry and degradation potential (Andersen, 2006) and their easy, sensitive and reliable detection by UV spectrophotometer. Finally, the HPLC method of assay was chosen because it is currently the most frequently used separation technique, and is capable of resolving complex chemical mixtures including the crude extract of traditional medicines (Ye, *et al,* 2006).

3.3.5. Compilation of certificate of analysis

A certificate of analysis (CoA) is a list of analytical tests, acceptance criteria, and results obtained on a specific product (USP, 2009) and forms an important part of any manufacture and quality assurance programme. Because the Phela capsules were intended for a clinical study, a CoA was required (WHO, 2005b). In this study, the CoA was compiled from the results obtained in the pharmaceutical analysis of various batches of raw materials and the herbal capsule dosage form as described under 3.3.2.to 3.3.4.

Chapter 4

Methods

In this chapter, the process used to select the criteria and specifications that can be applied to assess the pharmaceutical quality of a herbal product such as Phela capsules are presented. In addition, the materials, equipment, methods and procedures used to evaluate the pharmaceutical quality of the finished Phela capsules are presented.

4.1. Chemicals and equipment

The following chemicals were used:

Acetonitrile, water and methanol (HPLC-Grade, Burdick & Jackson, *Cape Town*, South Africa); double distilled water (Fi-streem; England); hydrochloric acid (32% w/w, Analar grade*, Cape Town*, South Africa) "KIMIX"; potassium dihydrogen phosphate (KH_2PO_4) Anala- R® (99.5-100.5%). Hard vegetable gelatine capsules (Size 0; Colour: Yellow and Orange, Cape Town).

The following equipment was used:

Oven (figure 4.1., *Model P.A Luthbert & C0, South Africa);* milling machine (figure 4.1., *DCE Donaldson Limited, England*)**;** Automated capsule machine (figure 4.1.,"SCF-10", China), Balance:- "OHAUS" GA110 "Mettler" AJ 100; 0.45µm HV DURAPORE® MEMBRANE FILTERS; 5 ml syringes (PVA) "Promex"; Dissolution apparatus:- "VanKel" VK 700; UV-Vis Spectrophotometer:- "Hitachi" U-3200; microscope, light:- "Nikon Abbe 1.25" with an Olympus eyepiece; 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*);

Mechanical Tapping Device (*Chadwell Heath Essex, England*); The HR73 Halogen Moisture Analyzer (*METTLER TOLED, South Africa*); Ash machine (*Lasec, South Africa*) HPLC system:- "Agilent 1100 Series" fitted with: an Agilent 1100 Series quaternary pump, an Agilent 1100 series Diode Array and Multiple wavelength detector; HPLC column: (XTerra ® 5µ C-18, 4,6 x 150 mm, reversed-phase; Computer program in data-analysis:- Graph Pad PRISM "Version 5.00"

Oven	**Milling Machine**	**Automated Capsule Machine**
		

Figure 4. 1: Manufacturing Equipments

4.2. Methods and procedures

The following methods and procedures were used.

4.2.1. Selection of criteria, specifications and test methods for pharmaceutical quality of plant containing capsules

Information provided by pharmacopoeia (e.g. BP, etc) and various regulatory authorities (e.g. EMEA, WHO, etc) via literature and the internet was searched and assessed for criteria and specifications that may be used to determine the pharmaceutical quality of the Phela capsules. Search terms like "guidelines for assessment for herbal medicines", "quality control parameters for herbals'',

"specification for herbal drug preparations" and "acceptance criteria of herbal medicines" were used to look at the sites and locate the specifications, i.e. analytical test, method and accepted tolerance limits, which were generally advocated to assure quality of herbal preparations. The internet search was conducted between the months of August to September 2008 and two websites, viz. that of the European Medicines Agency (EMEA, 2004) and the World Health organization (WHO, 2000).

A summary of all the test procedures, methods, criteria and specifications found was drawn up and from this a list those procedures, etc that were deemed appropriate for the evaluation of the Phela capsule was compiled.

4.2.2. Manufacture of Phela capsules

Phela capsules containing the combination of four plants i.e. RM, CG, PT and S that were collected by IKS MRC in different regions of South Africa were manufactured. The fresh plant materials were collected in July 2008 After collection the plant materials were separated from earthy and other foreign material, washed with distilled water, the leaves, stems and roots separated from the other portions of plant material and dried at 30^{o}C in the industrial oven (figure 4.1) until each part retained a constant weight. Generally the individual Phela plant materials were reduced to one fourth of its weight upon drying. After this, each part (i.e. leaves, etc) was ground into a fine powder using an industrial milling machine (figure 4.1). The Phela mixture was then made up by mixing the four plants i.e. RM, CG, PT and S (and mainly the roots) in a ratio of 1:1:1:2 parts, respectively, and capsules of the mix prepared using an automated capsule machine (figure 4.1). Finally, the finished capsules were cleaned by polishing them and then placed in suitable airtight containers. In this study the final

capsules, the Phela mixture as well as the dried powder of the individual constituent plants were pharmaceutically evaluated.

4.2.3. Determination of the pharmaceutical quality of Phela capsules

The pharmaceutical quality of the capsule contents and the capsules itself were evaluated.

4.2.3.1. Determination of organoleptic properties of Phela capsule and contents

To determine the organoleptic features of the capsule the appearance, shape and dimension of the capsule as well as the odour, colour and taste of the dried mixed and individual constituent powders of Phela were characterized using the natural sense organs (i.e. by eye, nose and tongue). This was done to establish a few key identification characteristics for the capsules. For the physical features, we looked at the material, particle size, texture and the strength of the particles.

4.2.3.2. Determination of particle size and shape of Phela powders

Sieve and microscopic methods are the commonly used methods to determine particle size and shape and were used to determine these for the materials used in this study. In this case, the British Pharmacopoeia (BP, 2000) methods were used to determine the fineness**,** particle size and particle shape of the Phela powder mixture and the individual constituent plant powders. For this sieves No's 500, 355, 180, 125, 90 and 0 were netted in order of decreasing aperture size, and a receiving pan closely fitted at their bottom. An accurately weighed amount (about 10.0 g) of samples of the Phela powders were placed evenly upon the top-most sieve, the sieve arrangement shaken using the mechanical shaker until sifting was practically complete (around 40

minutes). Then, a soft cloth hairbrush was used to gently dislodge any powder material entrapped within the sieve openings. Thereafter the powder materials retained on each sieve size were weighed to the nearest 0.1 g and the proportion of powder materials retained on each sieve calculated as percentages of the total feed. These values were then compared with the BP specifications (BP, 2000) to establish the fineness classification of each powder.

To determine the shape of the particles of the mixture and individual powders a microscope was used to observe the particle shape and measure the size. The length and breadth of a selection of the particles in the view was measured with the micrometer, the readings recorded and the degree of sphericity calculated using the following equation

$$\mathbf{N = L/B} \qquad \text{equation} \qquad 4.1$$

Where: N = the degree of sphericity, L = particle length and B = particle breadth.

4.2.3.3. Determination of the density of Phela powders

To determine the density of the Phela powders a tester that essentially consists of a graduated cylinder placed on the tester platform was used (figure 4.2). In this apparatus a tapping action is generated by a camshaft, which lifts the platform and allows it to drop back to its original position. The normal speed of 100 taps per minute was used and taps were applied until a maximum packing condition was achieved. For the actual study, each powder was passed through the 710-micron sieve in order to break up agglomerates. Then it was weighed and poured, via a funnel, into a graduated cylinder. The volume of the powder was measured and the loose bulk

density calculated by dividing the weight of the powder by this volume. Thereafter, the level of powder in the graduated cylinder was checked after every 10 taps till there was no further reduction in the level. This was taken as the tapped volume which was used to calculate the tapped density. The measurements were done in triplicate for each Phela powder and an average value calculated. From the loose-packed and tapped densities, Carr's compressibility index was calculated using the following equation (Thalberg, *et al*, 2004).

$$\mathbf{HR = TD/BD} \qquad \text{equation} \qquad 4.2$$

Where HR = the Hausner ratio, TD = the tapped density and BD = the loose-packed bulk density of the powder.

Figure 4. 2: Apparatus for density measurement

4.2.3.4. Determination of flow properties of the Phela powders

To assess the flow properties of the Phela powders the angle of repose for the Phela mixture and individual constituent plant powers were determined as follows. About 10 g of sample powder was placed in a glass cylinder that is open at both ends (figure 4.3). The cylinder was then slowly and even lifted allowing the powder to freely run out the bottom end of the cylinder unto a flat surface forming a small pile of powder. Care was taken not to impose any stress on the powder in the cylinder and excess powder was removed from the cylinder. The height and radius of the heap were measured and the angle of repose calculated using the following equation

$$\tan \theta = h/r \qquad \text{equation} \qquad 4.3$$

where θ = the angle of repose, h = height of the conical mound (cm) and r = the radius of the conical mound of powder (cm) (Chandira, *et al*, 2010). Triplicate readings were made for each of the powders and the average reading calculated and recorded.

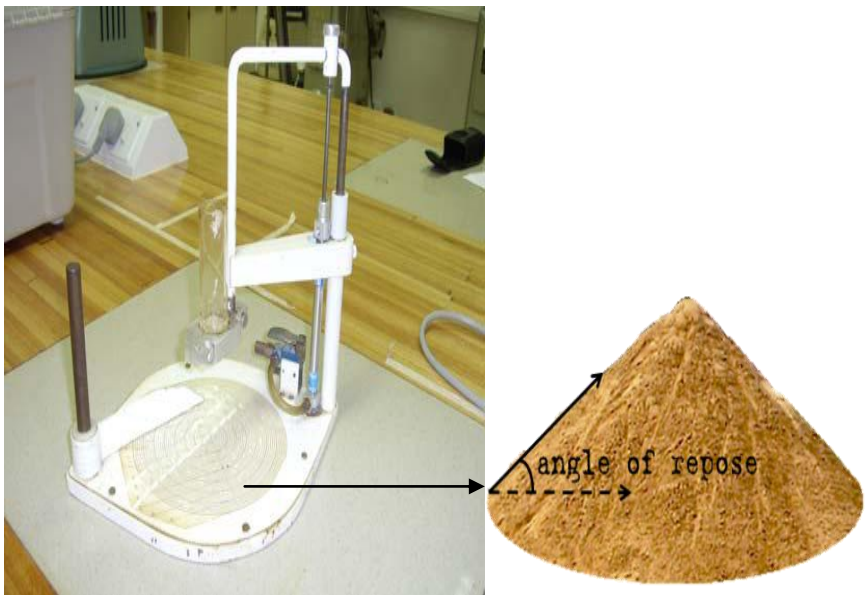


Figure 4. 3: Apparatus for measuring angle of repose.

4.2.3.5. Determination of total ash values of Phela powders

For the total ash testing, 2 g of the dried Phela plant or mixture powder material was heated in a tared crucible at 450 ^{0}C in a furnace until they were free of carbon (by checking color change). For those for which a carbon-free ash could not be obtained in this way, hot water was poured over the charred mass, the mixture filtered on ashless filter paper and the residue on the filter paper incinerated at 450 ^{0}C. Thereafter the powders were cooled, weighed and the percentage of ash calculated with reference to the mass of the air-dried drug.

For the acid-insoluble ash testing, the total ash of the relevant Phela powder was boiled for 5 minutes with 25 ml of 2M hydrochloric acid, the insoluble matter collected on an ashless filter paper, washed with hot water and then ignited and heated at 450 ^{0}C. Finally, the remaining ash was weighed and the percentage of acid-insoluble ash determined with reference to the mass of the air-dried drug. The individual powders were subjected to the same procedures and the results obtained were compared.

4.2.3.6. Determination of the moisture level of the Phela powders.

The moisture levels of the Phela capsule contents and individual powders were determined using a Halogen Moisture Analyzer HR73. The analyzer consisted of a halogen-heating unit, a sample plate and an electronic weighing balance and works on the thermogravimetric principle i.e. the moisture analyzer determines the weight of the sample at the start, the integral halogen-heating module then quickly heats the

sample and the moisture vaporizes. During the drying process, the instrument continually measures the weight of the sample and displays the reduction in moisture.

Figure 4. 4: HR73 Halogen moisture analyzer.

To determine the moisture level of the Phela powders six replicates of one gram of the individual powders and Phela mixture was placed on the balance plate, the analyzer started and the apparatus allowed to automatically calculating the weight of the moisture, from the following formula.

Moisture weight = Initial weight (Wet mass) – Final weight (Dry mass) - equation 4.4

4.2.3.7. Determination of the water-soluble extractable fraction of Phela powders.

To determine the proportion of extractable fraction of the Phela mixture and individual powders, about four grams of each powdered material was accurately weighed in a conical flask, 100 ml of water added and the mixture macerated for 6 hours while shaking frequently Thereafter, the mixture was allowed to stand for 18 hours before it was filtered and approximately 25 ml of the filtrate was transferred to a

tared flat-bottomed dish and evaporated to dryness at 105 ^{0}C for 6 hours. The residue was then cooled in a desiccator for 30 minutes, and weighed immediately. The proportion of extractable matter was determined by subtracting the weight of the residue from the total powder weight and expressing it as fraction of the total powder weight. The results obtained for the powder mixture was compared with that for the individual plants.

4.2.3.8. Determination of microbial contaminants of Phela powders.

To determine the level of microbial contamination of the Phela mixture, 10 g of samples of the individual powders and mixture were packed into sealed brown glass containers and subjected to the following microbial level tests: total microbial activity (TMA) or total viable count, *Escherichia coli* count, yeast and mould count, *Salmonella* count and *Enterobacteriaceae* and other Gram-negatives counts. These tests were done by *Swift Microlaboratories, Cape Town* using standard procedures for microbial testing. Finally, the level of microbial contaminants obtained was assessed according to the specifications set by WHO (WHO, 2007).

4.2.3.9. Determination of heavy metal contaminants in the Phela powders.

To determine the levels of heavy metal and pesticides, the Phela mixture and individual dried powders were subjected to the following pesticide and heavy metal level tests done by the South African Bureau of Standards (SABS) Laboratories, Cape Town and MARLAB, Cape Town: pesticides - organophosphate, organonitrate, organochloride and N-methyl carbamate and heavy metals - lead (Pb), cadmium (Cd), mercury (Hg), arsenic (As), nickel (Ni) and chromium (Cr).

4.2.3.10. Determination of HPLC fingerprints of the Phela powders.

An Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) comprising a quaternary solvent delivery system, an on-line degasser, an auto-sampler, a column temperature controller and photodiode array detector coupled with an analytical workstation was used to generate chromatographic fingerprints of the Phela powders. An Agilent XTerra ®MS C_{18} reversed-phase column (5 μm, 150 mm × 4.6 mm) and an Agilent Zorbax Extend C_{18} guard column (5 μm, 5 mm × 4.6 mm) maintained at 40 °C were used.

The sample solution was prepared by extracting 10 mg of dried Phela plant material with 10 ml of methanol and distilled water (50:50 v/v). The solution was sonicated for 15 minutes, centrifuged at 1000 rpm for 30 minutes, the supernatant collected and evaporated to dryness under a gentle stream of nitrogen at room temperature. The extracted material was re-dissolved into 5 ml water and acetonitrile (50/50 v/v) and the sample solution filtered through a 0.45- μm filter membrane before HPLC analysis. The sample solutions for the four individual plant materials and the Phela mixture were prepared in the same way and 20 μl of the extracts were injected on column.

Elution of the solute peaks were achieved with a mobile phase consisting of water-formic acid (A; 100:0.01, v/v) and acetonitrile (B) pumped at flow rate of 1ml/min and the following gradient, viz. initial condition of solvents A : B of 50:50 v/v; linearly changed to 40:60 v/v at 10 min and then to 20:80 v/v over the next 20 minutes. The detector wavelength was set at 300 nm and the chromatographic fingerprints of Phela and individual plant materials obtained were compared (in terms

of number, retention times and area percentage). The more prominent peaks (in terms of peak size and presence in the individual plant powders) were identified and subjected to spectral analysis to try and identify those with possible flavonoid spectral characteristics.

4.2.3.11. Determination of uniformity of weight of the Phela capsules.

The British Pharmacopoeia (BP, 2000e) method was used. According to this method not more than two of the individual weights (masses) of the capsules must deviate from the average weight (mass) by more than 7.5 % and none of the deviates by more than twice that percentage.

To determine the uniformity of weight of the manufactured capsules and its content twenty capsules were randomly selected from each of the manufactured batches. Each capsule was weighed and then completely emptied of its contents, the empty shells brushed to remove any remaining particles, and the empty capsule reweighed. The mass of the capsule contents was calculated by subtracting the mass of the empty capsule from the initial mass of the full capsule. The values for the 20 capsules were averaged. The percent deviation calculated and the results compared to the British Pharmacopoeia (BP, 2000) specifications.

4.2.3.12. Determination of the dissolution profile of Phela capsules.

The British Pharmacopoeia (BP) basket method (I) was used to determine the dissolution profile of the Phela capsules. Approximately 900 ml of buffer solution at pH 1.2 was used as dissolution medium to simulate dissolution in the stomach. The solution was degassed with helium gas before being introduced into the vessels in the dissolution apparatus and the apparatus allowed to warm up to 37±0.5 ^{0}C in the water

bath until a constant temperature was reached. One capsule was placed in each dry basket, the basket lowered into position and the apparatus immediately started at the rotation speed of 100 rpm.

At various time points, viz. at 0, 15, 30, 45, 60, 55 and 120 minutes after start, 5 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. The 5 ml withdrawn was immediately replaced with 5 ml of the buffer solution. The sampling probes were fitted with in-line 0.45 μm filters so that filtered samples were obtained. The UV absorbance of each sample was read at 280 nm (against a blank reference consisting of just the medium).

The sample giving the highest reading at 280 nm or the one at 120 minutes (with clear dispersion of the capsule contents) was taken as the 100 % released dissolved sample. The percent material dissolved at each time point was calculated and the average percentage material dissolved for the 6 Phela capsules at each time pointed plotted. From the graph, the time taken for 70 % of the capsule contents to dissolve was determined.

4.2.3.13. Determination of the stability of Phela capsules.

For this study, the manufactured Phela capsules were stored with packaging under one condition, viz. 40 ± 2^0 C and 75 ± 5 % relative humidity (RH) in a climate chamber (labcon). Some capsules were stored at 40°C and 75 % relative humidity (at site A) and some at 25^{o}C and ordinary room level relative humidity (at site B) and some at 5^0C and 70 % relative humidity (at site C, in a desiccator).At the start and every two

weeks samples of capsules were taken from each site of the storage condition and assessed for organoleptic properties and physical properties (i.e. gross physical nature, colour and odour of the powder content and overall size, shape and appearance of the capsule) and the level of selected markers.

The same HPLC conditions (see section 4.2.3.10) were used to assess the stability profile levels of Phela capsules, except that kinetex column was used. The spectrums of Phela capsules were compared in terms of retention time and peak heights after 24 weeks of storage.

Chapter 5

Results and Discussion

In this chapter, the results obtained during the investigation of the criteria and specifications that may be applied to determine the pharmaceutical quality of herbal capsule dosage forms and their use to assess the pharmaceutical quality of Phela capsules are presented and discussed.

5.1. Selection of criteria, specifications and test methods for pharmaceutical quality of plant-containing capsules.

The attributes, specifications, tests and test methods that are recommended by the BP, EMEA and WHO for the control of the quality of herbal products were reviewed and the results obtained are summarized in table 5.1. From this, a set of criteria, procedures, etc that were deemed appropriate for the evaluation of the Phela capsule was compiled.

Table 5. 1: A selection of criteria, tests, test methods and specifications for pharmaceutical quality of herbal dosage forms

Assessment criteria	*Tests*	*Method*	*Specification (Tolerance limit)*
	Raw material of herbal products		
Physical attributes	Organoleptic features (odour, colour & taste)	Visual, Olfactory	Consistency of odour, taste & colour
	Macroscopic features (size and shape)	as per BP [2000] and WHO	Must conform to herbarium reference material

	Microscopic	as per BP [2000] and WHO	Must conform to herbarium reference material.
Chemical identity	Chemical features presence &/or level of marker	TLC, HPLC or other internationally recognized methods	Presence and/or exact level of markers
Quantity of plant active(s)	Level of marker chemical constituent	Quantitative by GC, HPLC, etc	90-110% of label claim
Microbial contamination	Presence & level of contaminating fungus (yeast and mould)	Microbial test as per BP[1993], EMEA [2000], WHO [2007]	$< 1 X 10^4$ CFU/g or ml
	Total Aerobic Count	Microbial test as per BP[1993], EMEA [2000], WHO [2007]	$< 1 X 10^5$ CFU/g or ml
	Escherichia coli	Microbial test as per BP[1993], EMEA[2000], WHO [2007]	Absent
	Salmonella spp.	Microbial test as per BP[1993], EMEA[2000], WHO [2007]	Absent
	Staphylococcus aureus	Microbial test as per BP[1993], EMEA[2000], WHO [2007]	Absent
	Pseudomonas aeruginosa	Microbial test as per BP[1993], EMEA[2000], WHO [2007]	Absent
Heavy metal contamination	Level of Arsenic	Chemical test as per BP[1993], EMEA [2000], WHO [2007]	< 0.14 µg/kg b.w /day
	Level of Cadmium	Chemical test as per BP[1993], EMEA [2000], WHO [2007]	< 0.14 µg/kg b.w/day

	Level of Lead	Chemical test as per BP[1993], EMEA [2000], WHO [2007]	< 0.09 µg/kg b.w/day
	Level of total Mercury	Chemical test as per BP[1993], EMEA [2000], WHO [2007]	< 0.29 µg/kg b.w/day
Pesticide Contamination	Level and or presence of Pesticides (e.g. DDT)	Chemical test as per BP, EMEA, WHO [2007]	Conforms to Pharmacopoeial limits
Mycotoxins Contamination	Presence &/or level of Mycotoxins	Chemical test as per BP, EMEA, WHO [2007]	Aflotoxins < 20 ppb
	Herbal products/dosage forms		
Uniformity of Dosage form	Mass or content variation	As per BP [20006]	The % RSD for capsules is ±7.5%
Dissolution profile	Percentage released in set time performance test	As per BP [2000][5].	75% of label claim to be released in 45 minutes of dissolution testing.
Stability	Shelf life; expiry date of product; degradation half-life of chemical marker compounds level of (active or impurity).	as per , EMEA [2007], WHO [1996]	N/A

Only the information provided by the one pharmacopeia and two agencies , viz. the European Medicine Agency (EMEA, 2006), the World Health Organization (WHO, 1998) and the British Pharmacopoeia (BP, 2000) were looked at for the compilation of the above table and for the purpose of this study the BP, EMEA and WHO will

collectively be referred to as the agencies. These agencies seem to advocate similar criteria, tests, methods and tolerance levels (i.e. specifications) for the control of the quality of both pharmaceutical and herbal products. For the above table the assessment criteria for quality control of herbal medicine were separated into those focussing on the raw material attributes e.g. physical attributes, chemical identity, microbial, heavy metal, pesticides, etc contamination and finished product e.g. dissolution, stability, content uniformity (in terms of quantity of plant active), etc.

According to the requirements of the 3 agencies a list of the physical attributes of the raw material (and finished products and/or their contents) must be compiled based on the organoleptic and micro- and macroscopic physical features. For this the senses (i.e. visual or olfactory) and a variety of tests (e.g. using a BP method) can be used. For herbal products the tolerance level for these attributes must typically conform to that of herbarium reference material e.g. be consistent in odour, taste and colour to the latter.

Another important characteristic for quality control is the chemical identity of the herbal material or product. For this a test for the level or presence of marker chemical compounds found in the material can be done using internationally recognised methods, such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), etc. Some of the methods are more preferred than others. For example HPLC have advantages of speed of assay, automation, improved accuracy and precision over TLC and this is why it is nowadays more frequently employed in quality control. Finally, the specification level for this chemical identity

attribute of herbal material typically should indicate the presence or exact level of a chemical marker(s).

Probably the most important quality criterion for herbal products might be the level of active constituents they contain. Various quantitative analysis methods i.e. HPLC, GC, etc can be used to identify and obtain the levels of chemical marker compounds in the plant material that may or may not be plant active(s). For quality control the tolerance limits for the quantity of active ingredients in normal pharmaceuticals is typically set at 80 % to 120 % (or some narrower range) of the label amount (esp. if tested by HPLC). The same can thus be advocated for herbal products. Thus for herbal products the levels of target marker(s) or active constituent(s) should be well within a range set as the acceptance criteria for each marker(s).

Apart from the constituent chemicals of the herbal material the presence/absence of extraneously introduced materials such as microorganisms, heavy metals toxins, pesticides and adulterants that could end up in the herbal materials is also an important determinant of the quality of herbal material. All three agencies recommend that herbal materials be tested for a list of microbial contaminants, which include *E. coli, Salmonella spp, Staphylococcus aureus, fungi and molds, etc,* using typical methods also given in or by the BP, EMEA or WHO. Typically the quality control specification with respect to microbial contamination is based on the level of contaminants allowed to be present e.g. the colony levels of yeast and mould should be less than 1×10^4 CFU/g or ml and that for total aerobic counts $< 1 \times 10^5$ CFU/g or ml) or the complete absence of the contaminant e.g. *E. coli, Salmonella,* etc. should be

absent upon testing. The BP, EMEA and WHO generally advocated the same microbial contamination tolerance levels.

For heavy metal contamination such as lead, mercury, etc the BP, EMEA and WHO generally advocate the same tests, test methods and the tolerance levels. In addition there are tests for pesticides and mycotoxins (e.g. test for the presence of DDT and aflotoxins) required to confirm the quality of herbal products. Specifically, the levels of mycotoxins (e.g., aflotoxins) should be below 20 ppb and the presence of pesticides must conform to specific pharmacopoeial limits. Overall, the three agencies agree on the set of criteria and methods and specifications that are required to confirm the quality of an herbal product as far as contamination is concerned. The issue of intentional adulteration of herbal products with pharmacologically active pharmaceutical products is a related issue of quality but was not addressed in this project.

As far as dosage forms of herbals are concerned, the first important quality criterion for an herbal product should be that of mass and content uniformity. The test for mass uniformity entails is based on the use of a representative sample of 20 units that are individually weighed, the average mass determined and the specifications that not more than two of the individual masses deviate from the average mass by more than one percentage deviation (PD) and none deviates by more than twice that percentage (Martin, *et al,* 1998). According to the British Pharmacopoeia (BP, 2000f), 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 % (Ma, 2006).

Another important criterion of quality of especially herbal products is the rate at which it or its marker compounds dissolve i.e. its dissolution profile (BP, 2000e). The dissolution test and dissolution specification criterion is thus important for the quality control of herbal products. The test requires the determination of the percentage release of the active constituent into the dissolution medium over time typically using a method and specification criterion given in the BP. For instance, the tolerance limit set by the BP for a rapidly dissolving product is that 75 % of the label amount has to be released within 45 minutes. Once dissolution specifications are set, the pharmaceutical product or herbal product should, as a mark of continued quality, comply with those specifications throughout its shelf-life.

Lastly, the EMEA and WHO recommend that a profile of the stability of the herbal product be provided (EMEA, 2006b; WHO, 1996). Appropriate stability test(s) should be used to determine the levels and degradation half-life of chemical marker compounds (active or impurity) and calculate the shelf life and expiry date of the product(s). Typically the methods used to establish the dissolution profile and/or quantitative levels of active constituent(s) can be used in these stability tests. Presently, there are no formally recommended specifications available for the stability of herbal products in the EMEA, 2007 and WHO, 1996, but the limits that denote acceptable stability may be derived from the profile obtained when the herbal material was assessed in previous or pre-formulation studies. The stability tolerance limits need to include individual and total upper limits for impurities and degradation products. Normally, in the case of conventional pharmaceuticals, impurities and degradation products in concentrations higher than 0, 1 % should be identified (MCC, 2006) and possibly the same could apply for herbal products.

Overall, the review of the recommendations of the BP, EMEA and WHO on the quality control of herbal products, as summarised in table 5.1, provided useful information of quality attributes, test methods and specifications on which the quality profile of the Phela capsules could be based. It was consequently concluded that an assessment of the physical attributes, chemical identity and quantity of plant active(s), microbial and other contaminant levels, content and mass uniformity, dissolution profile and stability profiles of the Phela capsules and/or its contents should provide a good idea of the quality of the Phela capsules (suitability for use in clinical trial).

5.2. Assessment of the pharmaceutical quality of Phela plant materials.

5.2.1. Organoleptic properties of Phela raw material and capsule contents.

The determination of organoleptic properties involves measurement of attributes such as capsule size, shape, colour, presence or absence of odour, taste, surface textures, physical flaws and consistency. In this study, the following organoleptic properties of the plant materials of Phela were assessed: physical appearance, odour and taste using the natural sense organs (e.g. eyes, nose, and mouth).

The results of the assessment of the organoleptic characteristics of the individual powders and the final Phela mixture are given in table 5.2. The milled dried plant material of plant RM and CG had similar physical appearance, colour, taste and odour. Plant S powder was dark brown in color, had a characteristic odour and irregular shape and size particles and was bitter in taste while Plant PT powder was yellow in color, had a smooth soft texture, was extremely bitter in taste and medicinal odour.

Table 5. 2: The organoleptic characteristics of individual powders and Phela mixture

Characteristics	*Plant RM*	*Plant CG*	*Plant PT*	*Plant S*	*Phela*
Colour	light brown	light brown	yellow	dark brown	Uniformly light brown
Physical Appearance	small particle powders. rough and brittle particle free-flowing	small particle powders. rough and brittle particle free-flowing,	small particle powders, smooth soft particle free-flowing,	large particle powders, even, rough texture particles non-flowing	small particle powder even rough and brittle particles free-flowing,
Taste	Bitter	bitter	extremely bitter	bitter	bitter
Odour	characteristic plant odour	characteristic plant odour	medicinal odour	senna odour	characteristic medicinal odour

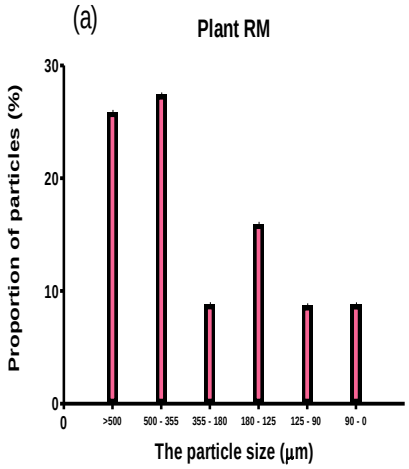
Not surprisingly the entire mixture had light brown colour reflecting the organoleptic features of its components, even rough and brittle particles, a bitter taste and unpleasant odour. The features, especially the taste and colour, of the final Phela mixture may result in poor patient acceptability. Fortunately, the two characteristics were however masked by using a capsule dosage form that alleviated these unpleasant effects.

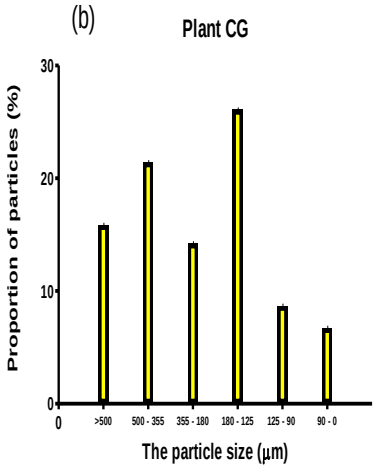
5.2.2. Particle size and shape of Phela raw material and final mixture.

Sieve and microscopic methods are the commonly used methods to determine particle size and shape (BP, 2000). The results of the determination of the particle size of the Phela mixture and individual powders are given in appendix 1 and summarized in table 5.3 and figure 5.1.

Table 5. 3: Percentage of Phela plant powder retained on each sieve size

Sieve size(µm)	*Plant RM (%)*	*Plant CG (%)*	*Plant PT (%)*	*Plant S (%)*	*Phela (%)*
500	8.79	6.7	6.01	6.33	7.62
355	8.7	8.67	7.68	15.63	9.61
180	15.93	26.06	18.33	33.39	24.44
125	8.8	14.2	17.22	11.05	8.27
90	27.41	21.4	39.31	17.09	22.66
0	25.86	15.84	5.94	11.84	20.96
total	95.49	92.87	94.49	95.33	93.56





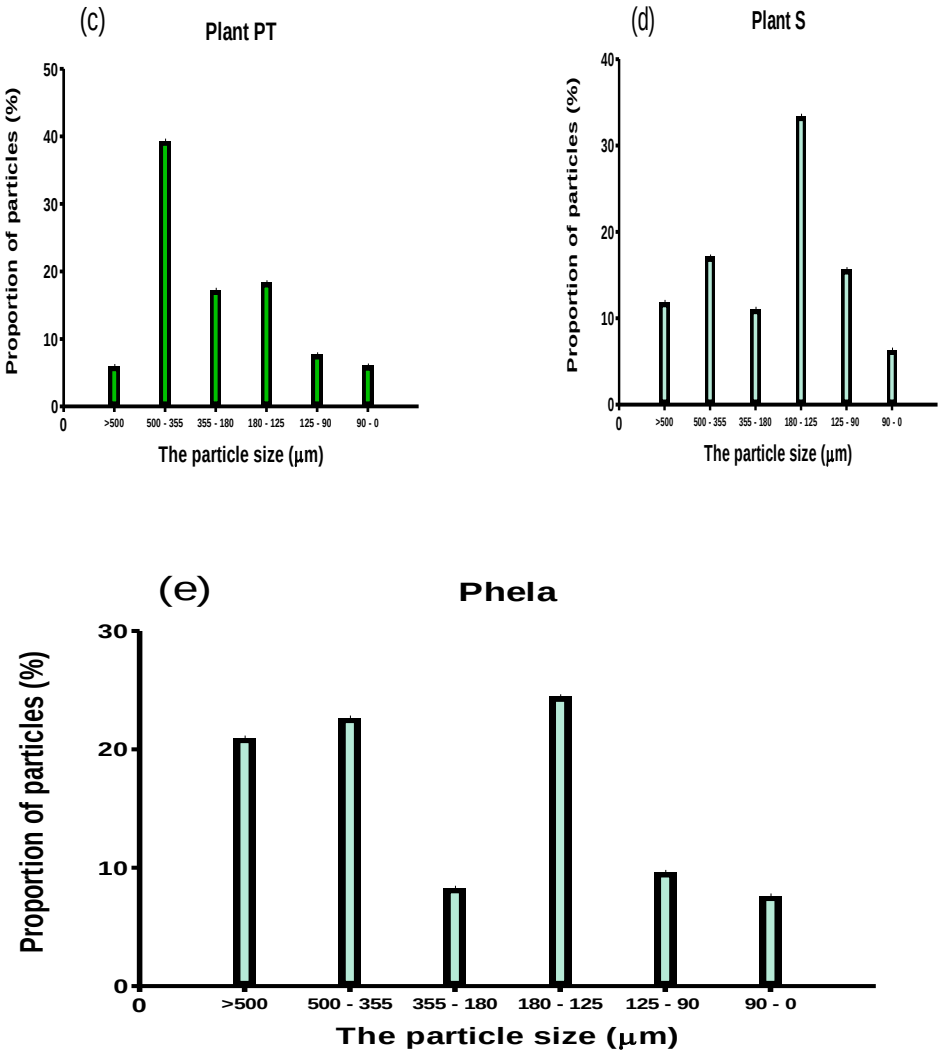


Figure 5. 1: **Distribution of particle size of the Phela powders passing through different sieve sizes. Histogram of powders of plants (a) RM, (b) CG, (c) PT (d) S and (e) Phela mixture.**

It appears that the powder of plants RM and CG had a bimodal particle size distribution where as that for plants PT and S were perhaps more skewed to the left and right, respectively. Overall, the individual plants were non-uniform in particle size and so was the powder of the final Phela mixture. Based on the BP (2000) criteria the particle size of the powders was thus not gradable. Such range in particle size may have an impact on the capsule manufacture, for example lead to non-uniform filling and variation in capsule weight and perhaps in variation of dissolution. Since the powders contained a large proportion of large particles (i.e. approx 40 % > 355

micron) additional sieving and removal of these bigger particles before encapsulation may be another option to consider to ensure capsules of uniform weight and content.

The results of the particle shape analysis are given in appendix 2 and in Table 5.4.

Table 5. 4: Degree of sphericity for particles of Phela powders

Powder material	*Degree of sphericity (θ)*
Plant RM	3.38 ± 2.32
Plant CG	3.60 ± 1.44
Plant PT	1.19 ± 0.63
Plant S	3.72 ± 1.88
Phela	3.17 ± 1.78

Generally, the numerical value for the degree of sphericity (θ) of a particle having the shape of a sphere is one (Wadell, 1932). Of the four plant powders, the particles of plant PT were essentially spherical (θ = 1.19 ± 0.63***)*** but that of the other three plants and the mixture had distinctly elongated and non-spherical (θ > 3). The particles of the mixture was thus expected to have relatively small surface area and their elongated shape could have led to non-uniform filling of the capsule, but additional grinding and sieving of the three plants or the mixture would, if needed, have remedied such problems. Overall, the non-uniform particle size and shape obtained was however not unexpected for the type of milling machine and plant material i.e. leaves, stems and roots, used. In contrast, for instance, when aqueous extract of plant material is used e.g. the *Artemisia afra* freeze-dried aqueous extract powder (Komperlla, 2004) and *Leonotis leonorus* (Ma, 2006), the particle size and shape obtained were much more uniform.

5.2.3. The density, compressibility and flowability of Phela powders.

Density measurements are methods commonly used to determine the flowability and compressibility of powders. The results of the density, compressibility and flowability tests are indicated in table 5.5.

Table 5.5: Summary of pharmaceutical properties of the Phela plant and final mixture powders.

Pharmaceutical property	Plant powder				
	Plant RM	**Plant PT**	**Plant CG**	**Plant S**	**Phela mixture**
Tapped Density (n = 3)	0.36 ±0.003	0.59 ±0.02	0.30 ±0.02	0.29 ±0.03	0.4 ±0.001
Carr's compressibility index (n =3)	2.70 ± 0.82	6.39 ± 1.79	3.9 ± 1.32	4.62 ± 1.55	3.98 ± 0.97
Angle of repose (^{0}) (n = 10)	39.233±3.85	39.41±1.85	35.91±3.24	38.16±4.59	37.92±1.28
Total ash (%) (n = 10)	6.78±0.41	4.23±0.18	6.73±0.16	3.56±0.14	5.80±0.1
Moisture content level (%) (n = 6)	9.29±0.31	8.58±0.43	9.31±0.06	10.29±0.53	9.77±0.08
Water-soluble extractable fraction (%) (n = 6)	39.61±4.74	77.98±5.28	43.18±1.69	32.79±2.87	47.43±3.80

The final mixture had a tapped density of 0.4 ± 0.001 and Carr's compressibility index of 3.98 ± 0.97. These values are dependent on factors such as particle size, size distribution and shape. For instance, for the powders with large spherical particles, such as those of the plant CG, RM and S, a maximum packing condition is easily reached with fewer taps, while for the plant PT powder the mobility provided by the tapping on the segregating system would allow the small particles to rearrange to form

the densest packing. The tapped density value for the mixture however suggested that this powder should not lead to inconsistent packing into capsules and should be easily compressible into a tablet dosage form. Moreover, the compressibility results suggested that the powder of the final mixture, as well as that of the individual plants, had excellent flowability (see table 5.6).

Table 5. 6: Relationship between compressibility and flowability (Well, 1988)

Compressibility index (Carr's index)	***Flow quality***
5-10	**Excellent**
12-16	**Good**
18-21	**Fair to passable**
23-35	**Poor**
33-38	**Very poor**
>40	**Very very poor**

The latter was confirmed by the angle of repose measurements (table 5.5) which showed that the powder of the final mixture (and the individual plant powders) was free flowing (and that of the individual plant powders fair passable) (see table 5.7). Taken together, the results thus showed that the final mixture powder had excellent/passable compressibility and flowability properties. If this had not been the case the addition of excipients such as the talc, silica, magnesium stearate, etc could have been considered to improve the flowability properties of the final mixture. Finally, the compressibility and flowability results are in agreement with that obtained on freeze-dried aqueous extract powder of the *Artemisia afra* (Komperlla, 2004).

Table 5. 7: Carr classification of powder flowability based on Angle of Repose (Well, 1988)

Description Angle of repose	
Very free flowing	*25-30º*
Free flowing	*30-38º*
Fair to passable flow	*38-45º*
Cohesive	*45-55º*
Very cohesive	*>55º*

5.2.4. Total ash values of Phela powders.

Ashing involves an oxidation of the components of the products. The ash of any organic material is composed of their non-volatile inorganic components. Controlled incineration of crude drugs results in an ash residue consisting of an inorganic material i.e. metallic salts and silica (Siddique, *et al*, 2010). A high ash value is indicative of contamination, substitution and adulteration or carelessness in preparing the crude drug for marketing. Unwanted material from direct contamination such as sand or earth is immediately detected by considering the ash value.

The results obtained for the total ash value determination of the plant powders are given in table 5.5. Since there are no other data or reference values for the total ash, acid-insoluble ash and sulphated ash tests of the four individual plants (e.g. in monographs, etc of the individual plants) and the final mixture the results obtained in this study were thus taken as standards for the batch of material.

5.2.5. The moisture level of the Phela powders.

The moisture levels obtained for the individual powders are given in table 5.5. The level for the Phela mixture was 9.77±0.08 % indicating that the powder of the mixture was not particular hygroscopic and had been properly dried before manufacturing. The moisture content of the Phela mixture and the individual plant powders were comparable to that of *M. longifolia* aqueous extract powder (8.398±0.1357 %) (Haiqui, 2006) but this is the first data for the individual powders. The result should be useful for future reference when Phela capsules are made.

5.2.6. The water-soluble extractable fraction of Phela powders.

The results of the determination of water extractable matter obtained for the plant and final mixture powders are given in table 5.5. The Plant PT powder had the highest water extractability (77.98±5.28 %) plant S had the lowest (32.79±2.87 %) and the Phela mixture the average of the two (47.43±3.80 %). Because the mixture was extracted in water the extracted material is expected to be soluble and based on the descriptions given in table 5.8, to be soluble to very water soluble (BP, 2000). More important is the fact that the 47.43±3.80 % yield of water extractable material means that almost 50 % of the plant material is water soluble and would be found in the traditional liquid dosage forms of Phela.

Table 5.8: Common descriptive phrases of solubility and the corresponding quantitative solubility ranges as per B.P (2000)

Descriptive Phrase	*Approximate quantities of solvent by volume for 1 part of solute by weight.*
Very soluble	less than 1 part
Freely soluble	from 1 to 10 parts
Soluble	from 10 to 30 parts
Sparingly soluble	from 30 to 100 parts
Slightly soluble	from 100 to 1000 parts
Very slightly soluble	from 1000 to 10,000 parts
Practically insoluble	more than 10,000 parts

5.2.7. Microbial contaminants levels of Phela powders.

The microbial contamination test results for the four plant powders are given in the appendix 3 and summarized in table 5.9. No *Staphylococcus. aureus, Pseudomonas aeruginosa*, *Salmonella species* and *Escherichia coli* were detected in any of the Phela powders and the microbial contamination testing results were all within the WHO specification (WHO, 2007).

Table 5. 9: Microbial results of plant powders and Phela mixture

Plant powders	*Pseudomonas*	*Staphylococcus*	*E. coli*	*Salmonella spp*
Plant RM	Negative	Negative	Negative	Negative
Plant PT	Negative	Negative	Negative	Negative
Plant CG	Negative	Negative	Negative	Negative
Plant S	Negative	Negative	Negative	Negative
Phela Mixture	Negative	Negative	Negative	Negative

5.2.8. Heavy metal and pesticides contaminants in the Phela powders.

The results of heavy metal and pesticides level tests are shown in appendix 4 and 5, respectively. The Phela mixture on average contained <0.5 and 4.2 mg/kg of Cd and Pb, respectively. No traces of organophosphate, organonitrate, organochloride and N-methyl carbamate pesticides were detected. The heavy metal levels for Phela mixture were well within the limits specified by the WHO (2007) i.e. 10 mg/kg for Pb and 0.3 mg/kg for Cd. Overall, as far as heavy metal and pesticide contamination were concerned, the Phela powder complied with the WHO specifications and were thus safe for consumption.

5.2.9. HPLC fingerprints of the Phela powders.

A comparative study of chromatographic fingerprints of Phela powders was conducted. Copies of the individual chromatographic fingerprints of Phela powders obtained for plants S, CG, RM and PT are indicated in appendix 6 and that for the Phela mixture indicated in figure 5.2. A wavelength of 300 nm appeared to be the one where the most peaks for each plant material was obtained.

All the peaks observed were first checked to see if they represented single or multiple compounds using the photodiode array detector and the HPLC system's software (see figure 5.2). The Phela mixture had five distinct peaks (A to E), three of which showed reasonable resolution and two peaks, C and D, which overlapped. Although different solvent gradient systems were tried, it was not possible to separate the co-eluting peaks C and D. It was also not possible with the spectral analysis to conclusively prove that each of the peaks represented single compounds only, but except for peak

B, it is reasonable to assume that each peak represented either a single compound or mixture of structurally very similar compounds.

Peaks A to D had distinct UV-absorbance maxima at 293.5, 292.1, 290.1 and 295.4 nm respectively, while Peak E had a distinctive high intensity of absorbance's at 253.1 nm and low absorbance at 349.4 nm (figure 5.2E). The latter type of spectrum (i.e. absorbance maxima in 240 to 285 nm region – band II - and 300 to 400 nm region – Band I) is usually associated with that of flavonoids like that of sutherlandioside D (Avula, *et al*, 2010), suggesting that at least peak E may be the result of the presence of one or more flavone type structures. The position of band I in flavones is between 304 – 550 nm while flavonols absorbs in the range of 352 – 385 nm (Malešev, *et al,* 2007). The band II peak are generally assumed to be due to absorbance of benzoyl system ring A while band I is the results of absorbance due to the cinnamoyl system ring B (Bhat, *et al,* 2005). Further identification and analysis of the compounds represented by peaks A to E will however only be possible with other techniques such as LC-MS. Nevertheless these peaks (A to E) in the Phela HPLC fingerprint can still be a useful characteristic to use in the quality control of this plant medicine.

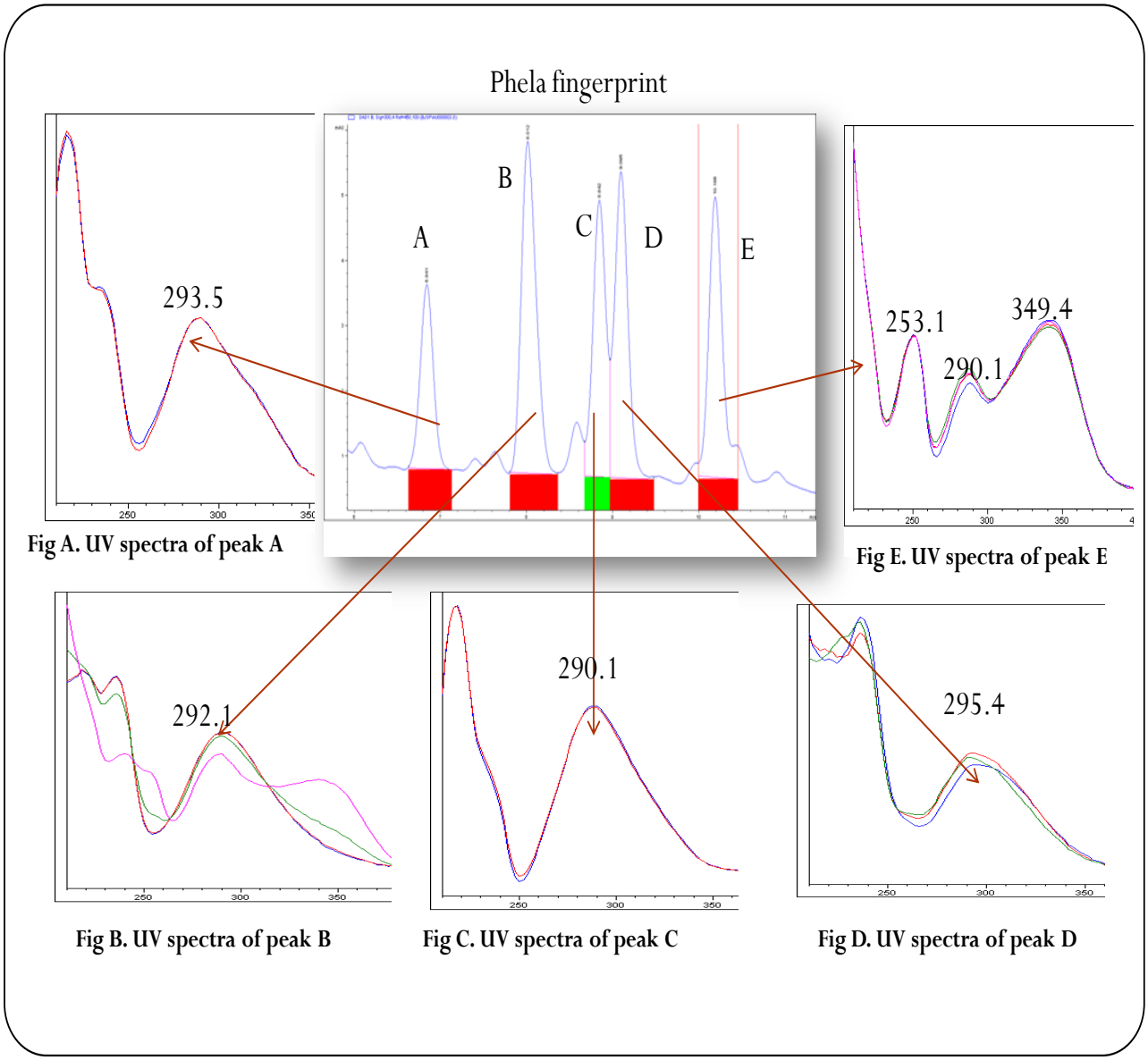


Figure 5. 2: **HPLC fingerprint of Phela mixture eluted with acetonitrile/water-formic acid mixtures and ultraviolet spectra of five selected individual peaks (A-E). For the fingerprint UV detection at 300 nm was used and spectrum over 200 to 350nm.**

In addition to the above qualitative analysis, all the peaks having an area percentage greater than 1.5 of the total area and retention greater than 7 min were also identified and used for the comparison of the fingerprints and the results obtained are given in table 5.10 and appendix 6. As mentioned earlier, the Phela mixture had six peaks that met these criteria of having retention time > 7 minutes and area > 1.5%.

Table 5.10: Summary of main peaks in HPLC fingerprints of Phela materials. Only peaks having retention time > 7 min mins and area > 1.5% are recorded.

Plant material	Main Peaks (Retention time, Rt > 7 min and area > 1.5 %)												
	Peak A		Peak B		Peak C		Peak D		Peak E		Peak F		Peak G
	Rt (min)	Area (%)	Rt (min)	Area (%)	Rt (min)	Area (%)	Rt (min)	Area (%)	Rt (min)	Area (%)	Rt (min)	Area (%)	Rt (min)
Phela mixture	7.3	1.5	7.78	2.9	11.05	5.62	12.68	3.54	13.16	3.51	14.21	3.5	
CG	7.29	0.9	7.77	3.2	11.04	6.05	12.68	3.51	13.75	5.9	14.21	4.7	
RM	7.27	1.7									(15.65)	1.2	16.53
PT							12.69	--	13.76	--	14.21	---	
S	7.36	4.6	(10.05)	2.6	(11.92)				(13.93)	3.2			16.55

The fingerprint of the plant CG had six peaks that also appeared in the fingerprint of the Phela mixture while that for plants RM and S had only one of these peaks in common with that of the mixture. The PT fingerprint had indications of presence of 3 of the 6 peaks (A to F) but the areas did not meet the > 1.5 % inclusion criterion. It has been noted that there other additional peaks (10.05, 11.92, 13.93 and 15.65) minute which are not prominent in the final mixture. The fingerprints of plant RM and S each also contained at least one additional peak which was not evident or very prominent in the fingerprint of the final mixture (under the conditions used in this study). Collectively, this qualitative analysis suggested that the six peaks (A to F) in the fingerprint of the mixture reasonably reflected the possible presence (peak retention time and, possibly peak size) of the 4 individual constituent plants and might thus form a reasonable criterion on which the quality control of the Phela mixture could be based.

5.2.10. Conclusion on the Phela plant powders.

Collectively, the results (from section 5.2.1 to 5.2.2) indicated that the Phela plant mixture powder was organoleptically, a uniformly light brown in colour, had a bitter taste and characteristic medicinal odour which could be masked by a capsule dosage form. The 4 plant powders had irregular shapes and sizes and the final mixture was not easily gradable in terms of particle size and shape. The four crude plant powders (RM, PT, CG, S) and the mixture had similar flow properties (i.e. angles of repose of 39.233±3.85°, 39.41±1.85°, 35.91±3.24°, 38.16±4.59° and 37.92±1.28°, respectively) and acceptable moisture content levels (i.e. 9.28±0.31 %, 8.58±0.43 %, 9.31±0.06 %, 10.29±0.53 % and 9.77±0.08 % respectively) which was unchanged in final mixture. The fraction of water soluble matter was very high for plant PT (77.99±5.82 %), low for plant S (32.79±2.87 %), RM (39.61±4.74 %), CG (43.18±1.69 %) and for the Phela mixture (47.43±3.80 %). The heavy metal, pesticides and microbial contaminant levels were well within acceptable WHO standards and the HPLC fingerprints indicated five compounds or peaks that could used to assess the quality of Phela mixture.

The overall these results indicated that Phela powders could thus be easily compressed into a tablet dosage form and that the powders had a good flowability, which would make filling them into capsules non-problematic. Moreover, the contaminant levels were well within the generally accepted regulatory criteria and the capsules were thus be safe for consumption. Finally, an HPLC fingerprint containing 6 distinctive peaks with the retention time > 7minutes and area percentage > 1.5 % was an appropriate quality control specification for the Phela mixture powder.

5.3. Assessment of the pharmaceutical quality of the finished Phela capsules.

The finished Phela capsules were analyzed for uniformity of weight, dissolution profile and stability.

5.3.1. Uniformity of weight of the Phela capsules.

The results of the uniformity of weight of the Phela capsules determination are given in appendix 7. The full capsules on average weighed of 0.42 ±0.01 g, with RSD of 2.38 % and contained 0.32±0.01 g of plant formulation (contents) with RSD of 3 %. The average deviations in weight for capsules were 2.801±1.45 %. According to the British Pharmacopoeia (BP, 2000e), 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 the Phela capsules met the British Pharmacopoeia specifications.

5.3.2. Dissolution profile of Phela capsules.

The dissolution test of a herbal product such as Phela capsules measures the rate of release of soluble constituents from such dosage forms. In the present study the amount released and going into solution in the dissolution medium was measured by monitoring the UV absorbance of the solution at 280 nm at various times after exposure to the dissolution medium. From these readings the cumulative amount and percentage of Phela soluble ingredients released was calculated and plotted over time. The results of the dissolution studies on Phela capsules are given in appendix 8 and is summarised in figure 5.3. In the pH 1.2 dissolution medium the release and dissolution of the soluble ingredients of Phela was slow with 50.5 ± 6.76 % (n=6) of the Phela content dissolving in 45 minutes and 90.82 ± 2.17 % after 75 minutes.

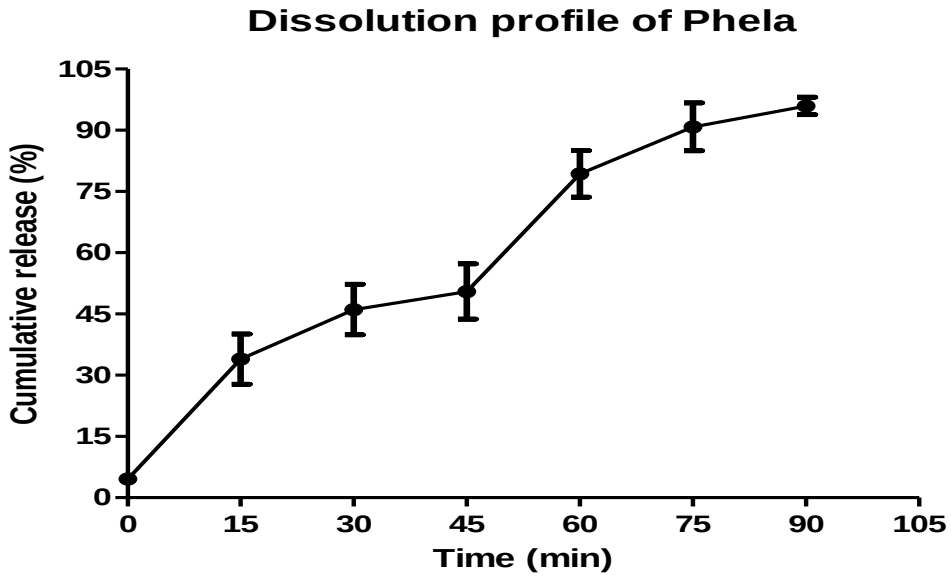


Figure 5. 3: **Dissolution profile of Phela capsules (n=6). Dissolution conditions: basket method; 900ml pH 1.2 hydrochloric acid buffer; 37±0.5℃; samples quantitated by UV assay at 280 nm.**

Official dissolution specifications have been set for very few herbal products. The USP 29, contain the dissolution specifications for some botanical products, e.g. ginger capsules - not less than 60 % of the content of 6-gingerol ($C_{17}H_{26}O_4$) capsules is dissolved in 60 minutes (USP, 2006); chondroitin sulfate sodium tablets - not less than 75 % of the labeled amount of chondroitin sulfate sodium is dissolved in 60 minutes (USP, 2006); milk thistle capsule or tablets - not less than 75 % of the labeled amount of silymarin as silybin ($C_{25}H_{22}O_{10}$) is dissolved in 45 minutes (USP, 2006) and glucosamine tablets - not less than 75 % of the labeled amount of glucosamine ($C_6H_{13}NO_5$) is dissolved in 45 minutes (USP, 2006). The EMEA proposes that a disintegration test may substitute for a dissolution test if the active ingredient is known to be highly soluble in aqueous media at pH values typical of the gastro-intestinal tract (EMEA, 2005). On the other hand, the Food and Drug Administration (FDA) in their guidelines for dietary supplements leaves any specific requirements for

dissolution or disintegration unaddressed (FDA, 2007). This overall lack of dissolution standards for herbal products may be partly due to the chemical complexity of botanicals (Jin, *et al*, 2007) and/or because few of these products are officially registered.

In the present study, the Phela capsules exhibited substantially slow dissolution suggesting that they will only release soluble active ingredients slowly in the stomach. It must however be noted that the assay method used (i.e. UV absorbance at 280 nm) was not very specific and neither was a selective ingredient(s) monitored. Monitoring the dissolution of specific Phela ingredients and using a more sensitive and selective validated assay method such as HPLC may produce more specific data on which a quality control dissolution specification for these Phela capsules could be based.

It must also be kept in mind that the dissolution profile of the Phela capsules may be quite different at pH 4.5 and 6.8. Thus the *in vivo* bioavailability or absorption profile of its active ingredients can not necessarily accurately be predicted from the present dissolution data. The present dissolution data is however most useful as a product quality or consistency indicator (e.g. to establish capsule uniformity and batch to batch consistency, etc) and the non-selective UV assay an inexpensive, convenient and easy method to use to establish/confirm that criterion. The present dissolution data is the first one of Phela and can thus be used to attain and monitor the consistency in the quality of the Phela capsules to be used in the proposed clinical trials on the herbal product. A dissolution specification of not less than 60 % of Phela content dissolved in 60 minutes appears to be appropriate for the Phela capsule (as for the USP specification for the ginger capsules).

5.3.3. Stability of the Phela capsules.

Stability studies are designed to give an insight into the drug degradation mechanism, half-life (one-half the original concentration) and expiry date (t_{90}, shelf-life) estimation (Álvarez-Lueje. 2005). The purposes of stability testing are to provide evidence on how the quality of a drug substance or medicinal product varies with time under the influence of a variety of environmental factors, such as temperature, humidity, and light, and to establish a re-test period for the drug substance or a shelf life for the medicinal product and recommended storage conditions (EMEA, 2007). For the present study, the selected chemical markers indicated as compound A to E in (Fig. 5.4) were used in the assessment of the stability of the Phela capsules.

Two batches of Phela capsules were stored at room temperature and in the climatic chamber for six months, respectively. The results for the first batch are indicated in appendix 9.1 and summarized in table 5.11. The first batch of Phela capsule powders stored at room temperature were assessed for organoleptic features and were found to remain unchanged in size, shape, colour (light brown) and odour. On average, the five compounds of Phela (A to E) had lost 9.68 %, 9.07 %, 8.97 %, 9.25 % and 8.39 % of their original levels after 24 weeks of storage, respectively. Less than ten percent degradation of compounds A to E occurred, suggesting an average shelf-life of more than two years for the Phela capsules under these ambient test conditions. Although this result should be confirmed by study over a full 2-years period, the current information can already be used for future reference when Phela capsules are made.

Table 5.11: Average values of the compounds peak heights obtained in the stability study after 24 weeks of storage under ambient condition. (N=3)

Time (weeks)	Peak Heights (ave ± SD, n =3) (mAUFs)				
	Comp. A	Comp. B	Comp. C	Comp. D	Comp. E
0	4.1±0.1	5.4±0.2	5.8±0.2	6.5±0.4	5.6±0.5
6	3.99±0.1	5.3±0.5	5.7±0.1	6.4±0.1	5.4±0.3
14	3.98±0.2	5.1±0.2	5.4±0.1	6.2±0.3	5.2±0.2
24	3.97±0.5	4.9±0.3	5.2±0.2	6.01±0.3	4.7±0.2

The second batch of Phela capsules was stored in a plastic container under high temperature (40 ± 2 ^{0}C) and relative humidity (75 ± 5% RH) conditions for six months and samples taken every two weeks and analysed by HPLC. Although the capsules, after six months of storage, appeared sticky the organoleptic features of its contents appeared unchanged. The HPLC chromatograms were analysed and peaks exhibiting a typical flavonoids spectrum were identified, their retention times recorded and peak heights compared.

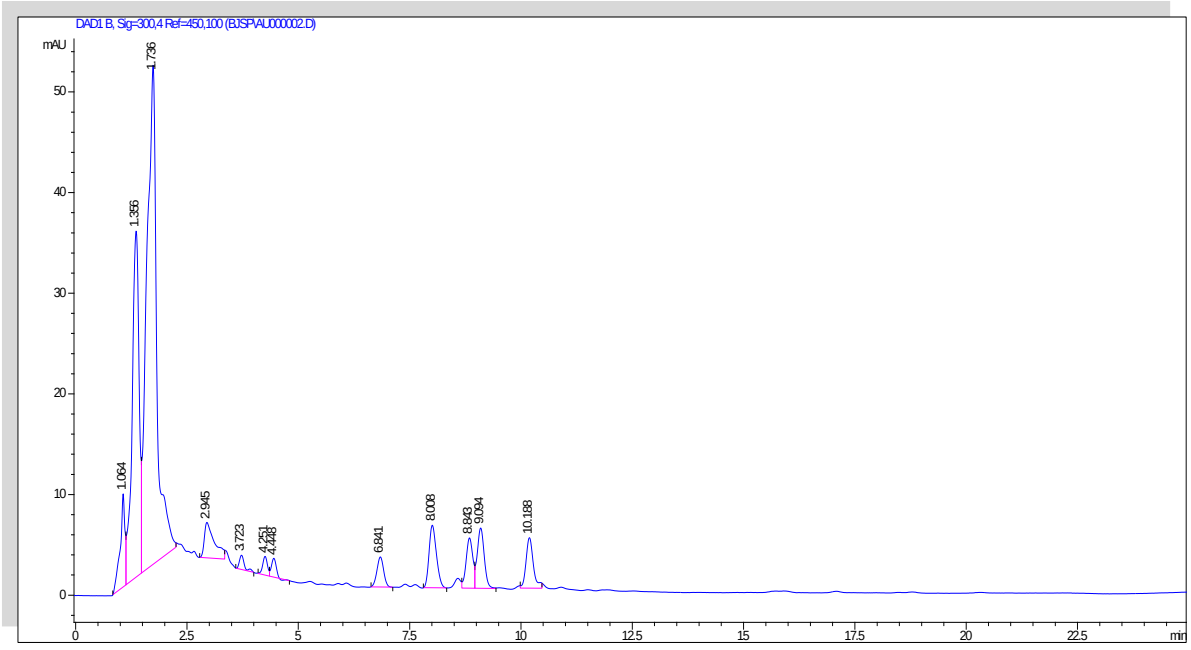


Figure 5. 4: A typical spectra of Phela material showing the peak heights and retention times of flavonoids after storage for 24 weeks.

Figure 5.4 is an example of a typical chromatogram obtained for the Phela mixture and an overlay of 5 peaks (i.e. A to E) representing single compounds with typical flavonoid spectrum is shown in figure 5.5. The peaks representing compounds A to E had retention times of 6.84±0.4, 8.008±0.3, 8.84±0.5, 9.09±0.3 and 10.188±0.1 minutes, respectively. After 24 weeks storage, compounds A to E lost about 50 %, 57.41 %, 63.79 %, 70.77 % and 82.14 % of their contents, respectively (see figure 5.6 and Appendix 8.1). After six months of storage, the selected compounds A to E degraded by more than 50 %. The same study of (Shah, *et al*, 2005) revealed that the formulations of A, B, and C also exhibited more than 50 % degradation of hypericin compared to its theoretical value and more than 30 % degradation of pseudohypericin. The change in the heights of peaks (or compounds) A to E over the 24 weeks of storage period are shown in figure 5.6.

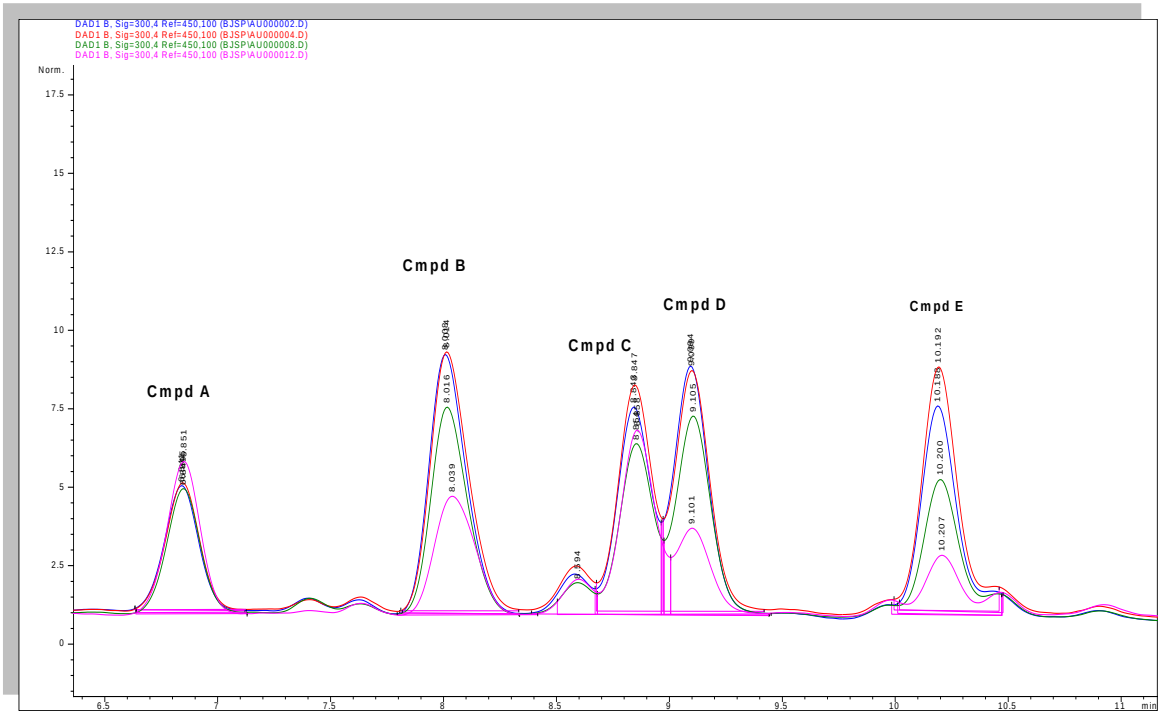


Figure 5. 5: **Overlay of HPLC fingerprints of Phela powder after different periods of storage at 40 C and 75% relative humidity. 4 fingerprints of capsules taken at 2 week interval are depicted.**

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). On average, the five compounds had lost 22 % and 70 % of their original levels after 12 and 24 weeks of storage, respectively, at the elevated temperature and humidity conditions. Ten percent degradation of compounds A to E occurred after 5.3, 6.3, 5.6, 4.1 and 5.5 weeks, respectively, suggesting an average shelf-life (i.e. t_{90}) of 6.1±0.8 weeks for the Phela capsules under these accelerated test conditions. Although the active constituents of Phela are not known, the possibility is strong that the active constituents might show similar instability trends.

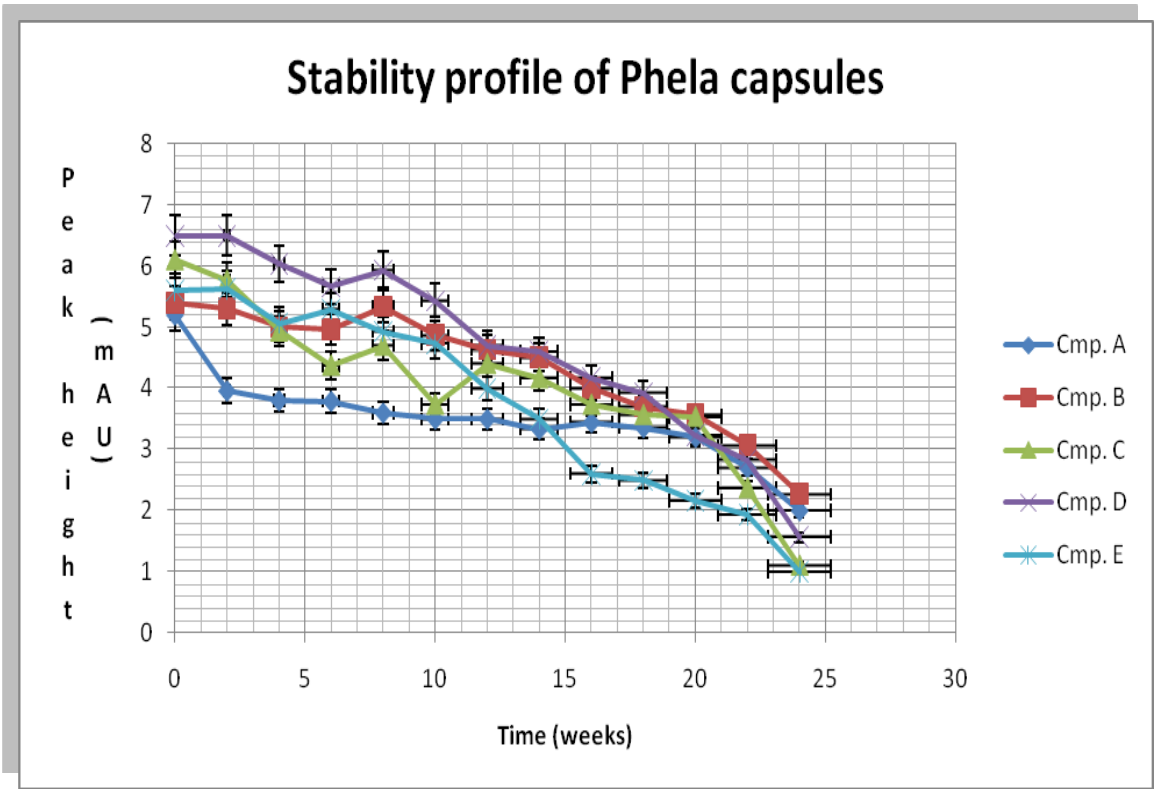


Figure 5. 6: **Stability profile of Phela capsules over 24 weeks of storage at 40°C and 75% RH conditions measuring a change in HPLC peak heights for 5 compounds (A to E) after 2 weeks.**

The overall results of the stability study thus suggest that the contents of the capsules of Phela were stable under room temperature condition but not under high humidity and temperatures and that under such conditions the capsules would have no practical shelf life. It can also be concluded that the flavonoids, by being affected by the high temperature and humidity conditions could be used for the assessment of stability. Clearly the Phela capsules must not be stored under elevated temperature and humidity conditions, and the customary accelerated stability testing methods used for pharmaceuticals cannot be applied for this medicinal plant product. When kept under normal ambient conditions the capsules were however reasonably stable and should thus be viable for clinical trials and stored under ambient conditions.

5.3.4. Conclusion on Phela capsule.

Overall, Phela capsules had an acceptable average weight of 0.42 ±0.01 g, which did not deviate from the set specification as required by the BP (2000). This means that Phela powders passed a uniformity of weight test. Although Phela capsules had not released 60 % of its contents at 60 minutes, the average amount of the material released for the six capsules of Phela were 50.5 ± 6.76 %. The manufactured capsules maintained their general appearance and physical properties under the humid conditions for six months of storage. The stability results obtained collectively indicated that the manufactured capsules essentially had no practical shelf life under accelerated condition but did have a practical shelf-life of more than two years stored under ambient condition.

5.4 Certificate of Analysis (CoA)

A Certificate of Analysis is a report usually sent to the customer at the time of shipping the finished goods. It contains information related to quality i.e. the quality characteristic, its specification or limit and the results obtained. Based on the results obtained in this study the CoA shown in table 5.12 can be drawn for the present Phela capsules. The detailed information on test/methods/procedure is indicated in the previous sections in this paper.

Although the results for particle size and shape and the degree of sphericity did not meet the required specifications, it did not complicate much in capsule filling of capsules since the capsules weights were well within the required specifications. The dissolution profile of Phela capsules did not meet the required specification (i.e. by

releasing 60 % of the contents in 60 minutes, the next batch of Phela capsules should meet the dissolution require. Apart from the test/procedure indicated for Phela in table 5.12, there are other additional tests that can be explored (e.g. determination of tannins, assay chemicals, impurities, quantification of peaks etc) for material that could be presence/absence and also are important determinants to the quality of herbal material. As soon as above mentioned problems are realised then should not be a problem when submitting the certificate of analysis for clinical studies.

Probably the most important aspect of clinical studies is CoA. ICH requires before the start of any clinical trial that the product must have a CoA that must have met specifications of analysis that ensure its quality, purity, and identity as required by the pharmacopoeias and drug regulations established for herbal drugs (ICH).

Table 5. 12: Certificate of analysis for Phela capsules

Product: Phela **Dosage form: Capsule**
Batch no: P240574 **Capsule size: 0**

Characteristic	Test/Method/Procedure	Specifications	Results	Conclusion
Formulation:	See section 4.2.2. for method	A capsule containing mixture of 4 plants powders i.e. plant RM, CG, PT and S. *for property reason exact plant names and amount not given.	Plant PT Plant CG Plant PT Plant S	**Complies** **Complies** **Complies** **Complies**
Identity: **Capsule**	Organoleptic Features (i.e. colour, odour, shape and size) by. visual, olfactory & tactile senses	Consistency of odour, taste & colour	Yellow and brown capsule half's, Biconvex, Oval shape. Size 0	**Conforms**
Identity: **Phela material**	Macroscopic features (size and shape)	Uniform in shape and size: 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.	Non-uniform and not gradable	**Did not comply**
Identity: **Phela material**	See section 4.2.3.2. for method	Sphericity (θ): < 2	3.17 ± 1.78	**Did not comply**
	See section 4.2.3.5. for method	Ash values: Range (5 – 6)%	5.80±0.1%	**comply**
	See section 4.2.3.3. for method	Carr's compressibility index: Range (5-10)	3.98 ± 0.97	**comply**
	See section 4.2.3.4. for method	Angle of repose: Range (38-45)º	37.92 ± 1.28^{0}	**comply**
	See section 4.2.3.6. for method	Moisture content level: ≤ 10%	9.77±0.08%	**comply**
	See section 4.2.3.7. for method	Water-soluble extractable fraction: Range (45 – 60)%	47.43±3.80%	**comply**

(table 5.12 continues)

Levels of Microbial Contaminants	Microbial test as per BP[1993]	Absence of *E. Coli* (i.e.0 CFU/G) Absence of *Salmonella* (i.e. 0 CFU/G) Absence of Staphylococcus (i.e. 0 CFU/G) Yeast and Moulds < 100 CFU/G	No growth No growth No growth No growth	**Conforms** **Conforms** **Conforms** **Conforms**
Levels of Pesticides	Chemical test as per BP, EMEA, WHO [2007]	Absence of Organophosphate N/A Organonitrogen N/A Organochloride N/A N-Methyl Carbamate N/A (N/A = no official specification available)	 No residue detected No residue detected No residue detected No residue detected	 **Conforms** **Conforms** **Conforms** **Conforms**
Levels of Heavy metals :	Chemical test as per BP[1993], EMEA [2000], WHO [2007]	Limits Arsenic 3 mg/kg Lead 10 mc/kg Mercury 3 mg/kg Cadmium N/A	 As = 1.1 mg/kg Pb = 6.4 mg/kg Hg = 1.0 mg/kg Cd = 0.5 mg/kg	 **Complies** **Complies** **Complies** **Complies**
Uniformity of mass of dosage units	As per BP 2000[6]	± 7.5 % (Test Eur. Ph 5th Ed)		**Complies**
Average mass	As per BP 2000[6]	235.1±7.5%	222.4 mg	**conforms**
Dissolution	As per BP 2000[5]	Not less than 60% of Phela capsules dissolved in 60 minutes in artificial gastric juices without enzymes	50.5±6.76	**Did not conform**
Stability	as per EMEA [2007], WHO [1996]	≥ 2 years stored under ambient conditions.	≥ 2 years	Conforms

Chapter 6

Conclusions and Recommendations

The aims and objectives of this study were to establish (from literature) a series of criteria and specifications that are appropriate to establish the pharmaceutical quality of plant-containing capsules such as Phela, assess the pharmaceutical quality of the finished Phela capsules, assess the pharmaceutical quality of the "formulated" Phela raw material and to provide a certificate of analysis for the finished capsules.

The following hypotheses were postulated for the study, viz. that:

Firstly, the capsule dosage form of Phela plant material would meet most of the general pharmacopoeial quality specifications,

Secondly, the water extractable plant material contents of Phela capsules would be rapidly dissolving and meet the BP specification of 75 % of the extractable plant material dissolving in the acidic solution at pH 1.2 within 45 minutes, and

Thirdly, that the Phela plant materials contain flavonoid-like compounds that will be susceptible to degradation and may be suitable as marker compounds to monitor the stability of the Phela capsules during storage.

From the results obtained the following conclusions could be drawn:

1. There is a fairly good agreement, between various regulatory agencies, on the characteristics that could be used to assess the quality of herbal products as well as the methods and quality indication specifications (or tolerance limits) to use. Consequently it was concluded that the quality of the Phela capsules could be assessed by looking at the characteristics and specifications suggested by the World Health Organisation and in the British Pharmacopoeia and United States Pharmacopoeia. This entails an assessment of the physical attributes, chemical identity and quantity of plant active(s), microbial and other contaminant levels, content and mass uniformity, dissolution profile and stability profile of the Phela capsules and/or its contents which would provide a good idea of the quality of the Phela capsules (and its suitability for use in clinical trial).

2. As far as pharmaceutical quality of the finished Phela capsules are concerned, the manufactured capsules conformed to the uniformity of weight specification as set by the BP. The capsules looked elegant and generally had a good physical appearance even after storage under humid conditions, but, under these conditions, unfortunately had no practical shelf life.

   The Phela capsules did not dissolved fully as expected in the dissolution media and only released about 50.5 ± 6.76 % of its contents in 45 minutes. As such, the Phela capsules therefore did not meet BP specifications of a fast releasing

capsule i.e. 75 % of the extractable plant material dissolving in the acidic solution at pH 1.2 within 45 minutes. .Phela capsules were concluded to have slow drug delivery potential.

3. The formulated Phela raw material generally was of good pharmaceutical quality, particularly in terms of flowability, moisture content levels and the heavy metal, pesticides and microbial levels which were all within the generally accepted specifications. There was an uneven distribution in particle size, but this did not appear to affect the uniformity of weight in the final dosage form and although the powders had a bitter taste, the formulation of powders into the capsules helped to mask the taste. The raw material thus conformed to most of the specifications but uneven particle size might be a problem (especially in large scale manufacture and/or irregular dissolution and/or *in vivo* bioavailability of the active constituents).

4. The Phela capsules contained five selected flavonoid-like compounds (named compound A to E) that were useful as chemical markers to assess the stability profile of the manufactured Phela capsules. These marker compounds can be assayed by HPLC method but the latter was not fully validated in the present study.

5. Finally, a certificate of analysis for the finished capsules was produced which generally suggested that the Phela capsules conforming to the CoA should be of consistent pharmaceutical quality and thus suitable for use in a clinical trial.

In addition to the above conclusions the following recommendations can be made based on the findings of this study

Firstly, since the effect that the wide particle size distribution might have on the release and in vivo bioavailability of the actives from the Phela capsules attention may have to be given to reducing the wide particle size distribution of the Phela powders. This should be achievable by better milling and sieving during the production of the dry individual powders.

Secondly, it might also be worthwhile to consider the use of extracts of the Phela plant powders obtained by using organic solvents (aqueous-alcoholic) in the Phela capsules. This may lead to substantially better dissolution rate that would be similar to that of obtained with the traditionally used decoction dosage form and possibly also improved in vivo bioavailability of the actives of the Phela plant materials.

Thirdly, a one or more of the flavonoid peaks observed in the Phela material should be better identified (by LC-MS), a validated HPLC method established to quantitate it and the latter used to properly establish the stability of Phela and for use instability studies of pharmaceutical preparations containing Phela subjected to deterioration by hydrolysis, oxidation and chemical decarboxylation.

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APPENDICES

Appendix 1

Particle size data for the four Phela plant and mixture powders (see section 4.2.3.2 for method).

Appendix 1.1 Particle size distribution of plant RM

Serial no	Sieve size (micron)	Weight of plant powder retained (g)	Plant powder retained on sieve (%)*
1	500	0.879	8.79
2	355	0.87	8.7
3	180	1.593	15.93
4	125	0.88	8.8
5	90	2.741	27.41
6	0	2.586	25.86
total		9.549	95.49

*** Total amount of powder recovered as a percentage of the 10.02 g of sample introduced.**

1.2 Particle size distribution of plant CG

Serial no	Sieve size (micron)	Weight of plant powder retained (g)	Plant powder retained on sieve (%)
1	500	0.67	6.7
2	355	0.867	8.67
3	180	2.606	26.06
4	125	1.42	14.2
5	90	2.14	21.4
6	0	1.584	15.84
total		9.287	92.87

*** Total amount of powder recovered as a percentage of the 10.04 g of sample introduced.**

1.3 Particle size distribution of plant PT

Serial no	Sieve size (micron)	Weight of plant powder retained (g)	Plant powder retained on sieve (%)
1	500	0.601	6.01
2	355	0.768	7.68
3	180	1.833	18.33
4	125	1.722	17.22
5	90	3.931	39.31
6	0	0.594	5.94
total		9.449	94.49

*** Total amount of powder recovered as a percentage of the 10.06 g of sample introduced.**

1.4 Particle size distribution of plant S

Serial no	Sieve size (micron)	Weight of plant powder retained (g)	Plant powder retained on sieve (%)
1	500	0.633	6.33
2	355	1.563	15.63
3	180	3.339	33.39
4	125	1.105	11.05
5	90	1.709	17.09
6	0	1.184	11.84
total		9.533	95.33

*** Total amount of powder recovered as a percentage of the 10.07 g of sample introduced.**

1.5 Particle size distribution of plant Phela

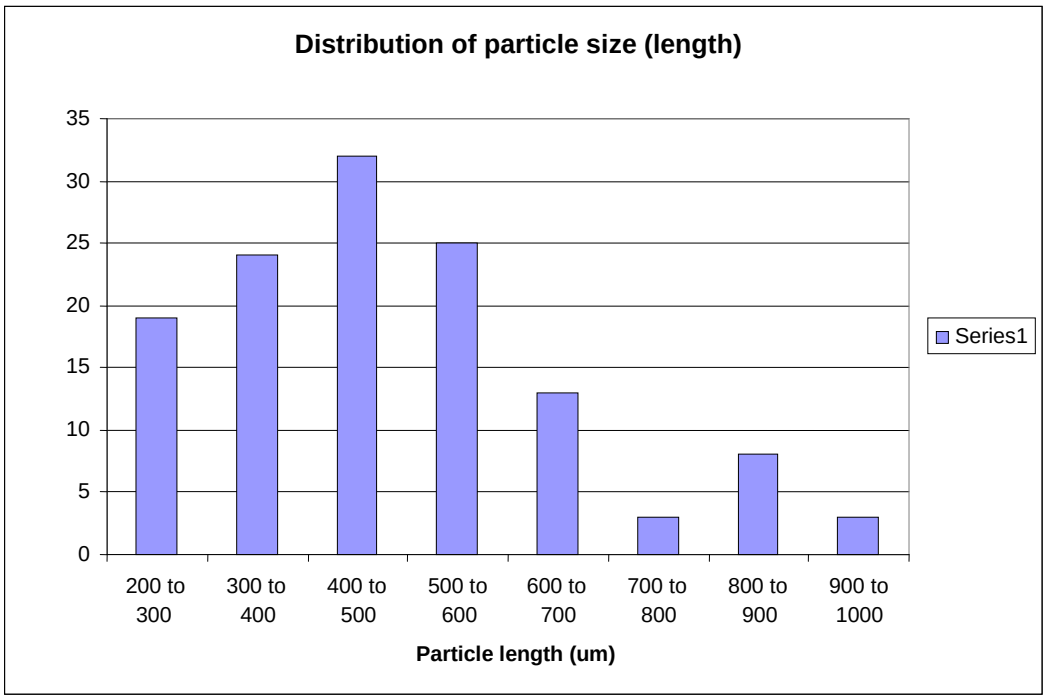
Serial no	Sieve size (micron)	Weight of plant powder retained (g)	Plant powder retained on sieve (%)
1	500	0.762	7.62
2	355	0.961	9.61
3	180	2.444	24.44
4	125	0.827	8.27
5	90	2.266	22.66
6	0	2.096	20.96
total		9.356	93.56

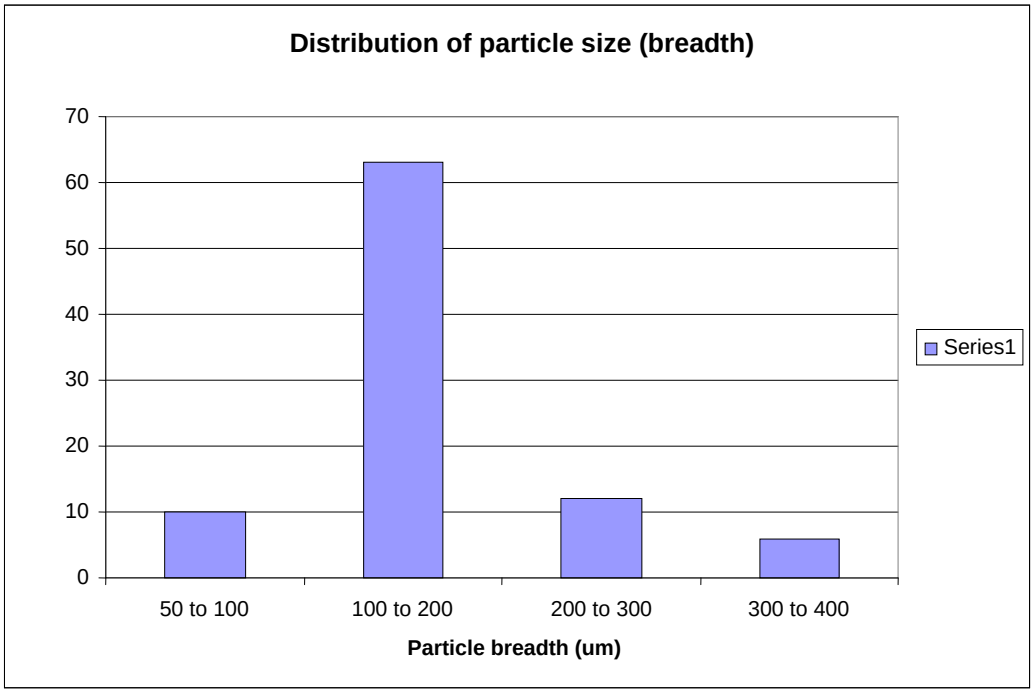
*** Total amount of powder recovered as a percentage of the 10.05 g of sample introduced**

Appendix 2

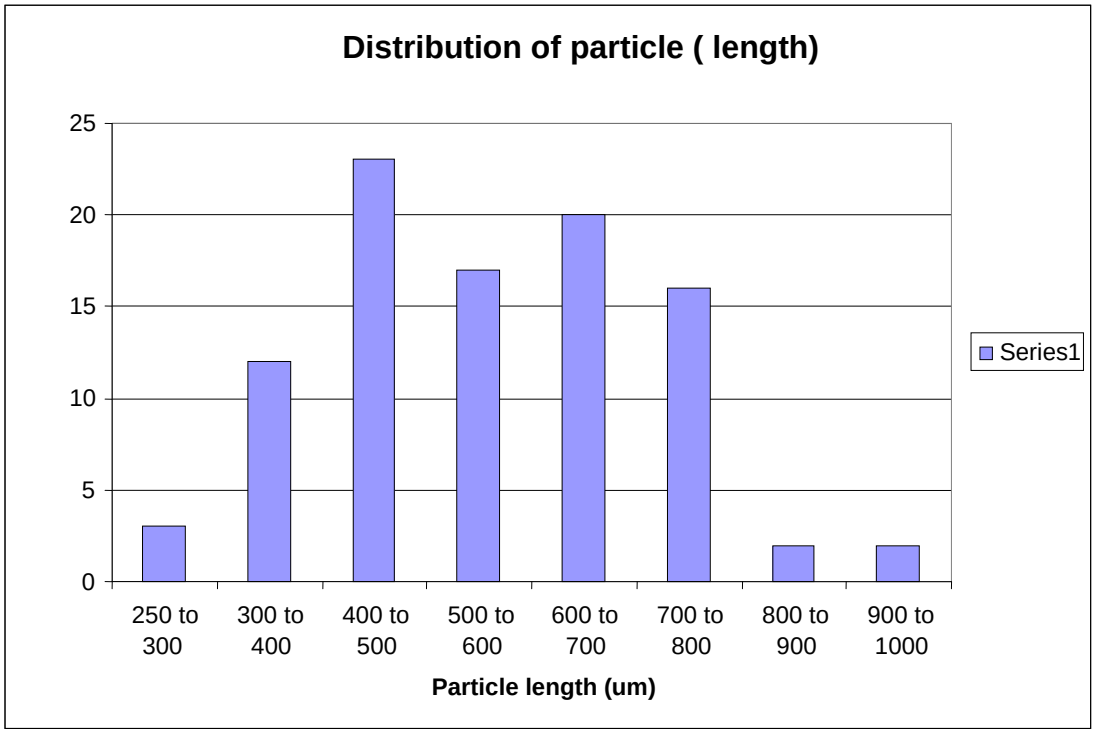
Particle shape data for the four Phela individual plant and mixture powders (see section 4.2.3.2 for method)

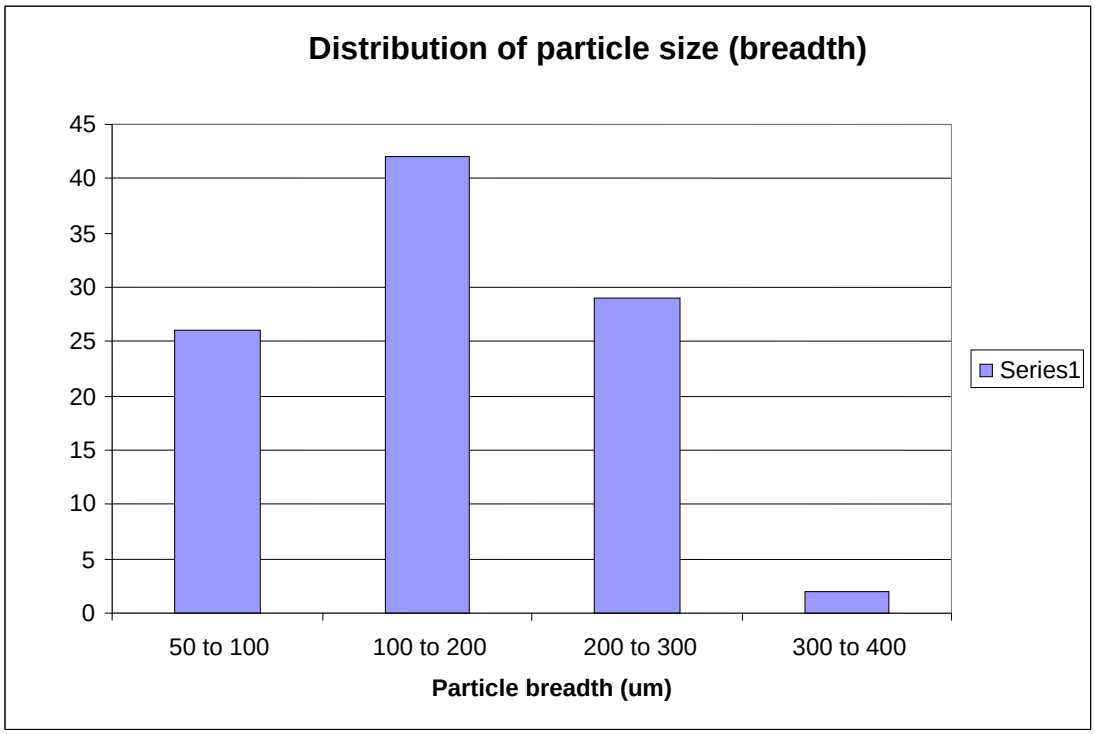
Append 2.1 Numbers of particles of Plant RM with different length (A) and breadth (B). The N = length / breadth for each particle was calculated and averaged to give the degree of sphericity (θ) = 3.38 ± 2.32 (n = 100 particles)



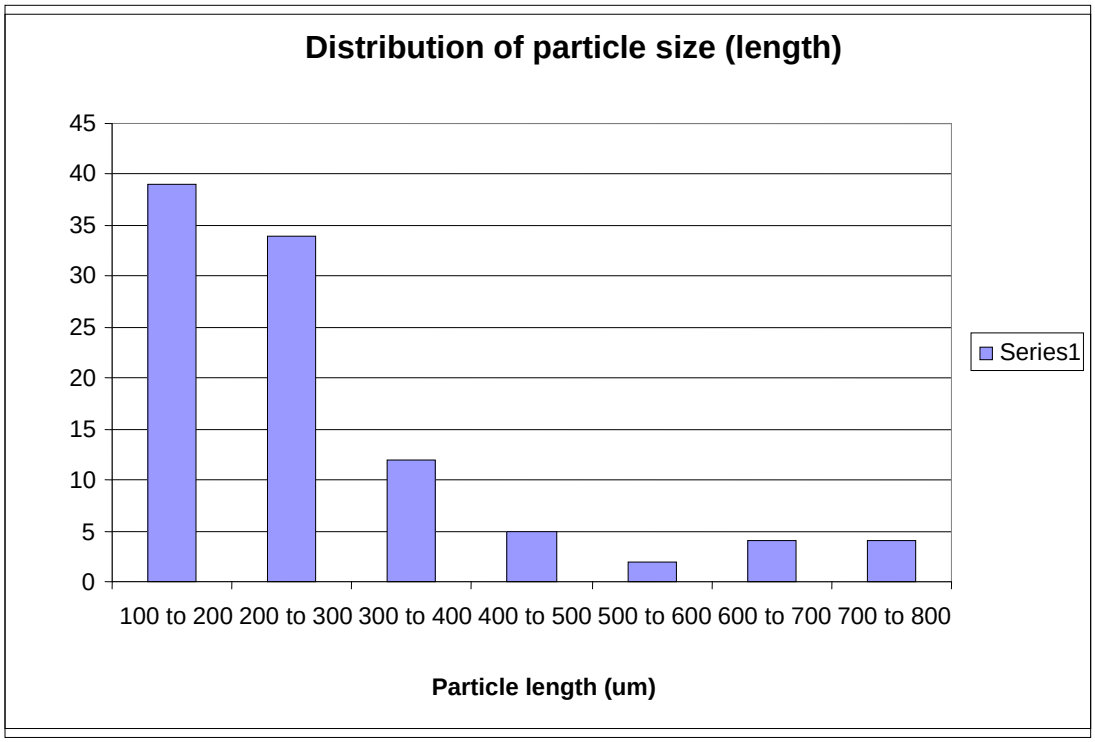


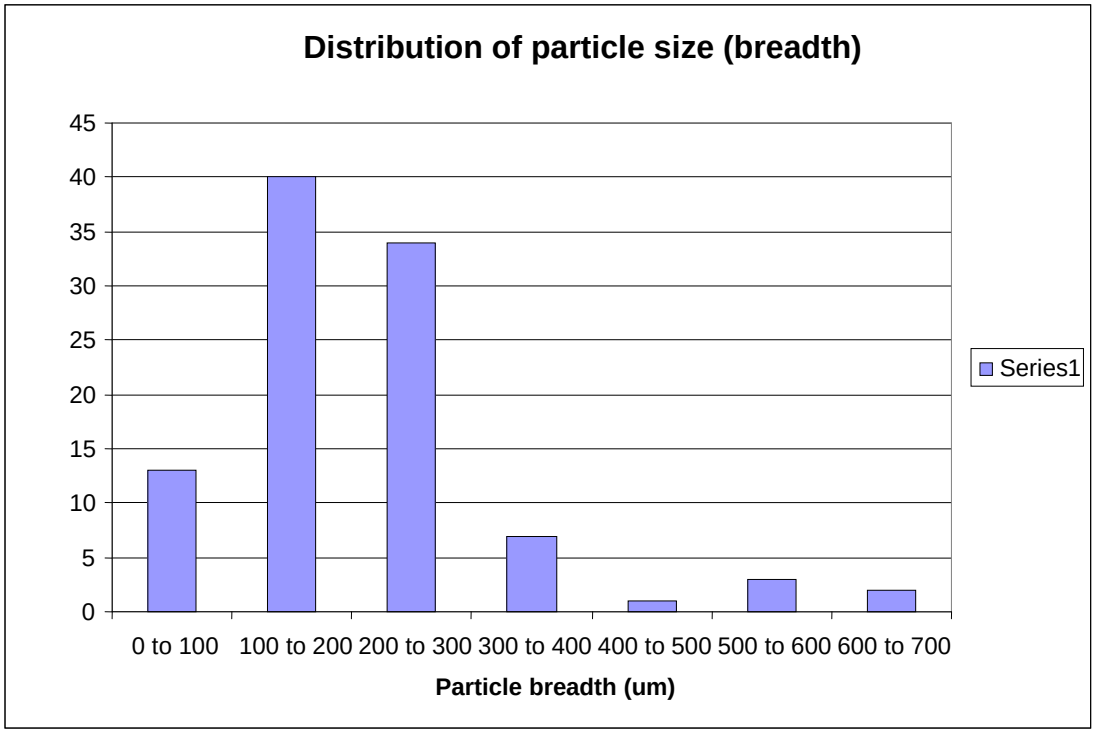
Append 2.2 Numbers of particles of Plant CG with different length (A) and breadth (B). The N = length / breadth for each particle was calculated and averaged to give the degree of sphericity (θ) = 3.60 ± 1.44 (n = 100 particles)



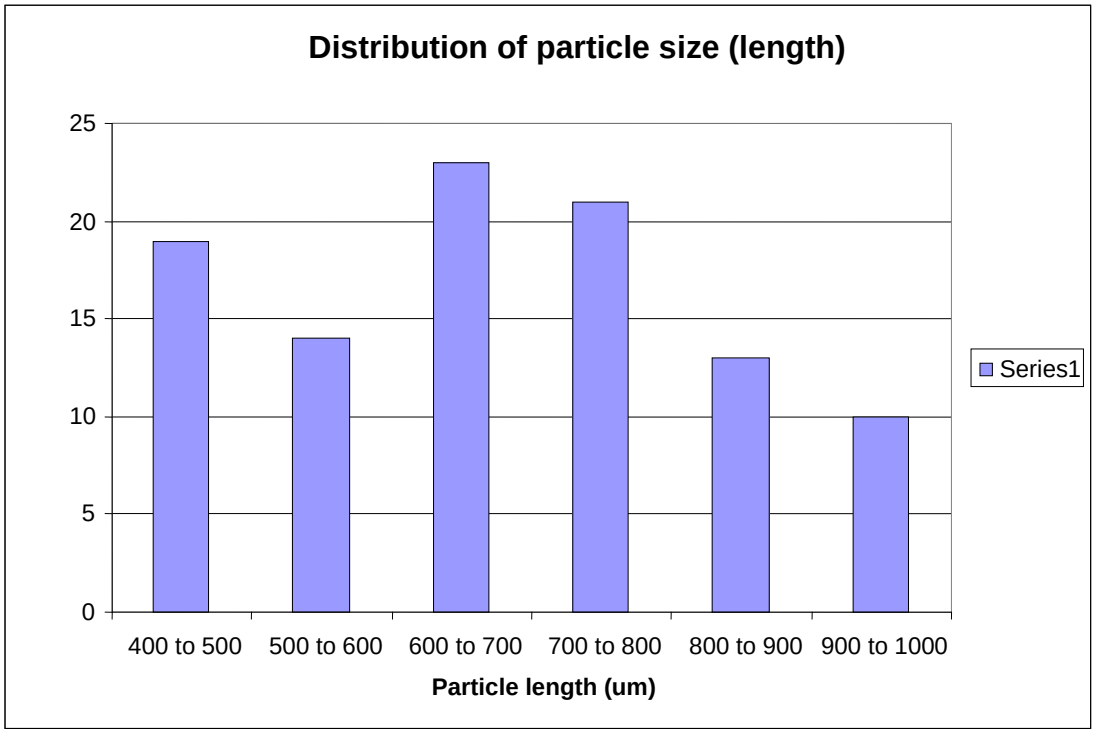


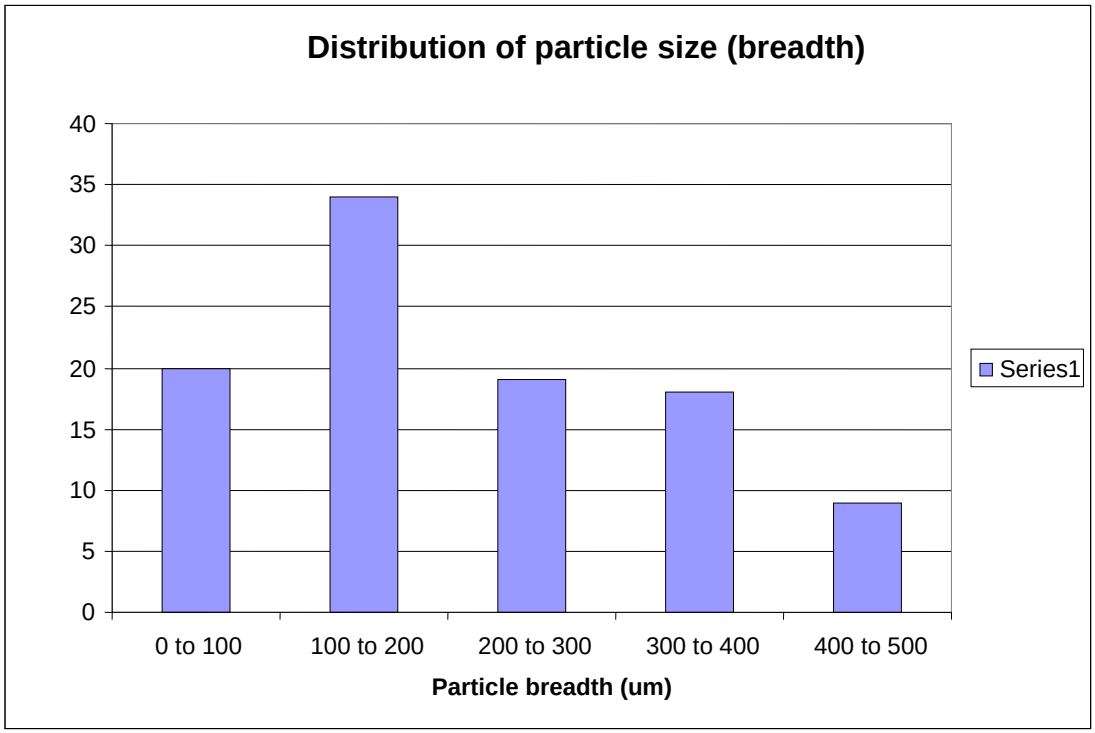
Append 2.3 Numbers of particles of Plant PT with different length (A) and breadth (B). The N = length / breadth for each particle was calculated and averaged to give the degree of sphericity (θ) = 1.19 ± 0.63 (n = 100 particles)



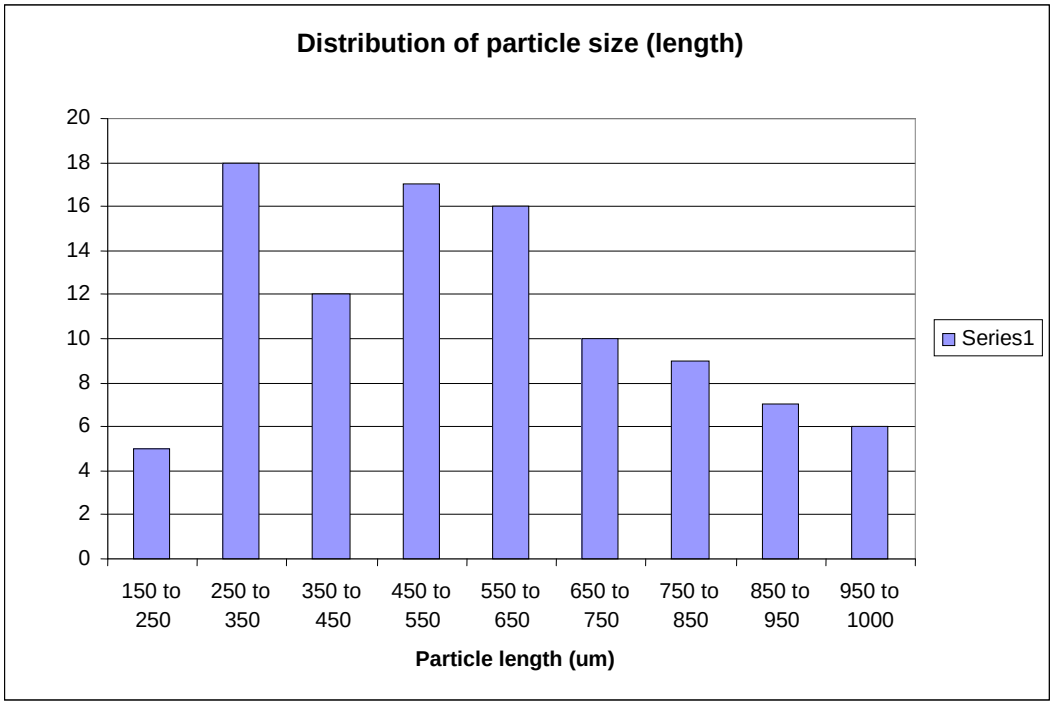


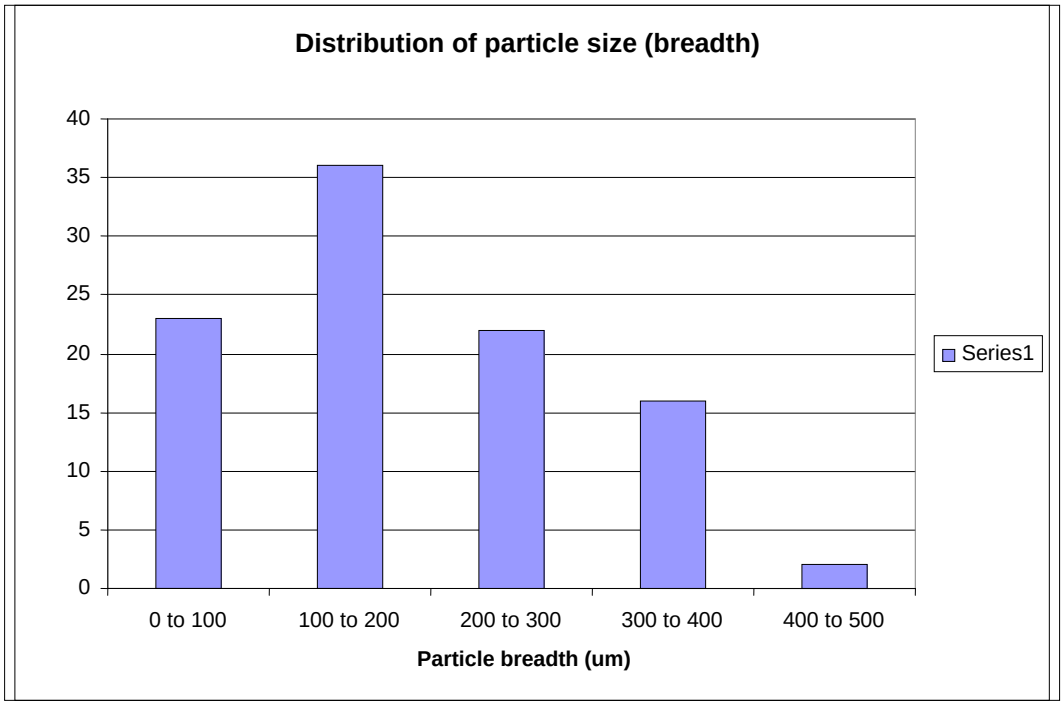
Append 2.4 Numbers of particles of Plant S with different length (A) and breadth (B). The N = length / breadth for each particle was calculated and averaged to give the degree of sphericity (θ) = 3.72 ± 1.88 (n = 100 particles)





Append 2.5 Numbers of particles of Phela mixture with different length (A) and breadth (B). The N = length / breadth for each particle was calculated and averaged to give the degree of sphericity (θ) = 3.17 ± 1.78 (n = 100 particles)





Appendix 3

Microbial contaminant level results for the Phela plant powders.

S A N A S
ACCREDITED LABORATORY T0800

MICRO REPORT
ADDENDUM

Swift MICRO LABORATORIES (Pty) Ltd
7 Warrington Road ▪ Claremont ▪ Cape Town ▪ 7708
Tel: +27(21)683 8436 / 08613 SWIFT ▪ Fax: +27(21)683 8420
E-mail: info@swift.co.za ▪ Web: www.swift.co.za

MRC - MEDICAL RESEARCH COUNCIL
FRANZIE VAN ZYL AVENUE
PAROW
7599

ATTENTION: BRIAN SEHUME

DATE: 20/08/08
DATE RECEIVED: 12/08/08
DATE TESTED: 13-20/08/08
REQ NO: CT 96219/08
PAGE 1 OF 2

SAMPLE TYPE: PRODUCTS
METHOD NO: SWJM 17; 42; 45; 46; 53

SAMPLE TYPE	TEST TYPE	DET TIME (HRS)	BACT.COUNT CFU/gram
Products			
Plant R.M	*Escherichia coli*	No Growth	No Growth
	Pseudomonas		No Growth
	Salmonella		Absent/25g
	Staphylococcus aureus		No Growth
Plant C.G	*Escherichia coli*	No Growth	No Growth
	Pseudomonas		No Growth
	Salmonella		Absent/25g
	Staphylococcus aureus		No Growth
Plant P.T	*Escherichia coli*	No Growth	No Growth
	Pseudomonas		No Growth
	Salmonella		Absent/25g
	Staphylococcus aureus		No Growth

/2....

TMA = Total Microbial Activity/Total Viable Plate Count
Limit of detection of Conventional Plate Count Methods = 10 CFU, unless otherwise specified.
A test report relates only to the specific item submitted for testing. It furnishes or implies no guarantee whatsoever, in respect of a similar item that has not been tested.
Method numbers refer to in-house methods. Standard test method references available on request.
Detection times only relevant to certain test methods, where Malthus Systems are applicable.

Plant S	*Escherichia coli*	No Growth	No Growth
	Pseudomonas		No Growth
	Salmonella		Absent/25g
	Staphylococcus aureus		No Growth

SEAN SWATTON
LABORATORY MANAGER

BRENDA DU TOIT
LABORATORY MANAGER

Appendix 4

Certificate of analysis of Phela : heavy metal levels

AMENDED CERTIFICATE OF ANALYSIS

Our ref: H:\USERS\MARLAB\REPORTS\Malr1116A

Report Number: MALR1116A

04 May 2008

Medical Research Council
P.O. Box 19070
Tygerberg
7505

Attention Mr Brian J Sehume

CHEMICAL ANALYSIS: plant samples (Order No.: 84221)
Samples received: 19/04/2008
Analysis completed: 30/04/2008 Fe analysis completed on 04/05/2008
Sample description: Dried & grounded plant material in sealed plastic pill vials.

Results

		1st Batch	Latest batch
Lab	No	11371	11372
Sample	Id	Phela A	Phela B
As	in mg/kg	*1.0*	*1.1*
Cd	in mg/kg	*<0.5*	*<0.5*
Co	in mg/kg	*<0.5*	*<0.5*
Cr	in mg/kg	*25.5*	*6.3*
Cu	in mg/kg	*24.2*	*7.3*
Fe	in mg/kg	*1737.0*	*514.0*
Hg	in mg/kg	*<1.0*	*<1.0*
Mn	in mg/kg	*57.0*	*29.5*
Ni	in mg/kg	*3.4*	*1.2*
Pb	in mg/kg	*6.4*	*2.0*
Zn	in mg/kg	*88.6*	*28.8*

Andrew Pascall
MARINE ANALYTICAL SERVICES

Sebastian Brown
MARINE ANALYTICAL SERVICES

Page 1 of 2

Appendix 5

Certificate of Analysis of Phela: pesticide residue levels

	Your ref.:	PO 00499280
	Our ref.:	17/36/8
Medical Research Council of South Africa	**Enquiries:**	P Broere
Attention: Renee Street	**Tel:**	+27124286341
PO Box 19070 Tygerberg Cape Town 7505	**Date**	09/09/2008

Fax nr: 021 955 6584

DETERMINATION OF PESTICIDE RESIDUES

Dear Renee Street

Enclosed please find our report No 2418 / C 752 on the determination of pesticide residues.

Yours faithfully

WS Louw
Manager: Chromatographic Services

TEST REPORT

SABS Commercial (Pty) Ltd

SABS

Report No: *2418 / C 752*

METHOD OF TEST - SABS Inhouse method 029/2006

MULTI-RESIDUE) A MULTI-RESIDUE METHOD FOR THE ANALYSIS OF PESTICIDE RESIDUES IN PRODUCE				
Pesticide group	Sample preparation	Technique used	Limit of quantitation (LOQ)	Recovery range measured (%)
Synthetic Pyrethroids *		GC-ECD	0.02 mg/kg	57 - 132
Organochlorine (OC's) and related pesticides *		GC-ECD	0.01 mg/kg	53 - 139
Organophosphorus pesticides (OP's) *		GC-FPD / NPD	0.02 mg/kg	50 - 106

All pesticides reported are confirmed by GCMS
* See attached list of pesticides included in the analysis

SAMPLE DESCRIPTION AND RESULTS - SABS Inhouse method 029/2006

MULTI-RESIDUE - FRUIT & VEGETABLES			
Commodity	Sample description	Pesticide	Concentration mg/kg
Plant material, dry powde	Sample 1, Dicoma Anomala, June 2008	No residues detected	
Plant material, dry powde	Sample 2, Polianthes Tuberosa, June 2008	No residues detected	
Plant material, dry powde	Sample 3, Cleodendron Glabrum, June 2008	No residues detected	
Plant material, dry powde	Sample 4, Senna, June 2008	No residues detected	
Plant material, dry powde	Sample 5, Ratheca Mycoides, June 2008	No residues detected	
Plant material, dry powde	Sample 6, Ambrosia, June 2008	No residues detected	

WS Louw
Manager: Chromatographic Services

P Broere
Principal Test Officer
Technical Signatory

This test was performed by SABS Commercial (Pty) Ltd.

This report relates only to the specific sample(s) tested as identified herein. It does not imply SABS approval of the quality and/or performance of the item(s) in question and the test results do not apply to any similar item that has not been tested. (Refer also to the complete conditions printed on the back of official test reports.)

Opinions and interpretations expressed herein are outside the scope of SANAS accreditation

T0270

SABS Inhouse method 029/2006

MULTI-RESIDUE) A MULTI-RESIDUE METHOD FOR THE ANALYSIS OF PESTICIDE RESIDUES IN PRODUCE

Sub-method **Organochlorine (OC's) and related pesticides ***

Technique **GC-ECD**

Pesticide	*Recovery (%)*	*Note*
Aldrin		
Azoxystrobin		
BHC (Lindane) Gamma-	57	
BHC Alpha-	62	
Bromopropylate		
Chlordane, cis and trans	64	
Chlorfenapyr		
Chlorothalonil	60	
DDD pp'-		
DDE pp'-	63	
DDT pp'-		
Dieldrin		
Endosulfan	91	Sum of alpha- and beta-endosulfan and endosulfan sulphate
Endrin	60	
Fenpropathrin	57	
Heptachlor		
Heptachlor Epoxide	53	
Imazalil		
Indoxacarb		
Iprodione	115	Limit of Quantitation (LOQ) = 0.05mg/kg
Kresoxim-methyl		
Prochloraz	70	
Procymidone		
Trifloxystrobin	60	
Vinclozolin	139	

Sub-method **Organophosphorus pesticides (OP's) ***

Technique **GC-FPD / NPD**

Pesticide	*Recovery (%)*	*Note*
Bromophos methyl-	60	
Chlorpyrifos ethyl-	60	
Chlorpyriphos methyl-	61	
Diazinon	56	
Dichlorvos	75	Includes Trichlorfon
Fenthion	50	
Malathion	56	
Methidathion	76	
Mevinphos	106	

Parathion	54	
Parathion methyl-	59	
Pirimiphos methyl-	53	
Profenofos	78	
Prothiophos	60	

Sub-method **Synthetic Pyrethroids ***

Technique **GC-ECD**

Pesticide	*Recovery (%)*	*Note*
Cyfluthrin		
Cyhalothrin Lamda-	57	
Cypermethrin	132	
Deltamethrin	57	
Fenvalerate		

Appendix 6

HPLC fingerprints of the four plant materials and Phela mixture

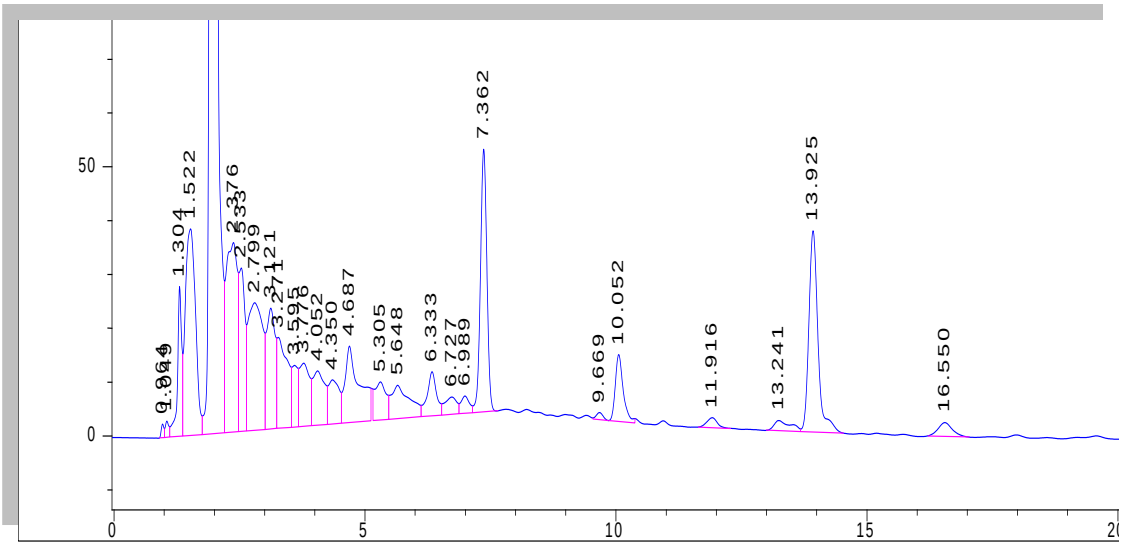


Figure A6. 1: HPLC fingerprint for extract of plant S. UV detection wavelength set at 300 nm

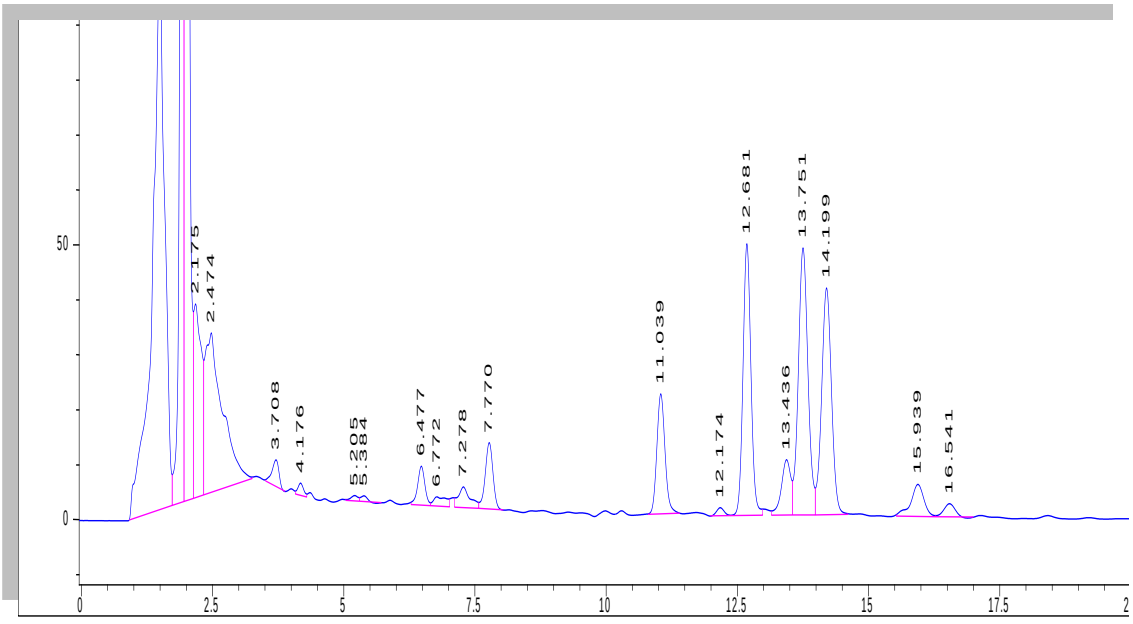


Figure A6. 2: HPLC fingerprint for extract of CG. UV detection wavelength set at 300 nm

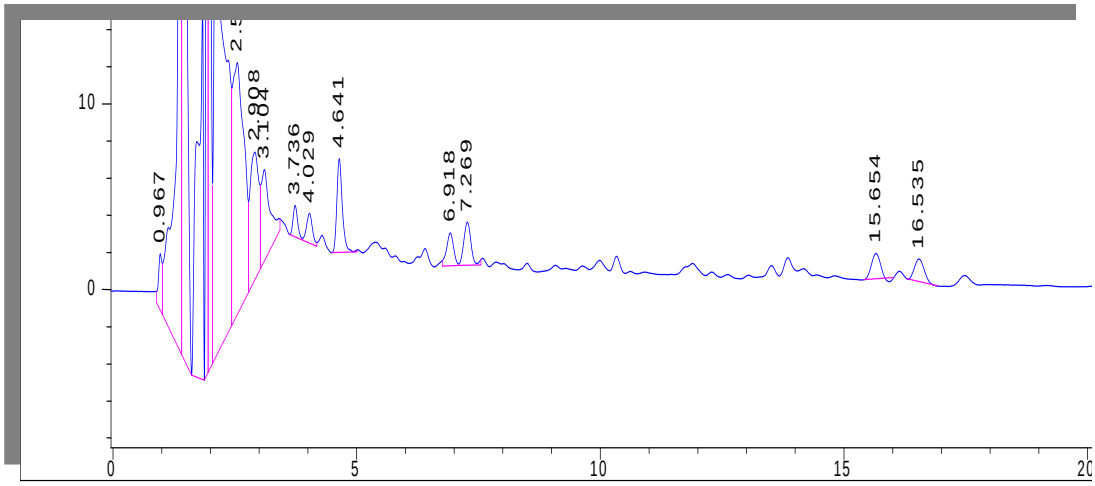


Figure A6.3: HPLC fingerprint for extract of RM. UV detection wavelength set at 300 nm

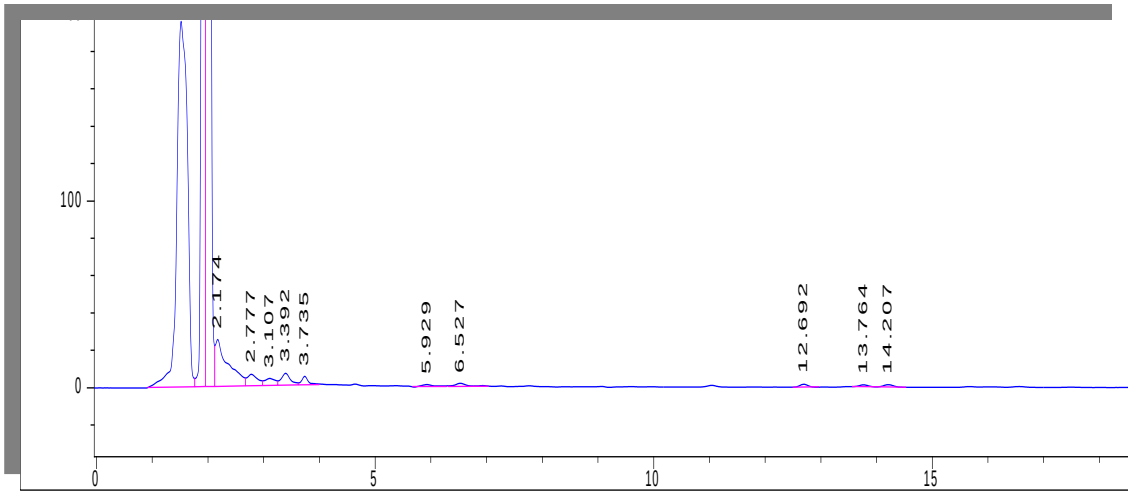


Figure A6. 4: HPLC fingerprint for extract of PT. UV detection wavelength set at 300 nm

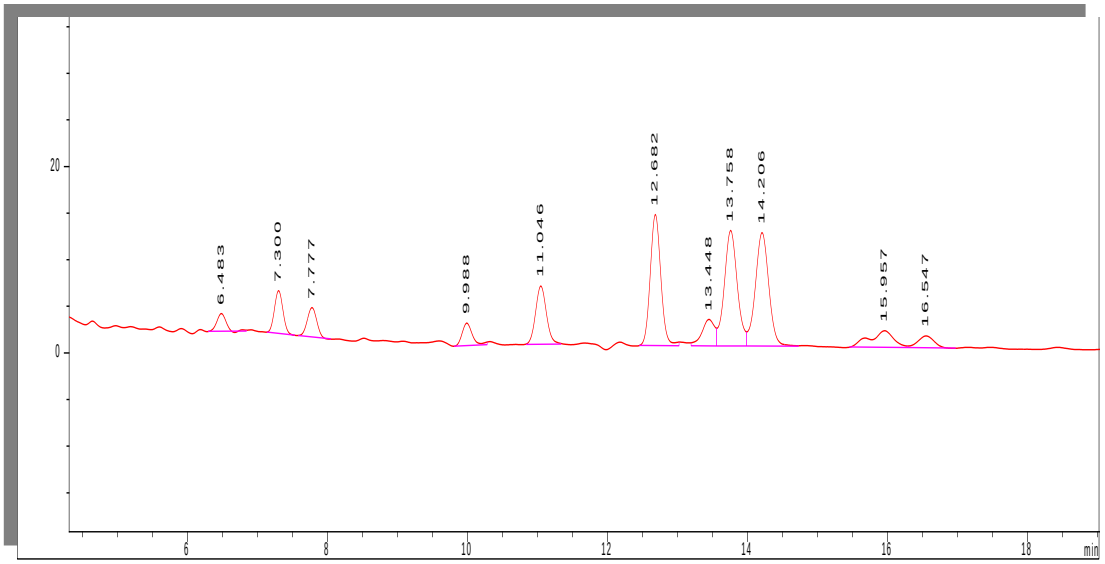


Figure A6. 5: HPLC fingerprint for Phela extract. UV detection wavelength set at 300 nm

Appendix 7
Weight uniformity of for Phela capsule

Table A7: Weight uniformity test results for Phela capsules

	Weight of whole capsule (mg)	*Weight of emptied capsule- shell (mg)*	*Weight of capsule-contents (mg)*	**Deviation In weight from average (%)*
1	0.401	0.0932	0.3078	4.41
2	0.4236	0.0936	0.3300	2.48
3	0.41	0.0947	0.3153	2.08
4	0.421	0.0956	0.3254	1.06
5	0.428	0.0959	0.3321	3.14
6	0.431	0.0933	0.3377	4.88
7	0.402	0.0977	0.3043	5.5
8	0.417	0.0967	0.3203	5.2
9	0.4295	0.0954	0.3341	3.76
10	0.411	0.0943	0.3167	1.65
11	0.4098	0.0941	0.3157	1.96
12	0.423	0.0952	0.3278	1.61
13	0.4302	0.0973	0.3329	3.39
14	0.409	0.0911	0.3179	1.27
15	0.412	0.0959	0.3161	1.83
16	0.4275	0.0968	0.3307	2.70
17	0.421	0.0947	0.3263	1.34
18	0.4001	0.0924	0.3077	4.44
19	0.4101	0.0955	0.3146	2.30
20	0.4211	0.0958	0.3253	1.02
Mean	0.42	0.09	0.32	2.80
SD	±0.01	±0.002	±0.01	±1.45
relative standard deviation		1.76	3.00	

*Deviation in weight (g) = [Weight of content (g) - Average weight of content (g)] 100 % / Average of contents

Appendix 8
Dissolution profile of Phela Capsules

Table A8: Percentage drug release of six Phela capsules over time in a dissolution medium at pH 1.2 and 37 ^{0}C.

Time (min)	*Capsule 1(%)*	*Capsule 2 (%)*	*Capsule 3 (%)*	*Capsule 4 (%)*	*Capsule 5 (%)*	*Capsule 6 (%)*	*Ave*	*SD*
0	3.92	4.64	4.4	4.98	4.75	4.93	4.60	±0.39
15	28.43	36.17	37.13	43.61	27.44	30.93	33.95	±6.17
30	36.88	46.84	49.12	47.77	54.51	41.28	46.07	±6.19
45	40.61	51.19	56.38	51.19	58.59	45.02	50.5	±6.76
60	76.08	84.48	84.01	79.78	82.11	69.49	79.32	±5.72
75	86.68	95.18	93.02	97.85	90.47	81.74	90.82	±5.88
90	94.84	99.98	96.49	94.79	93.87	95.79	95.96	±2.17
120	100	100	100	100	100	100		

Appendix 9

Chromatograms and "flavonoid or selected" peak heights obtained in the stability study

Figure A9.1: HPLC chromatogram of capsule stored at 40 ^{0}C & 70 % RH and analysed every after 2 weeks. Heights of peaks at Rt = (6.845, 8.014, 8.950, 9.104 and 10.198) min representing flavonoids are monitored.

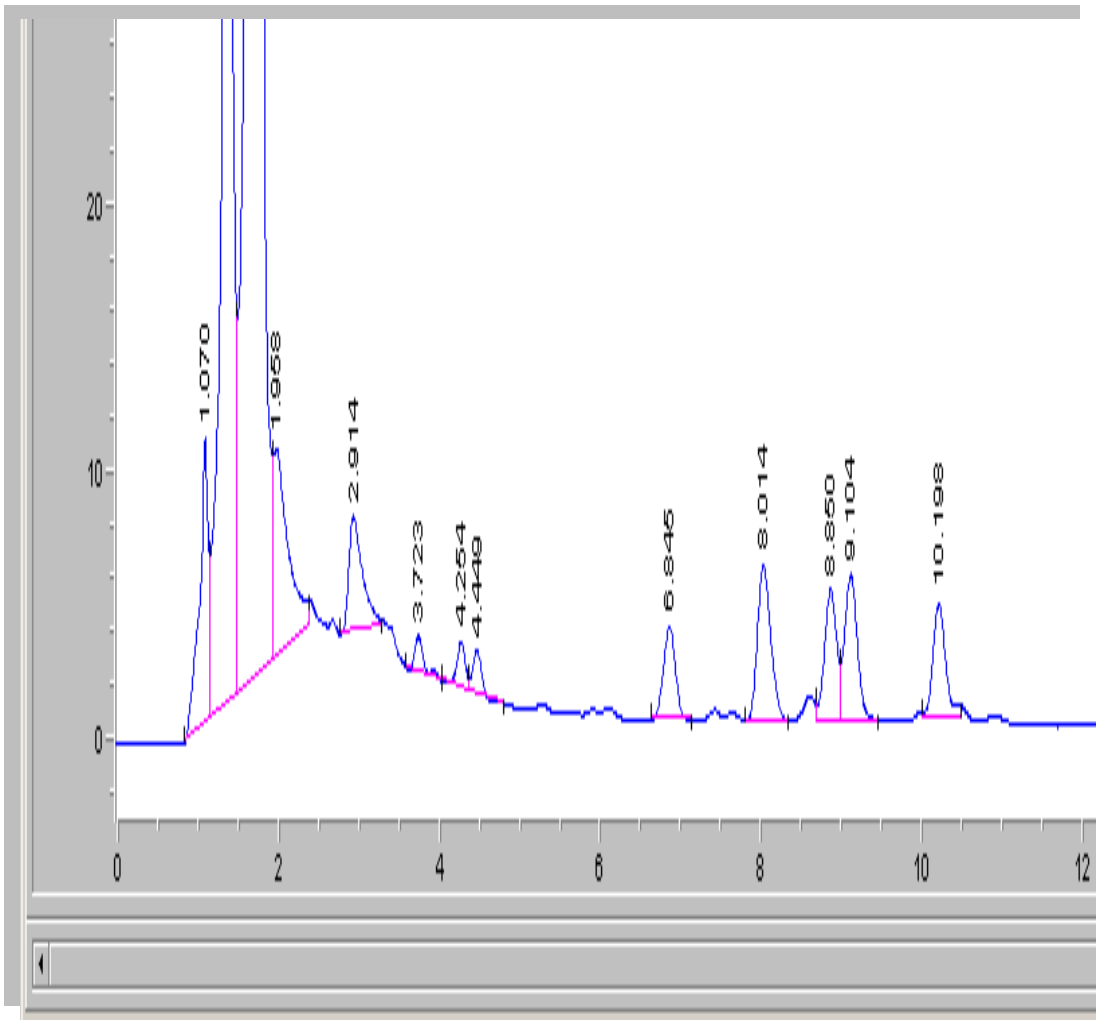


Table A9.1: Average values of the compounds peak heights obtained in the stability study after 24 weeks. (N=3)

Time	*Comp. A*	*Comp. B*	*Comp. C*	*Comp. D*	*Comp. E*	*Average*
0	4±0.1	5.4±0.2	5.8±0.2	6.5±0.4	5.6±0.5	5.46
2	3.96±0.1	5.3±0.2	5.8±0.2	6.5±0.2	5.6±0.4	5.432
4	3.8±0.1	5.0±0.1	4.9±0.1	6.03±0.2	5.1±0.1	4.966
6	3.79±0.1	5.0±0.1	4.4±0.2	5.7±0.2	5.3±0.2	4.838
8	3.6±0.1	5.3±0.1	4.7±0.1	5.9±0.1	4.9±0.1	4.88
10	3.5±0.1	4.9±0.1	3.7±0.2	5.4±0.1	4.7±0.1	4.44
12	3.5±0.1	4.6±0.3	4.4±0.1	4.7±0.2	4±0.1	4.24
14	3.3±0.1	4.5±0.2	4.2±0.1	4.6±0.1	3.5±0.2	4.02
16	3.4±0.1	4±0.1	3.7±0.2	4.2±0.3	2.6±0.2	3.58
18	3.4±0.1	3.7±0.1	3.6±0.1	3.9±0.1	2.5±0.1	3.42
20	3.2±0.2	3.6±0.2	3.5±0.1	3.2±0.3	2.2±0.2	3.14
22	2.7±0.6	3.1±0.1	2.4±0.8	2.8±0.1	1.9±0.3	2.58
24	2±0.1	2.3±0.3	2.1±0.2	1.9±0.3	1±0.2	1.6