Acute and chronic toxicity of the flavonoid- containing plant, *Artemisia afra* in rodents

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

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ABSTRACT

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Despite being one of the most popular traditional plant medicines used in South Africa, very little is known about the safety and efficacy of *Artemisia afra*. The aim of this study was to investigate the possible toxicity of the flavonoid-containing plant, *Artemisia afra* and especially to establish the safety of the aqueous extract of this plant after acute and chronic administration to mice and rats respectively. To realize this objective, the following were determined: (1) the adverse effects caused by the dried aqueous extract of *Artemisia afra* in mice and rats, (2) the LD_{50} for the aqueous extract of the plant administered acutely in mice using the traditional method of Litchfield and Wilcoxon and a computer-based (AOT425statPgm) method, (3) the doses (i.e. LD_{50}) of the plant causing adverse effects after oral and intraperitoneal acute administration, (4) the general toxicity profile obtained after chronic oral dosing with the extract in rats and (5) the correlation between the plasma levels of luteolin, plant doses and toxic effects obtained after the chronic oral administration of the plant extract to rats. It was hypothesized that: (i) the flavonoids such as luteolin contained in *Artemisia afra*, if given in high enough doses may be associated with some adverse effects; (ii) the dose of the plant at which such adverse effects occurred would depend on both the route of administration (oral vs. intraperitoneal) as well as the duration (acute or chronic) of exposure to the plant material; (iii) the new computer program-based method (i.e. AOT425statPgm) for testing acute toxicity of environmental pollutants, can advantageously be used to evaluate the acute toxicity of *A. afra* extract, and (iv) plasma levels of luteolin could be used as a marker to monitor *A. afra* toxicity and/or its bioavailability (ingestion) in rats.

Plant material (i.e. leaves & aerial parts) was collected from Montague Museum, Western Cape Province, South Africa and the freeze-dried aqueous extract prepared. Mice were

administered single oral doses of *A. afra* (0.175 to 24 g/kg) or intra-peritoneal doses (0.175 to 5,5g/kg) and were monitored for mortality and toxic symptoms for two weeks. The LD_{50} values for each route were determined according to the methods of "Litchfield and Wilcoxon" and the "Acute Oral Toxicity (Guideline 425) statistical program" (AOT425statPgm). In another study, rats were given oral doses (0,1 or 1g/kg) of *A. afra for*: (a) three months (92 days), after which blood was withdrawn for measurement of several haematological and biochemical parameters and plasma levels of luteolin by HPLC assay, and selected tissues inspected for histopathological changes, or (b) seven days, after which the levels of luteolin in the blood of rats were measured. In addition, the luteolin level in the *Artemisia afra* aqueous extract was determined using HPLC.

The LD_{50} of *A. afra* extract after acute intraperitoneal injection in mice was 27% of that after oral administration (i.e. 2450 mg/kg vs. 8960mg/kg). The AOT425statPgm method, while giving the same symptom profile and LD_{50} results and having the same study time to conduct, however involved substantially fewer mice, in fact only 20 and 50% of that used in the Litchfield and Wilcoxon method for the acute p.o. and i.p. toxicity testing, respectively. In the chronic toxicity study, all the rats survived the duration of the treatment (i.e. LD_{50} was much higher than 1000mg/kg), with no significant changes in physical signs, haematological and biochemical parameters, except for a transient decrease in aspartate aminotransferase (AST) activity. There were also no significant differences in the organs weights, and the histopathological results showed normal architecture suggesting no morphological disturbances. The luteolin levels in the *Artemisia afra* and rat plasma were 0,923± 0,015 µg/mg extract and less than 0,2 µg/ml plasma, respectively.

Collectively, the results indicate that acute doses of *A. afra* are relatively non-toxic in mice irrespective of the route of administration used, chronic doses of *A. afra* are very safe in the rat, that the AOT425statPgm is a potentially useful tool for the evaluation of acute toxicity of plant medicines and, finally, that luteolin may be a good marker for *A. afra* bioavailability, but not likely for its toxicity.

DECLARATION

I, James Tshikosa Mukinda, declare that "**Acute and chronic toxicity of the flavonoid-containing plant, *Artemisia afra* in rodents**" is my own work, that it has not been submitted for any degree or examination to any other University, and that all sources that I have used or quoted have been indicated and acknowledged by means of complete references.

Signature: **December 2005**

"The greatest use of life is to spend it for something that will outlast it."

James, W.

Dedicated to my wife to be, our progeny, my brothers and sisters.

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Chapter 1

INTRODUCTION

In the absence of an efficient primary health care system, traditional medicine occupies a central place in the provision of health care, especially among rural communities of developing countries. The strong historical bond between plants and human health is well substantiated by plant species' diversity and related knowledge of their use as herbal medicines (Tabuti et al., 2003). In addition, it is attributable to the accessibility and affordability of herbal medicines (Steenkamp, 2003).

In Western and African folk medicine, several species of the genus, *Artemisia* are used for their claimed healing properties and the curing of specific ailments. In Europe, the plants of the genus, *Artemisia* have been used as remedies for chronic fever, swellings and inflammation of the liver (*A. absinthium)*; as antihelmintic and emmenagogue *(A. abroatanum)*; as efficacious antimalarial, antispasmodic *(A. annua)*; and as decongestant and laxative agents *(A. maritima)*. In the Mediterranean region, they are known for their anti-inflammatory, anti-allergenic, anti-infectious and parasiticidal effects (Pappas and Sheppard-hanger, 2004). In South Africa, the study of the indigenous knowledge indicates the widespread common use of *Artemisia afra* for numerous ailments- mainly as a remedy for chest conditions, coughs, colds, heartburns, hemorrhoids, fevers, malaria, asthma, diabetes mellitus and other conditions (Dyson, 1998; Hutchings et al., 1996; Iwu, 1993; Roberts, 1990). In addition, leaves are commonly smoked by some tribes to help release phlegm, and ease and soothe a sore throat and coughing at night (Roberts, 1990).

Artemisia afra, family *ASTERACEAE,* is in fact a multi-stemmed perennial shrub with grayish- green feathery leaves, commonly found in most areas of South Africa, where it is known as "Wildeals" (Afrikaans), " Umhlonyane" (Xhosa, Zulu), "Lengana"(Sotho) (Roberts, 1990; Van Wyk et al., 2000). Importantly, the plant *Artemisia afra* has been shown to display a wide spectrum of biological and pharmacological activities, which provide experimental support for the empiric ethno-pharmacological use of this plant in traditional medicine. Results of investigations conducted on the aqueous extract of

Artemisia afra have indicated that the plant has bronchodilator and possibly anti-inflammatory activities (Harris, 2002), as well as anti-histaminic and narcotic analgesic properties (Hutchings et al., 1996). Other assays carried out on the ethanolic extract and dichloromethane extract have shown the plant to have *in vitro* hypotensive and anti-tuberculosis effects, respectively (MRC and SAHealthinfo, 2004).

The merit of the empirical use of *Artemisia Afra* has also been corroborated by the isolation and identification of several active compounds, including flavonoids (apigenin, hesperetin, kaempferol, luteolin and quercetin) and volatile oil, which has been said to contain mainly 1, 8- cineole, α-thujone, ß-thujone, camphor and borneol (Waithaka, 2004; Van Wyk et al., 2000). Also present in the plant are terpenoids as well as coumarins and acetylenes, although their contribution to the biological activity of *Artemisia afra* is not yet known (Van Wyk et al., 2000). Similarly, very little is known about the toxicity of this plant.

The volatile oil of *Artemisia afra* has been isolated by Van der Lingen and is said to be as toxic as oil of Sabine, producing hemorrhagic nephritis, degenerative changes in the liver and pulmonary oedema, after experimental administration to rabbits (Watt and Breyer–Brandwijk, 1962). When given experimentally via the oral route to rabbits and guinea pigs, the volatile oil of *Artemisia afra* produced haemorrhagic nephritis and sometimes abortion in pregnant animals (Watt and Breyer–Brandwijk, 1962). Moreover, it has been reported that the toxic and hallucinogenic effects of this volatile oil are attributed to thujone, so that overdoses or continued use over long periods are potentially harmful (Van Wyk et al., 2000). Furthermore, the aqueous extract of *Artemisia afra* has also been shown to be cytotoxic to Hela cells *in vitro* (SA Healthinfo, 1999).

It was also found that *Artemisia afra* contained several flavonoids, *inter alia*, apigenin, hesperetin, kaempferol, luteolin and quercetin (Waithaka, 2004). It is well known that such flavonoids have many potential activities, e.g. anti-oxidant, anti-inflammatory and anti-allergic activities (Harborne et al., 2000). Additionally, they have chemo-preventive activity against skin cancer (e.g. apigenin); inhibitory effects on chemically induced

mammary gland, urinary bladder and colon carcinogenesis in laboratory animals (e.g. hesperetin); and anti-carcinogenic and platelets anti-aggregatory effects (e.g. quercetin) (Erlund, 2002; Waithaka, 2004). Furthermore, the flavonoid luteolin has been shown to exhibit anti-mutagenic and anti-tumorigenic activities (Shimoi et al., 1998) as well as vasodilatory and potent anti-platelet activities (Harborne et al., 2000). It is also a promising agent for use in ophthalmology for the prevention and treatment of cataract and vascular eye disorders (China Great vista chemicals, 2002).

While noting the apparently beneficial health effects of flavonoids, the results of a few studies in both bacterial and mammalian experimental systems have, however, also indicated their adverse effects. Quercetin has been identified as a potent topoisomerase II inhibitor at low concentrations, a mutagenic agent and an inhibitor of thyroid peroxidase that is essential for thyroid hormone synthesis (Skibola and Smith, 2000). Luteolin has been shown to be slightly more toxic towards isolated rat hepatocytes; and apigenin having inhibitory effects on Hela tumor cell proliferation (Moridani et al., 2002). Nothing, however, has so far been reported that might specifically implicate the flavonoids (especially luteolin) of *Artemisia afra* in the possible adverse effects of this particular plant medicine.

In fact, notwithstanding the wide spread use of this indigenous medicine in South Africa, no systematic study has so far been done on the toxicity of *Artemisia afra*. In particular, in a recent application to the Medicine Control Council (MCC) to conduct a clinical study on the effect of *Artemisia afra* in asthmatic patients, the absence of safety data in humans was noted as a shortcoming of the application (Van Wyk. A et al., 2003). A study on the toxicity of the plant in animals may help to partially correct this shortcoming.

Therefore, the aim of the present study was to investigate the possible toxicity of the flavonoid-containing plant, *Artemisia afra* and to specifically establish the safety of the aqueous extract of this plant by focusing on its acute and chronic toxicity in mice and rats respectively. To realize this objective, I consequently proposed to determine the adverse effects that a dried aqueous extract of *Artemisia afra* may cause in mice and rats; to

determine the LD_{50} for the aqueous extract of the plant administered acutely in mice using a traditional method (of Litchfield and Wilcoxon) and new computer program-based method (AOT425statPgm method that may offer distinct advantages over the traditional method); to compare the potential doses (i.e. LD_{50}) of the plant causing adverse effects after oral and intraperitoneal acute administrations; to determine the general toxicity profile that results from chronic oral dosing of *Artemisia afra* in rats; and to assess whether the plasma levels of flavonoids (especially luteolin) possibly correlate with (a) the dose of *Artemisia afra* ingested and (b) any of the toxic effects that may be observed after chronic ingestion of *Artemisia afra.*

The report of this work consists of the following sections:

Chapter two: A literature review section where information relevant to this study is briefly presented.

Chapter three: Reveals the Work Plan, the objectives, hypothesis and the study approach, which led to the studies of the acute and chronic toxicity of the flavonoid-containing plant, *Artemisia afra,* in rodents.

Chapter four: This chapter only deals with the acute toxicity study. It covers the methodology that led to the generation of data, describes the equipment and materials that were used and finally, presents the results and their discussion.

Chapter five: In this chapter information on the chronic toxicity study is presented, viz the materials, equipment and methods that were used, the results that were obtained and a discussion of the results.

Chapter six: In this chapter, various conclusions are drawn and finally synthesized into one general conclusion. Recommendations are also presented thereafter.

Chapter 2

LITERATURE REVIEW

The present study is an investigation of the possible toxicity of the flavonoid-containing plant *Artemisia afra* and a safety evaluation of the aqueous extract of the plant, focusing on its acute and chronic toxicity in mice and rats, respectively.

This chapter provides an overview of (1) toxicity and the various definitions of key toxicity terms used in this report, (2) the current use and importance of herbal medicines, their traditional dosage forms and mode of administration of herbal medicines as well as some specific aspects of their toxicity, viz. possible causes, prevalence and the evaluation of herbal toxicity, (3) *Artemisia afra*, its botany, main medicinal uses and dosage forms as well as its pharmacological activities and major chemical constituents, (4) the flavonoids with focus on their classification and biological activities and (5), the flavonoid luteolin, with focus on its activities and aspects of its toxicity and the analytical techniques that are suitable for the determination of its levels in plant material and blood plasma.

2.1. Overview of toxicity and definitions of key terms

2.1.1. Definition of toxicity

Toxicity is defined as "the potential of a substance to exert a harmful effect on humans or animals, and a description of the effect and the conditions or concentration under which the effect takes place" (Health & safety, 2004).

In order to support an application for a clinical trial or for the registration of a new drug, it is necessary to satisfy legislation that requires that certain data should be produced from a variety of toxicological investigations that show the safety profile of the compound to which humans may be exposed. Therefore, in the majority of cases of evaluation of the toxicity of most substances, rodents and non-human primates are first

used in preclinical animal safety studies before further studies are done in humans. These animals are mainly used because of their biological similarity to humans that allows them to be regarded as the suitable metabolic models for humans in a broad range of investigations (Loomis and Hayes, 1996; Pascoe, 1983).

In general, toxicity testing methods can be divided into two categories: The first category comprises tests that are designed to evaluate the overall effects of compounds on experimental animals. Individual tests in this category differ from each other basically in regard to the duration of the test and the extent to which the animals are evaluated for general toxicity. These tests are classified as acute, prolonged and chronic toxicity tests (Loomis and Hayes, 1996). The second category of tests consists of those that are designed to evaluate specific types of toxicity in detail. The prolonged and chronic tests do not detect all forms of toxicity, but they may reveal some of the specific toxicities and indicate the need for more detailed studies. Thus, this second category of tests has been developed for the determination of effects of compounds on the fetus in a pregnant animal (teratogenic tests), on the reproductive capacity of the animals (reproduction tests), on the genetic system (mutagenic tests), for the determination of the ability of agents to produce tumors (tumorigenicity and carcinogenicity tests), etc. (Loomis and Hayes, 1996; Timbrell, 2002).

In this report, the focus was on the first category of toxicity testing methods, viz. acute and chronic toxicity tests. The primary concern was to determine how toxic the aqueous extract of *Artemisia afra* might be after acute administration to mice. Secondary, what would be the target organ for that toxicity and whether there would be any correlation between the toxic effects and the flavonoids (i.e. luteolin) contained in the plant materials after chronic oral administration to rats.

Acute and chronic toxic effects differ principally from each other with respect to the amount of chemical compound involved and the time intervening before the effect is seen (Timbrell, 2002). Acute effects are normally observed soon after exposure and result from the uptake of large amounts of poison, generally as a single dose. On the other

hand, chronic effects are often detected over an extended period of time during which exposure may be continuous or intermittent, though obviously at levels which are too low to produce an acute effect (Loomis and Hayes, 1996; Pascoe, 1983).

2.1.2. Acute toxicity

Acute toxicity has been defined as "the ability of a substance to cause severe biological harm or death soon after a single exposure or dose; or any poisonous effect resulting from a single short-term exposure to a toxic substance" (Association of Vermont Recyclers, 1996).

An acute toxicity test is a single test that is conducted in a suitable animal species and may be done for essentially all chemicals that are of any biologic interest. Its purpose is to determine the symptomatology consequent to administration of the compound and to determine the order of lethality of the compound. The test consists of administering the compound to the animals on one occasion (Loomis and Hayes, 1996; Timbrell, 2002; Pascoe, 1983).

Furthermore, acute toxicity tests are those designed to determine the effects, which occur within a short period after dosing. They serve to establish the lethal dose range of the test substance and provide prompt warning if a highly toxic compound is being dealt with (Poole and Leslie, 1989). They also provide information on the limiting toxicity arising from the pharmacological effects of the compound on target organs and, often, on the maximum dose to be used in subsequent sub-acute studies (chronic studies). This latter information is particularly important for predicting the amount of chemical required for future toxicological studies (Poole and Leslie, 1989).

The initial procedure, in an acute toxicity test programme, is to test a series of range-finding single doses of the compound in a single animal species. This necessitates selection of a route of administration, preparation of the compound in a form suitable for administration via the selected route and selection of an appropriate experimental animal

species (Poole and Leslie, 1989). Generally, even if the intended use of the compound does not involve the oral or parenteral routes, at least the oral route is used, in addition to other routes, so that comparison with other related compounds can be made. Additionally, such testing may also indicate the viability of the oral route for use in subsequent, more extensive and prolonged toxicity studies (Loomis and Hayes, 1996). Normally, the significance and use of the data that are obtained are limited to those routes of administration that were used in the actual experiment.

As usual, all initial acute toxicity tests are performed on either rats or mice because of the low cost, the availability of the animals, and the fact that abundant reference toxicologic data for many compounds in these species are available (Loomis and Hayes, 1996). In addition, these animals generally metabolize compounds in a similar manner to humans and the compounds (including metabolites) may have similar pharmacodynamics in the animals and humans. Before the experiment is performed, a total number of animals of similar body weight and same sex, or equal numbers of both sexes, are selected and randomly assigned to test (treatment) and control groups. After exposure to single doses of the test compound (or treatment), the animals are monitored for a minimum of 24hrs for any clearly recognized effect (such as changes in locomotor activity; bizarre reactions; sensitivity to pain, sound and touch; changes in social interaction; aggressive behavior; convulsions; paralysis, etc.) seen, as an index of toxicity, shortly or/and consistently after the administration of the chemical (Timbrell, 2002; Loomis and Hayes). However, the most easily recognized and certainly the most significant of effects is that of death and this outcome is usually used as a primary measure of acute toxicity. If the animals appear to be healthy at the end of 24hrs, they are monitored at daily intervals for at least a further one to two weeks for the appearance of delayed toxicity (Loomis and Hayes, 1996; Pascoe, 1983; Timbrell, 2002).

In the rodents, three types of acute toxicity studies may be performed. For the first type of study, it is usual to establish the maximum tolerated dose (i.e. the highest dose after which the animals recover completely from all effects of the chemical) and the minimum lethal dose for the compound (or treatment). The second type of study is the single dose

study to establish the target organ(s) for toxicity while the third type of study is for the determination of the precise LD_{50} or median lethal dose. The results of the latter type of study may be required, in most countries, for a clinical trial's certificate or even for a product licence (Poole and Leslie, 1989; Timbrell, 2002; Pascoe, 1983).

Usually, to establish a LD_{50}, at least four dose levels are used, with 5-10 male animals plus 5-10 females per treatment group. The animals are given a single dose of test compound and, at the end of 14-days observation period, the major organs and abnormal tissues of the surviving animals are collected and subjected to histopathological investigation. The LD_{50} and its confidence limits are calculated from the lethality data using probit analysis (Pascoe, 1983). Since a great range of concentrations or doses of various chemicals may be involved in the production of harmful effects, the LD_{50} has been used by some authors to devise categories of toxicity on the basis of the amounts of the chemicals necessary to produce harm. An example of such a categorization, along with the respective lethal doses, is given in the following Table.

Table 2.1. Classification of toxicity based on LD50 dose ranges.

Category	LD50 (mg/kg)	LD50 (mg/kg)	Classification
Extremely toxic	1 or less	< 5	Super-toxic
Highly toxic	1 to 50	5- 50	Extremely toxic
Moderately toxic	50 to 500	50- 500	Very toxic
Slightly toxic	500 to 5000	500- 5000	Moderately toxic
Practically non-toxic	5000 to 15000	5000- 15000	Slightly toxic
Relatively harmless	More than 15000	> 15000	Practically non-toxic
(Loomis and Hayes, 1996)		(Pascoe, 1983)	

2.1.3. Chronic toxicity

Chronic toxicity is defined as "the capacity of a substance to cause poisonous health effects in humans, animals, fish and other organisms after multiple exposures occurring

over an extended period of time or over a significant fraction of an animal's or human's lifetime "(Association of Vermont Recyclers, 1996).

The purpose of the chronic toxicity test is to investigate the harmful effects that foreign compounds that are introduced to animals in repeated doses or in continuous exposure over an extended period of time may produce (Poole and Leslie, 1989). The dose levels of compounds used usually range from a very low fraction of the therapeutically effective dose (i.e. somewhere in the range of the ED_{50} for the compound in that species, or of the same order as the anticipated human therapeutic dose range) to doses that approach the maximum non-lethal dose (as established in rodent acute toxicity studies) (Poole and Leslie, 1989; Loomis and Hayes, 1996).

Different approaches to dose ranging studies are applied depending on the species being used. The procedures used can vary, but usually involve exposing the experimental animals (in typical group sizes of two to five animals/sex/group) to various doses of the test compound, i.e. from the maximal non-lethal dose (determined in the acute studies) down to doses in the pharmacological dose range. Clinical chemistry and haematological parameters are then measured at the start of the study (i.e. within 48 hours after the first dose) and at the end of the study, along with full histopathology analysis of all abnormal tissues plus the major organs (such as the heart, liver, kidneys, lungs and brain of the animals), at least at the end of the study (Poole and Leslie, 1989; Timbrell, 2002).

2.1.4. Toxic effects

Toxic effects are defined as "harmful responses of a biological system to a toxic compound, and death of cells or the whole organism are the major response." (Timbrell, 2002)

In all the cases, the toxic effects are usually manifested either in an acute or a chronic manner, and occur mostly as a result of an acute or chronic exposure to toxic compound by oral ingestion, inhalation or absorption following skin contact (Timbrell, 2002). The

toxic effects are seen as (1) signs or reflection of a disturbance of the normal activities of enzymes that perform essential biochemical roles in all forms of life; (2) alteration of the normal activities of plasma membrane that regulate the exchange of nutrients and metabolites between the cell and its surroundings and (3) the disturbances of other normal cell activities, e.g. RNA and DNA synthesis, growth, division and general metabolism at all levels of organization from sub-cellular to organ and organ system (Pascoe, 1983; Timbrell, 2002).

The way in which the toxic agent is introduced into the body also plays a significant role.

2.1.5. Routes of administration

This term refers to the way in which drugs or compounds are introduced to animals or humans. To evaluate toxicity of a compound in animals various routes may be used, but two most commonly used modes of administration for animals studies are via intra-peritoneal injection or the oral route (Poole and Leslie, 1989)

2.1.5.1. Intra-peritoneal injection

This is one of the methods of dosing, which may occasionally provide information about local as well as systemic toxicity. To give drugs by intraperitoneal dosing, the animal is laid on its back and the abdomen shaved. This area is thoroughly cleansed and, using an appropriate syringe and needle, the abdominal wall is punctured. To ensure minimal danger of perforation of abdominal viscera, the injection should be made rostral and lateral to the bladder at an angle of about 15º to the abdomen. The depth of penetration should not exceed 5mm (Poole and Leslie, 1989; Waynforth, 1980).

2.1.5.2. Oral administration

The oral route is probably one of the most common means by which a chemical enters the body. In short, the oral administration is the form of administration involving the

gastrointestinal tract, which may be viewed as a tube going through the body, starting at the mouth and ending at the anus. Although it is within the body, its contents are essentially exterior to the body fluids. Most orally administered chemicals can otherwise have a systemic effect on the organism only after absorption has occurred from the mouth or the gastrointestinal tract. Oral administration of chemicals that are rapidly absorbed from the gastrointestinal tract would theoretically expose the liver to concentrations of the agent that would not be obtained if other routes of administration were used (Loomis and Hayes, 1996). Furthermore, if a compound entered the enterohepatic cycle, at least a portion of the compound would be localized in the organs involved in the cycle. Compounds that are known to be toxic to the liver would be expected to be more toxic following oral administration on repeated occasions, whereas their administration by other routes may be less hazardous (Loomis and Hayes, 1996; Waynforth, 1980).

2.2. Current use and importance of herbal medicines

A World Health Organisation survey indicated that about 70-80% of the world's populations rely on non-conventional medicine mainly of herbal sources in their primary healthcare. This is especially the case in developing countries where the cost of consulting a western style doctor and the price of medication are beyond the means of most people (Chan, 2003; Dyson, 1998). In South Africa, it is also currently estimated that 70-80% of the population, visiting traditional healers such as sangomas, inyangas and Rastafarians, rely on herbal medicines for health care (Dyson, 1998). In addition, the South African territory is considered to be a "hotspot" for biodiversity with more than 22,000 plant species occurring within its boundaries. This represents 10% of the World's plant species although the land surface of South Africa makes up less than 1% of the earth (Coetzee et al., 1999). Despite this enormous richness in plant species, still only approximately 3000 of these species of plants are today used as medicines. Of these, only 350 species are the most commonly used and traded medicinal plants with very few of them actually having been studied for their potential therapeutic properties (Van Wyk et al., 2000; Coetzee et al., 1999). Use of herbal medicines is thus, widespread in the world and in South Africa, but still very little is known about these medicinal plants.

A medicinal plant is factually any plant which in one or more of its parts contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of direct therapeutic agents (Sofowora, 1982). On the other hand, the knowledge and practice of herbal medicine, is assumed to be an acquisition of hundreds years of trial and observation handed down from generation to generation verbally or in writing. Specifically, in African traditional medicine, a treatment usually consists of a complex mixture of several extracts or the active principles of plants and often only one of the ingredients is responsible for the claimed therapeutic activity (Sofowora, 1982; Tomlinson and Akerele, 1998; Waithaka, 2004).

Plants were once a primary source of all the medicines in the world and they still continue to provide mankind with remedies. Examples include "Quinine" obtained from *Cinchona spp* and used against malaria (antiprotozoal); "Taxol" from *Taxus brevifolius*, used as anticancer and "Atropine" from *Datura* spp, used as mydriatic and antispasmodic. Today, still about one–quarter of all marketed orthodox pharmaceutical medicines is either derived from plant sources or from derivatives of secondary plant metabolites (Van Wyk et al., 2000; Chan, 2003). However, presently the rate for the successful discovery of new drugs from natural sources is not very promising. Several factors are responsible for this state of affairs of which the major factor is the triumph of synthetic-chemistry by which natural extracts have, in the pharmaceutical industry, been replaced with synthetic molecules that often has no connection to natural products. Nevertheless, various chemicals and biotechnological products of plant origin are being screened by major multinational pharmaceutical industries in the hope of finding new cures for diseases that are better than current therapies in terms of effectiveness, selectivity and reduced side effects (Chan, 2003; Raskin et al., 2002).

Other factors that sustain the low rate of success of herbal medicines as a potential source of new drugs are, firstly, the challenge and difficulty of acquiring well-documented plant material and the non-scientific process by which folk therapy systems operate, i.e. herbal medicine is often accused of being more based on faith, (Waithaka, 2004). Secondly, and perhaps one of the greatest arguments against herbal medicine today, is the lack of

scientific proof of its efficacy. Thirdly, witchcraft and the evil aspects associated with herbal medicine also discredit this form of medicine. Finally, further shortcomings of herbal medicine are the imprecise diagnosis that is often made by practitioners of this type of medicine and the absence of precise dosages for many herbal treatments (Sofowora, 1982).

In recent years, however, it has been noted that plants are arguably poised for a comeback as sources of human health products. The hope for this comeback is rooted in the unique and newly appreciated properties of phytochemicals *vis-à-vis* conventional new chemical entities-based pharmaceuticals, and are based on (1) the enormous propensity of plants to synthesize mixtures of structurally diverse bioactive compounds with multiple and mutually potentiating therapeutic effects; (2) the low-cost and highly scalable protein and secondary metabolite biomanufacturing capacity of plants; (3) the perception that because of the history of human use and co-evolution of plants and humans, phytochemicals provide a safer and more holistic approach to disease treatment and prevention (Raskin et al., 2002). Although the above properties of plants have been known for a long time, the ability to better exploit the uniqueness of plant therapeutics was only recently acquired as a result of the dramatic advances in metabolic engineering, biochemical genomics, chemical separation, molecular characterization and pharmaceutical screening. A challenge for phytochemical-based botanical therapeutics is to integrate the ability to identify and genetically manipulate complex biosynthetic pathways in plants with better characterization of genetic targets for the prevention and treatment of complex diseases (Raskin et al., 2002; Chan, 2003).

Notwithstanding the above, to date, the medicinal plant preparations are, in most cases, still used in their traditional dosage forms with ingredients that are poorly defined.

2.2.1. Traditional dosage forms and mode of administration of herbal medicines

When considering the traditional dosage forms of herbal medicine, several additional factors are also noteworthy. Firstly, the most common methods for administering traditional medicines are orally, sublingually, rectally, topically, nasally, smoking, steaming and bathing (Van Wyk et al., 2000). Secondly, the method of preparation of the traditional dosage form is most of the time also critical, since it deals with the amount of fresh or dry plant material to be used, the addition of appropriate volumes of solvents such as water or alcohol and additional activities such as boiling for a specified length of time or partial burning to achieve a desired colour (Van Wyk et al., 2000). The additional activities are usually intended to neutralize certain toxins or to increase the extraction of the active compound in the aqueous form of medicines. The method of administration and the method of preparation of the herbal dosage form can be expected to have a significant impact on the effectiveness of the various traditional dosage forms of herbal medicine used.

The following are examples of preparations commonly employed for therapeutic purposes via various specific routes of administration: (1) e*nemas, which are aqueous or oily solutions or suspensions,* intended for rectal injection.; (2) the *decoction,* the extract of any plant drug obtained by boiling, as well as the *infusion* which is an extract prepared by macerating the crude drug for a short period of time in cold or boiling water, both intended for administration by mouth; (3) *snuffs*, preparations of finely powdered, dried medicinal plants that can be drawn up into the nostrils through inhalation;(4) *inhalants, powder for licking, under the skin implantations, bath mixtures, poultices, balms, internal cleansing solutions and lotions* are intended either for bathing with, rubbing into incisions, anointing, inhaling as smoke, licking or applying to skin or nibbling on (Van Wyk et al., 2000; Sofowora, 1982).

Among other dosage forms not normally traditionally used but also very convenient are herbal tablets and capsules. Despite being quite useful, tablets may, however, contain

fixed formulations that may not readily be adaptable to the individual's specific needs. Capsules appear to be a more convenient form to take powdered plant drugs because they mask the unpleasant tastes or textures of the powdered forms of plant drugs and this is a distinct advantage for delivery of plant ingredients into the gastrointestinal tract (Waithaka, 2004).

Given the variety of traditional dosage forms, methods of preparation and modes of administration used with herbal medicine, as well as the general non-professionalism many of the traditional medicinal practitioners display with respect to these activities and the impact that these processes may have on the effective use of herbal products, much concern has recently emerged over aspects of the efficacy, safety and quality of treatment methods and products obtained from herbal or natural sources. Particularly, there is a concern that many herbal practitioners continue to use plants (and dosage forms and routes of administration) for the treatment of diseases without any knowledge of the toxicity profiles or safety of their plant materials (Chan, 2003; Waithaka, 2004).

2.2.2. Toxicity aspects of use of herbal preparations

Currently, there is an ongoing world-wide "green" revolution which is mainly premised on the belief that herbal remedies are safer and less damaging to the human body than synthetic drugs (Williamson et al., 1996). Many writers claim that it is assumed that "all things natural are good" (Gaillard and Pepin, 1999) and, generally, the extensive traditional use of herbal products is not assumed to be based on a comprehensive well-documented logic, but rather on empirical wisdom accumulated over many years, often arrived at through trial and error and transmitted orally from generation to generation. This traditional methodology has enabled those herbal medicines producing acute and obvious signs of toxicity to be well recognized and their use avoided. However, the premise that "traditional use of a plant for perhaps many hundreds of years establishes its safety does not necessarily hold true" (Medicines Control Agency-UK, 2002). The more subtle and chronic forms of toxicity, such as carcinogenicity, mutagenicity, and hepatotoxicity, may well have been overlooked by previous generations and it is these

types of toxicity that are of most concern when assessing the safety of herbal remedies (Williamson et al., 1996; Tomlinson and Akerele, 1998).

Safety should be the overriding criterion in the selection of medicinal plants for use in health care systems (Tomlinson and Akerele, 1998). As with all forms of self-treatment, the use of herbal medicinal products also presents a potential risk to human health. As far as medicinal plants go, there are a few main categories of safety concerns.

- Firstly, there is the extrinsic or non-plant associated category where the patient may be at risk of toxicity as a result of exposure to (1) accidental or deliberate contaminants present in the herbal product or to (2) substitutes of herbal material.
- Secondly, we have the intrinsic or plant associated category of safety concerns. In this category, the patient may be exposed to potentially toxic substances naturally present in the herbal products or materials.
- Thirdly, there is the category of safety concerns related to interactions when the use of synthetic prescription drugs is combined with herbal medicines.
- Finally, another category of safety concerns arises where specific patient groups may be at risk, e.g. pregnant or nursing mothers, children and the elderly (Tomlinson and Akerele, 1998; Medicines Control Agency-UK, 2002).

There can thus be several causes of toxicity with herbal products.

2.2.2.1. Causes of toxicity with herbal products

All chemicals may be considered toxic under certain conditions, e.g. even pure water when inhaled is rapidly absorbed across the lung alveoli to cause lysis of red blood cells. But some chemicals present a greater hazard than others (Pascoe, 1983). A large number of plants contain appreciable levels of biosynthetically produced chemical substances and many of these have either been reported to be toxic to humans or are predictably toxic based on extensive animal or *in vitro* studies (Tomlinson and Akerele, 1998).

Toxicity with medicinal plant products may arise in various ways, but in general two categories of causes can be distinguished:

- In the first category, as previously mentioned, the toxicity may be as a result of exposure to intrinsic ingredients of some medicinal plants. Examples of some more important classes of ingredients implicated here include: p*yrrolizidine alkaloids,* which are said to be hepatocarcinogens; a*ristolochic acid I*, said to be mutagenic and carcinogenic; *phorbol esters,* which are tumor promoters and vesicant to the skin; *carboxyactractyloside*, a deadly toxic compound; a*mygdalin*, a cyanogenic compound with many undesired effects; etc. (Gaillard and Pepin, 1999; Tomlinson and Akerele, 1998). In addition, several studies conducted on flavonoids indicate that, besides their apparently beneficial health effects, they may also induce mutagenicity and genotoxicity (e.g. quercetin) in both bacterial and mammalian experimental systems (Skibola and Smith, 2000).

- The second category of causes of toxicity of herbal medicines is more extrinsic or non-associated with the plant active constituents. In this category, the toxicity is a result of exposure to plant products contaminated with excessive or banned pesticides, microbial contaminants, heavy metals or chemical toxins, or with substituted ingredients. The pesticide, heavy metal and microbial contaminants may be linked to the source, collection or processing of the herbal materials (e.g. in contaminated environments). Chemical toxins may arise due to incorrect storage conditions or chemical treatment during storage (Chan, 2003; Gaillard and Pepin, 1999; Tomlinson and Akerele, 1998). Some of these environmental factors can be controlled by implementing standard operating procedures that lead to good agricultural, good laboratory, good supply and good manufacturing practices for producing medicinal products from herbal or natural sources (Chan, 2003).

Given the above, what then is the current prevalence of toxicity with herbal products?

2.2.2.2. Prevalence of toxicity with herbal products

Different retrospective studies done over the last 20 years indicated that the incidence of deaths occurring due to exposure to plants (as a proportion of total patients poisoned by traditional plant medicine) was about 1.5% in France, 5% in Belgium, 6.5% in Italy, 7.2% in Switzerland and 6% in Turkey (Gaillard and Pepin, 1999). The total number of deaths due to exposure to plants throughout the world however, is very difficult to establish and must certainly be underestimated since all cases of such deaths were, from analytical and forensic points of view, not always well documented and thus, rarely published. Nevertheless, it seems that death due to plant poisoning might be more important than other causes of poisonings. For instance, in South Africa, 2% of the people admitted for acute poisoning died compared to 15% of the patients poisoned with traditional plant medicine (Gaillard and Pepin, 1999).

From published reports, it appears that side effects or toxic reactions, of any form but associated with herbal medicines, are rare (Tomlinson and Akerele, 1998). This may be because herbal medicines are generally safe, that adverse reactions following their use are underreported, or because the side effects are of such a nature that they are not reported (Tomlinson and Akerele, 1998; Gaillard and Pepin, 1999).

There is, therefore, a need for the public to have an understanding of the risks posed by herbal medicines so as to ensure that such products are used safely. Particularly, as highlighted above, the safety of some herbal products is compromised by lack of suitable control of the quality of herbal medicines and the absence of appropriate herbal use information for patients. To appropriately inform and protect the public, the herbal products must, however, first be evaluated for its toxicity.

2.2.2.3. Evaluation of herbal toxicity

Herbal toxicity can be evaluated by (1) observing human or animal populations exposed to the plant material, (2) administering the plant medicine to animals under controlled

conditions and observing the effects *(in vivo)* and (3) exposing cells, sub-cellular fractions or single-celled organisms to the plant material *(in vitro)* (Timbrell, 2002). In this report the focus is more on the *in vivo* model.

Ethically, toxicity of a compound cannot randomly be evaluated in humans. Nevertheless, the exposure of humans to toxicity with herbal materials, as highlighted previously on point 2.2.2.1, may occur accidentally when these are part of their therapeutic activities or intentionally as with drugs and food additives (Chan, 2003). In such cases, the accidents resulting from this type of exposure may, if well monitored and recorded (i.e. by measuring substances and their metabolites in body fluids and using biological indices of pathological change), provide important information about the toxicity of a plant material in humans. However, acquiring such data is often difficult and rarely complete, and the latter is the main reason why procedures for animal toxicity testing have been maintained as a successful alternative for evaluating the harmfulness of compounds for humans (Timbrell, 2002; Tomlinson and Akerele, 1998).

As previously reported in sections 2.1.2 and 2.1.3, to date, the majority of data on the toxicity of chemicals, including drugs and herbal medicines, is gained from experimental studies done in animals (*in vivo*). The data so acquired are used for the risk assessment and safety evaluation of drugs (or herbal medicines) prior to human exposure. Because animal tests can be carefully controlled with the exact known doses being used, the quality of the data obtained is generally reliable (Timbrell, 2002). The number of animals used should be enough to allow statistical significance to be demonstrated and the application of humane conditions and proper treatment of the animals are essential, for scientific as well as ethical reasons, to help ensure that the data are reliable and robust (Timbrell, 2002; Chan, 2003).

In a toxicity study, the animal species selected will depend partly on the type of toxicity test, existing data available and also on ethical and financial considerations. The most common species used are rats and mice for reasons of size, accumulated knowledge on

these species and cost, besides the similarity of their metabolism to that of humans (Timbrell, 2002; Loomis and Hayes, 1996).

In the present study, the concern was on the evaluation of the toxicity of *A. afra* after the administration of the plant material to rodents. As part of the effort of quality assurance of herbal products, scientific experiments were to be carried out to assess the safety of this medicinal plant using the rodent as an alternative mammalian species

But what is *Artemisia afra*?

2.3. *Artemisia afra* – important traditional medicinal plant

Artemisia afra is known as a "cure all" traditional remedy in South Africa and its neighbouring countries due to the multiplicity of ailments for which it is used (Roberts, 1990). The present section traces what is known about the botany, current medicinal uses, the pharmacological effects and active ingredients of the plant.

2.3.1. Botany of Artemisia afra

2.3.1.1. Taxonomy

Artemisia afra belongs to:

Division: ***Magnoliphyta***
Class: ***Magnoliopsida***
Sub class: ***Asteridae***
Order: ***Asterales***
Family: ***Asteraceae***
Genus: ***Artemisia***
Species: ***A. afra***

Figure: 2.1. *Artemisia afra* in its natural habitat at Montague museum

Major African tribes like the Zulu, Xhosa, Tswana and Sotho have different vernacular names for *A. afra* and for the colonists it resembled European wormwood (*Artemisia absinthium*). The popular names for *A. afra* include:

- Wild wormwood, als, wild als, African wormwood (English)
- Wilde als, als, alsem (Afrikaans),
- Iliongana, lengana (Pedi, Tswana)
- Umhlonyane (Xhosa)
- Mhlanyane (Zulu)
- Zengana (Southern sotho)

(Van Wyk et al, 2000; Dyson, 1998; Hutchings et al., 1996; Iwu, 1993; Roberts, 1990).

2.3.1.2. Botanical description

Artemisia afra is a medium sized clump-forming perennial herb, rarely exceeding 2m in height. It has a ribbed stem, is much- branched and woody, shortly rhizomatous, and is an asset to any garden. Its leaves are finely-divided, silver- grey due to the presence of fine hairs, up to 80mm long x 40mm wide; alternately arranged, oval in shape and aromatic (Van Wyk et al., 2000; Dyson, 1998; Hutchings et al., 1996; Roberts, 1990). It produces pale yellow tubular florets, with few outer female and inner bisexual florets, occurring in an elongated racemose panicle. The capitula are small, receptacle flat and naked. The African wormwood flowers between March and July, and the seeds are produced from August to November. The fruits are about 1mm long, somewhat 3-angled and slightly curved with a silvery-white coating (Van Wyk et al., 2000; Hutchings et al., 1996; Roberts, 1990).

The aerial parts of *A. afra* have the strong characteristic odour of wormwood and a bitter taste. The leaves are the important components of the plant used, but sometimes roots are also used (Van Wyk et al., 2000).

Finally, *A. afra* is very drought resistant and hardy, very quick and easy to propagate, and will grow in any soil just needing occasional watering and cutting back (Hutchings et al., 1996).

2.3.1.3. Geographical habitat and distribution

According to the FAO (Food and Agriculture Organisation of the United Nations) report, *Artemisia afra* is a herb growing in the high land areas of Eastern and Southern Africa at altitudes ranging between 1500 and 3000m where the soils range from volcanic ash, loamy sands, to sandy or calcareous clay loams of volcanic or granitic origin (Iwu, 1993).

The plant grows in the South and Eastern regions of the continent and has been located in Ethiopia, Kenya, Tanzania, Zaire, Zambia, Zimbabwe, Angola and the Republic of South Africa (Iwu, 1993; Watt and Breyer –Brandwijk, 1962). In South Africa, it usually grows in rocky mountainous areas along forest margins and stream sides and its natural distribution extends from the Northern and Eastern Transvaal to the Western Cape, except the Northern Cape.

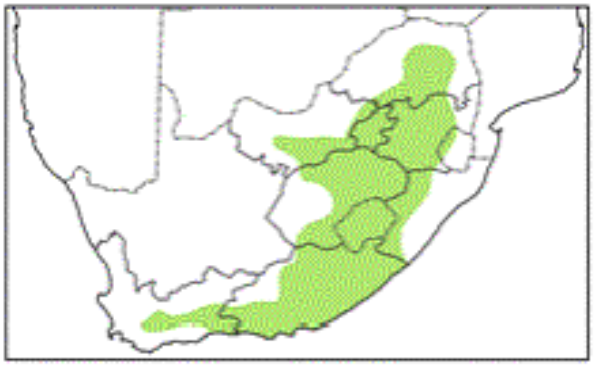


Figure: 2.2. – Map of *A. afra* distribution in South Africa.
(Adapted from SATMERG Pharmacopoeia, Monographs Project, 1999).

2.3.2. Medicinal uses

Artemisia afra (wormwood) got its name from its ability to get rid of worms. Beside this anthelmintic property, it is widely used for numerous other ailments – mainly, cough,

cold and influenza, as well as fever, loss of appetite, colic, headache, earache and malaria, amongst others. In fact, the plant is used for various respiratory and gastro-intestinal ailments including croup, whooping cough, dyspepsia, and as antihelmintic and emetic. In addition to the aforementioned uses, the plant has been used for gout, to keep urine free from sugar in a case of diabetes mellitus, for menstrual chill after childbirth, bathing hemorrhoids, measles, gumboils, toothache, heartburns, bronchitis and asthma (Dyson, 1998; Hutchings et al., 1996; Roberts, 1990).

A variety of traditional dosage forms are employed when *A. afra* is used for the above-mentioned ailments.

2.3.3. Traditional dosage forms of A. afra

In South African traditional medicine, *Artemisia afra* is mostly taken in the forms of a decoction or infusion as the usual preparation. It is also taken in the form of steam or vapour arising from boiling the leaves in a water pot. In addition, fresh leaf rolls are used for inhalation purposes. The infusions or decoctions are made with variable quantities of fresh leaves and, as reported by Watt and Breyer –Brandwijk (1962), various forms of these preparations are used by the different tribes to treat different diseases. The most popular traditional method for preparing a tea (infusion) or decoction is to add a quarter cup of fresh leaves to one cup of boiling water, allow it to stand and steep for 5 minutes, then strain and sweeten the filtrate with honey or sugar to mask the bitter taste. Thereafter, the tea is sipped at a set times (to ease all types of cold, cough, heartburn and bronchial disorders) or used as a gargle (e.g. for a sore throat) (Roberts, 1990).

Another popular traditional method of preparing an infusion is to pour two litres of boiling water over one cup of fresh leaves and stems, allow the mixture to stand for an hour and then straining the mixture (Roberts, 1990). This filtrate may then be used as a wash for heamorrhoids and in the bath to bring out the rash in measles, to soothe fevers, to wash wounds, sores, rashes, bites, and stings, and as an eyebath diluted with warm water to soothe red, smarting eyes (Roberts, 1990).

On the other hand, an infusion called '*Strong brew*' is made from quarter cup of leaves and one and a half or two cups of boiling water, which is allowed to stand, steep and draw for 10 minutes before it is strained. This filtrate is used as a mouthwash for gumboils and mouth ulcers, or can be dropped gently into the ear to relieve earache. Another popular preparation is '*Wilde als brandy,'* which is an old standby kept in the medicine chest to treat colds, coughs and chest ailments, as well as indigestion and stomach cramps. It is made from one bottle of brandy, one cup of wilde als leaves, a quarter cup thyme, half a cup mint leaves, one cup of sugar, one thumb length piece of ginger and a quarter cup of rosemary. All the ingredients are pushed into the bottle of brandy and shaken well daily for a month. Thereafter, it is either strained or allowed to grow stronger with age by leaving the herbs in it. Doses of one Tablespoon in water are taken at a time. This "magic potion" is still made today and is a much respected and popular medicine among rural people in South Africa (Roberts, 1990).

For inhalation purposes, the popular method of preparation is to place two or three cups of leaves into a bowl and pour enough boiling water over it to cover it. Then a towel tent is made over the patients head, the bowl held under his/her nose and the patient instructed to inhale deeply until the brew cools. This traditional method of administering *Artemisia afra* in steam or vapour form provides an excellent inhalant to use for bronchitis, blocked nose, tight chest, asthma and chest colds. (Roberts, 1990).

Besides the above mentioned dosage forms of *Artemisia afra,* the Venda and Twana people also make a solution (wash) of the plant for skin ailments, and use the warmed leaves to draw out pimples and boils by applying them as a poultice. The warmed leaf is also used as an excellent and a soothing poultice over a painful neuralgia, mumps swellings and on a sprained or strained muscle, and bound over the stomach for babies with colic. In addition, the *Artemisia afra* leaves may be rolled and inserted into the nostrils to alleviate headache and stuffy nose or a fresh leaf tip may be inserted and packed in the gaps of the teeth to relieve toothache. Dried leaf is also smoked to relieve throat irritation, ease congestion, release phlegm and soothe a sore throat and coughing at

night (Watt and Breyer –Brandwijk, 1962). Finally, the Zulu people make the infusion of *Artemisia afra* by grinding up the leaves and adding hot water, and then give this as an enema to children with worms and constipation (Roberts, 1990; Watt and Breyer – Brandwijk, 1962).

To date, few studies have been done to verify the quality and effectiveness of the above described traditional dosage forms of *A. afra* when it is employed for the various medicinal uses (mentioned under 2.3.2). Analysis of the chemical constituents of *A. afra* and studies on the pharmacological effects of the plant and/or some of its constituents may, however, throw some light on this matter.

2.3.4. Pharmacological effects and chemical constituents of A. afra

As already mentioned, only very few pharmacological investigations have been done on *A. afra* using mainly different extracts. Thus, studies using the aqueous extracts of *Artemisia afra* have indicated that the plant has bronchodilator and anti-inflammatory activities (Harris, 2002), and preliminary tests done by Hutchings et al.(1996) using the same type of extracts have shown that *Artemisia afra* may have narcotic analgesic and antihistaminic activity. Other assays carried out on the ethanolic extract and dichloromethane extract have shown the plant to have *in vitro* hypotensive and anti-tuberculosis effects, respectively (MRC and SAHealthinfo, 2004).

The above mentioned pharmacological activities and any other pharmacological effects of *A. afra* must be linked to its chemical constituents and it has been shown that the plant contains volatile oil, terpenoids, coumarins, acetylenes, scopoletin, flavonoids as well as triacontane and umbelliferone etc. The volatile oil has been shown to contain 1, 8-cineole, α-thujone, β-thujone, camphor and borneol as major constituents and to have definite anti-microbial and anti-oxidative properties. The toxic and hallucinogenic effects known to occur with the use of this plant have been associated with thujone. Overdose or continued use of *Artemisia afra* over long periods can thus, be potentially harmful (Watt and Breyer –Brandwijk, 1962; Van Wyk et al., 2000; Tang et al. ,2000).

Although terpenoids, coumarins, acetylenes, scopoletin, as well as triacontane and umbelliferone have been shown to be present in *A. afra*, their contribution to the biological activities of the plant is not yet known (Van Wyk et al, 2000; Iwu, 1993).

Artemisia afra also contains flavonoids, a group of phytochemicals well-known for their many biological activities (Harborne, 1973). For instance, aqueous extracts of *Artemisia afra* have been shown to contain the flavonoids luteolin, kaempferol, apigenin, hesperetin and quercetin (Waithaka, 2004) and these flavonoids have been said to be responsible for some of the pharmacological activities exerted by other *Artemisia* species and other medicinal plants. For example, some flavonoids (including luteolin and its derivatives) found in *Artemisia copa* exhibited anti-tumoral, anti-inflammatory and analgesic activities (Mino et al., 2004) while the flavonols (quercetin, kaempferol and their glycosides) present in the aerial parts of *Artemisia annua* were associated with the antioxidant activity of the plant (Cai et al., 2004). Flavonoids (quercetin and its derivatives) isolated from *Artemisia capillaris* were shown to have significant anti-platelet aggregation activity (Wu et al., 2001). While the establishment of the pharmacological activities of flavonoid-containing plants such as *Artemisia afra* is still a challenge to face, based on even the limited information presently available, it does appear that the flavonoids with their associated pharmacological effects may be important constituents as far as validating the folk medicinal use of *Artemisia afra* is concerned.

But what exactly is known about the flavonoids?

2.4. Flavonoids

Flavonoids are phenolic compounds widely present in plants and foods of plant origin (Markham, 1982; Harborne, 1973; Nuutila et al., 2002). The term, phenolic compound, embraces a wide range of chemical constituents possessing an aromatic ring bearing one or more hydroxyl substituents in common. It is estimated that about 2% of all carbon photosynthesized by plants is converted into flavonoids or closely related compounds.

They are virtually ubiquitous in green plants in which they are often responsible for the coloration of flowers, fruits and leaves. Their occurrence is however not restricted to the latter organs but include all parts of the plant, i.e. roots, wood, bark and nectar. As such, flavonoids constitute one of the most characteristic classes of compounds that are likely to be encountered in any work involving plant extracts (Markham, 1982; Harborne, 1973).

2.4.1. Chemistry and classification

Flavonoids exist in a large range of chemical structures. They contain fifteen carbon atoms in their basic nucleus, arranged in a C_6-C_3- C_6 configuration consisting of two aromatic rings (A and B) linked by a three carbon unit which may or may not form a third ring (C) (Markham, 1982), (Figure 2.3).

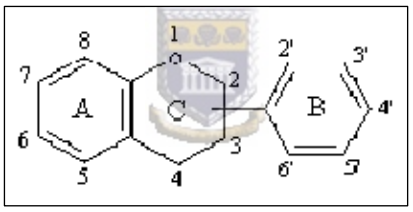


Figure: 2. 3. General structural formula of flavonoids

The flavonoids can exist in the free aglycone (i.e. flavonoids without attached sugars) form or conjugated glycoside form (flavonoid aglycone attached to one or more sugars) (Markham, 1982). Depending on the chemical bondage between the aglycone and sugar moiety, two types of glycosides are found:

- Flavonoid *O* –glycosides in which one or more of the hydroxyl groups is bound to a sugar or sugars by an acid labile hemiacetal bond. The effect of glycosylation is to render the flavonoid less reactive and more water (sap) soluble.
- Flavonoid *C*–glycosides in which the sugars are attached directly to the benzene nucleus by a carbon – carbon bond. They are typically acid resistant.

According to the substitution patterns of ring C, the oxidation state of the heterocyclic ring and the position of ring B, flavonoids can be classified into various sub-groups. The major sub-groups are the anthocyanidins, flavonols, flavanones, flavones and isoflavonoids.

Anthocyanidin is an extended conjugation made up of the aglycone of the glycoside, anthocyanins (Figure 2.4), (Waithaka, 2004). Next to chlorophyll, anthocyanins constitute the most important group of plant pigments visible to the human eye (e.g. dark colours present on red fruit such as plums strawberries, black berries, etc.). Furthermore, the anthocyanidins and their glycosides constitute a large family of differently coloured compounds and occur in countless mixtures in practically all parts of higher plants. They are of great economic importance as fruit pigments and thus, are used to colour fruit juices, wine and some beverages (Friedli, 2004; Waithaka, 2004).

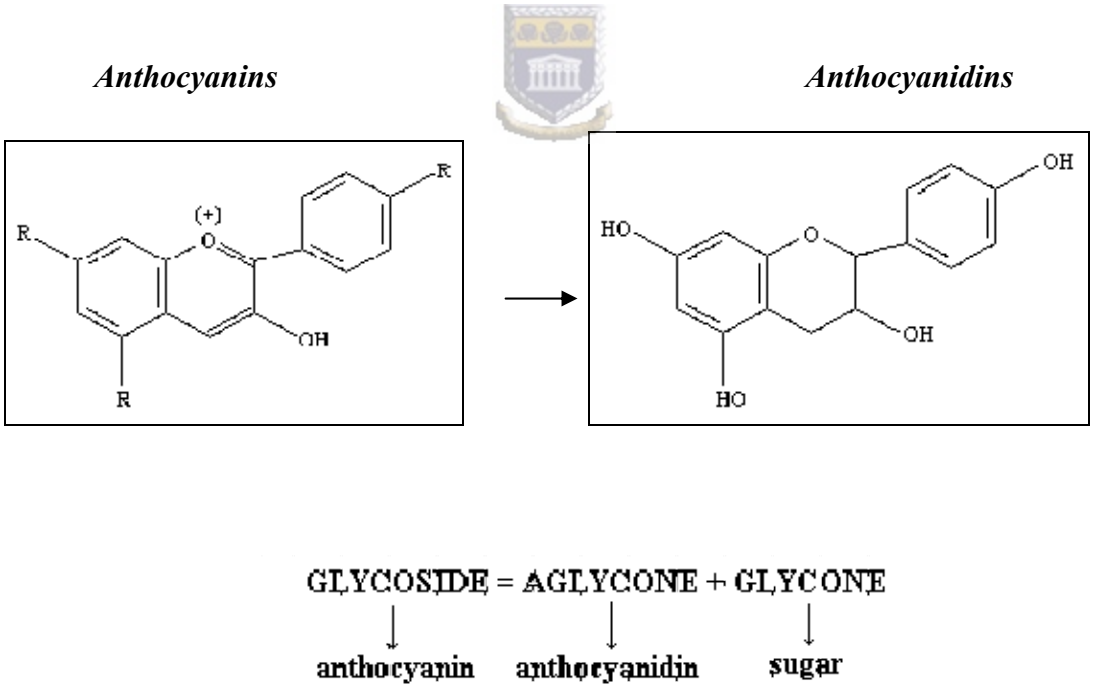


Figure: 2.4. General structures of anthocyanin and their aglycone forms. R = sugar moiety

Flavonols (Figure 2.5) are generally found in angiosperms and include, e.g. quercetin (present in black tea, broccoli, garlic, tomatoes, etc.), kaempferol and myricetin (present in French bean, carrot, Chinese cabbage, etc.) (Miean and Mohamed, 2001)

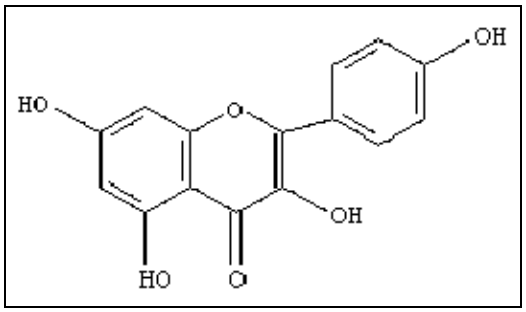


Figure: 2.5. The structure of flavonols

Flavanones (Figure 2.6) can be found in *citrus sp*. and examples include hesperetin, hesperidin, etc. (Benavente-Garcia et al, 1997).

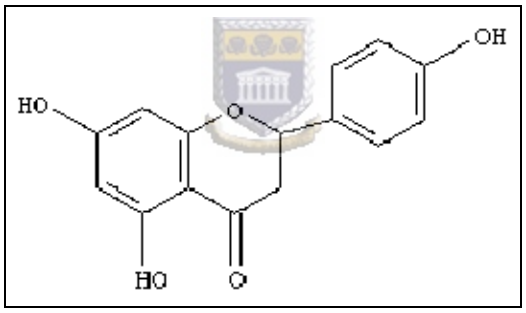


Figure: 2.6. The structure of flavanones

The main **flavones** (Figure 2.7) are apigenin and luteolin and they are generally found in herbaceous families, e.g. Labiatae, Umbelliferae, Compositae, and in *Citrus sp* and aromatic plants (Friedli, 2004; Benavente-Garcia et al., 1997; Waithaka, 2004).

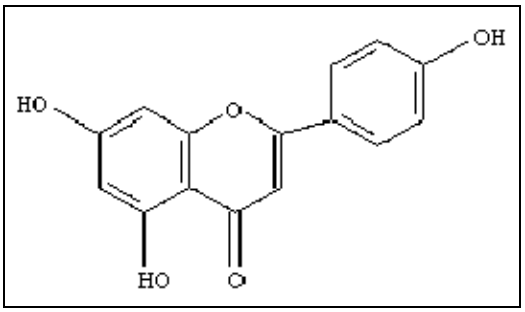


Figure: 2. 7. The structure of flavones

Finally, while most of the afore-mentioned flavonoids (i.e. the flavanones, flavones, flavonols, and anthocyanins) have ring B in position 2 of the heterocyclic ring, in the last group of flavonoids i.e. the **isoflavonoids**, ring B occupies position 3 (Figure 2.8).

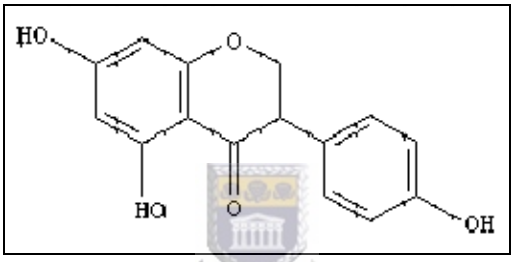


Figure: 2.8. The structure of isoflavonoids

Additionally, depending also on the chemical bondage between the aglycone and sugar moiety, flavonoids have been shown to exhibit many potential activities (Aherne and O'Brien, 2002). The flavonoid aglycone forms are known to exhibit pharmacological activities more than the glycoside forms (Komperlla, 2005; Markham, 1982).

2.4.2. Biological activities of flavonoids

Flavonoids display a wide range of biological activities. These include anti-oxidation, anti-inflammation, anti-platelet, anti-thrombotic action, and anti-allergic effects (Miean and Mohamed, 2001); inhibition of carcinogenic cell invasion, effect on coronary heart disease, anti-proliferative effects, anti-microbial and anti-mutagenic effects (Benavente-

Garcia et al., 1997). In addition, they can also inhibit enzymes such as prostaglandin synthase, lypoxygenase, cycloxygenase and those closely related to tumorigenesis, and induce detoxifying enzyme systems such as glutathione S-transferase (Miean and Mohamed, 2001).

In terms of activity, luteolin and apigenin have been reported to inhibit platelet aggregation in rabbits, to be chemo protective, anti-inflammatory-allergy agents (Benavente-Garcia et al., 1997; Harborne et al., 2000). Luteolin is also a vasodilator agent (Benavente-Garcia et al., 1997).

While the apparently beneficial health effects of flavonoids are noteworthy, the results of a few studies have also indicated their mutagenicity and genotoxicity in both bacterial and mammalian experimental systems. Thus, quercetin has been identified as a potent topoisomerase II inhibitor at low concentrations, a mutagenic agent and an inhibitor of thyroid peroxidase essential to thyroid hormone synthesis (Skibola and Smith, 2000); luteolin as being slightly more toxic towards isolated rat hepatocytes, and apigenin as presenting inhibitory effects on *Hela* tumor cell proliferation (Galati et al., 2001). These adverse effects are said to be due to their activity as pro-oxidants generating free radicals that damage DNA or their inhibition of DNA- associated enzymes such as topoisomerase (Skibola and Smith, 2000; Galati et al., 2001). High intakes of flavonoids may, due to their diverse pharmacological properties, thus, potentiate other deleterious effects, which may include alteration of drug- and amino acid metabolism. In addition, the flavonoids may also modulate the activity of environmental genotoxicants and alter the activity of other key metabolizing enzymes (Skibola and Smith, 2000).

In this study, the focus was more on luteolin found in *Artemisia afra* and its possible adverse effects.

2.5. Luteolin

Luteolin is the name given by Chevreul to a yellow crystalline substance he isolated for the first time as an active compound in *Reseda luteola L*, in' 1833. In 1896, Pekin also confirmed the presence of the same compound in weld (*Reseda luteola),* a species of plant (Cristea et al., 2003; Muganga, 2005).

Luteolin is a flavone (Figure 2.9) that is found in foods such as parsley, artichoke leaves, celery, peppers, olive oil, rosemary, citrus fruits, sage, thyme, and many others. It is found in glycosylated forms in green pepper, perilla leaf, celery, etc. and in the aglycone form in other vegetables e.g. perilla seeds (Miean and Mohamed, 2001; Shimoi et al., 1998). Generally, luteolin is found in plants in the form of luteolin-7-O-glucuronide, luteolin-5-O-glucuronide or luteolin-3'- glucuronide (Komperlla, 2005).

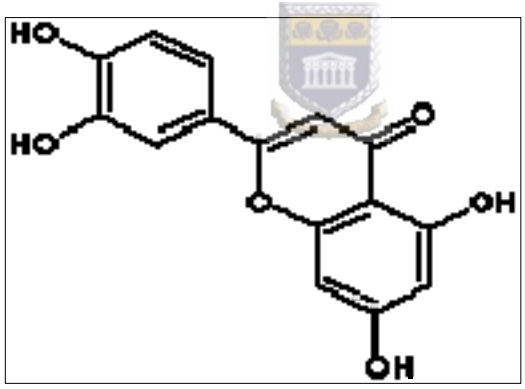


Fig 2.9: Luteolin (flavone) 3', 4' 5, 7- tetrahydroxy flavone. Formula: C15 H10 O6

As for all flavonoids, depending on the chemical structure of the compound, luteolin has been shown to exhibit various biological activities.

2.5.1. Biological activities of luteolin

"There are no safe drugs, only safe ways of using them" (Voltaire in Timbrell, J., 2002).

Luteolin may be a valuable emerging pharmacological agent owing to its many beneficial properties. It has antioxidant, anti-inflammatory, anti-allergic, anticancer activities and immune-modulating properties to suppress hyperactive immune systems (Shimoi et al., 1998; China Great Vista Chemicals, 2002; Mino et al., 2004). In addition, luteolin is an antimutagenic and antitumorigenic agent, an inhibitor of protein kinase C and a potent hypoglycemic agent that improves insulin sensitivity (Shimoi et al., 1998; China Great Vista Chemicals, 2002). Besides producing spasmolytic effects, this compound may, apparently, also help to promote healthy blood glucose levels, help in weight management for syndrome X, as well help the body withstand radiation and chemotherapy (China Great Vista Chemicals, 2002). In ophthalmology, luteolin is a promising agent for prevention and treatment of cataract and of vascular eye disorders (China Great Vista Chemicals, 2002). It is also an active inhibitor of different hyaluronidases, which modify hyaluronic acid, one of the polymers that account for the toughness and flexibility of cartilage and tendon (China Great vista chemicals, 2002).

Based on the above information, luteolin, thus, appears to be one of the potential pharmacological constituents that could contribute to the medicinal values of many plants. However, very little is known about the possible adverse effects of this compound.

2.5.2. Toxicity aspects of luteolin

Luteolin has been investigated *in vivo* and *in vitro* with the focus on its potential adverse effects. It is said to act as potent inhibitor of aromatase and 17 ß-hydroxysteroid oxidoreductase, which are enzymes involved in estrogen metabolism (Skibola and Smith, 2000). In addition, it was shown that luteolin-induced cytotoxicity in isolated rat hepatocytes correlated with mitochondrial membrane potential, and that it was also slightly more toxic than the polyphenol chrysin towards the *Hela* tumor cells (Moridani et

al., 2002). Furthermore, Kotanidou et al. (2002) reported that the intra-peritoneal administration of a 50-mg/kg dose of luteolin was lethal to three of three mice receiving it.

Apart from the above information, little is, however, known to date about the toxicology of excess flavonoid (luteolin) intake, while its beneficial attributes are commonly overemphasized (Skibola and Smith, 2000). Furthermore, if, as was the case with the present study, one wanted to determine the toxicity profile of a plant such as *Artemisia afra* that contains luteolin and attribute any of the effects to luteolin (or flavonoids), one also clearly had to link the toxic effects with high levels of this flavonoid. A suitable analytical technique for the assay of luteolin in plant material and biological fluids was, thus, needed.

2.5.3. Analytical techniques suitable for the determination of luteolin levels in plant materials and in blood plasma.

Literature reveals several efficient methods and techniques for the analysis of plant materials. The methods generally include steps for the separation, purification and identification of the many different constituents that may be present in plants.

As a typical flavonoid, luteolin can be extracted from plant materials using a variety of solvents, depending on its form, i.e., whether it is either the aglycone or glycoside. The flavonoid (luteolin) possessing a number of unsubstituted hydroxyl groups or a sugar is a polar compound and generally moderately soluble in polar solvents such as ethanol, methanol, butanol, acetone, dimethyl sulphoxide, water etc. The presence of an attached sugar tends to render the flavonoid more water-soluble and combinations of the above mentioned solvents with water are thus, better solvents for glycosides. In contrast, less polar aglycones such as flavonols, highly methoxylated flavones and others tend to be more soluble in solvents such as ether and chloroform (Markham, 1982). Besides this, the flavonoids may be also extracted from plant materials by the use of the aqueous

extraction methods, which usually are preferred in order to mimic the herbal practitioners' methods. This was the case in this study. Typically then, the plant materials are boiled with water for a half hour in order to induce an efficient extraction of the flavonoids.

Several techniques can be used to separate, identify and quantitate flavonoids, but among these, the high performance liquid chromatography (HPLC) appears to be the most adequate, popular and applicable technique for the analysis of flavonoids (Cristea et al., 2003). In fact, the technique is basically a form of column chromatography, which utilizes a column of packing material of small particle size and regular shape. In addition, the fineness of the packing and the high pressures are employed to achieve acceptable flow rates. Thus, the technique offers the researcher a method of quantitative analysis of a flavonoid-containing mixture at a high level of resolution and sensitivity. It is, in fact, the quantitative aspect of the analysis, in particular, which sets it apart from other chromatographic methods.

Wide ranges of solvent combinations for the elution of flavonoids in HPLC have been reported in the literature. Normally, solvents such as water-methanol, water-methanol-acetic acid (formic acid) and water-acetonitrile in varying proportions have been successfully used to chromatograph flavonoids (luteolin) (Markham, 1982).

Using the above principles as a basis, it was anticipated that a suitable HPLC assay could be developed that would not only allow the quantification of luteolin in plant materials, but might also be used to determine the level of luteolin in blood plasma samples of an animal that had ingested luteolin-containing *Artemisia afra*.

Chapter 3

WORK PLAN

3.1. Introduction

The present chapter traces the outline of the objectives, hypothesis and the study approach intended for the acute and chronic toxicity studies of the flavonoid- containing plant, *Artemisia afra* in rodents, namely mice and rats.

3.2. Objectives

The overall objective of the present study was to investigate the possible toxicity of the flavonoid-containing plant, *Artemisia afra* and specifically to establish the safety of the aqueous extract of this plant by focusing on its acute and chronic toxicity in mice and rats respectively.

To realize this objective it was decided:

- To determine the adverse effects that *Artemisia afra* aqueous extract may cause in mice and rats (two commonly used animals for toxicological studies),
- To determine the LD_{50} for *Artemisia afra* administered acutely in mice using the traditional method of "Litchfield and Wilcoxon" and the "AOT425statPgm method ",
- To compare the doses of the plant extract inducing lethality (i.e. the LD_{50}) after acute oral and intraperitoneal administration to mice,
- To determine the toxicity profile of *Artemisia afra* after chronic oral dosing in rats,
- And finally, to assess whether the plasma levels of flavonoids (esp. luteolin) possibly correlate with the dose ingested and/or any of the toxic effects that may be observed after chronic ingestion of *Artemisia Afra.*

3. 3.Hypothesis

"*All substances are poisons: there is none which is not a poison. The right dose differentiates a poison and a remedy"* (Poole and Leslie., 1989).

I hypothesized that: (i) the flavonoids such as luteolin contained in *Artemisia afra*, if given in high enough dose, may induce some adverse effects; (ii) the dose of plant at which such adverse effects occur would depend on both the route of administration (oral vs. intraperitoneal) used as well as the duration (acute or chronic) of exposure to the plant material; (iii) the new computer program-based method (i.e. AOT425statPgm) for testing acute toxicity of environmental pollutants, can advantageously be used to evaluate the acute toxicity of plant medicines, and (iv) plasma levels of luteolin could be used as a marker to monitor *A. afra* toxicity and/or its bioavailability (ingestion).

3.4. Study approach

3.4.1. Rationale for the selection of the plant material

Artemisia afra was chosen mainly because it is one of the oldest and best known of all indigenous medicines in South Africa (Roberts, 1990). It is a warming, cleansing and disinfecting herb used for a great number of ailments as stipulated in the previous chapter, and is still effectively used to date. In addition, this plant and its flavonoids are actually one of the subjects of the current research programme in the Pharmacology Discipline in the School of Pharmacy at University of the Western Cape. The main objectives of the research programme in this Discipline are to evaluate the claimed therapeutic effects of the plant material by means of pre-clinical and clinical studies, and to further develop a better dosage form of this plant. The study of its safety, first in a suitable animal model, forms a crucial part of the overall programme.

3.4.2. The form of the plant material used

In this study, the aqueous extract of the plant was used so as to remain as close as possible to the traditional methods used by herbal practitioners and generations of local people, and in that way, to assess the risk that might be incurred by people exposed to the mixture of chemical compounds (known and unknown) found in *Artemisia afra*.

3.4.3. Rationale for the use of the animal model for safety testing of the plant material

It is known that the selection of suitable animal species as experimental model will likely offer the opportunity of valid quantification and prediction of plant material as potential therapeutic agent or as potential toxicant in humans (Mdhluli, 2003).

The *in vivo* mice and rat toxicity models were selected for this study based on the following facts:

1. These two animal species are similar to humans in many of their biochemical responses to drugs, including the absorption, distribution, metabolism and excretion of drugs. Moreover, if interpreted with due care, the extrapolation of the biological results obtained with these animals to humans may be considered valid.
2. These test species are small and can, with practice, be easily handled, and may be housed in large numbers in a relatively small area. Also, being small animals, it was an economical and practical proposition to use large enough numbers in the experiment to provide the required statistical validity to the results.
3. *In vivo* testing is highly recommended as the first choice testing method for assessing acute and chronic toxicity of new chemical entities (Ankier and Warrington., 1989).

3.4.4. Rationale for the toxicity parameters measured

Normally, before a new chemical entity can be marketed as a drug its pharmacological effects must first be evaluated using animals. Its bioavailability must also be determined and toxicological studies performed to assess its acute and chronic toxicity, mutagenicity, carcinogenicity, etc. If the results of the efficacy and toxicity studies in animals are favourable, the drug is then tested in a few human volunteers, followed by pilot studies and large-scale clinical trials (Ankier and Warrington., 1989).

As is the case for many medicinal plants, *Artemisia afra* can, because of its richness in organic compounds having numerous biological activities (as seen in the previous chapter), be used for the same purposes as orthodox medicine. The chemicals confer to the plant the therapeutic values that sustain its multiple uses in humans. However, the therapeutically active individual compounds are always found in the plant(s) as a complex mixture with some other compounds having either beneficial and/ or toxic effects.

Therefore, to assess to the safety of *Artemisia afra,* an array of parameters were to be measured in the acute and chronic toxicity studies proposed.

In the acute study:

For this study, the main parameter to be measured was death of the mice after ingestion of *A. afra* via the oral and intraperitoneal routes. More specifically, the LD_{50}, i.e. the dose that would kill 50% of the dosed animals was to be determined. This parameter is a standard measure of the acute toxicity potential of biologically active material. From the LD_{50} for the acute dosing, doses for the subsequent chronic study in the rats could also be estimated. In addition to death, the overall effects that the plant had on the activity and behaviour of the mice would also be monitored.

In the chronic study:

Firstly, the body weight and food intake of rats were to be monitored to determine the effect of *Artemisia afra* on the growth of rats.
Secondly, various hematological parameters (viz. RBC, WBC, hemoglobin, haematocrits and platelets) were to be tested before and after the study to assess the effects the plant may have on the blood cells and the circulation.
Thirdly, various biochemical parameters (viz. aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, cholesterol and creatinine) were to be analyzed, before and after the administration of the plant, to determine if there were any plant-induced alterations in liver and renal functions.
Finally, the plasma levels of luteolin were to be determined to assess whether the plasma levels of flavonoids (esp. luteolin) correlated with the dose ingested and/or any of the toxic effects observed after chronic ingestion of *Artemisia Afra.*

3.4.5. Rationale for luteolin

Luteolin was chosen as the flavonoid to be studied in this investigation, firstly, on account of the fact that it has been found, along with other flavonoids, in the crude extracts of *Artemisia afra* and, secondly, because it seemed to be potentially reliable pharmacological marker agent with the claimed biological activities as mentioned in the previous chapter. However, not much has been reported in scientific literatures on the luteolin-containing plant, *Artemisia afra* and particularly, on its toxicity or safety.

It was thus, presumed that the study on the toxicity of *Artemisia afra* aqueous extract would fill this gap and, by carefully checking the correct concentrations or doses of the aqueous extracts that will induce toxic effects in animal species with the equivalent doses of luteolin, may provide more information that could help ensure the safe use of the plant.

3.4.6. Rationale for HPLC assay of luteolin level in plant and biological materials

High performance liquid chromatography (HPLC) was proposed as a suitable analytical method for the determination of the luteolin levels in the plant and biological materials, for the following reasons:

Firstly, HPLC assays offer many advantages such as simplicity, high-speed of separation and sensitivity to low concentrations.

Secondly, this technique shows the specificity in detection and compatibility with a wide range of organic solvents miscible with water, which would ease any solubility issues with the flavonoids.

Thirdly, it offers additional advantages of high reproducibility and repeatability of data thus, leading to reliable results.

Finally, the HPLC technique is eminently suitable for the analysis of low quantities of samples, as it was expected to be obtained in the plasma samples of the tested rats with *Artemisia afra* aqueous extract via chronic oral administration.

Chapter 4

ACUTE TOXICITY STUDY

4.1. Introduction

The present chapter describes the equipment, material, methods and procedures that were used in (1) the collection and preparation of the aqueous extract of *Artemisia Afra,* (2) the determination of the luteolin level in the plant material and (3) the acute toxicity study. The results obtained from the preparation and subsequent analysis of the plant extract, the identification and quantification of luteolin in the plant extract as well as the results from the acute toxicity study in mice are also reported and discussed. The chapter is broken down into three parts: Materials and methods, results and discussion, and conclusion.

4.2. Materials and methods

4.2.1. Materials and equipment

4.2.1.1. Chemicals, reagents and materials

The chemicals, reagents and equipment that were used during the preparation of the aqueous extract, analysis of the plant material and the acute toxicity study in mice are described below.

Chemicals: These consisted of acetonitrile, methanol (HPLC grade), ethyl acetate, acetic acid, hydrochloric acid, acetone and dimethylsulfoxide (DMSO). All these were solvents purchased from Merck (Darmstadt, Germany). Luteolin standard was purchased from Sigma (St. Louis, USA), sterile normal saline (0, 9% NaCl) solution was purchased from Kassbell pharmacy (a local pharmacy in Bellville, South Africa) and the distilled water used was prepared in the pharmacology laboratory and purified using the Analyst HP water purifier (Oxon, England).

Materials: Freshly picked *Artemisia afra* plant material (i.e. leaves & aerial parts) was purchased from Montague Museum (Western Cape Province, South Africa).

A voucher specimen number 6639 (file number 9358) was dried and stored at the herbarium of the University of the Western Cape. The BALB/C mice used in this study were purchased from the Animal Unit of the University of Cape Town (UCT), South Africa, (Fig. 2, Appendix XI).

4.2.1.2. Equipment

The following equipment was used in the preparation of the aqueous extract of *Artemisia afra* as well as in the HPLC assay and the acute toxicity study:

A ventilated oven (*Memmert®,* model 854); a balance (*Mettler®,* model PE 6000); a scientific balance (*Ohaus®,* model GA 110); heating plate *(Gerhardt-Bonn®,* model MAG-H); a minus 70 degree freezer (*Maldon* ®, model CFC U 85360, England); a freeze- dryer (*Virtis* ®, Freeze mobile model 125L); a water bath (*Labcon®,* model CDH 110 Maraisburg, South Africa); a centrifuge (*Multex MSE®,* England); desiccator; vacuum pump (Diaphragm vacuum pump, model GMBH, Germany); 9.0cm filter paper (*Whatman®,* England*)*; syringe filters 0,45µm Millipore (USA); Kimix centrifuge tubes; 2ml glass clear vials for auto sampler (*National scientific company)* with 0,2ml sample vial inserts (*Sun-Sri®)* and cannula tubes. All the small vials used in the HPLC assay were purchased from Anatech Instruments (Pty) Ltd (Cape Town, South Africa).

The chromatographic analysis was performed on the HPLC system comprising a programmable binary gradient pump (*Beckman®*, Gold Module 126), an auto sampler (*Beckman®,* Gold Module 507), a diode array detector (*Beckman®,* Gold Module 168) supplied by Beckman and operating under *Beckman®* - System Gold with 32-Karat ™ Gold software package (Fullerton CA, USA). The whole setup was controlled through a desktop computer. The column used in this study was the Synergy®, Hydro-reverse phase column (*Phenomenex 80A, USA*) having 4µm particle size and a column length of 250 x 4,60mm purchased from Anatech Instruments (Pty) Ltd (Cape Town).

4.2.2. Methods and procedures

4.2.2.1. Collection and identification of plant materials

The plant material was collected during its flowering period in the summer of 2004. The material was authenticated as *Artemisia afra (A. afra)* by Mr. Franz Weitz, a botanist in the Department of Botany at the University of the Western Cape (UWC) and a voucher specimen number 6639 (file number 9358) was deposited in the herbarium at UWC.

4.2.2.2. Preparation of aqueous extract

After collection, the wet plant material was stored overnight in the laboratory at room temperature. The following day, all the leaves were plucked from the stalks, weighed (wet weight) and washed with distilled water and thereafter, dried in the oven at 30˚c for 3 days. The dried leaves were again weighed (dry weight), slightly crushed (but not powered) by hand, packed in brown paper bags and stored in a dark cupboard at room temperature until further extraction. The percentage yield was calculated from the wet and dry weights.

The study plant material was extracted in a way that, as much as possible, mimicked the method traditional herbal practitioners have used, over generations, to extract their plant medicines. Generally, the South African herbal practitioners use about 1/2 liter of boiling water to extract one teaspoon (about 2,5 g) of *Artemisia afra* dry leaf powder (Muganga, 2005). However, in this study, the dried leaves (10 times the amount used by herbal practitioners) were suspended in distilled water (i.e. 50g dried leaves per 1 liter water) and the mixture boiled for 30 minutes (Fig 3: Appendix XI). The decoction obtained, was left to cool before it was filtered, transferred into round-bottom flasks and frozen. The samples were then dried over four days using the Virtis™ mobile freeze-dryer. Using the afore-mentioned procedure, several batches of the dried extract were prepared, which were then combined into a single final homogenous batch. This final batch was weighed (to determine the percentage yield), put into stoppered brown bottles and then sent to *Hepro Cape Gamma,* a facility providing sterilization by gamma irradiation

(radiation conditions: *mass:0, 5; dose kGy: 18; time: 2hrs*). The final dried aqueous extract was stored at room temperature in a dark cupboard until further bioassay.

4.2.2.3. HPLC assay

4.2.2.3.1. Preparation of the plant extract for HPLC assay

The analysis of the flavonoid luteolin in *Artemisia afra* extract was done using the HPLC system. For this, solutions of un-hydrolyzed and hydrolyzed plant extract were prepared. 25mg of the plant extract were initially dissolved in 200µl methanol and vortex mixed for 1min. To obtain the hydrolyzed plant extract, 4ml of 2N HCl was slowly added, and the acid-methanol plant mixture was heated in a water-bath at 80˚C for 40min and then cooled. To both un-hydrolyzed (containing of 200µl MeOH) and hydrolyzed plant mixtures, 5ml ethyl acetate was added (per sample) to extract the luteolin, the mixtures vortex-mixed for 3min and thereafter centrifuged for 10min at 3000rpm. The supernatant organic phase (ethyl acetate) was carefully collected into a test-tube and evaporated to dryness under a gentle stream of nitrogen gas. Then, the residue was reconstituted in 200µl of HPLC mobile phase solution, filtered and 20µl aliquots injected onto the HPLC column.

4.2.2.3.2. Procedure for the HPLC determination of the luteolin level in the plant

To quantify the luteolin level in the plant material, a stock solution of luteolin (3mg/ml) was made in dimethyl sulfoxide (DMSO) and kept protected from light in a -80ºC deep freezer until use. From this stock solution, work solutions of luteolin were prepared by making dilutions in the concentration range of 1µg -100µg/ml. 20µl aliquots of the latter were injected onto column.

The analysis was performed in reverse phase mode using isocratic elution. The mobile phase was composed of methanol and 1% acetic acid aqueous solution (70:30, v/v) and this was pumped, at a flow-rate of 1,0ml/min, through a Synergy®, Hydro-reverse phase column (*Phenomenex 80A, USA*) having 4µm particle size and a column size of 250x

4,60mm.The volume of injection was 20µl, the detection wavelength 345nm and the assay was done at room temperature (25˚ C). For all the runs of plant extract, the diode array detector was used to confirm the identification of the luteolin peak and was set to perform UV scans between 168 and 345 nm.

To identify the luteolin peak and its retention time, 20µl of standard solutions of luteolin at different concentrations (1, 2 and 5µg/ml) were injected five times each onto the HPLC column. The peak produced in the chromatogram of each run and its retention time, height and the UV spectrum for the peak were noted. This procedure was repeated several times within a day and on different days, under the same conditions until it was clear that the retention times obtained were consistent and constant.

To identify the luteolin in the plant material, the range of retention times for the peaks obtained with the standard solutions of luteolin was compared to the retention times obtained after the analysis of several samples of the plant material, some of which were spiked with luteolin and some un-spiked and all assayed under similar HPLC conditions. To determine the level of luteolin in the plant material, a series of samples of aqueous solutions of luteolin were assayed, a standard curve of peak height (Y) vs. the concentration of standard solution (X) ranging from 1- 50µg/ml plotted and the linear regression line determined using the method of least squares analysis. Once the height of the identified luteolin peak in the chromatogram of plant extract was known, the level of luteolin was calculated from the standard curve regression equation.

4.2.2.3.3. Validation of the HPLC assay

To validate the HPLC assay used in this study, the following parameters were assessed: the specificity, lowest limit of quantification (LLQ), linearity, reproducibility and recovery. The specificity of the assay was verified by comparing the spectra of the extracted luteolin to that obtained after injection of the standard solutions containing the pure luteolin. The LLQ of the assay was taken as the lowest level of luteolin quantifiable on the HPLC system producing a peak height at least three times higher than the base line

noise. This parameter was also used to express the sensitivity of the system. For the linearity of the assay, the correlation coefficient (R^2) for the standard curve of the peak height (Y) vs. the concentration (X) of luteolin over the range of 1- 50µg/ml was calculated. To evaluate the reproducibility of the assay, the heights of peaks obtained after extracting and injecting seven different concentrations of luteolin onto column were recorded five times within a day and over several days, and the relative coefficients of variation for each concentration level calculated to obtain the intra-day and the inter-day reproducibility of the assay. Finally, the recovery of the assay was assessed by comparing the height of the luteolin peak obtained after hydrolysis and extraction of standard solutions as a percentage of the peak height obtained with injection of the non-treated standard luteolin solution directly onto column. At least 5 replications per concentration of luteolin were used.

4.2.2.4. Acute toxicity study

4. 2.2.4.1. Animals and animal care

The study protocol was approved by the University of the Western Cape (UWC) Senate Ethics committee.

In this experiment, healthy BALB/C mice of both sexes and weighing 17 to 33g were used. The animals were divided into 22 groups of 6 and were, as closely as possible, matched for weight and size over the groups in order to reduce the variability of their responses to the *Artemisia afra* aqueous extract. All animals were pathogen free and purchased from UCT Animal Unit, where they had been bred under conventional conditions for research purposes.

Before use, the animals were kept for at least 5 days prior to dosing in the animal room at the School of Pharmacy (UWC), to allow for their acclimatization to the laboratory conditions. The animal room was well ventilated, the lighting regulated to reproduce a 12 hour cycle of day and night conditions and the temperature maintained at around 25˚C. All animals had free access to tap water and the same type of food, throughout the study,

except for the short fasting period before the oral administration of the single doses of *Artemisia Afra* aqueous extract. The selected individual animals were weighed and identified through marks made on their body with different colour permanent ink pens. Three mice of the same sex were housed per cage. Additionally, all cages were labeled with details of the individuals' sex and the dose administered.

4. 2.2.4.2. Treatments

Normal saline (0, 9% NaCl solution) was used as a vehicle for the *Artemisia afra* doses and a stock solution of 12g of dried extract in 15ml sterile normal saline was aseptically prepared and filtered. An appropriate volume of this solution was administered intra-peritoneally or orally as described below.
Two methods were used to test the acute toxicity of the *Artemisia afra*:

4.2.2.4.3. The Litchfield and Wilcoxon method

This procedure uses one of the oldest statistical methods for evaluating biological data and it allows one to carry out the necessary calculations within a short period of time without a calculating machine, and without having to resort to logarithms (Litchfield and Wilcoxon, 1949).

The range of *A. afra* doses used were based on that reported in the study of Hilaly et al.(2004) and the *A. afra* was administered via two routes, namely the intraperitoneal and oral routes. For the intra-peritoneal administration, the first 6 groups of 6 animals (3 males and 3 females) each were used. The 6 groups were randomly assigned to receive single doses of 0; 1,5; 2,5; 3,5; 4,5 or 5,5 mg of *A. afra* extract per gram body weight, per group. The single doses were administered in the morning and were based on the animals' body weights on that day as indicated in Table 4.1 and calculated in Table 4.2.

Table 4.1 Intraperitoneal injection of *A. afra* to mice. The Table showing the doses against the weights of mice in each group

Group	Dose (mg /g body weight)	Sex	Number of mice per dose	Weight per mouse (g)
1	0	Male	3	29,5 26,0 28.0
		Female	3	24,0 22,8 23,7
2	1,5	Male	3	28,6 31,1 31,3
		Female	3	24,7 22,9 22,2
3	2,5	Male	3	27,9 28,7 31,0
		Female	3	24,5 23,3 21,9
4	3,5	Male	3	25,2 21,1 23,5
		Female	3	23,2 22,8 22,8
5	4,5	Male	3	25,8 23,7 25,1
		Female	3	21,3 21,0 23,8
6	5,5	Male	3	28,3 27,0 26,4
		Female	3	23,8 23,5 23,3

The maximum amount of fluid injected per mouse was fixed at 200µl and normal saline was used to make up the calculated volume of injection to 200µl.

Table 4.2: Example of calculation of the amount of *Artemisia afra* injected per mouse

Dose (mg/g bodyweight)	Weight per mouse (g)	Amount plant extract required (mg)	Stock solution (µl)*	Saline added to make:
1,5	28,6	Dose x W= 42,9	42,9x 15/12= 53,6	200µl

*: **Stock solution was made of 12 g of dried extract in 15ml sterile normal saline**

To effect the actual intra-peritoneal administration, the mouse was taken out of its cage by its tail, placed on a flat surface and the palm of the free hand placed on its back. The thumb and the forefinger were passed around the neck, with the thumb going behind one forelimb ending up under the lower jaw. Once the animal was securely held and lifted, the injection using a 1ml syringe was made into the lower left quadrant of the abdomen. The tip of the needle (26G x1 inch) was first injected subcutaneously then thrust through the abdominal muscles, holding the syringe nearly vertical.

For the oral administration, another 6 groups of 6 mice each comprising animals of both sexes and weighing 18 to 24g were used. As was the case for the intraperitoneal, the mice in each group matched each other in terms of weight and sex. Each of the groups was then randomly assigned to receive a single dose of 0; 2; 4; 6; 8 or 10 mg of *Artemisia afra* per gram body weight via the oral route. The amount of the extract mixture administered per mouse was calculated in similar fashion as in the intra-peritoneal administration (see Table 4.2 for example of dose calculation). Again another batch of stock solution containing 12g *Artemisia afra* extract in 15ml sterile normal saline was aseptically prepared for this purpose, but this time the final volume of dose administered for each mouse was 300µl.

Since not all of the mice had died a day after the 0 to 10mg *A. afra* oral dosing, an additional 4 groups of 6 mice were given *A. afra* extract in order to obtain the LD_{50}. These mice had individual weights of 19 to 24g and the groups were given the higher single oral doses of 12, 16, 20 or 24mg of extract per gram body weight. The four higher

doses were contained in a final volume of 720µl of saline, and the dose was given in two oral administrations of 360µl each with a 2hour-interval between the administrations.

The procedure for effecting the oral administration was similar to that for the intra-peritoneal administration, except that in this case, the mouth of the mouse was gently prized and kept open with the thumb and forefinger on the jaw of the animal. Then a plastic cannula attached to a 1ml syringe containing the *Artemisia afra* mixture was inserted into the mouse's stomach via its mouth. When the cannula was in place, the plunger of the syringe was depressed and the contents injected into the animal's stomach. Care was taken to ensure that the animals did not choke. After both the oral and intra-peritoneal dosing, the animals were monitored as described in section 4.2.2.4.5 below.

4.2.2.4.4. The AOT425statPgm method

The "Acute Oral Toxicity (Guideline 425) statistical program" (AOT425statPgm), is a statistically based acute oral toxicity testing procedure prepared by the US Environmental Protection Agency (USEPA) (US Environmental Protection Agency, 2003). This testing procedure is designed to minimize the number of animals used to estimate the acute toxicity of chemicals, and provides an estimation of the LD_{50} and its confidence interval. In addition, the test allows the monitoring of signs of toxicity and establishes the testing sequence in response to these signs. The program consists of 2 tests; the main test is performed when the chemical is expected to be toxic or when little or no information about its toxicity is available while the limit test is used in cases where information indicates that the test substance is likely to be non-toxic below regulatory limit doses (2000 or 5000mg/kg) and uses a maximum of 5 animals (US Environmental Protection Agency, 2003). In this study the main test was used.

Firstly, 30 mice weighing between 20 and 30 g were selected and individually marked as described above. They were divided into the two sexes and groups of each sex were given *A. afra* via intra-peritoneal or oral route (i.e. eventually 4 groups or experiments). The animals were housed in groups of 6 until the administration of the dose. The test

substance which was administered to these groups was of the same formulation stated in section *4. 2.2.4.2.* (i.e. 12g aqueous extract in 15ml saline), and the required dose per mouse was administered in a final volume of 200µl. The *A. afra* was administered orally or intra-peritoneally as described under section *4.2.2.4.3* above.

Since the main test procedure of the *AOT425statPgm method* was used, the animals were dosed orally or intra-peritoneally following the ordered dose progression indicated by the computer program (US Environmental Protection Agency, 2003). Briefly, the first mouse received a dose suggested by the computer (a level below the best estimate of the LD_{50}) and was then monitored for up to 48 hours. If the animal survived, the computer would suggest another dose (usually higher by a factor of 3.2) for the next animal. The latter was then duly administered the new suggested dose and again monitored for 48 hours. If the animal died within 48 hours the computer would suggest another dose (typically one lower by the factor of 3.2). The typical dose progression factor of 3.2 was the default factor corresponding to a dose progression of one half-log unit. While the computer program allowed one to start with doses from 1.75; 5.5; 17.5; 55; 175; 550; 1750 or 5000 mg/kg, in this study, 175 mg/kg was selected as the starting dose. This dose was consequently administered to the first animal (i.p or orally) and thereafter, the subsequent doses to be administered were those suggested by the computer program. Each animal was carefully observed for up to 48 hours after each administration, and each time the outcome was fed into the computer program. The study finally ended when one of the stopping criteria were met i.e. 3 consecutive animals survived at the upper bound or 5 reversals occur in any 6 consecutive animals tested. At that time, the program will then generate a printout of the doses that were administered, the outcomes and an estimate of the LD_{50}, and its 95% confidence interval (US Environmental Protection Agency, 2003).

4.2.2.4.5. Monitoring of animal after Artemisia afra administration.

In both methods, after the administration of the *Artemisia afra* extract via either the intra-peritoneal or oral route, the general behaviour of the mice was continuously monitored for 1 hour after dosing, periodically during the first 24hours (with special attention given

during the first 4 hours), and then daily thereafter, for a total of 14 days. The times at which signs of toxicity (mortality) appeared were systematically recorded as well as all other observations that were made, including changes in the normal activity of animals and their individual weights following the treatment. All surviving mice were sacrificed (euthanised with diethyl ether) at day 14 and various tissues (brain, kidneys and liver) were collected, weighed and visually inspected for any histopathological changes.

4.2.2.5. Data and statistical analysis

In this study, conventional and standard statistical methods were used for data transformation and data analysis. To determine the luteolin level in the plant material, the computer software GraphPad Prism 4 program was used to plot the standard curves, to subject the luteolin peak heights vs. concentration data to linear regression analysis, to determine the levels of luteolin in the extract samples and to calculate various statistical parameters e.g. the mean ± standard deviation (SD), the co-efficient of variation (CV), and 95% confidence intervals (CI). For the calculation of the LD_{50}, the procedures described for the two methods, viz. the "Litchfield and Wilcoxon statistical method" (which uses the Student t-test, the $(Chi)^2$ test, 19/20 confidence limits, tables and nomographs) and the "Acute Oral Toxicity (Guideline 425) statistical program" (AOT425statPgm) method (which uses the method of maximum likelihood) were used. Differences between the $LD_{50's}$ for oral (p.o.) and intra-peritoneal (i.p.) route of administration were assessed as the ratio of the i.p/p.o. (LD_{50s} (%)).

4.3. Results and discussion

4.3.1. Introduction

The results obtained during, (1) the collection, identification and preparation of the plant material, (2) the identification and quantification of luteolin in the plant aqueous extract, and finally, (3) the acute administration of A*rtemisia afra* to the mice, are reported and discussed in this section.

4.3.2. Collection, identification and preparation of plant materials

More than 5kg of plant material was collected from Montague Museum, and was authenticated by Mr. Frans Weitz, a botanist in the Department of Botany at the University of the Western Cape (UWC). A voucher specimen was dried and stored at the herbarium under number 6639 (file number 9358).

Three days of drying at 30ºC in a ventilated oven were sufficient to dry the wet leaves (414,9g total) of *Artemisia afra* to a product with constant weight (200g) and the yield was 48,2%. When 200g of dry *Artemisia afra* leaves were extracted with water, the yield of the dried aqueous extract (39,9g) obtained after freeze-drying was 19,9%. And the overall yield of freeze-dried extract from wet leaves was 9,6%.

The *A. afra* wet material was as described in chap 2 section 2.3.1.2 while the dried leaves were feathery grayish green in colour as shown in Figure 4.1 and retained a distinct aromatic smell. On the other hand, the extract had a grayish light brown crystalline feathery appearance as shown in Figure 4.2 and was very hygroscopic.

Figure 4.1: Dried leaves

Figure 4.2: Freeze-dried aqueous extract

The yields obtained were reasonable and comparable to the results obtained in other similar experiments, viz, 46.7% yield from wet material to dried material and 11.9% from finely ground dried material to aqueous extract (Waithaka, 2004) and 14.22% yield from dried material to extract (Harris, 2002). Furthermore, the appearance and characteristics of the materials were also similar to that obtained in these previous studies.

For the acute and chronic toxicity studies, a total of 500 g of *A. afra* aqueous extract was prepared using approximately 55 liters of distilled water and 2700g of dried leaves (which was obtained from more than 5500g of wet leaves).

Before the *A. afra* aqueous extract was used in these studies, its luteolin level was determined using a validated HPLC assay.

4.3.3. HPLC assay of Luteolin in the aqueous extract of Artemisia afra

4.3.3.1. Development aspects of the HPLC assay

The optimal separation of the luteolin peak from that of other compounds found in the plant extract was achieved after different HPLC mobile phases were tested. These mobile phases comprised acetonitrile or methanol with water-acetic acid at different concentrations sufficient to control the pH and suppress the tailing problem generally encountered in chromatography of many flavonoids. The preliminary separations were performed on a Synergy® Hydro-reverse phase column (*Phenomenex 80A, USA*) having

4µm particle size and a column length of 250 x 4,60mm. From all the runs accomplished, it was determined that a mobile phase consisting of methanol (55- 70%) in 1% acetic acid (45- 30%), and an isocratic elution provided a good resolution for luteolin. These results were in accord with that found by Muganga (2005). Under these conditions, the retention time of luteolin varied from 4,5 to 4,9 min and in all cases, the luteolin peaks were easily detected at 345nm as shown below in Figures 4.3 and 4.4.

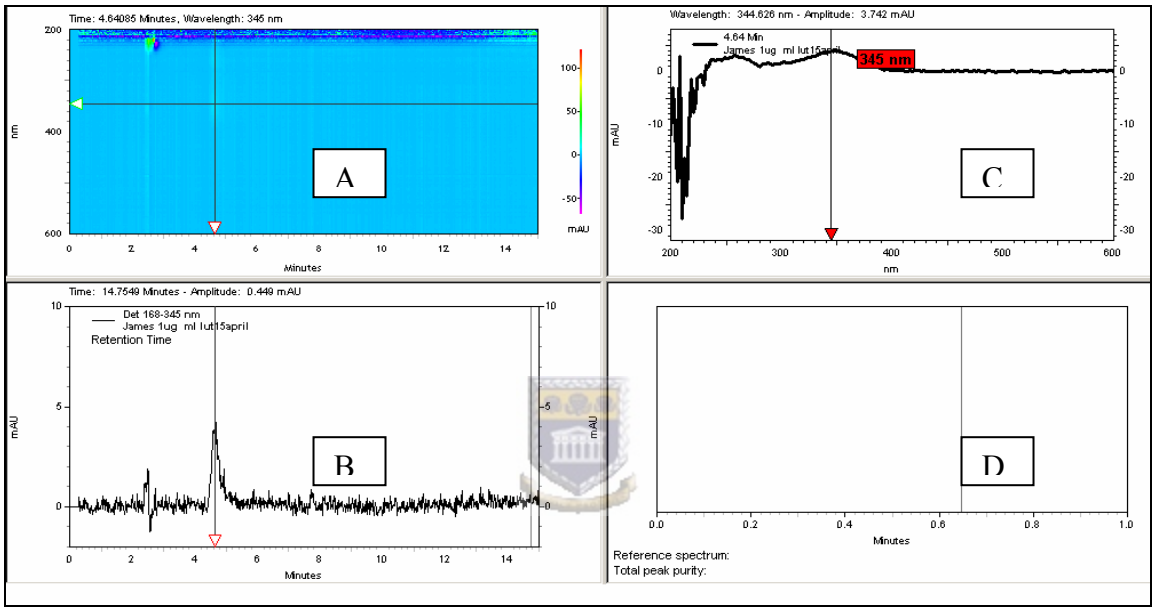


Figure 4.3: Representative UV spectrum of pure luteolin in aglycone form obtained using the diode array UV detector. This Figure represents the chromatogram of the lowest level of luteolin quantifiable (LLQ) after injecting 20 µl of 1 µg/ml luteolin solution onto column. The retention time was 4, 64 min.

Section A represents the UV light through the standard solution. The vertical and horizontal arrows indicate the retention time of luteolin and the optimal wavelength at which this compound was detected at the maximum peak level, respectively. The crossing point of arrows shows the luteolin spot in UV light, the time and the wavelength at which this compound was detectable in its pure state.

Section B represents the chromatogram of the standard luteolin solution. The arrow points the peak retention time and confirms the position of vertical arrow in section A.

Finally, section C represents the UV spectrum of luteolin in the standard solution. The vertical arrow points the optimal wavelength of luteolin (345nm) and confirms the position of the horizontal arrow in section A. And section D was not used, but its assigned purpose was to monitor the total peak purity.

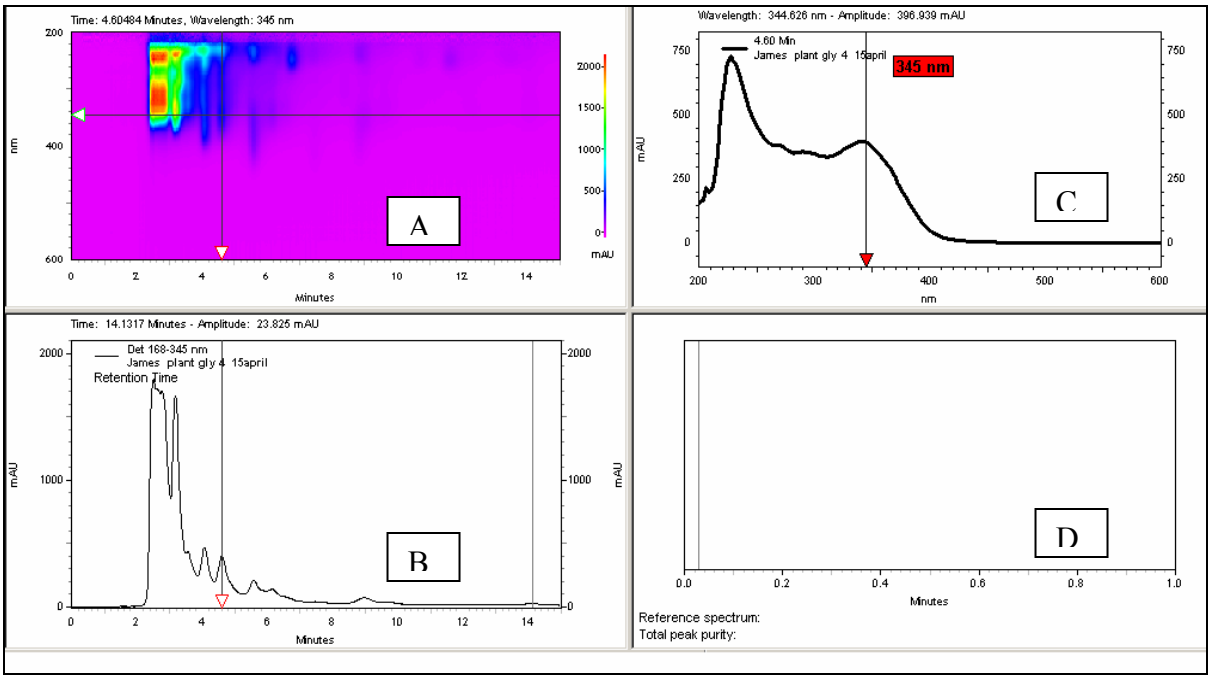


Figure 4.4: Representative UV spectrum of luteolin in hydrolyzed plant extract obtained using diode array UV detector. The retention time of luteolin was 4, 60 min; the extract was prepared as described in section 4.2.2.3.1. Similarly, section A shows the UV light through *A. afra* extract. The horizontal arrow indicates the optimal wavelength of luteolin as supported by the arrow on the UV spectrum in section C and the vertical arrow points the retention time of luteolin, which is confirmed by the arrow in the representative chromatogram of the *A. afra* aqueous extract in section B.

4.3.3.2. Validation of the HPLC assay

The results of the validation parameters for the HPLC assay of luteolin are given in Table 4.3 and Figures 4.5 and 4.6. The luteolin could be extracted from the plant matrix with 70,6 ± 1,0 % efficiency and under the HPLC conditions applied it had a low limit of quantification (LLQ) of 1 µg/ml (Appendix IV.1), a low limit of detection (LLD) of 20ng on column and a high intra-assay reproducibility (i.e. CV below 3%). Furthermore, the assay was linear (Figure 4.5) over the concentration range of 1 to 50 µg/ml with the regression equation being Y= 0,8006X + (0, 2530± 0, 2593) and correlation coefficient, R2 = 0, 9991.

Table 4.3: Results of the assay validation for the quantification of luteolin in the plant extract.

Retention time of luteolin	4,517 to 4,910 min
Low limit of quantification (LLQ)	1µg/ml
Sensitivity (LLD)	20ng on column
Linearity: • Slope • Intercept • Correlation coefficient (R^2)	 0.8006 ± 0.00986 0.2530 ± 0.2593 0,9991
Reproducibility: • Intra-assay (CV %) • Inter-assay (CV %)	 1.28% 2.65%
Recovery (mean± SD) CV %:	70,6 ± 1,0% 1.41%

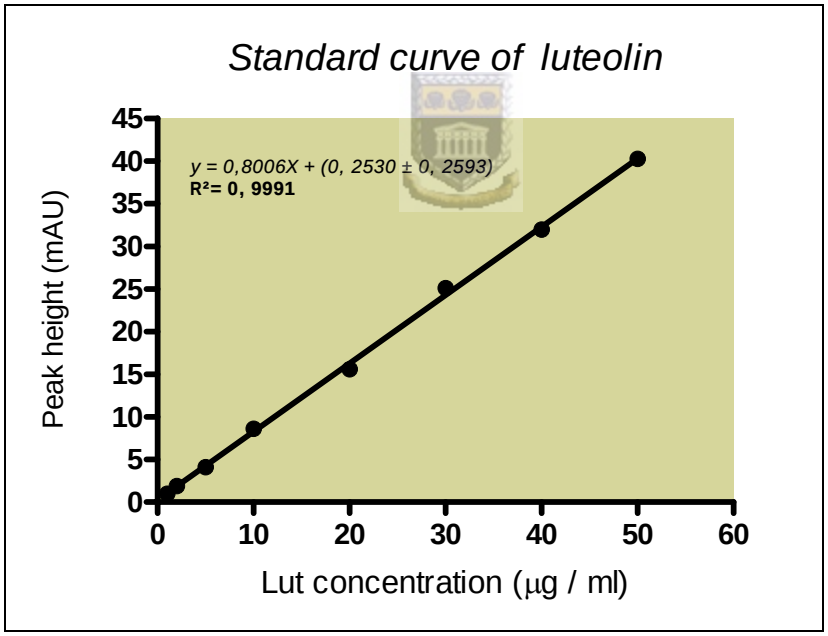


Figure 4.5: Standard curve of luteolin for the analysis of plant extract. Each data represents an average value of 6 replications (real peak height value = peak height x 4908). Example of an approximated standard curve is shown in Figure 4. 6

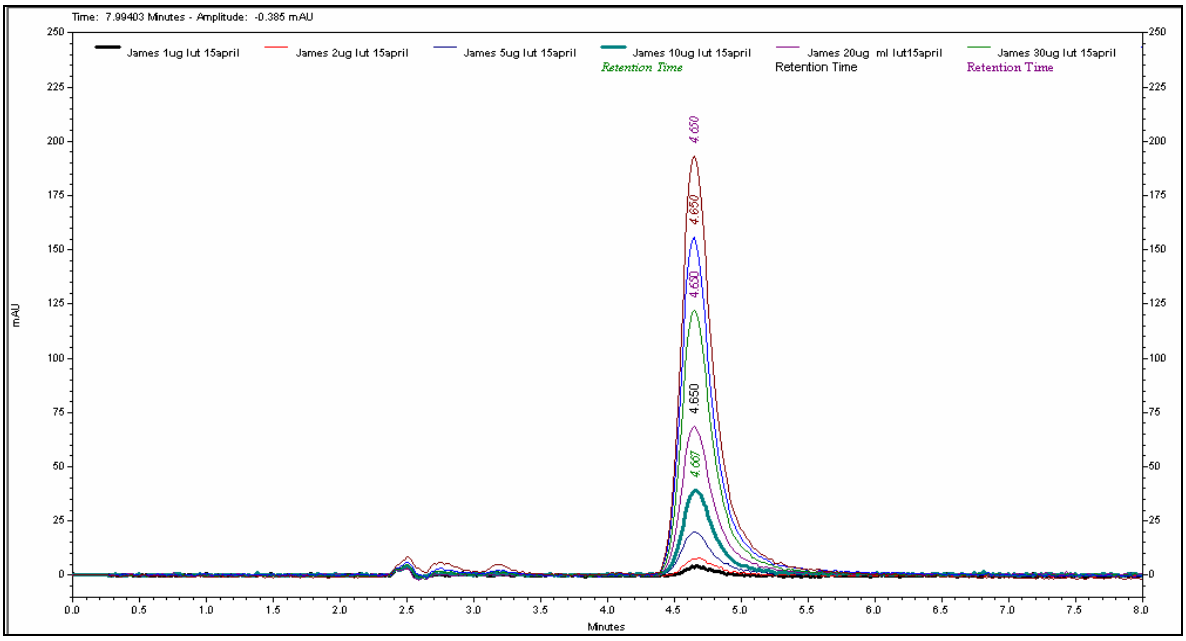


Figure 4.6: Overlay of chromatograms obtained with eight different concentrations of the standard solution of luteolin. This shows an approximated figure of the standard curve of luteolin in order to establish whether the compound presented a true identity, purity and reproducibility.

In this study, the absorbance of luteolin was read at 345nm (Figure 4.3 and Figure 4.4), which differed slightly from the 349nm maximum wavelength for luteolin recommended by Markham (Markham, 1982; Muganga, 2005) and the 340nm used by Waithaka for luteolin of *Artemisia afra* (Waithaka, 2004).

Overall the validation results indicated that the assay was sensitive and sufficiently reproducible for consecutive analysis of luteolin and linear over the luteolin concentration range of 1-50µg/ml. As such, the method was thus suitable to use for the assessment of the luteolin level in the plant extract.

4.3.3.3. Identification and level of luteolin in A. afra aqueous extract

The above described validated HPLC assay was used to identify and quantitate the levels of luteolin in the un-hydrolyzed and hydrolyzed *A. afra* extracts, and the results obtained and representative chromatograms are given in Figures 4.7 to 4.10 and Table 4.4.

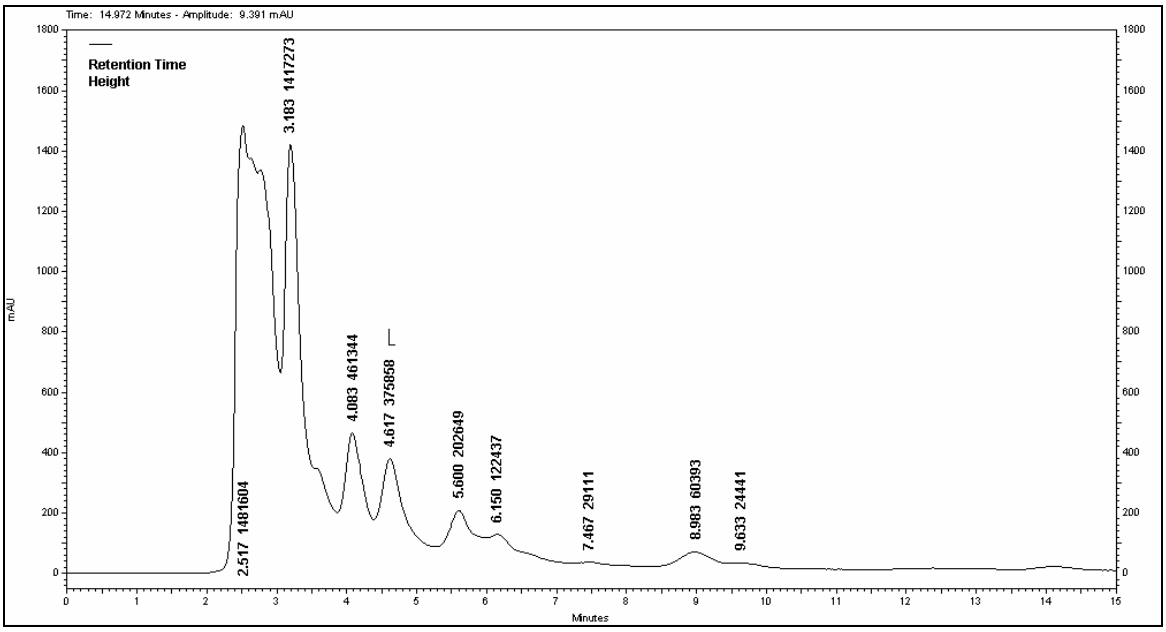


Figure 4.7: Representative HPLC chromatogram of hydrolyzed *Artemisia afra* extract. The retention time of luteolin (peak L) was 4,617 min

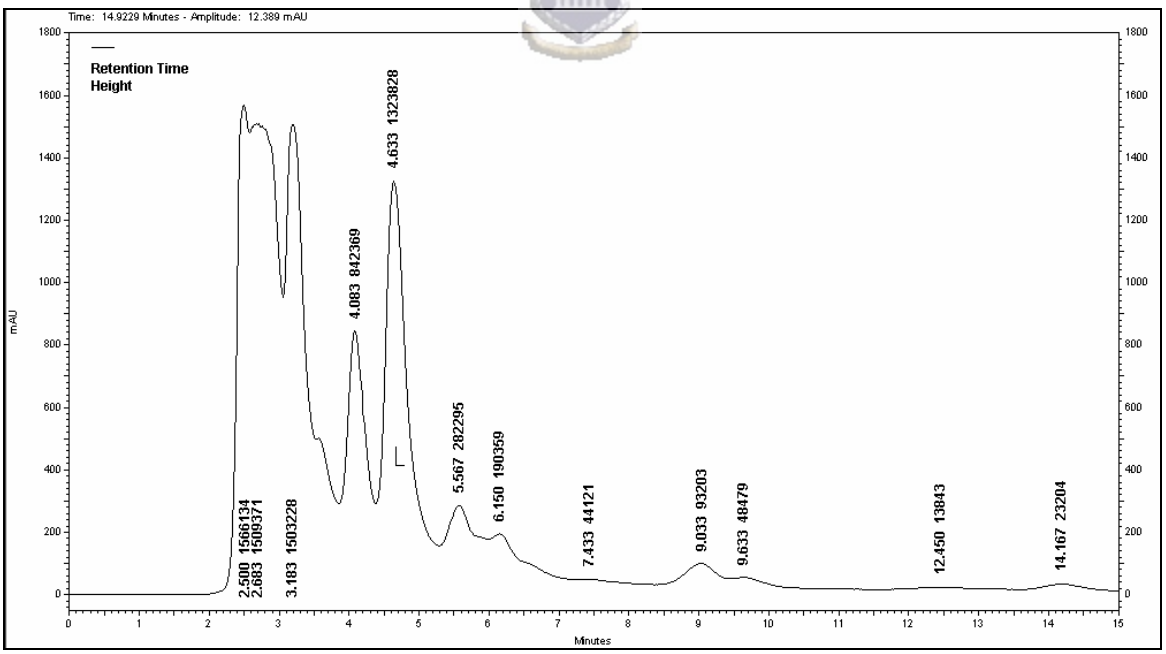


Figure 4.8: Representative HPLC chromatogram of hydrolyzed plant extract spiked with the standard solution of luteolin. The retention time of luteolin (peak L) was 4,633 min

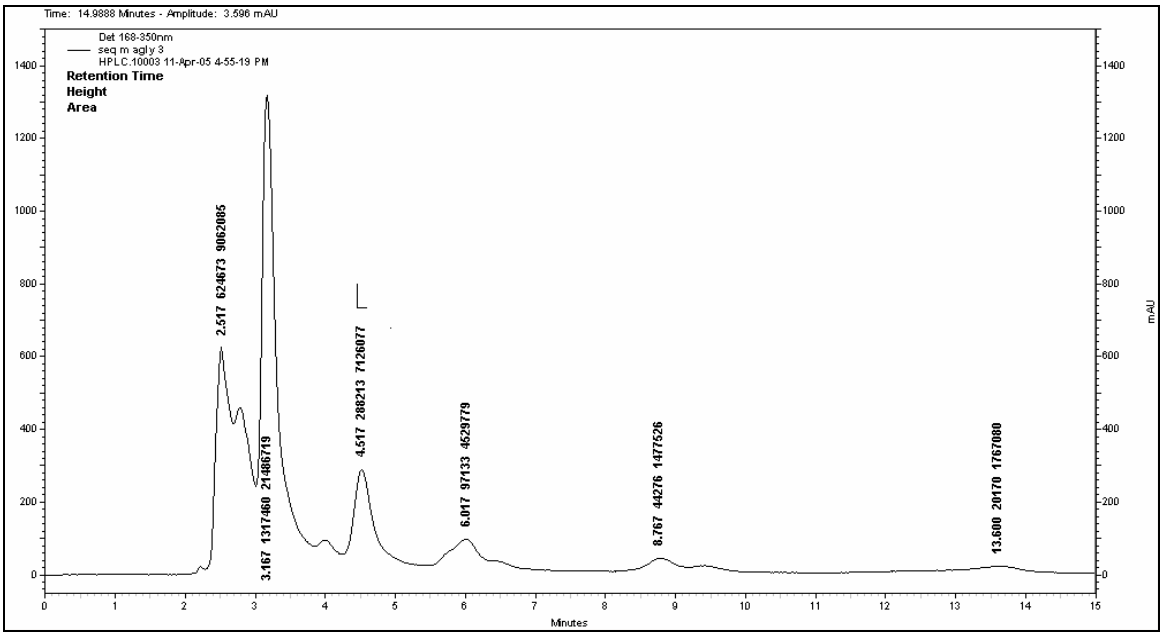


Figure 4.9: Representative HPLC chromatogram of un-hydrolyzed plant extract. The retention time of luteolin (peak L) was 4, 517 min

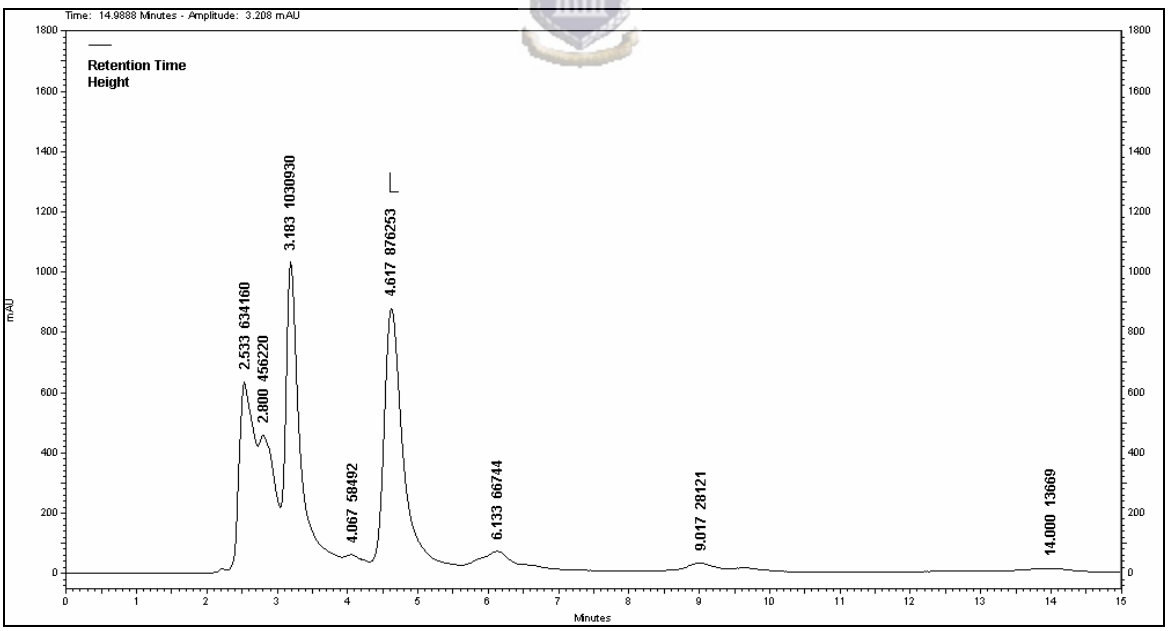


Figure 4.10: Representative chromatogram of an un-hydrolyzed extract of *Artemisia afra* spiked with the standard solution of luteolin. The retention time of the luteolin (peak L) was 4,617 min

Under the HPLC and specific isocratic elution conditions used, the peaks for several compounds in the hydrolyzed and un-hydrolyzed plant extract (Figure 4.7 and Figure 4.9, respectively) could be separated of which one was identified as luteolin (retention time 4,6 min) by spiking samples and diode array analysis (Figures 4.4; 4.7 to 4.10). The HPLC conditions thus allowed the quantification of luteolin without problems of interference from other UV-absorbing compounds. Furthermore, the chromatograms were strikingly similar to that found previously (i.e. Muganga, 2005) and clearly indicated the presence of appreciable amounts of luteolin in the aqueous extracts of *A. afra* (Table 4.4).

Table 4.4: Level of luteolin in the plant extract. Each value in the Table represents a mean ± SD of 7 replicates.

Plant extract	**Luteolin Concentration in plant extract (µg/25mg), Mean ± SD (n = 7)**
Un-hydrolyzed	11,911 ± 1,139
Hydrolyzed	23,075 ± 0,378

In fact, the level of luteolin was 0.923 ± 0,015µg/mg (n = 7) and 0.476 ± 0,045 µg/mg (n = 7) in the hydrolyzed and un-hydrolyzed aqueous extracts of *A. afra*, respectively. The amount of luteolin in the un-extracted plant material might, of course, have been even higher than the levels found in this study, since water, not necessarily the best solvent for flavonoid extraction, was used (Markham, 1982).

Overall, the HPLC results obtained thus confirmed the presence of luteolin among the major compounds in *A. afra* as has been found by other authors (Waithaka, 2004; Muganga, 2005; Komperlla, 2005). It is also well known that luteolin has a wide spectrum of pharmacological activities; and thus, could possibly play a role in the biological activity of the plant, as it was previously shown in at least one other study (Harris, 2002). Therefore, the investigation of the possible safety of the luteolin-containing *Artemisia afra* in the form of the aqueous extract of the plant in the rodents was thus well justified.

4.3.4. Acute toxicity of Artemisia afra in mice

The acute toxicity of the *A. afra* aqueous extract administered via the oral and intraperitoneal routes to mice were determined via two methods and the results obtained for each test are reported and discussed below.

4.3.4.1. Acute toxicity study of A. afra according to Litchfield and Wilcoxon method

The effects that acute oral and intra-peritoneal treatment with *A. afra* had on the mice, as determined using the Litchfield and Wilcoxon method, are summarized in Tables 4.5 to 4.7

The acute treatment with *A. afra* had definite effects in the mice as far as mortality was concerned. In both the intra-peritoneal and oral experiments, besides death, the treated mice also experienced a significant loss in body weight over the first 48hours, which was, however, regained during the following days. In addition, food intake was also, significantly reduced in the 48 hours following the administration. On the first day, the food intake in treated mice was reduced to 10-20% compared to that of the control mice and its improvement was recorded after 48 hours until the full recovery was attained after 96 hours in the surviving mice.

4.3.4.1.1. Effects of intra-peritoneally injected A. afra aqueous extract in mice.

For the testing of the acute toxicity of intra-peritoneally injected *A. afra* aqueous extract in the mice, 6 doses were selected (0, 1500, 2500, 3500, 4500 or 5500 mg/kg) and 36 mice of the both sexes were used, and the summary of results is as shown in Table 4.5.

Table 4.5: Effects of *A. afra* aqueous extract in mice after the intraperitoneal injection

Dose of *A. afra* extract (mg/ kg)	Sex	D/T	Mortality latency (h)	Symptoms of toxicity
0	Male	0/3	-	None
	Female	0/3	-	None
1500	Male	0/3	-	Hypo-activity, pilo-erection, loss of appetite
	Female	0/3	-	Hypo-activity, pilo-erection, loss of appetite
2500	Male	1/3	>120, <144	Hypo-activity, pilo-erection, Loss of appetite
	Female	2/3	>72, <120	Hypo-activity, loss of appetite, 0ne case of blindness
3500	Male	2/3	>12, <48	Dizziness, convulsion hyperventilation
	Female	3/3	>12, <36	Hyperventilation, syncope, convulsion, dizziness
4500	Male	3/3	>12, <24	Disorientation, convulsion hyperventilation,
	Female	3/3	>12, <24	Syncope, hyperventilation convulsion, disorientation
5500	Male	3/3	>1, <24	Syncope, hyperventilation convulsion, disorientation
	Female	3/3	>1, <12	Hyperventilation, syncope convulsion, disorientation

***A. afra*: *Artemisia afra*; D/T: dead/treated mice; (-): no toxic symptoms during the period of observation; mortality latency: time to death (in days) following the injection.**

After the intra-peritoneal injection of single doses of *A. afra* extract, there was a regular dose-dependent increase and decrease in mortality and mortality latency, respectively, in both sexes of mice. The first mouse died between 72 to144 hours after the injection of the 2500 mg/kg bodyweight dose. As the doses increased, the maximum frequency of death was observed within 1 to 24 hours after injection of the 4500 mg/kg and upward doses. In total, 20 of the 30 mice treated with single doses of *A. afra* died. Nine were males and eleven females. The dose of 2500 mg/kg induced mortality in 1 male and 2 females, the 3500 mg/kg was lethal to 2 males and to 3 of 3 female mice treated, and the 4500 mg/kg and upward doses, to all mice. This latter result suggested that female mice were more sensitive to intra-peritoneally administered *A. afra* than the male mice.

Other effects associated with the increasing doses of intra-peritoneally administered plant extract in the mice, included minor to severe hypo-activity, loss of appetite, pilo-erection and hyperventilation as well as dizziness, disorientation, convulsion, and syncope. The hypo-activity, loss of appetite, pilo-erection and hyperventilation were seen immediately after administration (stress conditions) while the effects of dizziness, disorientation, convulsion and syncope were recorded later on, were more pronounced at higher doses and persisted until death. One case of blindness was also recorded, but this could not be explained without further investigations.

The no-observed-adverse-effect levels (NOAEL) for intra-peritoneally administered *A. afra* in the mice were below 1500 mg/kg and the maximum tolerated dose (highest dose for which the mice recovered completely from all effects of *A. afra*) 1500 mg/kg. The minimum lethal dose (the lowest dose that induced the first mortality in mice) was 2500 mg/kg in male and probably < 2500mg/kg in female mice. The **LD_{50}** calculated according to the method of Litchfield and Wilcoxon was **2450 mg/kg** (19/20 confidence limits = 1750 to 3430 mg/kg) after i.p. dosing.

4.3.4.1.2. Effects of orally administered A. afra aqueous extract in mice.

For the testing of the acute toxicity of orally administered *A. afra* aqueous extract in the mice, 10 doses were selected (over range of 0 and 2000 to 24000 mg/kg), 60 mice of the both sexes used and the results were as shown in Table 4.6.

Table 4.6: Effects of *A. afra* aqueous extract in mice after the oral administration.

Dose of *A. afra* extract (mg/ kg)	Sex	D/T	Mortality latency (h)	Symptoms of toxicity
0	Male	0/3	-	None
	Female	0/3	-	None
2000	Male	0/3	-	None
	Female	0/3	-	None
4000	Male	0/3	-	Hypo-activity, pilo-erection
	Female	0/3	-	Hypo-activity, pilo-erection
6000	Male	1/3	>12, <24	Hypo-activity, convulsion, loss of appetite, dizziness
	Female	2/3	>12, <192	Hypo-activity, convulsion, hyperventilation, salivation
8000	Male	1/3	>12, <24	Hypo-activity, convulsion, hyperventilation, salivation
	Female	0/3	-	Hypo-activity, salivation, loss of appetite
10 000	Male	3/3	>12, <24	Hypo-activity, convulsion, hyperventilation, dizziness
	Female	2/3	>12, <24	Hypo-activity, convulsion, hyperventilation, dizziness
12 000	Male	1/3	>24, <36	Hypo-activity, convulsion, hyperventilation, dizziness
	Female	2/3	>12, <96	Hypo-activity, convulsion, loss of appetite, hyperventilation, salivation
16 000	Male	3/3	>12, <24	Hyperventilation, syncope, convulsion, salivation
	Female	3/3	>12, <24	Hyperventilation, dizziness, convulsion, hypo-activity
20 000	Male	3/3	>12, <24	Hyperventilation, dizziness, convulsion, salivation, syncope
	Female	3/3	>12, <24	Hyperventilation, dizziness, convulsion, hypo-activity
24 000	Male	3/3	>12, <24	Hyperventilation, dizziness, convulsion, syncope
	Female	3/3	>12, <24	Hyperventilation, dizziness, convulsion, syncope

After the oral administration of single doses of *A. afra* extract to the mice, there was an irregular dose-dependent increase in mortality in both sexes (Table 4.6). The first mice died between 12 to 192 hours after ingestion of the 6000 mg/kg dose (1of 3 males and 2

of 3 females). As the *A. afra* doses were increased, the mortality frequency remained constant for the male mice (1out of 3 treated) after the ingestion of the 6000, 8000 or 12000 mg/kg doses and for female mice (2 out of 3 treated) after the ingestion of the 6000, 10000 or 12000 mg/kg doses. The maximum frequency of death was observed after ingestion of doses of 16000 mg/kg and upwards in both sexes. In total, 30 mice (15 males and 15 females) out of 54 given *A. afra* extract via the oral route, died. The data thus suggested that the effects of orally administered *A. afra* extract in mice were not affected by sex.

The mortality latency was relatively constant over all the doses, and the other symptoms of toxicity recorded were similar to those found after the i.p injection of *A. afra* to the mice, and included minor to severe hypo-activity, pilo-erection, loss of appetite, hyperventilation, convulsion, dizziness and syncope, and salivation as the only additional symptom.

The no-observed-adverse-effect level (NOAEL) for *A. afra* administered via oral route was 2000 mg/kg, and the maximum tolerated dose (highest dose for which the mice recovered completely from all effects of *A. afra)* was 4000 mg/kg while the minimum lethal dose (the lowest dose that induced the first mortality in mice) was 6000 mg/kg and the **LD_{50}** calculated (according to the method of Litchfield and Wilcoxon) was **8960 mg/kg** (19/20 confidence limits = 5490 to 14600 mg/kg).

When the mortality rates of both intra-peritoneal and oral administration of *A. afra* to the mice were compared in terms of LD_{50}'s, it was found that the LD_{50} after i.p administration (2450 mg/kg) was 27% of that calculated after p.o administration (8960 mg/kg) (Table 4.7). This suggested that poor absorption and bioavailability from the gastrointestinal tract might have significantly affected the effects produced by and thus the safety of, *A. afra* in mice.

Table 4.7: The LD_{50} and 95% CI determined according to the method of Litchfield and Wilcoxon

Route of administration	LD_{50} value (mg/kg)	19/20 confidence limits (mg/kg)
Intra-peritoneal (i.p)	2450	1750 to 3430
Oral (p.o)	8960	5490 to 14600

Finally, for the determination of the LD_{50s} according to the method of Litchfield and Wilcoxon, a total of 96 mice (36 and 60 in i.p and p.o, respectively) were used. This number of animals used was clearly very high and it was hoped that the second method *AOT425 statistical program,* known for requiring a lower number of animals (as described in section 4.2.2.4.4) could be used as an alternative method in future for such studies with plants.

4.3.4.2. AOT425 statistical program

The study of the acute toxicity of *Artemisia afra* in the mice was repeated, but now using the AOT425 statistical program. Male and female mice were dosed in separated groups via intraperitoneal or oral route, and the results obtained by this computer program-driven method are summarized in Tables 4.8 to 4.11. (A summary of the complete test results is given in Appendix VI).

4.3.4.2.1. Effects of intraperitoneally administered A. afra aqueous extract in the mice.

For the testing of the acute toxicity of intra-peritoneally injected *A. afra* aqueous extract in the female mice, the computer program proposed four doses (175; 550; 1750 or 5000 mg/kg) and only 10 mice were used (Table 4.8).

Table 4.8: Acute toxicity study of *Artemisia afra* after intraperitoneal injection in female mice according to AOT425statPgm.

Dose (mg/kg)	O (Survived)	X (Died)	Total
175	1	0	1
550	2	0	2
1750	3	1	4
5000	0	3	3
All Doses	6	4	10

Estimated **LD_{50} = 1750 mg/kg**

95% PL Confidence interval is 1239 to 4450.
99% PL Confidence interval is 778.6 to 12800
(PL: profile likelihood)

Symptoms of toxicity recorded at 1750-5000 mg/kg: *hypo-activity, pilo-erection,* loss *of appetite, convulsion, syncope as well as dizziness and hyperventilation.*

There was a regular dose-dependent increase in the mortality frequency in the mice that were injected the single doses of *A. afra* aqueous extract, with the first mouse (1 out of 4) dying after the injection of the 1750 mg/kg dose and the maximum frequency of death (3 of 3) being observed after injection of the 5000 mg/kg dose. In total, 4 mice out of the 10 treated with the single doses of *A. afra* via i.p route, died.

Other symptoms of toxicity recorded after i.p. injection of the 1750 to 5000 mg/kg doses of *A. afra* to the female mice included minor to severe hypo-activity, loss of appetite, pilo-erection, convulsion, syncope, dizziness and hyperventilation. The no-observed-adverse-effect levels (NOAEL) for *A. afra* administered via the i.p route were below 550 mg/kg; the minimum lethal dose (i.e. the lowest dose that induced the first mortality in mice) was 1750 mg/kg and the estimated **LD_{50}** according to AOT425statPgm method was **1750 mg/kg** (95% CI = 1239 to 4450 mg/kg).

For the testing of the acute toxicity of *A. afra* after intra-peritoneal (i.p.) injection of the extract in the male mice, the computer program proposed the same doses as in the previously mentioned experiment, viz., 175; 550; 1750 or 5000 mg/kg, and only 8 mice were needed (Table 4.9).

Table 4.9: Acute toxicity study of *Artemisia Afra* after intraperitoneal injection in male mice according to AOT425statPgm.

Dose (mg/kg)	O (Survived)	X (Died)	Total
175	1	0	1
550	1	0	1
1750	3	0	3
5000	0	3	3
All Doses	5	3	8

Estimated **LD_{50} = 3129mg/kg**

Approximate 95% confidence interval is 1750 to 5000.
Approximate 99% confidence interval is 1750 to 5000

Symptoms of toxicity recorded at 1750-5000 mg/kg: *hypo-activity, convulsions, syncope, pilo-erection, loss of appetite, dizziness and hyperventilation.*

There were no deaths among the male mice that were injected single doses of *A. afra* aqueous extract below 5000 mg/kg. Death (3 mice) was only observed at the 5000 mg/kg dose, and in total 3 of 8 male mice died after the acute i.p doses of *A. afra*.

The other symptoms of toxicity recorded after injection of the 1750 to 5000 mg/kg doses to the male mice included minor to severe hypo-activity and loss of appetite, pilo-erection, convulsion, syncope, dizziness and hyperventilation. The no-observed-adverse-effect levels (NOAEL) for *A. afra* extract administered via the i.p route in male mice were below 550 mg/kg, the maximum tolerated dose (i.e. the highest dose for which the mice recovered completely from all effects of *A. afra*) was 1750 mg/kg and the estimated

LD_{50} according to AOT425statPgm method was **3129 mg/kg** (95% CI = 1750 to 5000 mg/kg)

When the results obtained for the male vs. female mice given i.p. *A. afra* were compared (i.e. Table 4.8 vs. Table 4.9), it was found that 10 female mice were used and 40% of them died while only 8 males were used of which 37,5% died. The dose of 1750 mg/kg was the minimum lethal dose of *A. afra* in female mice while being the maximum tolerated dose in the males. Furthermore, the LD_{50} in the female mice was 55,9% of that for the male mice. Taken together, this data suggested, and confirmed the previous observation made with the Litchfield and Wilcoxon method, that the female mice may be more sensitive to *A. afra* given via the intra-peritoneal route, than males. However, the other symptoms of toxicity found after i.p injection of *A. afra* were similar in both sexes.

4.3.4.2.2. Effects of orally administered A. afra aqueous extract in the mice.

For the testing of the acute toxicity of orally administered *A. afra* extract in the female mice, the computer program proposed the same four doses as for the i.p administration (175,550, 1750 or 5000 mg/kg), but only 6 mice were needed (Table 4.10).

Table 4.10: Acute toxicity study of *Artemisia afra* after oral administration to female mice according to AOT425statPgm.

Dose (mg/kg)	O (Survived)	X (Died)	Total
175	1	0	1
550	1	0	1
1750	1	0	1
5000	3	0	3
All Doses	6	0	6

Statistical Estimate based on long term outcomes:

The **LD_{50}** was greater than **5000 mg/kg**.

Symptoms of toxicity recorded at 5000 mg/kg: *hypo-activity*, *pilo-erection, loss of appetite, salivation and hyperventilation*

None of the female mice died at any of the oral doses (up to 5000 mg/kg) and only minor symptoms of toxicity such as hypo-activity, pilo-erection, loss of appetite, salivation and hyperventilation (i.e. stress symptoms), were observed.

The no-observed-adverse-effect level (NOAEL) for *A. afra* extract administered to the female mice via the oral route was 1750 mg/kg while the minimum lethal dose and the estimated **LD_{50},** according to AOT425statPgm method, was **> 5000 mg/kg.** This suggested that the female mice were not very sensitive to *A. afra* extract given via the p.o.route.

For the testing of the acute toxicity of orally administered *A. afra* extract in the male mice, the computer program proposed the same four single oral doses of the plant extract as in the previously mentioned experiment, viz. 175, 550, 1750 or 5000 mg/kg and again only 6 mice were needed (Table 4.11).

Table 4.11: Acute toxicity study of *Artemisia afra* after oral administration to male mice according to AOT425statPgm.

Dose (mg/kg)	O (Survived)	X (Died)	Total
175	1	0	1
550	1	0	1
1750	1	0	1
5000	3	0	3
All Doses	6	0	6

Statistical Estimate based on long term outcomes:

The **LD_{50}** was greater than **5000 mg/ kg**.

Symptoms of toxicity recorded at 5000 mg/kg*: loss of appetite, hyperventilation, hypo-activity, pilo-erection and salivation.*

None of the male mice died at any of the oral doses (up to 5000 mg/kg, the last recommended dose) and only minor symptoms of toxicity such as hypo-activity, pilo-erection, loss of appetite, salivation and hyperventilation (i.e. stress conditions), were observed.

The no-observed-adverse-effect level (NOAEL) for *A. afra* extract administered to the male mice via the oral route was 1750 mg/kg while the minimum lethal dose and the estimated **LD_{50}** according to AOT425statPgm method, was **> 5000 mg/kg**. As for the female mice, this data thus suggested that the male mice were also not very sensitive to *A. afra* given orally.

When the results obtained for the female vs. male mice given p.o. *A. afra* were compared (Table 4.10 vs. Table 4.11), it was found that both sexes of the mice had similar reaction to *A. afra* extract after the oral administration. The symptoms of toxicity were the same, viz. minor hypo-activity, pilo-erection, loss of appetite, salivation and hyperventilation (stress conditions), and the estimated $LD_{50's}$ did not differ significantly (>5000 mg/kg).

Additionally, when the results obtained for the mice administered either i.p or p.o. *A. afra* extract according to the AOT425statPgm method, were compared, the observations summarized in Table 4.12 were made.

Table 4.12: Summary Table of the acute toxicity results obtained using the AOT425statPgm

Route of administration	#: of mice	Sex	D/T	LD_{50} (mg/kg)	Symptoms of toxicity
Oral	6	♂	0/6	>5000	Loss of appetite, hypo-activity, hyperventilation, pilo-erection and salivation
	6	♀	0/6	>5000	
Intra-peritoneal	8	♂	3/8	3129	Hypo- activity, pilo-erection, loss of appetite, convulsion, dizziness, syncope, and hyperventilation
	10	♀	4/10	1750	

#: Number, D: dead, T: treated

- Firstly, significantly fewer animals were needed for the p.o than the i.p. test (12 vs. 18),

- Secondly, the mice were more sensitive to *A. afra* administered via the i.p than p.o route. In fact, the minimum lethal dose after i.p. injection was 1750 mg/kg (Table 4.8) while >5000 mg/kg after p.o. dosing (Tables 4.10 and 4.11), and the LD_{50s} after intra-peritoneal injection << 35 % and << 62% of that obtained after the oral administration in the female and male mice, respectively.
- Thirdly, the other post-administration symptoms produced by the aqueous extract of *A. afra* in mice were similar for both routes, except for the symptoms of convulsion, syncope and dizziness that preceded death after i.p. injection and salivation as the only additional symptom following the oral administration of the plant extract.

4.3.4.3. Comparison of the results obtained with both "Litchfield and Wilcoxon" and "AOT425statPgm"methods after administration of A. afra to mice.

In this study, no significant bodyweight losses or changes in weight of organs of mice treated with *A. afra* via either the intra-peritoneal or oral routes and monitored by the two methods, were observed. In addition, the gross analysis of organs' structure of the surviving mice showed normal architecture and colour compared to the control. A summary of the comparison of the rest of the results obtained with the "Litchfield and Wilcoxon" and "AOT425statPgm"methods is given in Table 4.13 and from that the following additional observations were made.

Table 4.13: Summary Table of the acute toxicity results obtained with both "Litchfield and Wilcoxon" and the "AOT425statPgm"methods after i.p or p.o administration of *A. afra* extract.

Method used	Route of administration	# of mice	Sex	D/T	LD_{50} (mg/kg)	Symptoms of toxicity
AOT425statPgm	Oral	6	♂	0/6	>5000	Loss of appetite, pilo-erection, hyperventilation hypo-activity and salivation
		6	♀	0/6	>5000	
	Total	***12***	♂/♀	***0/12***	***>5000 ****	
	Intra-peritoneal	8	♂	3/8	3129	Hypo- activity, pilo-erection, loss of appetite, convulsion, dizziness, syncope, and hyperventilation
		10	♀	4/10	1750	
	Total	***18***	♂/♀	***7/18***	***2439,5 ****	
Litchfield & Wilcoxon	*Oral*	*60*	♂ & ♀	*15/30* *15/30*	*8960*	*Hypo-activity, loss of appetite, syncope, salivation, pilo-erection convulsion, hyperventilation and dizziness*
	Intra-peritoneal	*36*	♂ & ♀	*9/18* *11/18*	*2450*	*Hypo-activity, loss of appetite, syncope, pilo-erection convulsion, hyperventilation and dizziness* *+One case of blindness*

#: Number, D: dead and T: treated and *: average value

Firstly, for i.p *A. afra*, it was found that the number of mice of both sexes used in the AOT425statPgm method (18 mice) was only 50% of that used in the Litchfield and Wilcoxon method (36 mice). Further, the mortality frequency was 38,8 % and 55,5% in the AOT425statPgm and Litchfield and Wilcoxon methods, respectively; with a slightly higher number of female mice dying compared to male mice in both methods. There were no significant differences in the LD_{50s} for the i.p injected *A. afra* extract obtained with both methods and the other symptoms of toxicity were similar (Table 4.13), with only one unexplained case of blindness found when using the Litchfield and Wilcoxon method.

Secondly, for acute oral *A. afra*, the following observations (Table 4.13) could be made:

- AOT425statPgm method used fewer mice of both sexes (12), which were only 20% of the number used in the Litchfield and Wilcoxon method (60 mice).

- The overall mortality frequency obtained in the AOT425statPgm method (0%) was also dramatically less than that for the Litchfield and Wilcoxon method (50%).
- But, the LD_{50s} obtained with the 2 methods as well as the other symptoms of toxicity obtained after the oral administration of *A. afra* extract (with dizziness, convulsion, and syncope occurring just before death, in the Litchfield and Wilcoxon method) were essentially the same.

Finally, when all the acute toxicity results obtained after the i.p or p.o administration of *A. afra* aqueous extract to the mice and determined according to both the Litchfield and Wilcoxon and AOT425statPgm methods, were compared (Table 4.13), the overall conclusion that could be drawn was that the mice were more sensitive to i.p than p.o. administered *A. afra*, with the LD_{50} after i.p being 27% of that after p.o administration. This result may be the consequence of the poor absorption and bioavailability from the gastrointestinal tract that might have significantly affected the effects, and thus safety, of *A. afra.* In addition, the number of mice of both sexes used in p.o and i.p study and monitored by the AOT425statPgm method was respectively 20 % and 50% of that used in the Litchfield and Wilcoxon method.

Collectively, the results obtained with the AOT425statPgm method were thus in accord with that obtained with the Litchfield and Wilcoxon method. However, if account is taken of the number of mice used, then the results also will suggest that the AOT425statPgm was an efficient tool and good alternative method for use in future acute toxicity studies of plants.

4.3.4.4. Toxicity classification of the Artemisia afra extract

Loomis and Hayes (1996) and Pascoe (1983) have proposed, as shown in Table 4.14, systems for the classification of the toxicity of compounds based on their LD_{50} values. When the LD_{50}'s for *A. afra* obtained in this study (and depicted in Table 4.13) were assessed based on the afore-mentioned systems of toxicity classification, the

classification for *A. afra* aqueous extract administered via p.o or i.p routes given in Table 4.15 was obtained.

Table 4.14: LD_{50} range and Toxicity classification

Category	LD_{50} (mg/kg)	LD_{50} (mg/kg)	Classification
Extremely toxic	1 or less	< 5	Super-toxic
Highly toxic	1 to 50	5- 50	Extremely toxic
Moderately toxic	50 to 500	50- 500	Very toxic
Slightly toxic	500 to 5000	500- 5000	Moderately toxic
Practically non-toxic	5000 to 15000	5000- 15000	Slightly toxic
Relatively harmless	More than 15000	> 15000	Practically non-toxic
(Loomis, T.A and Hayes, 1996)		(Pascoe, 1983)	

Table 4.15: Toxicity classification of *Artemisia afra* aqueous extract

Method used	Route of administration	LD_{50} (mg/kg)	Classification
Litchfield & Wilcoxon	Oral	8960	***Practically non-toxic*** Slightly toxic
	Intraperitoneal	2450	***Slightly toxic*** Moderately toxic
AOT425statPgm	Oral	>5000	***Practically non-toxic*** Slightly toxic
		>5000	***Practically non-toxic*** Slightly toxic
	Intraperitoneal	3129	***Slightly toxic*** Moderately toxic
		1750	***Slightly toxic*** Moderately toxic

Based on the classification of Pascoe (1983), the aqueous extract of *Artemisia afra* was found to be (i) slightly toxic to mice when given via the oral route and moderately toxic when given via i.p injection while (ii) *practically non-toxic* and *slightly toxic* via oral and i.p route, respectively, according to the classification of Loomis and Hayes (1996). It is

well known that the intra-peritoneal injection route is one of the methods of drug administration, which occasionally provides information on local toxicity. However, since the human exposure to the crude extracts of *Artemisia afra* plant is very unlikely to occur by this route, it might be best, based on the results of this study and the more recent scale of Loomis and Hayes (Table 4.15) to describe *Artemisia afra* as being ***practically non- toxic***.

4.3.4.5. Assessment of luteolin as marker of the acute toxicity of A. afra extract

Although the results discussed in the previous section seemed to suggest that *A. afra* was practically non-toxic, some of the mice did die from the aqueous extract of the plant. Moreover, the component(s) responsible for the mortality manifestation were not known. Nevertheless, in this study, the level of luteolin in *Artemisia afra* was determined and, as previously calculated in section 4.3.3.3, found to be approximately 0.923 ± 0,015 µg/mg of hydrolyzed aqueous extract (n = 7). From this information, the amount of luteolin ingested with each dose could be calculated, and the *A. afra* LD_{50s}' doses and the lowest lethal dose for all the mice (LLDAM) could be given in terms of dose of luteolin. The data in Table 4.16 summarizes the equivalent amounts of luteolin in the afore-mentioned doses found in this study.

Table 4.16: Luteolin levels in the LD_{50} and LLDAM doses of *A. afra* used in the acute toxicity study.

Route of administration	Dose of *A. afra* extract (mg/kg)	Equivalent luteolin dose (mg/kg)*#
Intra-peritoneal	1750	1.6 ± 0,026
	2450	2.2 ± 0,0 36
	3129	2.9 ± 0,0 47
	4500 (LLDAM)	4.2 ± 0,067
Oral	>5000	> 4.6 ± 0,075
	8960	8.3 ± 0,13
	16000 (LLDAM)	17.3 ± 0,24

LLDAM: lowest lethal dose for all the mice, *: Amount of luteolin in hydrolyzed extract=0.923 µg/mg #: mg luteolin per kg mouse; n= 5.

All the mice that received a single intra-peritoneal dose of 4500 mg/kg of extract (Table 4.5) containing the equivalent luteolin dose of 4.2mg/kg as well as those that received a single oral dose of 16000mg/kg (Table 4.6) containing the equivalent of 17.3 mg/kg of luteolin, died.

There is very little information in the literature on the toxic or lethal levels of luteolin. Tshikalange et al. (2005) reported that at concentrations superior or equal to 250 µg/ml, luteolin did not show any toxicity against VK (Vervet monkey kidney) cells while Kotanidou et al. (2002) reported that the intra-peritoneal administration of a 50 mg luteolin /kg dose was lethal (to three of three mice receiving it). When this latter dose of luteolin is compared to the i.p. equivalent luteolin doses shown in Table 4.16, it can be seen that the highest equivalent luteolin dose (i.e. that contained in the lowest dose that proved lethal for all the mice) after i.p injection, viz. 4.2 ± 0,067mg/kg (n = 5) was only 8.4% of the dose of luteolin reported by Kotanidou et al (2002). The levels of luteolin in the plant doses injected into the mice in the present study was thus very low, and probably too low to induce death solely due to luteolin. More likely, the mortality caused by *A. afra* was due to additional one or more of the other chemical compounds present in the aqueous extract (some of which have been identified and referred to earlier in chapter 2, section 2.3.8) or due to additional yet unidentified compounds in the plant. Collectively, the results discussed in this paragraph thus suggested that *luteolin* was *not a good marker* for the acute toxicity profile of the flavonoid-containing medicinal plant, A*rtemisia afra,* in mice.

Note:

Finally**,** if an extrapolation of the above results of the effects of *A. afra* in mice is to be made to humans, then it may be said that, for any intoxication, a human being would need to ingest more than 4g of *A. afra* aqueous extract (or 20g of dried leaves) per kg body weight (i.e. the lowest dose that induced the first mortality in mice). Moreover, if the doses taken by humans are estimated from (1) the dose directions reported by Roberts (1990), who used a quarter cup of fresh leaves to prepare a decoction in 1cup of boiling water, (2) the calculation shown in Appendix VII and (3) the extraction yields given in

section 4.3.2 is taken into consideration, a human dose of approximately 50 mg of freeze-dried extract per kg body weight is obtained. And this latter dose is only **2,5%** of 2000 mg/kg dose the no-observed-adverse-effect-level (NOAEL) given in Table 4.6.and would represent an equivalent luteolin dose of only about 0,046-mg/kg bodyweight. Clearly, the usual doses of *A. afra* recommended for human use, even if under self-medication, should thus be quite safe. In fact, from the following Figure (Figure 4.11), it is suggested that only oral doses above 6 g/kg upwards might be lethal while doses up to 2 g/kg should be without any adverse effects, and those in the 2 to 4 g/kg range be tolerable doses (i.e. doses for which the recovery from all effects was assured).

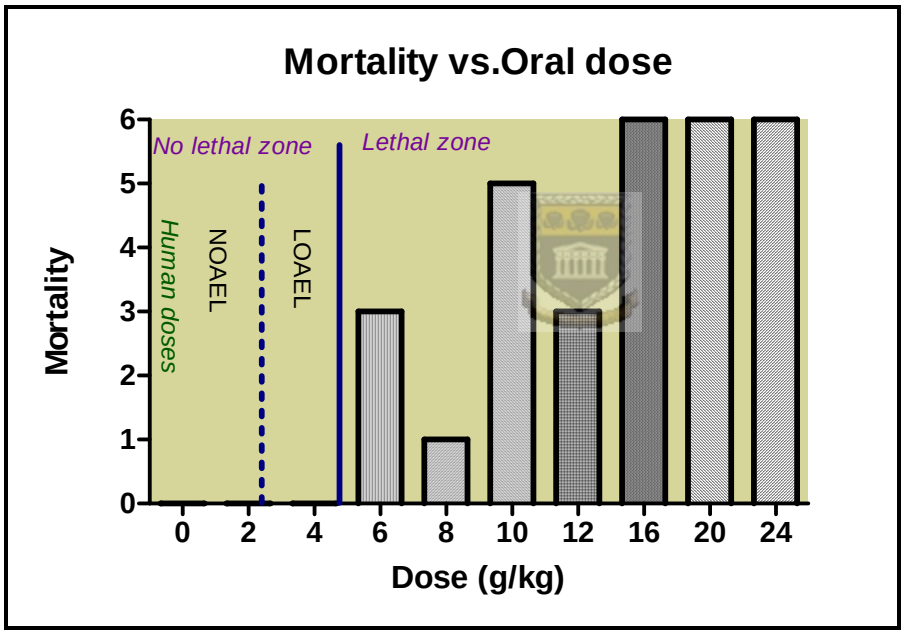


Figure 4.11: Relationship between dose and mortality. The dose and mortality data were taken from Table 4.6 used to depict the safety and lethality ranges after oral administration of *A. afra*. NOAEL: No-observed-adverse-effect-level, LOAEL: Low-observed-adverse-effect-level or maximum tolerated dose.

4.4. Conclusions

The aim of this study was to investigate the acute toxicity of the flavonoid-containing plant *Artemisia afra* and especially to establish the safety of the aqueous extract of the plant in mice. From the results obtained the following conclusions may be drawn:

- *A. afra* appeared to be practically non-toxic (LD_{50} = 8960 mg/kg) and slightly toxic (LD_{50} = 2450 mg/kg) when administered acutely to mice via the oral and intra-peritoneal routes, respectively. While female mice seemed to be more sensitive to acute intra-peritoneally administered *A. afra* than male mice, the same post-administration symptoms were produced by *A. afra* via the two routes, viz. minor to severe hypo-activity, pilo-erection, hyperventilation, loss of appetite, salivation, syncope, dizziness and convulsions.

- The AOT425statPgm was an efficient tool and good alternative to the Litchfield and Wilcoxon method for determining the acute toxicity of a plant medicine such as *A. afra.* While giving the same symptom profile and LD_{50} results and having the same study time to conduct, this method however used substantially fewer mice, in fact only 20 and 50% of those used in the Litchfield and Wilcoxon method for the testing of acute p.o. and i.p. toxicity, respectively.

- The luteolin in the aqueous extract of *A. afra* was not a good marker for determining the acute toxicity potential of this flavonoid-containing plant. Although the aqueous extract contained quantifiable levels of luteolin that could possibly play a role in the biological activity of this plant, the levels of luteolin calculated to be in the LD_{50} doses of the extract simply did not relate to the toxic luteolin level reported in the literature.

- Finally, since the doses of *A. afra traditionally* taken during self-medication or when recommended by herbal practitioners were far less (i.e. 2,5 % of non-observed adverse effect level or NOAEL) than the possible human toxic doses

predicted using data collected from the mice during the study, *A. afra* should, upon acute administration, also be relatively non-toxic in humans.

But what if it is administered chronically via the oral route, as is the traditional practice?

Chapter 5

CHRONIC TOXICITY STUDY

5.1. Introduction

In this chapter, the equipment, material, methods and procedures that were used in the chronic toxicity study and HPLC assay are described; thereafter, the results obtained pertaining to the effects of *A. afra* on animal behaviour, weight and food intake, a range of biochemical and haematological parameters and the plasma levels of luteolin obtained after the chronic administration of *Artemisia afra* to the rats and its correlation with the dose ingested are reported and discussed.

5.2. Materials and methods

5.2.1. Materials and equipment

5.2.1.1. Chemicals, reagents and materials

The chemicals, reagents and equipment used in the chronic toxicity study of *A. afra* in the rats and for the determination of the luteolin levels in the plasma samples were the same as that described in chapter 4 (section 4.2.1.1), except for the following supplementary elements:

Chemicals: Sodium acetate trihydrate, 10% buffered formalin, potassium phosphate and sodium pentobarbitone 6% solution purchased from Kyron Laboratories were used in addition to the previously described chemicals.

Reagents: Luteolin standard, B-glucuronidase (EC 3.2.1.31 type H-3) and sulfatase (EC 3.1.6.1 type H-2) were purchased from Sigma (St. Louis, USA).

Materials: The same batch of *Artemisia afra* aqueous extract described in sections 4.2.2.1 - 4.2.2.3 was used in the chronic toxicity study while male Wistar rats were

obtained from the Animal Unit of the University of Cape Town (UCT) (Fig. 1, Appendix XI).

5.2.1.2. Equipment

Besides the equipment previously described in section 4.2.1.2, EDTA tubes (Becton-Dickson vacutainer: purple top), gel tubes (SST: yellow top) and sodium fluoride tubes (grey top), all supplied by *PathCare*-Vet Laboratory (Ltd) in Cape Town, South Africa, were also used for the blood collection.

The chromatographic analysis of plasma samples was performed using two HPLC systems. The first system consisted of a single HPLC pump (*Beckman®,* Module 110B), a manual injector (*Alter Vent*® 210) and a programmable UV detector (*Beckman®,* Module 166), all coupled via an Analogue Interface (*Beckman®,* module 406) to a PC and controlled with the Gold Chromatography data system. The second system comprised an isocratic pump (Thermo separation product, *Spectra Series* P100), an UV detector (Thermo separation product, *Spectra Series* UV 150) and an auto sampler (Thermo separation product, *Spectra Series* AS 100), all functioning under control of Delta chromatography data system (Delta I0-280). The column used in this study on both aforementioned systems was the Synergy®, Hydro-reverse phase column (*Phenomenex 80A, USA*) having 4µm particle size and a column length of 250 x 4,60mm and purchased from Anatech Instrument (Pty) Ltd (Cape Town).

5.2.2. Methods and procedures

5.2.2.1. Chronic toxicity study of A. afra

5. 2.2.1.1. Animals and animal care

The protocol for this study, along with the acute toxicity study, was approved by the UWC Senate Ethics Committee. The main study sample of twenty-four adult male rats (weighing 230-270g) was housed in groups of 6 in a rat cage, under the same conditions as described previously for the mice (section: 4.2.2.4.1). There were 4 groups of 6

animals each, viz. a control group, a low dose treatment (100mg/kg) group and two high dose treatment (both 1000mg/kg) groups. The individual rats were identified by marks on their tails made with different coloured permanent ink and through cage markings indicating the group designation and treatment dose. Before the initiation of dosing, the rats were left for 7 days to acclimatize to laboratory conditions, Thereafter, baseline readings of the weights of all the animals were taken and samples of blood collected from 6 sacrificed individuals.

A further 9 animals were used in a shorter 7-day study. They were divided into 3 groups of 3 each and housed care for in the same way as described above for the animals used in the main study.

5. 2.2.1.2. Treatments

The animals in the main 92-day study were randomly assigned to 3 treatment groups to receive 100, 1000 or 1000mg/kg *Artemisia afra* aqueous extract and one control group (no *A. afra*). The doses were selected on the following basis. Firstly, they were based on the directions reported by Roberts (1990), who used a ¼ cup of fresh leaves in 1cup of boiling water to prepare a decoction. Taking the yields reported in section 4.3.2 into consideration, it was estimated that this produced the human dose of approximately 50 mg of freeze-dried extract per kg body-weight. The low treatment dose for the rat was then taken to be double of this dose i.e. 100 mg/kg. Secondly, in the acute toxicity study in the mice, a LD_{50} of 8960 mg/kg was found for the orally administered plant extract. Assuming that there would be no or little difference in *A. afra* effects in mice and rats, the high treatment dose was then consequently fixed at approximately 10% of the LD50 i.e. 1000 mg/kg. The *A. afra* was dispersed in sterile normal saline (0, 9% NaCl solution as vehicle) at a concentration of 12 g dried aqueous *A. afra* extract in 15 ml saline. This stock solution was freshly prepared every week and kept in 4^{o}C cold room and protected from light in between use. Each morning, for 3 months, each animal received the treatment dose or vehicle in an adjusted final volume of 0,5 ml.

In addition to the above experiment, a second 7- day study was conducted. This was to determine whether the time of blood collection could affect the luteolin levels. For this short study 9 rats were randomly assigned to 3 groups of 3 rats each and they were given no *A. afra* (control group), 100 mg/kg (low dose) or 1000 mg/kg (high dose) doses of *A. afra*, respectively, via oral gavage daily for 7 days.

5. 2.2.1.3. Handling of animals and general parameters monitored

The *A. afra* or saline vehicle was administered orally as follows. Firstly, the rat was held very firmly by the skin of the neck and back so that the head was kept immobile and in line with the back. Then, a 18 gauge needle encased in a plastic cannula and attached to the 1ml syringe, was passed into the mouth as far to one side as possible (i.e. not centrally), and after locating the entry to the oesophagus, was pushed gently into the stomach, where the contents of the syringe was discharged through the needle, first slowly (to confirm intra-gastric passage) and then fairly rapidly as was described by Waynforth (1980).

To determine the effect of the *Artemisia afra* on the general wellbeing of the rats, all animals were monitored for any deviations in normal behaviour, coat condition, discharge, movements and mortality on a daily basis during the 3-month period of study. In addition, their daily food intake was also monitored. The body-weight (in gram) of each rat was recorded on day 0 and at weekly intervals throughout the course of the study and the average body-weights for the groups calculated.

5.2.2.2. Blood collection and measurement of haematological and biochemical parameters

5.2.2.2.1. Blood collection and analyses

In the 92-day study, groups of animals were sacrificed at day 0 (control group baseline) and for control and treatment groups at the end of the 3-month period of the study. On day 92, the animals received the last treatment dose early in the morning after a one-hour

fast. A further 1 to 2 hours after the dosing, each animal was anaesthetized with intra-peritoneal injection of sodium pentobarbitone 6% solution (40mg/kg body-weight) and blood samples collected via cardiac puncture. Only the control, low treatment dose and one of the high treatment dose groups were sacrificed on day-92. The second high treatment dose group of rats was sacrificed on day-93 to measure the luteolin level one day after administration (i.e. steady state trough level). Approximately 5ml of blood were collected from each rat at stipulated times and divided into several aliquots for the haematological, biochemical and HPLC analyses.

The determination of the full blood count and the biochemical parameters were performed at a large commercial laboratory (PathCare, Cape Town, South Africa), which is accredited by the South African National Accreditation Systems (SANAS). The HPLC assay to determine the luteolin levels was performed in the pharmacology laboratory in School of Pharmacy (UWC).

For the shorter 7-day study only the luteolin levels in the blood were monitored. For this 5 ml of blood was collected from each animal, 45 minutes after the last dose of *A. afra* was given. From this blood plasma was prepared and stored frozen at -84 ˚C until it was assayed for luteolin content.

5.2.2.2.2 Haematological analyses

The blood samples for the heamatological tests were collected into vacuum tubes containing EDTA as anticoagulant. The collected samples were sent within one and a half hours after collection to PathCare laboratory, where the following haematological parameters: red blood cells (RBC), haemoglobin, haematocrit, white blood cells and platelets were determined on a fully automated analyzer (CELL-DYN 3700, Abbott Laboratories, Santa Clara, CA, USA) using the 'Combination laser light scattering and impedance counting analytical method'. The values obtained for each parameter were reported as the average for the group per dose

5.2.2.2.3. Biochemical analyses

Another aliquot of the sampled blood was collected into sodium fluoride and SST (gel) tubes for the determination of plasma glucose level and the other biochemical parameters, respectively. The blood chemistry tests were performed on an auto-analyzer (Beckman Coulter CX7, Fullerton, CA, USA) using different methods, viz. a kinetic rate method for the enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), the modified rate Jaffe method for creatinine; the oxygen rate method for glucose, and the timed endpoint method for total cholesterol (PathCare manual, 2005). The values obtained for each parameter were reported as the average for the group per dose.

5.2.2.2.4. Histopathological analyses.

The wet organs viz. kidneys, liver and brain of each rat in the main study were also isolated, weighed and visually inspected for any histopathological changes.

5.2.2.3. Determination of the luteolin level in the plasma of the rats.

In this project, 2 sets of plasma were assayed for luteolin, viz those from rats in the main (92-day treatment) and the shorter (7-day treatment) studies.

5.2.2.3.1. Preparation of samples for HPLC assay.

For the HPLC assay, the blood samples, freshly collected by cardiac puncture into vacutainers containing EDTA anticoagulant, were gently mixed and immediately centrifuged at 1500 rpm for 15 min at room temperature. Aliquots (approximately 500µl) of the clear upper layer of plasma were transferred using a Pasteur pipette, to labeled 2ml graduated micro-centrifuge tubes with flat top cap, and stored at –84 ˚C until they were analyzed.

To determine the luteolin level in the plasma, the frozen samples were thawed and then subjected to acid (to precipitate proteins and hydrolyze luteolin in the plasma) or enzyme hydrolysis before extraction with ethyl acetate as detailed below.

To one series of duplicate Kimix^{TM} glass tubes containing 200 µl of thawed plasma samples, 200 µl of methanol (used to denature the proteins and removed luteolin from clogged protein mass) and 200 µl of 2N HCl were added. To another series of tubes each containing 200 µl of blank plasma, different amounts of luteolin (for standard curve) and the same volumes of methanol and acid were added. Then, all the tubes were vortex-mixed for 1min, heated for 30 min in water bath at 40˚C and afterwards cooled to room temperature. Thereafter, 4 ml of ethyl acetate was added to each tube and the samples vortex-mixed for 1min and centrifuged at 2500 rpm for 10 min at room temperature. The centrifuged tubes were placed in a dry-ice acetone bath and the unfrozen supernatant organic phase (ethyl acetate) carefully collected into cleaned glass tubes and concentrated to dryness using a gentle stream of nitrogen gas. The residue was reconstituted in 200 µl methanol, the solution filtered through a micro syringe filter and 23 & 50 µl aliquots thereof respectively injected into the column of the Gold System and Delta System HPLC systems described under 5.2.1.2.

For the enzymatic hydrolysis, work solutions of sulfatase (800units/ml) and B-glucuronidase (21600 units/ 1ml) were prepared in the appropriate buffer from the stock solutions of 4,080 units/ml for sulfatase and 145,700 units/ml for B-glucuronidase. Forty units of sulfatase (contained in 50 µl of 1M sodium acetate buffer; PH 5, 0) and 1080 units of B-glucuronidase (contained in 50µl of potassium phosphate buffer; PH 6, 8) were added to 200 µl of thawed plasma. In another set of tubes, some containing the blank plasma that was spiked with different concentrations of luteolin, the same amounts of enzymes were added. All the samples were incubated at 37˚C for 1 hour and then cooled to room temperature. Thereafter, 200 µl of acetone (to precipitate proteins) were added and the samples vortex-mixed before 4ml ethyl acetate was added, the samples vortex-mixed one more time and centrifuged at 2500 rpm for 10 minutes at room temperature. The supernatant organic phase (ethyl acetate) was carefully collected into cleaned glass

tubes and concentrated to dryness using a gentle stream of nitrogen gas. The residue was reconstituted in 200 µl methanol, the solution filtered through a micro syringe filter and 23 & 50 µl aliquots thereof respectively injected into the column of the Gold System and Delta System HPLC systems.

5.2.2.3.2. HPLC determination of the luteolin level in the plasma.

A stock solution of luteolin (1mg/ml) was prepared in dimethyl sulfoxide (DMSO). This solution was diluted with methanol to prepare work solutions containing 0,5 to 10µg/ml of luteolin. The analytical standards were used to assess the linearity, recovery and reproducibility of the assay.

Plasma calibration standards, containing 0,5 to 5µg/ml of luteolin were also prepared individually by adding aliquots of the luteolin-work solutions to blank plasma as described in the previous section. Replicate samples of the luteolin in plasma standards were freshly prepared on each day of analysis.

The HPLC assay was performed in reverse phase mode using isocratic elution. The optimum separation of the luteolin was achieved with a mobile phase composed of methanol and 1% acetic acid aqueous solution (70: 30 v/v) & (55:45 v/v) on the Gold System and Delta System HPLC systems, respectively, flowing through a Synergy®, Hydro-reverse phase column at a flow-rate of 1,0ml/min and at room temperature. The volume of samples injected was 23 µl & 50 µl on Gold and Delta HPLC systems, respectively, and the UV detector wavelength was set at 345nm.

To identify the luteolin peak and its retention time the procedure as described in the previous chapter, section 4.2.2.3.2, was followed: For the luteolin in the plasma, firstly, the range of retention times for the peaks obtained with the standard solutions was compared to those obtained after analysis of samples of the plasma, which were spiked or un-spiked with luteolin and analyzed under similar HPLC conditions. Secondly, a standard curve of peak area (Y) vs. the concentration of standard solution (X) over range

of 0.5 to 5 µg/ml was plotted. Once the area of the identified luteolin peak in the chromatogram of a plasma sample was known, the level of luteolin was calculated from the regression equation of the standard curve.

5.2.2.3.3. Validation of the HPLC assay.

The same procedures described in section 4.2.2.3.3 for the plant material were used to validate the HPLC assay for luteolin in the plasma samples (i.e. the specificity, lowest limit of quantification (LLQ), linearity, reproducibility and recovery), except that in this case the peak areas were used instead of peak heights.

5.2.2.4. Statistical analysis.

In this study, the values of the various measured data or statistical parameters calculated were expressed as mean ± SD, coefficient of variation, and/or 95% confidence intervals. The Microsoft Excel software package 2000 (version 5.1) was used for raw data transformation and analysis. Data was then transferred into the GraphPad Prism 4™ graphics software package. The latter was used to prepare graphs and to perform statistical comparisons between the data for the control and treatment groups of rats. The one-way ANOVA followed by the Dunnett's test and Student t-test was used to compare differences in the growth, the haematological and biochemical parameters of the control and the treated rats, with $p \leq 0.05$ set as the level of significance. Finally, the least squares method was used for the linear regression analysis of the luteolin plasma concentrations and to plot the standard curve. The levels of luteolin in the plasma of treated rats were then calculated from the linear equation.

5.3. Results and discussion.

The results obtained on the general effects, and the effects on various haematological and blood biochemical parameters and plasma luteolin levels obtained after chronic oral administration of *Artemisia afra* to the rats, are reported and discussed in this section.

5.3.1. Chronic toxicity of A. afra in rats.

5.3.1.1. Effect of chronic administration of A. afra on the general behaviour of the rats

A summary of the results of the mortality and gross symptoms of toxicity seen in the rats treated with *A. afra* over 92 days is given in Table 5.1.

Table 5.1 Mortality and gross symptoms of toxicity observed after chronic oral administration of plant extract to the rats.

Dose of *A-A* extract (mg/kg)	Mortality and gross symptoms of toxicity in various groups of rats				
	Number of rats/dose	Sex	D/T	Mortality latency (day)	Symptoms of toxicity
0	6	Male	0/6	-	None
100 (LD)	6	"	0/6	-	None
1000 (HD_1)	6	"	0/6	-	Partial hypo activity, diarrhea, salivation
1000 (HD_2)	6	"	0/6	-	Partial hypo activity, diarrhea, salivation
Total	24		0/26		

D: dead; **T:** total number; ***A-A***: *Artemisia afra,* LD: low dose; HD_1: high dose 1; HD_2: high dose 2

Firstly, there was no noticeable deviation in the behaviour of the rats treated with the low dose (100 mg/kg) compared to that of the control (no dose) group and essentially all the treated rats remained healthy during the 3-month period of chronic oral ingestion of *Artemisia afra*. Only the groups receiving the high dose (1000 mg/kg) manifested minor symptoms of toxicity, comprising intermittent, diarrhea, salivation and partial hypo-activity. Moreover, no deaths occurred with any of the doses up to 1000 mg/kg given over 92 days indicating that **the LD_{50}** for chronic dosing with *A. afra* was **higher than 1000 mg/kg.**

5.3.1.2. Effect of chronic ingestion of Artemisia afra on the bodyweight, food intake and feed conversion efficiency of the rats

In rodents, a decrease in food and water consumption is an important sign of deterioration of health or an indicator of poor health, and generally results in loss of bodyweight (Ullman-Cullere and Foltz, 1999). Changes in the body-weight have also been used as an indicator of adverse effects of drugs and chemicals (Hilaly et al., 2004).

In the present main study, the rats, each morning, for 3 months, received aqueous extract of *A. afra* or saline by gastro-gavage, and a summary of the changes in their body-weight, food intake and food conversion efficiency that occurred in this period is given in Tables 5.2 to 5.5 and Figures 5.1 to 5.4.

Table 5.2: The effect of *A. afra* on the change in body weight of rats over the dosing period

Time of measurement (Days)	*Body mass of rats ((g) mean ± SD) (n =6)*			
	Control group (0 mg/kg dose)	**LD group** (100mg/kg dose)	**HD_1 group** (1000mg/kg dose)	**HD_2 group** (1000mg/kg dose)
D0	265,87 ± 15,12	267,30 ± 8,57	264,79 ± 8,07	261,03 ± 11.14
D2	275,03 ± 14,50	275,60 ± 14,14	278,43 ± 12,13	273,27 ± 11,10
D9	312,67 ± 18,01	307,67 ± 16,60	314,86 ± 18,63	302,29 ± 17,11
D16	321,42 ± 17,13	318,23 ± 19,31	333,71 ± 21,35	325,45 ± 20,15
D23	366,94 ± 20,21	357,98 ± 20,08	351,63 ± 20,58	356,63 ± 23,08
D30	391,70 ± 20,25	376,94 ± 22,8	365,94 ± 17,8	367,29 ± 15,96
D37	413,10 ± 20,87	403,33 ± 24,61	391,70 ± 2161	390,00 ± 18,80
D44	431,20 ± 23,52	418,23 ± 26,41	405,41 ± 25,41	413,24 ± 20,20
D51	444,60 ± 22,45	425,13 ± 18,06	415,40 ± 18,66	422,44 ± 23,36
D58	446,30 ± 22,92	426,41 ± 17,46	423,56 ± 19,46	412,93 ± 28,31
D65	453,54 ± 22,02	425,11 ± 21,05	419,74 ± 20,15	418,43 ± 27,36
D72	460,20 ± 24,53	434,33 ± 17,12	432,70 ± 19,22	421,41 ± 27,56
D79	484,19 ± 26,53	450,32 ± 19,97	458,60 ± 18,92	448,66 ± 25,83
D86	494,40 ± 28,02	481,25 ± 17,04	485,60 ± 21,03	451,07 ± 28,83 **
D92	496,07 ± 27,84	477,82 ± 21,27	482,28 ± 20,20	438,01 ± 29,79**
Growth rate Per day	**2.342 ± 0.18**	**2.078 ± 0.17**	**2.082 ± 0.12**	**1.792 ± 0.19**

D: day; LD= low dose; HD_1= high dose 1; HD_2 = high dose 2,
**** = Significantly different from the control (P<0.05); paired student t-test.**

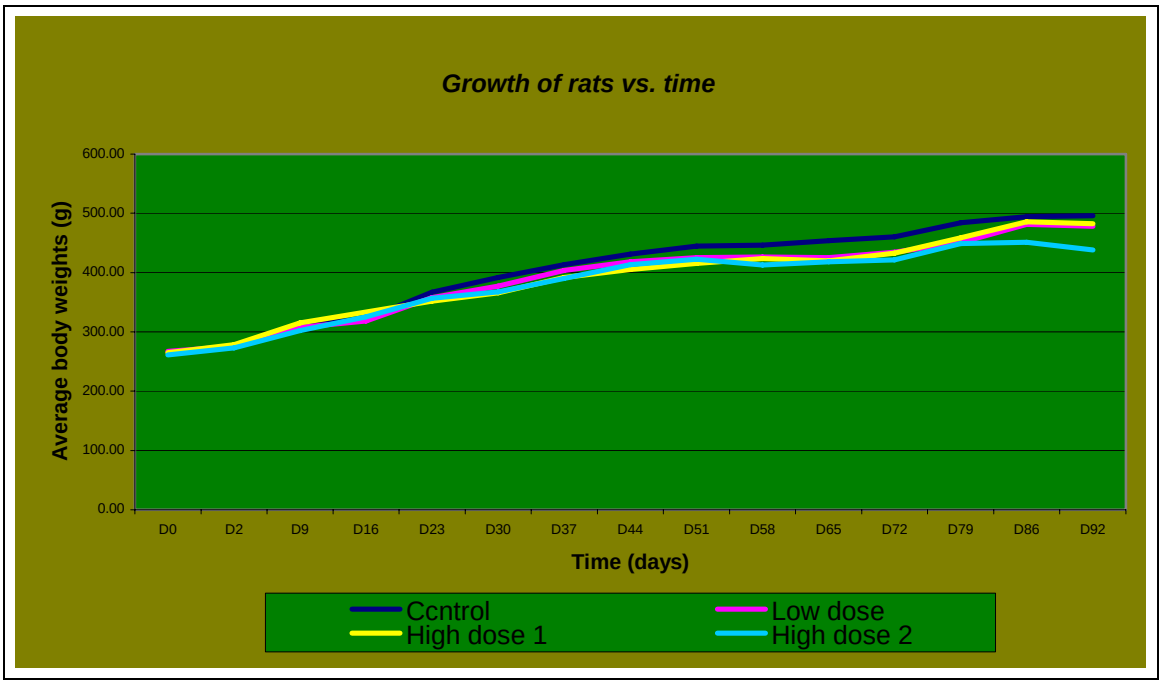


Figure 5.1: Graph of daily body-weight of control rat and rats treated with *A. afra.* Rats received daily oral doses of 0 (control= blue); 100 (LD= violet) and 1000 (HD_1= yellow and HD_2 = cyan) mg/kg of *Artemisia afra.*

Overall the rats grew at rates of 2.342 ± 0.18; 2.078 ± 0.17; 2.082 ± 0.12; 1.792 ± 0.19 grams per day for the control, low dose, high $dose_1$ and high $dose_2$ groups, respectively (Table 5.2). After 92 days of exposure to *A. afra,* there was no significant difference ($p > 0.05$, paired student t-test) in the overall growth and growth rate of all the treated groups compared to the control group of rats, except, in the case of the second high dose group of rats (HD_2). In this group, HD_2, a significant decrease in growth, relative to that of the control group, was observed in the last two weeks of treatment (Figure.5.1). These results suggested that the 3 month long chronic oral ingestion of *Artemisia afra* did not significantly affect the weight of the rats.

Table 5.3: The effect of *A. afra* on weekly food intake of rats dosed orally for 3 months.

Time of measurement (Days)	*Average weekly food intake per individual rat (g)*			
	Control group (g)	LD group (g)	HD_1 group (g)	HD_2 group (g)
D 2	81.89	85.06	81.46	82.89
D 9	275.73	284.16	259.89	247.64
D16	263.19	276.37	249.39	242.54
D 23	270.31	269.10	252.19	245.20
D 30	238.83	247.21	214.96	216.73
D37	249.54	256.46	239.67	226.13
D 44	204.73	205.77	188.34	182.47
D 51	194.09	200.39	183.87	182.10
D 58	193.97	176.96	179.53	171.31
D 65	212.76	214.43	184.47	183.89
D72	167.89	167.53	152.83	157.77
D79	185.13	206.00	147.14	162.89
D86	190.41	204.28	164.22	161.11
D92	137.74	131.77	127.42	113.27
Total	2866.20	2925.48	2625.36	2575.94
Average Daily food intake	**31.15**	**31.80**	**28.54**	**28.00**

D: day; LD= low dose *A. afra*, 100 mg/kg; HD_1= high dose *A. afra* 1, 1000 mg/kg and HD_2 = high dose *A. afra* 2, 1000 mg/kg.

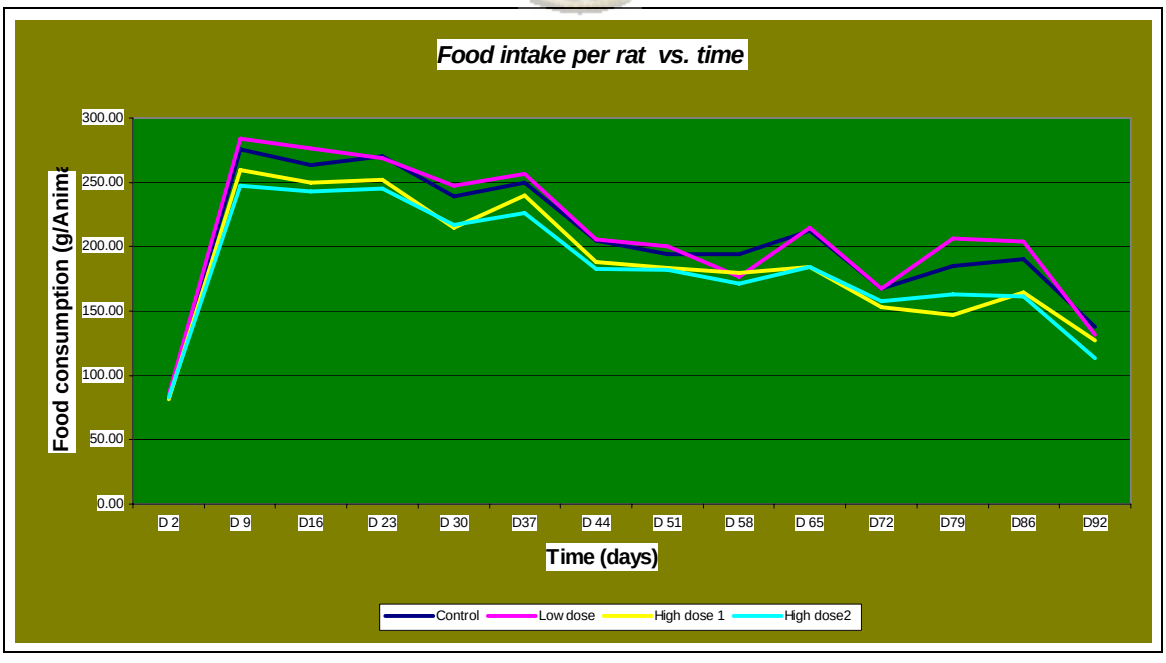


Figure 5.2: Daily food intake of rats. The amount of food consumed per rat in groups of rats given daily doses of 0 (control = blue), 100 (violet), and 1000 (yellow and cyan) mg/kg of *A. afra* over 92 days is shown.

At the end of the 3 month-period of study, the total amount of food consumed per rat, in the group that received 100 g/kg (LD) was 2% higher, and for the 1000 mg/kg (i.e. HD1 and HD2) dose group about 8 and 10 % lower than that of the control group (Table 5.3 and Figure 5.2), but these differences were not statistically significant ($p > 0.05$). Similarly there were no significant differences in the daily body-weight gained (per rat) and in feed conversion efficiency (i.e. body weight gain / food consumption x 100%), between the control and treated groups of rats (Table 5.4 and Figure 5.3; Table 5.5 and Figure 5.4). Collectively, these findings indicated that food intake and utilization in the rat were not affected by chronic ingestion of *Artemisia afra.*

Table 5.4: The effect of *A. afra* on weekly body-weight gain of rats. Groups of rats were given daily oral doses of *A. afra* over 3 months.

Time of measurement (Days)	*Body weight gained per rat (g)*			
	Control group	LD group	HD_1 group	HD_2 group
D 2	9.16	8.30	13.64	12.24
D 9	37.64	32.07	36.43	29.02
D16	8.75	10.56	18.85	23.16
D 23	45.52	39.76	17.92	31.18
D 30	24.76	18.96	14.31	10.66
D37	21.40	26.39	25.76	22.71
D 44	18.10	14.90	13.71	23.24
D 51	13.40	6.90	9.99	9.20
D 58	1.70	1.29	8.16	-9.51
D 65	7.24	-1.30	-3.82	5.49
D72	6.66	9.21	13.00	3.00
D79	23.99	15.99	25.86	27.24
D86	10.21	30.93	27.00	2.41
D92	1.67	-3.43	-3.32	-13.06
Total	**230.20**	**210.52**	**217.49**	**176.98**
Daily BW gained	**2.50**	**2.29**	**2.36**	**1.92**

D: day; LD= low dose *A. afra*, 100 mg/kg; HD_1= high dose *A. afra* 1, 1000 mg/kg and HD_2 = high dose *A. afra* 2, 1000 mg/kg.

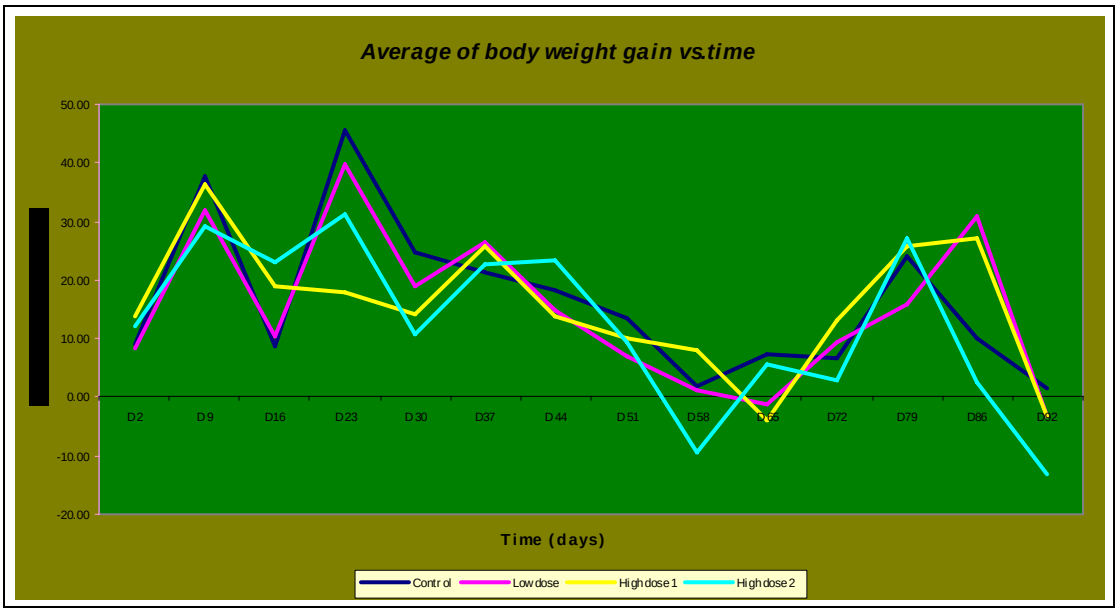


Figure 5.3: **The effect of *A. afra* on weekly body-weight gain of rats. Daily body weight gained (g) per rat given 0 (control= blue line), or 100 (LD= violet line) or 1000 (HD_1=yellow and HD_2 =cyan line) mg/kg *A. afra* daily over 3 months were recorded. Data points calculated from average daily body weights.**

Table 5.5: The feed conversion efficiency in control and treated groups of rats administered *A. afra*

Time of measurement (Days)	*Food conversion efficiency (%)* = body weight gain (g) / food intake (g) x 100.			
	Control group (g)	LD group (g)	HD_1 group (g)	HD_2 group (g)
D 2	11.19	9.76	16.75	14.77
D 9	13.65	11.29	14.02	11.72
D16	3.32	3.82	7.56	9.55
D 23	16.84	14.77	7.11	12.72
D 30	10.37	7.67	6.66	4.92
D37	8.58	10.29	10.75	10.04
D 44	8.84	7.24	7.28	12.74
D 51	6.90	3.44	5.43	5.05
D 58	0.88	0.73	4.55	-5.55
D 65	3.40	-0.61	-2.07	2.98
D72	3.97	5.50	8.51	1.90
D79	12.96	7.76	17.57	16.73
D86	5.36	15.14	16.44	1.50
D92	1.21	-2.61	-2.61	-11.53
Daily conversion	**1.17**	**1.02**	**1.28**	**0.95**

D: day; LD= low dose *A. afra*, 100mg/kg; HD_1= high dose *A. afra* 1, 1000mg/kg and HD_2 = high dose *A. afra* 2, 1000mg/kg.

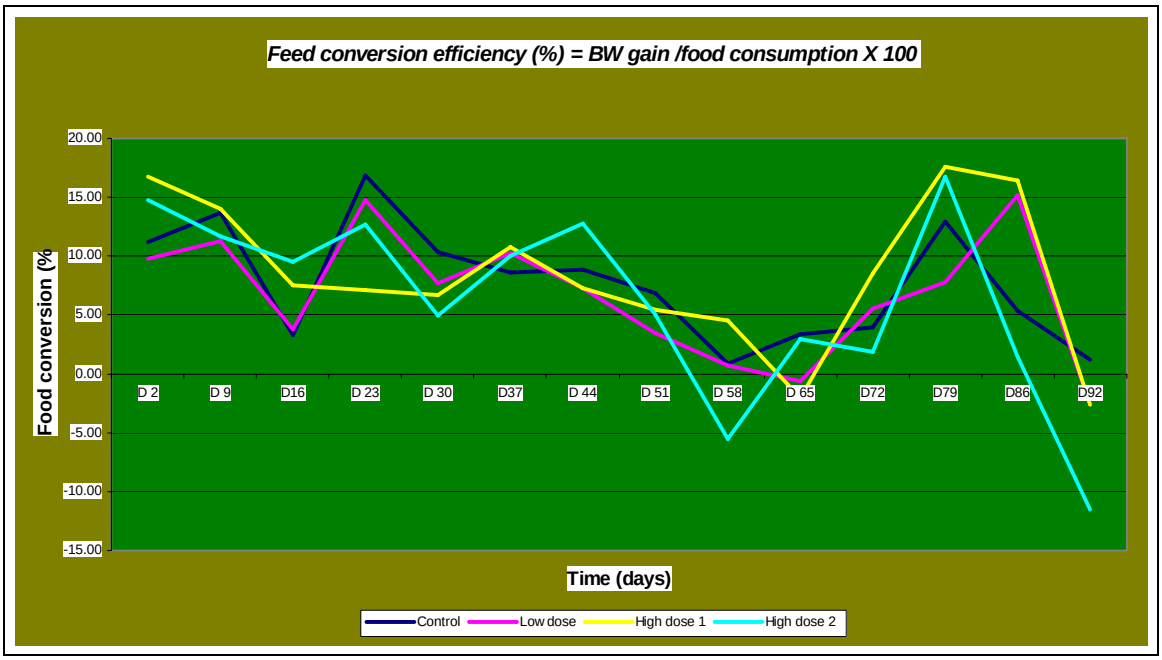


Figure 5.4: The feed conversion efficiency of rats given *A. afra*. Groups of rats (n=6) were given 0 (control, blue line), or 100 (LD = violet line) or 1000 (HD_1= yellow and HD_2 =cyan line) mg/kg daily dose *A. afra* over 3 months, food conversion efficiency at various times calculated from food consumption and weight gain.

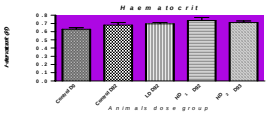
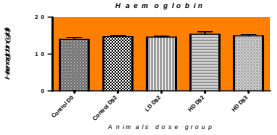
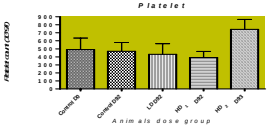
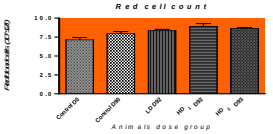
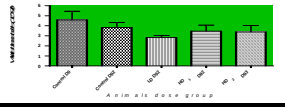
Changes in body-weight have been used as an indicator of adverse effects of drugs and chemicals (Hilaly et al., 2004). Since, no significant changes were observed in the behaviour of the rats, the body-weight, food intake and utilization of food in the treated groups (LD, HD_1 and $HD_{2)}$ compared to that of the control group, it suggested that at the oral doses administered, *Artemisia afra* extract had no effect on the growth of rats.

For 7-day study, the administration of *A. afra* extract was done by oral gavage as described in the first study. After seven-day period of the treatment, no significant difference was observed in the animals' growth and food intake between the control and the treated groups (Fig. 1 in Appendix X).

5.3.1.3. Effect of chronic oral administration of Artemisia afra on the haematological indices of the rats.

To determine the effect that *A. afra* would have on the haematological indices of the rats, approximately 5 ml of blood was collected from each animal after the chronic oral administration of the plant extract. 3 ml of blood was taken for the haematological tests and biochemical test and the remainder was kept for the HPLC assay. The results obtained for the blood analyses are summarized in Table 5.6.

Table 5.6: Effects of *Artemisia afra* extract on haematological indices of rats after 3 month-period of daily intake

Parameter	Effect of *Artemisia afra* extract on haematological indices in various groups of rat (expressed as mean ± SD, n = 6)					
	Control at D0	**Control at D92**	**LD at D92**	**HD_1 at D92**	**HD_2 at D93**	**Representative graph of each parameter for each dose group.**
Haematocrit (l/l)	0,63 ± 0,047	0,68 ± 0,070	0,70 ±0,043	0,74 ± 0,088	0,71 ± 0,047	
Haemoglobin (g/dl)	14,00 ±1,10	14,73 ±0,73	14,60 ±0,68	15,38 ± 1,60	14,93 ±0,85	
Platelet (10^9/l)	490 ± 354	469 ±273	431 ±324	391 ±190	742 ±312	
R BC (10^{12}/l)	7,1 ± 0,69	7,9 ± 0,68	8,3 ± 0,50	8,9 ± 0,91	8,6 ± 0,41	
WBC (10^9/l)	4,6 ± 2,2	3,8 ± 1,3	2,8 ± 0,48	3,5 ± 1,5	3,4 ± 1,6	

(Detailed values are given in Appendix VIII); D0 = day 0; D92 = day-92; D93 = day 93 and Control = 0; LD = low dose, 100 mg/kg; HD_1 and HD_2 = high dose, 1000 mg/kg *A. afra*

While the haematopoietic system is one of the most sensitive targets for toxic compounds (Harper, 1973), there were no significant ($P > 0.05$, t –test) differences in the haematocrits, mean cell haemoglobin concentration, and platelet, red cell and white cell counts of the control versus the treated rats. These results therefore indicated that 3-month administration of *Artemisia afra* had no effects on the circulating cells nor interfered with their production. The haemoglolobin and the RBC levels were not affected suggesting that haemolytic anemia and polycythemia, (that are characterized by decreases and increases in RBC count, haematocrits and hemoglobin, respectively (Mdhluli, 2003)), were not likely to be induced by *A. afra* in this species. The platelet levels were also not adversely affected indicating that the plant extract also did not affect the production of platelets nor induced thrombocytopenia, the latter normally being the first evidence of drug-induced toxic effects on haematopoiesis (haematogenesis) (Mdhluli, 2003). The levels of white blood cells, (which serve as scavengers that destroy microorganisms at infection sites, remove foreign substances and debris that results from dead or injured cells (Miller and Harley, 1996; Mdhluli, 2003; Guilhermino, 1998)), were also not changed suggesting that the aqueous extract of *Artemisia afra* was also not toxic to the immune system and did not affect leucopoiesis. Collectively, all the results suggest that the chronic ingestion of the aqueous extract of *Artemisia afra* did not alter the haematological parameters of the rats.

Furthermore, the actual values for the haematological parameters, (Hb, platelet and RBC), in both the treated and non-treated rats after the 3 month chronic ingestion of *Artemisia afra,* were compared to the "normal" values given by Waynforth (1980) and copied in Table: 5.7.

Table 5.7: Haematological reference indices for rats, according to Waynforth (1980)

Haematological parameter	Normal Value
1. Haematocrit (packed cell volume; ml/100 ml	47
2. Haemoglobin (g/100 ml)	15,7
3. Red blood cells (no / ml)	8×10^9
4. White blood cells (no/ ml)	$1,3 \times 10^7$
5.Platelets (no/ml)	$5,3 \times 10^8$

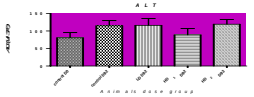
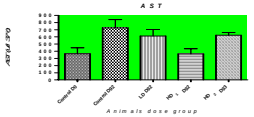
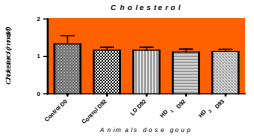
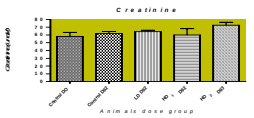
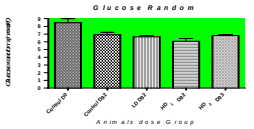
The values given in this Table are intended to act only as a guide (reference). They apply most closely to an adult rat about three months old, unless specifically stated, not to any particular strain of rat (Waynforth, 1980).

Most of the parameter values obtained in the present study appeared to agree with these "normal" values. From Table 5.6 it can be seen that the recorded haemoglobin levels of 14 to 15.38 g/ 100 ml (g/dl); RBC of 7.1 to 8.9 x 10^9 /ml (or 10^{12} /l) and platelets of 3.91 to 7.42 x10^8 /ml (or 391 to 742 x10^9/l) compared well to the "normal" reference values of 15.7 g/100 ml; 8x10^9/ml and 5.3 x10^8/ml, respectively, given in Table 5.7. However, the haematocrit mean-values ranged from 0, 63 to 0, 74 l/l, or 63 to 74 ml/ 100 ml and were thus 34 to 57% higher than the "normal" value, while the WBC of 0.28 to 0.4 6 x 10^7/ml (or 2.8 to 4,6 x10^9/l) were 3 to 4 times lower than the tabulated reference value. But because the haematocrit and the WBC values for the control and treated rats at day 0 (control D0) and day 92 (ControlD92 and treated 92 groups) did not differ significantly, it means that the deviation of the recorded values (Table 5.6) from the "normal" tabulated values (Table 5.7) may most likely be due to differences in the strain of animals used (rather than age). No further conclusions can however be drawn from the present data.

5.3.1.4. Effect of chronic oral administration of Artemisia afra on the biochemical indices of rats.

The results of the biochemical analyses of the blood plasma and serum samples are shown in Table 5.8.

Table 5.8: Effects of chronic daily p.o. *Artemisia afra* extract on selected biochemical parameters in blood of rats.

Parameter	Effect of *Artemisia afra* extract on biochemical indices in various groups of rat (expressed in mean ± SD, n = 6)					
	Control at D0	Control at D92	LD at D92	HD_1 at D92	HD_2 at D93	Representative graph of each parameter for each dose group
ALT(u/l)37^{0}C	80,50±36,57	114,8±33,10	116,0±43,41	88,83±45,78	119,5±33,13	
AST(u/l)37^{0}C	365,5±202,3	725,5±283,6*	613,0±222,2	361,7±178,9*	624,3±84,66	
Cholesterol (mmol/l)	1,3± 0,54	1,2 ±0,20	1,2 ±0,20	1,1± 0,19	1,1 ±0,15	
Creatinine (µmol/l)	58 ±13	62 ± 6,3	64 ±4,4	60 ±20	73 ±9,5	
Glucose (mmol/l)	8,5 ±1,3	6,9 ± 0,81	6,6± 0,36	6,1 ±0,93	6,8 ± 0,34	

D0 = day 0; D92 = day-92; D93 = day 93 and Control = 0; LD = low dose, 100mg/kg; HD_1 and HD_2 = high dose, 1000mg/kg *A. afra*
***: Significantly different from the control at day 92. (Detailed values are in Appendix IX)**

The results (Table 5.8) showed that, despite small differences between the groups, there were no statistically significant (t-test; $p > 0.05$) differences for virtually all of the biochemical parameters (i.e. serum ALT activities and cholesterol, creatinine and glucose levels) between the levels for the control group at day 0 and that for control group and treatment groups at day 92 or 93. However for the serum AST activities, there was a significant increase in the levels for the control and two of the treatment groups (LD and HD_2) at D92 and D93, when compared with the level in the control group at day zero. This may imply that AST activity levels increased with time. There was however no difference in the level at D0 and D92 for one of the high dose groups (HD_1) i.e. group when blood was taken shortly (1 to 2 hrs) after the last dosing (Table 5.8). But when levels were taken a day later (i.e. 25 hrs after the last dosing), much higher levels were obtained. The significance of this result is unclear and any attempt at interpretation would be very speculative.

Many compounds are metabolized in the liver, but if too many demands are made on this organ's capacity, the continued function of its cells is no longer ensured (Mdhluli, 2003). It is known that the liver and kidneys play significant roles in various metabolic processes. The liver plays an important role in xenobiotic function; and the kidneys are the main organs involved in drugs elimination, and, therefore, particularly exposed to the toxic effects of exogenous compounds (Bidhe and Ghosh, 2004). It was thus important to investigate the effect of *Artemisia afra* on the function of these organs.

The transaminases (AST and ALT) are useful enzymes as biomarkers predicting possible toxicity (Rahman et al., 2001). Any damage to the parenchymal liver cells will result in elevations in both these transaminases (Wolf et al., 1972). On the other hand, AST found in the serum is of both mitochondrial and cytoplasmic origin and if it is raised that can be taken as a first sign of cell damage that lead to the outflow of the enzyme into the serum (Mdhluli, 2003). The AST level results obtained in this study (viz. a dose dependent decrease in the AST levels recorded for the control and treatment groups at D92) seem to suggest that at higher doses *A. afra* did not adversely affect the cell mitochondria; in fact may even stabilized these organelles (e.g. brought level back to the day 0 levels).

However, this appeared to be an acute, short lasting response because a day (i.e. on day 93) after the last dose, the higher AST levels returned.

The liver also is the site of cholesterol disposal or degradation, as well as its major site of synthesis. It controls glucose synthesis and can generate free glucose from hepatic glycogen stores (Kaplan et al., 1995). The results in Table 5.8 showed that the chronic oral administration of *Artemisia afra* did cause significant changes in glucose and cholesterol levels in the control and the treated rats. *A. afra* thus did not adversely affect lipid and carbohydrate metabolism of the rats. Furthermore, there was no effect on the levels of transaminases ALT and creatinine, good indicators of liver and kidney functions, respectively (Hilaly et al., 2004). Collectively, all the results suggest that the chronic ingestion of the aqueous extract of *Artemisia afra* did not induce alterations in the blood biochemical parameters or damage to the liver and kidneys of the rats.

5.3.1.5. Effect of chronic oral administration of Artemisia afra on the organs of rats.

At the end of the study-period, the organs of both the control and treated rats were isolated, weighed and visually inspected for any histopathological changes in the tissue architecture and colour. The results obtained are shown in Table 5.9 and Figure 5.5.

Table5. 9: Wet weight (in gram; average ± SD) of organs of rats chronically dosed with *A. afra*.

Dose group	Wet weight (in gram; average ± SD; n=5)		
	Liver	Kidney (single)	Brain
Control	14,7 ±0,49	1,6± 0,08	3,2 ± 0,22
Low dose (100mg/g *A. afra)*	13,9± 0,38	1,54± 0,07	2,97± 0,19
High dose 1 (1000mg/kg *A. afra*)	14,4± 0,57	1,52± 0,10	3,02± 0,25
High dose 2 (1000mg/kg *A. afra*)	13,6± 0,59	1,50± 0,09	2,88± 0,12

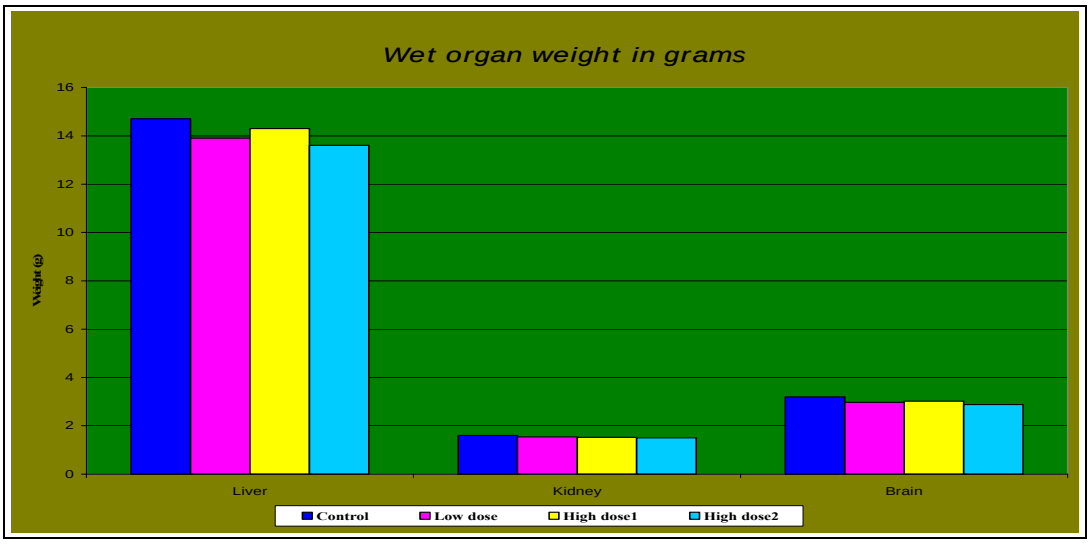


Figure 5.5: Weight of the wet organs of rats after chronic *A. afra* doses. Rats were given 0 (blue), 100, (LD=violet), or 1000mg (HD_1=yellow and HD_2=cyan) mg/kg aqueous extract of *A. afra* p.o for 3 months. There was no significant difference (t-test, P> 0.05) between control and treatment groups.

Upon visual histological examination the organs of both treated rats and the control showed normal architecture, no colour changes and no morphological disturbances, indicating that the chronic oral doses of *A. afra* extract administered had no effect on the organs of the rats and was well tolerated over the 3-month study period. The *A. afra* extract can therefore be considered as material that should be safe for use in oral formulations.

5.3.2. *Levels of luteolin in rats' plasma after chronic ingestion of Artemisia afra.*

The blood obtained from rats given *A. afra* daily for 3 months and rats given *A. afra* for 7 days were analyzed for luteolin content using an HPLC assay. Aspects of the assay that was developed and the luteolin plasma levels obtained in these 2 studies are described and discussed below.

5.3.2.1. The HPLC assay for luteolin in the rat plasma.

The HPLC assay for the quantitation of luteolin in the plasma of the rats was validated for its specificity, lowest limit of quantification (LLQ), linearity, reproducibility and recovery, and the results obtained for the assays used in the 92 -day and 7-day studies are summarized in Table 5.10 and Figures 5.6 and 5.7.

Table 5.10: Validation parameters of the HPLC assay for luteolin in the plasma of rats (n=4).

Parameter	92-day study (Main study)	7-day study
Luteolin concentration range (µg/ml)	0,5 to 5	0,5 to 5
Retention time of luteolin (min)	4.8 to 5.3	7, 5 to 8,2.
Low limit of quantification (LLQ) (µg/ml)	0.5µg/ml	0.5µg/ml
Sensitivity (LLD)	11.5ng on column	25 ng on column
Linearity:		
• Slope (mAU.ml /µg)	106000 ± 3300	114600 ± 2881
• Intercept (mAU)	2770 ± 9070	5845 ± 7923
• Correlation coefficient (R^2)	0,994	0,996
Reproducibility:		
• Intra-assay (CV %)	0,79	0,84
• Inter-assay (CV %)	1,29	1,50
Recovery at 5 µg/ml (mean ± SD; n = 3)	71,3 ± 2,3%	69,36 ± 3,7%

As shown in Table 5.10, for the main study, the recovery at level of 5µg luteolin per ml plasma was 71.3 ± 2.3%, indicating that the accuracy of the method was satisfactory. The limit of quantification (i.e. LOQ = the lowest nominal concentration of the control sample that could be measured in five replicates) of luteolin was 0.5 µg/ml solution or 11.5ng on column and the intra and inter-assay CV < 2 % indicating that the assay had acceptable sensitivity, precision and accuracy. (Appendix IV.1). Furthermore, the standard curve of

luteolin in plasma was linear over the concentration range of 0.5 to 5.0 µg/ml (or 11.5 to 115.0 ng on column) with the regression coefficient of R^2 = 0,994 (Figure 5.6).

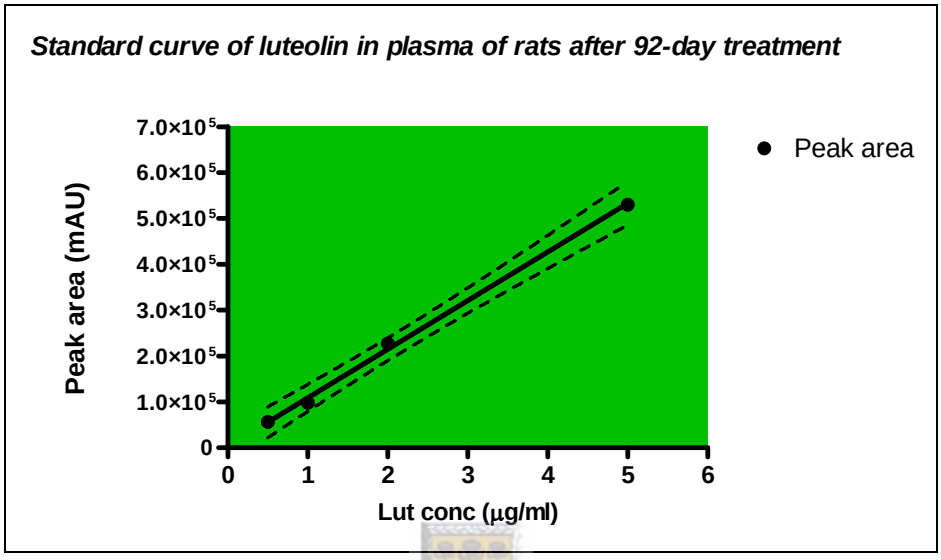


Figure 5. 6: Standard curve of luteolin in the plasma of rats after 92-day treatment with *A. afra*

The second HPLC system (Delta system), as described in section 5.2.1.2, was used for the 7-day experiment and the analysis was performed on the same column (Synergy®, Hydro-reverse phase column). The assay sample preparation and validation of the method were handled in the same way as in the first experiment. The HPLC assay for quantification of luteolin in the plasma was validated and the results were as shown in Table 5.10 and Figure 5.7. This time the retention time of luteolin was between 7.5 to 8.2

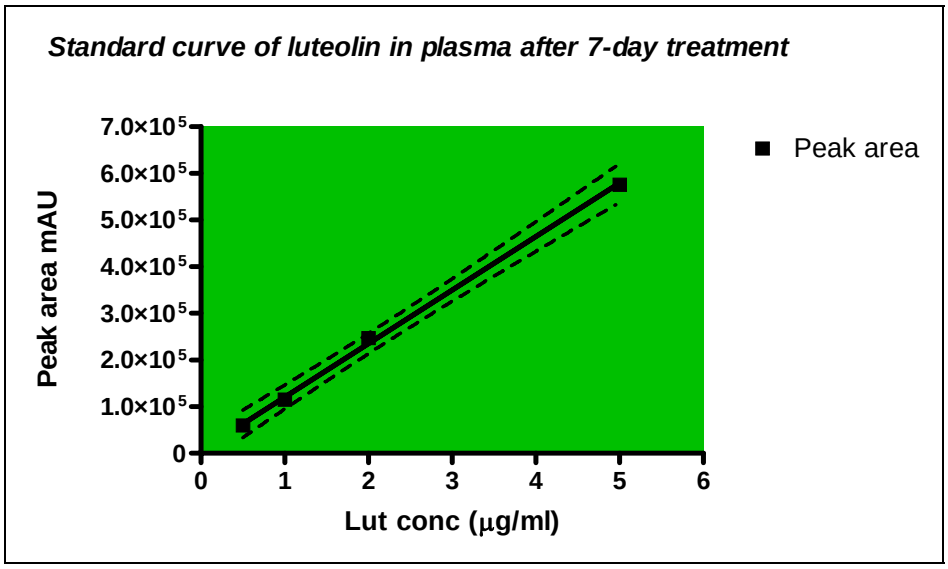


Figure 5. 7: Standard curve of luteolin in the plasma of rats after 7 days of dosing with *A. afra*.

minutes with no interference from plasma peaks, the coefficient of variation low (0,84 to 1,50 %), recovery (at 5 µg/ml) acceptable at 69,36 ± 3,7% and the standard curve linear over the concentration range of 0.5 to 5 µg luteolin per ml plasma (or 25 to 250 ng luteolin on column) with regression coefficient of $R^2 = 0{,}996$ (Figure 5.7).

5.3.2.2. The luteolin levels in the plasma of the rats.

Using the afore-mentioned HPLC assays and standard curves (i.e. the regression equations), the levels of luteolin in, firstly, day 0 and day 92 plasma of the control rats (i.e. rats not receiving *A. afra*), the day 92 plasma of the rats receiving the low dose (100 mg/kg *A. afra* per day) and rats receiving the high dose (1000 mg/kg *A. afra* per day) and day 93 plasma of the second group of rats receiving the high dose (1000 mg/kg *A. afra* per day) and, secondly, the day 7 plasma of rats in the control, low dose (100 mg/kg) and high dose (1000 mg/kg) groups were determined using the Gold and Delta HPLC systems, respectively.

In the main study, the chromatograms of acid hydrolyzed blank plasma spiked with luteolin (5 µg/ml) showed a distinct peak at 4.93 minutes (Figure 5.8), while the acid-

hydrolyzed plasma of the control animals had no detectable levels of luteolin at both day 0 (Figure 5.9) and at day 92 (Figure 5.10). Neither was luteolin detected in the day 92 acid-hydrolyzed plasma samples of rats in the low dose (100 mg/kg *A. afra*) group (Figure 5.11).

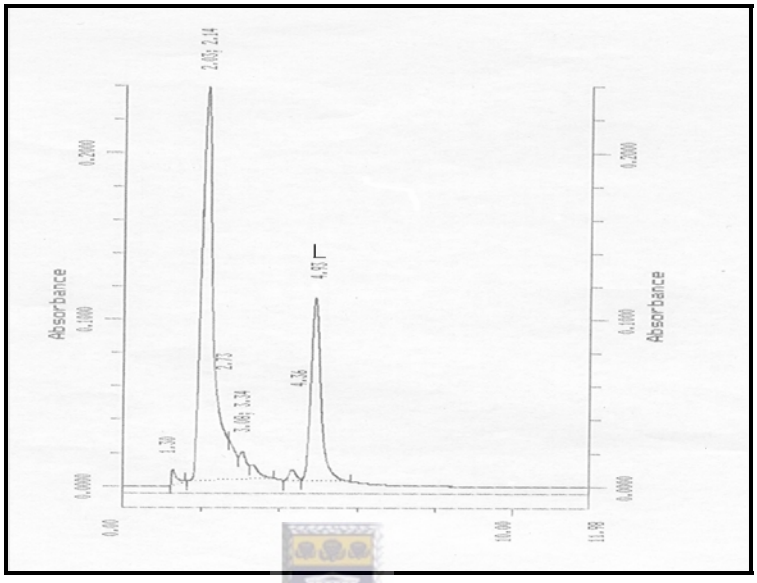


Figure 5.8: Representative chromatogram of blank plasma spiked with 5µg/ml luteolin. L: luteolin peak, retention time 4.93 min

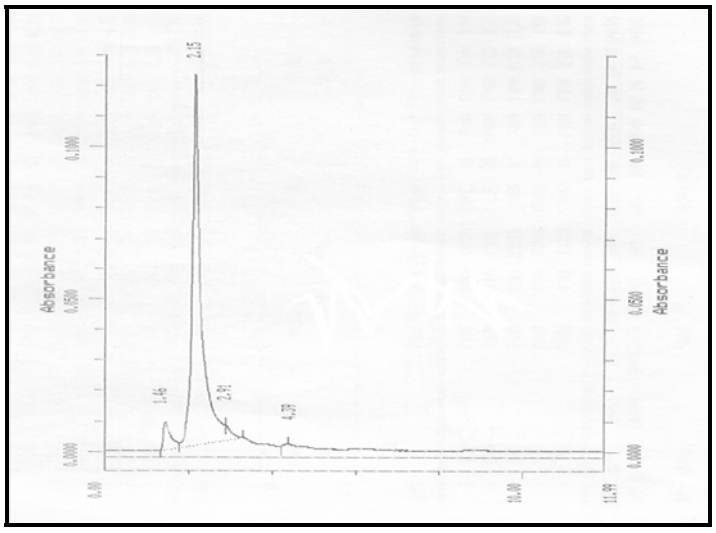


Figure 5. 9: Representative chromatogram of the day 0 plasma of rat in the control group. No luteolin peak at 4.93 minutes

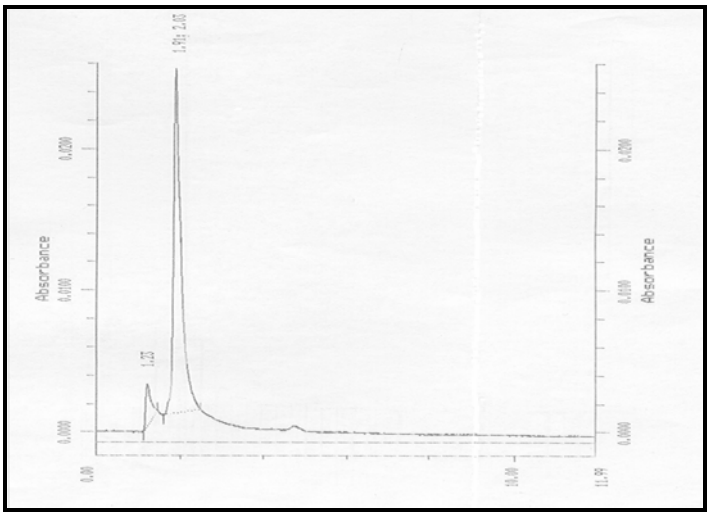


Figure 5. 10: Representative chromatogram of the day 92 plasma of rat in the control group. No luteolin peak at 4.93 minutes

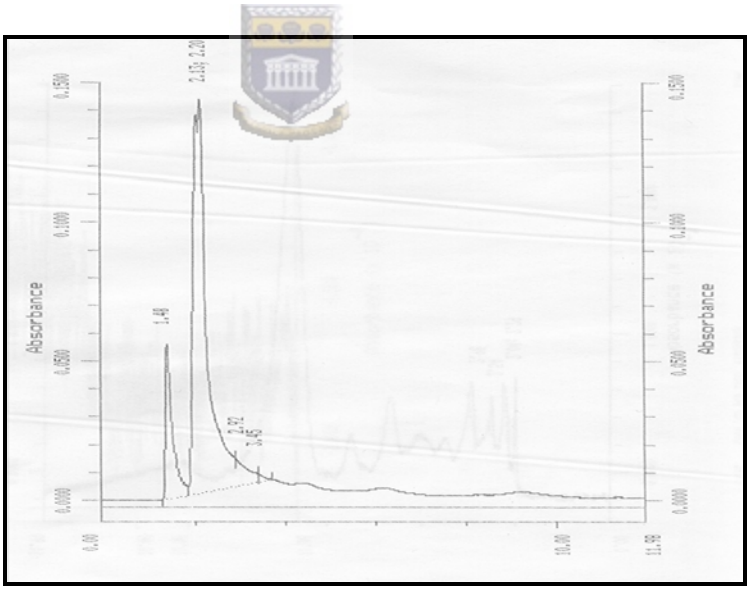


Figure 5.11: Representative chromatogram of day 92 plasma of rat treated with the low dose of *A. afra* (100mg/kg per day). No luteolin peak at 4.93 minutes.

There were, however, clear peaks for luteolin in the acid hydrolyzed day-92 plasma of the rats treated daily with the high dose (1000 mg/day) of *A. afra* (Figure 5.12).

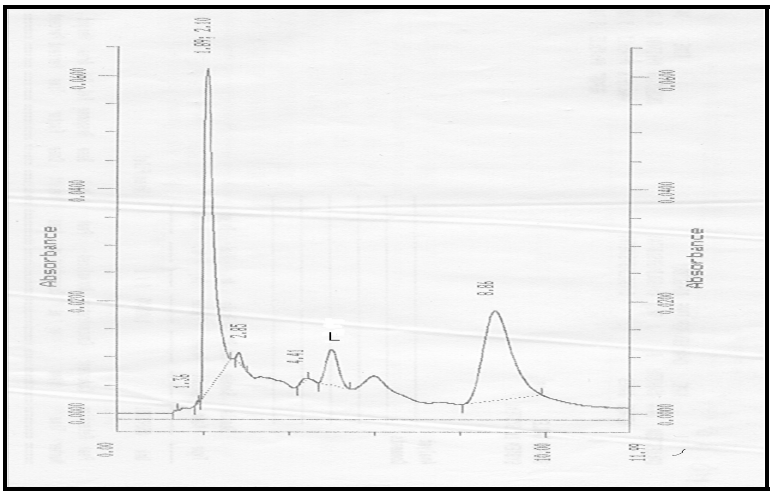


Figure 5.12: Representative chromatogram of day 92 acid hydrolyzed plasma of rat treated with the high dose (1000 mg/kg) of *A. afra.* The luteolin (L) peak found at retention time: 4, 95 min

Albeit much lower, there were also detectable luteolin peaks in the enzyme-hydrolyzed day 92 plasma of the rats treated on the high dose (1000 mg/kg) of *A. afra* (Figure 5.13).

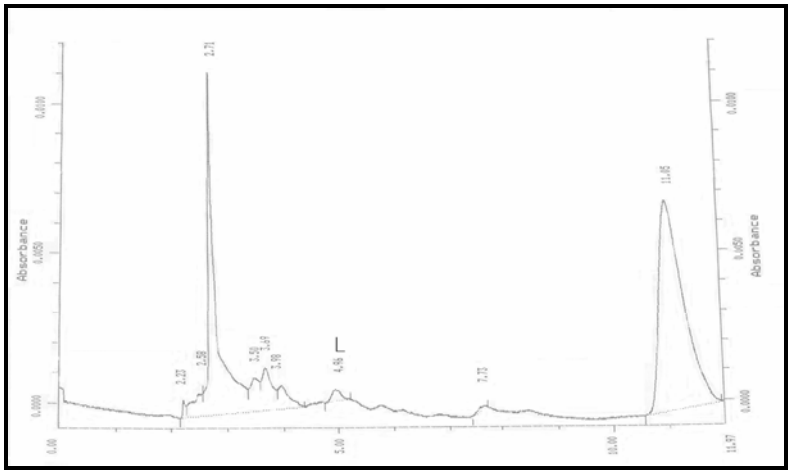


Figure 5.13: Representative chromatogram of day 92 plasma sample of rat treated daily with high dose (1000 mg/kg) of *A. afra* and subjected to enzyme-mediated hydrolysis. Luteolin (L) appears at retention time: 4. 96 minutes.

Finally there were no detectable luteolin in the day 93 (i.e. one day after last dose on day 92) plasma samples of the rats given the high dose (i.e. 1000 mg/kg) *A. afra* (Figure 5.14). These samples were acid-hydrolyzed.

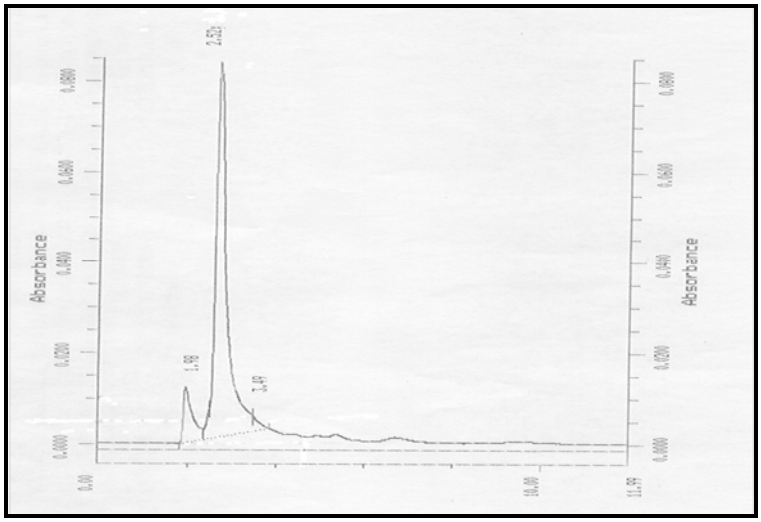


Figure 5.14: Representative chromatogram of plasma sample one day after the oral administration of *Artemisia afra* at high dose (1000 mg) was stopped (D93), luteolin was not detectable.

The representative chromatograms of the blank and treated plasma samples for the rats used in the 7-day study are shown in Figures 5.15 to 5.20.

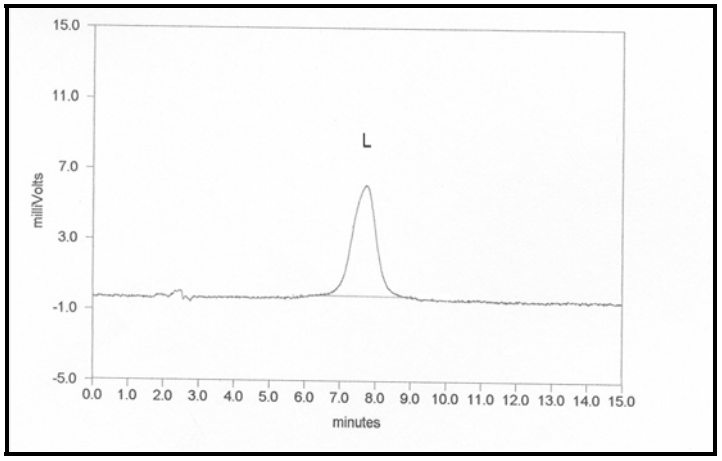


Figure 5. 15: Representative chromatogram of luteolin standard solution. Luteolin peak (L) appeared at 7,8 min

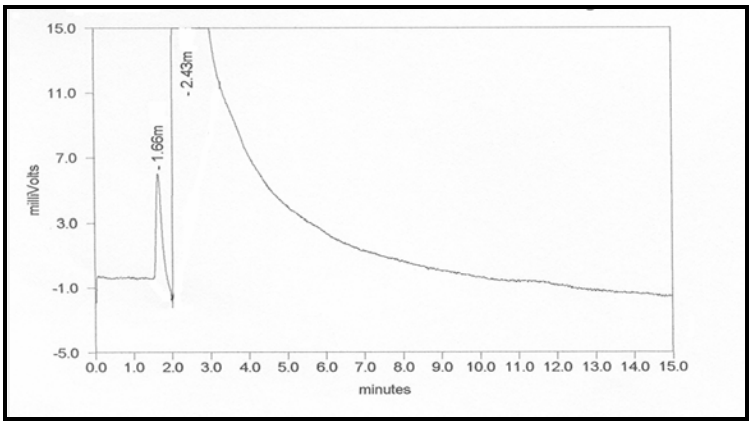


Figure 5. 16: Representative chromatogram of the control plasma sample at day 7. No luteolin peak at 7.8 min

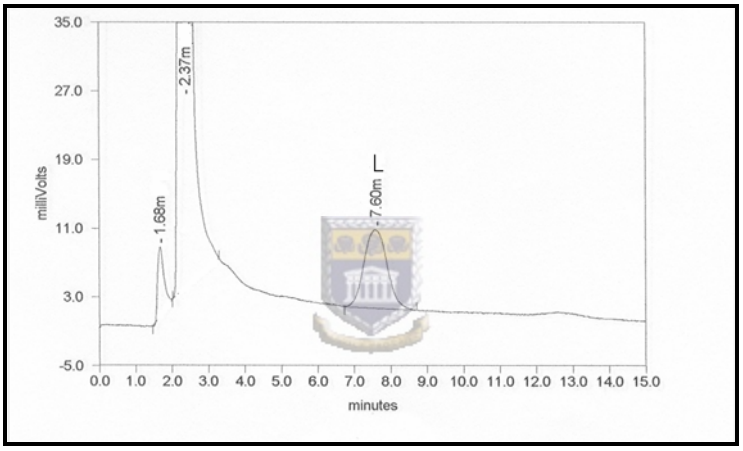


Figure 5. 17: Chromatogram of blank plasma spiked with 2µg/ml luteolin. Luteolin peak (L) appeared at 7.6 min

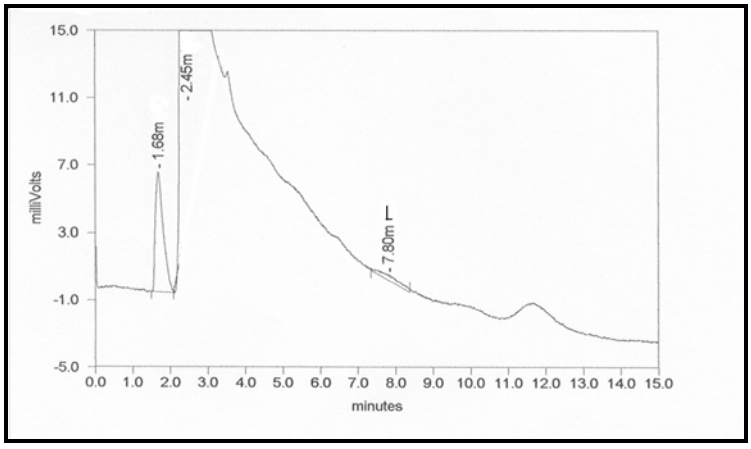


Figure 5. 18: Chromatogram of plasma sample after 7days of oral treatment of *Artemisia afra* at low dose (100mg/kg). Very small peak of luteolin (L) appeared at 7.8 min

The luteolin levels in the plasma samples of the rats after 7day treatment with high dose of *A. afra* (1000 mg/kg) were very low and again highly variable. The Figures 5.19 and 5.20 below give an example of the variation in luteolin levels displayed by 2 rats of the same dose group, from which the blood samples were collected at the same time interval.

A summary of the quantitative luteolin results obtained in both the 92-day and 7-day studies are given in Table 5.11.

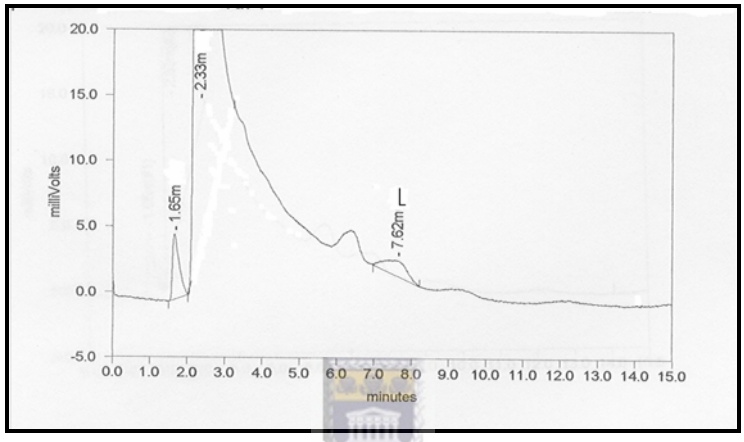


Figure 5. 19: Chromatogram of rat$_1$ plasma collected after 7days following the oral administration of *A. afra* (1000 mg/kg dose). Luteolin peak (L) at retention time: 7.62min

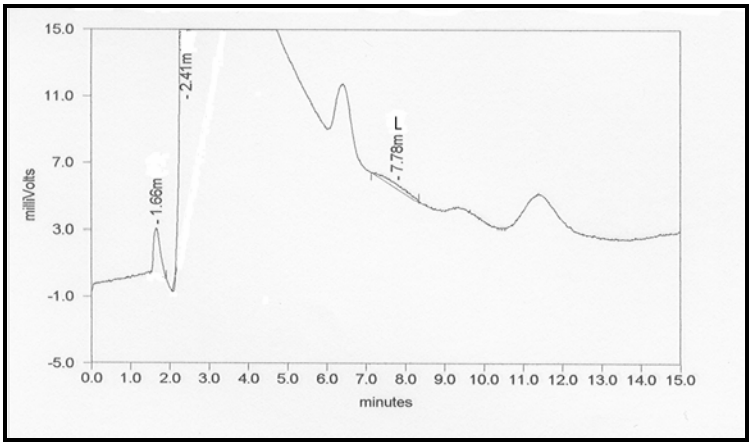


Figure 5.20: Chromatogram of rat$_2$ plasma collected after 7days following the oral administration of *A. afra* (1000 mg/kg dose). Luteolin peak (L), retention time: 7.78min.

Table 5.11: Luteolin levels in the hydrolyzed plasma of rats after 92 days and 7 days of oral administration of *Artemisia afra* extract.

Plasma sample	Luteolin concentration (ng/ml) (Mean± SD)
Control at Day 0 (D0)	NQ
Control at Day 92 (D92) *	NQ
Low dose (100 mg/kg *A. afra*) at Day 92 *	NQ
Low dose (100 mg/kg A. afra) at *Day 7* **	*41,16 ± 25,02* (n=3)
High dose (1000 mg/kg A. afra) *at Day 7* **	*152, 23± 30,68* (n=3)
High dose (1000 mg/kg *A. afra*) at Day 92 *	132,67 ± 31,98 (n =4)
High dose (1000 mg/kg *A. afra*) at Day 92 * (Enzyme-mediated hydrolysis)	47,12 ± 17,72 (n = 4)
High dose (1000 mg/kg) *A. afra*) at Day 93 ***	NQ

NQ = Not quantifiable; * = sample taken 1 to 2 hrs after last dose; ** = sample taken 45 min after last dose; * = sample taken 25 hr after last dose.**

In general, there were no detectable luteolin in the day 92 plasma of rats given low dose (100 mg/kg) *A. afra*. For the higher dose, the plasma luteolin levels found in the 92 day study were very low and highly variable viz. 0.133 ± 0.032 µg/ml plasma for the 1000 mg/kg *A. afra* dose, while the levels for the enzyme-hydrolyzed samples were even lower viz. 0.047 ± 0.018 µg/ml plasma after the same dose was given. All the day 92 samples were of course taken 1 to 2 hr after the ingestions of the last dose of *A. afra.* There was however also no detectable luteolin in the 93-day samples i.e. those collected 25 hours after the last dose of *A. afra.*

Artemisia afra contains luteolin in glycoside form, which when given orally, could be hydrolyzed to the aglycone form through the action of acid in the gastrointestinal tract and/or the hydrolytic enzymes of the host (rat) or resident intestinal microorganisms in the host. It is generally assumed that flavonoids such as luteolin are primarily absorbed in the aglycone form with subsequent conversion to conjugate metabolites, which appear in the circulation. In the present 92-day dose study, no luteolin could be extracted from the un-hydrolyzed plasma for all the dose groups, suggesting that there were no free luteolin

in the plasma or that the levels thereof were far below the detection level of the present assay. The fact that the hydrolyzed samples did have measurable luteolin level renders support to the first suggestion, viz that most of the luteolin in the plasma is in an acid-labile conjugate form. Glucuronidase and sulphatase enzyme-mediated hydrolysis also lead to measurable luteolin levels suggesting that some of the luteolin in the plasma was also in an enzyme-labile conjugate form. The fact that no luteolin could be detected 25 hours after the last dose strongly suggested that little accumulation of luteolin appeared to occur with chronic dosing and/or that rapid elimination of the ingested luteolin or its conjugate metabolites took place in the rat.

To test whether the time of sampling after the last dose might significantly affect the luteolin levels obtained, 9 rats were dosed with *A. afra* for 7 days and plasma samples collected on the 7th day at 45 minutes after the last dose. The results obtained and given in Table 5.11 showed that, after 7-day treatment with *A. afra***,** the plasma luteolin levels were very low and also highly variable viz. 0.041 ±0.025 µg /ml plasma for the 100 mg/kg *A. afra* dose and slightly higher viz. 0.152 ± 0.030 µg/ml plasma for the 1000 mg/kg (high) dose.

When the luteolin levels obtained in the high dose (1000mg/kg) groups for the 92-day and 7–day experiments are compared there was no significant difference between the levels. However, for at the low dose (100mg/kg) group levels of luteolin were detected in the 7-day treatment experiment while no quantifiable luteolin was found for the same dose group in the 92-day study. These findings suggested that the chronic ingestion of *A. afra* firstly gives very low and highly variable plasma levels of luteolin in rats with little evidence of substantial accumulation over time (compare 7 day vs. 92 day levels). Secondly, the time of sample collection after *A. afra* last dose appears to have a significant effect. For instance, for the 7 day experiment i.e. where samples were collected 45 minutes after the dose, levels of luteolin could be detected for the low dose while this was not the case in the 92 day experiment when samples were taken later i.e. 1 to 2 hours after the last dose. Even for the higher dose (i.e.1000mg/kg), the luteolin levels after the 7-day treatment (45 minutes post last dose) were slightly higher than that obtained in the 92-day (i.e.. 1 to 2hrs post dose). Both observations suggest that rapid

elimination of luteolin occurred and that was the most significant factor affecting the luteolin levels attained after chronic dosing with *A. afra.*

But how did the luteolin levels relate to the luteolin ingested in the form of the plant material. In Table 5.12 the levels of luteolin contained in each dose of *A. afra* and the plasma levels that were obtained are given.

Table 5.12: Luteolin level in both plant extract and hydrolyzed plasma samples of rats after the chronic ingestion of *A. afra.*

Material	Luteolin level	Equivalent luteolin dose ingested per rat per day
A. afra aqueous extract	0.923 ± 0.015 µg/mg	-
Plasma samples LD at D92	NQ	92.3 ± 1.5 µg /kg bm
Plasma samples HD at D92	0.133 ± 0.032 µg/ml	923 ± 15 µg/kg bm
Plasma samples LD at D7	0.041 ± 0.025µg/ml	92.3 ± 1.5 µg /kg bm
Plasma samples HD at D7	0.152 ± 0.030µg/ml	923 ± 15 µg/kg bm

HD= high dose (1000mg/kg); LD = low dose (100mg/kg); bm = body mass and D= day

As expected the plasma level increased as the dose was increased, but the limited data does not however allow any further deductions on the possible dose vs. plasma level relationship for luteolin from the *A. afra* extract. What it however does is to allow one to conclude that constituents of the *A. afra* were absorbed by the rat given chronic oral doses of the aqueous extract and that luteolin was a reasonable marker for such bioavailability.

Finally because the chronic ingestion of *A. afra* at doses up to 1000mg/kg/day in the rat did not produce any significant adverse effects to which the luteolin levels could be correlated, no further comment can also be made on this aspect. Except to note that, for this study, the amount of luteolin in each daily dose i.e. 92.3 to 923 µg/kg was, of course, substantially lower than the 50mg/kg dose that had been reported as a toxic dose for luteolin in the literature (Kotanidou et al, 2002), which may help to explain the lack of toxicity of *A. afra.*

5.4 Conclusions

The objectives of this study were to determine the toxicity profile of chronic doses of *Artemisia afra* in rats and to assess whether the plasma levels of luteolin correlates with (a) the dose ingested and (b) any of the toxic effects observed after chronic ingestion of the plant extract. From the results obtained the following conclusions could be drawn:

- Chronic oral administration of *Artemisia afra* has a low toxicity potential with LD_{50} much higher than 1000 mg/kg. While minor intermittent diarrhea, salivation and partial hypo-activity were associated with the chronic ingestion of daily dose of up to 1000 mg/kg *A. afra* in rats, various growth, haematological and blood biochemical parameters, and organs of these animals were not altered by the plant extract.

- The chronic oral administration of *Artemisia afra* to rats gives very low and highly variable plasma levels of luteolin with little evidence of any accumulation over time. As expected the plasma level increased with the increase in the administered dose, but because of limited data no further deductions on the possible dose *versus* plasma level relationship for luteolin from the *A. afra* extract could be drawn. In addition, there appeared to be no correlation between the luteolin levels in the plant dose and any of the adverse effects, primarily because few of the latter were actually produced by the plant doses used.

- Chemical constituents of *A. afra* were well absorbed in rats given chronic oral doses of the aqueous extract of the plant and luteolin was a reasonable marker for such bioavailability.

Finally and overall, the results of the chronic study suggest that, at the chronic doses traditionally used by humans, whether in self-medication or not, the aqueous extract of *A. afra* should be relatively safe to use.

Chapter 6

CONCLUSIONS

The aim of this study was to investigate the possible toxicity of the flavonoid-containing plant *Artemisia afra* and, especially, to establish the safety of the aqueous extract of this plant by focusing on its acute and chronic toxicity in mice and rats, respectively. To realize this objective, it was decided to, firstly, determine the adverse effects that a dried aqueous extract *Artemisia afra* may cause in mice and rats; secondly, determine the LD_{50} for the aqueous extract of the plant administered acutely in mice using a traditional method (of Litchfield and Wilcoxon) and new computer program-based method (AOT425statPgm); thirdly, compare the potential doses (i.e. LD_{50}) of the plant causing adverse effects after oral and intra-peritoneal acute administration; fourthly, determine the general toxicity profile obtained with chronic oral dosing of rats with the extract; and, finally, assess whether the plasma levels of luteolin observed after chronic ingestion of the plant extract correlated with the equivalent dose ingested and/or any of the toxic effects in the rats. The primary hypotheses to be tested in this study were that the dose of the plant material at which adverse effects would occur depended on both the route of administration (i.p and p.o) used as well as the duration (acute or chronic) of exposure to the plant, that the new computer program-based method (i.e. AOT425statPgm) for testing acute toxicity of environmental pollutants, can advantageously be used to evaluate the acute toxicity of plant medicines and that plasma levels of luteolin could be used as a marker to monitor *A. afra* bioavailability (ingestion) and/or its toxicity.

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

1. *A. afra* appears to be practically non-toxic (LD_{50} = 8960 mg/kg) and slightly toxic (LD_{50} = 2450 mg/kg) when administered acutely to mice via the oral and intra-peritoneal routes, respectively.

2. Acute administration of *A. afra* to mice via the p.o and i.p routes induces the same symptoms in both sexes (viz. minor to severe hypo-activity, pilo-erection,

hyperventilation, loss of appetite, salivation, syncope, dizziness and convulsions), although, female mice may be more sensitive to acute intra-peritoneally administered *plant extract* than male mice.

3. The AOT425statPgm is an efficient tool and good alternative to the Litchfield and Wilcoxon method for determining the acute toxicity of a plant medicine such as *A. afra.* While giving the same symptom profile and LD_{50} results and having the same study time to conduct, this method uses substantially fewer mice.

4. The luteolin level in the freeze-dried aqueous extract of *A. afra* is not a good marker for determining the acute toxicity potential of this flavonoid-containing plant. Although the aqueous extract contains quantifiable levels of luteolin, these levels are substantially lower than that reported in the literature relating to luteolin toxicity.

5. When chronically administered to rats via the oral route, the aqueous extract of *A. afra* has a very low toxicity potential (i.e. LD_{50} higher than 1000 mg/kg). Chronic ingestion of the plant in doses up to 1000 mg/kg only produced adverse effects of minor intermittent diarrhea, salivation and partial hypo-activity and no alteration in the growth, haematological and blood biochemical parameters, and organs of these animals.

6. After chronic ingestion of the freeze-dried aqueous extract of *A. afra*, the levels of luteolin in the plasma of rats are very low and highly variable with little evidence of any accumulation over time. The levels increase with the increase in the administered dose, but there was no correlation between the levels and any adverse effects for doses up to 1000 mg/kg.

Collectively, the results indicate that acute doses of *A. afra* are relatively non-toxic in mice irrespective of the route of administration used, chronic doses of *A. afra* are very safe in the rat, that the AOT425statPgm is a potentially useful tool for the evaluation

of acute toxicity of plant medicines and, finally, that luteolin may be a good marker for *A. afra* bioavailability, but not likely for its toxicity.

In addition, to the above conclusions the results also raised the following concerns. Firstly, although the chronic oral doses of *A. afra* extract used in this study did not produce any significant adverse effects in rats with which the luteolin plasma levels could be correlated, further studies using higher doses of the plant may be needed to arrive at a final definitive conclusion on this question.

Secondly, to confirm the non-toxic nature of *A. afra*, the effect that various factors such as the growth stage and maturity of the plant, the specific parts of the plant (leaves, roots, bark, flowers, seeds, etc), seasonal variation, storage conditions, etc may have on its chemical composition (luteolin and other compounds) and consequently its toxicity may also need to be investigated.

Thirdly, of course studies to determine the effects of *A. afra* on the foetus in a pregnant animal, on the reproductive capacity of the animals, on the genetic system and to determine the ability of this plant to produce tumors (tumorigenicity and carcinogenicity tests) are further tests that need to be done to complete the profile of this plant medicine, although the findings of the present study would suggest that it should not have a very high potential to produce some of these effects.

Overall, the results of this study provide valuable preliminary data on the toxicity profile of *Artemisia afra* that should be useful for the planning of future pre-clinical and clinical studies of this plant medicine.

REFERENCES

Aherne, S.A. and O'Brien, N.M. (2002). Dietary flavonols: Chemistry, food content, and metabolism. *J. Nutrition*, **18**: 75-81.

Ankier, S.I. and Warrington, S.J. (1989). Research and development of new medicines. *The Journal of International Medical Research*, **17**, 407- 416

Association of Vermont Recyclers (1996). School solid waste reduction guide www.vtrecyclers.org/WASTE KIT/definitions.htm

Beecher, G.R. (2003). Overview of dietary flavonoids: Nomenclature, occurrence and intake. *J. Nutrition*, **133**: 3248S-3254S

Benavente-Garcia, O., Castillo, J., Marin, F.R., Ortuno, A., and Del Rio, J. A. (1997). Uses and properties of citrus flavonoids. *J. Agricultural and Food Chemistry*, **45**:4505-4515

Bidhe, R.M and Ghosh, S. (2004). Acute and sub chronic (28-Day) oral toxicity study in rats fed with novel surfactants. *AAPS Pharm Sci:* 6(2) article **14**: 1-10 http://www.aapspharmsci.org

Cai, Y., Luo, Q., Sun, M., Corke, H. (2004). Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *J. Life Sciences*, **74**: 2157-2184.

Chan, K. (2003). Some aspects of toxic contaminants in herbal medicines. *J. Chemosphere* **52**, 1361-1371.

China Great vista chemicals/ chemical manufacturer & supplier (2002). Herb Extracts: Luteolin. *Am. J. Respir Crit Care Med*. www.greatvistachemicals.com /herb extracts /luteolin.htm

Coetzee, C., Jefthas, E. and Reinten, E. (1999). Indigenous plant genetic resources of South Africa. *J. Janick (ed.), ASHS press*, Alexandria, VA.

Cristea, D., Bareau, I., Vilarem.(2003). Identification and quantitative HPLC analysis of the main flavonoids present in weld (*Reseda luteola L.*). *J. Dyes and Pigments* **57**, 267-272.

Dyson, A. (1998). Discovering indigenous healing plants of the herb and fragrance gardens at Kirstenbosch National Botanical Garden. Cape Town, *National Botanical Institute, the Printing Press*: 9-10

Erlund, I. (2002). Chemical analysis and pharmacokinetics of the flavonoids quercetin, hesperetin and naringenin in humans. Academic dissertation. Department of Applied chemistry and Microbiology, *University of Helsinki*. Helsinki.

Friedli, G-L. (2004). Friedli enterprises: http://www.friedli.com/herbs/phytochem/flavonoids.html

Gaillard, Y. and Pepin, G. (1999). Poisoning by plant material: review of human cases and analytical determination of main toxins by high-performance liquid chromatography- (tandem) mass spectrometry. *J. Chromatography*, **733**: 181-229.

Galati, G., Moridani, M.Y., Chan, T.S., O'Brien,P.J. (2001). Peroxidative metabolism of apigenin and naringenin versus luteolin and quercetin: Glutathione oxidation and conjugation. *Free radical Biology and Medicine*, **30**:370-382

Graph Pad software Inc. (2003). Prism 4 ™ version 4.00 for windows, Graph Pad software, California, U.S.A. www.graphpad.com

Guilhermino, L., Soares, A. M.V.M., Carvalho, A.P., and Lopes, M.C. (1998). Acute effects of 3, 4-Dichloroaniline on blood of male wistar rats. *J. Chemosphere* **37**:619-632

Harborne, J.B. (1973). Phytochemical methods: A guide to modern techniques of plant analysis. London, *Chapman and hall*: 1-74.

Harborne, J.B., Williams, C.A. (2000). Advances in flavonoid research since 1992. *J. Phytochemistry*, **55**: 481-504.

Harper, H.A. (1973). Review of physiological chemistry, 14th ed. California, *Lange medical publications*: 185-402

Harris, L. (2002). An evaluation of the bronchodilator properties of *Mentha longifolia* and *Artemisia afra*, traditional medicinal plants used in the Western Cape. M. Thesis, Discipline of pharmacology. School of pharmacy, *University of the Western Cape*. Bellville

Health & Safety (2004). *Glossary of health & safety terminology* www.delta.edu/slime/glossary.html

Hilaly, J. E., Israili, Z. H., Lyoussi, B. (2004). Acute and chronic toxicological studies of *Ajuga Iva* in experimental animals. *J. Ethno-pharmacology*, **91**:43-50

Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A. (1996). Zulu Medicinal plants: An inventory. South Africa, *University of Natal press*, Scottsville: 327

Iwu, M.M. (1993). Handbook of African Medicinal plants. USA, Florida, *CRC Press*: 121-122.

Kaplan, A., Jack, R., Opheim, K.E., Toivola, B., and Lyon, A.W. (1995). Clinical Chemistry interpretation and techniques. 4th ed. USA, *Williams & Wilkins*: 155-333.

King, M. (1973). A medical laboratory for developing countries. London, Oxford *University press*: 7.1 - 7.30

Komperlla, M.K. (2005). The formulation and evaluation of rapid release Tablets manufactured from *Artemisia afra* plant material. M. Thesis, Discipline of pharmaceutics. School of pharmacy, *University of the Western Cape*. Bellville

Kotanidou, A., Xagorari, A., Bagli, E., Kitsanta, P., Fotsis, T., Papapetropoulos, A., Roussos, C. (2002). Luteolin reduces lipopolysaccharide-induced lethal toxicity and expression of proinflammatory molecules in mice. *Am. J. Respir. Crit Care Med*, **165**: 818-823

Li, L., Jiang, H., Wu, H., Zeng, S. (2005). Simultaneous determination of luteolin and apigenin in dog plasma by RP-HPLC. *J. Pharmaceutical and biomedical analysis*, **37**: 615-620.

Litchfield, J.T. J.R. and Wilcoxon, F. (1949). A simplified method of evaluating dose-effect experiments. *J Pharmacology and Experimental therapeutics,* **96**: 99-113.

Loomis, T. A. and Hayes, A.W. (1996). Loomis's essentials of toxicology. 4th ed., California, *Academic press*: 208- 245

Markham, K.R. (1982). Techniques of flavonoid identification. London, *Academic press*: 1-60.

Mdhluli, M. (2003). Toxicological and antifertility investigations of oleanolic acid in male vervet monkeys (*Chlorocebus aethiops*). PhD Thesis, Discipline of physiological sciences, *University of the Western Cape*. Bellville.

Medicines and Healthcare products regulatory Agency (2004). Safety and quality of traditional Chinese medicines: Questions and answers. *Herbal safety news*. UK. www.mhra.gov.uk.

Medicines Control Agency (MCA-UK) (2002). Safety of herbal medicinal products. *Herbal safety news*. UK. www.mhra.gov.uk.

Miean, K. H. and Mohamed, S. (2001). Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants. *J. Agric. Food Chem,* **49**:3106-3112.

Miller, S.A. and Harley, J.P. (1996). Zoology, 3rd edition. *Wm.C. Brown Publishers*, USA.

Mino, J., Moscatelli, V., Hnatyszyn, O., Gorzalczany, S., Acevedo, C., and Ferraro, G. (2004). Antinociceptive and anti-inflammatory activities of *Artemisia copa* extracts. Academic press. *J. Pharmacological research,* **50**: 59-63

Moridani, M.Y., Galati, G., O'Brien, P.J. (2002). Comparative quantitative structure toxicity relationships for flavonoids evaluated in isolated rat hepatocytes and Hela tumor cells. *J. Chemico-biological interactions*, **139**: 251-264

MRC & SA Healthinfo (2004). Traditional medicines database: www.mrc.ac.za/ Tramed3/Tramed3PlantPharmacologyDetails

Muganga, R. (2005). Luteolin levels in selected folkloric preparations and the bioavailability of luteolin from *Artemisia afra* aqueous extract in the vervet monkey. M. Thesis, Discipline of pharmacology. School of pharmacy, *University of the Western Cape*. Bellville.

Nuutila, A.M., Kammiovirta, K-M., Oksman-Caldentey, K.-M. (2002). Comparison of methods for the hydrolysis of flavonoids and phenolic acids from onion and spinach for HPLC analysis. *J. Food Chemistry*, **76**: 519-525

Pappas, R. and Sheppard- Hanger, S. (2004). *Artemisia arborescens*-essential oil of the Pacific Northwest: a high-chamazulene, low-thujone essential oil with potential skin- care applications. *Atlantic institute*, Reference manual www.atlanticinstitute.com/artemisia.pdf

PathCare (2005). Laboratory manual. Bellville, South Africa

Pascoe, D. (1983). Toxicology. England, London, *Edward Arnold limited*.1-60.

Poole, A., Leslie, G.B .(1989) A practical approach to toxicological investigations. 1st ed. Great Britain. *Cambridge University press*: 2, 30-117

Rahman, M.F., Siddiqui, M.K., Jamil, K. (2001). Effects of Vepacide (*Azadirachta indica*) on aspartate and alanine aminotransferase profiles in a sub chronic study with rats. *J. Human and Experimental Toxicology,* **20**: 243-249

Raskin, I., Ribnicky,D.M., Komarnytsky, S., Ilic, N., Poulev,A., Borisjuk, N., Brinker, A., Moreno, D.A., Ripoll,C., Yakoby, N. et al. (2002). Plants and human health in the twenty-first century. *Trends in biotechnology*, **20** N0.12: 522-531

Roberts, M. (1990). Indigenous healing plants. South Africa. *Southern Book*: 226-228

SA Healthinfo (1999). UWC Pharmacopoeia monograph project; *Artemisia Afra* herba: http://www.sahealthinfo.org/traditionalmeds/monographs/artemisia.htm

Shimoi, K., Okada, H., Furugori, M., Goda, T., Takase, S., Suzuki, M., Hara, Y., Yamamoto, H., Kinae, N. (1998). Intestinal absorption of luteolin and luteolin 7-*O*-ß-glucoside in rats and humans. *FEBS Letters*, **438**: 220-224

Shimoi, K., Saka, N., Nozawa, R., Sato, M., Amano, I., Nakayama, T., Kinae, N. (2001). Deglucuronidation of a flavonoid, luteolin monoglucuronide, during inflammation. *American Society for Pharmacology and Experimental Therapeutics*, **29**:1521-1524

Skibola, C.F. and Smith, M.T. (2000). Potential health impacts of excessive flavonoid intake. *Free radical biology & medicine,* **29**: 375-383.

Sofowora, A. (1982). Medicinal plants and traditional medicine in Africa New York, *John Wiley and sons*: 1-7; 67-105.

Springfield, E.P., Eagles, P.K.F. and Scott, G. (2005). Quality assessment of South African herbal medicines by means of HPLC fingerprinting. *J. Ethno-pharmacology*, **3706**: 1-9

Steenkamp, V. (2003). Traditional herbal remedies used by South African Women for Gynaecological complaints. *J. Ethno-pharmacology*, **86**: 97-108

Tabuti, J.R.S., Lye, K.A., Dhillion, S.S. (2003). Traditional herbal drugs of Bulamogi, Uganda: plants, use and administration. *J. Ethno-pharmacology*, **88**:19-44

Tang, H.Q., Hu, J., Yang, L., Tan, R.X. (2000). Terpenoids and flavonoids from *Artemisia* species. *Planta medica*, **66**:391-393.

Tanira, M.O.M., Ali, B.H., Bashir, A.K. and Chandranath, I. (1996). Some pharmacologic and toxicologic studies on *Rhazya stricta* Decne in rats, mice and rabbits. *Gen. Pharmac*, **27**: 1261-1267

Timbrell, J. (2002). Introduction to toxicology. 3rd ed., London, *Taylor & Francis*: 163-179.

Tomlinson, T.R. and Akerele, O. (1998). Medicinal plants their role in health and biodiversity. Philadelphia, *University of Pennsylvania press*: 29-40

Tshikalange, T.E., Meyer, J.J.M., Hussein, A.A. (2005). Antimicrobial activity, toxicity and the isolation of a bioactive compound from plants used to treat sexually transmitted diseases. *J. Ethno-pharmacology*, **96**:515-519.

Ullman-Cullere, M.H. and Foltz, C.J. (1999). Body condition scoring: A rapid and accurate method for assessing health status in mice. *The American association for laboratory animal Science*: 319-323

US Environmental Protection Agency (USEPA) (2003). OECD guideline for testing of chemicals: Acute oral toxicity (Guideline 425) statistical program (AOT425statPgm) http://www.epa.gov/oppfead1/harmonization.

Van Wyk, B- E., Van, O.B. and Gericke, N. (2000). Medicinal plants of South Africa. 2nd ed. *Tien Wah Press*, Singapore: 44

Van Wyk, A., Mugabo, P., Syce, J. (2003). A pilot study on mild to moderate Asthmatics subjects to test the bronchodilatory effect of the herbal plant *Artemisia afra*. Clinical study protocol. *University of the Western Cape*. Bellville

Waithaka, J. (2004). The evaluation of markers for quality control studies of flavonoid-containing medicinal preparations. M. Thesis, Discipline of pharmacology. School of pharmacy, *University of the Western Cape*. Bellville

Watt, J.M. and Breyer-Brandwijk, M.G. (1962). The medicinal and poisonous plants of Southern and Eastern Africa. 2nd ed. London, *Livingstone*: 199-202.

Waynforth, H. B. (1980). Experimental and surgical technique in the rat. London, *Academic press*: 17-68

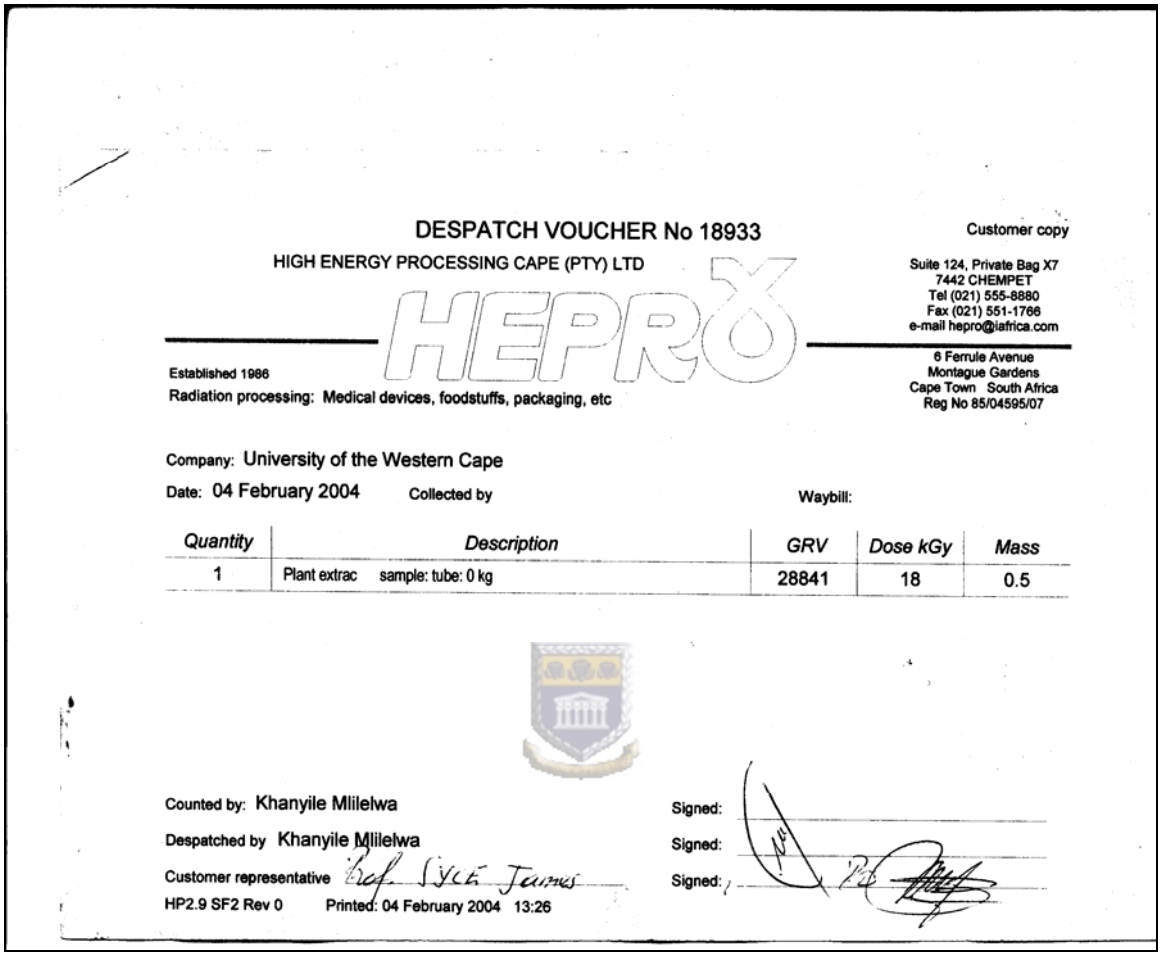
Williamson, E.M., Okpado, D.T., Evans, F.J. (1996). Selection, preparation and pharmacological evaluation of plant material. England, *John Wiley& sons*: 1-25

Wolf, P.L., Williams, D., Tsudaka, T., and Acosta, L. (1972). Methods and Techniques in clinical chemistry. USA, *John Wiley & Sons*: 132-196, 375-383

Wu, T-S., Tsang, Z-J., Wu, P-L., Lin, F-W., Li, C-Y., Teng, C-M., and Lee, K-H. (2001). New constituents and antiplatelet aggregation and anti-HIV principles of *Artemisia capillaries. J. Bioorganic & Medicinal chemistry*, **9**: 77-83

APPENDICES

Appendix I: **Certificate of irradiation of *A. afra* aqueous extract from HEPRO (PTY)**



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Reg No 85/04595/07

Company: University of the Western Cape

Date: 04 February 2004 Collected by Waybill:

Quantity	Description	GRV	Dose kGy	Mass
1	Plant extrac sample: tube: 0 kg	28841	18	0.5

Counted by: Khanyile Mlilelwa

Despatched by Khanyile Mlilelwa

Customer representative Prof Sylvia James

HP2.9 SF2 Rev 0 Printed: 04 February 2004 13:26

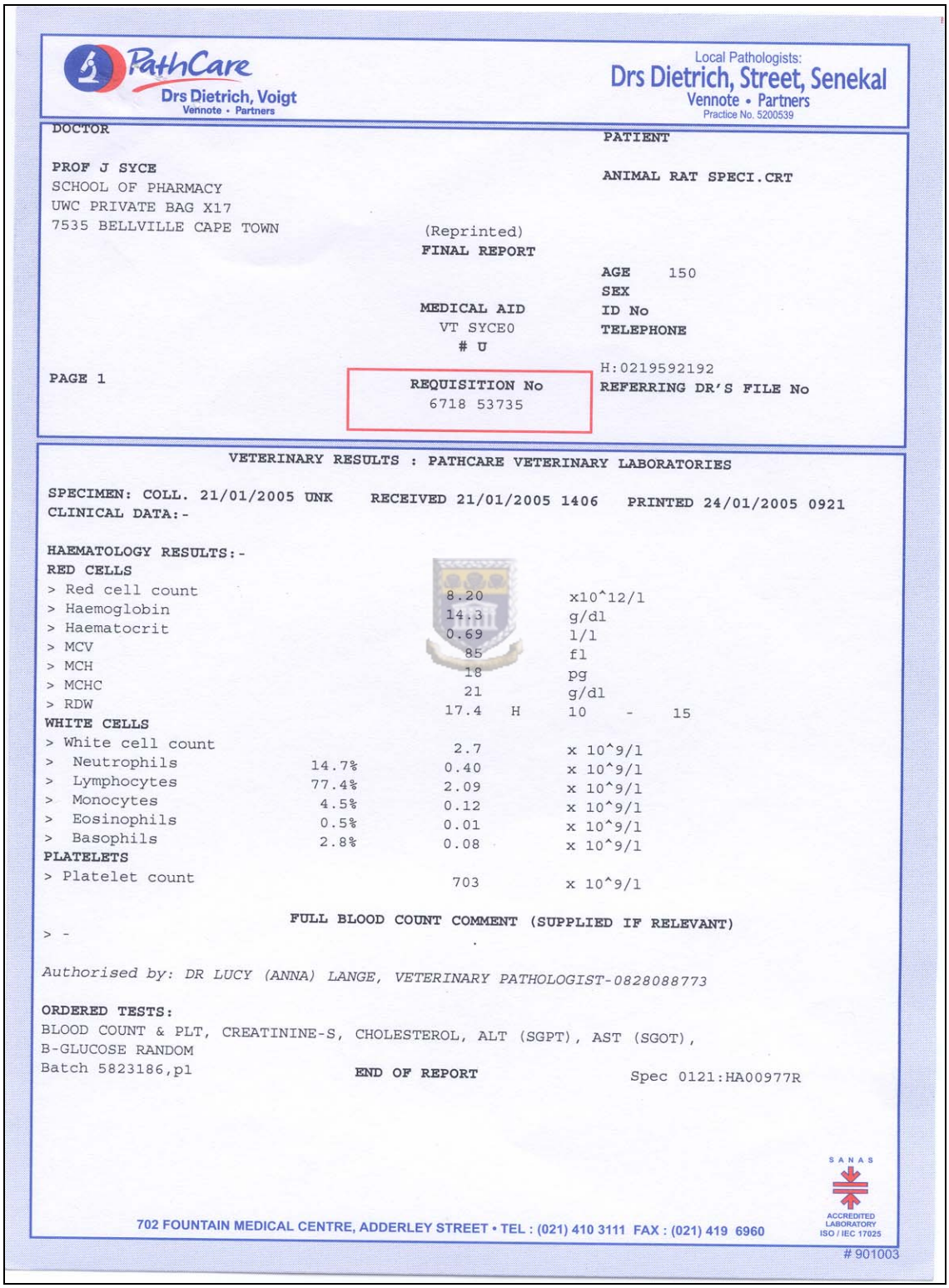
Signed:

Signed:

Signed:

Appendix II

Sample of haematological test report of rats' blood from PathCare
(After chronic oral administration of *A. afra*)



PathCare
Drs Dietrich, Voigt
Vennote • Partners

Local Pathologists:
Drs Dietrich, Street, Senekal
Vennote • Partners
Practice No. 5200539

DOCTOR

PROF J SYCE
SCHOOL OF PHARMACY
UWC PRIVATE BAG X17
7535 BELLVILLE CAPE TOWN

(Reprinted)
FINAL REPORT

PATIENT

ANIMAL RAT SPECI.CRT

AGE 150
SEX
ID No
TELEPHONE

MEDICAL AID
VT SYCE0
U

H:0219592192

PAGE 1

REQUISITION No
6718 53735

REFERRING DR'S FILE No

VETERINARY RESULTS : PATHCARE VETERINARY LABORATORIES

SPECIMEN: COLL. 21/01/2005 UNK RECEIVED 21/01/2005 1406 PRINTED 24/01/2005 0921
CLINICAL DATA:-

HAEMATOLOGY RESULTS:-

RED CELLS				
> Red cell count		8.20		x10^12/l
> Haemoglobin		14.3		g/dl
> Haematocrit		0.69		l/l
> MCV		85		fl
> MCH		18		pg
> MCHC		21		g/dl
> RDW		17.4	H	10 - 15
WHITE CELLS				
> White cell count		2.7		x 10^9/l
> Neutrophils	14.7%	0.40		x 10^9/l
> Lymphocytes	77.4%	2.09		x 10^9/l
> Monocytes	4.5%	0.12		x 10^9/l
> Eosinophils	0.5%	0.01		x 10^9/l
> Basophils	2.8%	0.08		x 10^9/l
PLATELETS				
> Platelet count		703		x 10^9/l

FULL BLOOD COUNT COMMENT (SUPPLIED IF RELEVANT)

> -

Authorised by: DR LUCY (ANNA) LANGE, VETERINARY PATHOLOGIST-0828088773

ORDERED TESTS:
BLOOD COUNT & PLT, CREATININE-S, CHOLESTEROL, ALT (SGPT), AST (SGOT),
B-GLUCOSE RANDOM
Batch 5823186,p1

END OF REPORT

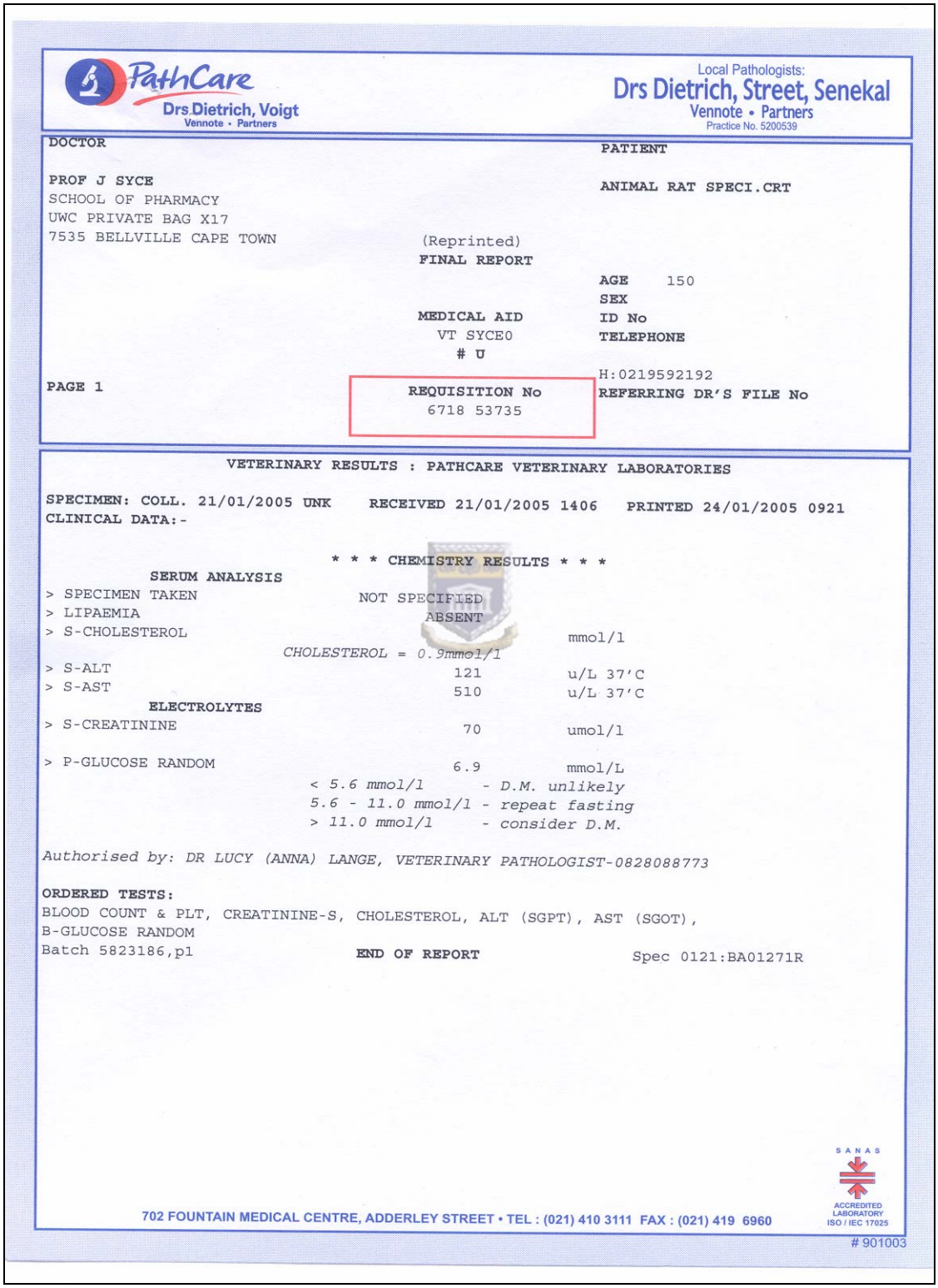
Spec 0121:HA00977R

SANAS
ACCREDITED LABORATORY
ISO / IEC 17025
901003

702 FOUNTAIN MEDICAL CENTRE, ADDERLEY STREET • TEL : (021) 410 3111 FAX : (021) 419 6960

Appendix III

Sample of biochemical test report of rats' blood from PathCare
(After chronic oral administration of *A. afra*)



PathCare
Drs Dietrich, Voigt
Vennote • Partners

Local Pathologists:
Drs Dietrich, Street, Senekal
Vennote • Partners
Practice No. 5200539

DOCTOR

PROF J SYCE
SCHOOL OF PHARMACY
UWC PRIVATE BAG X17
7535 BELLVILLE CAPE TOWN

(Reprinted)
FINAL REPORT

MEDICAL AID
VT SYCE0
U

PAGE 1

REQUISITION No
6718 53735

PATIENT

ANIMAL RAT SPECI.CRT

AGE 150
SEX
ID No
TELEPHONE

H:0219592192
REFERRING DR'S FILE No

VETERINARY RESULTS : PATHCARE VETERINARY LABORATORIES

SPECIMEN: COLL. 21/01/2005 UNK RECEIVED 21/01/2005 1406 PRINTED 24/01/2005 0921
CLINICAL DATA:-

* * * CHEMISTRY RESULTS * * *

SERUM ANALYSIS		
> SPECIMEN TAKEN	NOT SPECIFIED	
> LIPAEMIA	ABSENT	
> S-CHOLESTEROL		mmol/l
CHOLESTEROL = 0.9mmol/l		
> S-ALT	121	u/L 37'C
> S-AST	510	u/L 37'C
ELECTROLYTES		
> S-CREATININE	70	umol/l
> P-GLUCOSE RANDOM	6.9	mmol/L

< 5.6 mmol/l - D.M. unlikely
5.6 - 11.0 mmol/l - repeat fasting
> 11.0 mmol/l - consider D.M.

Authorised by: DR LUCY (ANNA) LANGE, VETERINARY PATHOLOGIST-0828088773

ORDERED TESTS:
BLOOD COUNT & PLT, CREATININE-S, CHOLESTEROL, ALT (SGPT), AST (SGOT),
B-GLUCOSE RANDOM
Batch 5823186,p1

END OF REPORT

Spec 0121:BA01271R

SANAS
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901003

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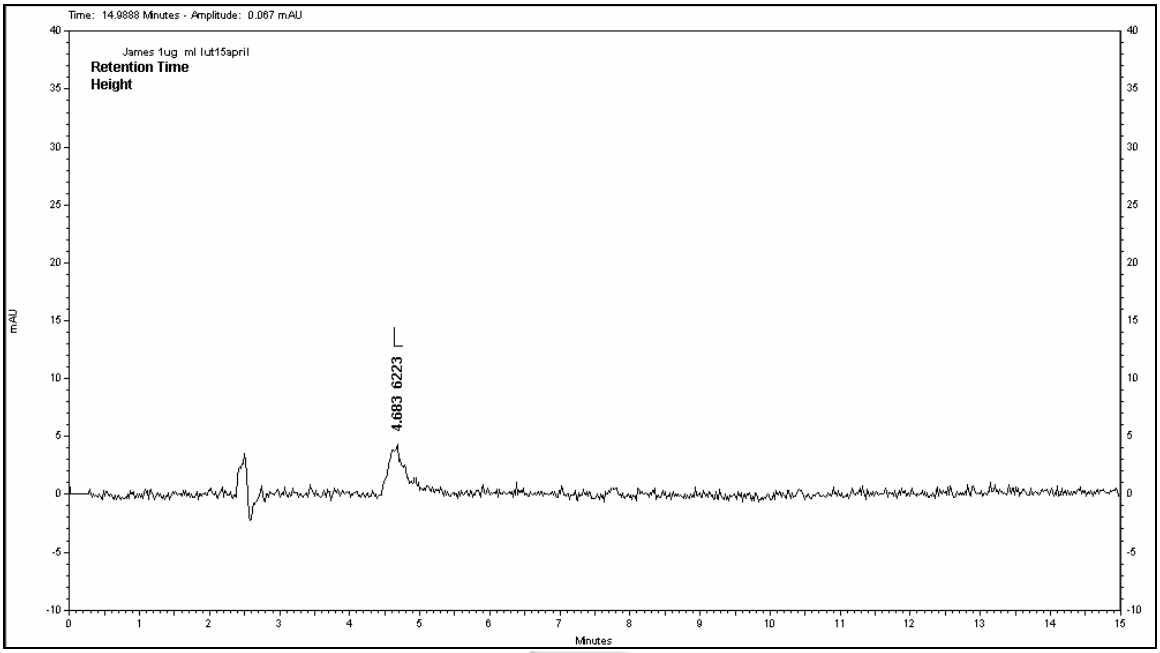
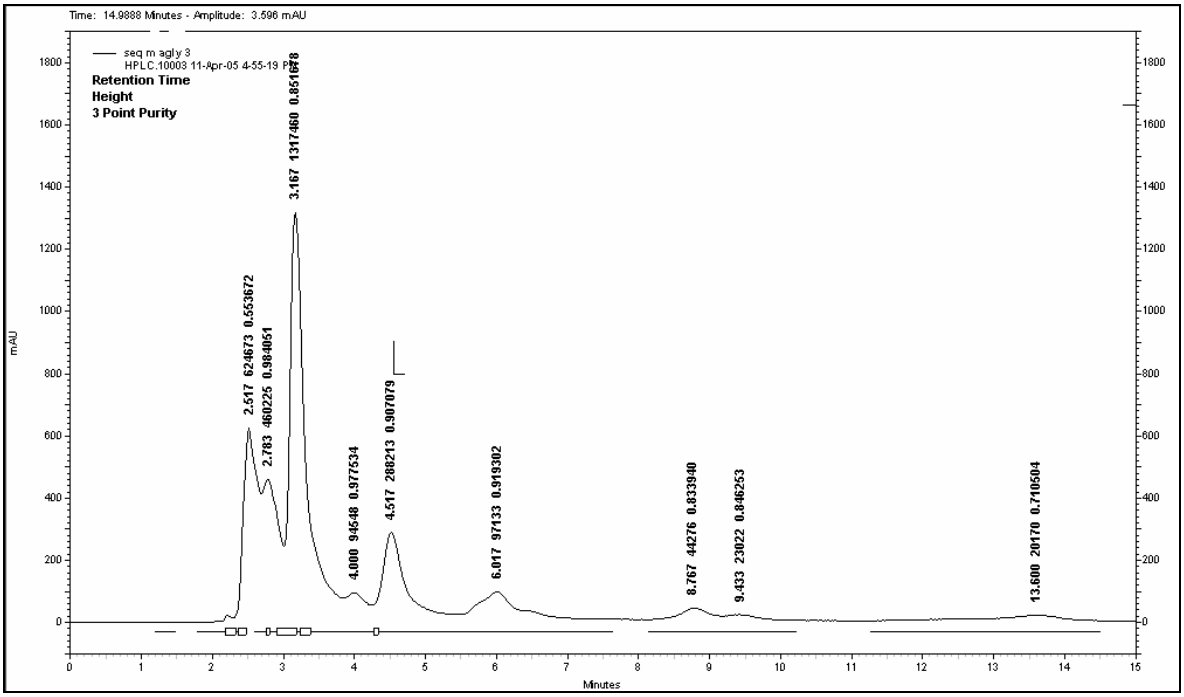
Appendix IV

Appendix IV.1: Representative HPLC chromatogram of the lowest level of luteolin quantifiable (LLQ: peak L)

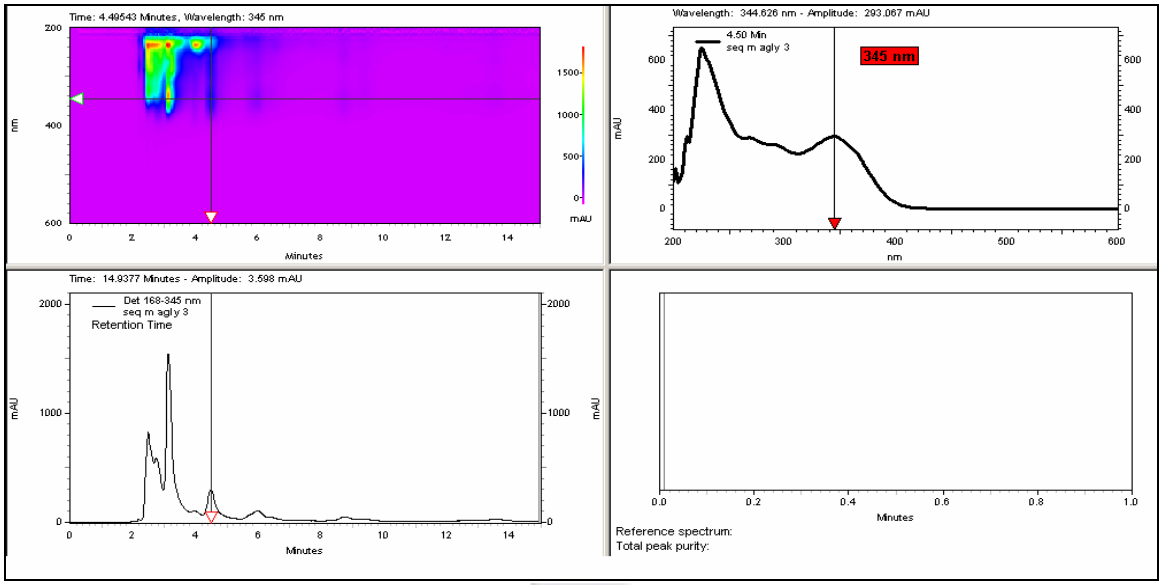


APPENDIX IV.2: Representative HPLC chromatogram of luteolin in the un-hydrolyzed plant material. The straight line under peaks indicates their purity

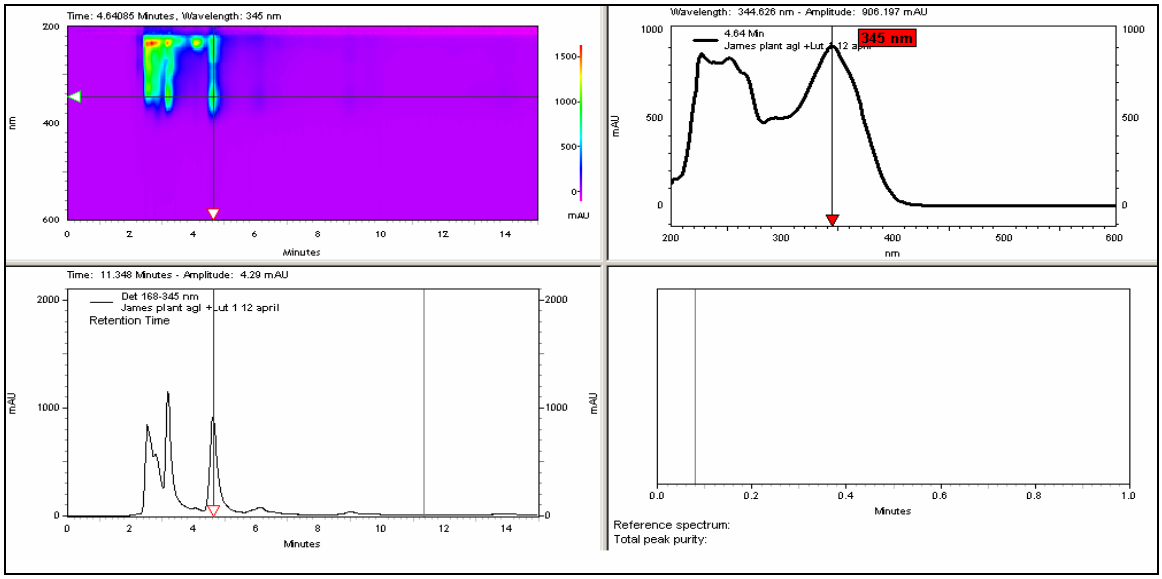


Appendix V

Appendix V.1: Representative UV spectrum of luteolin, in un-hydrolyzed plant extract, obtained using diode array UV detector. The sections of this Figure are described as shown in section 4.3. 3.1.



Appendix V.2: Representative UV spectrum of luteolin, in un-hydrolyzed plant extract spiked with the standard, obtained using diode array UV detector. The sections of this Figure are described as shown in section 4.3.3.1.



Appendix VI

Print out of results of acute toxicity test of *A. afra* according to the "AOT425 statistical program"

AOT425statPgm (Version: 1.0) Test Results and Recommendations
Acute Oral Toxicity (OECD Test Guideline 425) Statistical Program

1. Test/Substance***: Acute Toxicity of Artemisia afra after intraperitoneal injection in female mice***

Test type: Main Test
Limit dose (mg/kg): 5000
Assumed LD50 (mg/kg): Default
Assumed sigma (mg/kg): 0.5

Recommended dose progression: **5000, 1750, 550, 175**, 55, 17.5, 5.5, 1.75

DATA:

Test Seq.	Animal ID	Dose (mg/kg)	Short-term Result	Long-term Result
1	1	175	O	O
2	2	550	O	O
3	3	1750	O	O
4	4	5000	X	X
5	5	1750	O	O
6	6	5000	X	X
7	7	1750	X	X
8	8	550	O	O
9	9	1750	O	O
10	10	5000	X	X

(X = Died, O = Survived)

Dose Recommendation: The main test is complete.

Stopping criteria met: LR criterion.

SUMMARY OF LONG-TERM RESULTS:

Dose	O	X	Total
175	1	0	1
550	2	0	2
1750	3	1	4
5000	0	3	3
All Doses	6	4	10

Statistical Estimate based on long term outcomes:

Estimated **LD50 = 1750 mg/kg** (The one dose with partial response).
95% PL Confidence interval is 1239 to 4450.
99% PL Confidence interval is 778.6 to 12800

2. Test/Substance: ***Acute Toxicity of Artemisia Afra after intraperitoneal injection in male mice***

Test type: Main Test
Limit dose (mg/kg): 5000
Assumed LD50 (mg/kg): Default
Assumed sigma (mg/kg): 0.5

Recommended dose progression: **5000, 1750, 550, 175**, 55, 17.5, 5.5, 1.75

DATA:

Test Seq.	Animal ID	Dose (mg/kg)	Short-term Result	Long-term Result
1	1	175	O	O
2	2	550	O	O
3	3	1750	O	O
4	4	5000	X	X
5	5	1750	O	O
6	6	5000	X	X
7	7	1750	O	O
8	8	5000	X	X

(X = Died, O = Survived)

Dose Recommendation: The main test is complete.

Stopping criteria met: 5 reversals in 6 tests. LR criterion

SUMMARY OF LONG-TERM RESULTS:

Dose	O	X	Total
175	1	0	1
550	1	0	1
1750	3	0	3
5000	0	3	3
All Doses	5	3	8

Statistical Estimate based on long term outcomes:

Estimated **LD50 = 3129 mg/kg** (Based on an assumed sigma of 0.5).
Approximate 95% confidence interval is 1750 to 5000.
Approximate 99% confidence interval is 1750 to 5000

3. Test/Substance***: Acute Toxicity of Artemisia afra after oral administration to female mice***
Test type: Main Test
Limit dose (mg/kg): 5000
Assumed LD50 (mg/kg): Default
Assumed sigma (mg/kg): 0.5

Recommended dose progression: **5000, 1750, 550, 175**, 55, 17.5, 5.5, 1.75

DATA:

Test Seq.	Animal ID	Dose (mg/kg)	Short-term Result	Long-term Result
1	1	175	O	O
2	2	550	O	O
3	3	1750	O	O
4	4	5000	O	O
5	5	5000	O	O
6	6	5000	O	O

(X = Died, O = Survived)

Dose Recommendation: The main test is complete.

Stopping criteria met: 3 at Limit Dose.

SUMMARY OF LONG-TERM RESULTS:

Dose	O	X	Total
175	1	0	1
550	1	0	1
1750	1	0	1
5000	3	0	3
All Doses	6	0	6

Statistical Estimate based on long term outcomes:

The **LD50** is great than **5000 mg/ kg**.

4. Test/Substance***: Acute Toxicity of Artemisia afra after oral administration to male mice***

Test type: Main Test
Limit dose (mg/kg): 5000
Assumed LD50 (mg/kg): Default
Assumed sigma (mg/kg): 0.5

Recommended dose progression: **5000, 1750, 550, 175**, 55, 17.5, 5.5, 1.75

DATA:

Test Seq.	Animal ID	Dose (mg/kg)	Short-term Result	Long-term Result
1	1	175	O	O
2	2	550	O	O
3	3	1750	O	O
4	4	5000	O	O
5	5	5000	O	O
6	6	5000	O	O

(X = Died, O = Survived)

Dose Recommendation: The main test is complete.

Stopping criteria met: 3 at Limit Dose.

SUMMARY OF LONG-TERM RESULTS:

Dose	O	X	Total
175	1	0	1
550	1	0	1
1750	1	0	1
5000	3	0	3
All Doses	6	0	6

Statistical Estimate based on long term outcomes:

The **LD50** is great than **5000 mg/ kg**.

Appendix VII

Estimation of the average dose of *A. afra* usually taken by humans.

Roberts (1990) in "Indigenous Healing Plants" said that the usual preparation of *Artemisia afra* is in the form of a tea or decoction (1/4 cup of fresh leaves to 1 cup of boiling water).

That means **1 cup of fresh leaves to 4 cups of water.**

1cup of water ≈ **230 ml**

Dried material / wet (fresh) material = **48.2%**

1 cup dried material = 16.8g that means, 1cup of wet (fresh) material= 16.8/0.482= 34.8g

This is to say **34.8 g** of wet material in **920 ml** of water **(≈ 35 g wet material in 1liter of water).**

The **dried extract powder yield ≈ 10%** of wet (fresh) leaves.

➔ 34.8g/100 x 10 = **3.5 g of dried extract powder in 1 cup of fresh leaves.**

We can thus say that the daily dose of *A. afra* in human = *3.5* g of dried extract powder. Assuming that the average bodyweight of a human is 70kg, the dose per kg body weight will then be: 3500/70 = **50 mg/kg bodyweight**.

Table 1: Summary of doses given, number rats per group and numbers of rats sacrificed at various times in the *A. afra* chronic toxicity study.

Dose per group (mg/kg)	Number of rats per dose group	Number of rats sacrificed at times (day)	
		D0	D90
0 100 1000	8 8 14	2 2 2	6 6 12
TOTAL	30	6	24
Used for		Blood analyses + HPLC	Blood analyses + HPLC

1. 100 mg/kg = double of the estimated dose taken by humans
2. 1000 mg/kg = 10% of the LD_{50} obtained after acute p.o administration of *A. afra* using the Litchfield and Wilcoxon method.

Appendix VIII

Results of the haematological analyses of rats' blood collected before and 92 or 93 days after p.o. administration of *A. afra* extract

A. Haematocrit

Table: 1

Animal	Control at D0	Control at 92	LD at D92	HD at D92	HD at D93
1	0,67	0,74	0,67	0,91	0,73
2	0,62	0,73	0,70	0,66	0,72
3	0,58	0,62	0,76	0,73	0,67
4	0,59	0,57	0,65	0,72	0,79
5	0,61	0,69	0,73	0,69	0,66
6	0,70	0,73	0,66	0,71	0,70
Mean	0,63	0,68	0,70	0,74	0,71
Std. Deviation	0,047	0,070	0,043	0,088	0,047

B. Haemoglobin

Table: 2

Animal	Control at D0	Control at 92	LD at D92	HD at D92	HD at D93
1	14,5	15,2	14,5	18,4	15,5
2	13,3	14,9	14,9	13,9	15,2
3	12,7	13,6	15,6	15,4	14,4
4	15,3	15,5	13,8	15,1	16,2
5	13,1	14,1	14,9	14,6	13,9
6	15,1	15,1	13,9	14,9	14,4
Mean	14,00	14,73	14,60	15,38	14,93
Std. Deviation	1,1	0,73	0,68	1,6	0,85

C. Platelet

Table: 3

Animal	Control at D0	Control at 92	LD at D92	HD at D92	HD at D93
1	871	222	727	609	858
2	237	603	147	435	749
3	110	288	163	150	1057
4	459	944	103	438	844
5	291	474	661	540	800
6	972	284	782	171	142
Mean	490	469	431	391	742
Std. Deviation	354	273	324	190	312

D. Red blood cells

Table: 4

Animal	Control at D0	Control at 92	LD at D92	HD at D92	HD at D93
1	7,75	8,43	8,22	10,60	9,11
2	7,09	8,53	8,60	8,04	8,55
3	6,43	7,68	9,01	9,11	8,08
4	6,49	6,87	8,02	8,68	9,09
5	6,97	7,58	8,57	8,28	8,51
6	8,16	8,55	7,60	8,70	8,35
Mean	7,1	7,9	8,3	8,9	8,6
Std. Deviation	0,69	0,68	0,50	0,91	0,41

E. White blood cells

Table: 5

Animal	Control at D0	Control at 92	LD at D92	HD at D92	HD at D93
1	3,6	3,6	2,8	2,4	1,9
2	6,2	5,5	3,2	2,4	2,5
3	3,1	2,1	2,3	3,5	5,5
4	6,8	3,2	2,9	6,4	5,4
5	1,5	3,5	2,3	3,1	2,6
6	6,3	5,1	3,5	3,0	2,4
Mean	4,6	3,8	2,8	3,5	3,4
Std. Deviation	2,2	1,3	0,48	1,5	1,6

Appendix IX

Results of the biochemical analyses of rats' blood collected before and 92 or 93 days after p.o. administration of *A. afra* extract.

A. ALT (serum)

Table: 6

Animal	Control at D0	Control at 92	LD at D92	HD at D92	HD at D93
1	45	-	145	77	128
2	111	148	160	64	102
3	45	143	88	107	132
4	102	121	132	78	165
5	126	72	-	37	124
6	54	90	55	170	66
Mean	80,50	114,8	116,0	88,83	119,5
Std. Deviation	36,57	33,10	43,41	45,78	33,13

B. AST (serum)

Table: 7

Animal	Control at D0	Control at 92	LD at D92	HD at D92	HD at D93
1	151	1239	768	287	628
2	537	712	949	255	465
3	158	595	540	463	635
4	614	751	566	342	717
5	477	382	557	159	637
6	256	674	298	664	664
Mean	365,5	725,5	613,0	361,7	624,3
Std. Deviation	202,3	283,6	222,2	178,9	84,66

C. Cholesterol (serum)

Table: 8

Animal	Control at D0	Control at 92	LD at D92	HD at D92	HD at D93
1	0,9	1,4	1,1	1,0	1,1
2	2,0	0,9	1,5	1,0	1,3
3	1,1	1,1	1,1	1,1	1,1
4	2,0	1,1	1,2	1,4	1,1
5	0,8	1,1	1,2	0,9	0,9
6	1,2	1,4	0,9	1,3	1,3
Mean	1,3	1,2	1,2	1,1	1,1
Std. Deviation	0,54	0,20	0,20	0,19	0,15

D. Creatinine (serum)

Table: 9

Animal	Control at D0	Control at 92	LD at D92	HD at D92	HD at D93
1	61	59	70	97	61
2	62	69	60	62	62
3	55	63	68	60	75
4	80	51	63	40	81
5	44	63	59	54	72
6	47	66	66	47	84
Mean	58	62	64	60	73
Std. Deviation	13	6,3	4,4	20	9,5

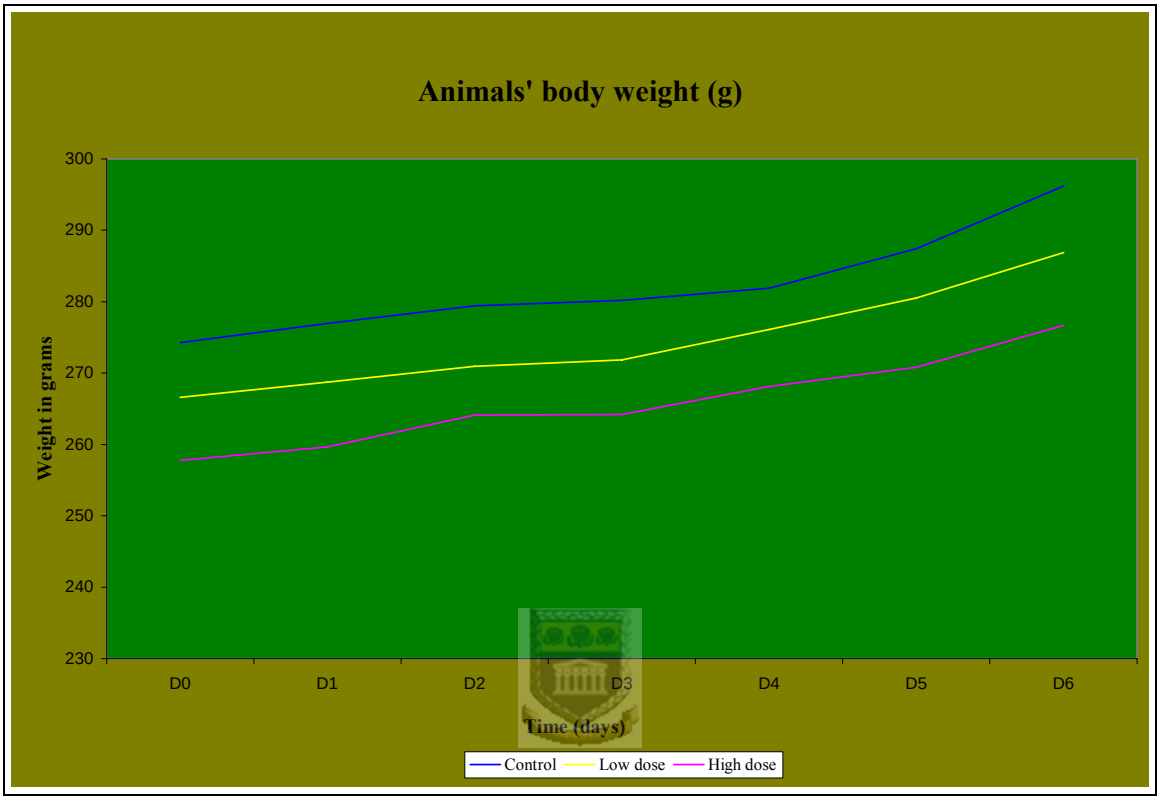
E. Glucose random (plasma)

Table: 10

Animal	Control at D0	Control at 92	LD at D92	HD at D92	HD at D93
1	9,4	6,1	6,9	4,5	6,9
2	8,0	7,3	6,0	7,1	6,3
3	7,5	6,3	6,9	6,8	7,0
4	6,9	6,5	6,5	6,4	7,0
5	8,6	8,3	6,6	5,7	7,2
6	10,4	7,0	6,9	5,9	6,5
Mean	8,5	6,9	6,6	6,1	6,8
Std. Deviation	1,3	0,81	0,36	0,93	0,34

Appendix X

Fig. 1: Growth of rats during 7-day period of oral administration of *Artemisia afra* extract.



Appendix XI

Figures: Experimental animals and preparation of plant extract.

Fig. 1: Male Wistar rats

Fig. 2: Balb/C mice

Fig. 3: Preparation of plant extract.

Brief introduction of the author

Mr. James Tshikosa Mukinda was born on 22nd October 1972 in Kolwezi, DR Congo. He completed high school education in July1993 in "Mathematics-Physics" at the 23rd Jean College in kolwezi. He enrolled at the University of Lubumbashi in October 1995 and obtained his first Degree in Agronomy in 1998. In 2000, he obtained the Honours Degree (Agricultural Engineer Degree) in Agronomy. From 2001-2002, he worked as researcher at the Centre of research on maize (CRM) in DR Congo.

In March 2004, he registered for his Masters Degree program in the School of Pharmacy at the University of the Western Cape.