DETERMINATION OF WITHAFERIN A CONTENT OF ROOT, STEM AND LEAF EXTRACTS OF *WITHANIA SOMNIFERA* (L) DUNAL COLLECTED IN KENYA

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DEPARTMENT OF PHARMACOLOGY AND PHARMACOGNOSY

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This thesis is my original work, and to the best of my knowledge, it has not been presented for award of any degree in any other institution of higher learning.

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DEDICATION

This work is dedicated to all the researchers in the efficacy, the effectiveness, the quality and the safety of phytomedicines.

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GLOSSARY OF SYMBOLS AND TERMS

BP - British Pharmacopoiea

CV - Coefficient of variation

h - Background noise range

H - Peak height

HPLC - High-pressure liquid chromatography

LOD - Limit of detection

LOQ - Limit of quantitation

RSD - Relative standard deviation

S/N - Signal-to-noise ratio

USP - United States Pharmacopoiea

RTA6 - *Withania somnifera* root sample from Ruai region, Nairobi County

RTA8 - Imported commercial formulation (powder) based on *Withania somnifera* root

RTA9 - Imported commercial formulation (capsules) based on *Withania somnifera* root

HPLC - High-pressure liquid chromatography

ABSTRACT

Introduction

Withania somnifera (L.) Dunal know as *Ashwagandha* belong to Solanaceae family. It is an annual herb growing in dry and arid soil as a wild plant. It is extensively used in most of the Indian herbal pharmaceuticals and nutraceuticals. It is widely cultivated in India and throughout the Middle East and is also found in eastern Africa. In Kenya, it grows wildly and is widely spread especially in drier areas.

The plant is well described in Ayurveda, the ancient Indian system of plant medicine for immunomodulation and anti-aging effect. The plant root is used as an anti-inflammatory, anti-tumor, anti-aging, anti-Alzheimer's disease, anti-stress, anti-oxidant, for mind or memory boosting, immunostimulant, as an aphrodisiac and for rejuvenation, as neuroleptic, for reducing blood sugar and cholesterol levels and as a diuretic.

Most of the beneficial pharmacological effects have been attributed to phytochemicals present in the plant known as withanolides of which withaferin-A is the most well studied and associated with. The quantity, the quality, the nature and the composition of phytochemical components in a plant are affected by environmental factors, and so those found in *Withania somnifera* are no exception.

Withania somnifera is a medicinal plant that is endangered due to over use. There is no data on the cultivation and adaptability of *Withania somnifera* in Kenya and how to make this nutraceutical an economically viable product. No product is available in Kenya based on *Withania* from Kenyan plants although there are imported products.

The objective of this study was to quantitatively determine the withaferin-A content of the root, stem and leaf extracts of *Withania somnifera* (L.) Dunal collected from different locations in Kenya.

Methodology

In this study, the plant was collected from different areas in Kenya where it is known to grow naturally. The powdered dry root, stem and leave materials of the plant were subjected to solvent extraction using composition of methanol-water (60:40).

Quantitative determination of withaferin-A in the samples was carried out using RP-HPLC performed isocratically with acetonitrile/ water (75:25) as the mobile phase. The column temperature was kept at 27 ^{o}C, flow rate and sample volume were set to 1.0 ml/min and 20 µl, respectively. All separations were monitored at 225 nm. Stability studies and efficiency of extraction of withaferin-A were also carried out.

Results and Discussion

The analysis of *Withania somnifera* root, stem and leaf confirmed the presence of withaferin-A in all parts of the plant but with significant differences in content. Withaferin-A content was highest in the leaves, with an average of 0.95 %w/w; the highest percentage recorded in a single leaf sample being 1.69 % w/w. This compound was lowest in stems, with an average of 0.29 %w/w, the highest content recorded in a single stem sample being 0.49 % w/w. The average withaferin-A content in the roots was 0.51 %w/w, with the highest content recorded in a single root sample being 0.77 %w/w. Two imported market products RT8 and RTA9 formulated from *Withania somnifera* root were analyzed. The percentage contents of withaferin-A in RTA8 and RTA9 were 0.16 % w/w in each. The Kenyan *Withania somnifera* plant collected from Limuru in Kiambu county showed the highest percentage withaferin-A content in the roots and stems, contents being 0.77 %w/w and 0.49 %w/w respectively, while the withaferin-A content in the leaves of the same plant collected in Limuru was the second highest (1.64 %w/w). Leaf samples collected from Narok town and environs in Narok county contained the highest withaferin-A content (1.69 %w/w).

On stability studies, withaferin-A showed to be more stable in a refrigerator (2-8 ºC) and easily degrades at room temperature. On efficiency of extraction methanol-water (60:40) was most efficient in extraction of withaferin-A compared with other solvents.

Conclusion and Recommendation

From the results of this research, the Kenyan *Withania somnifera* root appears had higher content of the pharmacologically active compound withaferin-A than the imported root formulations of the plant. The Kenyan plant also has more withaferin-A in the leaves than other parts.

Kenyan *Withania somnifera* could be endangered due to over use of the roots, whilst herbal pharmaceutical products derived from *Withania somnifera* leaves and stems products could offer useful alternatives to the *Withania somnifera* root products. Thus, *Withania somnifera* leaves and stems products merit further investigation.

Further studies should also be carried out to determine the effects of agro-ecological and pest infestation on the active compounds in *Withania somnifera.*

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. General background on herbal medicines and natural remedies.

Withania somnifera from the family Solanaceae, also known as Chepterekiat (Kipsigis), Ofuyaendwa (Luo), Mwanzo(Kamba), Hidigaga, Idigaga (Boran), Idi (Gabra), Murumbae (Kikuyu), Olasaiyet(Masai), Lopotwo (Pokot), Emotoe (Turkana) is widely spread in Kenya especially in drier areas [Kokwaro, 1993]. It is a woody herb or shrub 0.5-2 m with orange or red fruits. The plant is used widely by Kenyan people to alleviate many disease conditions. For example the root decoction is used for treating stomach-ache, especially gastric ulcer, treatment of colds in children, skin rashes, labour pain, gonorroea and general ill health. Although the other plant parts are used as source of the medicinal preparations, roots are the most commonly used parts, thus endangering the plant with extinction. In areas where it was originally found in Kenya, it can no longer be found in abundance [Beentje, 1994; Kokwaro, 1993].

Most of the uses recorded by literature cite widespread use of the plant in India (Ashwagandha, Indian Ginseng) where its roots have been used for thousands of years [Singh and Kumar, 1998]. It is extensively used in most of the Indian herbal pharmaceuticals and nutraceuticals. In the USA it is used in the rapidly growing market of nutraceuticals. The plant is used as an anti-inflammatory, anti-tumor, anti-aging, anti-stress, anti-oxidant, for mind or memory boosting, immunostimulant, as an aphrodisiac and for rejuvenation, as neuroleptic, for reducing blood sugar and cholesterol levels and is diuretic [Ray and Gupta,1994, Al- Hindawi *et al.,* 1992, Devi 1996]. According to Mishra *et al.,* (2000), the plant is an ingredient in many formulations prescribed for a variety of musculoskeletal conditions (e.g. arthritis and rheumatism), as a general tonic to increase energy, improve overall health and longevity, and prevent diseases in athletes, the elderly and during pregnancy. The roots contain flavonoids and many active ingredients of withanolide class.[Jayaprakasam *et al.,* 2003, Twaij *et al.,* 1989, Dredge *et al.,* 2003].

1.2. Nutraceuticals and Herbal Pharmaceuticals

Nutraceuticals can be defined as food or part of food or nutrient that provide healthy benefits including the prevention and treatment of a disease (DeFelice, 1989). Nutraceuticals can be also referred to as food ingredients that provide medical or health benefits beyond prevention and treatment of disease. The term "Nutraceutical" was coined from "Nutrition" and "Pharmaceutical" in 1989, by Stephen DeFelice, MD founder and chairman for Innovation in Medicine (FIM) [Evans, 2009; WHO, 2003].

Over the last few years , there has been a worldwide distinct shift in the healthcare paradigm, away from the treatment of symptom and towards a more holistic approach of both attaining and adopting a more preventive and functional approach to health care. More people are taking it upon themselves to preserve their health by adopting healthy lifestyles such as eating healthy foods and exercising regularly [Mwangi, 2012]. Nutraceuticals have become more relevant given the three main ills of life in the 21st Century: the globalization of unhealthy lifestyles, rapid unplanned urbanization and demographic ageing. These global trends have consequences on health, most notably seen in the universal rise of chronic non-communicable diseases such as heart disease, cancer, diabetes and mental disorders. For these diseases and many other conditions, nutraceuticals have much to offer in terms of prevention and treatment [Mwangi, 2012; European Union, 2005; WHO, 2003].

There are many botanical sources of nutraceuticals and herbal pharmaceuticals. Table 1.1 gives some examples of nutraceuticals and herbal pharmaceuticals, indicating their common names and botanical sources [Evans, 2009].

1.2.1. TABLE 1.1: Examples of Nutraceuticals and Herbal Pharmaceuticals

Nutraceutical	**Common name**	**Botanical source**
Ashwagandha	Ashwagandha	*Withania Somnifera*
Garlic	Garlic	*Allium sativum*
Ginger	Ginger	*Zingiber officinale*
Licorice	Licorice	*Glycyrrhiza glabra*
Milk thistle	Milk thistle	*Silybum marianum*
Valerian	Valerian	*Valeriana officinalis*
Ginkgo	Ginkgo	*Gingko biloba*
Ginseng	Ginseng	*Panax ginseng*
Aloe	Aloe	*Aloe species*,
Hypericum	Hypericum	*Hypericum perforatum*
Spirulina	Spirulina	*Arthrospira platensis*
Kavakava	Kavakava	*Piper methysticum*
Hawthorn	Hawthorn	*Crataegus pinnatifida*

1.2.2. Market and demand

The use of nutraceuticals, as an attempt to accomplish desirable therapeutic outcomes with reduced side effects, as compared with other therapeutic agents has met with great monetary success [European Union, 2005; WHO, 2003]. The preference for the discovery and production of nutraceuticals over pharmaceuticals is well seen in pharmaceutical and biotechnology companies [European Union, 2005]. The high cost of prescription pharmaceuticals and reluctance by some medical insurance companies to cover the cost of some drugs has led to the prevalence for the discovery and production of nutraceuticals over pharmaceuticals. Some of the pharmaceutical and biotechnology companies which commit major resources to the discovery and development of nutraceuticals include Monsanto (St. Louis, Mo, U.S.A.), Abbott laboratories (Abbott park U.S.A), Wamer – Lambert (Morris plains, NJ, U.S.A), Johnson and

Johnson (New Brinswick, NJ, U.S.A.) and Norvatis (Basel, Switzerland). The nutraceutical industry in the US is about $86 billion [European Union, 2005; WHO, 2003]. This figure is slightly higher in Europe and in Japan, represents approximately a quarter of the $6 billion total annual food sales. Forty seven percent of the Japanese population consume nutraceuticals. Even without specific financial figures, business reports continually suggest that the market is consistently growing [European Union, 2005; WHO, 2003; US Dietary Supplement Health and Education Act, 1994].

The last couple of decades witnessed phenomenal increase in Nutraceutical use worldwide. Today, nutraceutical is over a US$86 billion market with an ever increasing usage trend and increased preference for nutraceutical in developed countries like USA, Canada, England, Germany & Japan. Nutraceuticals, which provide health benefits and facilitate healing and prevention process of diseases, reduce the costs associated with healthcare and it is projected to be more than US$ 90 billion by the end of 2015 [US Nutraceuticals Market Analysis, RNCOS – Industry Research solution, May 2012; European Union, 2005; WHO, 2003; US Dietary Supplement Health and Education Act, 1994]. One possible explanation for the growth of nutraceuticals in the United States is the aging baby- boomer population. Other reasons include chronic diseases with poor therapeutic alternatives, desire for personalized medicines, large population trying to stave off the effects of aging, new focus on preventive medicine and also public perception that "natural is good" [WHO, 2003].

1.2.3. Challenges encountered in attempt to promote growth of nutraceuticals

Some challenges encountered in attempt to promote growth of nutraceuticals and herbal pharmaceuticals include, common belief that only imported herbals/botanicals are effective, little data on the cultivation and adaptability of popular herbals/botanicals and little data on how to make nutraceuticals a cash crop equal to the more traditional cash crops. Another serious challenge is the wide range of manufacturing processes with no attention to product extraction, effective "shelf-life", storage, ingredient standards or contamination. Lack of quality standards, weak quality control and poor product standardization practices are major setbacks in many countries. Other significant drawbacks to the promotion of growth of nutraceuticals include lack of ethno-botanical information on uses, lack of comprehensive survey on commercial value of

Kenyan medicinal plants and possible products, lack of public and private sector involvement in infrastructure that would facilitate value addition and processing opportunities, lack of scientific programs to identify potential uses and increase quality and intellectual property issues [WHO, 2003; Mwangi, 2012].

1.3. Factors influencing phytochemical components in a plant:

1.3.1. Environmental conditions

Plant growth and development and often the nature and quantity of secondary metabolites, are affected by temperature, rainfall, aspect, length of the daylight (including the quality of light) and altitude. Such effects have been studied by growing particular plants in different climatic areas and observing variations. A number of centres around the world are engaged in this research. The findings are illustrated by work on cannabis [El-Kheir and Ramirez, 1986; Evans, 2009] in which seeds of cannabis grown in England and rich in CBD (cannabidiol) and devoid of THC (tetrahydrocannabinol), when cultivated in Sudan started to produce THC in the first generation and in the second generation contained up to 3.3% THC with a further decrease (down to 0% in some plants) of CBD. However, it is impossible to control all the variables in such experiments, and special laboratories (phytotrons) have been constructed in which all the factors are independently controllable [Evans, 2009]. With investigations of this type, which involve phytochemical constituents, a meaningful expression of the results can present some difficulty. For example, a particular factor may lead to the development of a small plant which, when analyzed on a percentage dry weight basis, indicates a high proportion of metabolite, even though the overall yield per plant could be quite low. Similarly, certain nutrients may result in the production of large plants with a somewhat low analytical figure for constituents on a percentage dry weight basis, but yield per plant may exceed that of the control.

1.3.1.1.Temperature

Temperature is a major factor controlling the development and metabolism of plants. Although each species has become adapted to its own natural environment, plants are frequently able to exist in a consideration range of temperatures [Evans, 2009; Hornok, 1992]. Many tropical and subtropical plants will grow in temperate regions during summer months, but lack frost resistance to withstand the winter. In general, the highest temperatures are experienced near the

Equator, but as the temperature falls about 1ºC for every 200 m of elevation, it is possible in, say, Jamaica to have a tropical climate on the coast and a temperate one in the mountains. The annual variations in temperature are just as important as the temperature of the hottest month. At Singapore the annual range of temperature is as little as 1.5ºC, whereas Moscow, with its hot summers and cold winters, has a range of 29.3ºC. Night and day' temperature must also be considered, the variation often being considerable. In general, the formation of volatile oils appears to be enhanced at higher temperatures, although very hot days may lead to an excess physical loss of oil [Evans, 2009; Hornok, 1992; Martin and Woodcock, 1983]. The mean optimum temperature for nicotine production in *Nicotiana rustica* is 20ºC (lower at 11-12ºC and at 30ºC). Several authors have indicated that fixed oils produced at low temperatures contain fatty acids with a higher content of double bonds than those formed at higher temperatures.

1.3.1.2. Rainfall

The important effects of rainfall on vegetation should be considered in relation to the annual rainfall, its distribution throughout the year, its effect on humidity and its effect coupled with the water-holding properties of the soil. Variable results have been reported for the production of volatile oils under different conditions of rainfall and may in some instances be coupled with development of glandular hairs. Continuous rain can lead to a loss of water soluble substances from leaves and roots by leaching; this is known to apply to some alkaloid- (particularly Solanaceae), glycoside – and even volatile oil-producing plants. This could account for low yields of some active constituents in wet seasons from plants whose general condition appears to be good [Evans, 2009; Hornok, 1992; Martin and Woodcock, 1983].

1.3.1.3. Day-length and radiation characteristics

Plants vary much in both the amount and intensity of the light which they require. In the wild state the plant will be found where its shade requirements are met, and under cultivation similar shade must be provided. In certain cases research has shown that light is a factor which helps to determine the amount of glycosides or alkaloids produced. With belladonna, stramonium and *Cinchona ledgeriana* full sunshine gives a higher content of alkaloids than does shade [Hornok, 1992; Evans, 2009]. At Gif-sur-Yvette experiments indicated that with *Datura stramonium* Var.

tatula long exposure to intense light brought about a sharp increase in hyoscine content at the time of flowering. An important *in vivo* reaction in the formation of the antitumour alkaloids of *Catharanthus roseus* is exemplified by the dimerization of the indole alkaloids catharanthine and vindoline leading to vinblastine [Hirata and Loughrin, 1993]. Hirata and Loughrin [1993], demonstrated that irradiation of intact plants with near ultraviolet light in the range 290 – 380 nm (peak 370 nm) stimulates the synthesis of dimeric alkaloids, probably by inducing catharanthine oxidation as a trigger reaction. This observation has support from *in vitro* studies [Evans, 2009; Hornok, 1992; Martin and Woodcock, 1983].

It has been shown that under long day conditions peppermint leaves contain menthone, menthol and traces of menthofuran while plants grown under short day conditions contain menthofuran as a major component of the volatile oil. Furthermore a long photoperiod for young leaves activates the reduction pathway with conversion of menthone to menthol [Voirin andVerpoorte, 1990; Evans, 2009]. In studies on the day and night changes in the relative concentrations of volatiles from flowers of *Nicotiana sylvestris* and other species a marked increase (about tenfold) in aromatic compounds including benzyl alcohol was detected at night, whereas no increase in the volatiles (e.g. linalool, caryophyllene) originating from the melvalonic acid pathway (q.v) was noted [Loughrin and Constable, 1990; Evans, 2009].

The daily variation in the proportion of secondary metabolites is probably light-controlled. Type of radiation has been studied in respect to morphological development of the plant, but little information appears to have been reported concerning medicinal metabolites. Many plants initiate flowers only in certain day-lengths and where flowering is essential this factor must be carefully considered before planting in a new region. Presence or absence of light, together with wavelength range, have a marked effect on the secondary metabolite production of some plants in tissue culture [Evans, 2009; Hornok, 1992; Martin and Woodcock, 1983; Loughrin *et al.*, 1990].

1.3.1.4. Altitude

The coconut palm needs a maritime climate and the sugarcane is a lowland plant. Conversely, tea (1000-2000 m), cocoa (100-200 m), coffee (800-1800 m), medicinal rhubarb, tragacanth and cinchona require elevation. In the case of *Cinchona succirubra,* the plant grows well at low altitudes but produce practically no alkaloids. The bitter constituents of *Gentiana lutea* increase with altitude, whereas the alkaloids of *Aconitum napellus* and *Lobelia inflata* and the oil content of thyme and peppermint decrease. Other oil-producing plants may reach maximum at certain altitudes. Pyrethrum gives the best yields of flower-heads and pyrethrins at high altitudes on or near the Equator. It is therefore produced in East Africa and north-west South America. However, vegetative growth is more lush under irrigated conditions at lower altitude, so the propagation farms (for the vegetative multiplication of plants) are, in Ecuador, situated at lower levels than the final commercial farms [Evans, 2009; Hornok, 1992; Martin *et al.,* 1983].

1.3.1.5. Atmospheric composition

Although little work appears to have been done in this area concerning medicinal plants it has been reported that *Digitalis lanata* grown in greenhouses with a carbon dioxide-enriched atmosphere (1000 p.p.m. CO_2 during the whole of the growth period from April to November) produced 3.5 times the amount of digoxin per hectare than did the field cultivated plants [Stuhlfauth and Datta, 1990; Evans, 2009].

1.3.2. Soils

Different plant species vary enormously in their soil and nutrient requirements and this aspect has received considerable attention with medicinal plants. Three important basic characteristics of soils are their physical, chemical and microbiological properties [Evans, 2009].

Variations in particular size result in different soils ranging from clay, via sand, to gravel. Particle size is one factor influencing water holding capacity, and some plants (e.g. *Althaea officionalis)* which produce mucilage as a water retaining material contain less mucilage when grown in soil with high moisture content. In moist regions such as Western Europe, clay soils absorb water beyond their absolute capacity. The high moisture content makes them cold (i.e. they heat up slowly) and they are difficult to work on account of their stiffness. On the other

hand, in drier regions such as the Mediterranean such soils are much esteemed for their power of absorbing and retaining moisture [Evans, 2009; Martin and Woodcock, 1983; Hornok, 1992].
The basic soil type is modified by the presence of humus, organic fertilizers, chalk, lime, etc. Fine soils rich in humus and having a permeable substratum possess a degree of humidity which is generally favourable for plants. Sandy soils poor in humus and having a gravel subsoil are generally only suitable for xerophilous plants. On calcareous soils which are poor in humus, vegetation is mainly xerophilous; but if humus is present, the moisture-absorbing ability is much increased [Evans, 2009; Martin and Woodcock, 1983; Hornok, 1992].

Soils containing much humus and little lime are inclined to become acidic, while those with abundant lime are alkaline. Although particular species have their own soil pH tolerances (*Datura stramonium* 6.0-8.2, *Mojorana hortensis* 5.6-6.4), no marked influence of pH value within the tolerance range has been demonstrated for essential oils (*Mentha piperita*) and alkaloids (*Datura stramonium*). All plants require calcium for their normal nutrition but plants known as caliphobous plants (e.g. *Pinus pinaster* and *Datura purpurea*) cannot be grown on chalky soils, probably owing to the alkalinity. In other cases different varieties of the same species may grow on different soils [Evans, 2009; Martin and Woodcock, 1983; Hornok, 1992].

The effect of nitrogen-containing nutrients on alkaloid production has received considerable study (Solanaceous drugs including *Nicotiana*, Opium). Generally nitrogen fertilizers increase the size of the plants and the amounts of alkaloids produced. The effects of nitrogen on glycoside and essential oil contents appear variable; presumably in these cases the final result arises from the general effect of nitrogen on the plants metabolism. Nitrogen fertilizers have been shown to increase silymarin content of the fruits of *Silybum marianum* grown on reclaimed ground. Trace amounts of manganese are necessary for the successful production of *Digitalis purpurea* [Evans, 2009; Martin and Woodcock, 1983; Hornok 1992].

Little work appears to have been performed on the microbiology of soil with respect to secondary metabolism; Hardman *et al*. [1975], studied the response of *Trigonella foenum-graecum* (potential source of diosgenin) to field inoculation with *Rhizobium meliloti* 2101, in

their attempt to establish this species as a temperate crop. Soil bacteria of the genus *Agrobacterium* are finding application in the production of 'hairy root' cultures [Evans, 2009; Hardman *et al.,* 1975].

1.3.3. Cultivated and Wild Plants

Certain nutraceuticals and herbal drugs are now obtained almost exclusively from cultivated plants. These include cardamoms, Indian hemp, ginger and peppermint and spearmint for oil production, ceylon cinnamon, linseed, fennel and cinchona. In other cases both wild and cultivated plants are used [Evans, 2009; Hornok, 1992; Martin and Woodcock, 1983]. Some plants have been cultivated from time immemorial (e.g. flax, opium poppy and coca). Others are now grown because supplies of the wild plants are insufficient to meet the demand or because, owing to sparse distribution or inaccessibility, collection is difficult [Evans, 2009; Hornok, 1992; Martin and Woodcock, 1983].

Cultivation is essential in the case of drugs such as Indian hemp and opium, which are subject to government control, and in many cases it is advisable because of the improved quality of the drug which it is possible to produce. The improvement in the quality of the drug is due to the power to confine collections to species, varieties or hybrids which have the desired characters e.g. as in aconite, cinnamon, fennel, cinchona and valerian. The improvement may also be due to the better development of the plants owing to improved conditions of the soil, pruning, and the control of insect pests, fungi and humidity. In cultivated plants the availability and accessibility of better facilities for treatment after collection also contributes to the improvement in the quality of the drug obtained. For example, drying at a correct temperature in the cases of digitalis, colchicum, belladonna and valerian, and the peeling of cinnamon and ginger [Evans, 2009; Hornok, 1992; Martin and Woodcock, 1983].

For success in cultivation it is necessary to study the conditions under which the plant flourishes in the wild state and reproduce these conditions or improve on them. Small changes in ecology can affect plant products; thus, satisfactory rubber trees grow wild in the Amazon basin but cleared areas converted to rubber plantations have been a failure [Evans, 2009; Hornok, 1992; Martin and Woodcock, 1983].

1.3.4. Chemical Races/ Chemodemes

Chemical races or 'chemodemes are chemically distinct populations within a species and have similar phenotypes but different genotypes and as such are identical in external appearance but differ in their chemical constituents [Evans, 2009].

In recent years the plant kingdom has been subjected to extensive chemical investigation. Thousands of plant samples have been screened for substances of medicinal value or for suitable precursors of therapeutically active compounds. Many other plants have been studied chemically from the view points of natural treatments, plant resistance and biosynthesis of active constituents. From such observations, has emerged evidence of existence of 'chemical races' or 'chemodemes' [Evans, 2009].

Hegnauer [1975], emphasizes that the chemical race concept is distinct from local polymorphism, which involves genetically controlled chemical variation within a local population. Such genetically controlled chemical variations have obvious implications for the development of superior crude drugs [Eastwood and Douros, 1971; Evans, 2009].

Before the existence of a chemical race can be established, certain fundamental observations are necessary. A chemical analysis of a number of random samples of a particular species may show a variation between the samples but would be insufficient to demonstrate any genetical differences, since factors such as age, climate and soil can all exert profound effects on the result of the ultimate analysis. Samples of seed or clones from different plants must be raised together under uniform conditions and to exclude hybrids, which do not breed true, cultivation for a number of generations is desirable. It may then be possible to demonstrate that differences occur in either the nature or quantity of a particular constituent and that these differences are of a hereditary nature [Evans, 2009; Voirin and Verpoorte, 1990].

Such observations necessitate numerous assays and precise horticulture work, to which must be added the difficulties of dealing with plants which take long to mature. Nevertheless, the occurrence of chemical races in some species suggests itself without intensive investigation and

examples have been recognized for many years. The presence or absence of particular anthocyanin pigments in certain flowers gives rise to coloured or white forms, thus providing a visual guide for the examination of a chemical race. Other flowers contain mixtures of anthocyanin pigments and the formation of one pigment from another is dependent on the presence of specific genes. A similar situation exists in the yellow anthoxanthin series [Evans, 2009; Hegneur, 1975; Eastwood and Douros, 1971].

Some examples of plants exhibiting chemical races include alkaloids containing plants such as *Datura sanguinea*, *Dubuisia* species such as *D. myoporoides* and *D. leichharditii*; cyanogenetic glycoside containing plants such as *Prunus communis*; fixed oil containing plants such as Sunflower seed; cardiac glycoside containing plants such as *Digitalis purpurea, Digitalis lanata, Strophanthus sarmentosus*; anthraquinone containing plants such as *Rheum palmatum* and *Cassia angustifolia*; essential oil containing plants such as *Eucalyptus dives, Melaleuca bracteata , Cinnamomum camphora, Mellissa officionalis*; steroidal alkaloids producing plants such as *Solanum dulcamara*, *Solanum laciniatum*; and withanolides containing plants such as *Withania somnifera,* among many others [Evans, 2009; Hardman *et al.,* 1991].

In *Withania somnifera,* three chemotypes were discovered among 24 populations of the plant [Abraham *et al.,* 1968]. Chemotype I contained predominantly withaferin A (0.2% of the dry weight), which is the principle responsible for the plant's bacteriostatic and antitumour properties. Chemotype II contains a compound of similar structure, and Chemotype III a mixture of related compounds comprising a new group of steroidal lactones- the withanolides. The only morphological difference observed between the chemotypes was a difference in flowering time (12 days early) for chemotype III. Since then, other chemotypes of *Withania somnifera* have been reported from India and South Africa [Abraham and Moses, 1968; Evans 2009].

These examples serve to show that the occurrence of chemical races in plants, whether they are of natural origin or produced by plant breeding, can offer considerable scope for the improvement of the therapeutic value of the drug either by adjustment of the individual constituents or by increase in overall yield [Evans, 2009].

1.4. LITERATURE REVIEW: *WITHANIA SOMNIFERA* (L) DUNAL

1.4.1. Brief overview of the plant *Withania somnifera* (L)Dunal

Withania somnifera (L.) Dunal also known as *Ashwagandha* in India, belongs to Solanaceae family. It is an annual herb growing in dry and arid soil as a wild plant [Singh *et al.*,1998]. The roots are traditionally used for gastric ulcers, colds, skin rashes, labour pains and as a tonic [Mwangi, 2012; Kokwaro,1993]. *Withania somnifera* (L.) Dunal (Solanaceae), also commonly known as Winter cherry, is one of the most valuable plants of the traditional Indian systems of medicines, is used in more than 100 formulations of Ayurveda, Unani and Sidha, and is therapeutically equivalent to ginseng [Sangwan *et al.*, 2004]. It is extensively used in most of the Indian herbal pharmaceuticals and nutraceuticals and is well described in Ayuverda, the ancient Indian system of plant medicine with many advantageous properties such as liver tonic, aphrodiasic, purgative, diuretic, anti-ageing, anti-ulcer and adaptogenic [Sangwan *et al.*, 2004; Ray and Gupta, 1994; Mishra *et al.*, 2000; Mwangi, 2012].

Extracts and isolated compounds have shown broad spectrum of pharmacological activities such as antitumour, haemopoietic, anti-inflammatory, anti-ulcer, immunomodulation, antioxidant and anti-stress effects together with capacity to improve physical and mental health. Analgesic effects have also been reported [Ray and Gupta, 1994; Mishra *et al.*, 2000; Jayaprakasam *et al.*, 2003; Devis, 1996; Al-Hindawi *et al.*, 1992; Twaji *et al.*, 1989; Dredge *et al.*, 2003; Mwangi, 2012]. Other pharmalogical uses, applications and properties of the plant include hypnotic, anti-anxiety, anti-convulsion and brain stimulation activities, and the plant has been employed in the treatment of neurological disorders, geriatric debilities, arthritis, stress and behaviour-related problems [Schliebs *et al.*, 1973; Ray and Gupta, 1994]. Several modern molecular pharmacological studies have demonstrated linkage of these therapeutic actions to one or more withanolides present in the herb [Kinghorn *et al.*, 2004; Tohda *et al.*, 2005].

Phytochemically, the plant is unique in possessing the largest and structurally most diversified set of withanolides which are modified steroidal molecules based on an ergostane skeleton, named after the plant [Evans, 2009; Kinghorn *et al.*, 2004; Tohda *et al.*, 2005].

Chemical races or chemodemes do exist in *W. somnifera* and thus myriad of globally traded herbal preparations based on the plant are either poorly characterised from the phytochemical angle or are characterised with respect to just one or two withanolides, usually withaferin A. This is the predominant cause of vast compositional variations across the makes and batches of the herbal drugs and their phytoresources [Sangwan *et al*., 2004].

1.4.2. Detailed description of the plant *Withania somnifera* (L.) Dunal

Detailed description of the names, history, features, properties, distribution, chemistry, uses, pharmacology and scientific work done on this medicinal plant has been documented [Evans, 2009; Kokwaro, 1993; Ray *et al.,* 1994; Sangwan *et al.*,2004; Mishra *et al.,* 2000; Jayaprakasam *et al.,* 2003; Devis, 1996; Al-Hindawi *et al.,* 1992; Singh *et al.,* 1998]

1.4.2.1. Scientific names, Common names and Local names of the plant being studied

The scientific name of the plant is *Withania somnifera* (L.) Dunal, (synonym *Physalis somnifera* L.). It belongs to the Family Solanaceae [Evans, 2009]. The common names of *Withania somnifera* are Withania , aswaganda , winter cherry , Indian ginseng , ajagandha , kanaje Hindi , samm al ferakh , asgand (Hindi), amukkirag (Tamil), amangura (Kannada), asvagandha (Bengali), ashvagandha (Sanskrit), asundha (Gujarati), kuthmithi [Sangwan *et al.,* 2004; Mishra *et al.,* 2000; Jayaprakasam *et al.,* 2003; Devis, 1996; Al-Hindawi *et al.,* 1992, Singh *et al.,* 1998].

The local names of *Withania somnifera* in Kenya are Mwanzo (Kamba), Murambae(Kikuyu), Ofuyaendwa (Luo), Lapotwo (Pokot), Olasaiyet (Maasai), Chepterekiat (Kipsigis), Kipkogai (Marakwet) [Kokwaro, 1993].

1.4.2.2. Botanical description

W. somnifera (shown in Figures 1.1-1.5) is an erect, greyish, slightly hairy evergreen shrub that grows to about 1.5 m in height and has fairly long tuberous roots. The small and greenish-yellow flowers can be single or in clusters. The fruit is smooth, round, and fleshy, with many seeds; it is orange-red when ripe and enclosed in a membranous covering [Beentje, 1994; Evans, 2009; Kokwaro, 1993].

Figures 1.1-1.5 represents the Kenyan variety of *Withania somnifera* plant and the plant's botanical features.

Figure 1.1: ***Withania somnifera* (I)**

This photo shows the evergreen shrub that grows to about 1.5m in height, flowers in clusters and orange–red berries. The photo was taken in Limuru, Kiambu County.

Figure 1.2: ***Withania somnifera*** **(II)**

This photo shows the greyish hairy upper stem parts, alternate simple leaves with netted venation and entire leaf margin. The photo was taken in Limuru, Kiambu county

Figure 1.3: ***Withania somnifera*** **(III)**

This photo shows the greyish-green lower stem of the mature plant. This photo was taken in Narok Town environs , Narok County

Figure 1.4: ***Withania somnifera*** **(IV)**

This photo shows the unripe berries of the plant. The photo was taken of the plant under cultivation in Limuru, Kiambu County.

Figure 1.5: ***Withania somnifera*** **(V)**

This photo shows the ripe orange- red berries of the plant. Photo was taken in Narok Town environs, Narok County.

1.4.2.3. Distribution

It is widely cultivated in India and throughout the Middle East and is found in eastern Africa. [http://plants.usda.gov]. Two species, *Withania coagulans* Dunal and *Withania somnifera* Dunal, are found in India. In India, *W. coagulans* and *W. somnifera* are known by the same names in most of the regional languages. In the market, no distinction is made between the berries obtained from *W. coagulans* and *W. somnifera* . Both the species have identical macroscopical features of roots, such as smooth and whitish surface, and short and starchy fracture. The roots of the *W. coagulans* are however, characterized by the isolated groups of non lignified cells in the xylem tissues, calcium oxalate crystals in the phelloderm cells, and triseriate medullary rays [Sangwan, 2004; Evans, 2009].

In Kenya the plant is reported in Ruai and Syokimau areas in Nairobi County, Kyumbi and Salama areas in Machakos County, Makueni County, Naivasha area in Nakuru County, Baringo County, Kitui County, Narok County and Kajiado County [Kokwaro, 2012; Kenya National Herbarium, 2012]. It has also been reported in semi-arid areas of North Eastern and Northern Kenya like Mandera, Moyale, Turkana, Isiolo and Marsabit. It has also been reported in the Kenyan North Rift Region, semi-arid regions of South Rift and a few cases in Western Kenya as illustrated in Figure 1.10 [Beentje, 1994]. The photos of *Withania somnifera* in selected Counties in Kenya are shown in Figures 1.6-1.9.

Figure 1.6: ***Withania somnifera* (VI)**

Photo of the plant in Naivasha, Nakuru County.

Figure 1.7: ***Withania somnifera* (VII)**

Photo of the plant taken in Kathonzweni, Makueni County. The mature plant is showing ripe and unripe berries. The ripe berries are orange- red in colour

Figure 1.8: ***Withania somnifera*** **(VIII)**

The photo was taken in Narok Town environs, Narok County.

Figure 1.9: ***Withania somnifera*** **(IX)**

Photo of the mature plant under cultivation in Limuru, Kiambu County. Photo was taken in Limuru.

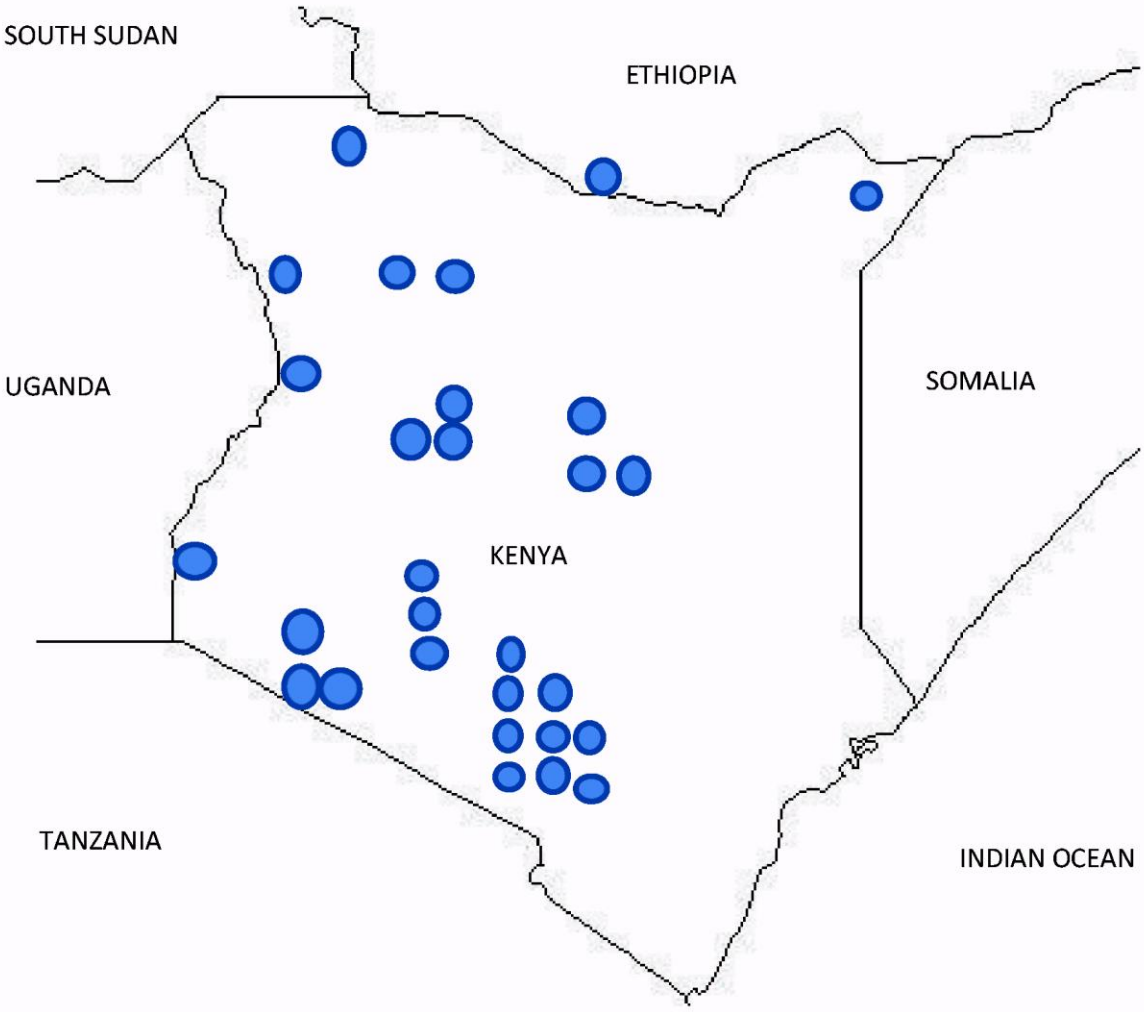


Figure 1.10: Distribution of *Withania somnifera* in Kenya [Beentje, 1994]

1.4.3. Pharmacological uses and applications of Ashwagandha.

The root of *Withania somnifera* commonly known as "Ashwagandha" is widely available as a commercial product in India. Ashwagandha has been used as an adaptogen, as a diuretic, as sedative, in the treatment of stomach ulcers, as a purgative, as a liver tonic, as an aphrodisiac, in management of senile debility and nervousness, in treatment of dropsy, treatment of cough and colds, treatment of skin rashes, alleviation of labor pains, management of asthma and haemorroids, management of poorly healing open wounds and in general ill health [Mwangi, 2012]. It is available in the United States as a dietary supplement [US Dietary Supplement and Education Act, 1994; Devi, 1996]. Trials supporting its clinical use are limited; however, many *in vitro* and animal experiments suggest effects on the immune and CNS systems, as well as in the pathogenesis of cancer and inflammatory conditions [Singh *et al.,* 1998; Al-Hindawi *et al.,* 1992; Jayaprakasam *et al.,* 2003]. In Kenya, commercial samples are imported from India in form of powder or capsules (Figure 1.11 and 1.12).

Ashwagandha also known as Indian Ginseng is also used in the management of rheumatoid arthritis. It boosts learning and memory and has been used in the management of Alzheimer's disease. It has anticonvulsant effects and has been used in management of seizures. It reduces serum cholesterol levels and has found application in various cardiovascular conditions like atherosclerosis. Analgesic, anti-inflammatory and anti-pyretic effects have been reported hence its use in alleviation of pain, rheumatoid arthritis and stress. It has been applied in the management of drug addiction. It has antimicrobial and antitumor effects hence useful in management of cancer patients. It has immune boosting effects hence is of high value in management of HIV/ AIDS patients. It has also been used as an aphrodisiac and as an anti-ageing agent [Sumantran *et al.,* 2008; Sumatran *et al.,* 2007; Singh *et al.,* 2008; Lu *et al.,* 2009; Kulkarni *et al.,* 2008; Zhao *et al.,* 2002; Choudhary *et al.,* 2005].

Figure 1.11. **Packaged *Ashwagandha* powder.**

Figure 1.12. Packaged *Ashwagandha* capsules.

(The photos in figure 1.11 and 1.12 were taken at Kunjus Herbal Products Ltd, in Ngara, Nairobi, Kenya).

1.4.4. History and traditional uses

The root of *W. somnifera* has for a long time been used to make the Ayurvedic tonic ashwagandha , which has been translated to "smells like a horse" [Ganzera *et al*., 2003]. Most of the uses recorded in literature cite widespread use of the plant in India under the names "Ashwagandha" and "Indian Ginseng" where its roots have been used for thousands of years [Singh and Kumar, 1998]. It has been extensively used in most of the Indian herbal pharmaceuticals and nutraceuticals. In USA it has been and is still being used in the rapidly growing market of nutraceuticals. Ashwagandha has been used as an adaptogen, diuretic, and sedative and is available in the United States as a dietary supplement. Other parts of the plant (eg, seeds, leaves) have been used as a pain reliever, to kill lice, and in making soap. The fresh berries have been used as an emetic [Ganzera *et al.,* 2003; Kulkarni and Dhir, 2008].

1.4.5. Local (Kenyan) ethnopharmacological applications

The plant *Withania somnifera* (L) Dunal is indigenous to Kenya [Beentje, 1994; Kokwaro, 1993]. It is found growing naturally in the wild, mainly in the hot semi-arid areas. The roots of this plant are dug out, washed, then pounded and the expressed sap used for treating stomachache, especially gastric ulcer. The root decoction is a good treatment for colds in children, skin rashes, excess bile, labour pains and general ill health. The roots may also be prepared by drying and grinding into a fine powder. A teaspoonful of this powder is suspended in a cup of tea or with honey and the preparation may be taken once or twice a day. Directly heated leaves are applied to various parts of the body as a pain killer. Root decoction is also used for treating gonorrhea [Kokwaro, 1993]

1.4.6. Phytochemistry

The principal bioactive compounds of *W. somnifera* reported in literature are withanolides, which are triterpene lactones. More than 40 withanolides and approximately 12 alkaloids and several sitoindosides have been isolated and identified from *W. somnifera* . The withanolides are reported to be structurally related to the ginsenosides of Panax ginseng, hence the common name "Indian ginseng" [Kulkarni *et al.,* 2008; Mishra *et al.,* 2000]. The chemical constituents for the roots, fruits, seeds, and stem include withanone; withaferin A; withanolides A, D, and G; and sitoindosides VII, VIII, IX and X [Ganzera *et al.,* 2003; Kulkarni *et al.,* 2008; Mishra *et al.,* 2000].

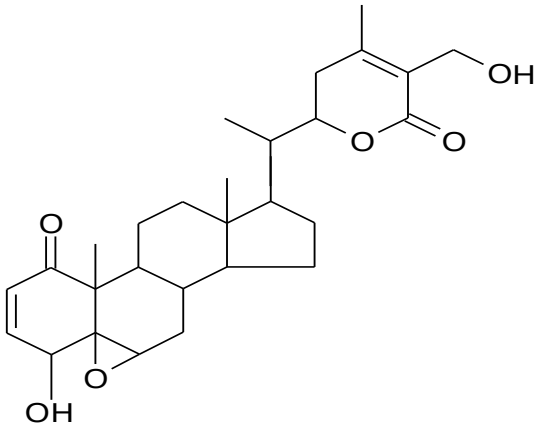
The withanolides in the plant, which have been described and evaluated, have manifested variations dependent upon cultivation and varieties [Zhao *et al.,* 2002; Choudhary *et al.,* 2005; Xu *et al.,* 2009]. Large amounts of iron are also found in the plant [Mishra *et al.,* 2000].

The leaves contain mainly the steroidal lactones, withanolides notably withaferin A which was the first withanolide to be isolated from *W. somnifera*. Nine structurally similar withanolides, namely, 27-hydroxy withanone, 17-hydroxy withaferin A, 17-hydroxy-27-deoxy withaferin A, withaferin A, withanolide D, 27-hydroxywithanolide B, withanolide A, withanone and 27-deoxywithaferin A have been identified in the leaves and roots of the plant [Choudhary *et al.,* 2005; Xu *et al.,* 2009; Mishra *et al.,* 2000].

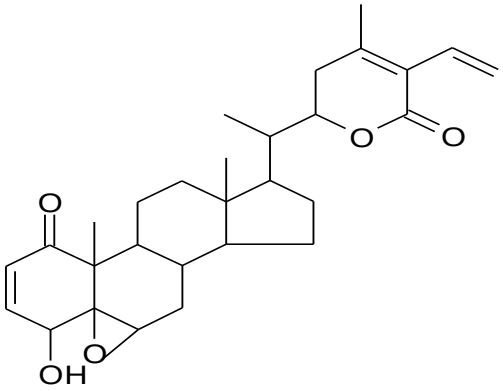
Withaferin A which is an ergostane-type steroidal lactone, is attributed to many of the pharmacological properties of *W. somnifera.* Other related bioactive compounds isolated in the plant include withanolide E, ashwagandhanolide which is a dimeric thiowithanolide, withasomniferols, and related glycosides called withanosides and sitoindosides [Zhao *et al.,* 2002; Choudhary *et al.,* 2005; Xu *et al.,* 2009]. The structure of withaferin A and selected withanolides are shown in Figure 1.13.

The yield of withaferin A in *Withania somnifera* varies. This could be explained by seasonal variations, environmental factors or different chemotypes of the plant [Evans, 1996; Hornok, 1992; Martin and Woodcock, 1983]. Investigations over the years, carried out on various sources of *Withania somnifera* plant material and concerning withaferin-A content have given differing

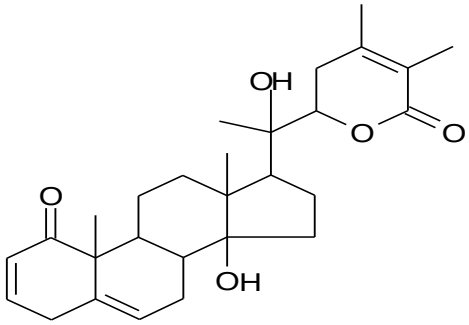
results [Evans, 2009]. Previous studies carried out by Israel scientists on the *Withania somnifera* found in that country reported withaferin-A content of 0.2% of the dry weight [Evans, 2009]. More recent studies on the Indian *Withania somnifera* plant have reported Withaferin-A content of 0.711% of the dry weight [Sangwan *et al*., 2006].



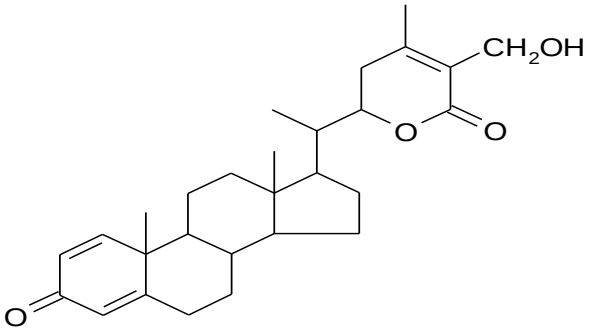
Withaferin-A



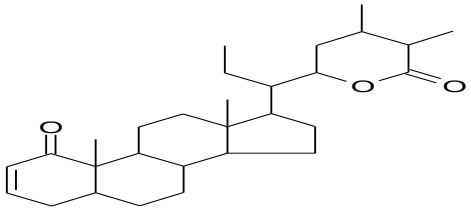
27-Deoxywithaferin-A



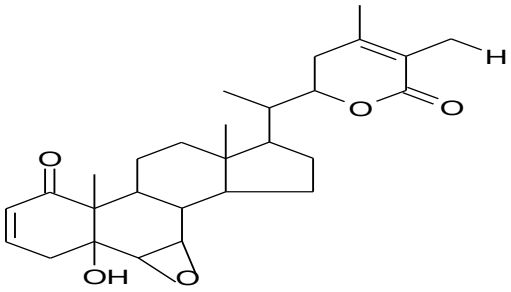
Withanolide G



Withasomidienone



Withanolide D



Daturametelin

Figure 1.13: Structures of some withanolides

In addition to their potential pharmacological value, withanolides have proved of interest because of their occurrence as chemical races of *Withania* and because of their structural variation in hybrids of different races [Evans, 2009].

1.4.7. Chemical races in *Withania somnifera*

Investigations over the years, carried out on various sources of plant material of *Withania somnifera*, and concerning the withanolides constituents, had given differing results, which were explained by the work of Abraham *et al.* [1968], on plants collected in Israel. Three chemotypes were discovered among 24 populations of *W. somnifera* [Abraham *et al.*, 1968]. Chemotype I contained predominantly withaferin A (0.2% of the dry weight), which is the principle responsible for the plant's bacteriostatic and antitumour properties. Chemotype II contains predominantly withanolide D, and Chemotype III a mixture of related compounds comprising a new group of steroidal lactones- the withanolides. The only morphological difference observed between the chemotypes was a difference in flowering time (12 days early) for chemotype III. Since then, other chemotypes of *Withania somnifera* have been reported from India and South Africa.

In a continuation of the Israel work on withanolides Eastwood *et al.* (1990), reported that a cross of a South African chemotype of *Withania somnifera* with Israel chemotype II produced three new withanolides not detected in either parent. The outstanding feature was the presence of new oxidizing system in the hybrid which apparently oxidizes ring A to a diketone in the presence of either the saturated or the unsaturated lactone. A further notable feature was the isolation of a compound having a saturated C2-C3 link, a feature which had only been noticed in a minor constituent of chemotype I [Hardman *et al.*, 1976; Evans, 2009].

1.4.8. Effects of environmental conditions and soils on withanolides of *Withania somnifera.*

No work appears to have been performed on the effects of environmental conditions as well as the effects of physical, chemical and microbiological properties of soils on withanolides production by the plant *Withania somnifera* [Evans 2009].

1.4.9. Pharmacology

1.4.9.1. Anti-inflammatory, analgesic and antipyretic effects.

In vitro and animal experiments suggest *W. somnifera* may possess anti-inflammatory properties. Cultures of cartilage from patients with osteoarthritis and rheumatoid arthritis have been used to demonstrate *W. somnifera 's* protective effects on chondroblasts [Sumantran *et al.,* 2008; Sumatran *et al.,* 2007; Singh *et al.,* 2008; Lu *et al.,* 2009]. Related effects on cytokines and transcription factors and suppression of nitric oxide have also been demonstrated [Kaileh *et al.,* 2007]. In experiments in rats with induced inflammation, decreased inflammation (paw volume), pain, and disability were noted, as well as an antipyretic effect after administration of *W. somnifera* root powder. The ulcerogenic effect of *W. somnifera* was lower than that of indomethacin [Mishra *et al.,* 2000; Rasool *et al.,* 2006; Khanna *et al.,* 2007; Al- Hindawi *et al.,* 1989; Agarwal *et al.,* 1999]. A small clinical study evaluating a combination therapy that included ashwagandha demonstrated decreased pain and disability in arthritis, while no changes were observed in the erythrocyte sedimentation rate [Kulkarni *et al.,* 2008; Mishra *et al.,* 2000]. In the study in which a polyherbal mixture was used for arthritis, *W. somnifera* 450 mg root powder was administered 4 times per day [Kulkarni, *et al.,* 1991].

1.4.9.2. Anticancer properties

In vitro and animal experiments have been conducted using whole plant extract, ethanol root extracts, aqueous and methanolic leaf extracts, individual withanolides, and withaferin A, with positive results, [Mishra *et al.,* 2000]. Human cancer cell line investigations include HL-60 leukemic and myeloid leukemia cell lines and bladder, breast, prostate, colon, kidney, gastric, and lung cancer cell lines. Mechanisms of action described include antiproliferative effects, apoptosis, radio-sensitization, mitotic arrest, antiangiogenesis and enhancement of cell defense mechanisms [Senthil *et al.,* 2007; Al-Fatimi *et al.,* 2005; Stan *et al.,* 2008; Malik *et al.,* 2007; Oh *et al.,* 2008; Srinivasan *et al.,* 2009]. Limited studies suggest withanone, withaferin A, and withanolide A have protective effects on glioma cell lines, as well as human fibroblasts and thereby slow senescence [Widodo *et al.,* 2009; Shah *et al.,* 2009].

Experiments in mice have demonstrated decreased lung adenoma tumor incidence with whole plant extract and complete regression of mouse sarcoma tumor with ethanol root extract, as well

as radio-sensitizing of carcinomas and increased apoptosis of human breast cancer cells by withaferin A, a steroidal lactone of *W. somnifera* [Mishra *et al*., 2000; Stan *et al.,* 2008].

Damage to the bladder by cyclophosphamide was ameliorated by *W. somnifera* extract, [Davis *et al.,* 2000] as was leukopenia induced by cyclophosphamide [Davis *et al.,* 1998].

1.4.9.3. Central Nervous System effects

In vitro studies and experiments in animals suggest Central Nervous System effects, including modulation of acetyl cholinesterase and butyrylcholinesterase activity, inhibition of calcium ion influx, blockade of gamma-aminobutyric acid receptors, modulation of 5-HT_1 and 5-HT_2 receptors, antioxidant activity and regeneration of neurites [Mishra *et al*., 2000; Choudhary *et al.,* 2005; Lu *et al.,* 2009] with some researchers suggesting potential applications in Alzheimer and Parkinson diseases [Shah *et al.,* 2009].

Degeneration of cholinergic neurons in the basal forebrain is associated with Alzheimer's disease. Drugs such as physiostigmine that inhibit acetylcholinesterase and hence decrease the breakdown of acetylcholine can bring improvement but this treatment has no effect on the underlying degenerative process. There is some evidence that drugs that inhibit gamma-aminobutyric acid receptors improve memory. Ashwagandha root powder has shown to produce significant improvement in senile dementia of Alzheimer type in studies carried out on elderly men over 85 years old. It has been suggested that the plant extracts improves memory and cognitive functions by both inhibition of acetyl cholinesterase and blockade of gamma-aminobutyric acid receptors [Kulkarni *et al.,* 2008; Zhao *et al.,* 2002; Choudhary *et al.,* 2005; Shah *et al.,* 2009].

Withania extract protected against pentylenetetrazol-induced seizures in a mouse anticonvulsant model when administered over a 9-week period [Kulkarni *et al.* 1996]. The same research group found the extract active in a rat status epilepticus model [Kulkarni *et al.,* 1998]. A depressant effect on the CNS was indicated by potentiation of pentobarbital effects on the righting reflex in mice, [Ahumada *et al.,* 1991] and a mild tranquilizing/relaxant effect in monkeys, cats, dogs, rats and mice by a total alkaloid extract from the plant roots [Mishra *et al*., 2000].

A further study of the extract found that it inhibited the development of tolerance to morphine in mice, while suppressing withdrawal symptoms precipitated by naloxone [Kulkarni *et al.*, 1997]. A withanolide-containing fraction reversed morphine-induced reduction in intestinal motility and confirmed the previous finding of inhibition of development of tolerance to morphine [Ramarao *et al.*, 1995]. The role of *W. somnifera* in the management of drug addiction has been suggested [Lu *et al.*, 2009].

An experiment supported the traditional Ayurvedic medicinal claim that the plant's use could be attributed to its boosting effects on learning and memory. Ibotenic acid-induced lesions in intact rat brain that led to cognitive deficit, as measured by performance in a learning task, were reversed by treatment with a withanolide mixture [Bhattacharya *et al*., 1995]. Limited trials in elderly populations using traditional combination therapies showed mixed results. In another study, enhanced body posture and increased strength and stability in limb movements was noted in elderly patients with long-term progressive degenerative cerebellar ataxia. In these elderly patients with long-term progressive degenerative cerebellar ataxia, ashwagandha 500 mg tablets were administered 3 times a day for 1 month (in combination), [Sriranjini *et al*., 2009].

1.4.9.4. Immunomodulatory effects

Withanolides inhibit murine spleen cell proliferation [Bahr *et al.*, 1982] and an extract of *W. somnifera* reversed ochratoxin's suppressive effect on murine macrophage chemotaxis [Dhuley, 1997]. Withanolide glycosides activated murine macrophages and phagocytosis and increased lysosomal enzymatic activity secreted by the macrophages, while also displaying antistress activity and positive effects on learning and memory in rats [Ghosal *et al.*, 1989]. Alpha-2 macroglobulin synthesis stimulated by inflammation was reduced by *W. somnifera* extract [Anbalagan *et al.*, 1985]. Similarly, the extract prevented myelosuppression caused by cyclophosphamide, azathioprine or prednisolone in mice [Ziauddin *et al*., 1996]. In a clinical study, ashwagandha 6 mL root extract administered twice daily for 4 days resulted in increased in CD4 expression, as well as activation of natural killer cells [Mikolai *et al*., 2009]. Additional effects on cytokines and the complement system, lymphocyte proliferation, and humoral and cell-mediated responses have been discussed [Rasool *et al.*, 2006].

1.4.9.5. Other effects

Animal experiments have been conducted to describe adaptogenic properties such as increased swimming endurance and reduced stress response) of *W. somnifera*. Aphrodisiac effects have also been demonstated in the root extracts. Anti ulcer activity has been demonstrated. It prevented gastric ulcers induced chemically or by stress in mice. [Singh *et al.,* 1982; Bhattacharya *et al.,* 1987; Grandhi *et al.,* 1994; Dhuley ,1998; Dhuley, 2000; Archana *et al.,* 1999; Panda *et al.,* 1999; Singh *et al.,* 2000, Bucci, 2000].

Effects on aging have been promoted, based on claims regarding increased hemoglobin, red blood cell count, hair quality and melanin levels in a non-peer-reviewed study conducted among healthy men. Serum cholesterol was also reduced and seated-stature improved in this study, [Widodo *et al.,* 2009]. Antimicrobial effects [Girish *et al.,* 2006] and antivenom activity via hyaluronidase inhibition have been described [Machiah *et al.,* 2006].

In the most of the phytochemical and pharmacological research studies carried so far on withanolides from *Withania somnifera,* withaferin-A is the most well studied active compound. Withaferin-A appears to occur in the plant in relatively higher proportions in the plant than the other withanolides hence it can act as a good marker for the standardization and quality control of *Withania somnifera* formulations [Sangwan *et al*., 2006; Ganzera *et al*., 2003; Evans, 2009].

Withaferin-A appears to act in conjunction or in synergy with the other withanolides to bring about the pharmacological effects already mentioned previously. This is in agreement with the established scientific fact that one of the tenets of herbal medicine is that the maximum effectiveness of the drug derives from the whole drug or its crude extract, rather than from the isolated components [Singh *et al.,* 2000; Evans, 2009; Ghosal *et al.,* 1989, Bahr *et al.,* 1982]

In conclusion, it is evident from the literature review that the phytochemistry and pharmacological properties of *Withania somnifera* have been extensively studied and this medicinal plant has demonstrated great potential as a nutraceutical and herbal pharmaceutical [Ganzera *et al*., 2003] Majority of the pharmacological effects have been associated with Withaferin A [Bahr *et al.,* 1982; Ghosal *et al.,* 1989]. However, it is clearly apparent that more phytochemical and pharmacological studies need to be carried out on the plant *Withania somnifera* to validate the established phytochemical and pharmacological properties and also to

come up with new possible phytochemical and pharmacological properties of the plant [Davis *et al.,* 2000; Davis *et al.,* 1998].

1.5. STUDY JUSTIFICATION

Withania somnifera is a medicinal plant that is endangered due to over use. In Kenya, the plant grows naturally in some semi-arid areas. The roots of this plant are used to make herbal medications by Kenyan traditional medical practitioners. The whole plant is uprooted to obtain the roots and thus there is need to promote sustainable use of the plant which is endangered due to overuse. Promoting the cultivation of this important medicinal plant would be of paramount importance to ensure sustainability of its use.

There is no data on the cultivation and adaptability of *Withania somnifera* in Kenya and how to make this nutraceutical an economically viable product. No studies have been done on the Kenyan plants to find if there are chemical varieties. The Withaferin A content for Kenyan plants is unknown. Majority of the pharmacological effects have been associated with Withaferin A. No commercial product is available in Kenya based on *Withania somnifera* from Kenyan plants although there are imported products in the market.

Health remains a key concern that needs new approaches. The plant holds great potential as herbal remedy for a host of health problems. As is evident from the literature, the potential of *Withania somnifera* to improve human health as a nutraceutical is very great due to its good health effects. The emergence of chronic diseases with poor therapeutic alternatives, desire for personalized medicines, large population trying to stave off the effects of aging and new focus on preventive medicine, places this plant as a very significant candidate for addressing these problems.

These studies may thus play a great role in determining the potential of economic exploitation of this plant. The economic gain, potential for employment and poverty alleviation for this high value plant is in line with the Kenya's vision 2030. It is expected that this study will provide the best source of *Withania somnifera* for cultivation in Kenya which could be commercially exploited for formulation of nutraceuticals and other pharmaceutical products.

1.6. OBJECTIVES

1.6.1 General objective

The objective of this study was to quantitatively determine the withaferin-A content of the root, stem and leaf extracts of *Withania somnifera* (L.) Dunal collected from different locations in Kenya.

1.6.2. Specific objectives

(i) To carry out preparation of plant extracts for analysis.

(ii) To validate the analytical method used in analysis of samples.

(iii) To quantitatively determine the withaferin-A content in the root, stem and leaf extracts of the plant and the imported *Withania somnifera* formulations.

(iv) To determine stability of withaferin-A at different temperature conditions.

CHAPTER TWO

METHODOLOGY

2.1. Plant collection, identification and preservation

The plant was collected from different areas where it is known to grow naturally. These areas were Kathonzweni in Makueni County, Naivasha in Nakuru County, Kitengela in Kajiado County, Kyumbi in Machakos County, and Ruai and Syokimau areas in Nairobi County, as shown in Table 2.1 below. The plant under cultivation in Limuru region in Kiambu County and in Ruai region in Nairobi County was also collected (also shown in Table 2.1).

The plant material was identified at the Department of Botany, University of Nairobi. Voucher specimens were deposited in the herbarium of the Department of Pharmacology and Pharmacognosy, School of Pharmacy, University of Nairobi, under Voucher numbers JM2011A1, JM2011A2, JM2011A3, JM2011A4, JM2011A5, JM2011A6, JM2011A7 and JM2011A10.

The locally available imported commercial formulations based on *Withania somnifera* were purchased in Kunjus Herbal Products limited, in Ngara, Nairobi (Table 2.2 below)

The roots, stems and leaves materials from *Withania somnifera* plant, and the two imported commercial preparations were used to prepare test samples.

Table 2.1: *Withania somnifera* Plant collected in Kenya.

Sample Designation	Part of plant Collected	Where collected County (region)
RTA1	Root	Nairobi (Syokimau)
LVA1	Leaf	Nairobi (Syokimau)
STA1	Stem	Nairobi (Syokimau)
RTA2	Root	Machakos (Kyumbi)
LVA2	Leaf	Machakos (Kyumbi)
STA2	Stem	Machakos (Kyumbi)
RTA3	Root	Makueni (Kathonzweni)
LVA3	Leaf	Makueni (Kathonzweni)
STA3	Stem	Makueni (Kathonzweni)
RTA4	Root	Nakuru (Naivasha)
LVA4	Leaf	Nakuru (Naivasha)
STA4	Stem	Nakuru (Naivasha)
RTA5	Root	Kajiado (Kitengela)
LVA5	Leaf	Kajiado (Kitengela)
STA5	Stem	Kajiado (Kitengela)
RTA6	Root	Nairobi (Ruai)*
LVA6	Leaf	Nairobi (Ruai)*
STA6	Stem	Nairobi (Ruai)*
RTA7	Root	Kiambu (Limuru)*
LVA7	Leaf	Kiambu (Limuru)*
STA7	Stem	Kiambu (Limuru)*
RTA10	Root	Narok (Town environs)
LVA10	Leaf	Narok (Town environs)
STA10	Stem	Narok (Town environs)

*****Under cultivation

Table 2.2: Imported commercial products based on *Withania somnifera*.

Brand name of commercial formulation	Part of the plant used to make the formulation	Country of origin	Manufacturer	Designation of the sample used in the analysis
Ashwagandha Churna powder	Root	India	Baidyanath	RTA8
Ashvagandha capsules	Root	India	Himalaya	RTA9

2.2. Materials, Reagents and Equipment

2.2.1. Materials and Reagents

Withaferin-A Reference Standard (99.4%) from Sigma-Aldrich Company Ltd (St. Lous, Mo, U.S.A.) and was purchased and imported through Kobian Scientific Kenya Ltd (Nairobi, Kenya).

Methanol and *n*- hexane were from Fischer Scientific U.K. Ltd (Loughborough, U.K.) while chloroform and acetonitrile were from Sigma Aldrich (Bangalore, India). Methanol was of HPLC grade while *n*-hexane was of analytical grade. Water was freshly distilled in the laboratory.

2.2.2. Equipment

Rotary vaporator, HPLC reverse phase column**,** Platform shaker, High-pressure vacuum pump and Analytical weighing balance.

2.2.3. Chromatographic system

A Shimadzu HPLC system (Shimadzu HPLC Class *VP* series) consisting of Prominence Liquid Chromatograph model LC- 20 AT *VP* pumps (Shimadzu Corporation, Kyoto, Japan), Prominence UV/ Vis detector model SPD-20A *VP* (Shimadzu Corporation, Kyoto, Japan), Prominence Column oven model CTO-10AS *VP* (Shimadzu corporation, Kyoto, Japan), Prominence Degaser model DGU-20A_3 *VP* (Shimadzu Corporation, Kyoto, Japan), Prominence Communications Bus Module model CBM-20A *VP* (Shimadzu Corporation, Kyoto, Japan). For all separations a Reverse Phase Gemni 5µ C-18 110A Phenomenex column (C-18 phase ,250 x 4.6 mm, 5 µm particle size) from Phenomenex (Tollance, CA, USA) was used. The HPLC system was equipped with Class *VP* series version 6.1 software (Shimadzu Corporation, Kyoto, Japan).

2.3. Preparation of the plant material for extraction

Collected roots, stems and leaves of the plant from the different locations were air dried at ambient temperatures in the shade for thirty days, and ground into fine powder. The powders were kept at room temperature in a dry place until used for extraction.

2.4. Preparation of the plant extracts

Five grams, ten grams and twenty grams each of air-dried powdered leaf, stem and root plant materials respectively were extracted three times with 20 ml each time with methanol-water (60:40) according to United States Patent No. US 7108870 B2 of Sep. 19, 2006, by Sangwan *et al*, 2006. The extractions were done in an Erlenmeyer flask by shaking on a platform shaker (10-30 RPM) for 8 hrs.

The extractions were recovered by filtration and the filtrates from the three extractions in each case were pooled and subjected to solvent-solvent partitioning with 3x60 ml *n*-hexane to remove pigments and fatty materials. The defatted and depigmented extract was subjected to solvent-solvent partitioning with 3x60 ml chloroform to recover withanolidal fraction including withaferin A in the chloroform layer. The chloroform fractions were pooled and evaporated to dryness. The dry residue of each plant extract was stored in a capped vial in a refrigerator at a temperature of between 2-8 ºC until the next process which was subjection to HPLC analysis. This extraction process was applied also to the procured commercial products based on *Withania somnifera*.

The extraction process of the plant material is illustrated in the flow chart in figure 2.1 below

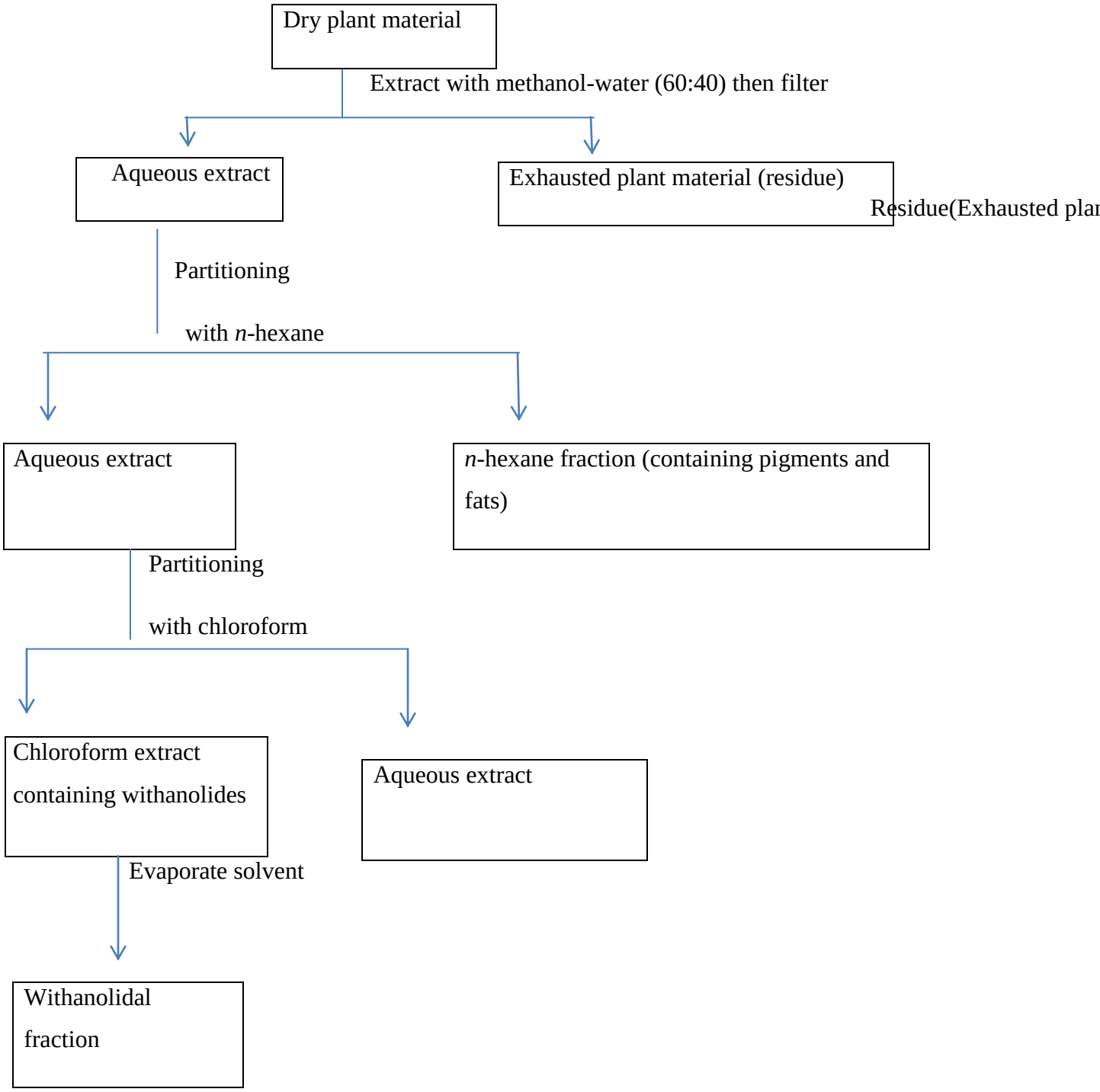


Figure 2.1. Flow chart of the extraction process of the plant material

2.5. Preparation of the samples for HPLC analysis

The residue (i.e. the dry powder) obtained from the extracts was dissolved in known volume (2.0 ml) of HPLC-grade methanol. Prior to use, all samples were filtered through a 0.22-µm Millex GV membrane filter model PF08535 (Carrigitwohill, Co Cork, Ireland) and 20 µl was subjected to high performance liquid chromatograhic (HPLC) analysis.

2.6. Sample injection

Rheodyne syringe (Model 7202, Hamilton) was used for injecting 20 µl of respective samples. Every sample solution was injected in triplicate. Relative standard deviations were below 2.0% for all experiments.

2.7. Calibration

Four milligrams of the standard compound (withaferin-A) was placed in one 5-ml volumetric flask and dissolved in methanol (stock solution). Six additional calibration levels were prepared by diluting this solution with methanol. The peak areas obtained from these solutions are presented in Table 3.2. Within the range of concentrations injected (800-30 µg/ml) the detector response was linear. The coefficient of determination (R^2) was 0.9966, prove of linearity. All data were recorded and processed by Millenium 32 software from Waters (Milford, MA, USA).

2.8. Analytical method.

Methods for determination of Withaferin A in *Withania species* have been developed and validated. Shaila *et al*, 2006 developed and validated a HPLC method suitable for direct determination of withaferin-A in *W. coagulans* and *W. somnifera* and investigated the distribution of this compound in different plant parts.

Quantitative determination of withaferin-A in the samples was undertaken according to the published method by Shaila *et al*, 2006. RP-HPLC was performed using mobile phase consisting acetonitrile (B) and water (A) whereby the mobile phase composition was acetonitrile/water (75:25). The separation temperature was kept at 27 ^{o}C, flow rate and sample volume was set to 1.0 ml/min and 20 µl, respectively. The detection and quantification were performed at a wavelength of 225 nm.

The mean peak areas at the mean retention times of the HPLC chromatogram of standard withaferin-A at an optimum wavelength of 225 nm was noted. The retention times and the peak areas of the HPLC chromatograms of roots, stems and leaves extracts of *W. somnifera* corresponding to standard withaferin-A at a wavelength of 225 nm were noted. The quantitative evaluation of withaferin-A in roots, stems, and leaves of *W. somnifera* was done through calculation by use of a calibration curve.

The method developed by Ganzera *et al.,* [2003], which was modified and adopted for this particular experiment, was subjected to validation protocols, in accordance to USP. Various analytical and statistical parameters were investigated for validation purposes. These parameters included linearity, accuracy, precision, sensitivity and ruggedness.

2.9. Validation parameters of analytical method

2.9.1. Precision.

The precision of the method was studied by injecting a single sample solution (the standard withaferin-A) five times and finding out the standard deviation and coefficient of variation.

2.9.2. Accuracy.

A recovery experiment was performed to confirm the accuracy of the method. Sample RTA6 (20.0 gm) was spiked with 1.00ml of the standard stock solution, and then extracted and analyzed under optimized conditions.

2.9.3. Linearity

Standard withaferin-A solutions of 0.010%, 0.020%, 0.025%, 0.040%, 0.050%, 0.060%, and 0.080% were used for studying the linearity. The peak areas obtained from these solutions were plotted on a linearity curve. The coefficient of determination (R^2) and regression equation for the standard compound (withaferin-A) were derived.

2.9.4. Sensitivity

The sensitivity of the method was investigated by determining the limit of detection (LOD) and the limit of quantitation (LOQ). This determination was based on signal-to-noise ratio (S/N) that

was performed by comparing measured signals of known concentrations of withaferin-A with those of blank. The S/N was calculated from the equation:

S/N=2H/h

Where H is the peak of interest and h is the range of the background noise in a chromatogram obtained after injection (British pharmacopoeia, 2012)

2.10. Efficiency of extraction

Four different solvents (methanol, water, chloroform and methanol/water (60:40) mixture were used to investigate the efficiency of extraction on sample RTA6. 20.0 g of sample RTA6 was extracted using each of the four solvents and quantification of withaferin-A carried out.

2.11. Stability of samples

Quantification of withaferin-A in the extract of sample RTA6 subjected to room temperature for two weeks was carried out and the results tabulated. Quantification of withaferin-A in the extract of sample RTA6 subjected to three freeze thaw cycles was also carried out and the results tabulated. Long term stability (30 days) was also carried out on the same sample and the results tabulated.

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1. Quantitative HPLC determination of withaferin-A content.

3.1.1. Percentage weights of the dry plant extracts obtained after extraction of each sample.

Table 3.1: Weights of the plant extracts

Sample	Weight of dry plant extract (g)	**Percentage weight of dry plant extract obtained (g/100g)
RTA1	0.2795	1.40
LVA1	0.0466	0.93
STA1	0.0601	0.60
RTA2	0.1222	0.61
LVA2	0.0587	1.17
STA2	0.0812	0.81
RTA3	0.1320	0.66
LVA3	0.0705	1.41
STA3	0.0785	0.79
RTA4	0.2771	1.39
LVA4	0.1080	2.16
STA4	0.0888	0.89
RTA5	0.0840	0.42
LVA5	0.0583	1.17
STA5	0.0511	0.51
RTA6	0.2101	1.05
LVA6	0.0312	0.62
STA6	0.0644	0.64
RTA7	0.2842	1.42
LVA7	0.1383	2.77
STA7	0.1120	1.12
RTA8*	0.0805	0.40
RTA9*	0.0762	0.38
RTA10	0.2724	1.36
LVA10	0.1439	2.88
STA10	0.0882	0.88

*Imported commercially available *Withania somnifera* products in the Kenyan market

(RTA8=Ashwagandha root powder sample, RTA9=Ashvagandha capsules. 20.0g of each of the commercial samples was used to carry out the extraction. The quantities of powdered dry plant material used in the solvent extraction process were 5.0 g for the leaf samples, 10.0 g for the stem samples and 20.0 g for the root samples. RT=Root, LV=Leaf, ST=Stem).

******Percentage weight of dry plant extract:

$$= \frac{\text{Weight (g) of dry plant extract obtained after extraction} \times 100}{\text{Weight (g) of dry powdered plant material used for extraction}}$$

3.1.2 Calibration

The peak areas counts obtained from the various concentrations of withaferin-A standard solutions are presented in Table 3.2. Calibration curve was plotted as the peak area versus concentration (Figure 3.1). Within the range of concentrations injected (800-30 µg/ml) the detector response was linear (Table 3.3).

Table 3.2: Calibration solutions for withaferin-A standard

Concentration of withaferin-A (%w/v)	Peak areas
0.010	27318
0.020	62228
0.025	68786
0.040	107370
0.050	138611
0.060	167796
0.080	212415

Table 3.3: Coefficient of determination (R^2), regression equation and limit of detection (LOD) for the standard compound (withaferin-A).

Compound	**R^2**	**Regression equation***	**LOD** (µg/ml)
Withaferin-A	0.9966	y=264.64*X* + 4322.2	20.00

*y=Peak area; *X*=Concentration (µg/ml)

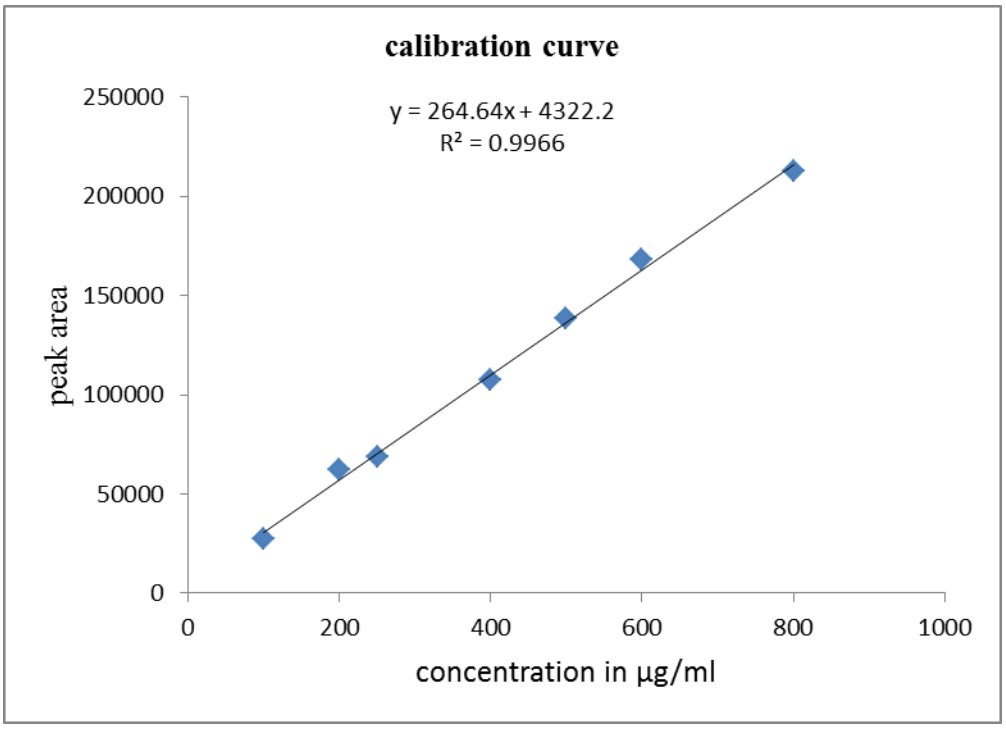


Figure 3.1: Calibration curve for the various concentrations of the standard (withaferin-A)

3.1.3. Validation parameters of analytical method

3.1.3.1. Precision.

The peak areas obtained by injecting a single sample solution (the standard withaferin-A) five times are reported in Table 3.4.

Table 3.4: Peak areas obtained by injecting replicate samples of 0.04% w/v of standard withaferin-A.

Concentration of Withaferin-A [%w/v]	**Peak areas**
0.04	107370
0.04	106956
0.04	107243
0.04	107502
0.04	106997

In the study on precision, the standard deviation and coefficient of variation were found to be 0.526 and 0.858 (Table 3.4). Low value of standard deviation and coefficient of variation were indicative of high precision of the method. All standards and samples were injected in triplicate. The resulting R.S.D. of less than 2.0% confirmed the precision of the method.

3.1.3.2. Accuracy

Accuracy of the method was confirmed by performing recovery experiments. Results for recovery of withaferin-A in sample RTA6 when the sample was spiked with a known amount of the authentic compound (spiked with 1ml of 0.08% w/v standard stock solution), extracted and analyzed are shown in Table 3.5. Compared to the theoretical amount, recovery of 98.75% for withaferin-A was obtained.

Table 3.5: Recovery of withaferin-A in sample RTA6.

Sample designation	Withaferin-A content of unspiked sample (µg/g) (*n=6) (Mean±S.D)	Withaferin-A content in the spiked sample (µg/g) (*n=6) Mean±S,D	Withaferin-A content recovered (µg/g) (*n=6) Mean±S.D.	Percentage recovery of withaferin-A
RTA6	4601±21	5391±43	790±34	98.75

*n=number of replicate samples injected; S.D. is standard deviation

3.1.3.3 Linearity

The peak areas obtained from various concentrations of the standard were plotted on a linearity curve and the coefficient of determination (R^2) and regression equation for the standard compound (withaferin-A) determined.

Linearity of the detector response for the standard compound (withaferin-A) was confirmed between 800.00 and 30.00 µg/ml, with a detection limit of at least 20µg/ml (section 3.1.2). It can be noted from the calibration curve that withaferin-A showed good linearity in the concentration range of 0.01%w/v - 0.08w/v %, with a coefficient of determination (r^2) of 0.9966

3.1.3.4. Sensitivity

The results for the sensitivity of the method which was investigated by determining the limit of detection (LOD) and the limit of quantitation (LOQ) are reported in Table 3.6

Table 3.6: Limit of detection and limit of quantitation of withaferin-A standard.

Compound	Limit of Detection	Limit of Quantitation (*n=6)	
	(µg/ml)	(µg/ml)	**C.V of peak areas
Withaferin-A reference standard	20	30	12.5

**C.V= Coefficient of Variation of the peak areas of the limit of quantitation

*n= Number of replicate samples injected.

The determination of limit of detection (LOD) and the limit of quantitation (LOQ) was based on signal-to-noise ratio that was performed by comparing measured signals of known concentrations of withaferin-A with those of blank. A signal-to-noise ratio of 3:1 was needed for establishing LOD while a signal-to-noise ratio of 10:1 with a coefficient of variation (CV) of less than 20% was needed for LOQ.

Signal-to-noise ratio of 3:1, which is acceptable for establishing LOD was obtained at analyte level of 20µg/ml. Signal-to-Noise ratio of 10:1 with a coefficient of variation of peak areas of less than 20% which is acceptable for establishing LOQ was obtained at analyte level of 30 µg/ml (Table 3.6). These values show that reliable quantitative results even at low solute concentrations are obtainable.

These analytical and statistical parameters determined with regard to method validation indicate the adequate reliability and reproducibility of this method within the analytical range

3.1.4. Efficiency of extraction

The results on efficiency of extraction whereby four different solvents (methanol, water, chloroform and methanol/water (60:40) mixture were used to investigate the efficiency of extraction on sample RTA6 are reported in Table 3.7.

Table 3.7: Comparison of the efficieny of withaferin-A extraction from sample RTA6 in four different solvents.

Solvent used for extraction	Methanol	Water	Chloroform	Methanol/water (60:40)
Content of withaferin-A obtained (µg/g) *(n=6) (Mean ±S.D.)	976.22±21.24	1602.32±25.78	502.73±22.27	4711.24±22.65
Percentage content of withaferin-A obtained (g/100g)	0.10	0.16	0.05	0.47

*****n= number of replicate samples injected; S.D. is standard deviation

Methanol-water mixture (60:40) showed the highest efficiency of extraction on sample RTA6 when compared with methanol alone, water alone and chloroform alone. Methanol-water mixture (60:40) was able to yield 4.83 times, 2.94 times and 9.40 times more withaferin-A content than methanol, water and chloroform respectively (Table 3.8).

3.1.5. HPLC chromatograms.

3.1.5.1. Standard withaferin-A.

The HPLC chromatogram of standard withaferin-A at the optimum wavelength of 225 nm, showed a mean retention time of 7.761min (Figure 3.2 and Appendix A).

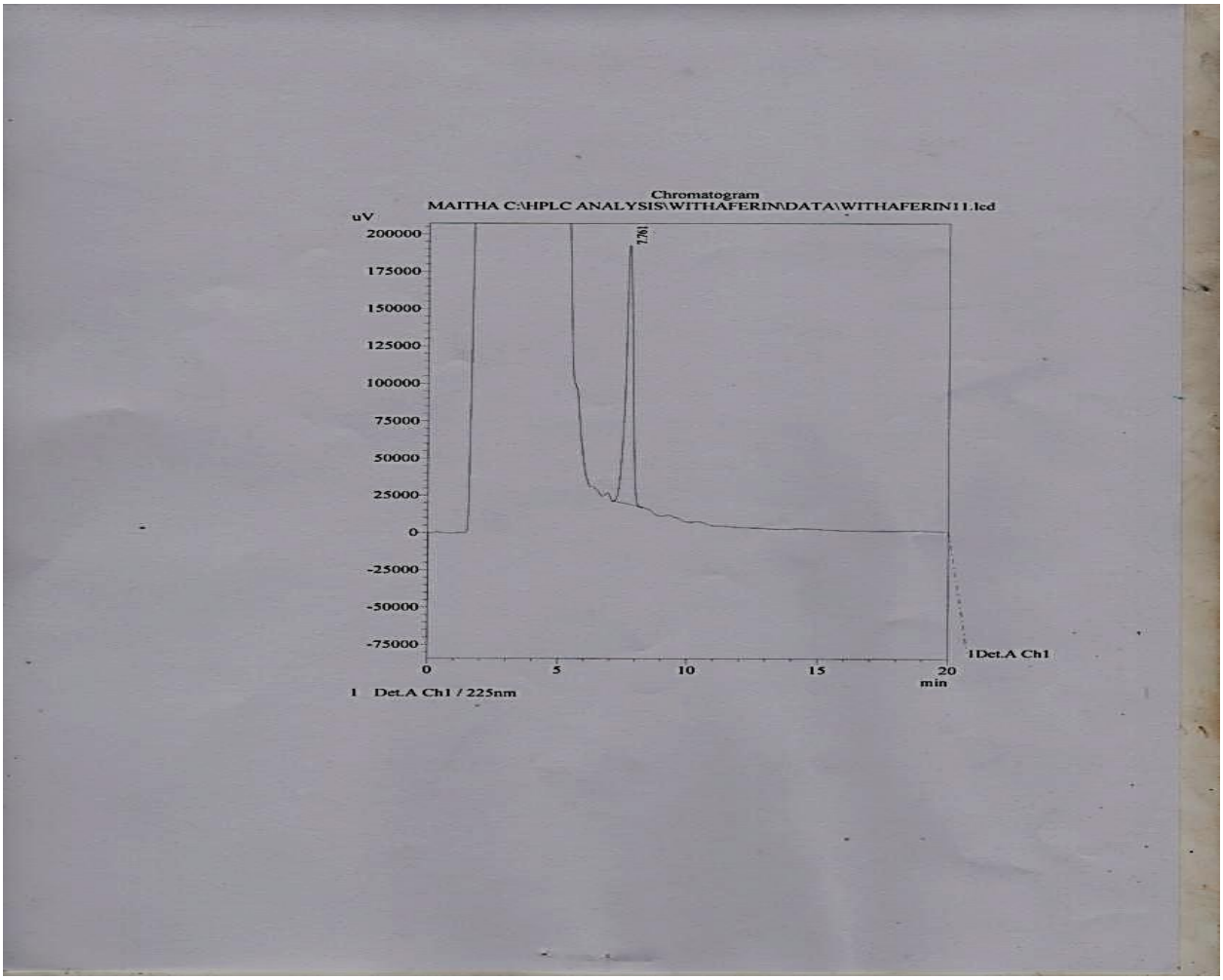


Figure 3.2: HPLC chromatogram of Withaferin-A reference standard

Chromatographic conditions: Column: Reverse Phase Gemni 5µ C-18 110A Phenomenex column (C-18 phase, 250 x 4.6 mm, 5 µm particle size), Column temperature: 27ºC. Mobile phase: Acetonitrile/ Water (75:25). Flow rate: 1ml/min. Detection: UV detection at 225nm.

3.1.5.2. Root extract

The HPLC chromatogram of root extract of *W. somnifera* corresponding to standard withaferin-A was shown at a retention time of 7.836 min, [Figure 3.3 and Appendix D]

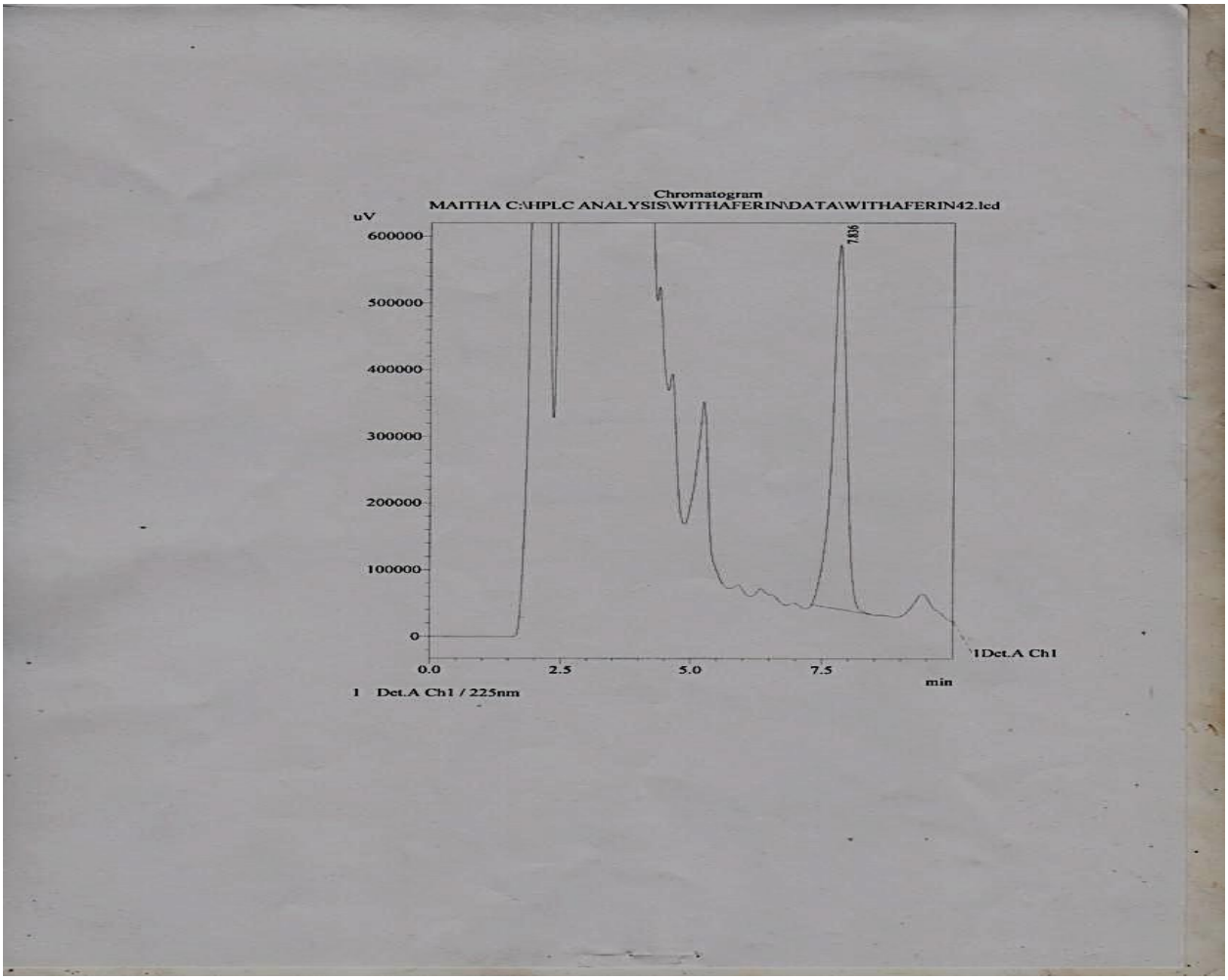


Figure 3.3: **HPLC chromatogram of *Withania somnifera* root**

Chromatographic conditions: Column: Reverse Phase Gemni 5µ C-18 110A Phenomenex column (C-18 phase, 250 x 4.6 mm, 5 µm particle size), Column temperature: 27ºC. Mobile phase: Acetonitrile/ Water (75:25). Flow rate: 1ml/min. Detection: UV detection at 225nm.

3.1.5.3. Leaf extract

The HPLC chromatogram of leaf extract of *W. somnifera* corresponding to standard withaferin-A was shown at a retention time of 7.596 min [Figure 3.4 and Appendix C].

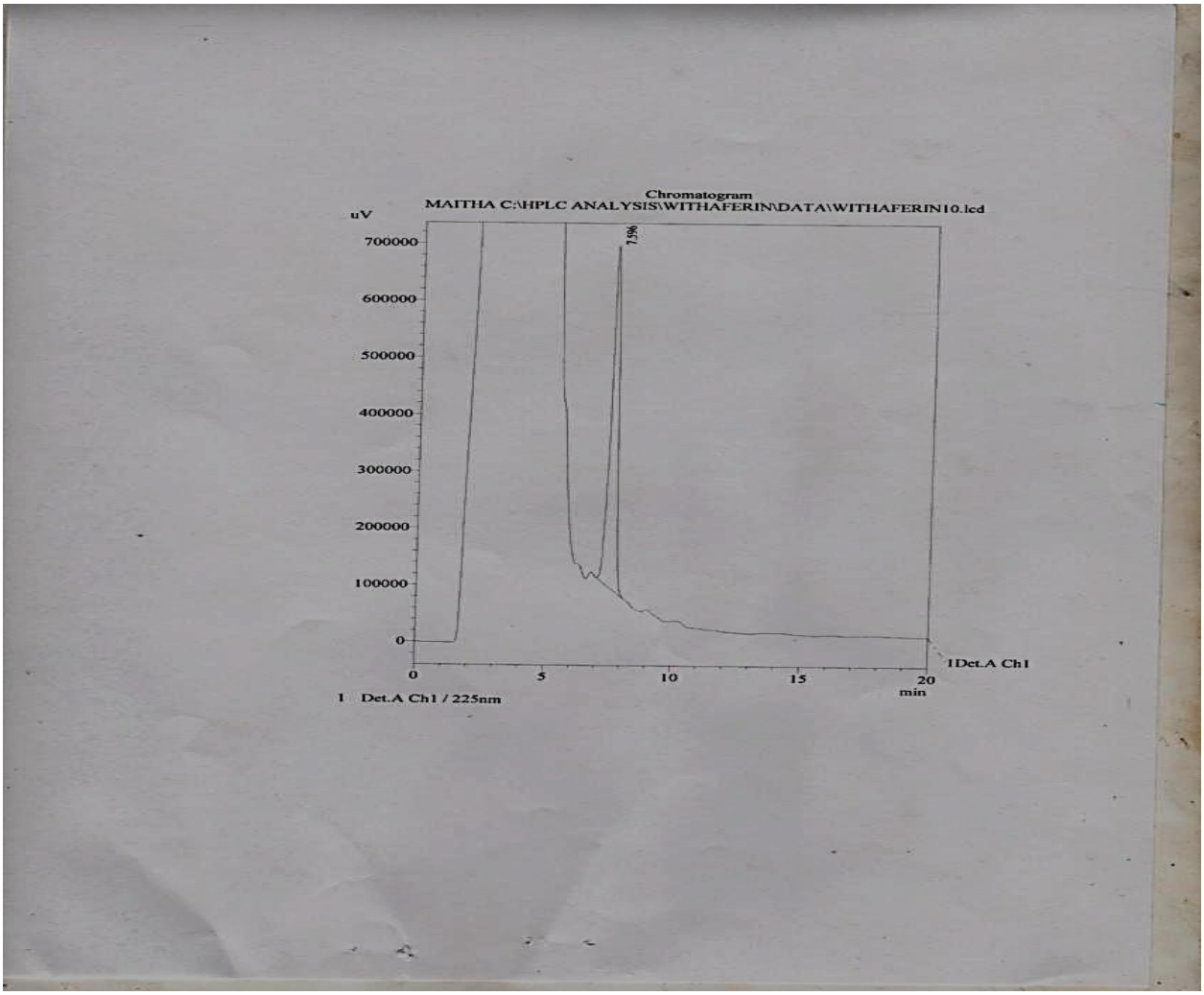


Figure 3.4: **HPLC chromatogram of *Withania somnifera* leaf**

Chromatographic conditions: Column: Reverse Phase Gemni 5µ C-18 110A Phenomenex column (C-18 phase, 250 x 4.6 mm, 5 µm particle size), Column temperature: 27ºC. Mobile phase: Acetonitrile/ Water (75:25). Flow rate: 1ml/min. Detection: UV detection at 225nm.

3.1.5.4. Stem extract

The HPLC chromatogram of stem extract of *W. somnifera* corresponding to standard withaferin-A was shown at a retention time of 7.793 min [Figure 3.5 and Appendix B]

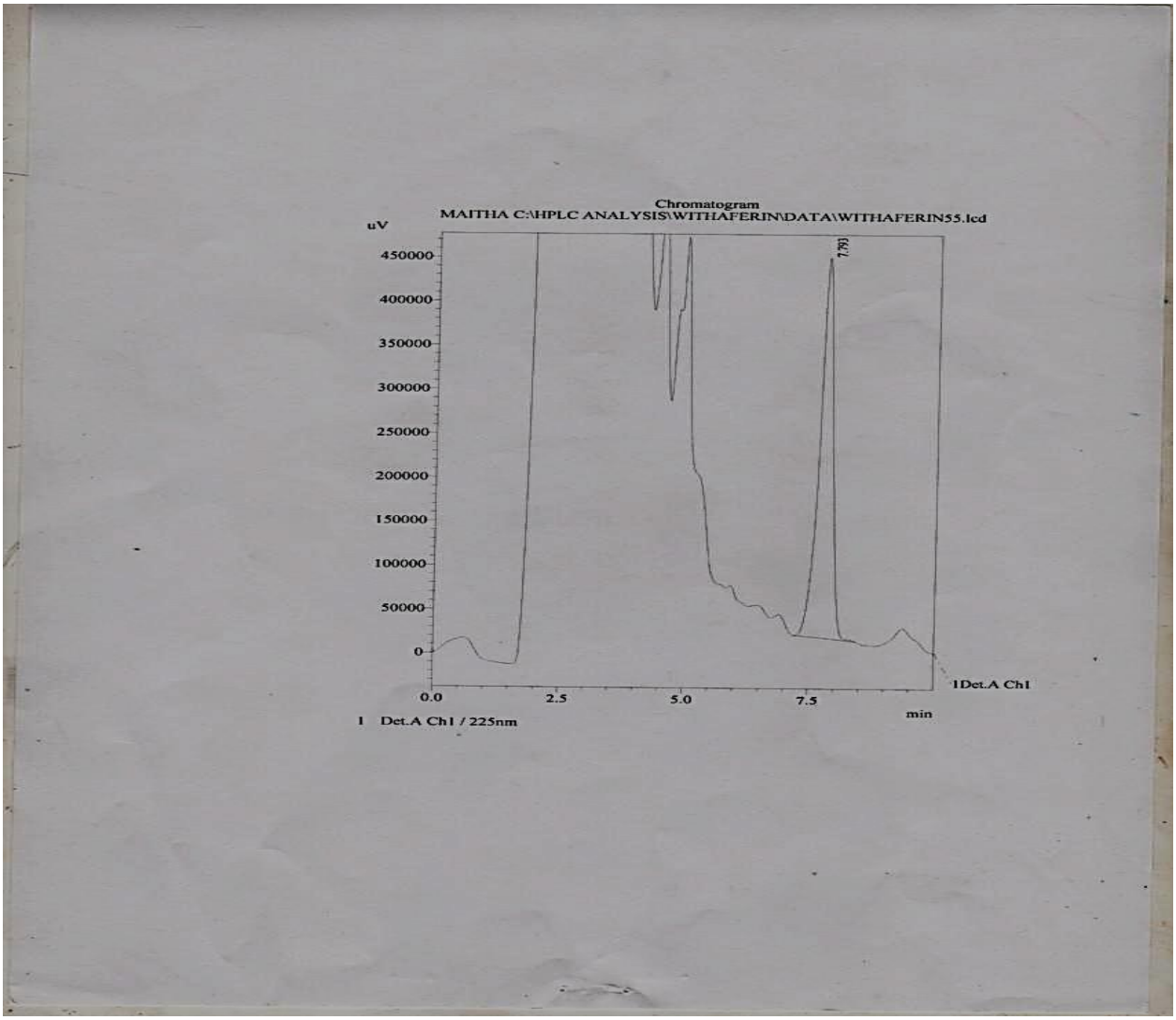


Figure 3.5: **HPLC chromatogram of *Withania somnifera* stem**

Chromatographic conditions: Column: Reverse Phase Gemni 5µ C-18 110A Phenomenex column (C-18 phase, 250 x 4.6 mm, 5 µm particle size), Column temperature: 27ºC. Mobile phase: Acetonitrile/ Water (75:25). Flow rate: 1ml/min. Detection: UV detection at 225nm.

3.1.6. Peak areas of withaferin-A in the plant extracts:

3.1.6.1. Leaf samples

Table 3.8: Peak areas of the stock and diluted solutions of *Withania somnifera* leaf extracts

Sample	Percentage weight of dry plant leaf extract obtained from dry leaf powder (g/100g)	concentration after dissolving the extract in 2.0ml methanol (mg/ml) (stock)	Peak areas obtained with the initial concentrations	Dilution factor (with methanol)	Concentration (mg/ml) of the extract obtained after the dilution	Peak areas obtained after the dilution
LVA1	0.93	23.30	2,552597	30	0.7766	126756
LVA2	1.17	29.35	3,954318	40	0.7338	103083
LVA3	1.41	35.25	6,652822	50	0.7050	102358
LVA4	2.16	54.00	8,868272	60	0.9000	145890
LVA5	1.17	29.15	3,287404	30	0.9710	149979
LVA6	0.62	15.60	1,385377	20	0.7800	133738
LVA7	2.77	69.15	9,224903	80	0.8644	139787
LVA10	2.89	71.95	12,035077	80	0.8994	143890

3.1.6.2. Stem samples

Table 3.9: Peak areas of the stock and diluted solutions of *Withania somnifera* stem extracts

Sample	Percentage weight of dry plant stem extract obtained from dry stem powder (g/100g)	concentration after dissolving the extract in 2.0ml methanol (mg/ml) (stock)	Peak areas obtained with the initial concentrations	Dilution factor (with methanol)	Concentration (mg/ml) of the extract obtained after the dilution	Peak areas obtained after the dilution
STA1	0.60	30.05	5,317282	60	0.5008	46250
STA2	0.81	40.60	7,307893	80	0.5075	48155
STA3	0.79	39.25	6,454168	100	0.3925	38278
STA4	0.89	44.40	9,770818	80	0.5550	63773
STA5	0.51	25.55	4,998482	50	0.5110	49229
STA6	0.64	32.20	5,344838	60	0.5367	49646
STA7	1.12	56.00	13,888177	100	0.5600	69265
STA10	0.88	44.10	7,984955	80	0.5512	60662

3.1.6.3. Root samples

Table 3.10: Peak areas of the stock and diluted solutions of *Withania somnifera* root extracts

Sample	Percentage weight of dry plant root extract obtained from dry root powder (g/100g)	concentration after dissolving the extract in 2.0ml methanol (mg/ml) (stock)	Peak areas obtained with the initial concentrations	Dilution factor (with methanol)	Concentration (mg/ml) of the extract obtained after the dilution	Peak areas obtained after the dilution
RTA1	1.40	139.75	20,390277	200	0.6988	102195
RTA2	0.61	61.10	10,141381	110	0.5555	57209
RTA3	0.66	66.00	10,659843	110	0.6000	68721
RTA4	1.39	138.55	19,951353	200	0.6928	100623
RTA5	0.42	42.00	7,681660	80	0.5250	55166
RTA6	1.05	105.05	15,117163	170	0.6179	75947
RTA7	1.42	142.10	22,460702	200	0.7105	106394
RTA10	1.36	136.20	18,561336	200	0.6810	97984

3.1.6.4: Locally available imported root powder formulations of *Withania somnifera*

Table 3.11: Peak areas of the stock and diluted solutions of *Withania somnifera* root extracts

Sample	Percentage weight of dry plant root extract obtained from dry root powder (g/100g)	concentration after dissolving the extract in 2.0ml methanol (mg/ml) (stock)	Peak areas obtained with the initial concentrations	Dilution factor (with methanol)	Concentration (mg/ml) of the extract obtained after the dilution	Peak areas obtained after the dilution
RTA8	0.40	40.25	7,403583	70	0.5750	64134
RTA9	0.38	38.10	6,414468	70	0.5443	63118

3.1.7. Concentrations of withaferin-A in the plant extracts.

3.1.7.1. Leaf samples

Table 3.12.: Concentrations of withaferin-A in the diluted and in the stock undiluted solutions of the leaf extracts.

Sample	Concentration of withaferin-A (in µg/ml) in the diluted samples as obtained from the calibration curve **(C)**	Dilution factor of the original solution of each sample **(D)**	Calculated concentrations of withaferin-A (in mg/ml) in the original undiluted solution **(C×D)**
LVA1	462.64	30	13.8793
LVA2	373.18	40	14.9275
LVA3	370.45	50	18.5225
LVA4	534.94	60	32.0967
LVA5	550.40	30	16.5119
LVA6	489.03	20	9.7805
LVA7	511.88	80	41.9507
LVA10	527.39	80	42.1910

3.1.7.2. Stem samples

Table 3.13: Concentrations of withaferin-A in the diluted and in the stock undiluted solutions of stem extracts.

Sample	Concentration of withaferin-A (in µg/ml) in the diluted samples as obtained from the calibration curve **(C)**	Dilution factor of the original solution of each sample **(D)**	Calculated concentrations of withaferin-A (in mg/ml) in the original undiluted solution **(C×D)**
STA1	158.43	60	9.5060
STA2	165.63	80	13.2506
STA3	128.31	100	12.8310
STA4	224.50	80	17.9598
STA5	169.69	50	8.4845
STA6	171.27	60	10.2760
STA7	245.40	100	24.5401
STA10	212.89	80	17.0314

3.1.7.3. Root samples

Table 3.14: Concentrations of withaferin-A in the diluted and in the stock undiluted solutions of root extracts.

Sample	Concentration of withaferin-A (in µg/ml) in the diluted samples as obtained from the calibration curve **(C)**	Dilution factor of the original solution of each sample **(D)**	Calculated concentrations of withaferin-A (in mg/ml) in the original undiluted solution **(C×D)**
RTA1	369.83	200	73.9660
RTA2	199.85	110	21.9830
RTA3	243.35	110	26.7680
RTA4	363.90	200	72.7789
RTA5	192.12	80	15.3700
RTA6	270.65	170	46.0106
RTA7	385.70	200	77.1403
RTA10	353.92	200	70.7845

3.1.7.4. Locally available imported root powder formulations of *Withania somnifera*

Table 3.15: Concentrations of withaferin-A in the diluted and in the stock undiluted solutions of the imported root powder extracts

Sample	Concentration of withaferin-A (in µg/ml) in the diluted samples as obtained from the calibration curve **(C)**	Dilution factor of the original solution of each sample **(D)**	Calculated concentrations of withaferin-A (in mg/ml) in the original undiluted solution **(C×D)**
RTA8	226.01	70	15.8209
RTA9	222.17	70	15.5521

3.1.8: Percentage content of withaferin-A in the *Withania somnifera* plant material.

3.1.8.1. Leaf samples

Table 3.16: Percentage content of withaferin-A in the *Withania somnifera* leaf material

Sample	Concentration of the stock leaf extract solution (in mg/ml) (C_0)	Concentration of withaferin-A in the stock leaf extract solution (in mg/ml (C_A)	Percentage content of withaferin-A in the leaf extract $\frac{(C_A \times 100)}{C_0}$	*Content (in g) of withaferin-A in the dry leaf extract (%content×wt of dry extract) =W	**Percentage content of withaferin-A (in g/100g) in the powdered leaf material $=\frac{W \times 100}{5.0\ g}$
LVA1	23.30	13.8793	59.56	0.0278	0.56
LVA2	29.35	14.9275	50.86	0.0299	0.60
LVA3	35.25	18.5225	52.55	0.0370	0.74
LVA4	54.00	32.0967	59.43	0.0642	1.28
LVA5	29.15	16.5119	56.64	0.0330	0.66
LVA6	15.60	9.7805	62.70	0.0196	0.39
LVA7	69.15	40.9507	59.22	0.0819	1.64
LVA10	71.95	42.1910	58.64	0.0844	1.69

*****Content (in g) of withaferin-A in the leaf extract=% content×weight (in g) of the dry extract

**Percentage content of withaferin-A (in grams per 100grams of dry plant leaf material

$$= \frac{\text{Content (in g) of withaferin-A in the dry leaf extract} \times 100}{\text{Weight (in g) of the dry leaf powder used for the extraction}}$$

3.1.8.2. Stem samples

Table 3.17: Percentage content of withaferin-A in the *Withania somnifera* stem material

Sample	Concentration of the stock stem extract solution (in mg/ml) (C_0)	Concentration of withaferin-A in the stock stem extract solution (in mg/ml (C_A)	Percentage content of withaferin-A in the stem extract $\frac{(C_A \times 100)}{C_0}$	*Content (in g) of withaferin-A in the dry stem extract (%content×wt of dry extract) =W	**Percentage content of withaferin-A (in g/100g) in the powdered stem material $=\frac{W \times 100}{10.0\ g}$
STA1	30.05	9.5060	31.63	0.0190	0.19
STA2	40.60	13.2506	33.13	0.0268	0.27
STA3	39.25	12.8310	32.69	0.0257	0.26
STA4	44.40	17.9598	40.45	0.0360	0.36
STA5	25.55	8.4845	33.21	0.0170	0.17
STA6	32.20	10.2760	31.91	0.0206	0.21
STA7	56.00	24.5401	43.82	0.0491	0.49
STA10	44.10	17.0314	38.62	0.0341	0.34

*****Content (in g) of withaferin-A in the stem extract=% content×weight (in g) of the dry extract
**Percentage content of withaferin-A (in grams per 100grams of dry plant stem material

$$= \frac{\text{Content (in g) of withaferin-A in the dry stem extract} \times 100}{\text{Weight (in g) of the dry stem powder used for the extraction}}$$

3.1.8.3. Root samples

Table 3.18: Percentage content of withaferin-A in the *Withania somnifera* root material

Sample	Concentration of the stock root extract solution (in mg/ml) (C_0)	Concentration of withaferin-A in the stock root extract solution (in mg/ml (C_A)	Percentage content of withaferin-A in the root extract $\frac{(C_A \times 100)}{C_0}$	*Content (in g) of withaferin-A in the dry root extract (%content×wt of dry extract) =W	**Percentage content of withaferin-A (in g/100g) in the powdered root material $=\frac{W \times 100}{20.0\ g}$
RTA1	139.75	73.9660	52.93	0.1479	0.74
RTA2	61.10	21.9830	35.97	0.0440	0.22
RTA3	66.00	26.7680	40.56	0.0535	0.27
RTA4	138.55	72.7789	52.53	0.1456	0.73
RTA5	42.00	15.3700	36.60	0.0307	0.15
RTA6	105.05	46.0106	43.80	0.0920	0.46
RTA7	142.10	77.1403	54.43	0.1543	0.77
RTA10	136.20	70.7845	51.97	0.1416	0.71

*****Content (in g) of withaferin-A in the root extract=% content×weight (in g) of the dry extract

**Percentage content of withaferin-A (in grams per 100grams of dry plant root material

$$= \frac{\text{Content (in g) of withaferin-A in the dry root extract} \times 100}{\text{Weight (in g) of the dry root powder used for the extraction}}$$

3.1.8.4. Locally available imported root powder formulations of *Withania somnifera*

Table 3.19: Percentage content of withaferin-A in the *Withania somnifera* root formulations

Sample	Concentration of the stock root extract solution (in mg/ml) (C_0)	Concentration of withaferin-A in the stock root extract solution (in mg/ml (C_A)	Percentage content of withaferin-A in the root extract $\frac{(C_A \times 100)}{C_0}$	*Content (in g) of withaferin-A in the dry root extract (%content×wt of dry extract) =W	**Percentage content of withaferin-A (in g/100g) in the powdered root material $=\frac{W \times 100}{20.0g}$
RTA8	40.25	15.8201	39.31	0.0316	0.16
RTA9	38.10	15.5521	40.82	0.0311	0.16

*****Content (in g) of withaferin-A in the root extract=% content×weight (in g) of the dry extract

**Percentage content of withaferin-A (in grams per 100grams of dry plant root material

$$= \frac{\text{Content (in g) of withaferin-A in the dry root extract} \times 100}{\text{Weight (in g) of the dry root powder used for the extraction}}$$

3.1.9. Summary of results : Withaferin-A content investigation in *Withania somnifera* plant collected from different locations in Kenya.

Table 3.20: Summary of the results of withaferin-A content investigation in *Withania somnifera* plant collected from different locations in Kenya.

Sample	Where collected: County (Region)	Percentage weight of dry plant extract obtained(g/100 g)	Concentration obtained after dissolving the extract in 2.0ml methanol (mg/ml)	Peak areas obtained from the original concentration (a)	Dilution factor (with methanol)	Concentration of the extract obtained after dilution (µg/ml)	Peak areas obtained after dilution (b)	Percentage content of withaferin-A* (g/100g)
LVA1	Nairobi (Syokimau)	0.93	23.30	2,552597	30	776.67	126756	0.56
LVA2	Machakos (Kyumbi)	1.17	29.35	3,954318	40	733.75	103083	0.60
LVA3	Makueni (Kathonzwen)	1.41	35.25	6,652822	50	705.00	102358	0.74
LVA4	Nakuru (Naivasha)	2.16	54.00	8,868272	60	900.00	145890	1.28
LVA5	Kajiado (Kitengela)	1.17	29.15	3,287404	30	971.00	149979	0.66
LVA6	Nairobi (Ruai)	0.62	15.60	1,385377	20	780.00	133738	0.39
LVA7	Kiambu (Limuru)	2.77	69.15	9,224903	80	864.37	139787	1.64
LVA10	Narok (Town)	2.88	71.95	12,035077	80	899.37	143890	1.69
Stem samples								
STA1	Nairobi (Syokimau)	0.60	30.05	5,317282	60	500.83	46250	0.19
STA2	Machakos (Kyumbi)	0.81	40.60	7,307893	80	507.50	48155	0.27
STA3	Makueni (Kathonzwen)	0.79	39.25	6,454168	100	392.50	38278	0.26
STA4	Nakuru (Naivasha)	0.89	44.40	9,770818	80	555.00	63773	0.36
STA5	Kajiado (Kitengela)	0.51	25.55	4,998482	50	511.00	49229	0.17
STA6	Nairobi	0.64	32.20	5,344838	60	536.67	49646	0.21

	(Ruai)							
STA7	Kiambu (Limuru)	1.12	56.00	13,888177	100	560.00	69265	0.49
STA10	Narok (Town)	0.88	44.10	7,984955	80	551.25	60662	0.34

Root samples

RTA1	Nairobi (Syokimau)	1.40	139.75	20,390277	200	698.75	102195	0.74
RTA2	Machakos (Kyumbi)	0.61	61.10	10,141381	110	555.45	57209	0.22
RTA3	Makueni (Kathonzwen)	0.66	66.00	10,659843	110	600.00	68721	0.27
RTA4	Nakuru (Naivasha)	1.39	138.55	19,951383	200	692.75	100623	0.73
RTA5	Kajiado (Kitengela)	0.42	42.00	7,681660	80	525.00	55166	0.15
RTA6	Nairobi (Ruai)	1.05	105.05	15117163	170	617.94	75947	0.46
RTA7	Kiambu (Limuru)	1.42	142.10	22,460702	200	710.50	106394	0.77
RTA10	Narok (Town)	1.36	136.20	18,561336	200	681.00	97984	0.71

The locally available imported commercial root formulations

RTA8	**Baidyanath Ltd (India)	0.40	40.25	7,403583	70	575.00	64134	0.16
RTA9	**Himalaya Ltd (India)	0.38	38.10	6,414468	70	544.28	63118	0.16

*(Content in g of withaferin-A in each sample/wt of the plant material used in extraction)×100

**Manufacturers of the commercial formulations.

RTA8=Ashwagandha root powder sample, RTA9=Ashvagandha root powder sample

Table 3.21. Comparison of the Percentage content of withaferin-A in *Withania somnifera* root, leaf and stem samples collected from different places.

	% of withaferin-A (in g/100 g)**		
Where collected County (Region)	Root sample	Stem sample	Leaf sample
Nairobi (Syokimau)	0.74	0.19	0.56
Machakos (Kyumbi)	0.22	0.27	0.60
Makueni (Kathonzweni)	0.27	0.26	0.74
Nakuru (Naivasha)	0.73	0.36	1.28
Kajiado (Kitengela)	0.15	0.17	0.66
Nairobi (Ruai)	0.46	0.21	0.39
Kiambu (Limuru)	0.77	0.49	1.64
Narok (Town environs)	0.71	0.34	1.69

**(Content in g of withaferin-A in each sample/wt of the plant material used in extraction)×100

Table 3.22: The mean, the median and the range of withaferin-A content (in g/100g) of the Kenyan root, stem and leaf samples analyzed.

	% of withaferin-A (in g/100 g)		
Samples	Mean*	Median*	Range*
Root samples	0.51	0.59	0.62
Stem samples	0.29	0.27	0.32
Leaf samples	0.95	0.70	1.30
All samples analyzed	0.58	0.48	1.54

*****These values don't include the two imported *withania somnifera* root formulations

3.2. Stability of samples.

Quantification of withaferin-A in the extract of sample RTA6 subjected to room temperature for two weeks is reported in Table 3.23. Quantification of withaferin-A in the extract of sample RTA6 subjected to three freeze thaw cycles is also reported in Table 3.24. Table 3.24 also gives results on long term stability (30 days) on sample RTA6.

Table 3.23: Room temperature stability of sample RTA6

Hours (days)	0 h	24 h (day 1)	48 h (day 2)	72 h (day 3)	96 h (day 4)	120 h (day 5)	144 h (day 6)
Quantity of withaferin-A obtained (µg/g); *(n=6) (Mean± S.D.)	4714±22	4632±48	4010±76	3802±60	3708±64	3584±44	3501±58
Percentage content of withaferin-A (g/100g);	0.47	0.46	0.40	0.38	0.37	0.36	0.35

168 h (day 7)	192 h (day 8)	216 h (day 9)	240 h (day 10)	264 h (day 11)	288 h (day 12)	312 h (day 13)	336 h (day 14)
3400±53	3322±76	3268±32	3132±81	3002±70	2870±85	2754±52	2652±66
0.34	0.33	0.33	0.31	0.30	0.29	0.28	0.27

*****n= number of replicate samples injected; S.D. is standard deviation

Table 3.24: Freeze and thaw stability, and long term stability of sample RTA6

	Freeze thaw stability Cycle 3 (48 h)	Long-term stability 30 days (room temperature)
Quantity of withaferin-A obtained (µg/g) *(n=6) (Mean± S.D.)	4612.65±22.21	1566.54±82.38
Percentage content of withaferin-A obtained (g/100g)	0.46	0.16

*****n= number of replicate samples injected; S.D. is standard deviation

3.3. Discussions

The goal of the present work was to quantitatively determine the withaferin-A content of *Withania somnifera* root, stem and leaf extracts collected from different places in Kenya. Quantitative determination of withaferin-A in the samples was undertaken according to the published method by Shaila *et al*., 2006. A simple solvent extraction technique consisting 60% methanol in water was utilized in the extraction of withanolides from the powdered dry plant material (Table 3.1). Chromatographic conditions were optimized to achieve the best separation and enhance the signal of Withaferin-A. Withaferin-A could be baseline separated from compounds of similar polarity in less than 10 min after all separation parameters were carefully assessed. RP-HPLC was performed isocratically with acetonitrile/water (75:25) as the mobile. The separation temperature was kept at 27 ^{o}C, flow rate and sample volume were set to 1.0 ml/min and 20 µl, respectively. All separations were monitored at 225 nm. For all separations a Reverse Phase Gemni 5µ C-18 110A Phenomenex column (C-18 phase ,250 x 4.6 mm, 5 µm particle size) from Phenomenex (Tollance, CA, USA) was used. Peaks were assigned by comparison of the retention times. Various analytical and statistical parameters were investigated for validation purposes. Stability studies and efficiency of extraction were also investigated.

3.3.1. Withaferin-A content determination in *Withania somnifera*

The analysis of *Withania somnifera* root, stem and leaf confirmed the presence of withaferin-A in all parts of the plant but with significant differences in their ratio (Table 3.16- 3.21). In all the samples analyzed, withaferin-A content was highest in the leaves, the highest percentage recorded in the leaves being 1.69 %.w/w This compound was lowest in stems the highest content recorded in the stems being 0.49 % w/w. The highest withaferin-A content recorded in the roots was 0.77 %w/w. Finally, two imported market products (Ashwagandha root powder and Ashvagandha capsules) were analysed. In all of these, the marker compound (i.e. withaferin-A) was detected. The percentage contents of withaferin-A in Ashwagandha root powder and Ashvagandha capsules were found to be 0.16 %w/w in each. The Kenyan root sample of *Withania somnifera* with the highest concentration of withaferin-A was RTA7 (0.77 % w/w) from the cultivated plant in Limuru, Kiambu county (Table 3.22). The *Withania somnifera* plant collected from Limuru in Kiambu county showed the highest average percentage withaferin-A content in the leaves, stems and roots (average of 0.97%w/w).

The variations in withaferin-A contents in the samples and the higher percentages of withaferin-A in samples LVA10, LVA7 (both leaf samples), STA7 (stem sample) RTA7 and RTA1 (both root samples) could be explained by seasonal variations, environmental factors or different chemotypes of the plant (which have been reported in the literature [Evans 2009, Hornok 1992, Martin and Woodcock, 1983]. The mean withaferin-A content in all the root samples analyzed was 0.51%w/w (units in g/100g), for all the stem samples analyzed was 0.29% w/w and the mean for all leaf samples analyzed was 0.95% w/w. The leaf had the highest mean withaferin-A content and stem the lowest.

Investigations over the years, carried out on various sources of *Withania somnifera* plant material and concerning withaferin-A content have given differing result as observed in the literature review (Evans, 2009). Previous studies carried out by Israel scientists on the *Withania somnifera* found in that country reported withaferin-A content of 0.2% w/w (Evans, 2009). More recent studies on the Indian *Withania somnifera* plant have reported Withaferin-A content of 0.711% w/w (Sangwan *et al*. 2006).

3.3.2. Stability of samples.

Significant degradation of withaferin-A in sample RTA6 stored at room temperature for two weeks was observed. After 2 weeks, the withaferin-A content had decreased to 56.25% of the original content (Table 3.23). The degradation was much lower in the sample subjected to freeze and thaw cycles. The withaferin-A content in the freeze thaw stability was 97.84% of the original content (Table 3.24). Long term stability after 30 days of storage at room temperature of sample RTA6 showed degradation of withaferin-A to values less than 33.22% of the original concentration (Table 3.24). These results show that withaferin-A is unstable and quickly undergoes degradation at room temperature and hence should be stored at low temperatures especially close to freezing temperatures.

CHAPTER FOUR:

CONCLUSIONS AND RECOMMENDATIONS

Health remains a key concern that needs new approaches. *Withania somnifera* plant holds great potential as herbal remedy for a host of health problems. In the modern world, the emergence of chronic diseases with poor therapeutic alternatives, desire for personalized medicines, large population trying to stave off the effects of aging and new focus on preventive medicines, have enormously resulted in extensive consideration of this plant for its great potential as a remedy for many health problems.

From the results of this research, the Kenyan *Withania somnifera* root had higher content of the pharmacologically active compound withaferin-A than the imported root formulations of the plant. The Kenyan plant can thus be used in the formulation of more potent, more efficacious, cheaper and more readily accessible drug formulations than the imported root formulations of this plant.

Since *Withania somnifera* is a medicinal plant that is endangered due to over use, nutraceuticals and herbal pharmaceutical products derived from *Withania somnifera* leaves and stems products could offer useful alternatives to the *Withania somnifera* root products as the leaves and stems have shown to also contain high levels of withferin-A. Thus, *Withania somnifera* leaves and stems products merit further investigation towards the development of efficacious and safe nutraceuticals and herbal pharmaceuticals.

Furthermore, it is recommended that further work on agronomical, ecological and pest infestation effects on the active compounds be carried out. More focus should be on the leaves as they were found to have the highest withaferin-A content. Focus on stem should also be encouraged to provide alternative source of the nutraceutical from root and leaves. Research should focus on the plant variety from Limuru, Narok and Naivasha as they had relatively high withaferin-A contents. More research work on the leaves geared towards substituting the roots as source of the plant products should be encouraged as well as studies on the leaves to compare other ingredients with those in the roots.

Further research work on the presence of chemical varieties in the plant also merit consideration. Investigation of the content of the active compounds at various stages of growth and development of the plant, together with seasonal variations of the active compounds, should also be encouraged so as to identify the appropriate stage of growth and season for collection and harvesting. Toxicological studies on the root, leaves and stem formulations need to be done.

Withaferin-A exhibited stability at low temperature hence formulations from *Withania somnifera* should not exposed to high temperatures. Stability of the formulations merit further investigation and further work on the formulation of nutraceuticals and other pharmaceutical products from the plant should be carried out. Methanol-water (60:40) should be adopted for extraction of plant material as it gave better results than methanol, water and chloroform.

It is recommended that the Pharmacy and Poisons Board formulates clear legal framework and policy geared towards the promotion of quality control and standardization of phytopharmaceuticals like *Withania somnifera* so as to ensure quality, safety, efficacy and acceptability of these products. It is further recommended that the government provides more funds to promote research in phytomedicines so as to scientifically validate the efficacy, potency and safety of these products. The government is advised to promote the cultivation, processing and value addition of useful medicinal plants and nutraceuticals like *Withania somnifera* both for local consumption and export. It is recommended that the government through the ministry of health considers the introduction of the scientifically validated beneficial herbal medicines into the mainstream healthcare system.

It is further recommended that the government undertakes a comprehensive survey of the commercial value of *Withania somnifera* and other medicinal plants and possible products in Kenya. It is recommended that Universities and Research Institutes work in cooperation and collaboration to promote research in this plant and other phytomedicines. There is need to undertake ethnopharmacological survey of *Withania somnifera* and other medicinal plants in Kenya so as to provide data base for future scientific research on herbal medicines and other phytopharmaceuticals. To achieve all these research goals on this plant and other beneficial medicinal plants, multidisciplinary approach will be of paramount importance.

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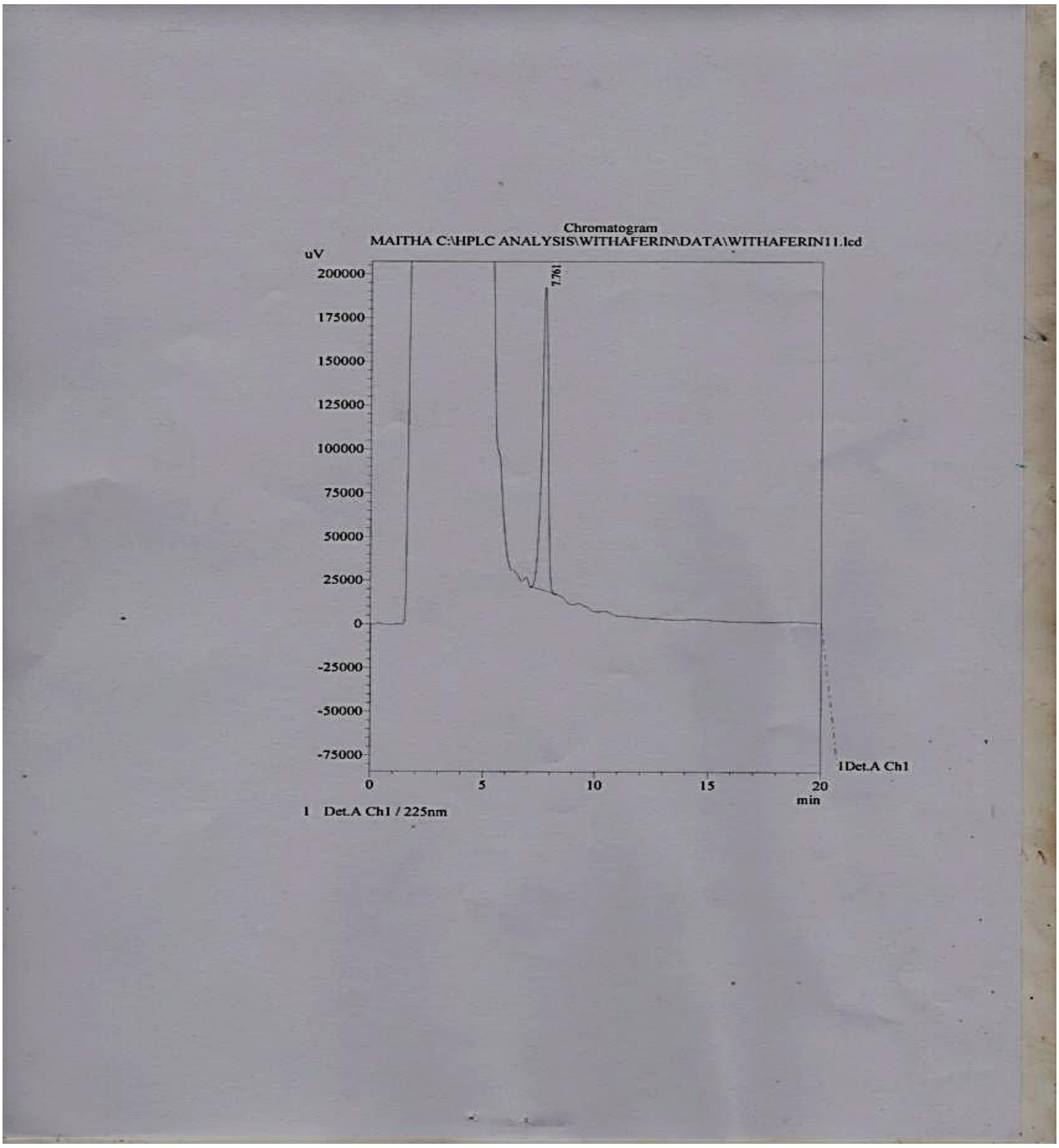
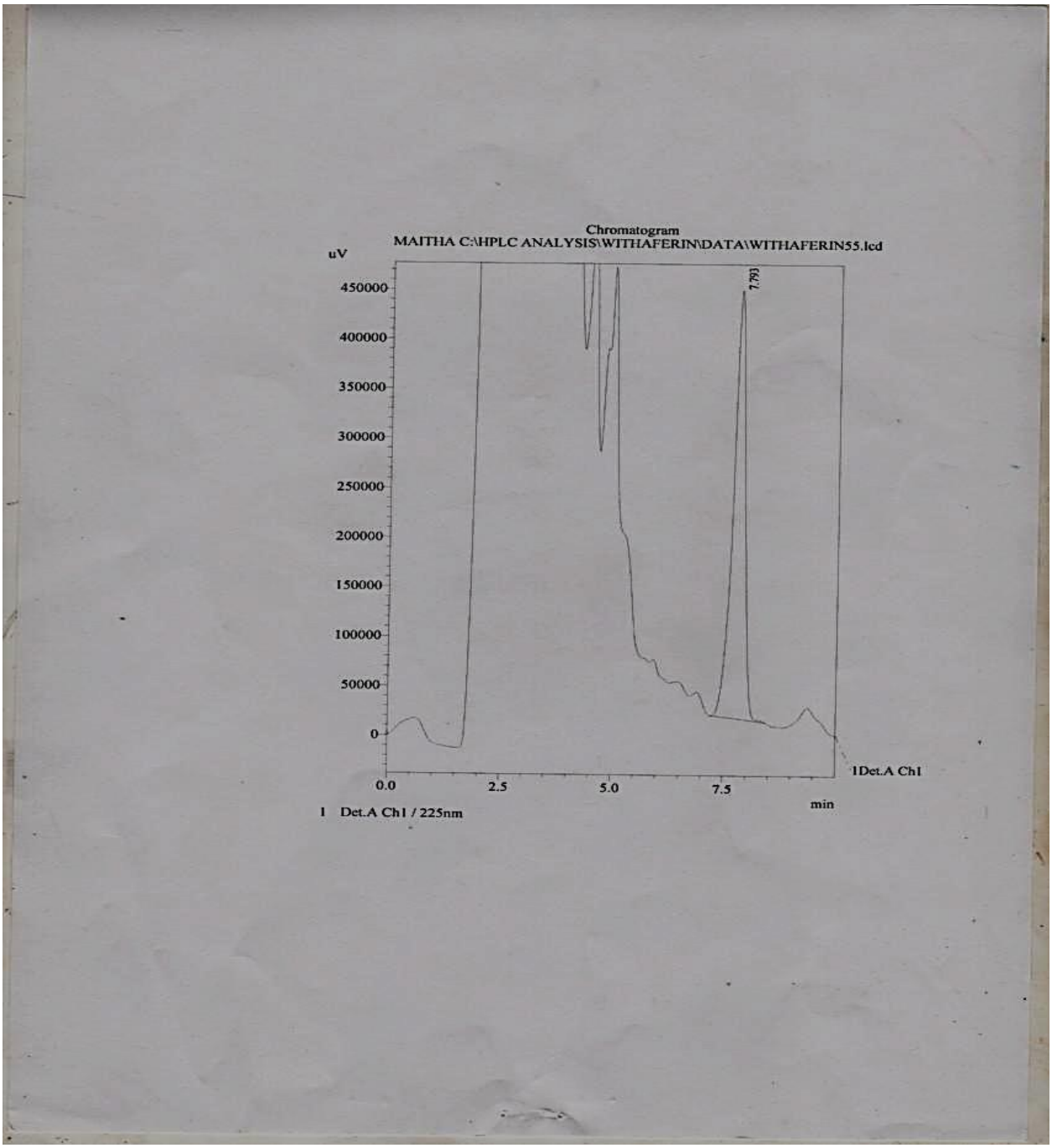
APPENDIX A



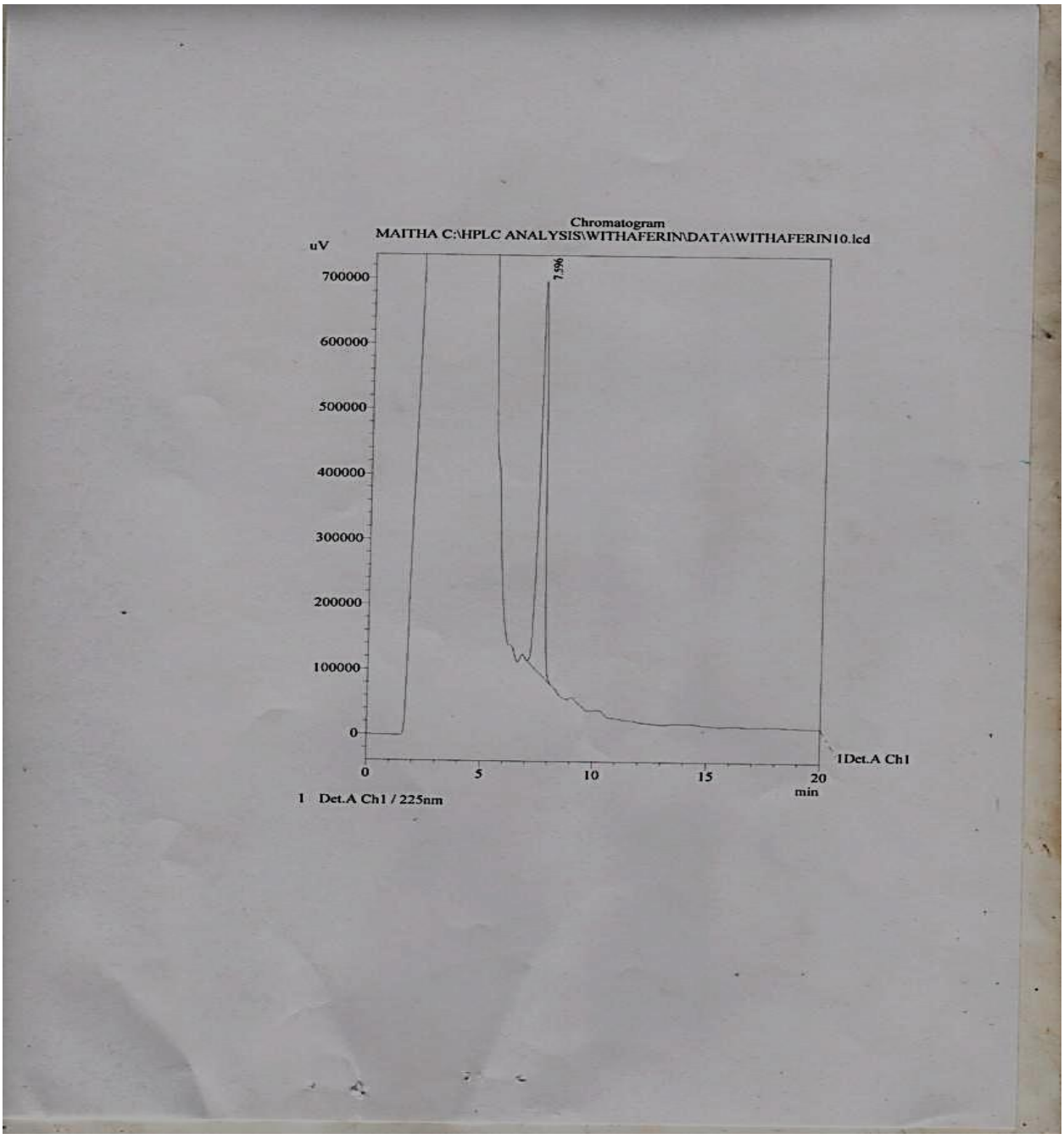
HPLC chromatogram of withaferin-A reference standard

APPENDIX B



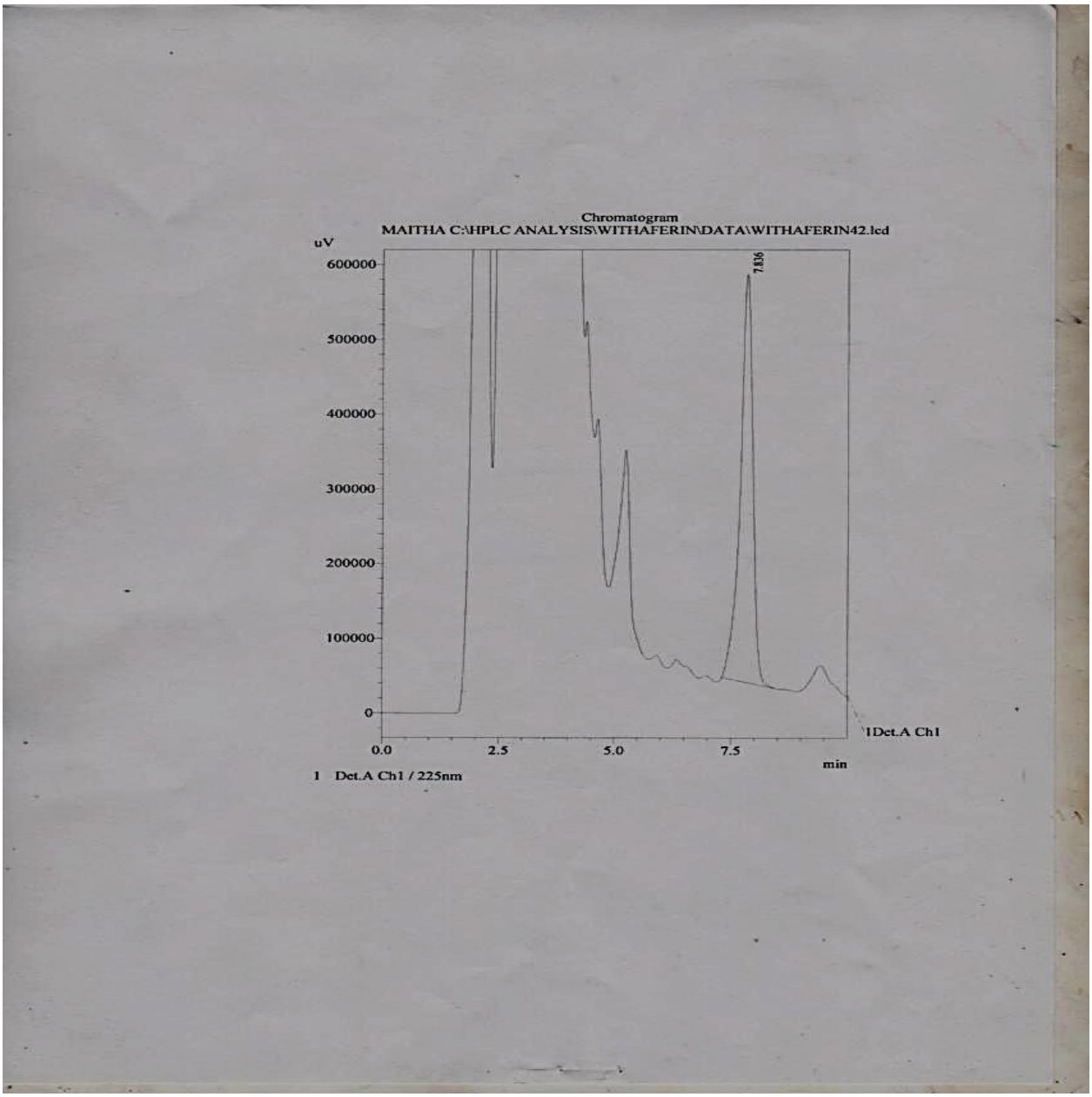
HPLC chromatogram of *Withania somnifera* stem extract

APPENDIX C



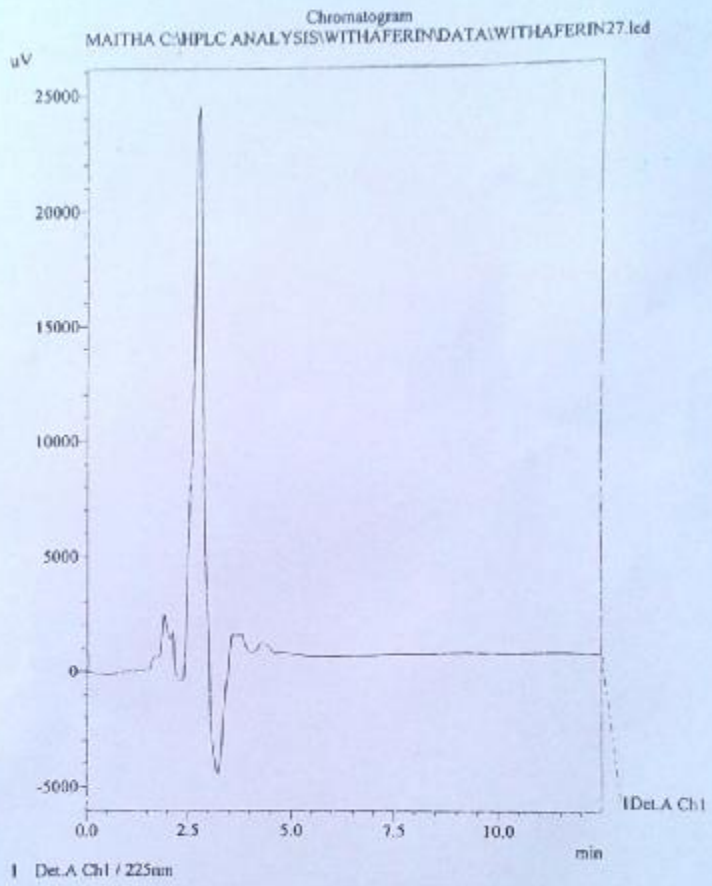
HPLC chromatogram of *Withania somnifera* leaf extract

APPENDIX D



HPLC chromatogram of *Withania somnifera* root extract

APPENDIX E



HPLC chromatogram of blank (methanol only)

APPENDIX F

Details of collection sites of *Withania somnifera*

1. **Nairobi County**

Locality: Syokimau region, 3km from Nairobi-Mombasa Road

Habitat: Open grassland **Frequency**: Abundant

2. **Kajiado County**

Locality: Kitengela, 2km east of Kitengela Town

Habitat: Open grassland **Frequency**: Occasional

3. **Machakos County**

Locality: Kyumbi area, 1km off Nairobi-Mombasa road

Habitat: Open grassland **Frequency**: Occasional

4. **Makueni County**

Locality: Kathonzweni region, 3km north of Kathonzweni market along Wote road

Habitat: Open grassland **Frequency**: Occasional

5. **Nakuru County**

Locality: Naivasha region-Kikopey area

Habitat: Open grassland, bushy area **Frequency**: Occasional

6. **Nairobi County**

Locality: Ruai region (Under cultivation)

Habitat: Cultivated alone in an open area **Frequency**: Occasional

7. **Kiambu County**

Locality: Limuru-Tigoni-region, next to Tigoni District Hospital

Habitat: Under cultivation in an open area **Frequency**: Cultivated in around half an acre.

8. **Narok County**

Locality: Narok Town enviros, 1km east of Narok Town, near Naivasha-Narok road

Habitat: Open grassland **Frequency**: Occasional