The Evaluation of Markers for Quality Control Studies of Flavonoid-Containing Medicinal Preparations

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A thesis submitted in partial fulfillment of the requirements for the degree Magister Pharmaceuticae in the Department of Pharmacology at the University of the Western Cape.

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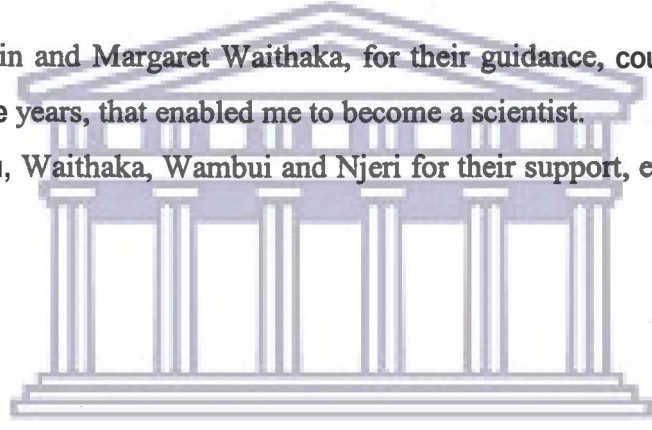
DEDICATION

To my parents Martin and Margaret Waithaka, for their guidance, counsel and constant vision throughout the years, that enabled me to become a scientist.

To Gathoni, Wanjiru, Waithaka, Wambui and Njeri for their support, encouragement and patience.



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DECLARATION

I declare that "The Evaluation of Markers for Quality Control Studies of Flavonoid-Containing Medicinal Preparations" is my own work, that it has not been submitted for any degree or examination in any other University, and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

Jane Waithaka January 2004

Signed

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ACKNOWLEDGEMENTS

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SUMMARY

In spite of the high utilization of South African traditional medicine, the therapeutic potential and the chemical components of many plant preparations are unknown. For the safe and effective use of these medicinal preparations the exact chemical composition of each preparation is required to verify the authenticity of the preparation and aid in the determination of the dose and consistency of use.

The overall objective of this study was to evaluate the suitability of flavonoids as markers for quality control studies of flavonoid-containing medicinal preparations. To realize this objective, it was necessary to develop an experimental assay that could determine selected flavonoid compounds in plant material and subsequently determine the suitability of these flavonoids for quality control studies of flavonoid-containing medicinal plant material. The flavonoid components of two medicinal plants namely, *Artemisia afra (A. afra)* and *Mentha longifolia (M. longifolia)* were investigated.

It was hypothesized that firstly, a suitable HPLC assay could be developed to simultaneously and qualitatively c quantitative'y detennine the flavonoids apigenin, hesperetin, kaempfer11\luteolin af quercetin. Secondly, that at least one of these flavonoids could be found in *'A. afra* ano M *longifolia-containing* preparations. Finally, that the monitoring of the levels of one or more of these flavonoids with the HPLC assay, could be used to establish and monitor the pharmaceutical integrity of such flavonoid-containing medicinal preparations.

Plants were collected from Montague Museum (South Africa), authenticated by a botanist and voucher specimens deposited in the Herbarium at UWC. The freshly collected plants were extracted using both aqueous and methanol extraction methods, then freeze-dried and stored. A high performance liquid chromatographic method (HPLC) was developed to determine and quantify the flavonoids as pure samples and in a plant sample matrix and mass spectrometry was used to confirm the identity of the peaks resolved by HPLC.

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The plant samples were analyzed as aglycones obtained after acid-hydrolysis of freeze-dried plant extract material. Identification of peaks was based on retention time, UV and mass spectra and UV peak areas were used for quantitation of the flavonoid contents. The chromatographic analysis was performed using a Hydro-synergy ® ODS reversed phase column and gradient elution with varied proportion of 2.2% (v/v) acetic acid-water (solvent A) and acetonitrile (solvent B) as the mobile phase and the solute peaks detected at ~ 290 nm and ~ 340 nm. The identification and quantitative determination of the markers in pure samples as well as in the plant preparations was performed, then the presence of the markers was used to authenticate the extracts and preparations of *A. afra* and *M longifolia* and, finally, the monitoring of the levels of the markers was used to assess the usefulness of the markers in determining the pharmaceutical quality of *Artemisia* and *Mentha*-containing preparations.

The HPLC assay developed was sensitive and reproducible and regression analysis revealed a linear relationship (correlation co-efficients 0.9995 - 0.9999) between the peak-area and concentrations of the flavonoid. The relative standard deviation for the flavonoid levels ranged between 3.46% (intra-day) and 4.46% (inter-day). Four of the five flavonoids were detectable in quantifiable levels in the *A. afra* and *M longifolia* plant material; apigenin (8.5 to 7.5 µg/g), hesneretin (10 to 1 µg/g), luteolin (11.5 to 50 µg/g) and quercetin (2 to 240 µg/g). All four flavonoids were suitable markers for evaluating the extracts of *A. afra* and *M longifolia,* while three of the markers (hesperetin, luteolin and quercetin) were useful for the authentication of the commercial preparations and the evaluation of the batch-to-batch differences of *Mentha* and *Artemisia-containing* medicinal preparations. Kaempferol was not considered suitable as a marker because it was difficult to analyze with the present HPLC assay.

This data provide evidence that hesperetin, luteolin and quercetin are suitable markers for the evaluation of the pharmaceutical integrity of flavonoid-containing medicinal preparations and, that the fourth flavonoid apigenin is only suitable for authenticating the extracts of *Mentha* and *Artemisia-containing* preparations. Kaempferol was unsuitable as a marker for the flavonoid-containing plant preparations investigated.

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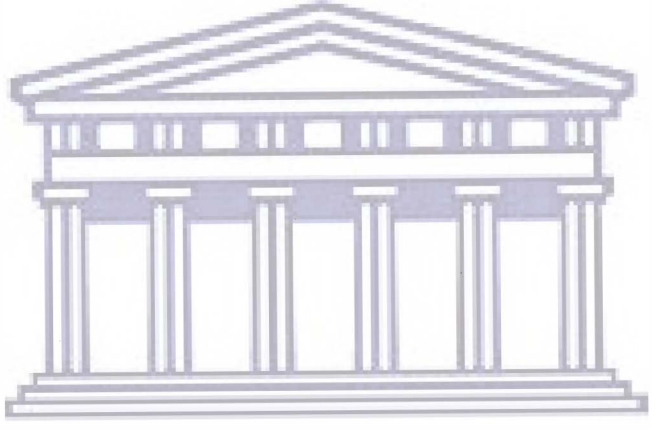

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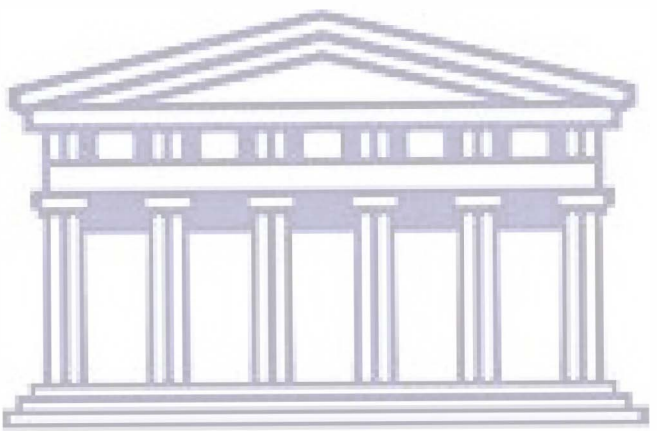

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Chapter 1

INTRODUCTION

Medicinal plant products in various forms have been available for the treatment of disease in both developing and developed countries for many years (Chan, 2003). Indigenous lmowledge on the use of two plants, ***Artemisia afra (A. afra)*** and ***Mentha longifolia (M. longifolia)*** in the Western Cape (South Africa), show the potential use of the plants for medicinal purposes.

The two plants are typically used for their anti-inflammatory and anti-asthmatic properties, and for related respiratory ailments (Hutchings et al, 1996; Van Wyk et al, 2000; Watt and Breyer Brandwijk, 1962). In addition, ongoing studies conducted in the Pharmacology Discipline (UWC) on the aqueous extracts of *A.afia* and ***M.longifolia,*** found the plants to have brochodilator and possibly anti-inflammatory activity (Harris 2002). The extracts of *A. afra* and *M. longifolia* are reported to contain flavonoids and flavonoid compounds (Hutchings et al. 1996; Sharaf et al. 1999; Van Wyk et al, 2000).

Flavonoids comprise one of the largest and most widely distributed group of polyphenols found in foods of plant origin (Harborne et al, 2000). The ***in vivo*** and ***in vitro*** investigation of flavonoids for anti-carcinogenic and anti-oxidant activity have been done with satisfactory results (Arai et al, 2000; Terao et al, 1999; Pietta et al, 2000; Sadik et al, 2003), and have proven activity of flavonoids against allergies, inflammation, free radicals, viruses and microorganisms, and enzymes (Basile et al, 1999; Harborne et al, 2000; Kimata et al, 1999).

In spite of the high utilization of South African traditional medicine, few studies have been done on ***A. afra*** and ***M. longifolia;*** therefore the scientific information on ,the therapeutic potential and the chemical components of the South African ***species*** of both plants is limited (Dyson, 1998; Van Wyk et al, 2000).

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Traditional medical practitioners use *A. afra* and *M. longifolia* without knowledge of the active ingredients and/or the mechanism of action. Nevertheless, for the safe and effective use of plant medicines it is important to establish the relationship between the chemical composition and therapeutic activity of many plant preparations. The exact chemical composition of each plant preparation is not only needed to verify the authenticity of each medicinal preparation but also determines the dose of the medicine to use, derives the consistency in use and, thus, responses or therapeutic activity.

However, in many instances the chemical structures of the active ingredients in a multitude of medicinal plants are not known (Muhizi, 2002), although the activity of many plants may be attributed to one or more of their chemical constituents. Therefore, one or more compounds or group of compounds e.g. the flavonoids found in the plants' (Hutchings et al, 1996; Van Wyk et al, 2000) could be used to assess the pharmaceutical consistency of the plant medicine. For example in the case of *A. afra* and *M. longifolia* flavonoids probably contribute to at least one of the plants' activities and for these plants to be employed in herbal medicine the consistency of the flavonoid content should be evaluated. To realize this goal, a suitable assay is required to indicate the presence of flavonoid compounds in the two medkinal plants.

Five flavonoids, apigenin, hesperetin, kaempferol, luteolin and quercetin constitute some of the compounds found in the crude extracts of *A. afra* and *M. longifolia* (Hutchings et al, 1996; Sharaf et al, 1999; Van Wyk et al, 2000). The HPLC analytical technique was proposed suitable for the assessment of these flavonoids in the plants, and several HPLC methods (Ficarra et al, 1990; Hasler et al, 1990; Keinanen et al, 1998; Merken et al, 2000; Palomino et al, 1996; Scheiber et al, 2001; Sladovsky et al, 2001; Romanova et al, 2000) could be adapted to analyze *A. afra* and *M. longifolia*.

The aims of this study were thus to (1), develop a suitable HPLC assay to indicate the presence of one or more of these five flavonoids in the plant material and (2), determine whether these select flavonoid compounds could be appropriate markers for the assessment of quality of preparations of the two medicinal plants.

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Chapter2

LITERATURE REVIEW

2.1 Introduction

The present study deals with the selection and determination of five appropriate flavonoid markers for *A. afra* and *M longifolia,* and the assessment of these selected flavonoids as markers for flavonoid-containing medicinal preparations.

This section provides an overview of (1), the current use of medicinal plant preparations, aspects of their dosage forms, safety and sample handling, and the role of (bio) markers in medicinal plant preparations (2), the botany, main traditional uses and active ingredients of *A. afra* and *M. longifolia* found in South Africa and (3), the chemistry and classification, occurrence, analysis and identification of flavonoids and, the potential role of five flavonoids as (bio) markers of medicinal plant material and their biological activities.

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2.2 Current use of medicinal plant preparations

Indigenous people employ many plant species as medicines. Currently, it is estimated that 80% of the people living in developing countries are almost completely dependent on traditional medical practices for their primary health care needs (Cordell, 2000; Tomlinson et al, 1998). Plants are considered medicinal if they possess pharmacological activity of possible therapeutic use. These activities are often discovered as a result of millennia of trial and error. Particularly in African medicine, a treatment usually comprises a complex mixture of several extracts of five or six plants and often only one of the ingredients is responsible for possible therapeutic activity (Barton et al, 1986).

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Of the 250,000 species of higher plants known to exist on earth, only a relative handful has been thoroughly studied for all aspects of potential therapeutic value in medicine (Cox, 1994; Cordell, 2000; Tomlinson et al, 1998), yet the plant kingdom has yielded 25% or more of drugs used as prescription medicines today. The earliest drugs were from plant extracts, however in the development of enhanced selectivity and reduction of side effects, the use for natural products declined (Havsteen, 2002). Current strategies or methods in the synthesis of drug products principally employ the use of synthetic and semi-synthetic substances that consume the major portion of research worldwide (Cordell, 2000; Havsteen, 2002).

The general neglect of plants as a source of potential drugs is due to a number of reasons. Firstly, the ability to acquire well-documented plant material is normally a challenge and folk therapies are often based on faith, mainly due to the non-scientific process by which these systems operate (Tomlinson et al, 1998). As a result ethno medical literature is not abstracted in a systematic manner and journals and books presenting information on the alleged medicinal applications of plants are difficult to locate in a simple way. Secondly, once a crude extract is discovered that has an interesting biological activity, a bioassay model must be used to monitor its chemical fractionation until the active principles are discovered. The eventual assay of crude extracts from the plants is therefore expensive and requires large amounts of extract (Cordell, 2000; Tomlinson et al, 1998).

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However there has been a resurgence of interest in complementary medicine within certain segments of the phannaceutical industry (Halkes, 2000), mainly due to the innovative development of *in vitro* bioassays and newer chemical assays. These assays, for example high performance liquid chromatography (Bloor, 2001) and mass spectrometry (Stobiecki, 2000), are mechanically driven and require minute amounts of plant material. The analyses of samples using these methods are relatively simple to perform with the procedures amenable to automation, leading to greatly reduced costs (Chan, 2003; Cordell, 2000).

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The drive to lower the cost of health care has made medicinal plants an attractive alternative to more expensive synthetic remedies and researchers around the world are increasingly turning to plants in the search for new medicines (Chan, 2003; Cordell, 2000; Tomlinson et al, 1998). The medicinal plant preparations available are presented in various dosage forms as either true phannaceuticals (capsules, tablets liquids, topical preparations) or with ingredients less well defined (tea, powder, oils).

2.2.1 Dosage forms of medicinal plant preparations

The most common routes for administering plant medicines are oral, sublingual, rectal, topical and nasal (Mills, 2000; Van W et al, 2000). The various oral dosage forms used to dispense herbal/plant preparations include liquids, tablets, powders, capsules, infusions and decoctions.

Liquid preparations are widely used because they are easy to prepare, involve minimal processing, and truly reproduee the chemical characteristics of the plant in a compact convenient form. Liquid preparations can be in form of linctuses, that are viscous liquids containing sugar and the medicinal ingredients possessing demulcent, expectorant sedative properties, or mixtures of medicaments either suspended or dissolved in water or some other aqueous vehicle. Infusions and decoctions are time honored methods for delivering oral doses of herbs and are mainly used where the active constituents of the plant are water soluble, because the process of preparation involves a short extraction time, usually 5-10 minutes in hot or cold water.

Herbal tablets are a convenient dosage form, however tablets contain fixed formulations that cannot be adapted to the needs of the individual patient. Capsules offer a more convenient way to give powdered plant drugs because they conceal the unpleasant tastes or textures pf powdered forms of plant drugs and provide a major advantage in delivery of plant constituents into the GIT.

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Increasing concerns have been expressed over the unprofessionalism of traditional medical practitioners, and to the efficacy, quality and safety of these traditional medicines. In contrast to this, many traditional medicinal practitioners continue to use plants without any knowledge of the toxicity profiles and/or safety of the use of these medicinal products (Chan, 2003, Halkes, 2000).

2.2.2 Safety aspects on the use of medicinal plant preparations

Safety should be the overriding criterion in the selection of plants for use in health services. Toxicity related to the use of medicinal plant products may have multiple causes, but in general two categories can be distinguished (Halkes, 2000).

The first category can be designated as extrinsic or non-plant associated whereby toxic effects or adverse reactions occur--because of accidental or deliberate contamination or substitution of the plant material described on the label. The second is more intrinsic or plant associated in nature. In this category, the plant material itself as an active ingredient produces the health risk (Chan, 2003; Halkes, 2000; Tomlinson et al, 1998).

It is not possible to establish the safety of medicinal plant preparations based on epidemiological studies because little published data exiitsrim countries where the major use of medicinal plants occurs. However, a survey of scientific literature (Raikes, 2000; Tomlinson et al, 1998) shows that medicinal plant preparations do not generally present a major problem with regard to toxicity.

According to these reports, side effects or toxic reactions associated with medicinal plant preparations are considered rare. This could be due to the fact that medicinal plant preparations are generally safe, side effects are underreported or, because the side effects are particularly mild in nature that they are not reported. In addition a considerable number of plants, especially herbs used in food and traditional medicinal plants, have a well-documented history of safe use (Halkes, 2000).

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Besides the safety aspects, comprehensive information regarding the identification of plant material, extracts obtained thereof and the chemical in$_{g\,r}$edient content of subsequent preparations should be known. The levels of chemically active ingredients are directly related to the physical conditions, harvesting periods and sample handling of the plant material.

2.2.3 Sample handling of plant material

Sample handling covers both the sample collection/storage and the sample-pre-treatment/cleanup. The selection, collection and identification of the plant material are the first steps in the preparation or the scientific investigation of plant material, followed by extraction and analysis of the plant material, or in the case of the formulation of a dosage form, the preparation, labeling and storage of the final medicinal product.

Plant selection should involve a literature survey of the floristic diversity of the area of interest. In general, during the collection of the plant material it is important to ensure that the specimens to be studied are healthy. Variations in collection site, plant age, plant organ used, climate, and soil type are factors that can affect the concentration levels of the chemical ingredients (Silva et al, 1998). An acknowledged authority must authenticate the botanical identity of the plants studied and at least three herbarium samples should be prepared (Harborne, 1991).

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At this stage, for the preparation of the medicinal dosage form, whole or parts of plants are dried and stored, or used in a fresh state. With modem medicines dry plant is stored with care to ensure active in$_{g\,r}$edients remain stable and that packing materials do not degenerate and contaminate the medicine (Van Wyk et al, 2000). The method of preparation is critical, and involves addition of volumes of solvents such as water and alcohol to an amount of fresh or dry plant material, and activities such as boiling or burning for a specified length of time, and finally the isolation and storage of final product.

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The extraction processes are more rigorous compared to the processes used in the preparation of medicinal plant dosage forms. However, conditions employed for extraction should be as mild as possible to avoid hydrolysis, oxidation, isomerization, thermal degradation and other chemical and biochemical changes in the sample (Tura et al, 2002).

Extraction methods can be classified as either organic (percolation, maceration) or polar (infusion, decoction, steam distillation). Organic solvents are thought to efficiently penetrate cell membranes permitting the extraction of high amounts of endocellular components, while the use of water normally extracts exclusively water-soluble components (Harborne, 1991; Silva et al, 1998).

Ideally, fresh plant tissues should be used for preparation of the plant extract and the extraction technique should take into account the traditional treatment methods and use. The fresh plant material should be plunged into boiling alcohol/water within minutes of collection or, alternatively freshly picked tissues stored dry in a plastic bag should remain in good condition until required for analysis.

However if immediate analysis is not possible it is important to dry plant material as quickly as possible with minimal heat, to avoid degradation of the active constituents (Harborne, 1991). The extract obtained should then be clarified by filtration and pre-treated further, concentrated, dried on a freeze drier and stored as powder.

In many instances, the precise mode of extraction and the solvent chosen for extraction/purification processes differ from one analyst to another. This dictates the type of chemical ingredient obtained in an extract, and the levels to which the compound is present. The chemical ingredient could be used to validate and control the quality of plant preparations, and hence prove useful as markers for the pharmaceutical profiles of medicinal plant preparations.

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2.2.4 Marken in medicinal plant preparations

Markers in analytical sciences can be defined as chemical compounds naturally contained or spiked into samples that add value to the analytical quality of sample, because they are more efficient to use than conventional measurement standards and analytes (Baena et al, 2002).

One group of markers is used for internal purposes, that is, to support analytical processes and in this way ensure the quality of results in a conventional way. The other group of markers is designed to provide information on the presence or concentration of specific analytes in samples. The majority of the latter markers occur either at constant or variable concentration and can be used as indicators that reflect the exposure, status or the effects to a product/preparation.

In plants the markers used to indieate the presence/concentration of analyte are generally contained within the plant material as an active chemical ingredient (Robards, 2003). However, due to the variation in the climatic conditions, soil type, and time of harvest and storage, the chemical ingredients in medicinal plant preparations may vary from batch-to-batch (Silva et al, 1998). The interference of such conditions should be minimized to maintain the impact of the chemical ingredients of these preparations independently and/or from batch-to-batch.

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Alternatively, a simpler approach to adopt for the preservation of the homogeneity of chemical ingredients of medicinal preparations in current use, is to set minimum levels for marker compounds in the plant raw material. Identifying and setting markers is a good starting point and choosing phytochemical classes of marker compounds like the flavonoids, rather than single individual chemical constituents could lessen uncertainties related to the chemical complexities of the active ingredients within medicinal plant preparations (Mills, 2000).

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In addition, testing one or more markers, or groups of markers in one plant can lead to better assessment of the chemical ingredients of a plant preparation (Robards, 2003; Mills, 2000). The presence and level of the markers typically would give an indication of the quality of the medicine and suggest the potential activity of the plant preparation.

A suitable marker/s of quality should be present in plant preparations in quantifiable amounts, extractable from plant material, stable in the plant under processing conditions of the raw material, selectively identifiable under set assay conditions, biologically active and non-toxic, and free from contamination concerns.

A group of compounds like the flavonoids, that are bioactive components of many plants can be used to give an indication of the quality of flavonoid-containing plant material. Testing for flavonoids, as set markers in plant material should give an indication of the quality of the preparation to ensure homogeneity and purity as well as infer the possible activity of such a preparation. For the validation of flavonoid-containing preparations, it thus is necessary to select one or more flavonoid compounds and propose their utility as markers for samples of a flavonoid nature for the present study.

Testing plant preparations for flavonoids could (1), identify the preparation/raw material (2), determine the effects of processing on the plant preparation (3), give an indication of the chemical content between batches and (4), show contamination and stability of active constituents.

Implementation of this strategy could be done with the development of a screening HPLC assay, adaptable for the assay of the marker/s in different types of plant material whether water soluble or alcoholic. In addition, the assay should adhere to the general requirements regarding validity, accuracy, reproducibility, simplicity and cost effectiveness.

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The flavonoid ingredients of two medicinal plants, *A. afra* and *M longifolia,* could be assayed using a suitable HPLC assay and utilized as possible markers for the phannaceutical quality control of preparations of these plants.

2.3 Plant preparations used in the study

2.3.1 Introduction

This section describes the botany, main traditional uses and active ingredients of the two medicinal plants, *A. afra* and *M longifolia* found in South Africa.

The importance of plant-derived medicine in South Africa is fairly evident and approximately 70 – 80% of South Africans are reliant on herbal medicines for health care. These healing practices are conducted using traditional healers such as *sangomas, inyangas and Rastafarians.* Several local South African plants used for their anti-asthmatic properties include, Wildkruisement *(Mentha longifolia)*, Bronkhors *(Salmolus valerad),* Teringtee *(Senecio gnaphalodes)* and Wildeals *(Artemisia afra),* (Watt and Breyer Brandwijk, 1962). Two of these plants namely *Mentha longifolia* and *Arlemisia afra* have been chosen for this study.

Indigenous knowledge on the use of two of these medicinal plants in South Africa, *Arlemisia afra (A. afra)* and *Mentha longifolia (M. longifolia),* show that the plants have been in use for their anti-asthmatic, anti-inflammatory properties and for related respiratory ailments (Dyson, 1998, Hutchings et al, 19996, Van Wyk et al, 2000, Watt and Breyer Brandwijk, 1962).

A. afra and *M longifolia* were chosen for this study, based on their claimed efficacy and pattern of use, as well as their availability and popularity amongst all the population groups. The research conducted on the use of these two plants in South Africa, and their active components are summarized in the section below.

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2.3.2 *Artemisia afra*

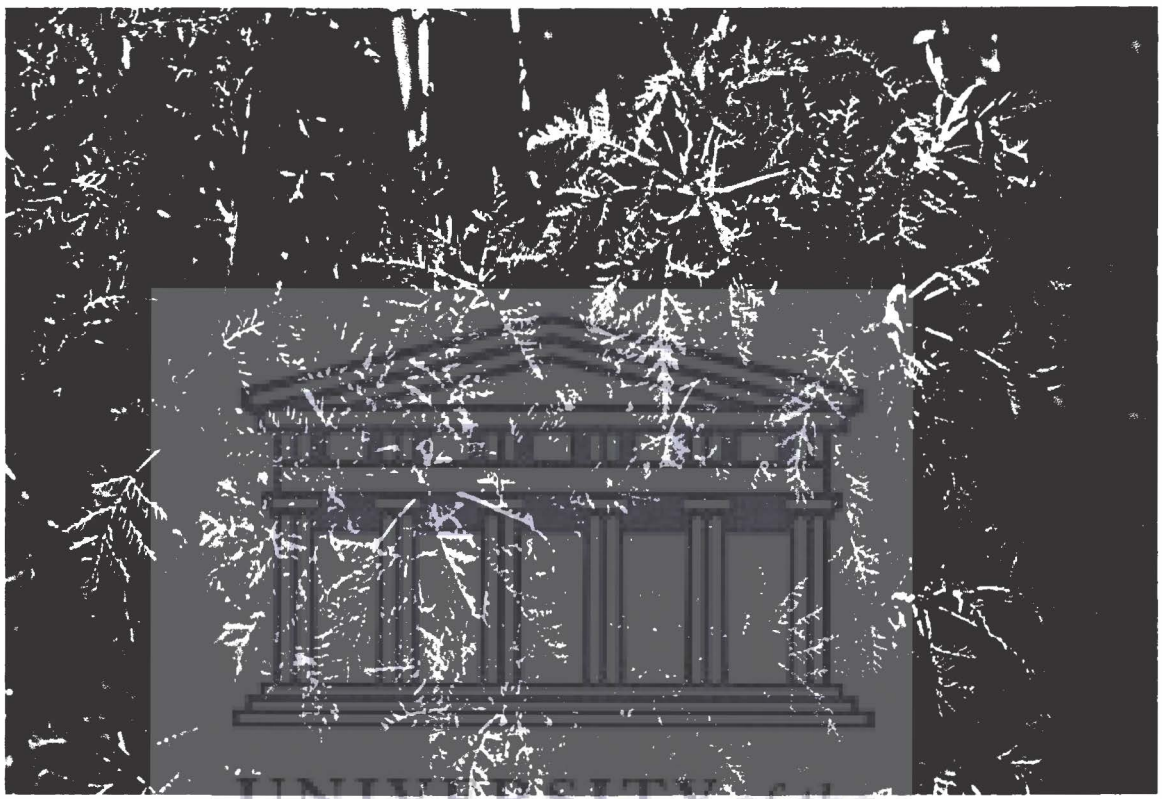

Fig 1: ***Artemisia afr,a (A. afra)*** **adapted from pharmacopoeia monographs project, 1999.**

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2.3.2.1 Taxonomy

Family	Asteraceae
Species	***afra***
Common names:	Wild wormwood, South African wormwood and African absinthe.
Indigenous names:	Lengana (Pedi), Lengana, zengana, (Southern Sotho) Umhlonyane (Xhosa), Mhlanyane (Zulu), and Wildeals (Afrikaans).

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2.3.2.2 Distribution

Grows in rocky mountainous areas throughout South Africa, but also in other parts of Africa. Most common in the Northern and Eastern Transvaal, Orange Free State, Kwazulu-Natal, and the Eastern and Western Cape Provinces.

2.3.2.3 Description

The feathery gray green shrub grows up to 2m in height and spread. The leaves are aromatic, fine gray-green, and make the giant look fem-like. The plant is covered with small, yellow-green, ball-shaped flowers in mid-summer.

2.3.2.4 Medicinal Uses

Wildeals is used to treat numerous ailments and is mainly indicated for coughs, colds and influenza. The plant is also used to treat fever, loss of appetite, colic, headache, earache, malaria and intestinal worms amongst other conditia (autchings et al, 1996; Van Wyk et al, 2000; Watt and Breyer-Brandwijk, 1962).

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2.3.2.S Bioactive Compounds

Flavonoids isolated from the plant include chrysosplenetin and a tetra-hydroxymethoxy flavone (Tang et al, 2000). Ten guaianolides and five glaucolides have been isolated (Hutchings et al, 1996, Tang et al, 2000). The essential oil contains cineol, thujone, camphor, bomeal (Hutchings et al, 1996, Van Wyk et al, 2000) and terpenoids; the sesquinterpene lactone arteminisin is responsible for the plants activity against malarial parasites (Tomlinson et al, 1998).

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2.3.3 *Mentha longifolia*

Fig 2: Mentha longifolia (M. lo1'gif:olia) adapted ti·om pbarmacoreia i:-onographs project, 1999.

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2.3.3.1 Taxonomy

Family	Lamiaceae
Species	*Longifolia*
Common names:	Spear mint, pennyroyal, and wild mint
Indigenous names:	Kwena, Kwena-ya-thaba (Sotho), Inxina, inzinziniba, (Xhosa) Ufuthane lomhlange (Zulu), and Kruisement (Afrikaans).

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2.3.3.2 Distribution

Common along streams in the Transvaal, also in the Cape Provinces, Orange Free State, Swaziland, Kwazulu-Natal and Lesotho.

2.3.3.3 Description

An erect perennial that grows up to 1 metre with 4-sided much branched reddish brown, densely hairy stems. The leaves are dark green, pungent smelling and smooth on top, lighter gray and hairy underneath. The plant grows small flowers white, pink or lilac in color.

2.3.3.4 Medicinal Uses

The plant is mainly used for coughs, colds, asllima and other respiratory ailments. The plant is indicated for headaches, fevers, hysteria, indigestion, flatulence, and painful menstruation, delayed pregnancx and lbr urinary tract infections (Hutchings et al, 1996; Van Wyk et al, 2000; Watt and Breyer-Bramtwjjk, 1962).

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2.3.3.S Bioactive Com11ounils

The composition is lmown 10 vary e,onside_rably for plants ootained from different localities but no information appears to be available on the South African plant (Van Wyk et al, 2000). Flavonoids isolated from *M. longifolia* include the glycosides of isorentin, vincenin, luteolin, tricetin, diosmetin, hesperetin, and the aglycones acacetin, luteolin and eriodictyol (Ali et al, 2002, Ghoulami et al, 2001, Hutchings et al, 1996, Sharaf et al, 1999, Van Wyk et al, 2000). The essential oil contains carvone, pinene, myricene, limonene, menthone, menthol and linalool (Hutchings et al, 1996, Van Wyk et al 2000).

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Flavonoids, which are ubiquitous to green plant cells, are found in practically all plants. Overall, the research done on *A. afra* and *M. longifolia* have shown the plant material to be rich in flavonoids compounds. These compounds are described below.

2.4 Flavonoids

2.4.1 Introduction

ht this section the chemistry and classification, occurrence, analysis and identification of flavonoids will be discussed, in addition to the potential role of five selected flavonoids as markers of medicinal plant material, and their biological activities will be assessed.

The term phenolic compound embraces a large array of chemical compounds possessing an aromatic ring bearing one or more of hydroxyl groups together with a number of other substituents. Flavonoids are the most abundant polyphenols in our diet and represent the single, most widely occurring group of phenolic phytochemicals (Bloor, 2001; Croft, 1998; Havsteen, 2002; Rice-Evans et al, 1997; Rice-Evans et al, 2001; Scalbert et al, 20oiJ. They are virtually ubiquitous in plants and are likely to be encountered in any work involving plant extracts.

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It is estimated that approximately 2% of all the carbon photosynthesized by plants is converted into flavonoids or closely related compounds (Scalbert et al, 2000; M 1982). Flavonoids arise from an initial reaction involving the condensation of a cinnamic acid, catalyzed by the chalcone synthetase enzyme. The chalcone formed in this initial reaction is then converted rapidly into a phenylbenzopyran, and further modifications lead to the formation of the different sub-classes within the flavonoid family (Bloor, 2001; Markham, 1982). The flavonoid group of compounds shares a basic C_6-C_3-C_6 structure and is the most diverse range of naturally occurring plant phenols.

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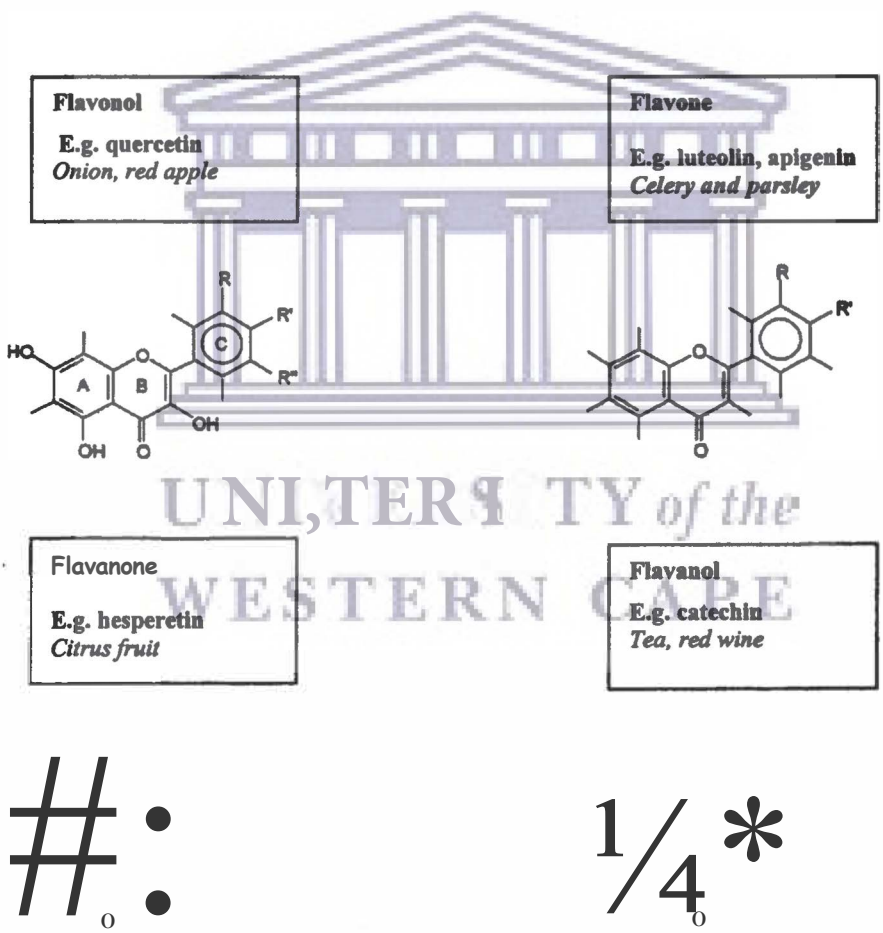
2.4.2 Chemistry and Classification

Flavonoids consist of two benzene rings (A and B) connected by a three-carbon unit, which may or may not form a third ring (C). Individual carbon atoms are labelled by a numbering system, utilising ordinary numerals for the A- and C rings, and primed numerals for ring B.

Flavonoids containing a hydroxyl group in position C-3 of ring C are classified as 3-hydroxyflavonoids (flavonols, anthocyanidins, leucoanthocyanidins and catechins), and those lacking it as 3-desoxyflavonoids (flavanones and flavones) Fig 3.



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JI& 3: The structural formula of some flavonoid classes. R represents the various substitutiou.

Classification within these two families is based on whether and how additional hydroxyl or methoxy groups are introduced into the different positions of the molecule.

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lsoflavonoids differ from the other groups; the B ring is bound to C-3 of ring C instead of a C-2. Anthocyanidins and catechins lack a carbonyl group on C-4 (Harborne, 1986; Havsteen, 2002; Markham, 1982; Merken et al, 2000; Rice Evans et al, 1997).

Flavonoids are present mainly as flavonoid O-glycosides in plants, in which one or more of the flavonoid hydroxyl groups is bound to a sugar or sugars by an acid-labile bond (Markham, 1982). The flavonoid aglycone fonn, where the flavonoid occurs without attached sugars, occurs less frequently in the plant. As glycosides, sugars may also be C-linked to the flavonoid fonning a flavonoid C-glycoside, in which case the sugars are directly attached to the benzene nucleus by a carbon-carbon bond, which is acid resistant. The range of flavonoid aglyconc involved in C-glycosylation is however very restricted, thus although isoflavones, flavanones and flavonols occur occasionally in C-glycosylated form, flavone C-glycosides are by far the most prevalent (Markham, 1982; Merken et al, 2000).

At least 8 different monosaccharides or combinations of these can bind to the different hydroxyl groups or C-link of the flavonoid aglycone and include glucose, galactose, rhamnose, xylose and arabinose. The effect of glycosylation of the flavonoid is to render the comPQund less reactive and more wate(:soluble, the latter property permitting the sto e of the flavonoid in the cell vacuole where most flavonoids are commonly found. The various sub-classes are further outlineci below.

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2.4.2.1 Anthocyanidins

Anthocyanidins and their glycosides (anthocyanins) are natural pigments responsible for the dark colors present on red fruit such as cherries, plums, strawberries, raspberries, black berries and black currants (Rice-Evans et al, 1997). An example of such a compound is cyanidin, a major constituent of dark red fruit berries like raspberries (Kong et al 2003).

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2.4.2.2 Flavonols

The most common flavonol is quercetin. Quercetin is present in many fruits, vegetables, and beverages. Quercetin is particularly abundant in onions (300mg/kg fresh weight) wine and tea (Manach et al, 1998; Scalbert et al, 2000). Other quercetin sources are apple skin, berries, black grapes and broccoli. The content varies greatly depending on the plant variety, culture conditions, and degree of ripeness and food processing.

Quercetin is present in plants in many different glycosidic forms with quercetin-3-rutinoside (rutin), being one of the most widespread forms. In onions the compound is bound to one or two glucose molecules (quercetin-4'-glucoside, quercetin-3, 4'-glucoside). Other flavonols present in the diet are kaempferol, found in leek, broccoli, grapefruit and tea, and myricetin and isorhamnetin (Harborne et al, 2000; Karakaya et al, 1999).

2.4.2.3 Flavanols

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The main flavanols are catechins, which usually occur as aglycones and are very abundant in tea. In black tea the quantity of catechins are reduced to about half of the original value, due to the oxidation of these compounds into more complex polyphenols during fermentation. They are also found in various fruits and vegetables such as apples, pears, grapes, and peaches and in red wine (Scalbert et al, 2000).

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2.4.2.4 Flavanones

The main dietary sources of flavanones are citrus fruits such as grapefruit and oranges (Croft, 1998, Rice-Evans et al, 1997). Citrus flavanones include hesperidin, diosmin, naringenin and hesperetin (Gil-Izquierdo, 2001).

2.4.2.S Flavones

The main flavones, apigenin and luteolin, are common in cereal grains and aromatic herbs like rosemary, parsley and thyme (Karakaya et al, 1999). Luteolin is predominantly found in lemon, olive and red pepper in addition to the sources stated above (Owen et al, 2003; Rice-Evans at al, 1997).

2.4.3 Occurrence

Flavonoids are present in significant amounts (0.5 – 1.5% of dry weight) in plants. However it is rare to find only a single flavonoid component in plant tissue and the compounds are often present in plant material as mixtures of different flavonoid sub-classes (Harborne, 1986; Harborne et al, 2000).

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Nutritionists estimate tha, the a\'erage intake of flavonoids by humans on a normal diet is 1- 2gm per day (Havsteen, 2002; Merken et al, 2000; Hollinan et al, 1997; Scalbert et al, 2000). However these values only cover five aglycones, therefore the total intake may reach several hundred mg/day (Terao, 1999).

2.4.4 Analysis and identification

The general methodology used for the analysis and identification of flavonoid compounds has been revolutionized by chromatography. High performance liquid chromatography (HPLC) and Mass spectrometry (MS) are highly sensitive and selective methods for the resolution, quantitative determination and identification of flavonoids (Bloor, 2001; Harborne et al, 2000; Stobiecki M, 2000). These modern

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techniques have become standard equipment for analysis and often yield excellent resolution and, retention times that are extremely reliable in the identification of individual flavonoids. Several studies show the effective use of these two methods for flavonoid analysis (Franke et al, 2002; Justesen et al, 1998; Romanova et al, 2000; Scheiber et al, 2000; Scheiber et al, 2001).

A Cls or Cs column containing octadecyltrichlorosilane is commonly used as the stationary phase. Eluting solvents often include combinations of organic solvent with water and small amounts of acids as buffers, which tend to improve separations considerably. Flavonoids eluted by HPLC are normally detected by means of a UV detector; two detectors can be used at the same time to monitor the elution at two wavelengths simultaneously.

The traditional method described in several pharmacopoeias and reviews, for the determination of flavonoids in a plant extract typically involves the acid-hydrolysis of the glycosides foliowed by chromatographic analysis (Bloor, 2001; Tura et al, 2002; Robards, 2003). Crude plant extracts should therefore be partli purified before HPLC analysis with the intention of characterizing the free flavoroid (aglycone) and minimizing interferences in the subsequent chromatography (Tura et al, 2002).

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A significant number of medicinal preparations containing flavonoids are currently in use and this study is particularly interested in the stanruirdization of medicinal plant preparations using flavonoids as markers to assess the pharmaceutical quality of plant material.

l.4.5 Flavonoids as marken

The flavonoids ultimately fulfill much of the criteria required for a marker compound/s, i.e. flavonoids are present in plant preparations in quantifiable amounts, are extractable from plant material, seem stable in the plant samples under processing conditions of the raw material, and are sufficiently selective as they can be identified under set assay

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conditions. In addition the flavonoids arc biologically active and free from contamination concerns.

To corroborate this overall view, it is known that flavonoids occur ubiquitously in the plant kingdom. Over 4,000 different flavonoids have been described (Hollmann et al, 1997). This makes the flavonoid group of compounds qualitatively and quantitatively one of the largest groups of natural products known (Harbome et al, 2000).

Flavonoids have a variety of biological effects in numerous mammalian systems, *in vitro* and *in vivo* (Harbome et al, 2000) and characterization methods for this group of compounds range from simple approaches to complete analysis of the absolute structure and configuration of a flavonoid moiety.

The toxicity aspects of flavonoids *in vivo* in mammals are unproven (Hollmann et al, 1997). Havsteen reviewed the evaluation of quercetin for mutagenicity concerns, and with the Ames test found that frequency of mutations induced by quercetin were exceptionally low, and absolved the flavonoid of any toxicity concerns (Havsteen, 2002). The risk of pathological consequences of mutation incurred by the consumption offlavonoids should therefore be considered low.

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It is thus proposed that five flavonoids, apigenin, hesperetin, kaempferol, luteolin and quercetin could be swtable arlcers for this family of, compounds. With a suitable HPLC assay to determine the quantity of markers in plant material, setting a minimum level for flavonoid components within preparations of this nature, could give an indication of the quality/pharmaceutical integrity of plant material. The flavonoids selected could possibly contribute to the efficacy of *A. afra* and M. *longifolia.* The biological activities of the five flavonoids are outlined below.

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2.4.6 Biological activities of selected markers

The most studied flavonoid is quercetin. Quercetin is reported to have anti-oxidant (Harbome et al, 2000; Manach et al, 1998; Sadik et al, 2003; Terao, 1999), anti-inflammatory (Rottelli et al, 2003), anti-proliferative (Benavente-Garcia et al, 1997), anti-atherogenic (Harbome and Williams, 2000; Benavente-Garcia et al, 1997; Hollmann and Katan, 1997) and anti-microbial (Benavente-Garcia et al, 1997) activities.

Quercetin

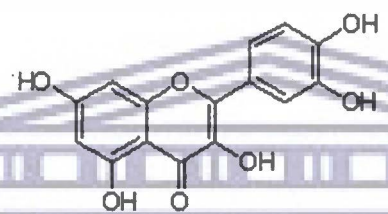

Fig 4: The chemical structure of quercetin (flavonol).

Kaempferol inhibits inflammation in croton-induced dermatitis, is a potentially active flavonoid on the vasculature, can cause inhibition of platelet aggregation, and is an effective anti-oxidant (Harbome et al, 2000).

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Kaempferol

Fig 5: The chemical structure of kaempferol (flavonol).

Hesperetin is mainly cancer-preventive, i.e. anti-carcinogenic, anti-tumor and anti-mutagenic. Benavente-Garcia (1997) showed the activity of hesperetin against acute myeloid leukemia, and as an anti-oxidant. Reviews on the role of hesperetin as an antioxidant include Gil-Izquierdo (2001), Harbome (2000). In addition the compound

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has analgesic, diuretic, anti-hypertensive, and hypo-lipidemic potential (Harbome et al, 2000; Kim et al, 2002).

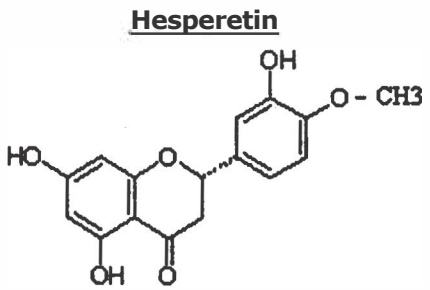
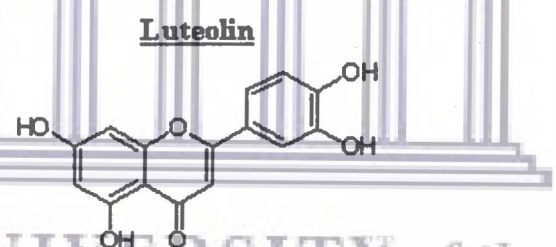


Fig 6: The chemical structure of hesperetln (flavanone).

According to Harbome (2000), flavonoids inhibit platelet aggregation, with apigenin and luteolin significantly inhibiting platelet aggregation in rabbits. Luteolin has vasodilator activity; this was proven when the compound depressed contractions induced in the rat aorta (Harborne et al, 2000).



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Fla 7: Chemical structure of luteolln (flavoneJ.

Apigenin, a less toxic and non-mutagenic flavone, is one of the most investigated flavonoids with promising chemo preventive activity against skin cancer (Romanova et al, 2000). Most of the recent advances in flavonoid research focus on apigenin and luteolin as chemo protective, anti-inflammatory and anti-allergy agents (Benavente-Garcia et al, 1997; Birt et a, 2000; Kimata et al, 1999).

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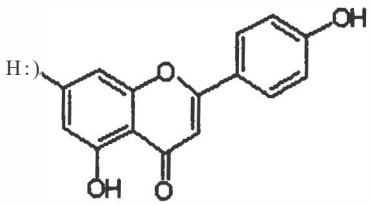
Apigenin



Fie 8: Chemical structure of Apigenin (flavone).

The mechanisms behind the effects of the flavonoids are largely unknown. Due to the anti-oxidant effects of most flavonoids and their ability to absorb UV light, they may act in all the stages of the carcinogenic process (Benavente-Garcia et al, 1997). However, in a number of human based snrdies, a direct relationship between a quercetin and lowered incidences of cancers is proof of the protective role of a flavonoid in the carcinogenic process. Furthermore, Birt (2001) has reported quercetin to be active in the prevention of caneer in experimental animals.

In general, the anti-oxidant activity of the flavonoids could be responsible for most of their cardiovascular activities on hypertension and atherogenesis, via their ability to inhibit platelet adhesion, aggregation and secretion. The inhibition of the cyclo-oxygenase and/or 5-lipoxygenase pathways of arachidonate metabolism could explain the anti-inflammatory and anti-allergy activities of the tive flavonoids (Harborne et al, 2000).

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In summary, although the epidemiological evidence regarding the association between flavonoids and the risk of disease may be difficult to interpret, most studies generally assess the compounds as biologically active, and the use of flavonoids for the prevention and cure of disease is already widespread (Havsteen, 2002). The assessment of plant preparations with regard to the apigenin, hesperetin, kaempferol, luteolin and quercetin content could enable analysts to roughly estimate the quality of the preparations of the medicinal plants, *A. afra* and *M. longifolia,* in addition to many un-screened medicinal preparations in use containing flavonoids.

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Chapter3

PLANOFWORK

3.1. Introduction

This chapter describes the objectives, hypothesis, and the study approach proposed for the evaluation of the suitability of apigenin, hesperetin, kaempferol, luteolin and quercetin for quality control studies of flavenoid-containing medicinal preparations.

3.2. Objectives

Among the secondary metabolites contamed in nlant medicines, flavonoid compounds are of major importance because these compounds make a significant contribution to the chemical content and intrinsic activity of the plant material. The overall objective of this project was to investigate the possibility that the flavonoid compounds apig hesperetin, kaempferol, luteolin and quercetin could be used as markers for the quality control of flavonoid-cohtaiirin'.g pl t material.

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To achieve this objective, it was essential to establisb,-whethcr any of these flavonoids were present in two, widely used indigenous medicinal plant species, namely A. *afra* and *M. longifolia,* whose activity was hypothesized to be due to the flavonoid content In order to fulfill this objective, a sensitive and reproducible assay to quantitate the flavonoids in these plants had to be developed, and the utility of this assay assessed for quality control studies, using several commercial flavonoid-containing medicinal preparations.

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3.3 Hypothesis

It was hypothesized that firstly, a suitable HPLC assay could be developed to simultaneously and qualitatively or quantitatively determine the flavonoids apigenin, hesperetin, kaempferol, luteolin and quercetin. Secondly, that at least one of these flavonoids could be found in *A. afra* and *M longifolia-containing* preparations. Finally, that the monitoring of the levels of one or more of these flavonoids by the HPLC assay, could be used to establish and monitor the pharmaceutical integrity of such flavonoid-containing medicinal preparations.

3.4 Study approach

3.4.1 Why choose these plants products?

Traditional medicine is widely practiced in South Africa and the traditional medicinal plants, *A. afra* and *M. longifolia* were chosen mainly because they have been used over many years by the local people in the Western Cape to treat coughs, colds and related respiratory disorders. These two plants form a major part of the current research programme in the [illegible] objective is to evaluate the claimed therapeutic effects of the extracts of these two plants by means of clinical and pre-clinicll studies, and in addition develop better dosage forms of these plants. The commercial plant P.reparations used for the study were chosen either because they contained *A. afra* or *M longi,folia* plant species, or because they were reported to contain one or more of the flavonoids used in this study.

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3.4.2 Why these five flavonoids?

The flavonoids form a large class of chemical compounds comprising of five prominent subclasses namely, anthocyanins, catechins, flavanones, flavones, and flavonols. These compounds represent one of the largest classes of secondary metabolites that have been easily isolated from many plant materials.

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The flavonoids, apigenin, hesperetin, kaempferol, luteolin and quercetin were specifically chosen for this study firstly because, they have been reported to be present in the crude extracts of *A. afra* and *M. longifolia* plant species. Secondly, these flavonoids are representative compounds of the three prominent flavonoid subclasses, flavanones (hesperetin), flavones (apigenin, luteolin) and flavonols (kaempferol, quercetin) that have a high presence in plant-based foods and crude plant.

Furthermore, a comprehensive coverage on the occurrence, structure variation and analysis of these flavonoids is extensively accessible in literature, and analysis of these flavonoids in literature show that the compounds have a well-known behavior under analytical conditions, with concentrations shown to remain constant throughout various analytical procedures. Therefore with these considerations in mind, the flavonoids selected were likely to be quantifiable in plant materials.

3.4.3 Why use an HPLC assay?

The development and use of a suitable HPLC assay was proposed as a suitable chromatographic technique to analyze the markers, for the following reasons.
Firstly, high performance liquid chromatography had the advantages of simplicity, sensitivity, high-speed separation, and the use of low quantities of sample. These reasons in combination with those of mass spectrometry f.e. selectivity and sensitivity with exceptional diagnostic ability; provided additional advantages for the validation of the analytes determined using the HPLC analysis. In addition, a varied number of standard phytochemical tests could be applied using the HPLC assay to generate reproducible and interpretable data for a quality control type of study.

3.4.4 How was the potential use as a biomarker established?

In this study the compounds were assessed for their suitability as markers based on firstly, whether or not, and the extent to which they were present in plant material. Secondly, on the ease and reproducibility of their assay, and thirdly, on the sensitivity

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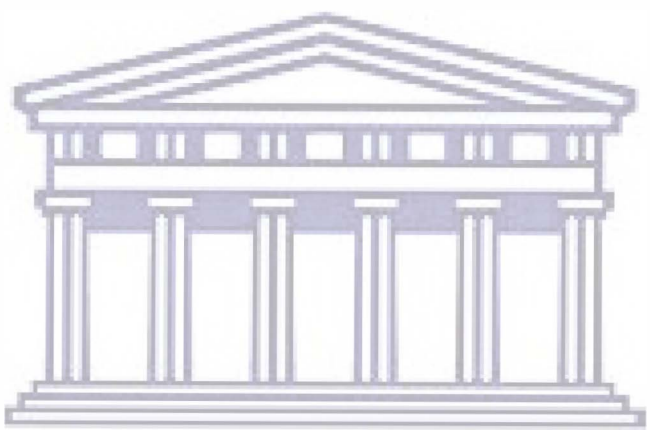
of the level of the marker to the processes of extraction and phannaceutical manufacture. This project aims to provide data which will assist in the development of sample handling and dosage form preparation strategies, that aim to improve the maintenance of the chemical and pharmacological integrity of flavonoid-containing plant material.

Establishing a suitable assay and a group of suitable biomarker compounds that are easy to analyze, pharmacologically active and relatively stable provides a suitable avenue to achieve this objective.



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Chapter4

METHODS

4.1 Introduction

This chapter describes the equipment, materials, methods and procedures that was used in, (1) the extraction of the plant materials in preparation for chromatographic analysis, (2) the development of an HPLC assay suitable for the identification and quantification of certain flavonoids and their verification by mass spectrometry (LC-MS) and, (3) the assessment of the utility of the assay for the identification and quantification of the flavonoids in plant material and the quality control of various flavonoids-containing medicinal plant materials.

4.2 Materials and equipment

4.2.1 Chemicals, reagents and materials

The following chemicals, reagents and materials were used in the extraction, chromatography, mass spectrometry and quality control studies.

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Chemicals: Methand, acetonitrile, ethyl acetate, diethyl ether, chloroform, hexane, acetic acid, formic acid and hyarochlori'c acid. All of tlie solvents used were of analytical or HPLC grade, and were purchased from Merck (Darmstadt, Germany). Distilled water was purified using a Purite, Analyst HP water purifier (Oxon, England).

Reagents: Apigenin, hesperetin, kaempferol, luteolin and quercetin standards were purchased from Sigma (St. Louis, USA).

Materials: *A. afra* and M. *longifolia* were purchased from Montague Museum, Voucher numbers 6634 and 6635, A. *a/ra* tablets were obtained from the Pharmaceutics dept, UWC (Komperlla et al, 2003).

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Asthmitea ®, *Arthritea* ® *and Floo-tea* ® commercial medicinal plant preparations prepared and packaged in S0gm packets by Sing Fefur Organic Herbs (South Africa) were purchased at Bellville Fruit and Vegetable market, (Westem Cape, South Africa).

Asthmitea®, a mixture of ***Lavendula species, Marrubium vulgare, Mentha species, Plantago off, Symphytum offand Urtica Urens species.***

Arthritea®, a mixture ***Achillea mill, Betulina barosma, Ruta graveolus, Symphytum officianalis and Urtica urens species.***

Floo-tea®, a mixture of ***A. afra, Betu/ina Barosoma, Marrubium vu/gare, Symphytum officianalis, Ruta graveolus and Urtica urens species.***

The ***Arthritea®*** commercial preparation was tested because this product was similar to an ***Artemisia-containing*** product. Four ***species (Betulina Barosoma, Ruta graveolus, Symphytum officianalis, Urtica urens)*** contained in it were present in ***Floo-tea®,*** which contained *Artemisia species*.

4.2.2 Equipment:

The following equipment was used in the preparation of the plant materials and assay samples:

An Ohaus scientific balance model GA 11O; Memmert oven model 854; syringe filters 0.45 µm Millipore (USA); Labaticon water Jatli modef GE>li 110 .(Maraisburg, South Africa); Virtis freeze mQ bil Freeze <iryer model 125L; Multex MSE centrifuge (England); Buchi Rotavapor model R 124 consisting of a Buehl water bath model B 481 vacuum and a Neuberger Laboport vacuum pump (Switzerland).

Chromatographic analysis was performed on a Beckman System Gold HPLC system with a 32-Karat ^{TM} software package (Fullerton CA, USA), consisting of a Beckman Gold Module 126 series programmable binary gradient pump, a Beckman Gold Module 507 series auto sampler with a Beckman Gold Module 168 series diode array detector. Mass spectrometric-liquid chromatographic analysis was performed on a TSP HPLC system, consisting of a TSP P2000 binary pump solvent delivery system, a TSP AS series auto sampler with a TSP UV2000 detector.

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Columns used for chromatographic analysis included: Inertsil® 5µm C18s (250 x 3mm), a Prodigy® 5µm C18 (150 x 3mm) and a Hydro-synergy® ODS 4µm column (250 x 4mm, Phenomenex, USA), protected by a guard column (4 x 3.0mm I.D) packed with the same stationary phase as the final analytical column (Hydro-synergy®).

4.3. Methods and procedures

4.3.1. Collection, identification and preparation of plant material

The plant material *A. afra* and *M longifolia,* was obtained from Montague Museum while they were flowering in the summer of--2002. A botanist at Kirstenbosch gardens authenticated the plants, and voucher specimens, voucher numbers 6634 and 6635, were deposited in the Herbarium at UWC. The freshly collected plants were stored in a cold room overnight. The next day all the leaves were removed, washed with distilled water and then dried in an oven at 30°C for 72 hours. The dried leaves were ground in an electric grinder and the ground material was packed into containers and sealed with poly film, then stored in a dark cupboard at room temperature, until required for extraction.

The commercial plant preparations *Asthmitea®, Arthritea®* and *Floo-tea®* were purchased early in 2003, and were purchased packaged in 50g labeled, airtight packets. The other plant materials, were assayed in this study included: *A afra* tablets made from aqueous extracts of the plant that were manufactured in the Pharmaceutics Discipline, UWC (Komperlla et al, 2003), aqueous and methanol extracts of *A. afra* and *M longifolia* prepared by students in the Pharmacology Discipline, (UWC). These plant materials were all obtained prior to the period of assay, between the month of April and August of 2003, when the assay was conducted.

4.3.2 Extraction of plant materials

The primary aim of the preliminary extraction step was to isolate the flavonoids present in the cell vacuole and to prevent enzymatic oxidation or hydrolysis of the flavonoids in the dried, ground plant material. Two main extraction procedures were used.

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4.3.2.1 Methanol extraction procedure.

Thirty gram each of ground *A. afra* and *M. longifolia* was exhaustively extracted with approximately 700ml methanol using a Soxhlet apparatus. Two hundred milliliter of distilled water was added to the methanol extract, and the hydro-alcoholic extracts were filtered and concentrated under vacuum at 40°C using a rotary evaporator to eliminate the methanol. The remaining aqueous solutions were washed exhaustively (x3) with chloroform to remove the lipids, chlorophyll and waxes. Thereafter, each extract was concentrated under reduced pressure to a small volume and freeze-dried to obtain a dry, flaky, pale yellow extract of *A. afra* or *M.longifolia.* All freeze-dried extracts were stored in sealed brown bottles in a dessicator until required for analysis.

4.3.2.2 Aqueous extraction procedure

In this procedure a 150g quantity of *Asthmitea®, A.afra* or *M. longifolia* was extracted using distilled water. The plant material was weighed and then immersed in 3L of cold distilled water, and boiled for 120 minutes. This infusion was filtered and then transferred into a round-bottomed flask. This infusion procedure was repeated three times with the same plant material, and then the infusions were combined and freeze-dried to obtain a fluffy black-brown extract of *Asthmitea®, A. afra* and *M. longifolia.*

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4.3.2.3 Preparation of the plant extracts for HPLC assay

For the HPLC analysis of the flavonoids in the plant material, solutions of the un-hydrolyzed dried extracts or hydrolyzed plant extract were prepared. For the former, typically 20 mg of dried extract was dissolved into 600 µl HPLC mobile phase solution, and. 20 µl aliquots injected onto the HPLC column. The hydrolyzed samples were prepared as described below.

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4.3.2.4 Acid-hydrolysis procedure

Extract powder (20 mg), was initially dissolved into 200 µl methanol and vortex mixed to thoroughly solubilize the powdered material. To this, 1.8 ml of 2 N HCl was added, to give a total volume of 2.0 ml. This acid-methanol solution was heated in a water bath maintained at 80°C for 40minutes, cooled, and then extracted with 2.0 ml of ethyl acetate. The ethyl acetate fraction was evaporated to dryness using a gentle stream of nitrogen gas, the residue re-dissolved into 600 µl of HPLC mobile phase solution, filtered and then 20 µl aliquots injected onto the HPLC column.

4.3.2.5 Preparation of flavonoid-containing preparations for HPLC assay

In addition to the dried aqueous or methanol plant extracts of *Asthmitea®*, *A. afra* and *M longifolia,* the flavonoids were determinetl in *A.afra* tablets prepared from an aqueous extract of the plant, *Asthmitea®*, *Arthritea®* and *Floo-tea®*. Hydrolyzed and un-hydrolyzed solutions of these samples were prep as follows:

To prepare un-hydrolyzed plant sample sqlutions; *A. afra* tablets were ground into a powder in a mortat an! pestle d twenfy grams of *A. afra* tablet powder, or mixture of leaves and stems of *Asthmitea®*, *Arthritea®* or *Floo..tea®* Wffti each dissolved in 600 µl of HPLC mobile phase solution, filtered and 20 µl aliquots injected onto the HPLC column.

To prepare the hydrolyzed plant samples the acid-hydrolysis procedure described in section 4.3.2.4 was used. The plant samples for hydrolysis therefore consisted of 20 mg *A. afra* tablet powder, or 20 mg mixture of leaves and stems of *Asthmitea®*, *Arthritea®* or *Floo-tea®* dissolved into a 2.0 ml acid-methanol solution. The residue obtained for each sample after hydrolysis, was re-dissolved into 600µ1 of HPLC mobile phase solution, filtered and 20 µl aliquots injected onto the HPLC column.

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A HPLC assay was developed and confirmed for the qualitative or quantitative determination of apigenin, hesperetin, kaempferol, luteolin and quercetin. In this section only the final procedures used are reported, the development aspects are reported in section 5.3.

4.3.3.1 General HPLC procedure

First, stock solutions of apigenin (4.54 mg/ml), hesperetin (5 mg/ml), kaempferol (5mg/ml), luteolin (5.15mg/ml) and quercetin (5mg/ml) were made up in dimethyl sulfoxide. All the stock solutions prepared were protected against light, kept refrigerated at 2° C, and thawed prior to use. Working solutions for apigenin, hesperetin, kaempferol, luteolin and quercetin were made up each day by making dilutions of aliquots of the stock solutions. The working solutions were prepared in the concentration ranges of approximately 3.125 µg/ml – 200 µg/ml and 20 µl aliquots were injected onto column.

The mobile phase consisted of 2.2% (v/v) acetic acid-water (solvent A) and acetonitrile (solvent B) run at a flow rate of 1ml/min. The analysis was performed in reverse phase mode using gradient elution which was obtained using the following conditions: 35% B (14 min), 35 to 80% B (3min), 80% B (5min), 80 to 35% B (Smm); and 35% B (2min). The peaks produced during analysis were monitored at ~ 290 nm and ~ 340 nm, and peak identification was further confirmed using a diode array detector set to perform UV scans between ~ 220 - ~ 400 nm.

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4.3.3.2 Identification of peaks

To establish the retention time for the flavonoid standards, firstly, a standard solution of apigenin, hesperetin, kaempferol, luteolin and quercetin was prepared as a ture. Thereafter 20µl aliquots were injected six times onto the HPLC column.
Five peaks were produced and for each of these five peaks the retention time and the UV spectra was noted. This procedure was repeated several times, under the same HPLC conditions until it was clear that the peak retention times obtained were consistent. Thereafter, independent solutions of apigenin, hesperetin, kaempferol, luteolin and quercetin were injected, their retention times noted, and for each flavonoid, the average ± SD of the retention time calculated. This information was used to assign each of the five peaks in the mixture to the relevant flavonoid.

To identify the flavonoid peaks in the plant material, the retention times of the peaks obtained in the flavonoid samples were compared to those obtained when plant material was subjected to analysis under similar HPLC conditions. On identification of apigenin, hesperetin, kaempferol, luteolin and quercetin peaks in the plant material, the concentrations of these compounds in the plant were assessed.

For this quantitative evaluation, calibration curves of the peak area (Y) vs. the concentration (X, µg/ml) of standard solutions for each flavonoid in the concentration range of approximately 3.125 µg/ml - 200 µg/ml were plotted. Once the peak area for the identified flavonoid peak in the plant was known, the concentration of the flavonoid was calculated from the calibration curve.

4.3.3.3 Validation of assay

To validate the assay the following were measured: linearity, precision and recovery.
To assess the linearity of the assay, calibration curves of the peak area (Y) vs. the concentration (X, µg/ml) of standard solutions of apigenin, hesperetin, kaempferol,

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luteolin and quercetin flavonoid standards prepared in the concentration range of 3.125µg/ml-100µg/ml were plotted and subjected to regression analysis using the least squares method.

To assess the precision of the assay, the peak areas of solutions of the flavonoid standards (apigenin, hesperetin, kaempferol, luteolin and quercetin) were determined six times on the same day and six times on the following day. The relative standard deviations were calculated and were used to express the intra-day and inter-day variability (relative standard deviations). To assess the sensitivity of the assay, the lowest concentrations for each flavonoid that generated a peak with a height that was at least three times higher than the base line noise was used to the provide limits of quantitation.

4.3.3.4 Mass spectrometry

Mass spectrometric analysis of the flavonoids was used to characterize the flavonoid standards and positively identify and verify the UV qualitative or quantitative analysis of flavonoid compounds detected in *Asthmitea®*, *A. afra* and *M longifolia* plant material.

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4.3.3.S General Mass spectrometry procedures

Working solutions of apigenin, hesperetin, luteolin and quercetin standards (corresponding to approximately 50 µg/ml), were prepared using the refrigerated stock solutions described in section 4.4.1.1. The working solutions were transferred into injector vials and 20-µl aliquot of these working solutions were injected on column. The mobile phase consisted of 2.2% (v/v) acetic acid-water (solvent A) and acetonitrile (solvent B) run at a flow rate of lmVmin, similar to the HPLC/UV procedure (section 4.3.3.1).

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The peaks produced were monitored at). 340 nm, followed by mass spectrometric measurements after electrospray ionization conducted in the negative mode, and the capillary temperature maintained at 220°C. The target analytes were monitored by observing either their deprotonated molecular ions [M-Hr, or in combination with their respective ions following collision-induced dissociation of the deprotonated molecular ions.

4.3.3.6 **Identification of flavonoids**

Initially using LC/MS, the flavonoids apigenin, hesperetin, luteolin and quercetin were screened for their individual deprotonated molecular masses, the latter expressed as [M-Hr defined as the mass of the molecular ion minus proton, or the *m/z* of the deprotonated molecular ion. The following deprotonated molecular ions were monitored: *m/z* 269 (apigenin), *m/z* 301 (hesperetin and quercetin), = *mlz* 28S (luteolin).

The flavonoids were further characterized by collision activated dissociation mass spectrometry (MS/MS), whereby individual deprotonated molecular ions were monitored in combination with their fragment ions after applying collision energies between the ranges of 32 - 43% to break up the parent molecular ion.

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Unequivocal confirmation of the flavonoids was obtained through collision-induced dissociation (CID) of the deprotonated molecular ions to yield characteristic product ion. mass spectra that served as fingerprints for each of the flavonoids. This two-stage mass spectrometric process allowed for the identification of these flavonoids in ***Asthmitea*** ®, ***A.afra*** and ***M longifolia*** plant samples.

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4.3.4 Pharmaceutical quality control analysis of plant material

The aim of this part of the study was to detennine the pharmaceutical quality of the selected flavonoid-containing preparations using the developed HPLC assay.
Specifically the assay for selected markers was to be used to assess, (1), whether or not the markers were present in the plant preparations, (2), the effects that extraction and/or processing might have on the level of the markers and (3), the consistency of the markers in various batches of plant materials. The results from this analysis were used to determine which of the five flavonoids is the best marker.

4.3.4.1 Preparation of plant samples

In this section, all the samples...were analyzed as solutions of the un-hydrolyzed and/or hydrolyzed products of the plant samples, and were prepared as described in section 4.3.2.4. Identification and quantitative determination of the markers in the plant preparations was performed as described in section 4.3.3.2.

4.3.4.2 Determination of markers in plant samples

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For the assessment of the presell of the markers; five extracts of *A. afra,* four extracts of *M longifolia* and one extract of *Asthmitea®,* prepare<! by different students for their individual research projects; and the commercial preparations, *Asthmitea* ®, *Arthritea* ® and *Floo-tee* ® were tested. The presence of the marker in the plant material was established by determining whether or not one or more peaks produced in the analysis of the plant preparation could be assigned to any one of the markers. This was done by comparing the retention times of the peaks with those obtained in the chromatograms of the flavonoid standards under similar chromatographic conditions. The marker was established absent if none of the peaks produced in the plant preparation corresponded to that of the flavonoid standards.

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4.3.4.3 Effect of processing on marken

To determine the effects of extraction, the variations in the levels of markers for five extracts of *A. afra,* four extracts of *M longifolia* and one extract of *Asthmitea®* prepared by different students for their individual research projects were assessed.

The level of marker in each plant sample was determined by calculating (from the obtained peak area), the concentration of the compound from the calibration curve of the relevant flavonoid standard. The variations in the level of the marker between the extracts were expressed as a percentage change (±SD) and were used to identify any significant differences between the plant extracts.

4.3.4.4 Batch-to-batch consistency of markers

To assess the usefulness of the markers in the evaluation of batch-to-batch variation, two batches of *A. afra* tablets prepared from an aqueous extract of the plant, and three batches of *Asthmitea®* were used. In each case, duplicate hydrolyzed or un-hydrolyzed samples of the plant preparation (tablet or dried herbs) were subjected to HPLC analysis, the peak areas obtained and the concentration (in ng/20 mg of hydrolyzed mass) of the marker determined from the calibration curve of the flavonoid standard. The mean concentrations of the markers were calculated and the differences assessed as a percentage change (±SD) of the flavonoid concentration between the batches.

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4.3.4.S Data and statistical analysis

All experiments were perfonned in at least three replicates. Data was transferred from the 32-Karat™ software package (Beckmann, 1999) into the Graphpad prism™ program (Graphpad, 2003). The latter graphics program was used to plot calibration graphs and fit the data to regression analysis using the least squares regression analysis.

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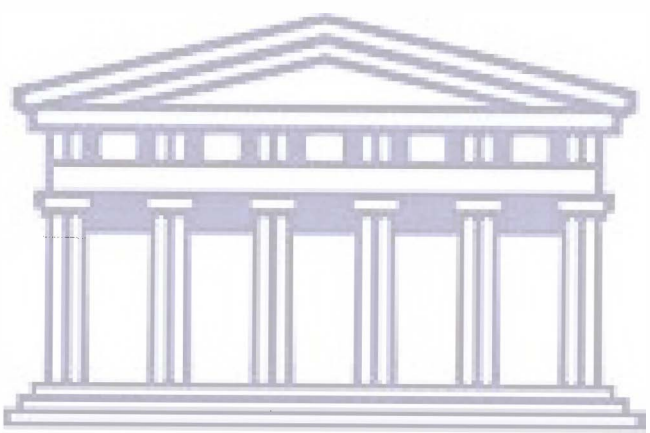
The program was also used to transform the raw data into percentages and to calculate the mean ± SEM, mean ± SD, co-efficient of variation, and 95% confidence limit for each data point. To analyze the variations of the mean± SD, data was subjected to non-parametric tests and these values were expressed in terms of the percentage change (± SD).



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Chapters

RESULTS AND DISCUSSION

S.1 Introduction

This chapter reports the results obtained in (1), the preparation of the plant material and subsequent preparation of the plant samples for HPLC analysis (2), the development and validation of a suitable HPLC assay for the simultaneous analysis of apigenin, hesperetin, kaempferol, luteolin and guercetin, (3) the validation of the identity of these flavonoid markers using mass spectrometry, and finally (4), the assessment of the use of these flavonoid marker compounds for pharmaceutical quality control studies.

5.2 Preparation of plant materials for assay

A botanist verified the medicinal plant material collected and voucher specimens were deposited at the Herbarium at the University of the Western Cape.

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The collected *A. afra* leaves (2015.8 g) were dried to a constant weight of 942.5 g and 46.7 % yield. The *M. longifglia* leaves (582.9 g) were dried to a constant weight of 390.3 g and 66.95 % yield. The dry leaves were ground to 739.1 g and 351.3 g powder for ***A. afra*** and ***M. longifolia*** respectively.

When thirty-gram aliquots of ***A. afra*** and ***M. longifolia*** were extracted with water and methanol using a soxhlet apparatus, the plants gave pale brown and yellow-brown extracts respectively. The yield from 30 g of the finely ground ***A. afra*** material was 3.59 g, ***(11.9 % yield),*** and 4.71g ***(15.7 % yield)*** for a corresponding quantity of M. ***longifolia.*** The ***A. afra*** extracts were slightly more hygroscopic than the ***M. longifolia*** extracts, however the physical appearance of all the extracts was acceptable.

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Both the extraction yields for *A afra and M longifolia* were reasonable and comparable to yields obtained in similar studies done by other researchers (Ali et al, 2002; Harris, 2002; Sharaf 1999).

The analysis of the flavonoids in the plants involved the chemical hydrolysis of the flavonoid glycoside compounds in the extracts. In this study, an acid hydrolysis method was employed and required the incorporation of a quantity of methanol prior to the addition of acid for hydrolysis, which was necessary to dissolve the extract.

Incomplete hydrolysis of the glycoside/s was observed for all the extracts when the proportion of methanol used was equal to that of hydrochloric acid (1:1), or when methanol was not used. The reason for incomplete hydrolysis of the glycoside was unclear, however it was likely that when methanol was not used the plant material did not completely solubilize in dilute hydrochloric acid.

It was difficult to obtain a constant concentration of the flavonoid when the incompletely hydrolyzed samples were analyzed, as the peak areas for the markers were very inconsistent ef F · · '=r analyzed at each of the compounds maximal wavelength. This irregularity was particularly apparent when more than one peak was contained under the peak assigned for a certain marker.

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The extraction conditions were thus investigated furllier and the most favorable conditions for hydrolysis of the flavonoid glycosides were those observed when a combination of hydrochloric acid: methanol (10:1) was used, followed by the extraction of the free flavonoid (aglycone) with ethyl acetate. The subsequent analysis of completely hydrolyzed samples consistently produced pure peaks that were generally symmetrical.

The general method currently used for determining the amount of flavonoid in a plant extract involves the hydrolysis of the flavonoid glycoside (s), followed by the extraction of the free aglycone using an organic solvent (Hasler et al, 1990; Harbome 1991;

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Markham, 1986; Tura 2002). According to Harbome (1991), hydrolysis should occur in 30 to 40 minutes. For instance Hasler (1990) describes the complete hydrolysis ofrutin, which occurred within 30 minutes with a combination of hydrochloric acid and methanol.

ht the present study the experimental conditions of dissolution of plant material into hydrochloric acid: methanol (10:1), hydrolysis in a water bath at 80°C for 40 minutes, followed by the final extraction of the aglycones with ethyl acetate proved quite successful and acceptable for *A. afra* and *M. longifolia*. Next the extracted flavonoid aglycones had to be quantified with a suitable assay.

S.3 Development and validation of the HPLC assay

S.3.1 Development aspects

To achieve optimal separation of apigenin, hesperetin, kaempferol, luteolin and quercetin, several mobile phase combinations, comprising methanol or acetonitrile with water-acid were tested. Most flavonoids are ionizable, therefore acid was added to the mobile phase to control the pH and suppress the tailing problem encountered in the chromatography of many flavonoids. The preliminary separations were conducted using a Prodigy® Cs column 5µm (150 x 3mm).

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Initially, each flavonoid was prepared as an aqueous solution in methanol-water and analyzed using acetonitrile/methanol (B) in water-acetic/formic acid (A) as the mobile phase. From these runs, it was determined that a mobile phase consisting of acetonitrile 65% (B) in water-acetic acid (98:2, v/v, A), and isocratic elution, provided a good resolution for all the five flavonoids individually. The flavonoids were then prepared as a mixture in this mobile phase and analyzed under similar isocratic conditions.

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Analysis of the five compounds as a mixture raised an apparent problem; two pairs of peaks overlapped namely, luteolin with quercetin, and apigenin with kaempferol. Moreover, other mobile phases based on methanol or acetonitrile gave comparable results with regard to these paired peaks.

In order to solve this problem, the use of longer columns was investigated and the effectiveness of the following columns were compared: an Jnertsil ® Sμm C_{18} (250 x 3mm), a Prodigy ® 5μm Cls (150 x 3mm) and a Hydro-synergy ® ODS 4μm column (250 x 4mm). The Hydro-synergy ® ODS column is end-capped with a hydrophilic agent, which the manufacturer claims gives better performance under aqueous conditions compared to other C_{18} columns. This column provided the best separation of the five flavonoids and consequently was selected as the column of choice for the assay. To avoid any carry over effects on the column, an additional 16 minutes of run time and a gradient were introduced into the method in order to flush and re-equilibrate the column between injections.

However, under the improved ideal conditions the peaks for apigenin and kaempferol still remained persistently unresolved. Merken (2000), using a combination of methanol and acetonitrile observed a similar partial resolution between apigenin and kaempferol. He however was doubtful that this combination of flavohoijls would be observed in the same foods.

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There are fewer published reports on the identification of kaempferol glycosides compared to that of apigenin in the extracts of *M longifolia.* Bearing this in mind as well as the difficulty encountered in resolving the kaempferol and apigenin peaks, the kaempferol was subsequently excluded from the mixture of flavonoids to be assayed in the present study.

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The final conditions of the assay settled on are as described in section 4.3.3.1 and involves gradient elution with the combination of 35% acetonitrile (B) and 65% water-acetic acid (2.2 v/v, A) at lmVmin. Under these conditions luteolin, quercetin, apigenin and hesperetin were resolved at 6.71 min, 7.11 min, 11.27 min and 12.87 min, respectively (Fig 9).

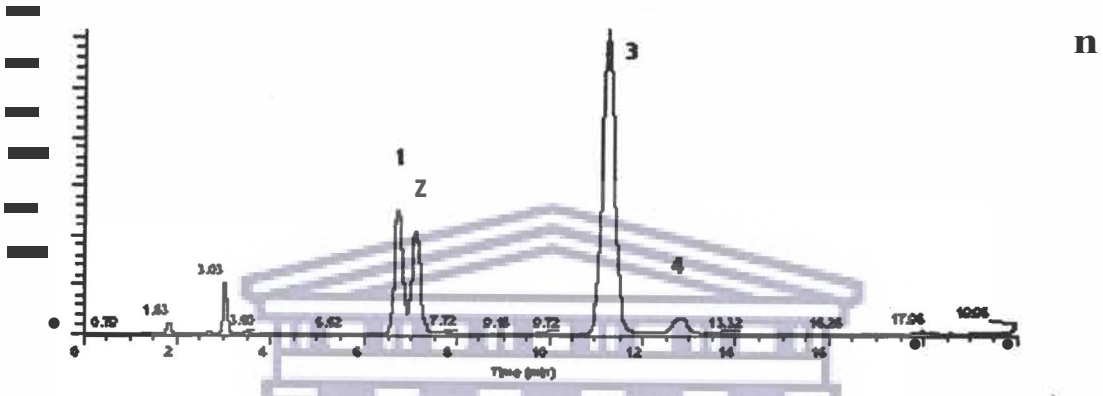

Fig 9: The typical HPLC chromatogram of a solution of apigenin (3), hesperetin (4), luteolin (1) and quercetin (2), flavonoids. Chromatographic conditions described in text (section 4.3.3.1).

Generally, these chromatographic findings are comparable to what found in previous studies on the flavonoids dcme y Hasler (1990), Keinanen (1998), Merken (2000), Palomino (1996), and Sladovsky (2001). The findings incl de, (1), the superiority of the results obtained when etomtrile instead of methanol is used ,2), the more effective suppression of flavonoid tailing observea with acetic acid compared to that of formic acid and (3), the effective use of a longer C_{18} column to improve separation.

Overall, it was thus evident that not all the selected flavonoids could be simultaneously determined in one run. In particular this partial resolution is likely to occur with flavonoids having such a closely related chemical structure. It was thus unlikely that kaempferol would be useful as a marker in this study, in view of the fact that it would prove challenging to identify the compound in a sample matrix. Furthermore, there was a probability that kaempferol could be absent in the extracts of *M longifolia.*

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From the above it was clear that assay conditions had been duly finalized for the simultaneous analysis of four of the five-flavonoid compounds. Next the assay had to be tested and proven reproducible and precise to see if it might be useful in assessing the suitability of the four flavonoids as markers for the extracts of *A. afra* and *M longifolia.*

S.3.2 Validation of assay

The results for of the experiments to validate the assay are summarized in Table 1. The intercepts and slope of the linear regression line for the calibration curves of all the reference standards are described, in addition to the concentration range and sensitivities obtained for the flavonoids.

Table 1: Linearity and sensitivity analytical characteristics for the calibration curves of four selected flavonoids determined by HPLC.

Compound	Concentration (µg/ml)	Slope *(b)*	Intercept *(a)*	r	Sensitivity (ng)
Apigenin	6.25 – 50	[illegible]	-0.2639±0.22	0.9998	62.5
Hesperetin	12.5 – 200	[illegible]	[illegible]	[illegible]	125
Luteolin	6.25 – 100	[illegible]	[illegible]	[illegible]	62.5
Quercetin	6.25-100	0.0213±0.02	-0.5689±0.25	0.9985	62.5

Abbreviations: r, correlation co-efficient

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The absorbance of the four flavonoids were read at 290 nm, A 340 and A 370 nm and the linear regression lines (table 1) and the calibration plots (appendix I - II) are shown for the wavelengths at which each flavonoid showed the highest absorbance.

The regression analysis revealed linear relationships between the peak area and concentration for the four flavonoids over the ranges indicated. All intercepts for the calibration plots were found not significantly different from the origin ($P > 0.05$). The correlation co-efficient for each flavonoid was reported to be between 0.9995 - 0.9999. Values for the limits of quantification ranged from 62.5 ng for apigenin, luteolin and hesperetin to 125 ng for hesperetin.

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These values are acceptable and compared favorably to those obtained by Hasler (1990), Lin (2001), Merken (2000), and Sladovsky (2001) whose sensitivity limits on column for apigenin, luteolin and quercetin were approximately between 50 - 80 ng.

The intra-day and inter-day variation studies indicated that the relative standard deviations were less than 3.46% and 4.46%, respectively. The proposed assay was thus sufficiently reproducible and sensitive for the simultaneous determination of apigenin, hesperetin, luteolin and quercetin. Accordingly, this method was deemed proficient for the assessment of the flavonoids as pure samples, and when present in a plant sample matrix.

5.3.3 Identification and verification of flavonoid compounds

5.3.3.1 Identification of flavonoids in pure sample

The identities of the peaks assigned to the four flavonoids, apigenin, hesperetin, luteolin and quercetin were validated using Mass Spectrometric analysis (MSIMS). A summary of the retention time (HPLC) and the deprotonated molecular ion (MSIMS) data used to identify each flavonoid are shown in Table 2.

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Table 2: Deprotonated molecular ions and retention times used to identify flavonoid compounds in mass spectrometry and HPLC analysis.

Flavonoid	Parent Ion M/z	Retention time (min)
Apigmin	269	11.27
Hesperetin	301	12.87
Luteolin	285	6.77
Quercetin	301	7.11

Abbreviation: m/z mass-charge-ratio.

Four peaks were produced in the MS/MS analysis of a mixture of apigenin, hesperetin, luteolin and quercetin (Fig 10). The retention times for these peaks corresponded to those found in the HPLC analysis of a mixture of the flavonoids (Fig 9).

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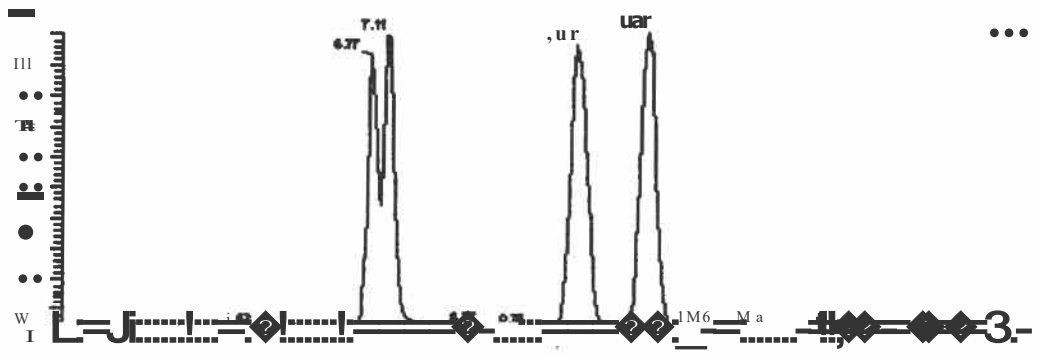
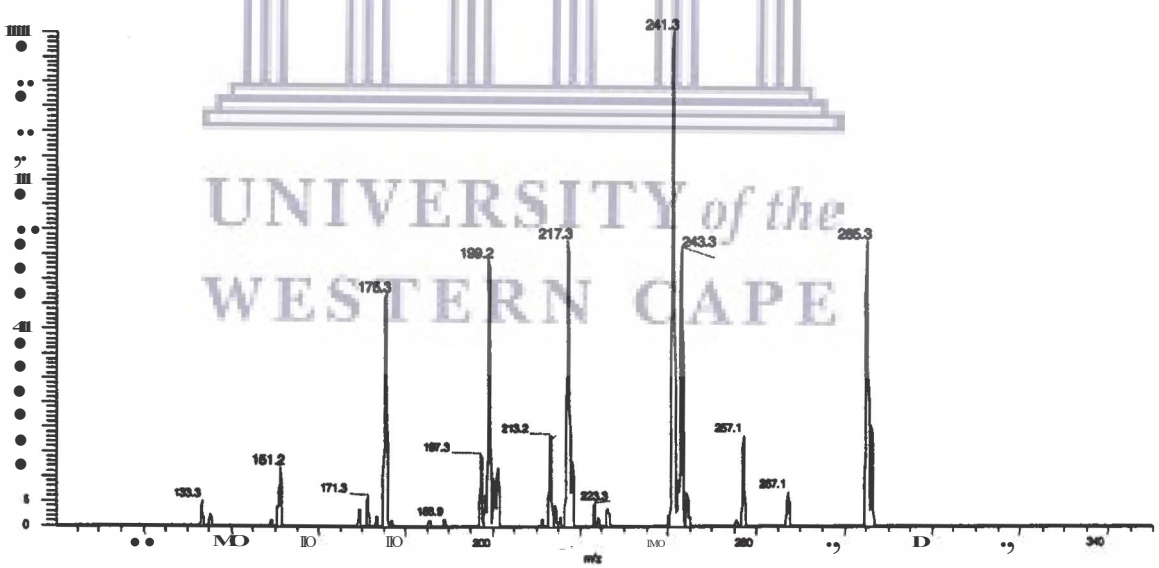


Flt 10: The typJcal Ion chromatogram of • solution of four 0.vonoida ualy7.ed uslq MS/MS. Chromato1nphlc conditions described In text (seccion 4.3.3.l).

The main ion peak for each flavonoid was monitored in combination with the fragment peaks that represented the breakdown of the compound. The typical mass spectra produced for all the four flaveneids are shown in fi 11 - 14.



Fi& 11: Product Ion mass spectrum ofluteoUn, Mlz 285 Identified by MS/ MS

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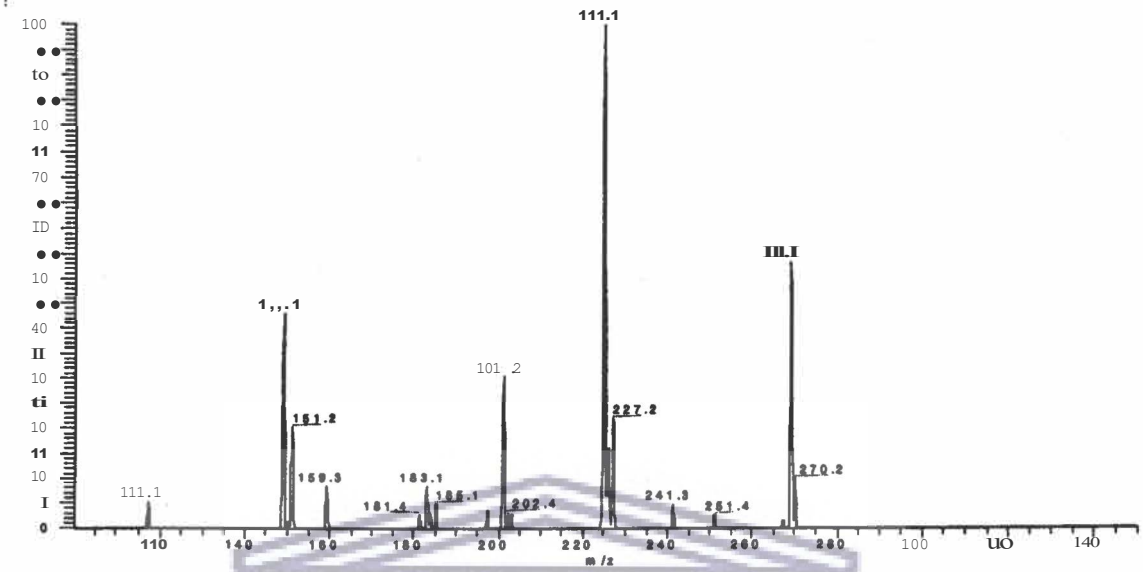

Fig 12: Product ion mass spectrum of apigenin, *m/z* 269 identified by MS/MS

The deprotonated melecular ion m/2 28S and fragment ion peaks *m/z* 243, 241, 217, 199, 175 and 151 were detected for luteolin (Fig 11), and the main ion peak *m/z* 269 and fragment ion peaks *m/z* 227, 225, 201, and 149 for apigenin (Fig 12).

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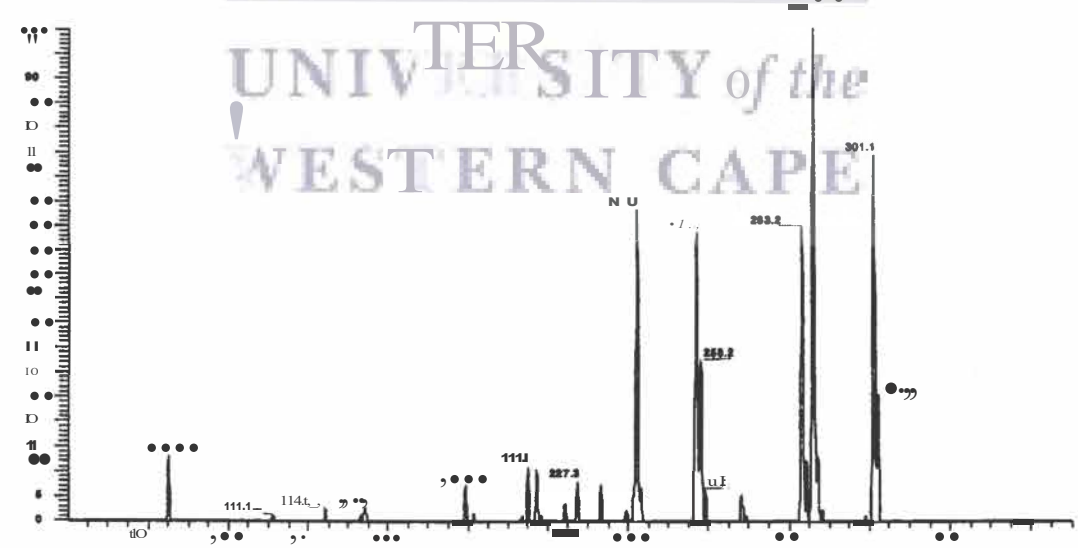

Fig 13: Product Ion mass spectrum of hesperetln, MI: 301 Idendfied by MS/ M S

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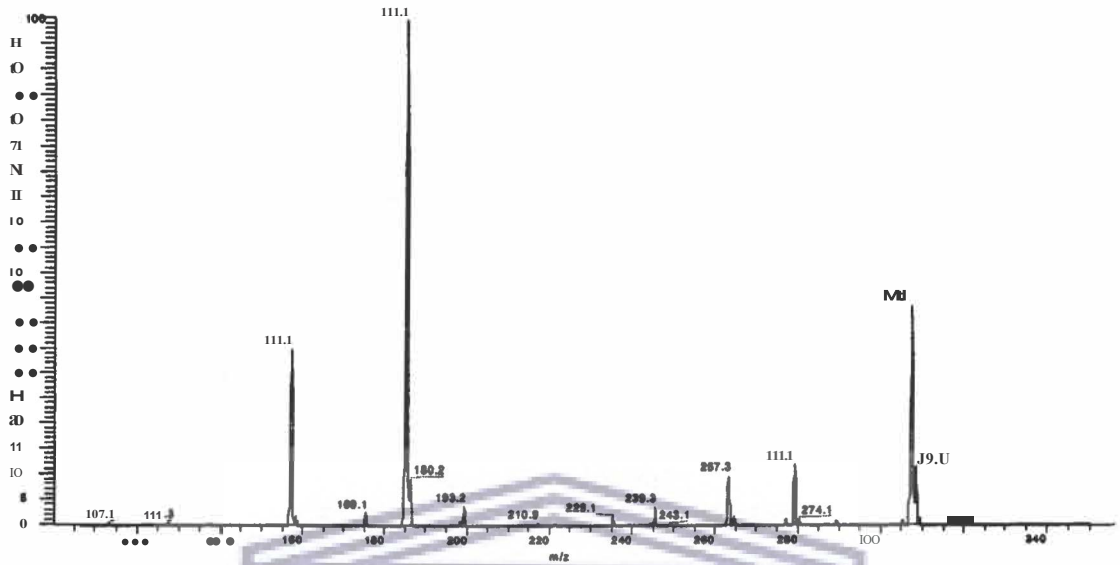

Fig 14: Product ion mass spectrum of quercetin, *m/z* 301 identified by MS/ MS

Both quercetin (Fig 13) and hesperetin (Fig 14) mass spectra had a similar main ion peak *m/z* 301. However, although the mass ion peak for both of these compounds was similar, the differences in the fragment ions peaks were evident, with hesperetin having ions *m/z* 286,257, 242 and 217 and those for quercetin *m/z* 273, 179 and 151.

The mass spectra of the four flavonoids, apigenin, hesperetin, luteolin and quercetin were similar to the mass spectra normally found in previous studies on flavonoids (Ameer, et al, 1996; Franke, et al 2002; Hirleaki et al, 1998; Schefber, et al 2000). These studies analyzed flavonoid compounds in the negative mode MS/MS, estimated similar data for the deprotonated molecular ion *m/z* (285, 269 and 301) and presented mass spectra peaks analogous to those obtained in this study.

In general from the MS/MS data obtained in the present study, it was evident that the four flavonoids could be reliably detected when compounds were assayed. This could be achieved when analyzing an aqueous solution of an individual flavonoid, or a solution of one or more flavonoids prepared as a mixture. It was thus considered feasible to use this assay to detect these flavonoids when they are present in plant samples.

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5.3.3.2 Identification of Oavonoids in a sample matrix

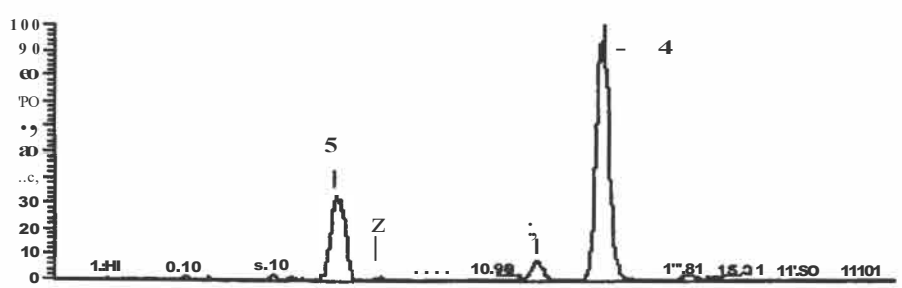
The plant products were first extracted [section 4.3] and hydrolyzed [section 4.3.2.4] in for chromatographic analysis. The products of hydrolysis were then assessed using the HPLC assay developed. The chromatograms for these samples showed multiple peaks and one or more of these peaks were assigned to the four flavonoids. The exact identities of some of these peaks were however conflicting and MS/MS was required to resolve and validate the identities of the peaks. For this pwpose the mass spectra were generated for the extracts of *Asthmitea®*, *A. afra* and *M /ongifo/ia* and the results obtained outlined in Table 3 and further discussed below.

Table 3: Confirmation of the identity of flavonoid markers in a sample matrix by MS/MS.

Plant extracts	Apigenin	Hesperetin	Luteolin	Querceda
Asthmited>	–	+	+	+
A.afra	+	–	+	+
M longifolia	+	+	–	+

Abbreviadons: - not detected in sample, + flavonoid marker identified in sample. Limit of detection 62.5ng/20mc plant extract mass.

Three of the four markers were detected in each ofilie'pl i sam les. Apigenin was not detected in *Asthmitea* ®, he eretin was not detected in *A. afra* and luteolin was not detected in *M longifolia.* Quercetin was present in all the samples. The prominence of the ion peaks generated in the total ion chromatograms (Fig 15 - 17) of the plant extracts can be taken to indicate the degree to which the flavonoid is present.



F11 15: The total Ion chromatogram of an aqueous extract of *M. longifoli.a* using MS/MS analysis showing the prevalence of apigenin (3) hesperetin (4), quercedn (2) Ions, and a luteolin-like compound (5).

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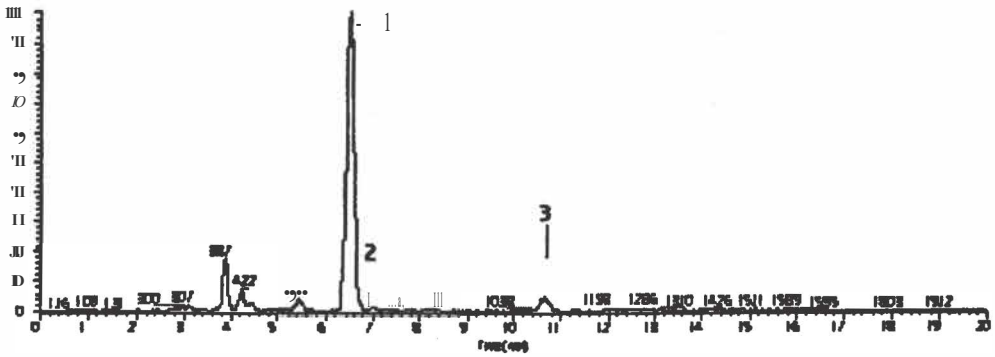

Fig 16: The total Ion chromatogram of a methanol extract of *A. afra* showing the prevalence of apigenin (3), luteolin (1) and q u e (2) ions.

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Fil 17: The total Ion chromatognm of an aqueous e:itnct of Asthmiua ® usln1 MS/MS showing the prevalence of Hesperetla (4) and luteolin (1) loaL

Generally the peaks generated for the flavonoids in the plant spectra (18 - 21) were similar to those produced for the corresponding pure flavonoid (Fig 11 - 14).

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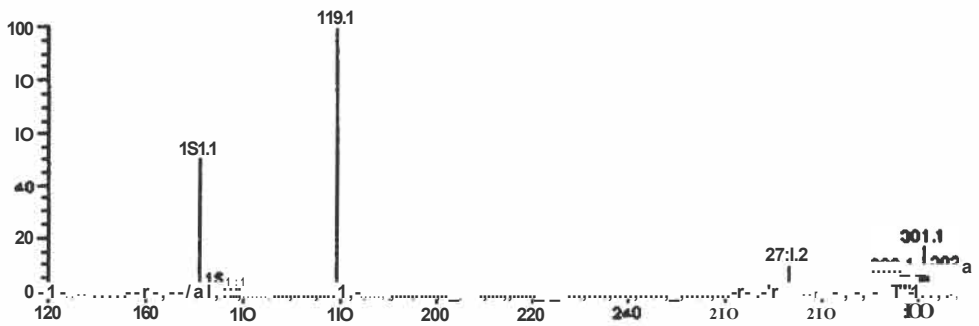

Fig 18: The D W I spectrum of quercetin u detected In an extnct *M. longifOU..*

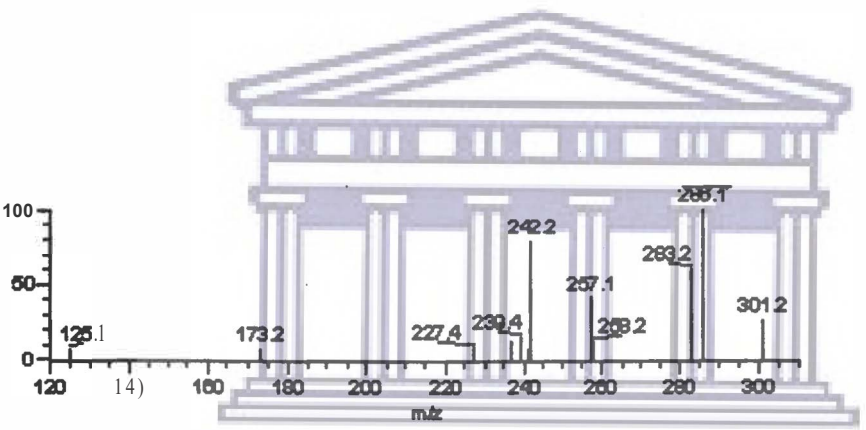

Fig 19: The mass spectrum of hes Pfretln **as detected In an ex**tnctofAsthmlta®

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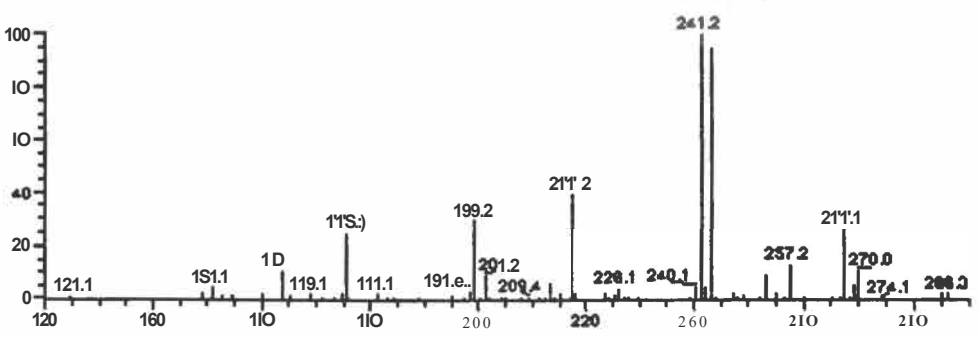

Fl1 20: The m u s spectrum of luteolln as detected In an extnctA. 11fr._

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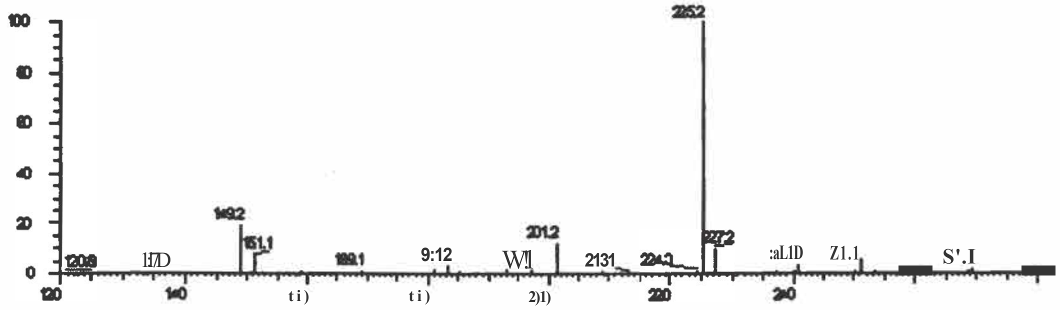

Fig 21: The mass spectrum ofaplgenln as detected In an extract.A. 11/r&

In addition to the four compounds, another compound (5) was detected in the extracts of *M. longifolia* (Fig 15). This compound could not be assigned to any one of the markers, but was imilar in structure to luteolin and also equally eluted at 6.77 min, the retention time used for the identification of luteolin.

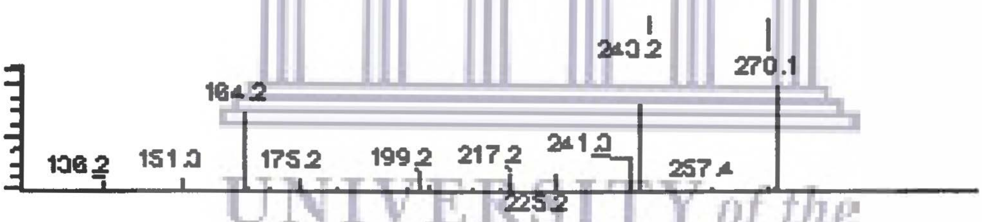

Fig 22: The mass spectrum of compound 5 as observed in the MS/MS analysis an aqueous extract of *M. longifolui.*

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The mass spectrum of compound *5* (Fig 22), had fragment peaks similar to luteolin *m/z* 151, 175, 199, 225, 257. However, on closer examination of the spectra additional peaks *m/z* 164 and 270 were noted, and an inverted ion abundance ratio of two of the peak fragments *m/z* 241 and 243 were observed. In addition, the main ion peak *mlz* 285 for authentic luteolin was noticeably absent in the mass spectra of compound 5. For these reasons, this compound could not be assigned to any of the markers and as a result is reported as a luteolin-like compound

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The results of the MS validation experiments showed that all four flavonoids could be identified in a plant-sample matrix prior to and after hydrolysis with no significant change in retention time. In addition the mass spectra obtained in this study were comparable to spectral determinations done on flavonol, flavone and flavanone compounds by other researchers (Ameer et al, 1996; Avallone et al, 2000; Hideaki et al, 1998).

The presence of the glycosides of apigenin, hesperetin, and luteolin has been shown in the extracts of *A. afra* and *M longifolia* (Hutchings et al, 1996; Sharaf et al, 1999; Van Wyk et al, 2000). However, in contrast to tliese r rts our findings indicate the absence of luteolin in all the extracts examined and the surprising possibility of a luteolin-like compound and quercetin in *M. /ongifolia,* flavonoids previously undetected in the extracts this plant.

Overall, the MS/MS analyses facilitated the appropriate identification of the peaks assigned to apigenin, hesperetin, luteolin and quercetin in plant sampies that correlated with prior HPLC analysis, and provided further proof that the assay was dependable and could be used to successfully identify flavonoid compounds in a sample matrix.

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Finally the markers were all shown to be present in the plant samples, and to different extents. Therefore, if the levels of apigenin, hesperetin, luteolin and quercetin could be ascertained in various extracts and preparations of *A. afra* and *M. longifolia,* it could be possible to use these compounds as suitable markers and the assay for the assessment of flavonoid-containing plant preparations.

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5.4 Assessment of flavonoid markers in the plant samples

5.4.1 Results for A. afra

In this section the abbreviations, Klb, Lb, Mh, Ray, Wnjy, and Hnk represent the aqueous or methanol extracts that students at the discipline of Pharmacology (UWC) had prepared, as part of their individual projects. The flavonoid content detected in each *A. afra* extract was expressed in ng/20 mg of crude or hydrolyzed mass of various extracts and given in table 4.

Table 4: Concentradon of marker (ng.120mg plaat material mean :!:SD) found in A. afra plant extracts.

	Aqueous extracts		Methanol extracts	
Marken	Crude	Hydrolyzed	Crude	Hydrolyud
Crude A. afra powder				
Apigenin	--			
Hespcretin		--		
Luteolin		139.3±0.024		
Quc:rcetin	--			
Klbextract			Wnjy extract	
Apigenin	69.27±0.066	74.12±1.577	1077±0.0240	117.9±0.016
Hespc:retin	--	--	--	--
Lutcolin	121()f.002	317.9±0.053	650.6±0.045	1113::1::0.002
Quc:rcetin	--	290.7±0.0240	--	1\44::t:0.055
Lb extract				
Apigenin	--	85.78:i:0.071		
Hespcretin	--	--		
Luteolin	109±5.6	317.9±0.053		
Quc:rcetin	--	115:0.03		
Mb extract				
Apigenin	24.77:0.099	52.93::1::0.035		
Hespcretin	--	--		
Lutcolin	127.0±0.007	272.4::1::0.85		
Quc:rcetin	--	105.5:0.01		
Ray extract				
Apigenin	--	27.51:i::0.063		
Hespcretin	--	--		
Lutcolin	78.8:3.9	230.9±0.35		
Quercetin	--	213.6::1::0.565		

Abbreviadons: - not detected in sample, C - un-hydrolyzed samples, **H - hydrolyzed samples.**

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Three of the four markers, apigenin, luteolin and quercetin, were identified in *A. afra.* None of the preparations contained the fourth marker hesperetin, while only one flavonoid namely, luteolin was detected in the crude powdered form of the plant material. Luteolin was the only flavonoid that was detected frequently in the aqueous extracts analyzed, unlike apigenin or quercetin that were occasionally absent in some of the samples.

The concentrations of the three flavonoids detected in *A. afra* preparations significantly increased after hydrolysis of the samples, compared to the levels found in crude extracts prior to this procedure. This was erobablf. due to the possible break down of glycoside compounds, with the subsequent release of the free flavonoid (aglycone). In the Ray and Wnjy extracts, luteolin increased by at least 90% after hydrolysis, suggesting that the unhydrolyzed samP-le extract JX!Ssibly contained substantial amounts of luteolin glycosides.

Whether this was a single glycoside or several different glycosides of luteolin was difficult to say from the present data, but the dramatic increase in luteolin suggested more than one glycoside. Furthermore, no scientific information on the identification of luteolin or its glycosides in *A. afra* were available.

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Quercetin was not aetected in any of the unhydrolyzed preparations. According to Harbome (2000), quercetin mainly occurs as the glycoside in nature. The result obtained for *A. afra* seemed to concur with this i.e. quercetin was only present as glycosides in *A. afra* and was only released after hydrolysis.

Generally the preliminary acid-hydrolysis of samples is the traditional approach to the structural elucidation and characterization of glycosides. The objective being to cleave any sugar/s bound to a flavonoid (Bloor, 2001; Hasler et al, 1990; Keinanen et al, 1998; Tura et al, 2002). Overall this concept was thus certainly well demonstrated by the results obtained for apigenin, luteolin and quercetin in *A. afra.*

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There is very little scientific information available on the occurrence of flavonoids in *A. afra.* However Tang (2000) isolated 6 flavones in *Artemisia species,* two of which were revealed to have a tetra-hydroxyl structure closely related to apigenin and luteolin.

In the present study two additional flavones, apigenin and luteolin were identified in the extracts of *A. afra,* in addition to the flavonol compound, quercetin. According to our knowledge these three flavonoids have not previously been reported in *A. afra,* and are now reported for the first time.

In summary, at *least* two of the four markers under investigation were identified for each of the *A. afra* extracts. Luteolin was the most consistent marker identified, although the three compounds namely, apigenin, luteolin and quercetin might also be acceptable markers. This superiority of luteolin as a marker was reinforced by the fact that it was consistently detected with ease in both crude and hydrolyzed plant samples, and at similar levels to that of apigenin and quercetin. For these reasons, luteolin is proposed as the most suitable marker for the extracts and preparations of *A. afra.*

Quercetin on the other hand might be a useful marker to indicate hydrolysis of glycosides contained in *A. afra.* If it is detected in crude unhydrolyzed *A. afra* material, then that material may have been exposed to conditions where hydrolysis could have taken place.

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S.4.2 Results for *M. longifolia*

The results indicating the presence or absence of flavonoids in *M longifolia* are shown in table 5. The flavonoid content of the samples analyzed was expressed in ng/20 mg of crude or hydrolyzed mass of the various extracts.

Three of the four markers namely, apigenin, hesperetin and quercetin were identified in *M longifolia.* Apigenin and quercetin were found in all the extracts, hesperetin in three of the extracts, while luteolin was not detected in any of the samples.

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Table 5: Quantitative Identification of the flavonold marken (n&f20 mg extract ma•) In *M. longifolill* plant extracts.

	Aqueou1 extractl		Methanol extracts	
Markers	Crude	Hydrolyzed	Crude	Hydro)yml
Crude *M. longifolill* powder				
Apigcnin	165.4±0.091			
Hesperetin	–			
Lutcolin	–			
Quercctin	120.6±0.01			
Klb extract			**Wnjy extract**	
Apigcnin	365.1±0.129	168.6±0.041	761±0.178	280.4±0.156
Hesperetin	–	–	144.1±0.047	279±0.0069
Lutcolin	–	–	–	–
Quercctin	294±0.038	936±0.113	1040±0.0029	1156.15::t:2.4
Ray extract			**Hnk extract**	
Apigcnin	906.2±0.012	502.3±0.041	–	1153:0.014
Hesperetin	222.1±0.001	222.2±0.028	64.08±0.047	279.8±0.006
Lutcolin	–	–	–	–
Quercetin	42.557±0.01	42.557±0.01	–	4795:0.038

Abbreviations: – not detected in sample, C- un-hydrolyzed samples, H- hydrolyzed samples.

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Apigenin and quercetin appeared- to be superior markers for M. *longifolia.* They were found in crude powdered and extracted form o f the plant, unhydrolyzed or hydrolyzed while hesperetin was present at much lower levels than the two aforementioned.

For *M longifolia,* quercetin was present in unhydrolyzed material indicating that unlike suggested in *A. afra* it was present in the aglycone form in this plant. There was however much difference in the levels o f this marker found in the different extracts, for example some extracts (Hnk, Wnjy) contained extremely high quantities o f the marker i.e. 240 µgig and 57 µgig for Hnk and Wnjy respectively, while others (Ray, crude powder) contained less than 5µg-quercetin/g o f plant extract.

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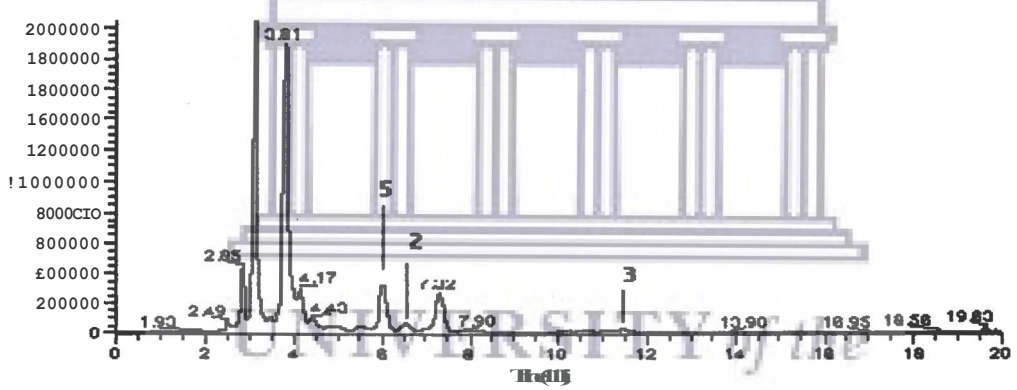
Both quercetin and hesperetin could be effective markers for M. */ongifolia* preparations because they were detected in M. *longifolia* extracts. However apigenin was probably a superior marker for *M. longifolia* because the apigenin content of most samples analyzed was similar and any differences between extracts were considered reasonable and logical compared to that found with hesperetin or quercetin.

The most interesting discovery from this data was however the identification of a luteolin-like compound in the extracts of M. */ongifolia.* This compound (peak 5), and the three markers, apigenin (peak 3), luteolin (peak 1), and quercetin (peak 2) are shown in the typical chromatogram of a *M. longifolia* extract (Fig 23).



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Fie 23: The UV chromatogram of a methanol extract of M. longifolia using HPLC. Apigenin (3) and Quercetin (2) peaks are Identified in addition to a luteolln-llke compound (5).

A significant number of luteolin glycosides have been reported in studies conducted on the *Mentha species* (Hutchings et al, 1996; Van Wyk et al, 2000). Sharaf (1999) in the investigation of the flavone glycosylation isolated luteolin 7-O-glucoside in the aerial parts of M. */ongifolia* and Ghoulami (2001) verified this finding by isolating the 7-O-glucoside and luteolin in one extract.

None of the extracts investigated in the present study however contained authentic luteolin; instead most of the extracts appeared to contain a luteolin-like compound as one of the major components. It was unclear whether this new compound was an

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actual compound present in the M. *longifolia* plant material. The latter scenario however seems unlikely particularly considering all the HPLC and MS/MS conditions remained constant, the compound is possibly some unknown isomeric structure to luteolin.

There are several studies, which report on the prevalence of the glycosides of apigenin, hesperetin and luteolin in the extracts of M. *longifolia* (Ali et al, 2002; Hutchings et al, 1996; Ghoulami et al, 2001; Sharaf et al, 1999; Van Wyk et al, 2000). The findings in the present study that identified apigenin and hesperetin in *M. longifolia* thus compares favorably with existing knowledge. The present study however also indicates the presence of two new compounds, quercetiii: and a luteolin-like compound, which are now reported for the first time.

In a majority of the *M. longif!)lia* extracts inv.estigated in this study, three of the markers namely, apigenin, hesperetin and quercetin were identified regularly; they were thus likely to be good markers for this plant. It should therefore lie reasonably simple to quantify either of these markers, and use them to assess the extracts and preparations of *M. longifolia.*

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5.4.3 Pharmaceutical control analysis of plant prepar.ations

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In this section the results of the pharmaceutical analysis of *A. afra* and *M. longifolia* preparations are reported. This analysis involved the assessment of the levels of markers (1), in plant material obtained after extraction (2), in commercial preparations of the two plants and (3), the assessment of the use of the markers to indicate differences in batches of plant preparations.

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5.4.3.1 Levels of markers in plant extracts

The levels of the markers in the extracts of ***A. afra*** and ***M /ongifolia*** were compared in this section. The plant extracts were obtained from students in the discipline of Pharmacology UWC as part of their individual research projects. Generally, ***A. afra*** and ***M /ongifolia*** plant material was collected at Montague Museum. However the collection period differed between some students. The plant material used for Hnk, Klb and Wnjy samples was collected in the summer of 2002, while Mh and Ray plant material was collected in the summer of 2003. The aqueous extracts were obtained by extracting the plant material with distilled water and the methanol extracts were obtained by extracting 30 g of finely ground powdered plant material in a soxhlet apparatus.

Four extracts each of *A. afra* and *M. longifolia* were analyzed. For assay purposes 20 mg of each extract was hydrolyzed and the products of hydrolysis were then analyzed using the HPLC assay developed. The results indicating the level of the markers after aqueous and methanol extraction of *A. afra* and *M. longifolia* are shown in Fig 24, 26 and Fig 25, 27 respectively.

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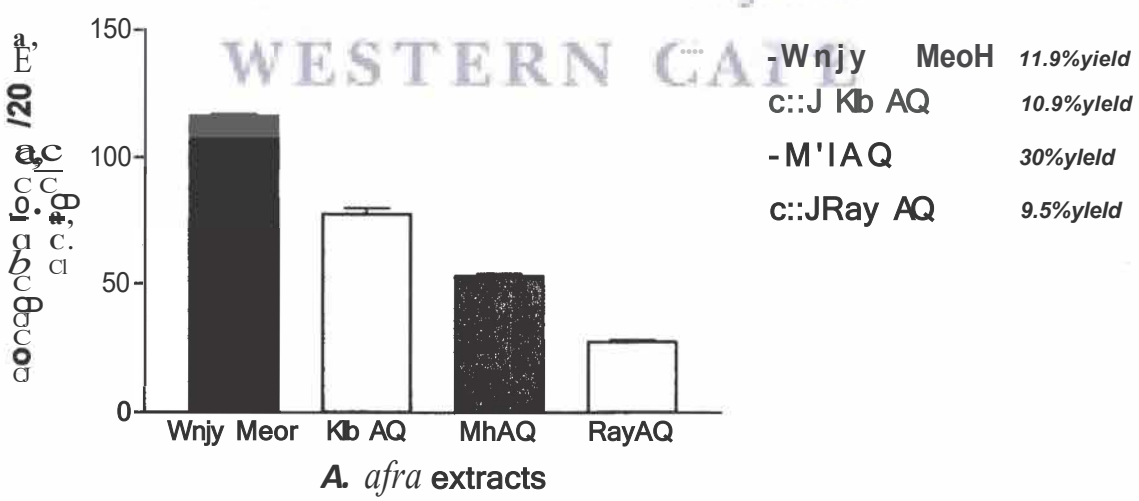

Fig 24: Apigenin content in A. *afra* extracts (n=3). Graph depicts apigenin levels obtained after aqueous and methanol extraction procedures, the extraction yield from plant extract is specified.

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The apigenin levels (Fig 24) detected in *A. afra* extracts ranged from 30-to 120-ng/20 mg (1.5 to 6 µgig). The single methanol extract (Wnjy 11.9% yield) contained 120 ng/20mg (> 6 µgig) of apigenin. The apigenin in the aqueous extracts ranged from approximately 30 to 70 ng-apigenin/20 mg (1.5 µgig to 3.5 µgig). The extraction yields of the *A. afra* extracts ranged from 9.5 to 30% and accordingly the levels of apigenin detected in these extracts were different.

However it was expected that extracts with a similar extraction yield could possibly contain apigenin in the same range. Two aqueous extracts Klb (10.9% yield) and Ray (9.5% yield) with extraction yields within the same range were compared. These aqueous extracts were both prepared using 150 g quantities of dried leaves. However the apigenin detected in the extracts was different with Klb containing 3.5 µgig and Ray aqueous extracts contaioing 1.5 µg/g of apigenin. These quantities were different by approximately 40%. This suggested that ilie quantity of apigenin contained in the extracts could vary even when the mode of extraction and the yield were comparable, and the differences observed were possibly related to the levels found in the original plant material.

Extracts with higher extraction yields were anticipated.ho ICOntain higher levels of apigenin. However this was not the case. The Mh aqueous extract that had an exceptionally high extraction yield of 30% contained 2.5 µg-apigenin/g. This quantity of apigenin was approximately in the same range as that found in the Klb and Ray aqueous extracts (1.5 µgig to 3.5 µgig) although the extraction yields of these extracts were smaller (at approximately 10%) compared to that of Mh extract. This suggested that an improved extraction yield was not a guarantee of detecting a high quantity of apigenin.

The use of powdered material enhanced the extraction yield ofWnjy (11.9% yield) and Mh (30% yield) extracts compared to Klb (10.9% yield) and Ray extracts (7.3% yield). However, although it was evident that the use of the powdered material for extraction improved the extraction yield, the apigenin content detected was not elevated to a large

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extent for the former extracts. This suggested that the original plant material, whether in powder or in the form of leaves, contained different levels of apigenin. This further reinforced the suggestion that an improved extraction yield was not a guarantee of a high quantity of apigenin.

The level of apigenin in the methanol extracts (6 µgig) was approximately 50 % higher compared to that found in the aqueous extracts (3 µgig). Although apigenin was higher in the Wnjy extract it was unlikely that the use of methanol as a solvent was the sole factor responsible for the higher apigenin level contained in the methanol extract Since the Wnjy methanol extract was prep_ared :using a soxhlet apparatus it was possible that this extraction methoo in- combination with methaiiol was more efficient in the extraction of flavonoids than immersing plant material in boiled distilled water. This possibly resulted in tile-difference in the levels of one oi more of the markers.

The levels of apigenim were ilifferent in the case of extracts prepared from plant material collected during different seasons. The Wnjy (11.9% yield) and Klb (10.9% yield) extracts whose plant material was obtained in the early summer of 2002 contained 6 µgig and 3 µ g of apigenin respective!}', and were the two highest levels of apigenin obtained. The plant material for Mh (30- yie d .and Ra)! (7.3% yield) extracts was collected in 2QQ3 and these two extracts con ed 2.5 and 1.5 µg-apigenin/g respectively, whicli was less than that found in Wnjy and Klb extracts. It was thus possible that the original plant material contained different levels of apigenin and could have resulted in the differences observed in the apigenin levels.

Scientific information on *A. afra* is limited and no reports on the identification of apigenin in the South African *species* are available. From the results found for the levels of apigenin in the *A. afra* extracts and the extraction yields in this study it would appear that the original plants that were extracted contained different levels of apigenin and an efficient mode of extraction and/or an organic solvent was likely to improve the quantity of apigenin detected in the extracts obtained thereof.

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The quantities of apigenin found in the ***M longifolia*** extracts are shown in figure 25. The apigenin concentration ranged from 170 to 1150 ngl20 mg (8.5 to 57.5 µgig), with the Hnk methanol extract (6.3% yield) containing the highest concentrations of apigenin 1150 ngl20 mg (50 µgig) and Klb aqueous extract (8.2% yield) the lowest quantity of approximately 170 ngl20 mg (8.5 µgig).

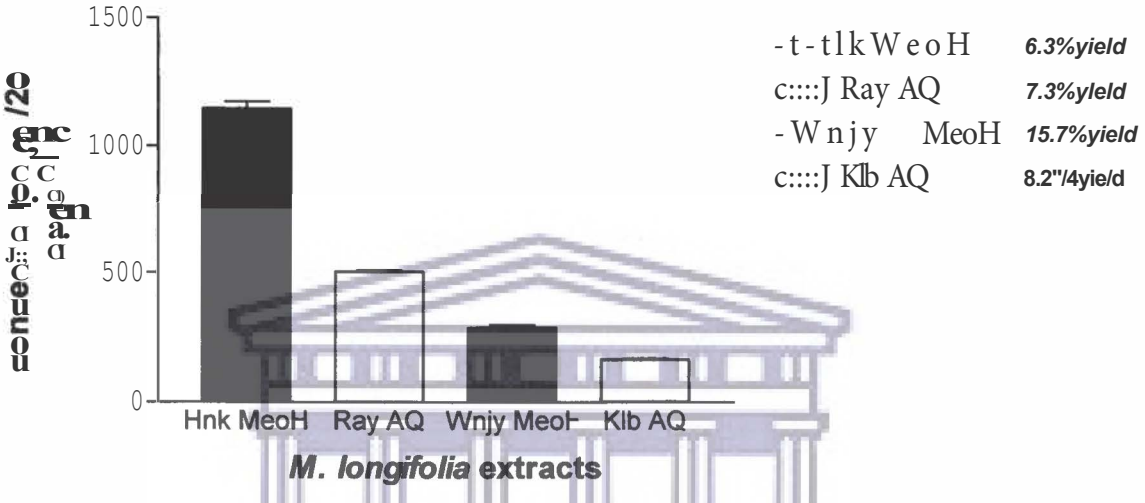

Fig 25: Apigenin content in *M. longifolia* extracts (n=3). Graph depicts apigenin levels obtained after aqueous and methanol extraction procedures, the extraction yield from plant extract is specified.

The extraction yields for the *M. longifolia* extracts ranged between 6 to15% and were quite similar. The revels ofraP,ifenin were however fairly different. Similarly although plant material for Hnk (6.3% yleld), Klb (8.2%) and -Wnjy (15.7%) extracts was collected at the same tiwe, 'te pigenin; levels of all iliree aorresponding extracts were different with levels of apigenin being 50 µgig, 8.5 µgig and 14 µgig for Hnk, Klb and Wnjy respectively. Further, Wnjy and Hnk methanol samples were collected at the same time and extracted with the same method of extraction using methanol. However when compared an inverse relationship in the quantity of apigenin was observed between these two samples of 12.5µglg vs. 50 µgig respectively. This was a clear indication that the plant material originally collected contained different quantities of apigenin.

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Although Hutchings (1996) and Van Wyk (2000) reported on the identification of apigenin and apigenin glycosides in ***M /ongifolia,*** data provided in these reviews was not quantitative. Generally from the findings in this section, it seems likely that most of the original plant material contained different amounts of apigenin, and subsequently the quantity of this marker in the extracts was varied.

The concentrations of the second marker namely, quercetin in the aqueous and methanol extracts of ***A. afra*** were found as being approximately 100 to 1450 ng /20 mg (5 to 72.5 µgig) and as shown in fi$_{gur}$e 26.

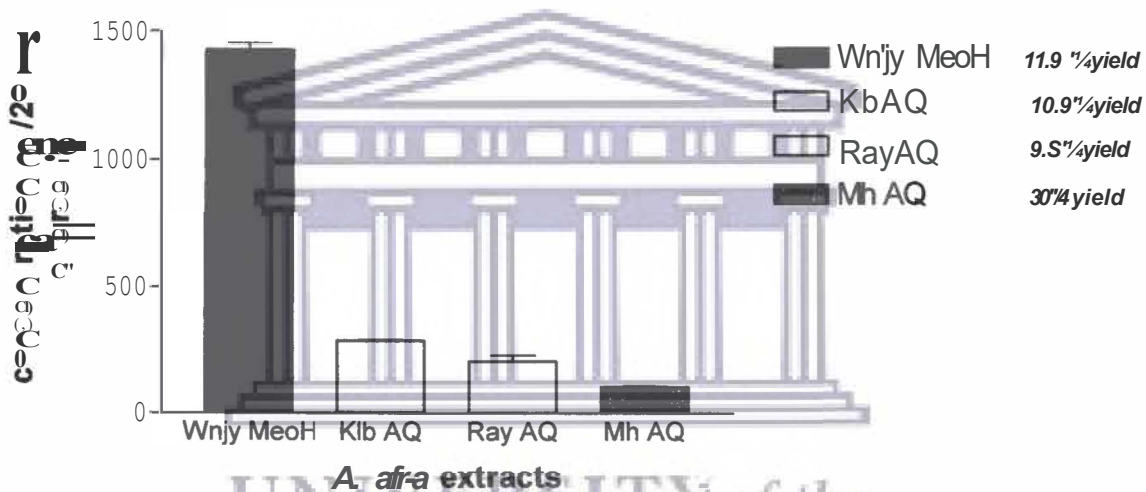

Fig 26: Quercetin content in *A. afra* extracts (n=3). Graph depicts quercetin levels obtained after aqueous and methanol extraction procedures, the extraction yield from plant extract is specified.

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The extraction yields of the ***A. afra*** extracts ranged from 9.5 to 30%. The Wnjy methanol extract (11.9% yield) contained 72 µg-quercetin/g that was the highest quantity of quercetin obtained in any of the extracts. The aqueous extracts contained 2.5 µgig to 14.5 µgig of quercetin. The yields of two of the aqueous extracts Klb (10.9%) and Ray (9.5%) were fairly similar and, equally the levels of apigenin found in these extracts were in the same range at 14.5 µgig and 10.6 µgig respectively. These two extracts were prepared in a similar manner using 150 g quantity of dried leaves. The similarity in the mode of extraction was likely to be responsible for the analogous range of quercetin detected.

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Accordingly, an increase in extraction yield was expected to improve the quantity of quercetin in the plant extract. However, although the Mh aqueous extract (30% yield) extract had the highest extraction yield only 5 µg-quercetin/g was detected in this extract and was the lowest quantity of quercetin detected in any of the extracts. This suggests that an improved extraction yield was not directly related to the levels of quercetin detected in the plant extract.

However, the high levels of quercetin detected in the Wnjy methanol extract (11.9% yield) could be attributed to the use of a more efficient solvent and method of extraction. The Wnjy methanol extract was prepared using methanol and a soxhlet apparatus, which were likely to be more efficient for extracting flavonoid compounds from plant material, compared to the infusion of plant material with polar solvent. Valant-Vetschera (2002) investigated nine *Artemisia species* for their flavonoid composition and found that the flavonoid content of these *species* included quercetin and quercetin glycosides as some of their components. However no quantitative data or information on the flavonoid content of the *afra species* was provided. It was therefore not possible to make any comparisons between his findings for quercetin to those found in the present study.

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The findings for quercetin in A. *afra* extracts would suggest that quercetin levels could be detected in the same range when similar extraction methods were employed. In addition, the quercetin in *A. afra* extracts was possibly better extracted using methanol as an extraction solvent In addition, improving the extraction yield was unlikely to improve the levels of quercetin detected. However in general from the variations found in the levels of this marker it was possible that the *A. afra* plant material originally contained different levels of quercetin.

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In the *M longifolia* extracts, the quercetin levels ranged from 40-to 4800-ngl20 mg (2 to240 µgig). The quercetin detected in the methanol extracts (Hnk, Wnjy) was above 50 µgig. However the aqueous extracts (Klb, Ray) contained less than 50 µgig of quercetin. These results are shown in figure 27.

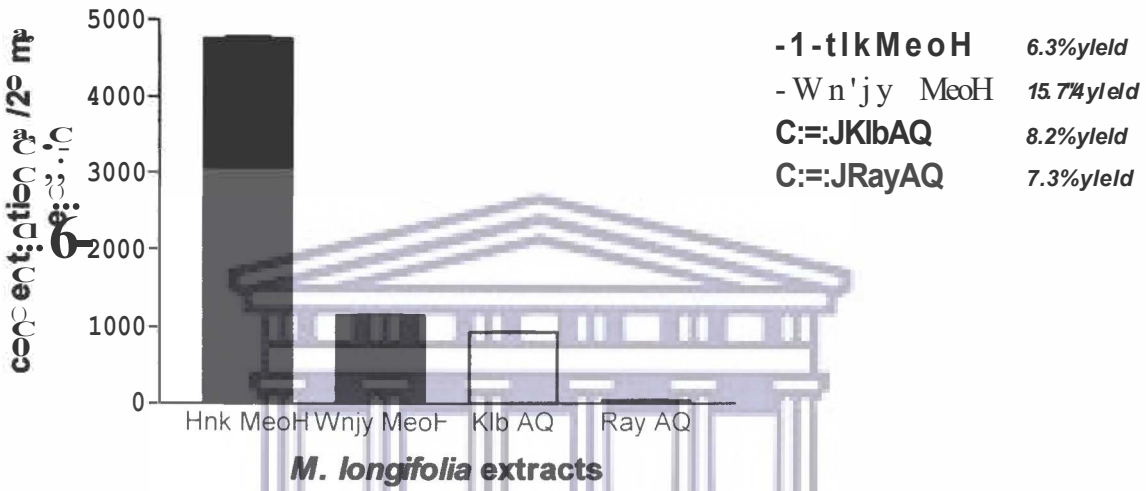

Fig 27: Quercetin content in M. longifolia extracts (n=3). Graph depicts quercetin levels obtained after aqueous and methanol extraction procedures, the extraction yield from plant extract is specified.

The *M longifolia* exA-acts had slmilar extraction yield af appmxirnately 10% however; the quantities of quercetil) d t ct d were different. The Hnk methanol extract with an extraction yield of 6.3% contained the most quercetin (240 µgig), which was greater by at least 50% compared to, that found in the three other extracts (Wnjy, Klb, Ray).

In general the methanol extracts Hnk (6.3% yield), Wnjy (15.7% yield) contained greater than 50 µg-quercetin/g compared to Ray (7.3% yield) and Klb (8.2% yield) aqueous extracts that contained less quercetin at approximately 2µglg and 57.5 µgig for Ray and Klb respectively. The two methanol extracts (Hnk, Wnjy) were prepared using 30 g of finely powdered plant with a soxhlet apparatus. In contrast for the Klb and Ray extracts the leaves of *M longifolia* were used.

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The variations in the levels of quercetin detected were possibly related to the use of different extraction methods. Unfortunately quercetin is not among the flavonoids that have been reported in *M longi.folia* therefore it was difficult to corroborate or compare these findings with any previously reported data.

All the *M longi.folia* plant material was collected in the summer of 2002 with the exception of Ray plant material that was collected in the summer of 2003. However, the levels of the Hnk, Klb and Wnjy extracts were different. A geographical or climatic reason could not therefore be invoked as the main factor behind the variations in the level of quercetin found in the different extracts because the plant material collected at similar climatic conditions contained different levels of quercetin. This rather suggested the possibility that the original plant material contained different levels of markers and the reasons for these variations were unclear.

From these findings for the quercetin levels in *M. longifolia* it was reasonable to suggest that the original plant material probably contained different levels of the marker. However more [illegible] *gifolia* methanol extracts compared to that extracted into the aqueous extracts of this plant. Furthermore, the use of an efficient extraction method for flavonoids in plant [illegible] possibly results in the detection of improved levels of quercetin in the subsequent plant extract Generally this suggests that the levels of quercetin were subject to exiraction methods and/or solvent used in this process.

The results obtained for the analy s is of hesperetin and luteolin samples in the extracts of *A. afra* and *M longifolia* are summarized in table 4 and *5* in section 5.41, 5.42.

Luteolin was not present in *M. longi.fo/ia,* while hesperetin was absent in *A. afra,* which made the samples that contained these two markers few in number. The luteolin content of the aqueous extracts of *A. afra* namely, Mb (30% yield), Klb (11.9 yield), Ray (9.5% yield) ranged between 10 to 15 µgig, and were lower compared to that of 50 µg-luteolin/g found in the single methanol extract, Wnjy (10.9% yield) investigated. This

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quantity of luteolin was exceptionally high compared that that found in the aqueous extracts. However, the Wnjy extract of *A. afra* was the only methanol extract analyzed and it was thus difficult to determine if this value was standard for methanol extracts of this plant

Tang (2000) in an investigation of *Artemisia species* isolated 2.7 mg/g of a tetra-hydroxyl structured fl.avone compound using petroleum ether. The compound isolated was structurally similar to luteolin, and the quantity of this tetra-hydroxyl compound found is equally high compared to that of luteolin found in the methanol extract of A. *afra* 50 µgig (0.05 mg/g). This sugges_ts that the luteolin of *A. afra* is better extracted in the methanol/organic solvents compared to extraction into an aqueous solvent and hence the level of this marker may be altered by extraction and the use of different solvents.

In the case of hesperetin in *M. longifolia,* 11 µg/g of hesperetin found in one aqueous Ray extract (7.3% yield) was similar to quantities of hesperetin (14 µgig) detected in the Wnjy (15.7% yield) and Hnk (6.3% yield) methanol extracts. This suggested that the levels of hesperetin in different extracts were similar and that the hesperetin levels were unaffected by extraction or solvent use, and the original plants probably contained similar amounts of this marker.

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Generally, from the above-mentioned results it was concluded that firstly, the levels of three of the four markers namely, apigenin, luteolin and quercetin were different in the extracts of *A. afra* and *M. longifolia* investigated. This suggested that the original plant material possibly contained different levels of these markers. However, although the original plant material contained different amounts of these markers the levels of apigenin, luteolin and quercetin in the plant extracts were affected by extraction or solvent use.

Secondly the levels of hesperetin were found to be unaffected by extraction and/or solvent use in the extraction process. An additional significant finding was the fairly high levels of some of the markers, in the methanol samples of *A. afra* and *M.*

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longifolia, compared to the levels found in the corresponding aqueous extracts and, the improved flavonoid content of plant extracts that were subjected to an efficient organic extraction. According to Tura (2002) who reviewed the extraction of flavonoids, ideally any flavonoid glycoside present in the plant material should freely dissolve into the water or methanol solvent when subjected to a water or methanol extraction. However, although the glycoside may be soluble in both water and methanol the use of these two different solvents to prepare extracts showed differential effects on the level of flavonoid extracted. This was possibly because methanol is a less discriminatory solvent compared to water and therefore retained a greater quantity of glycoside compounds compared to the quantity of these compounds extracted into water.

From the latter findings it was tempting to suggest that alcohol may be the solvent of choice to provide a maximal extraction of flavonoids, for the plant material used in this study. Considerations should however be given to the diversity of plant material tested in the present study, with particular emphasis on the varied origin of plant material, different methods of extraction and the multiple number of researchers involved. All of these factors could result in the subsequent losses of various extraction products/markers.

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In summary, one of the four markers namely; hesperetin was unlikely to be affected by different extraction processes in *A. afra* and *M. longifolia* plant material. The levels of apigenin, luteolin and quercetin were however varied in the different extracts, signifying that extraction and/or solvent use in the extraction process affected the levels of these markers. The levels of apigenin, luteolin and quercetin could however prove immensely useful in assessing the influence of extraction processes on medicinal preparations. The elevated quantity of one or more of these markers could indicate that an organic solvent was the extraction solvent. Similarly extraction with a polar solvent could be suggested by the detection of low quantities of a marker in the plant preparation.

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5.4.3.2 Levels of marker compounds in commercial preparations

In this section several commercial preparations of *A. afra* and *M longifolia* were analyzed for the presence of markers with the objective to assess if any of the markers might be suitable to use in the evaluation of the quality of such preparations. From the findings in section 5.4.1 and 5.4.2, it was concluded that luteolin could be a suitable marker for preparations containing *Artemisia,* while apigenin, hesperetin and quercetin could be appropriate markers for *Mentha-containing* preparations.

Four commercial preparations were analyzed. Two of the four preparations namely, *Floo-tea* ® and *A. afra* tablets were specified to contain *Artemisia.* The *A. afra* tablets were prepared from an aqueous extract of the plant (Mh, 30% yield). The third preparation investigated namelJ *Athl"itea* ® :was similar to tlie *Artemisia-containing* commercial preparation *Floo-tea* ®. Four of the six plant *species (Betulina Barosoma, Ruta graveolus, Symphytum officianalis, Urtica urens)* contained in *Floo-tea* ® were present in *Arthritea* ®. The faurth preparation investigated was *Asthmitea* ®, which contained the *Mantha species.* Th·s commercial Irenaration consisted of a mixture of stems and leaves of seven plant *species.* Two preparations of *Asthmitea* ® were analyzed, the first was an aqueous extract prepared from -50· g of the *Asthmitea* ® commercial preparation while the second sample consist of 20 mg of the mixture of the stems and leaves of *Astlimitea* ®.

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In summary the aim of the analysis of the four preparations was to establish the presence of (1), luteolin in the *Artemisia species* preparations and (2), apigenin, hesperetin and quercetin in the *Mentha species* preparations. Twenty-milligram samples of the preparations were analyzed and the results are presented in table 6.

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Table 6: Quantitative Identification of the flavonoid marken In the commercial preparatlom of A. ajr„ and M. */,ongifolill* [n=3].

Marken	Artemis/a species prep Hydrolyzed (ng/lO mg)	Mendul species prep Hydrolyzed (ng/20 mg)
A afra tablets		Asthmlua ® extract
Apigenin	129.6:0.01	–
He„perdin	--	76.2±0.0009
Luteolin	208.6:i:0.007	59.8:i:0.138
Qucrcctin	397:i:0.008	–
Arthrlua ® preparation		Asthmlua ® preparatioa
Apigenin	–	--
Hespa-etin	–	87.65±0.255
Luteolin	21 0±0.001	38.43±0.08
Qucrcctin	162±0.03	187±0.12
Floo-tea **® preparation**		
Apigenin	--	
He„perdin	--	
Luteolin	37.45±0.164	
Quc:rcetin	109±0.025	

Abbreviations: - not detected in sample.

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First, if we consider the level of luteolin in the *A. afra* preparations we observed that the *A. afra* tablets and *'Atthritea* ® both contained approximately 200 ng-luteolin/20 mg (10 µgig). This quantity of luteolin was comparable to that found in the extracts of *A. afra* (15 µgig) described in section 5.4.1. However in *Floo-tea* ®, the other *Artemisia*-containing preparation, the level of luteolin detected was significantly lower, at 40 ngl20 mg (2 µgig). This was lower by approximately 13% compared to quantities found in the aqueous *A. afra* extracts. The *F/oo-tee* ® preparation however contained seven plant *species* compared to *Arthritea* ® that had fewer (5) plant *species.*

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It was therefore possible that the incorporation of several species into this preparation could have diluted the *A. afra* presence and hence the levels of luteolin detected in the preparation. Nevertheless, the presence of luteolin in the preparations at least substantiates the inclusion of *A. afra* in *Arthritea* ® and *Floo-tea* ®.

In general the levels of luteolin detected in all the *Arthritea* ® and *Floo-tea* ® preparations were low as was expected since the *Artemisia* included in these commercial preparations consisted of un-extracted and unprocessed raw plant material and not extracts in which the concentration of luteolin might have been concentrated.

In the assay of the *A. afra* tablets 50 mg samples of the tablet were used to establish the presence of luteolin and hence confirm the *Artemisia* content of the tablets. The luteolin levels found in the *A. afra* tablet (10 µg/g) corresponded to the levels found in the Mh aqueous extract (13 µg/g) used in the preparAtion of the tablet. This suggested that the tablet manufacture rocess, which involved the addition of several adjuncts and the alteration of the extract into a compact dosage form, did not alter the luteolin levels.

The measurement of luteolin (in the original material and in the final product) thus seems a viable process and marker to use for the quality control for the pharmaceutical process of manufacture of the tablets con g *A. afra*.

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The finding that luteolin was present in the *Arthritea* ®, *A. afra* tablets and *Floo-tea* ® appeared to authenticate the *Artemisia* content of these preparations. In addition it was satisfactory to note that hesperetin, a marker that was absent in the extracts of *A. afra* (see section 5.4.1), was also predominantly absent in the *Artemisia* medicines further supporting this conclusion that *Artemisia* was indeed a constituent of the plant preparations.

The second part of the analysis involved the analysis of the levels of apigenin, hesperetin and quercetin in the *Mentha-containing* preparation *Asthmitea* ®.

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Hesperetin was present in both the crude and extract preparation of *Asthmitea* ®, while quercetin was only detected in the crude *Asthmitea* ® preparation. Both apigenin and quercetin were absent in the aqueous extracts of *Asthmitea* ®.

The quercetin content of the *Asthmitea* ® preparations was 187 ngl20 mg (9.35 µgig), a quantity that was comparable to that found in the aqueous *M longifolia* extracts of Klb (8.2% yield) and Ray (7.3% yield) that contained between *45* to 2.1 µgig of quercetin. The presence of quercetin in these preparations seemed to confirmed that the *Mentha species* may be one of the components of the *Asthmitea* ® preparation. Moreover this marker was present at levels approximately similar to that found in the aqueous extracts of *M longifolia.* Collectively, these results suggested that the quercetin content could be used to not only authenticate the *Mentha* presence in the preparation but could possibly also be used as a marker to assess the pharmaceutical uniformity of *M /ongifo/ia* preparations.

The hesperetin levels found in the *Asthmitea* ® extracts and preparations ranged between 76 to 87 ng/20 mg (3.8 to 4.3 µg/g). This quantity of hesperetin was less (by approximately 30%) than that found in the *M longifolia* aqueous (sample Ray 7.3% yield) and methanhl (samples Hnk 6.3% yield and, WQj 5.7% yield). The extracts contained approximately 11 to 13.9 µg-hesperetin/g.

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This lower quantity of hesperetin found in the preparation compared to that in the *M longifolia* extracts, may have been due to the dilution factor caused by the presence of the other plant *species* also found in the plant preparation. Nevertheless, the presence of hesperetin in *Asthmitea* ® even though lower than that found in the aqueous extracts of the *M longifolia,* still served to suggest that the *Mentha* was one of the *species* contained in this preparation.

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In addition to the above markers, luteolin was also found in both the *Asthmitea* ® extract and preparation even though prior analysis of the *Mentha* extracts, confirmed by mass spectroscopic analysis (see section 5.3.3.2), appeared to indicate luteolin was not a chemical component of *M. /ongifolia.* It was thus not clear why the *Asthmitea* ® preparations contained luteolin. In addition, why apigenin, a marker found to be present in the *M. longifolia* extracts was absent in the *Mentha-containing Asthmitea* ® preparation was also unclear. Van Wyk (2000) has reported that although little information is available on the South African *Mentha species* the composition of the *Mentha species* from different localities is known to vary considerably.

It is therefore possible that the identification of luteolin, a flavonoid earlier found to be absent in *Mentha SIJFCies* extracts in this study and, the absence of apigenin that was expected to present, might suggest that the *Mentha* plant material used for the *Asthmitea* ® preparation was different in origin to that used in the preparation of the extracts used in this study. Alternatively, the incorporation of the other *species* found in the plant preparation may have been responsible for luteolin being present, and/or by virtue of a dilution factor, the hesperetin, being found at lower than exp.es:ted levels and the apigenin being undetectable.

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In summary, from the data obtained in this section on the nresence of markers in *Artemisia* and *Mentha speci ontaining* plant preparations, it can be concluded that (1) luteolin was a reliable marker to authenticate the presence of *Artemisia* species in plant preparations (2), hesperetin and quercetin might also be usefuVgood markers for the assessment of *Mentha* preparations and (3), apigenin, however, seemed an unsuitable marker for establishing/confirming the presence of *Mentha /ongifolia* in Mentha-containing preparations (although it may still be immensely useful for the evaluation of the extracts of *M. longifo/ia* as established in the section 5.4.2).

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S.4.3.3 Levels between batches

The experiments in this section aimed to test the usefulness of the markers in the identification of batch differences in *A. afra* tablets and *Asthmitea* ®. Three batches of *Asthmitea* ® were purchased prior to the period of assay and stored under similar cool and dry conditions, protected against light until required for analysis. The assay of these preparations involved random sampling of 20 mg quantities from each plant package.

Two batches of *A. afra* tablets were analyzed. The plant material used in the preparation of the tablet batches differed in origin i.e. tablet batch-one plant material was collected from Kirstenbosch Botanical Gardens and tablet batch-two plant material from Montagu Museum. Tablet batch-one was prepared first and because the tablets were highly hygroscopic the tablets produced were not of high quality. The second batch of tablets was not hygroscopic and the physic appearance of these tablets was acceptable. The results of the assessment of the usefulness of the markers in identifying batch-batch differences are summarized in table 7.

Table 7: Batch-to-Batch assessment or the flavonold markers In *A. afra* and *M. longlfolill* plant extncts.

Batch	Apigenin ng/20 mg	Hesperetin ng/20 mg	luteolin ng/20 mg	Quercetin ng/20 mg
A.sthmitea ® preparatlo111				
Asthmitea ® Batch 2	–	87.65±0.255	38.43±0.08	187±0.12
Asthmitea ® Batch 3	–	–	140.7±0.01	362±0.11
Asthmitea ® Batch 4	–	–	210±0.105	299±0.062
A.afra crude tablets				
C-Batch 1	–	–	31.7±0.01	207±0.001
C-Batch2	–	–	60.2±0.017	651±0.175
A..afra hydrolysed tablets				
H-Batch 1	129.6±0.01	–	208.6±0.007	397±0.008
H-Batch2	–	–	*290.8±0.00S*	713±0.002

Abbreviations: - not detected In sample, C - un-hydrolyzed samples, H - hydrolyzed samples.

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The *Asthmitea* ® preparations contained *Mentha species;* these preparations were therefore assayed for the presence and level of apigenin, hesperetin and quercetin. Apigenin was not detected in any of the batches, while all the batches contained luteolin and quercetin. Hesperetin was detected in one of the three batches (batch two).

The levels of quercetin detected ranged from approximately 9 to 18.5 µgig which were comparable to that found in the K.lb and Ray aqueous extracts of *M. longi,folia* (2 to 46 µgig). The levels of quercetin found in the batches were different and it was likely that this was a result of the random sampling from different parts of the package. The presence of quercetin in the batches however confirmed the presence of the *Mentha species.* The monitoring the level of quercetin was likely to be of use in establishing the chemical homogeneity of *Mentha*-containing preparations.

Hesperetin was £)und in batch two at approximately 4.5 µg/g. This quantity when compared to that found in the *M. longifolia* aqueous extracts (K.lb, Ray; 10 µg-hesperetin/g) was lower.

However, the incorporation of more than one plant *species* in the *Asthmitea* ® possibly resulted in the dihilion of the *Mentha* content. Accordu, If tJijs uld possibly be the responsible for the reduction.,and/or the complete absence of he§Peretin in the batches. Monitoring the level of this marker seemed appropriate for determination of the chemical homogeny of preparation, and the differences between batches of *Mentha*-containing preparations.

Luteolin was contained in all the batches of *Asthmitea* ®, which was uncharacteristic of the preparations of *Mentha species,* investigated in this study. However these findings are equivalent to the findings in section 5.4.3.2 (pg 71) that established luteolin in the extracts and preparations of *Asthmitea* ®. The luteolin detected was accredited to one or more of the other *plant species* present in the *Asthmitea* ® preparation that were in greater abundance compared to that of the *Mentha species.*

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Apigenin was not detected in any of the batches and for this reason seemed unsuitable as a marker for *Mentha-containing* preparations. The levels of apigenin in the *Mentha-species* were likely to be exceptionally low and could not be detected in a sample matrix consisting of one or more *plant species.* This evidently disqualified the usefulness of the marker in the identification of batch-to-batch differences of *Mentha-containing* plant preparations.

Generally most of the variations in the levels of the markers in the batches of *Asthmitea* ® were possibly due to the random sampling from the dosage form consisting of leaves and stems interspersed in powder. In addition the incorporation of a number of plant *species* in *Asthmitea* ® were likely to reduce the quantity of *Mentha species* in the preparation and hence result in the reduction of one or more of the markers in the preparation. The random samplings in combination with the use of multiple plant components were evidently not without drawbacks. The difference in dose administration from batch one to another could possibly double or triple the ingestion of the active ingredient/s. It thus was concluded that quercetin and h Peperetin were useful markers in the determination of the batch-to-batch differences of *Mentha-containing* preparations.

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For the *A. afra* tablet analysis two batches were assayed. Luteolin was present in both tablet batches when in the hydrolyzed as well as when in the crude form. The luteolin levels in the tablet batches were however different and varied by approximately 50%. Tablet batch one contained luteolin ranging from approximately 397-ng/20 mg (19.8 µgig) for the hydrolyzed tablet and was less compared to that found in tablet batch two 713-ng/20 mg (35.6 µgig) for the product of hydrolysis. The preservation or maintenance of the active ingredients in tablet batch one was possibly compromised as a result of the hygroscopic nature of the tablet. This subsequently was likely to have caused the alterations in the levels of luteolin detected. Alternatively the use of different plant material to prepare the extracts probably affected the luteolin levels detected in batch one compared to that detected in batch two.

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Leow (2000), in the review of the quality and standardization of medicinal products stresses that the nature of herbal products to a substantial extent are determined not only by the raw materials used in their preparation but through the process of their preparation.

The results of the differences in the marker levels between batches concur with this view. Due to the inappropriate dosage form of *Asthmitea* ® the markers were found to be present at different levels in the *Asthmitea* ® commercial samples. In addition luteolin levels differed from batch-to-batch in poorly manufactured tablets. However when plant medicinal preparations were-standardized in the form of a high-quality tablet, the differences in the occurrence and levels of the marker compounds were less distinct

Generally the sample handling aspects of plant collection, extraction of plant material, and the effects of improper storage by the manufacturer, could affect the integrity of the chemical ingredients in these products. In addition, currently standardized products can be manufactured using modern analytical methods. These products are of a constant quality and provide a standard dose each time the medicinal product is administered.

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From the findings in the batch-to-batch analysis, it was concluded that three of the four markers namely, hesperetin, luteolin and quercetin were useful in the assessment of the differences in preparations from batch-batch. The three markers could successfully identify and standardize the preparations of *Mentha* and *Artemisia species.* This shows that the markers could be used to simultaneously authenticate preparations as well as determine any discrepancies between batches of commercially prepared powders, teas or tablets made from any of the two *plant species.* However the fourth marker, apigenin was not suitable for the evaluation of differences from batch-to-batch for *Mentha*-containing preparations.

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In summary, in the evaluation of apigenin, hesperetin, luteolin and quercetin for pharmaceutical quality control studies of plant preparations the following conclusions were made. (1), The levels of hesperetin were unaffected by the extraction processes, while the levels of the three other markers apigenin, luteolin and quercetin were altered by extraction and/or solvent use in the extraction process (2), Monitoring the levels of hesperetin, luteolin and quercetin was useful in authenticating *Mentha-and-Artemisia*-containing medicinal preparations. However, apigenin was not a suitable marker for confirming the presence of either of these plant *species* in plant preparations and (3), the monitoring of the levels of hesperetin, luteolin and quercetin was useful in the evaluation of the batch-to-batch differenc of *Mentha-and-Artemisia-containing* medicinal preparations. However, apigenin was not suitable for determining the chemical homogeny of different batches of plant material.

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Chapter6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The objectives of this study were to develop a suitable HPLC assay to evaluate apigenin, hesperetin, kaempferol, luteolin and quercetin in *A. afra* and *M longifolia,* and with the assay developed, determine the suitabilitx of flavonoids as markers for the quality control of fla-v_onoid ntainin g medicınal preparations.

From the results of the above study it was concluded that:

1) A suitable, sensitive and rqproclucible HPLC assay was developed for the qualitative or. quantitative determination of four of the five selected flavonoids namely, apigenin; hesperetin, luteolin and quercetin.

2) The four flavonoids were oetecfuble in quantifiable levels in *A. afra* and *M longifolia* pl t material and, were suitable markers for evaluating the extracts *A. afra* and *M longifolia.*

- Apigenin, hesperetin, quercetin and a luteolin-like compound were detected in *M longifolia* plant material.
- Apigenin, luteolin and quercetin were detected in *A. afra* plant material.
- Kaempferol could not be detected using the HPLC assay developed.

3) Three of the four flavonoids namely, hesperetin, luteolin and quercetin were suitable markers for the pharmaceutical quality control of flavonoid-containing medicinal preparations.

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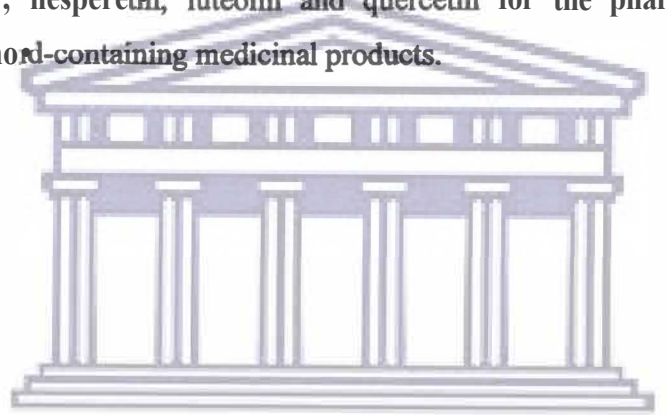
- Monitoring the levels of hesperetin, luteolin and quercetin were useful in authenticating *Mentha-and-Artemisia-containing* medicinal preparations.
- Monitoring the levels of hesperetin, luteolin and quercetin were useful in the evaluation of the batch-to-batch differences of *Mentha-and-Artemisia-containing* medicinal preparations.

In general, with the HPLC assay developed it is thus possible to use apigenin, hesperetin, luteolin and quercetin as markers to successfully evaluate the chemical integrity of *A. afra* and *M longifolia* extracts and consequently, use three of these markers namely, hesperetin, luteolin and quercetin for the pharmaceutical quality control of flavonoid-containing medicinal products.



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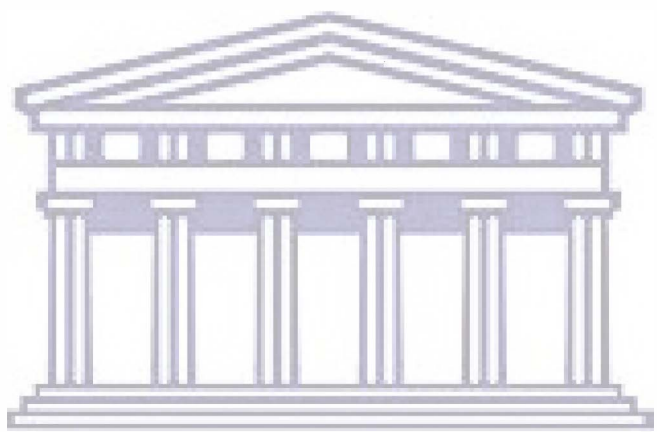

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APPENDIX I

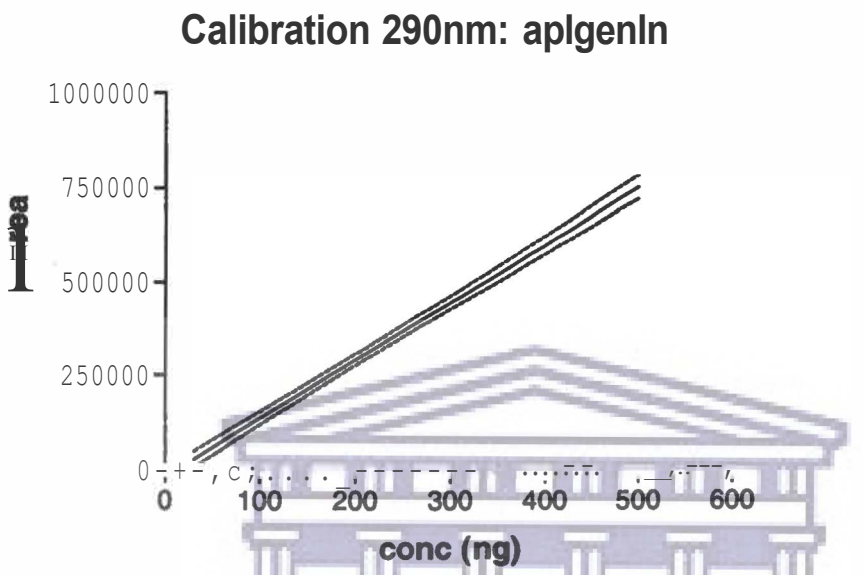

Fig 28: Calibration curve for apigenin

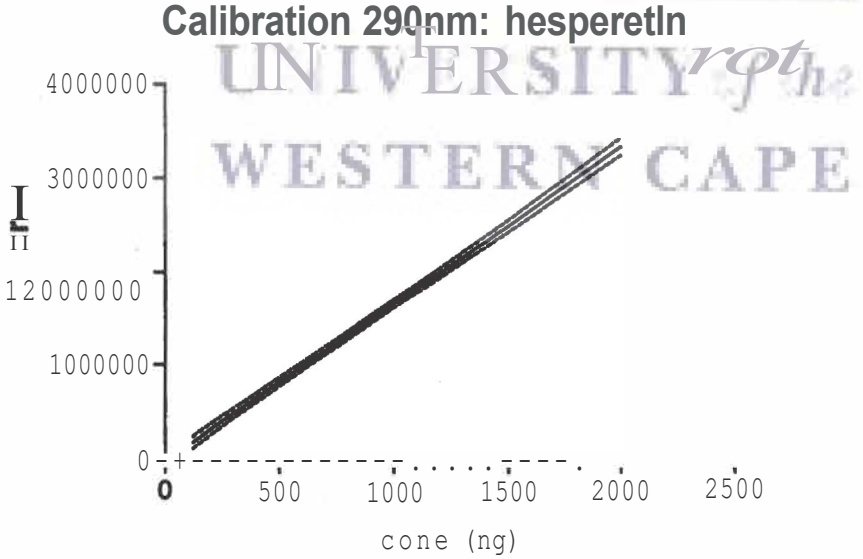

Fig 29: Calibration curve for hesperetin

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APPENDIX II

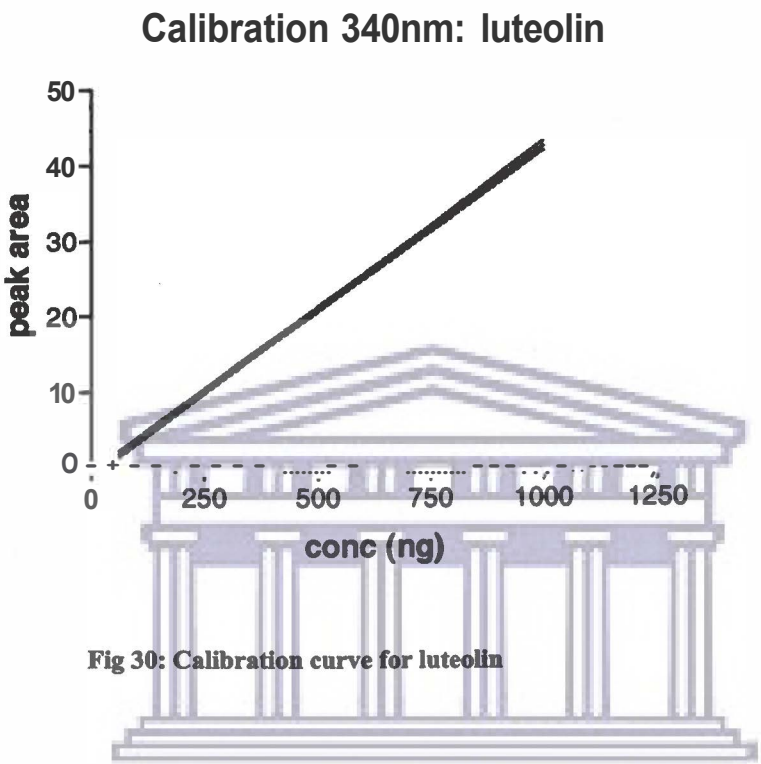

Fig 30: Calibration curve for luteolin

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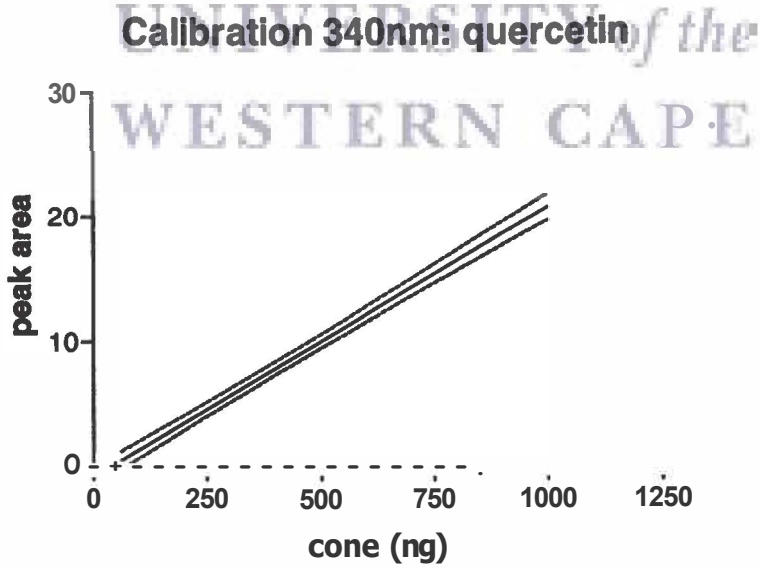

Fig 31: Calibration curve for querceda

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