An assessment of *Carpobrotus acinaciformis* and *Cissampelos capensis* as potential antimicrobial agents and their effects on animal metabolism

By

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An assessment of *Carpobrotus acinaciformis* and *Cissampelos capensis* as potential antimicrobial agents and their effects on animal metabolism

Keywords

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STATEMENT

I declare that "An assessment of *Carpobrotus acinaciformis* and *Cissampelos capensis* as potential antimicrobial agents and their affects on animal metabolism" is my own work, only submitted to the University of the Western Cape, with all research resources used in this project duly acknowledge by means of complete references.

Andrea Bowie
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Chapter 1: OVERVIEW

Medicinal plant history

The definition of a medicinal plant (as formulated by the WHO) is a plant that consists of either one or more plant parts with therapeutic properties that can be used in the development of drugs (Sofowora 1982). Various medicinal plants have been used over the years for their healing properties, and it can even be said that this is an ancient practice (Cowan 1999). Plant-derived medicines have been part of traditional healthcare in most parts of the world for thousands of years (Palombo & Semble 2001; Salie 1998)). Presently, approximately 75% of the world population make use of plants and its extracts (Salie 1998; Abelson 1990). Despite this widespread use of medicinal plants, only a fraction has been tested. Many plant-derived natural products must be investigated for their pharmacological activity (Mendelson & Balick 1998; Gentry 1993). Hence it is of utmost importance to study medicinal plants for safety and efficacy (Eloff 1998), since there is still an infinite number that must be studied and discovered (Abelson 1990; Sofowora 1982).

Hippocratus (in the late fifth century B.C.) was a physician who strongly believed in herbal remedies to treat many ailments and recorded the use of approximately 300 to 400 medicinal plants (Cowan 1999; Sofowora 1982; Schultes 1978). It is also believed that the Egyptians (1500 B.C) made use of medicinal plants as some records excavated from ancient Egyptian tombs stated (Sofowora 1982). The *De Materia Medica*, a medicinal plant catalogue of about 600 medicinal plants, was compiled by the Greek Dioscorides (A. D. 60) and was used by Europeans as a standard reference during medieval times (Cowan 1999, Sofowora 1982). The works of the physician Galen were used in the middle ages but many of his records were destroyed. In spite of these unfortunate circumstances, present allopathic and homeopathic systems have been derived from the works of Galen (Sofowora 1982).

The Bible contains descriptions of approximately 30 healing plants with Frankincense and Myrrh which were used as mouthwashes because of their acclaimed antiseptic properties (Cowan 1999). However, the belief of Christians is that the only cure for diseases (heaven-sent punishments) was repentance and prayer (Cowan 1999).

This forestalled Western advances in the understanding of medicinal plants, with much of the documentation of plant pharmaceuticals being destroyed or lost (Cowan 1999; Sofowora 1982). During this time the Arabian physicians continued with their research and added many new discoveries made.

The medieval times proved to be a slow progressive medicinal period for the Europeans, who at the time, based their findings on the urinal state of patients as well as astronomy (the state of the stars at that given time). The influence of the church (repentance and prayer prescriptions) was also great at the time (Sofowora 1982). Moreover, the Doctrine of Signatures was used at this time, since it was believed that God made the healing herbs with specific characteristics associated with the illness for which it could be used, e.g. the plant Eyebright (*Euphrasia officinalis* L.) was used for poor sight. The spot on its corolla resembles an eye (Sofowora 1982).

Anthropologists believe that the plants were initially used as part of religious rituals in Africa. This is still common practice in some African areas. Another theory states that the discovery of medicinal plants was quite accidental as man perhaps observed the effects of these plants when consumed by domestic animals. To this day, some herbalists test their remedies on domestic animals to assess the toxicity. On the other hand, hunters may also be the discoverers of herbal remedies in African countries. Their knowledge could have been gained while hunting for game. They may have noted when a specific animal that was wounded escaped capture and ate a certain plant that served as an antidote (Sofowora 1982).

Antibiotic resistance

Presently it is common practice to make use of medicinal plants for ailments. About 3300 million people make use of medicinal plants in a regular basis. This is especially the case since the emergence of antibiotic resistance (Salvat *et al.* 2001; Palombo & Semble 2001; Monroe & Polk 2000; Davies 1994). Indiscriminate use of antibiotics has led to resistance of an alarming number of bacterial strains (Martini & Eloff 1999; Salie 1998; Salie *et al* 1996). An increase in the number of resistant strains has been reported (Monroe & Polk 2000).

This resistance to anitbiotics occurred after the huge problem in 1977 caused by penicillin resistance and multiresistant pneumococci in South-African hospitals (Eloff 1998). Berkowitz called the emerging of drug resistance a medical catastrophe (Eloff 1998). Leggiardo states that effective regimens may not be available to treat some enterococcal isolates, which is of utmost importance to develop new antimicrobial compounds for these, and other organisms (Eloff 1998; Martini & Eloff 1998).

This state of affairs compels us to a search of alternative sources due to the constant need for new effective therapeutic agents that is safe to use (Palombo & Semble 2001).

Safety of medicinal plants

The number of medicinal poisoning cases is another reason for in-depth experimentation with medicinal therapeutic agents. A number of previous reports indicates that the use of certain traditional remedies can result in serious illnesses such as liver and renal failure and can even lead to death (Salie 1998; De Smidt 1991). Plants containing pyrrolizidine alkaloids are especially harmful as they cause hepatic failure in humans. More than 280 species with these biologically active compounds have been listed by the World Health Organization (Salie 1998). Many botanical supplements also lack thorough efficacy and safety trials as well as standardization and quality standards (Kressmann *et al.* 2002).

It is essential that suitable diagnostic assays have to be developed, the toxicological actions of these compounds must be investigated and an attempt to devise antidotes for the compounds must be made (Bye & Dutton 1991).

Overexploitation

The overexploitation of traditionally used plants is hastily leading towards extinction, which is a great concern for all ecologists (Van der Watt & Pretorius 2001). Necessary actions need to be taken to ensure the conservation of our precious medicinal plants (Salie 1998). With the exponential population growth severe, demands are being placed on already depleted sources and have caused the eradication of certain species (Salie 1998; Bye & Dutton 1991).

The plant kingdom is a potential great source for new effective therapeutic agents but may be lost to all as overexploitation and eventually extinction plays a big role in the study of these medicinal plants. The fynbos represents such an example with its diverse richness (Salie 1998; Salie *et al* 1996). In order to discover new natural medicinal agents, a thorough investigation of in-depth cultural knowledge, research into the nature of the drug and chemical analysis as well as pharmacotoxicological tests need to be done (Salie 1998; Anton & Rivier 1991).

Fynbos

The flora of the western Cape is commonly known as the Cape Fynbos (Salie 1998; Salie *et al* 1996). The Cape Fynbos stretches over a nutrient poor area of about 90,000 km^2. It comprises of a richness of more than 8,600 species (Elmqvist 1999; Salie 1998; Salie *et al* 1996). Most of the endemics belong to the Ericaceae, Preoteaceae, Rutaceae, Rhamnaceae, Fabaceae, Polygelaceae and Rosaceae. It consists of shrubby dry vegetation with little tree species and normally occurs in areas with a rainfall ranging between 250-300mm annually (Salie 1998).

What is exceptional about this vegetation is its beta-and gamma-diversity. Two adjoining areas may only have a small amount of species in common, e.g. the Koggelberg and the Cape Peninsula share less than half of their species (Elmqvist 1999). Taking all this into consideration, it is clear that Cape Fynbos represents a potential source of new anitmicrobial agents.

Plants included in study

Southern Africa contains approximately 10% of the world's plants, but they have not been studied intensively (Eloff 1998). Noristan assessed more than 300 southern African plants. These studies showed that 31% had high activity, 48% medium activity and 21% showed no activity when tested (Martini & Eloff 1998; Eloff 1998).

This study focuses on *Cissampelos capensis and Carpotbrotus acinaciformis* as possible alternative and effective indigenous plant remedies that have anti-infective value.

Cissampelos capensis

Cissampelos capensis belongs to the *Menispermaceae* family. It is commonly known in Afrikaans as "Dawidjiewortel". This plant is a sprawling or climbing shrub with woody, twining stems. It has ovate or trowel-shaped leaves. Flowers are normally green in colour, auxiliary and velvety. These flowers form clusters and are followed by orange berries. Few species occur in temperate areas and are more common in tropical and subtropical regions. The plant is widely distributed in the western region of South Africa. Approximately 7 genera and 13 species are found in southern Africa. It is known for its medicinal value, especially in the Western Cape (Van Wyk *et al* 1997).

Rhizomes and roots are the plant parts that are used for medicinal purposes. Fresh or dry rhizomes are taken. The medicine is normally prepared as a brandy tincture, but infusions and decoctions can also be made of it. It is commonly used for bladder problems, as a blood purifier, for boils and syphilis, diarrhoea, colic and cholera.

Rhizomes are also taken for diabetes, TB, and stomach and skin sores (Van Wyk & Gericke 2000; Van Wyk *et al* 1997).

Inhalation of the smoke from burning a dry rhizome aids in headaches and its sedative properties relieve pain when taken as an infusion. (Van Wyk & Gericke 2000).

The rhizomes of a number of studied *Cissampelos* species are rich in bisbenzyltetrahydroisoquinoline-type alkaloids. Cissampareine is an example of such a biologically active alkaloid (Van Wyk & Gericke 2000; Van Wyk *et al* 1997).

Very little information is available on *C. capensis,* but it is likely that the antimicrobial activity of this medicinal plant is due to the same or similar alkaloids (Van wyk *et al* 1997).

Carpobrotus acinaciformis

Carpobrotus acinaciformis belong to the Mesembryanthemaceae family and most of the genera and species occur in the southwestern parts of Africa. It is commonly known as the sour fig, Hottentots fig, "Elandsvye", "Gouna", "strand-vy", "suurvye" in Afrikaans and "Guakum" (Khoi-San) (Watt & Breyer-Brandwijk 1962). *Carpobrotus* species normally range from the Western Cape up to KwaZulu-Natal. *Carpobrotus edulis* is presently propagated globally and is occasionally used to stabilize sandy banks. The distribution of *Carpobrotus acinaciformis,* however, is more restricted to the Western Cape. This very succulent perennial forms large mats covering the ground. The trailing stems are either green or maroon to yellow. This colour is influenced by the season. The astringent leaves are robust with a triangular shape and it contains malic acid, citric acid and calcium salts (Watt & Breyer-Brandwijk 1962). They are opposite one another and green in colour with slightly reddish edges. Flowers of *Carpobrotus acinaciformis* are generally purple. Its stems can reach lengths of up to two metres long (Goldbladtt & Manning 2000; Leistner 2000; Van Wyk *et al* 1997).

C. acinacifomis leaf juice is used as a mouthwash and gargle, and as a wash for sores. It can also be made into a lotion and applied to burns and scalds (Watt & Breyer-Brandwijk 1962). The leaf pulp is also used for its medicinal values and can be taken orally or applied externally. This plant is generally used for throat, skin and eye infections, in the treatment of pulmonary tuberculosis, other chest conditions as well as diarrhoea (Van Wyk & Gericke 2000; Van Wyk *et al* 1997; Watt & Breyer-Brandwijk 1962).

The aim of this project is to perform *in vivo* and *in vitro* studies to investigate the:

- Potential antimicrobial properties of *Cissamelos capensis* and *Carpobrotus acinaciformis.*
- Effect of *Cissamelos capensis* and *Carpobrotus acinaciformis* on animal metabolism.

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Chapter 2

The analyses of *Cissampelos capensis* from the Western Cape as a novel antimicrobial candidate and its effect on animal metabolism

Abstract

C.capensis is known to possess anti-infective properties that reside in its rhizome but not much scientific information exists to prove its efficacy and safety. The main objective of this study was to investigate this medicinal herb at an *in vivo* and *in vitro* level to assess its effectiveness. Sections were made of the rhizome using a sledge-microtome. This was performed to determine where the active compounds might reside. Atomic Absorption Spectrophotometry (AA) was used to measure the elemental concentrations within the *C.capensis* rhizome and the soil from which it was collected. K^+ (6.21mg/g) was the highest element in the plant and Ca^{2+} (10.79mg/g) the highest element in the soil. In order to assess the *in vitro* effects of the plant, the fresh and dried rhizome extract of *C.capensis* was tested against four pathogens. These included the *C.albicans, S.aureus, P. aeruginosa* and *M.smegmatis*. *S.aureus* was most affectively inhibited by all the concentrations of the extract. The fresh extract was highly active against *M.smegmatis,* and the 30mg/ml, 40 mg/ml and 80 mg/ml solutions inhibited its growth. The dried extract was less active and only the 80 mg/ml solution showed activity against *M.smegmatis*. Only the 80 mg/ml of the fresh rhizome extract showed activity against *C.albicans*. No activity was apparent against *P. aeruginosa.* To assess the in vivo effects of the *C.capensis* extracts, 20 Wistar rats were divided into a control group (n=10) receiving water and an experimental group (n=10) that consumed the herbal medicine. Metabolic readings were taken of the two groups over 24 hours at days 15, 30 and 45. These parameters included animal mass, food consumed, water intake and urine excreted. The animal mass showed a significant difference between the control and experimental groups at days 15 ($P<0.05$), day 30 ($P<0.05$) and day 45 ($P<0.01$). Food consumed was significantly higher for the experimental group at day 45 ($P<0.01$). At day 45 the control group had a significantly higher water intake ($P<0.05$) and its urine volume excreted was significantly increased ($P<0.0001$). The whole blood samples analyzed for red blood cell and white blood cell parameters indicated that *C.capensis* extracts had no measurable effect on the blood biochemistry of the animals. The analysis of the histological sections of the ovaries and liver showed no macro architectural damage. The data obtained indicate that *C.capensis* has antimicrobial activity.

1 Introduction

Antimicrobial resistance is one of the grave threats to human health and it is rapidly increasing (Salvat *et al.* 2001; Davies 1994; Palombo & Semble 2001; Monroe & Polk 2000; Amabile-Cuevas *et al.* 1995; Frontiers in Biotechnology 1994). In fact, abuse of antibiotics is the major cause of resistance of many pathogens (Martini & Eloff 1998, Salie *et al.* 1996). An example of this is a study performed by Sanglard *et al.* (1996) that showed that continual use of the azole antifungal fluconazol by candida-infected patients resulted in resistance.

An example of such a harmful pathogen is *Candida albicans* that occurs naturally as normal flora on the mucous membranes and in the gastrointestinal tract (Kaiser 2002). It is responsible for various infections, which are generally termed as Candidiasis (Kaiser 2002). Studies have shown that a fully functional FAS2 gene is necessary for successful candida infection in animal models with both orophyrangeal and systemic candidiasis (Zhao *et al.* 1996; Zhao *et al.*1997). One such infection is commonly known as vaginal thrush.

People who are immunosuppressed, or have received antibacterial therapy for a prolonged period of time are normally vulnerable to *Candida* infection (Fredkin & Jarvis 1996). In most cases, women who are diabetic, pregnant, taking oral contraceptives, or having menopause are also more susceptible to vaginitis or vaginal thrush (Kaiser 2002). Other general illnesses, tight fitting pants, nylon underwear, humid weather and travelling (prolonged sitting) can also result in *Candida* infection.

These infections can be treated with antimicrobials that are able to inhibit the growth of other microorganisms (Dictionary of Science, 1999). Antimicrobials are divided into 2 categories i.e., bactericidal (killing bacteria) and bacteriostatic (preventing any bacterial growth). These drugs usually act on one of the following: Cell wall synthesis, protein synthesis, cytoplasmic membrane permeability and nucleic acid synthesis and it is antimetabolic.

With the alarming increase of antibiotic resistance, more efforts are being made for the search for alternative antimicrobial agents (Eloff 1999). There is a constant need for new effective therapeutic agents (Palombo & Semple 2001). In 1984, approximately 25% of prescribed drugs issued in the USA and Canada were derived from or fashioned after plant natural products (Eloff 1998). Noristan performed studies on more than 300 southern African plants of which 31% showed high activity, 48% medium activity and 21% no activity against various pharmacological parameters (Martini & Eloff 1998; Eloff 1998). Even so, very little chemical work has been done on the diverse South-African floral kingdom (Eloff 1998).

It is believed that plant-derived antimicrobial compounds stunt the growth of bacteria by a different mechanism than the antibiotics that are commonly currently in use (Van der Watt & Pretorius 2001; Eloff 1999; Eloff 1998). Due to its different action it may have important clinical value in the treatment of bacterial strains that have become resistant to existing therapeutic agents (Van der Watt & Pretorius 2001; Eloff 1999; Eloff 1998).

This study focuses on *Cissampelos capensis* as a possible alternative and effective indigenous plant remedy that has anti-infective value. Potential anti-microbial agents from this medicinal herb could well inhibit the growth of harmful microorganisms such as *Candida albicans* amongst others that are detrimental to the female reproductive health.

The aim of this study was to assess the anti-infective value of *C.capensis* and to evaluate its effect on animal metabolism.

2 Methods and materials

2.1 Ethics

Ethical approval for this investigation was obtained from the University of the Western Cape.

2.2 Plant distribution

The first recording made in the Western Cape was of *Cissampelos capensis*. It was sighted at an altitude of 55m. The site is located at 34°21'58S21°25'21E. The populations were infrequent and none were in flower. One root sample was collected in the Pauline Bohnen Reserve at an altitude of 60m at 34°21'54S21°25'26E. No leaves were collected and no herbarium samples were collected.

2.3 Plant Anatomy

The plant sample that was collected was placed in FAA (Formaldehyde, Acetic acid and Alcohol) for later anatomical studies. Cross sections were made of the leaves of the plant using a freeze-microtome. The leave was cut into smaller sections that were used for sectioning. Liquid CO_2 gas and Hamilton's freeze solution was used to freeze the leave sample and sections were made with the attached blade ranging from 15-25 microns. The sections were then fixed on slides, stained with Safranin and Alcian blue and viewed under a microscope. A DP11 Olympus photomicroscope was used to capture digital photos containing images of the vascular bundles of the species. This was performed to assess the possible location and type of active ingredient in the plant organ used as herbal medicine.

2.4 Phytochemistry and soil analysis

2.4.1 Extraction Methods

A portion of the plant specimens that was collected was fixed in methanol for later extraction. The remaining specimens were dried in the oven at 30°C-40°C. This was done in order to derive extracts from a fresh sample as well as a dried sample to assess if there was any significant difference in the secondary compound characteristics.

2.4.2 Fresh sample

The methanol was filtered from the samples and put into round-bottom flasks and then rotary evaporated. Samples were placed in the methanol for a second time and blended. This was filtered, added to the first extract and evaporated again using a rotary evaporator to eliminate the remaining methanol. The extracts were frozen in a freezer, freeze-dried with a freeze-dryer and stored in the 5°C cooler room for later analysis.

2.4.3 Dry plant and soil sample

The plant and three soil samples that were collected were stored in a Memmert UML70 oven at a temperature of 30°C-40°C and 70°C respectively. After the plant material was dried it was ground with a grinder and the soil samples was sieved to get rid of the access waste.

Four grams (4g) of each soil sample and 0.8g of the ground dried plant samples were weighed out and put into long glass flasks for digestion:

2.4.4 Digestion procedure

- The plant and soil materials were digested using an acid digestion method (Allen *et al.* 1976):
- The digestion mixture was prepared as follows- 0.42g Se and 14g $LiSO_4$. H_2O were added to 350ml of H_2O_2. The flask was put in a container with ice before the 420ml of concentrated H_2SO_4 was added to the mixture in order to keep it cool.
- 4.4ml and 8.8ml of the digestion mixture were added to the soil and plant materials respectively.
- The flasks were placed in the digestion block and heated to a temperature of 220°C.
- The heat was increased by 50°C for every 30 minutes until a temperature of 350°C was reached.
- After a period of 20 minutes it was increased to 380°C at which the digestion was continued until a clear and colourless or apple green solution was obtained.

- The soil and plant samples were diluted with distilled water and filtered into a 100 ml volumetric flask.
- This was topped up to a 100 ml with distilled water and stored in white plastic bottles for further use.
- Blank solutions (H_2SO4) were also prepared using the same method.

2.5 Atomic Absorption Spectrophotometry (AA)

This procedure was performed to analyze the elemental concentrations in the digested soil and plant samples using a Unicam Solaar M Series Atomic Absorption Spectrophotometer. Standards were prepared to analyze the soil for Ca^{2+}, K^+, Mg^{2+}, Na^+ and Cu^{2+} concentrations. An air acetylene flame was used to determine all the elemental concentrations.

1000 mg/L Merck Ca^{2+}, Na^+, Mg^{2+}, K^+, Cu^{2+}, stock solutions were used to prepare the standards used in AA analysis. This was topped up with 1% H_2SO_4 in a volumetric flask in order to obtain a concentration of 100 mg/L. These were used to prepare standards within a range of 0.05 mg/L – 50 mg/L.

2.5.1 Biological sample preparation

Samples used in the AA were diluted from 15 ml to 75 ml with distilled water.

2.6 UV/Visible Spectrophotometry

Phosphorus (P)

- P was analyzed as in Murphy and Riley (1962):
- 10ml of the sample solutions was pipetted into 50ml volumetric flasks.
- The solutions were diluted.
- 8ml of Murphy and Riley reagent was mixed with the solution.
- The solutions were then left for an hour.
- The absorbance of each sample was read at 700nm using the UV/Visible Spectrophotometer.

2.7 pH analyses

pH measurements were also determined for each soil sample as well as the urine samples that were collected from the animal models at a later stage. A PHM83 Autocal pH-meter was used.

2.8 Antimicrobial evaluation

2.8.1 Herbal extract

A fraction of the plant samples collected was stored in methanol for approximately 48 hours. The methanol was filtered from the samples and put into round-bottom flasks and then rotary evaporated. Samples were placed in the methanol for a second time and blended. This was filtered, added to the first extract and evaporated again using a rotary evaporator to eliminate the remaining methanol. The extracts were frozen in a chest freezer, freeze-dried with a freeze-dryer and stored in the 5°C cooler room.

2.8.2 Micro-organisms

The two bacteria, *Staphylococcus aureus* (ATCC29213), *Pseudomonas aeruginosa* (ATCC 27853) and the yeast *Candida albicans* (ATCC 10231) were obtained from the Department of Medical Biosciences at the University of Western Cape. *Mycobacteria smegmatis* was obtained from Tygerberg hospital, in Tygerberg city. The agar plates containing the cultures were stored in a cooler room at 5°C until the onset of the antimicrobial experiment.

2.8.3 Disc diffusion

Individual nutrient broths containing, *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans* and *Mycobacteria smegmatis* were streaked onto nutrient (Difco) and mycobacteria 7H11 (Difco) agar plates respectively. A sterilized swab was inserted into the suspension and was used to make streaks on the agar plates. The spreading over the dish was made in three different directions to ensure an even-growing bacterial and fungal mat.

Sterilized nine-mm filter paper discs were impregnated with 50µl of methanol and aqueous extractions varying in concentrations. These were placed at equidistant spots on the inoculated agar plates. Methanol and water discs were used as controls.

Amphotericin B served as positive control for *C. albicans* and Ciprofloxacin was used for the three bacterial strains. The positive controls were supplied by the companies Bristol-Myers Squibb and Bayer respectively. Different plant extract concentrations were prepared. The concentrations were 10mg/ml, 20mg/ml, 30mg/ml, 40mg/ml and 80mg/ml. The plates were incubated at 37°C for 24 hours with the exception of *Mycobacteria* that required a growth period of 48 hours. The extracts were tested in triplicate. At the end of the incubation period, inhibition zones of the different plant extract concentrations were recorded by measuring the growth-free zones between the discs and the bacteria or yeast. Sensitivity of the zone size was measured at a minimum of 1mm.

2.9 Animal studies

2.9.1 Animal groups

20 Wistar female rats weighing between 150 g and 200 g were used as animal models. These were obtained from the Medical Research Council (MRC). The rats were divided into two groups, each consisting of 10 rats. Groups included 1) Control Group, 2) Group fed with *C. capensis* extract. Rat models were chosen for the laboratory experimentation because it is an internationally accepted animal model for both medical and other types of research.

The four-week old female rats were housed in cages with wire-mesh floors, two per cage. The room temperature was kept at 23°C and a day-and-night light cycle was maintained. A diet consisting of food pellets and distilled water was available *ad libitum*. The two-week acclimatization period were followed by gavaging and metabolic runs every 15 days.

2.9.2 Preperation of plant extract and administration

C.capensis

- 5g of the dried rhizome was boiled in 500ml distilled water on a hotplate for approximately 15 minutes. The specific dosage administered and preparation of the herbal medicine were as prescribed and recommended by the herbalists selling these medicinal plants and remedies.
- Once the solution was cooled it was filtered and the filtrate (used as medicine) was stored in a fridge. Medicine was prepared at a weekly interval.
- The dosage was related to body weight. This was derived from the dosage prescribed for human consumption by traditional herbalists where an average weighing individual (75kg) consumed three cups (250ml) of the herbal remedy per day. Expressed in grams this was equivalent to 1ml of the medicine per 100g. The average weight of the female rats was 200g thus resulting in a dosage of 2ml/200g, also expressed as 2mg/0.2kg.

2.10 Metabolic studies and tissue histology

Metabolic data were collected every second week over 45 days. The rat weight was recorded before and after the 24-hour metabolic run. Only 40g food pellets and 60ml distilled water were available during a run. At the end of the run, the food and water consumption were measured, and urine volume and stool wet weight were recorded. Urine and stool samples were prepared for elemental analysis with the latter being dried in a Memmert UL 40 oven at 90°C for 24 hours to determine the dry weight as well.

Baseline readings of all three groups were taken before the plant extracts was administered. The medicine was fed to the experimental groups via gastric gavaging using bulb-tipped needles. A dosage of 2ml of the *C.capensis* filtrate was administered every morning. The control group received distilled water instead of medicine. This was followed by metabolic measurements that were taken at days 15, 30 and 45.

At the end of the experimental period, the rats were sacrificed under chloroform-ether anesthesia. Approximately 3 ml of blood was collected by cardiac puncture and collected in an EDTA vacutainer and analyzed via a coulter counter. Blood parameters included red blood cell counts (RBC), haemoglobin (Hb), haematocrit (Ht), mean cell volume (MCV), mean cell heamoglobin (MCH), mean cell haemoglobin count (MCHC) and the RDW (red cell diameter).

The white blood cell count (WBC), neutrophils, lymphocytes, monocytes, neutrophil abs count (NAC), lymphocyte abs count (LAC), monocyte abs count (MAC) and the platelets were also analyzed.

Furthermore, a number of organs including the ovaries and liver were collected for analyses. These were preserved in Bouin's solution. Small sections of these tissues were processed in a histokinette for approximately a 22-hour period and embedded in wax. Histology was performed on the ovaries and liver using a microtome and sections were made at 5 microns. These two organs were selected because it is most vulnerable and susceptible to toxicity and serves as an indicator of any toxic changes in the system.

The sections were then put onto slides, stained using a Heamotoxylin and Eosin stain and mounted with DPX. The liver and ovary slides of the experimental group were then compared to slides of the control group and textbook pictures of normal and healthy livers and ovaries to assess its appearance.

2.11 Statistics

Data was analyzed using the 2000 Microsoft Excel Statistical package. Due to the significant difference at the baseline for a number of parameters, baseline corrections (eliminating the big differences in body weight) were made to the data for the respective collections made over the experimental period, after which statistical analysis was applied.

Control and experimental animal groups were compared with one another and a minimum significance of $P<0.05$ was accepted as significant for all metabolic parameters using the Mann-Whitney test. Baseline corrections were not applied to blood data, which was directly compared. In this regard, significant differences were also determined at a minimum level of $P< 0.05$ with the Mann-Whitney test.

2.12 Results

2.12.1 Herb histology

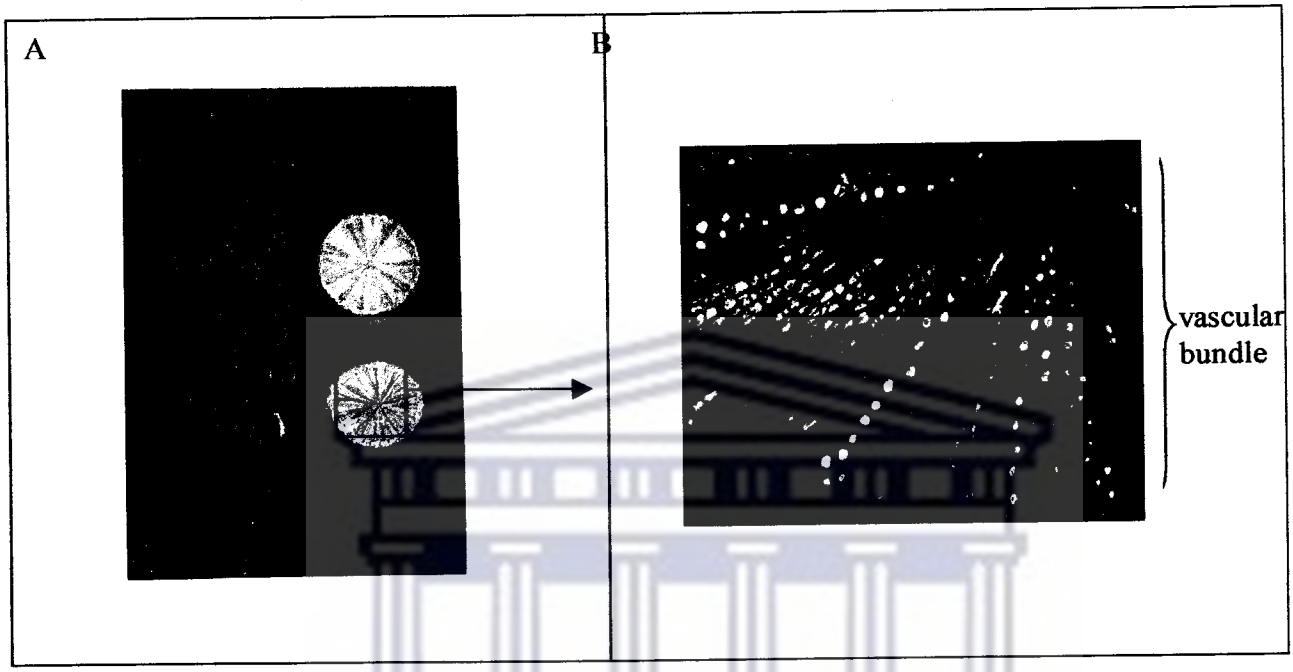


Fig. 1A and B: *Cissampelos capensis* rhizome and a cross section *Cissampelos capensis* at 200x magnification.

2.12.2 Elemental analyses

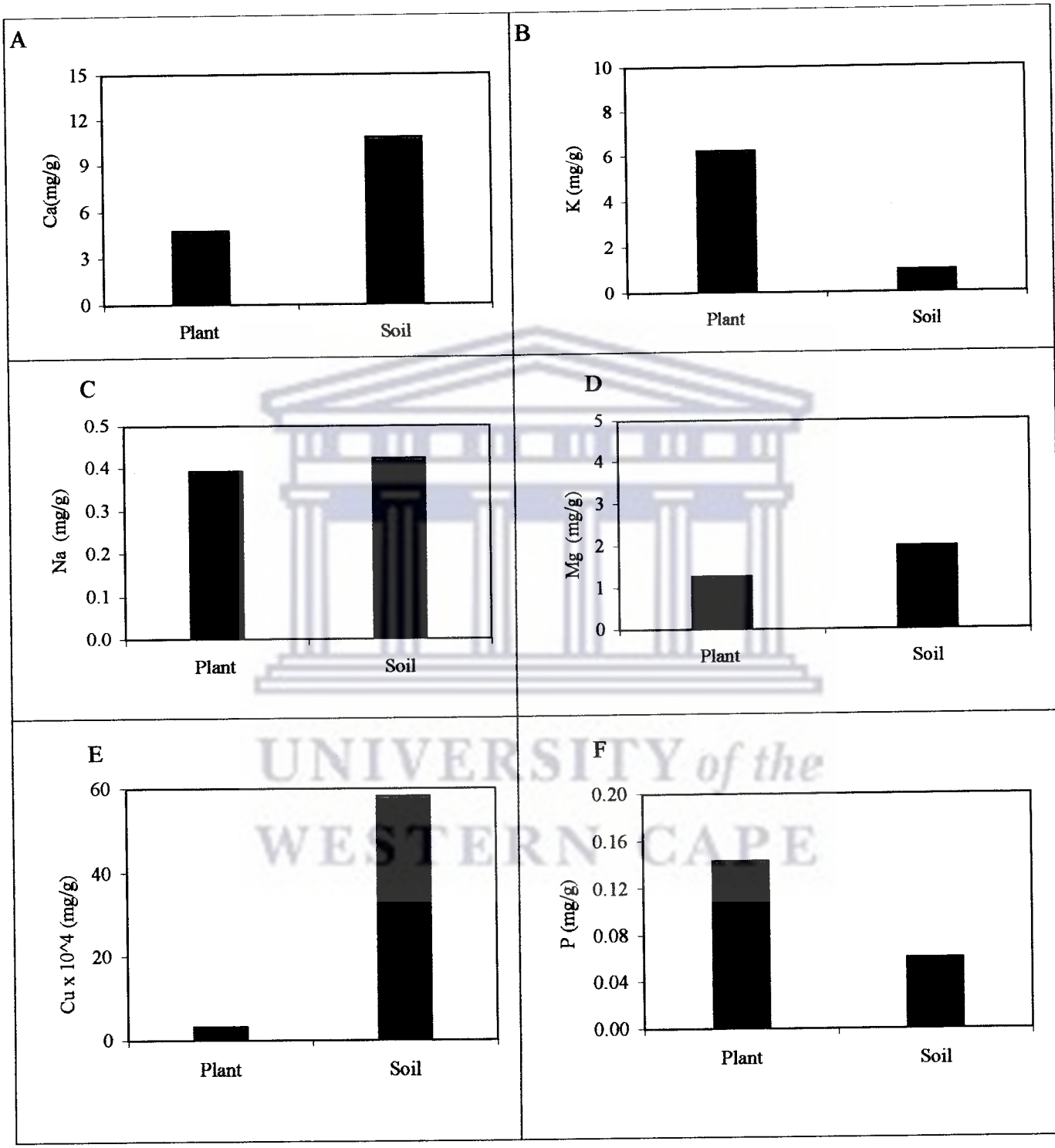


Fig. 2A-F: The different elemental levels in *C.capensis* rhizome (n=1) and the soil (n=3) in which it occurred.

Elemental analyses performed done on one *C.capensis* rhizome sample and three soil samples from where it was collected. K (6.21mg/g) was the highest element in the plant and Ca (10.79mg/g) the highest element in the soil. Cu concentrations were lowest on both the rhizome sample and the soil samples.

2.12.3 Antimicrobial assessment

Table1:
Antibiotic activity of *C.capensis* against four microorganisms (mm)

Plant	[ ]	*C.alb* [a]		A[b]	*P.aer*[a]		Ci[b]	*S.aur.*[a]		Ci[b]	*Msmeg.*[a]		Ci[b]
		fresh	dry		fresh	dry		fresh	dry		fresh	dry	
-	20	-	-	10	-	-	6	1	-	12	-	-	9
-	30	-	-	10	-	-	5	1.6	-	11	1.5	1	9
	40	-	-	9	-	-	5	1.5	1.5	10	1.5	2	9
	80	4	-	10	-	-	6	5	3	11	2	6	8

[a]*Candida albicans (C.alb), Pseudomonas aeruginosa (P.aer), Staphylococcus aureus (S.aur) and Mycobacteria smegmatis (M.smeg)*

[b]Amphotericin (A), Ciprofloxacin (Ci).

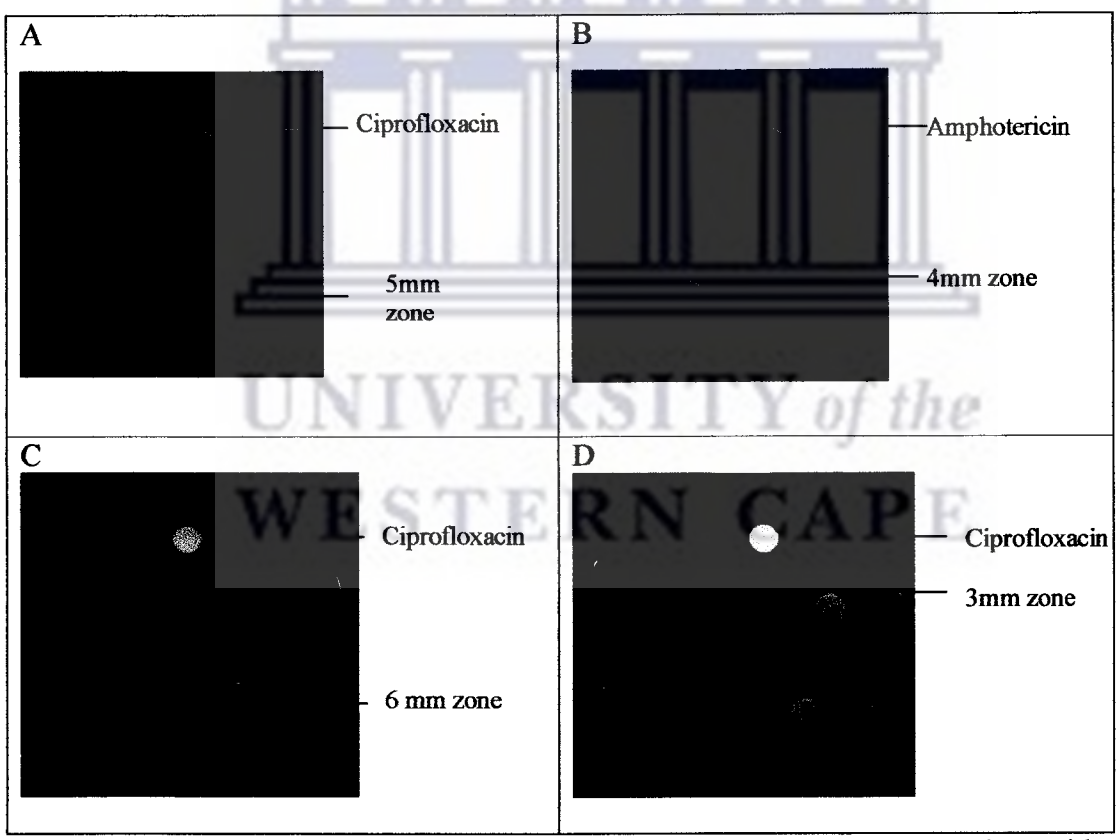


Fig. 3A *S.aureus* plate with *C.capensis* discs(80mg/ml); (B) *C.albicans* plate with *C. capensis* discs(80mg/ml); (C) *M.smegmatis* plate of dried *C.capensis* sample(80mg/ml); (D) *S. aureus* plate of dried *C.capensis* sample(80mg/ml).

C.capensis showed activity against all of the organisms with the exception of *P.aeruginosa.* The fresh rhizome extract was most effective against S.aureus. Only the highest concentration of the fresh rhizome sample had an inhibitory effect on *C.albicans.* The dried rhizome showed the highest inhibition against *M.smegmatis* (6mm zone). No activity for the tested concentrations was apparent against *P.aeruginosa.*

2.12.4 Metabolic outcomes

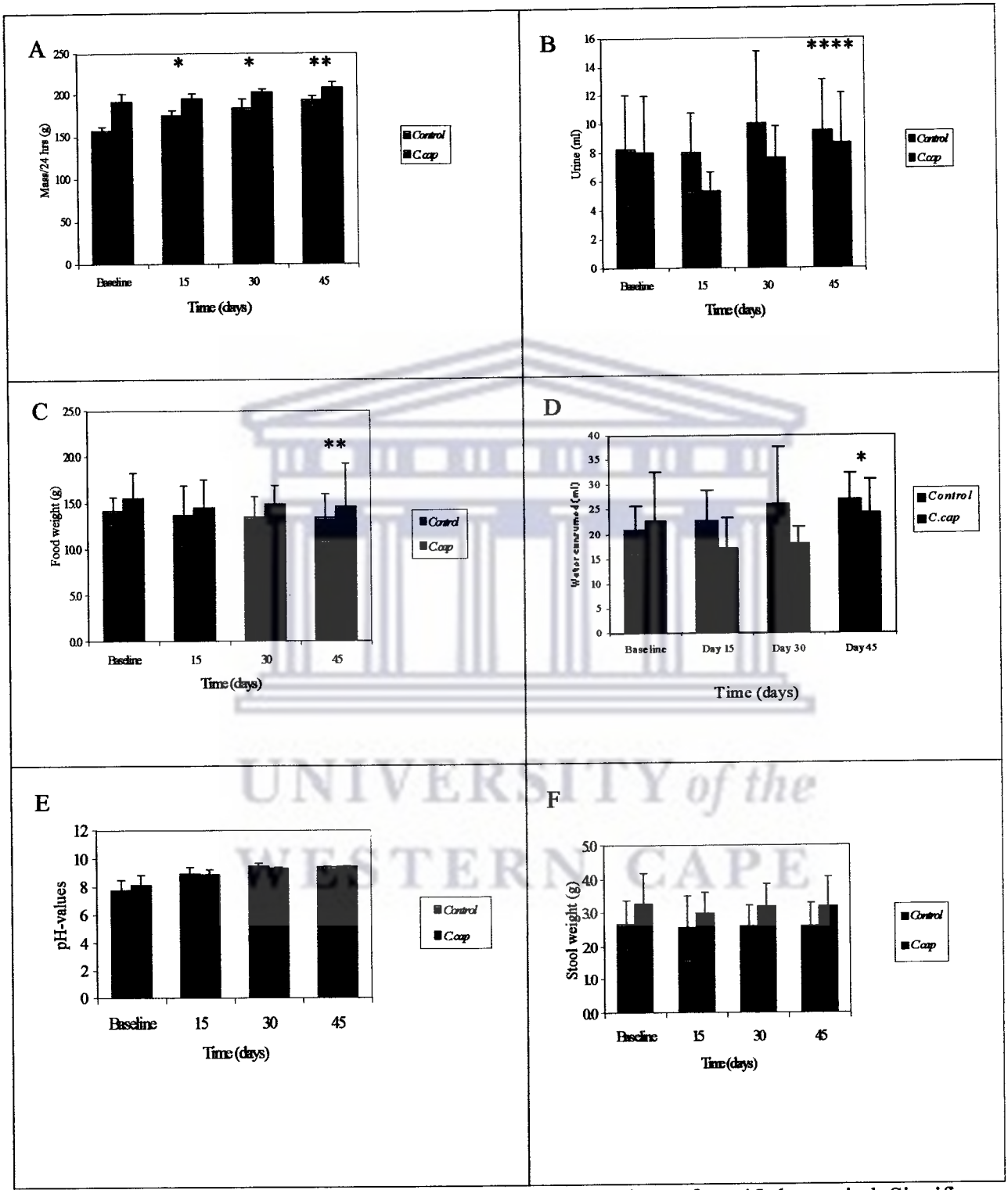
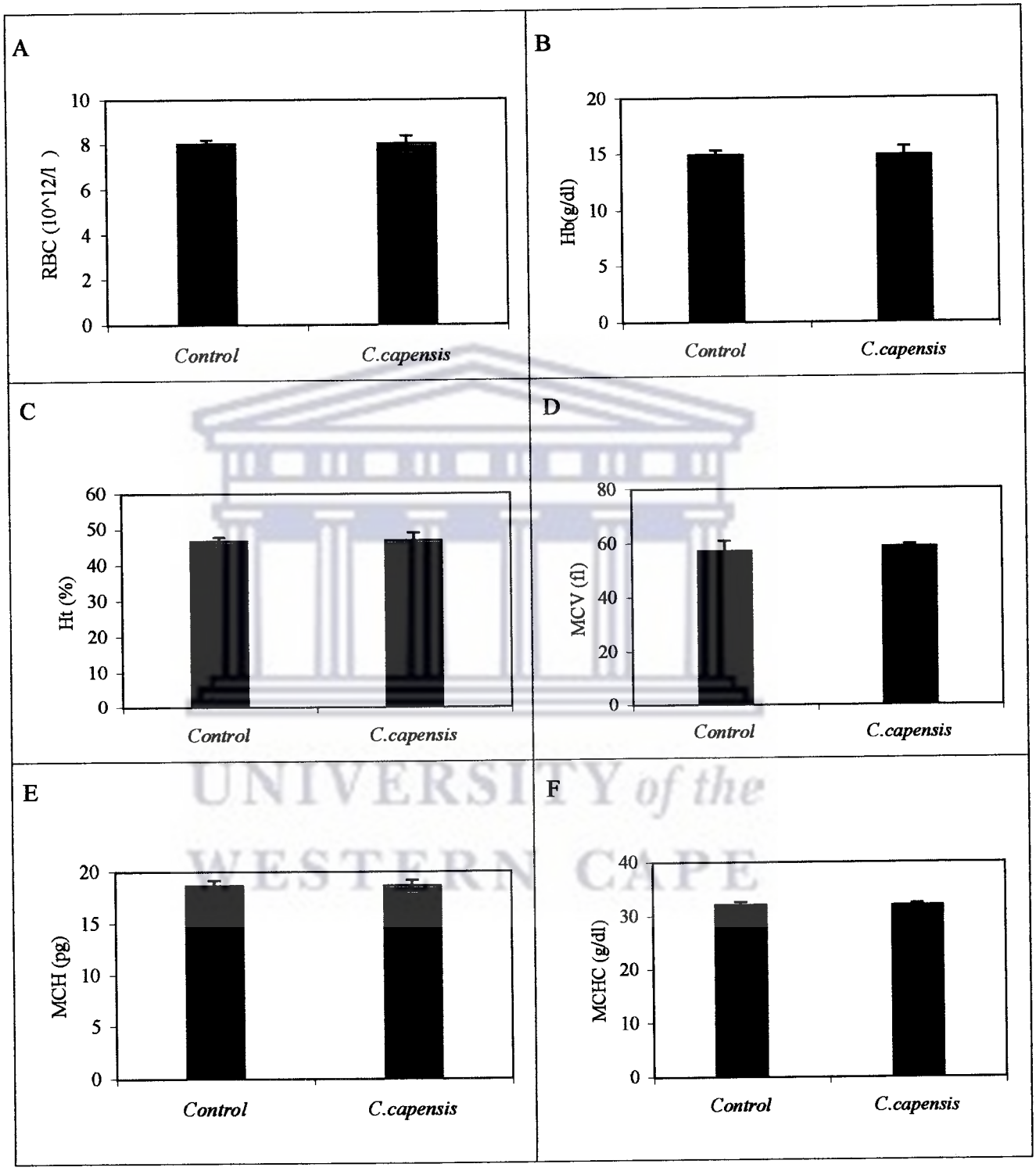


Fig. 1a – f: Metabolic parameters that were measured over 24 hours for a 45-day period. Significance were measured at*P<0.05,**P<0.01,***P<0.001,****P<0.0001.Panels with no stars: P>0.05.

The animal mass showed difference between the control and experimental groups at days 15 ($P<0.05$), 30 ($P<0.05$) and 45 ($P<0.01$). A significantly higher food intake was noticeable for the experimental group at day 45 ($P<0.01$). At day 45 the experimental group had a significantly higher water intake ($P<0.05$) whilst its urine excretion was significantly more ($P<0.001$).

2.12.5 Hematological profiles



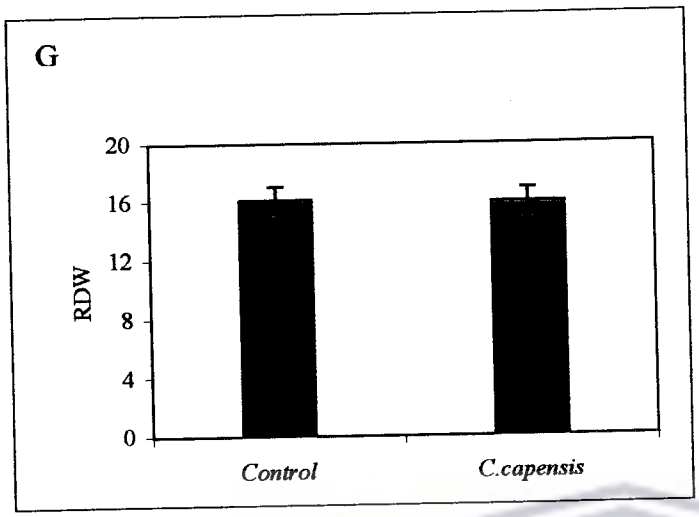
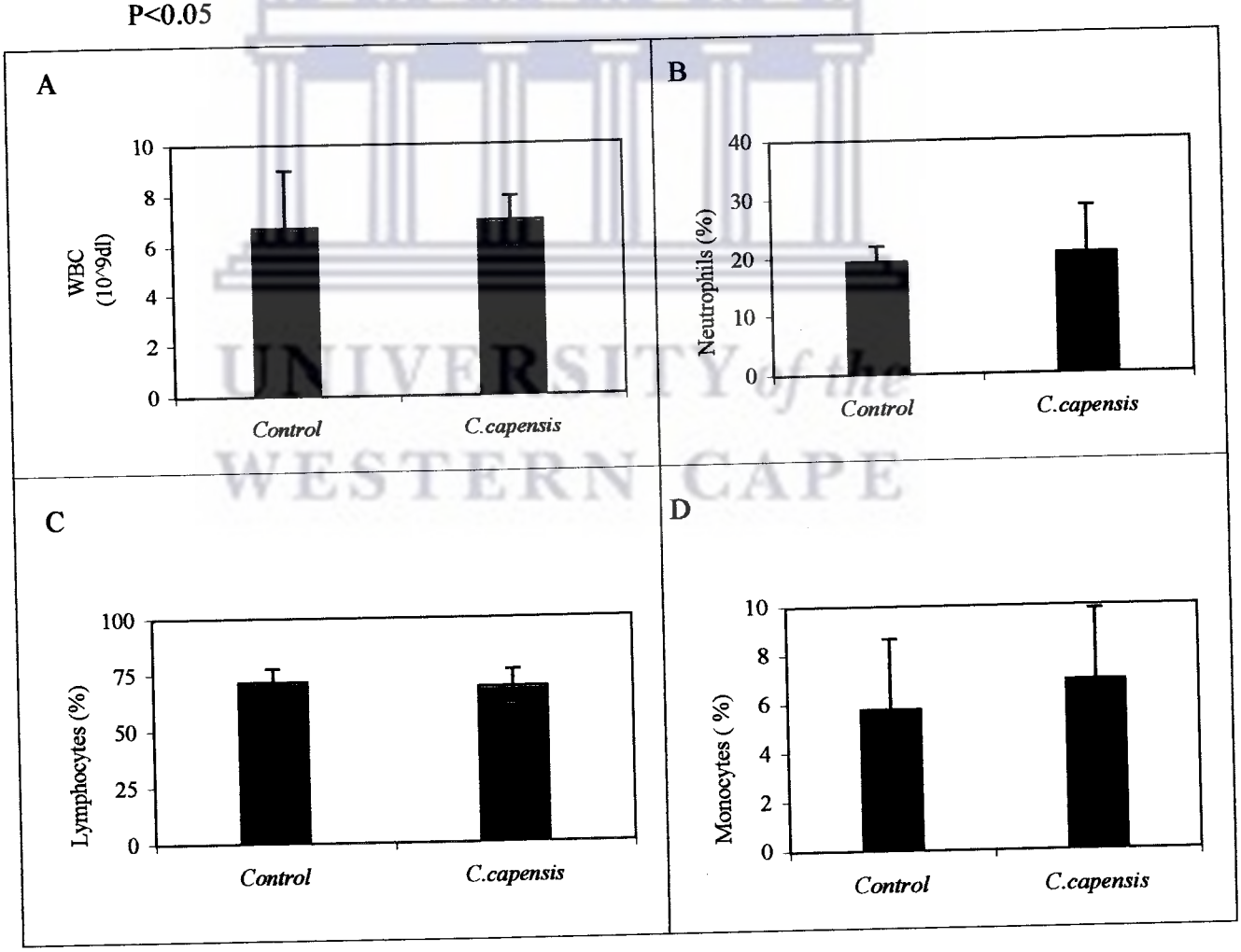
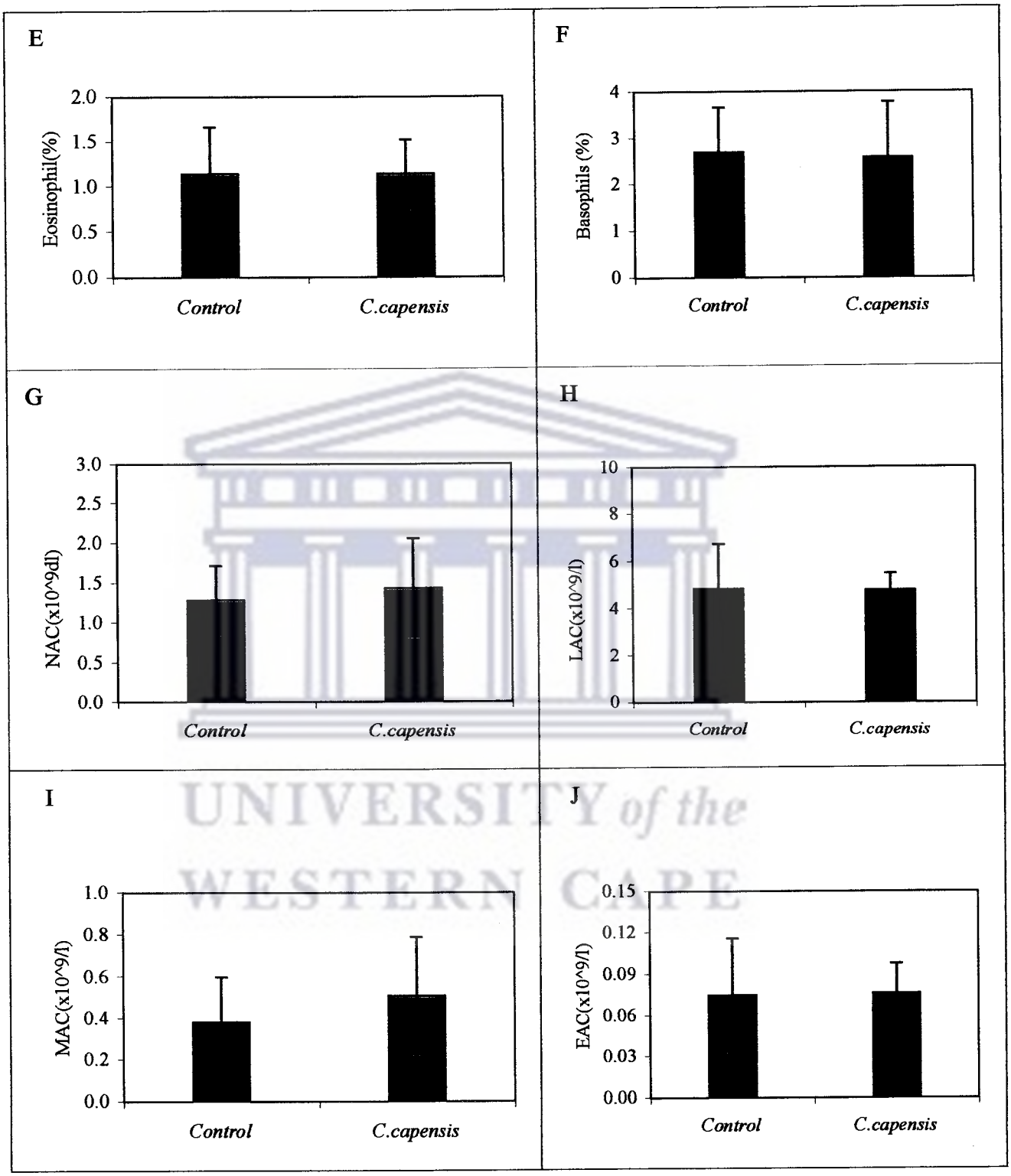


Fig. 4A-G: RBC parameters measured in whole blood samples of control and experimental groups. None of the parameters showed any significant differences at the minimum level of P<0.05





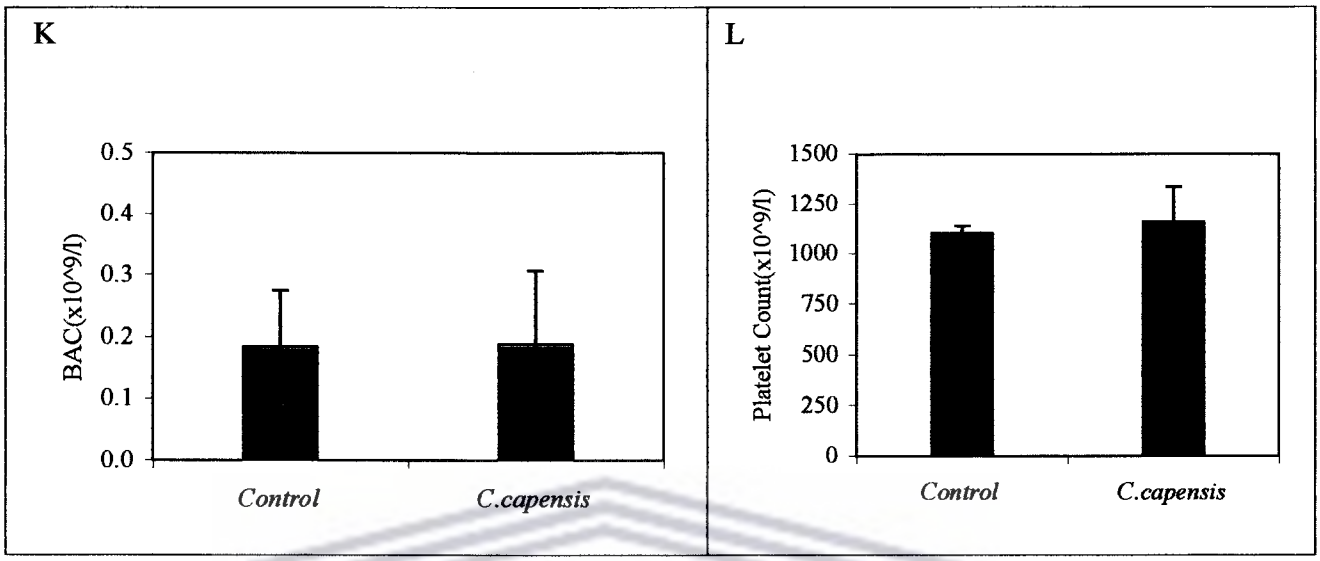


Fig. 5A-L: WBC parameters measured in whole blood samples of control and experimental groups. None of the parameters showed any significant differences at the minimum level of $P \leq 0.05$

All of the red blood cell and white blood cell parameters measured showed no significant differences between the control rat group receiving water and the experimental rat group that was fed the herbal medicine consisting of the *C.capensis* filtrate. Significance were measured at a minimum level of $P < 0.05$

2.12.6 Animal histology

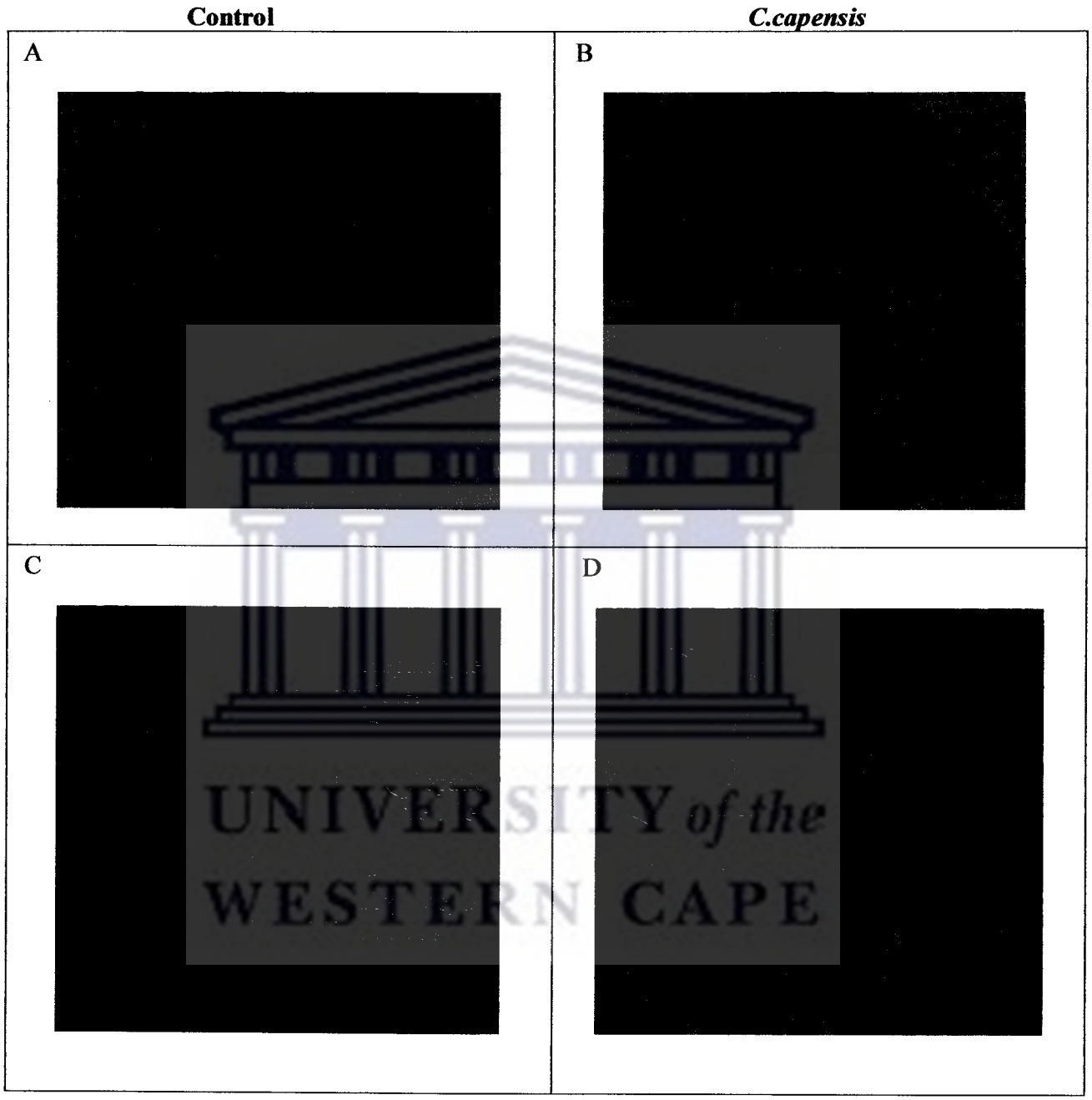


Fig. 6 (A) Ovarian cross section of control group (40x magnification); (B) Ovarian cross section of experimental group (40x magnification); (C) Liver cross section of control group (200x magnification); (D) Liver cross section of experimental group (200x magnification).

The analyses of the histological sections of the ovaries and liver showed no macro architectural damage. After comparison of the slides to both textbook pictures, descriptions and other slides of normal and healthy ovarian and liver cross-sections of the control group, no apparent differences could be detected. There was specific focus on these two organs because of its vulnerability to toxic variations in the system and its susceptibility to any toxicity.

1.13 Discussion

From this study investigation it is clear that *C.capensis* extracts have strong anti-infective value and a favourable safety profile. It is believed that the anti-infective activity of the *C.capensis* plant is due to bisbenzyltetrahydroisoquinoline-type alkaloids, for example cissampareine (Van Wyk & Gericke 2000; Van Wyk *et al.* 1997). These bioactive compounds reside in the rhizome. Sections were made from the rhizome of the plant. In Fig. 1B one can clearly see the rays and xylem.

Many elements are essential to plant life and it may also influence the antimicrobial properties of the plant. Soil is the source of all mineral elements present in plants (Williams 1963). In this case the alkalinity of the soil had an influence on the elements. Mineral availability in soils is affected by their effective concentrations in the soil solution (Reid & Horvath 1980). Alkaline soil tends to be more abundant in most elements (Hartmans 1970). From the elemental analysis performed on the rhizome of the plant and the soil in which it occurred, it was clear that Ca^{2+} (10.79mg/g) was the highest element in the alkaline soil (Fig. 2A). Ca^{2+} absorption is normally impaired by acidic soils. It has a vital role in the cell wall of the plant. K^+ (6.21mg/g) was most abundant in the rhizome sample (Fig.2B). K^+ is known as a quality nutrient and is absorbed in larger quantities by plants, which explains its abundance in the rhizome. Na^+ levels did not vary much between the plant and soil sample (Fig. 2C). It is not an essential element therefore it is not required in large quantities. Mg^{2+} is normally impaired by alkaline soil, thus it was slightly higher in the plant in comparison to the soil (Fig. 2D). Cu^{2+} had the lowest elemental concentration in both the plant and soil sample (Fig. 2E). The alkaline soil played a role in the availability making it less available. The plant sample contained higher P-levels (Fig. 2F), but it had the second lowest elemental concentration in the plan. P is normally readily available in alkaline soils but due to the plant's low phosphorus requirements, it is absorbed in small amounts.

From the four pathogens that were assessed, the gram-positive bacteria *Stapholococcus aureus* was most easily inhibited by both fresh and dried samples of *C.capensis* (Fig. 3A, Table 1).

Only the highest concentration (80 mg/ml) of the fresh sample showed efficacy against *C. albicans* (Fig. 3B, Table 1). No activity was evident against gram-negative bacteria *Pseudomonas aeruginosa* (Table 1) but it was highly active against *Mycobacterium smegmatis* (Fig. 3C, Table 1. The fresh sample showed more then dried sample. Fig. 3E shows the activity of the dried sample at a concentration of 80 mg/ml. This can be explained by the following.

Previous antimicrobial studies that were performed on gram-positive and gram-negative bacteria showed similar results (Chariandy *et al.* 1999). Research has shown that gram-negative organisms, particularly *P. aeruginosa*, are more resistant than gram-positive ones (Palombo & Semble 2001; Kudi *et al.* 1999; Paz *et al* 1995; Vlietnick *et al.* 1995). Gram-negative bacteria have a tendency to show higher resistance to antibiotics (Van der watt & Pretorius 2001). This feature could possibly be explained by the variation in the cell wall structures between gram-positive and gram-negative bacteria. Gram-negative bacteria have an outer membrane that serves as a barrier, prohibiting many environmental substances, including antibiotics (Palombo & Semble 2001). This outer membrane is semi permeable, acting as a coarse sieve that helps retain certain enzymes and prevents some toxic substances, e.g., Penicillin (Kaiser 2002). The cell wall of bacteria consists of a semi rigid, tight knit molecular complex called peptidoglycan. In order for antibiotics to work effectively they must inhibit the normal synthesis of petidoglycan, causing the cell wall to burst due to osmolysis (Kaiser 2002).

Biomedical research requires animal models in the case of *in vivo* experiments that are aimed at assessing the safety of potential therapeutics. *In vivo* tests in many cases remain the stepping-stone between antimicrobial, antiviral, antiparasitic and anti-cancer *in vitro* tests and the demonstration of corresponding activity in human clinical trials (Elmqvist 1999).

The metabolic data indicated that the control group weighed significantly less ($P<0.05$ and $P<0.01$) then the experimental groups (Fig. 4A). Baseline corrections were made to eliminate this difference. Urine excreted was significantly more by the control group on Day 45 at $P<0.0001$ (Fig. 4B).

The control groups drank significantly more water on Day 45, being considerably higher in comparison with the Baseline and Day 15 readings, which can explain the increased urine excretion. Food consumption showed a significant difference at $P<0.01$ at Day 45 (Fig. 4C). Since the experimental group was larger then the control group, their food consumption resulted in significantly higher food intake. The animals in the control group significantly drank more water ($P<0.05$) at Day 45 (Fig. 4D). No significant changes were noted for the pH of the urine samples (Fig. 4E). pH-levels remained alkaline. Stool excreted showed no significant differences (Fig. 4F). In essence, no major differences could be noted in the metabolism of the two groups. The increased urine excretion on Day 45 of the control group clearly correlates with its increased water intake on the same day. The experimental group being larger in size and therefore requiring more energy could explain the higher food consumption of this group.

Blood cells are divided into three major groups: red blood cells (Erythrocytes), white blood cells (Leucocytes) and platelets (Thrombocytes). They are formed via haemopoiesis in the bone marrow. Erythrocytes primarily function in oxygen and carbon dioxide transport within the vascular system. Leucocytes play an important role in the defense and immune systems and act outside blood vessels. Platelets are essential in the control of bleeding contributing to blood cascades.

Different blood parameters were measured from whole blood samples. These included Red Blood Cells (Fig. 5A-G), White Blood Cells (Fig. 6A-K) and Platelets (Fig. 6L). These comprised of the RBC, Hb, Ht, MCV, MCH, MCHC, RDW, WBC and neutrophils (constitute the first line of defense against invading organisms), lymphocyte (may function in the production of other blood cells and contribute DNA, enzymes or some other vital cellular constituent to the multipotential reticulum cell), monocyte (serve as scavengers of a wide variety of particulate materials and digest bacteria with a lipoid capsule), eosinophils (play a role in allergic reactions), basophil (deliver heparin at sites where vascular obstruction occurs), NAC, LAC, MAC, EAC, BAC and the platelet count. No significant differences were found between control and experimental group for these parameters.

Cross sections of the ovary and liver were studied to assess the *in vitro* effects of the herbal extract. These two organs were specifically chosen and assessed due to its sensitivity to any changes in toxicity levels in its surroundings and for insight on the effect of these herbal remedies on reproductive health. The ovary of the control and experimental groups showed no vital differences at a macro-architectural level (Fig. 7A and B). A single, focally pseudostratified layer of modified peritoneal cells making up the surface epithelium covers ovaries. These cells can either be flat, cuboidal or columnar. Histochemical studies have shown that glycogen, acid and neutral mucopolysaccharides occur within the surface epithelium. The cross-section of the ovaries was compared to other normal and abnormal slides; slides made of the control group's organs that also served as a reference as well as textbook pictures. No obvious differences could be noted in the macroarchitecture of the ovaries.

The hepatic lobule is the functional unit of the liver. Cross-sections of the control and experimental group's organs were compared with each other as well as textbook pictures and other slides of normal healthy liver cross-sections. The liver also showed no major differences between the experimental and the control group (Fig. 7C and D).

From the study it is apparent that *C.capensis* have anti-infective value against a number of harmful pathogens. The parameters investigated (metabolic, blood and histological) to assess the safety profile of the herb indicated that the herb in the current study caused no negative effects. It will however be favourable to embark on further scientific experimentation to ensure its safety profile and to assess its mode of action.

Chapter 3

The study of *Carpobrotus acinaciformis* as a possible antimicrobial agent and its effect on animal metabolism

Abstract

C.acinaciformis is believed to have antimicrobial activity but not a lot of scientific information is available for this specific species. Anatomical cross sections were made of the leaf of the plant. These cross sections showed a tannin cell/gland situated next to the vascular bundle. A high tannin content is a common feature of *Carpobrotus* species. Elemental levels also tend to affect the anti-infective qualities of plants. The plant and soil samples that were collected and analyzed indicated that Ca^{2+} was the highest element in the soil and Na^{+} was the highest element present in the leaf of *C.acinaciformis*. In the current study *C.acinaciformis* was tested for any antimicrobial activity against *C.alcbicans, S.aureus, P.aeruginosa* and *M.smegmatis* using the disc diffusion method. The 30mg/ml, 40mg/ml and 80mg/ml concentrations of the dried leave extract successfully inhibited the growth of *S.aureus*. Both the fresh and dried leave extract showed medium activity against *M.smegmatis* at the 80mg/ml concentration. To asses the plant at an in vivo *level* the dried extract were fed to Wistar rats (n=10) that were compared to a control group (n=10) receiving water over a 45-day period. Metabolic readings were recorded at 15-day intervals. Metabolic measurements included rat mass, food consumption, water intake, urine excreted, urine pH and stool mass. Significant differences were measureed at a minimum level of $P<0.05$. Food consumption was significantly more for the experimental group on day 45 ($P<0.05$). Urine excretion showed a significant increase for the control group on day 15 ($P<0.05$) and day 30 ($P<0.05$). pH of urine samples were significantly different on day 15 ($P<0.05$), day 30 ($P<0.0001$) and day 45 ($P<0.0001$). Stool mass was significantly higher for the experimental group on day 15 ($P<0.01$) and day 30 ($P<0.05$). Whole blood samples were collected and analysed for RBC (Red blood cells), WBC (White blood cells) and platelets. The results showed that the RDW and neutrophils were significantly higher in the control group at $P<0.05$ and $P<0.01$ respectively. Histological sections was also made of the ovaries and the liver of the animal groups. No obvious macro architectural differences were noted.

The results indicated that *C.acinaciformis* have moderate anti-infective value but additional in-depth studies is necessary to assess its safety and possible mode of action.

1 Introduction

The use of herbal medicines to combat a wide range of infectious diseases has been practiced for as long as one can remember (Abelson 1990). It may not always have been as prominent during the modern age but as of late "going back to our roots", literally and figuratively, seems to be the more viable and safer option at hand.

This is especially applicable ever since the abuse of conventional medicine is causing antibiotic resistance globally. Lately the increase of infections with resistant microorganisms has led to heightened interest in the discovery of new antimicrobial compounds (Salvat et al. 2001; Palombo & Semple 2001; Monroe & Polk 2000; Davies 1994). More and more resistant strains are arising. This follows the massive problem in 1977 caused by penicillin resistance and multiresistant pneumococci in South-African hospitals (Eloff 1998). The emerging of drug resistance was labelled as a medical catastrophe by Berkowitz (Eloff 1998). According to Leggiardo, effective regimens may not be available to treat some enterococcal isolates, which is of utmost importance to develop new antimicrobial compounds for these and other organisms (Eloff 1998; Martini & Eloff 1998).

As recorded by the World Health Organization approximately 80% of the population in developing countries make extensive use of traditional medicine (Eloff 1998). Plants are known to have many biologically active compounds and numerous plants with tested antimicrobial properties (Palombo & Semple 2001; Cowan 1999).

With present numbers of ineffective, and in most instances, unaffordable drugs on the rise, the search for effective alternative sources that are more affordable is of utmost importance (Watt & Pretorius 2001; Salie et al 1996). Natural products extracted from plants are a potential source (Watt & Pretorius 2001; Farnsworth 1994).

Natural plants and their derivatives have been used in most parts of the world for thousands of years and still are very popular to this day (Palombo & Semple 2001; Van der Watt & Pretorius 2001; Chariandy *et al* 1999). Southern Africa contains approximately 10% of the world's plant diversity but only a small portion of this has been studied intensively (Eloff 1998).

The Cape Fynbos consist of an infinite number of species that is rich in secondary metabolites and may be the solution to the problem that we are currently faced with. One of these species include *Caprobrotus acinaciformis*

The aim of my study is to test the plant for any antimicrobial activity and to investigate it at an *in vivo* level on animal metabolism.

2 Materials and Methods

2.1 Ethics

Ethical approval for this investigation was obtained from the University of the Western Cape (UWC).

2.2 Plant distribution

The plant specimen was collected in the Western Cape region. *Carpobrotus acinaciformis* was dry and no flowers were observed. It occurred around an altitude of 60m at 34°21'54S21°25'26E in the Pauline Bohnen Reserve. This specimen was dried out and was predominantly reddish. No other populations were noted. No herbarium samples were collected.

22.3 Plant Anatomy

The plant sample that was collected was placed in FAA (Formaldehyde, Acetic acid and Alcohol) for later anatomical studies. Cross sections were made of the leaves of the plant using a freeze-microtome. The leave was cut into smaller sections that were used for sectioning. Liquid CO_2 gas and Hamilton's freeze solution was used to freeze the leave sample and sections were made with the attached blade ranging from 15-25 microns.

The sections were then fixed on slides, stained with Safranin and Alcian blue and viewed under a microscope. A DP11 Olympus photomicroscope was used to capture digital photos containing images of the vascular bundles of the species. This was performed to assess the possible location and type of active ingredient in the plant organ used as herbal medicine.

2.4 Phytochemistry and soil analysis

2.4.1 Extraction Methods

A portion of the plant specimens that was collected was fixed in methanol for later extraction. The remaining specimens were dried in the oven at 30°C-40°C. This was done in order to derive extracts from a fresh sample as well as a dried sample to assess if there was any significant difference in the secondary compound characteristics.

2.4.2 Fresh sample

The methanol was filtered from the samples and put into round-bottom flasks and then rotary evaporated. Samples were placed in the methanol for a second time and blended. This was filtered, added to the first extract and evaporated again using a rotary evaporator to eliminate the remaining methanol. The extracts were frozen in a freezer, freeze-dried with a freeze-dryer and stored in the 5°C cooler room for later analysis.

2.4.3 Dry plant and soil sample

The plant and three soil samples that were collected were stored in a Memmert UML70 oven at a temperature of 30°C-40°C and 70°C respectively. After the plant material was dried it was ground with a grinder and the soil samples was sieved to get rid of the access waste.

Four grams (4g) of each soil sample and 0.8g of the ground dried plant samples were weighed out and put into long glass flasks for digestion:

2.4.4 Digestion procedure

- The plant and soil materials were digested using an acid digestion method (Allen *et al.* 1976):

- The digestion mixture was prepared as follows- 0.42g Se and 14g $LiSO_4$. H_2O were added to 350ml of H_2O_2. The flask was put in a container with ice before the 420ml of concentrated H_2SO_4 was added to the mixture in order to keep it cool.
- 4.4ml and 8.8ml of the digestion mixture were added to the soil and plant materials respectively.
- The flasks were placed in the digestion block and heated to a temperature of 220°C.
- The heat was increased by 50°C for every 30 minutes until a temperature of 350°C was reached.
- After a period of 20 minutes it was increased to 380°C at which the digestion was continued until a clear and colourless or apple green solution was obtained.
- The soil and plant samples were diluted with distilled water and filtered into a 100 ml volumetric flask.
- This was topped up to a 100 ml with distilled water and stored in white plastic bottles for further use.
- Blank solutions (H_2SO4) were also prepared using the same method.

2.5 Atomic Absorption Spectrophotometry (AA)

This procedure was performed to analyze the elemental concentrations in the digested soil and plant samples using a Unicam Solaar M Series Atomic Absorption Spectrophotometer. Standards were prepared to analyze the soil for Ca^{2+}, K^+, Mg^{2+}, Na^+ and Cu^{2+} concentrations. An air acetylene flame was used to determine all the elemental concentrations.

1000 mg/L Merck Ca^{2+}, Na^+, Mg^{2+}, K^+, Cu^{2+}, stock solutions were used to prepare the standards used in AA analysis. This was topped up with 1% H_2SO_4 in a volumetric flask in order to obtain a concentration of 100 mg/L. These were used to prepare standards within a range of 0.05 mg/L – 50 mg/L.

2.5.1 Biological sample preparation

Samples used in the AA were diluted from 15 ml to 75 ml with distilled water.

2.6 UV/Visible Spectrophotometry

Phosphorus (P)

- P was analyzed as in Murphy and Riley (1962):
- 10ml of the sample solutions was pipetted into 50ml volumetric flasks.
- The solutions were diluted.
- 8ml of Murphy and Riley reagent was mixed with the solution.
- The solutions were then left for an hour.
- The absorbance of each sample was read at 700nm using the UV/Visible Spectrophotometer.

2.7 pH analyses

pH measurements were also determined for each soil sample as well as the urine samples that were collected from the animal models at a later stage. A PHM83 Autocal pH-meter was used.

2.8 Antimicrobial evaluation

2.8.1 Herbal extract

A fraction of the plant samples collected was stored in methanol for approximately 48 hours. The methanol was filtered from the samples and put into round-bottom flasks and then rotary evaporated. Samples were placed in the methanol for a second time and blended. This was filtered, added to the first extract and evaporated again using a rotary evaporator to eliminate the remaining methanol. The extracts were frozen in a chest freezer, freeze-dried with a freeze-dryer and stored in the 5°C cooler room.

2.8.2 Micro-organisms

The two bacteria, *Staphylococcus aureus* (ATCC29213), *Pseudomonas aeruginosa* (ATCC 27853) and the yeast *Candida albicans* (ATCC 10231) were obtained from the Department of Medical Biosciences at the University of Western Cape. *Mycobacteria smegmatis* was obtained from Tygerberg hospital, in Tygerberg city.

The agar plates containing the cultures were stored in a cooler room at 5°C until the onset of the antimicrobial experiment.

2.8.3 Disc diffusion

Individual nutrient broths containing, *Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans* and *Mycobacteria smegmatis* were streaked onto nutrient (Difco) and mycobacteria 7H11 (Difco) agar plates respectively. A sterilized swab was inserted into the suspension and was used to make streaks on the agar plates. The spreading over the dish was made in three different directions to ensure an even-growing bacterial and fungal mat.

Sterilized nine-mm filter paper discs were impregnated with 50µl of methanol and aqueous extractions varying in concentrations. These were placed at equidistant spots on the inoculated agar plates. Methanol and water discs were used as controls.

Amphotericin B served as positive control for *C. albicans* and Ciprofloxacin was used for the three bacterial strains. The positive controls were supplied by the companies Bristol-Myers Squibb and Bayer respectively. Different plant extract concentrations were prepared. The concentrations were 10mg/ml, 20mg/ml, 30mg/ml, 40mg/ml and 80mg/ml. The plates were incubated at 37°C for 24 hours with the exception of *Mycobacteria* that required a growth period of 48 hours. The extracts were tested in triplicate. At the end of the incubation period, inhibition zones of the different plant extract concentrations were recorded by measuring the growth-free zones between the discs and the bacteria or yeast. Sensitivity of the zone size was measured at a minimum of 1mm.

2.9 Animal studies

2.9.1 Animal groups

20 Wistar female rats with weights ranging between 150 g and 200 grams were used as animal models. These were obtained from the Medical Research Council (MRC). The rats were divided into two groups, each consisting of 10 rats. Groups included 1) Control Group, 2) Group fed with *C. acinaciformis* extracts.

Rat models were chosen for the laboratory experimentation because it is an internationally accepted animal model for both medical and other types of research.

The four-week old female rats were housed in cages with wire-mesh floors, two per cage. The room temperature was kept at 23°C and a day-and-night light cycle was maintained. A diet consisting of food pellets and distilled water was available *ad libitum*. The two-week acclimatization period were followed by gavaging and metabolic runs every 15 days.

2.9.2 Medicine preparation and administration

Fresh plant material was collected in the Pauline Bohnen Nature Reserve in Stilbaai. This was dried in an oven at 40°C and prepared as follow:

C.acinaciformis

- 25g of the diced, dried leaves were boiled in 500ml distilled water for duration of 15 minutes. The specific dosage administered and preparation of the herbal medicine were as prescribed and recommended by the herbalists selling these medicinal plants and remedies.
- Once the medicines were cooled it was filtered and the filtrate (used as medicine) was stored in a fridge. Medicine was prepared at a weekly interval.
- The dosage used was related to body weight. This was derived from the dosage prescribed for human consumption by traditional herbalists where an average weighing individual (75kg) consumed three cups (250ml) of the herbal remedy per day. Expressed in grams this was equivalent to 1ml of the medicine per 100g. The average weight of the female rats was 200g thus resulting in a dosage of 2ml/200g, also expressed as 2mg/0.2kg.

2.10 Metabolic studies and tissue histology

Metabolic data were collected every 15 days over a 45-day period, when the animals were placed in metabolic cages. The rat weight was recorded before and after the 24-hour metabolic run. Only 40g food pellets and 60ml distilled water was available during a run.

At the end of the run the food and water consumption were measured and urine volume and stool wet weight were recorded. Urine and stool samples were prepared for elemental analysis with the latter being dried in a Memmert UL 40 oven at 90°C for 24 hours to determine the dry weight as well.

Baseline readings of all three groups were taken before the medicine was administered. The medicine was fed to the experimental groups via gastric gavaging using bulb-tipped needles. A dosage of 2ml (related to bodyweight) of the *C.acinaciformis* filtrate was administered every morning. The control group received distilled water instead of medicine. This was followed by metabolic measurements that were taken at days 15, 30 and 45.

At the end of the experimental period the rats were sacrificed under chloroform-ether anesthesia. Approximately 3 ml of whole blood was collected by cardiac puncture and collected in an EDTA vacutainer. The blood samples analyzed via a coulter counter. Blood parameters included red blood cell counts (RBC), haemoglobin (Hb), haematocrit (Ht), mean cell volume (MCV), mean cell heamoglobin (MCH), mean cell haemoglobin count (MCHC) and the RDW (Red Cell Diameter). The white blood cell count (WBC), neutrophils, lymphocytes, monocytes, neutrophil abs count (NAC), lymphocyte abs count (LAC), monocyte abs count (MAC), eosinophil abs count (EAC) and the platelet were also analyzed.

Furthermore, a number of organs including the ovaries and liver were collected for analyses. These were preserved in Bouin's soloution. Small sections of these tissues were processed in a histokinette for approximately a 22-hour period and embedded in wax. Histology was performed on the ovaries and liver using a microtome and sections were made at 5 microns. These two organs were selected because it is most vulnerable and susceptible to toxicity and serves as an indicator of any toxic changes in the system.

The sections were then put onto slides, stained using a Heamotoxylin and Eosin stain and mounted with DPX. The liver and ovary slides of the experimental group were then compared to slides of the control group's organs and textbook pictures of normal and healthy livers and ovaries to assess its appearance.

2.11 Statistics

Data was analyzed using the 2000 Microsoft Excel Statistical package. Due to the significant difference at the baseline for a number of parameters, baseline corrections (elimination of the significant differences in rat weight) were made to the data for the respective collections made over the experimental period, after which statistical analysis was applied.

Control and experimental animal groups were compared with one another and a minimum significance of $P<0.05$ was accepted as significant for all metabolic parameters using the Mann-Whitney test.

Baseline corrections were not applied to blood data, which was directly compared. In this regard, significant differences were also determined at a minimum level of $P< 0.05$ with the Mann-Whitney test.

2.12 Results

2.12.1 Herb histology

Fig. 1 A *Carpobrotus acinaciformis*

Van Wyk et al. 1997

Fig. 1 B Cross section of the leaf of ***Carpobrotus acinaciformis***

Magnification-200x

Fig. 1C Cross section of the leaf of ***Carpobrotus acinaciformis***

Magnification-200x

2.12.2 Elemental profiles

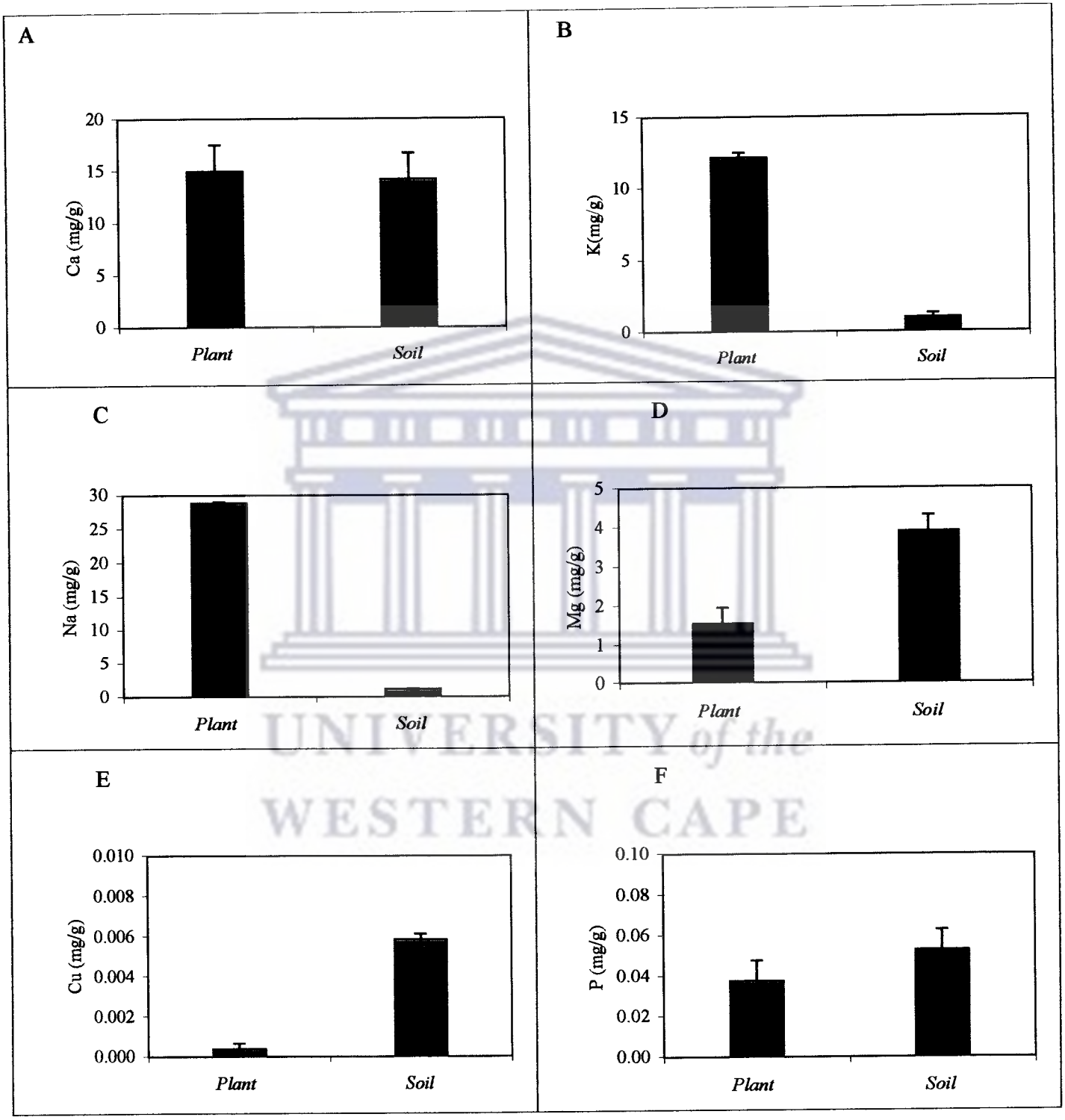


Fig. 2A-F: Elemental levels of the leaves of *C.acinaciformis* (n=1) and the soil in which it occurred (n=3).

Elemental measurements were taken in one plant sample and three soil samples. The analyses of the plant and soil samples that were collected indicated that Ca was the highest element in the soil and Na was the highest element present in the leaf of *C.acinaciformis.* In turn Cu was the lowest element present in both the plant and the soil sample.

2.12.3 Antimicrobial assessments

Table1:Antibiotic activity of *C.acinaciformis* against four microorganisms[a] (mm)

Plant	Extract	[ ]	*C.alb*			*P.aer*			*S.aur*			*M.smeg*		
			fresh	dry	Am[b]	Fresh	Dry	Ci[b]	fresh	dry	Ci[b]	fresh	Dry	Ci[b]
C.acinaciformis	MEOH	30	-	-	8	-	-	6	-	<1	7	-	-	12
		40	-	-	8	-	-	5	-	1	7	-	-	13
		80	-	-	9	-	-	6	-	2	7	-	1	13
	Water	-	-	-	7	-	-	6	-	-	8	1	-	14

[a]*Candida albicans (C.aclb), Pseudomonas aeruginosa (P.aer), Staphylococcus aureus (S.aur)* and *Mycobacteria smegmatis (M.smeg)*

[b] Amphotericin (Amp), Ciprofloxacin (Ci)

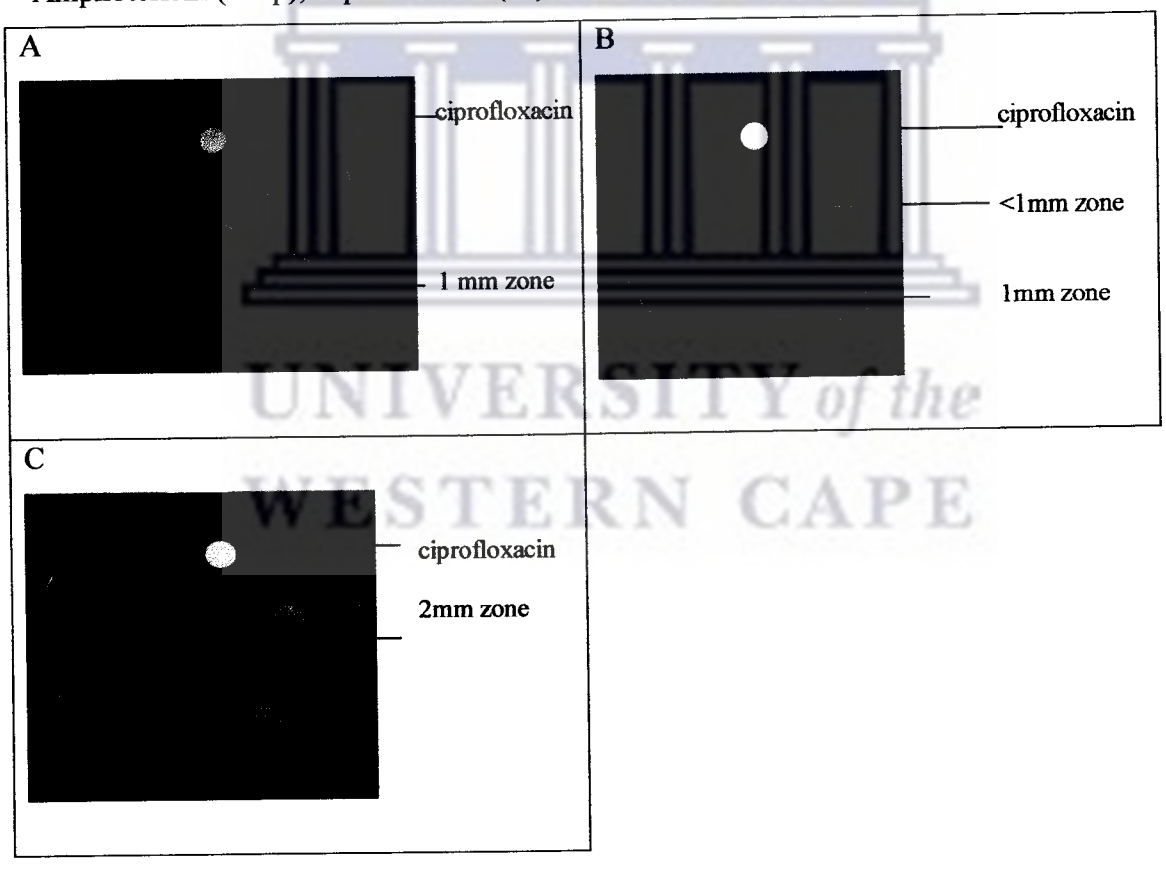


Fig. 2 (A) *M. smegmatis* plate with *C.acinaciformis* (80mg/ml); (B) *S.aureus* plates of dried *C.acinaciformis* (30 & 40mg/ml); (C) *S.aureus* plates of dried *C.acinaciformis* (80mg/ml).

The antimicrobial results of *C.acinaciformis* showed minimal activity against *M. smegmatis* and *S.aureus*. *C.acinaciformis* is a very fleshy plant that allowed for the preparation of both a methanolic as well as a water-based extract of the fresh fleshy leave. The water extract was less effective against the organisms then the methanol extract and only the 80mg/ml concentration was active against *M. smegmatis*.

2.12.4 Metabolic outcomes

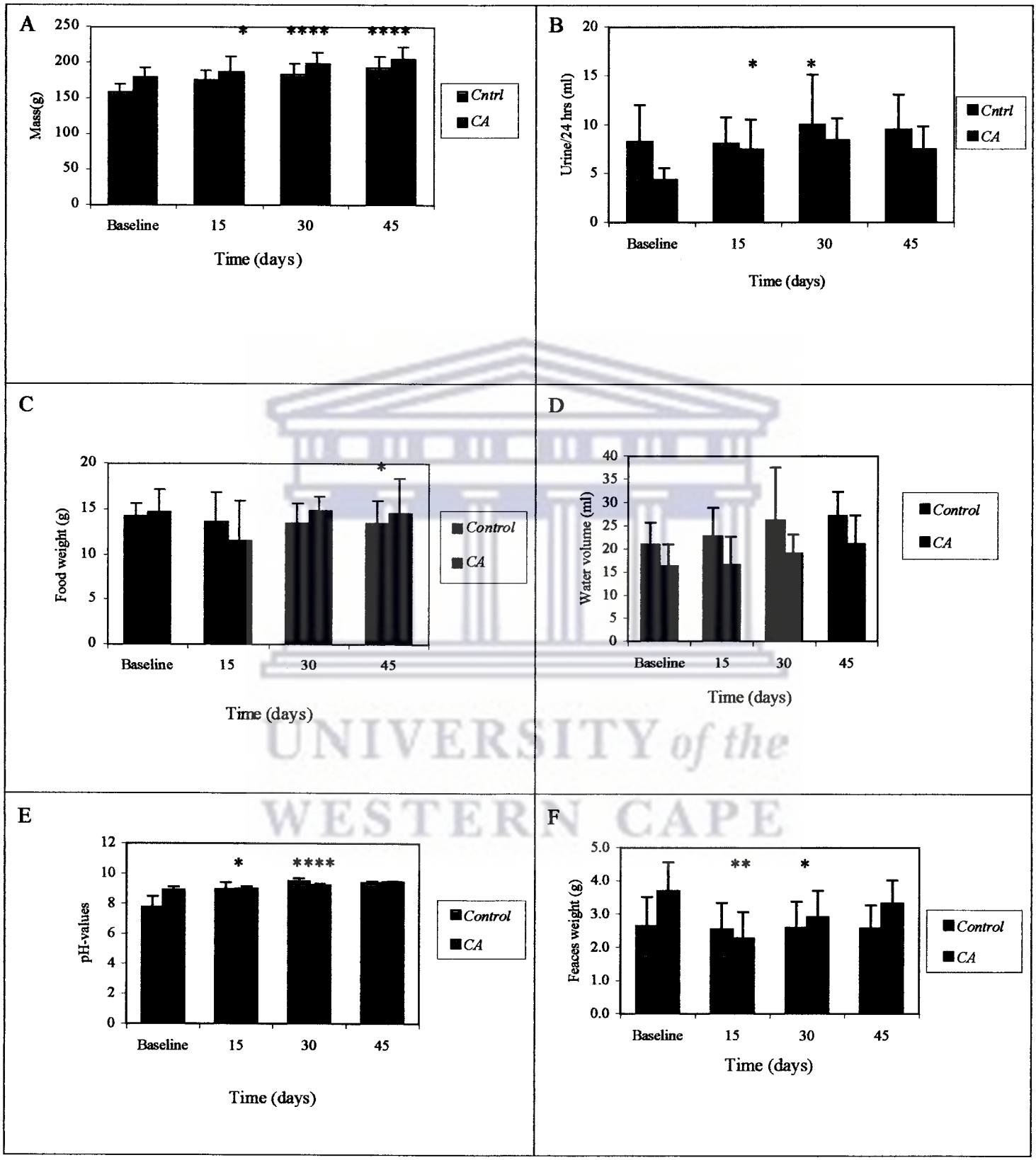
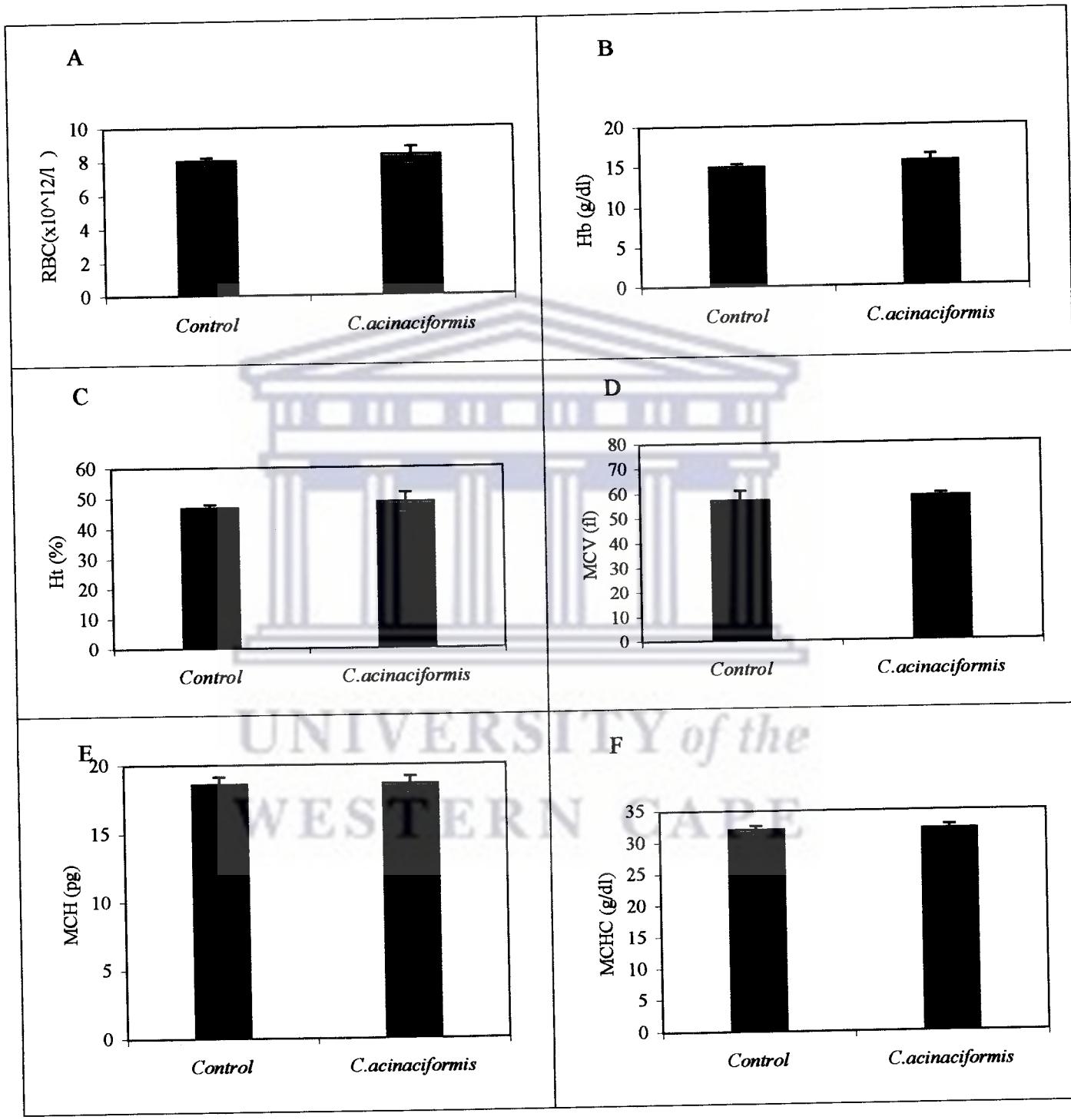


Fig. 2a-f: Different metabolic parameters measured in control and experimental groups over 24 hours for 45 days. Significance in relation to control and experimental group: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Significant differences were measured at a minimum level of $P<0.05$. Food consumption was significantly higher on day 45 ($P<0.05$). The control group's urine excretion had significant increases on day 15 ($P<0.05$) and day 30 ($P<0.05$). pH of urine samples were significantly different on day 15($P<0.05$), day 30 ($P<0.0001$). Stool mass showed significance on day 15 ($P<0.01$) and day 30 ($P<0.05$).

2.12.5 Hematological profiles



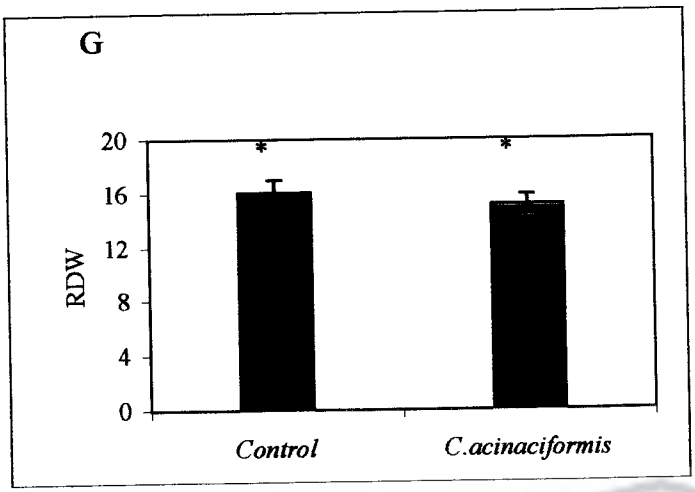
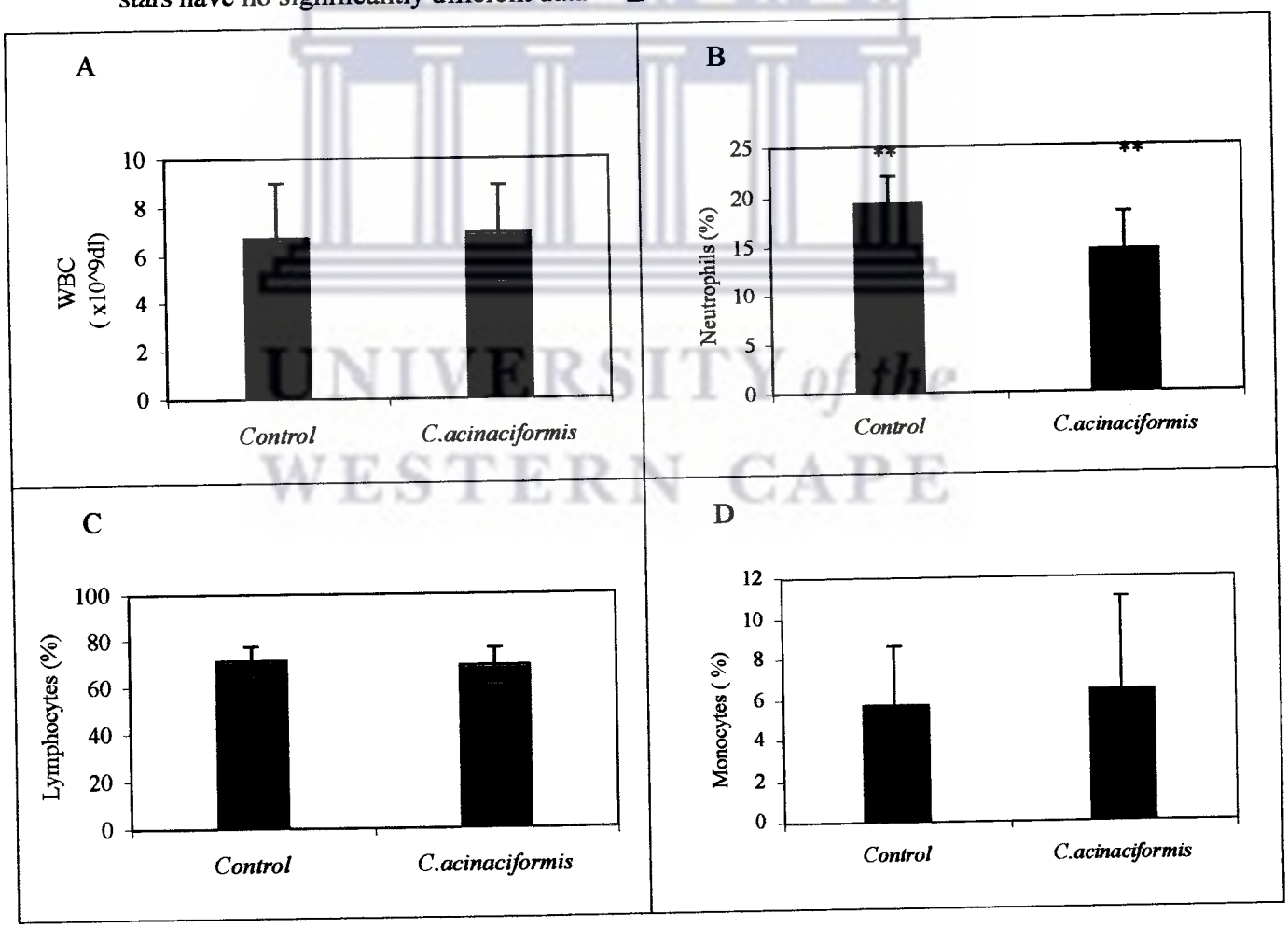
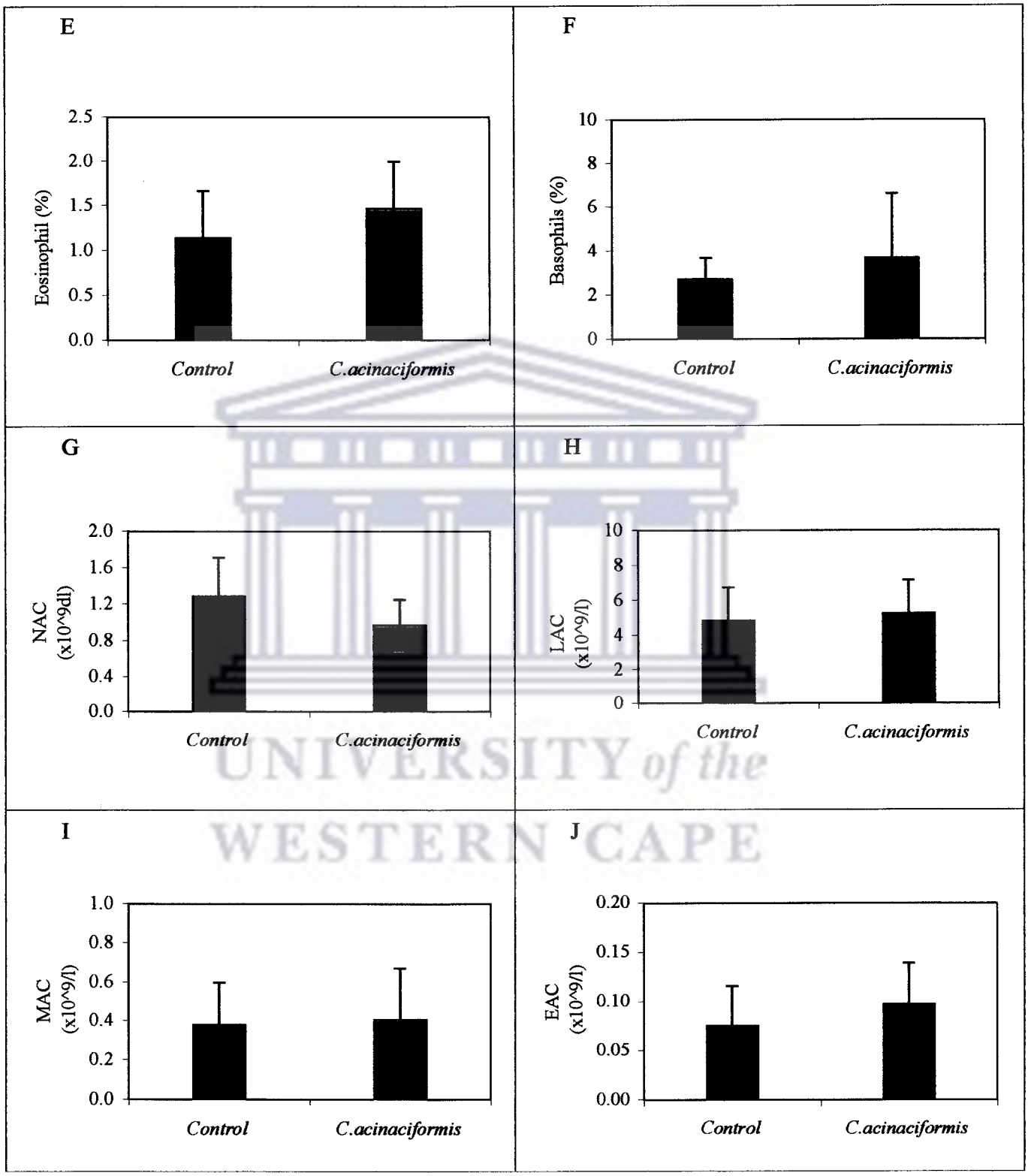


Fig. 5A-G: RBC parameters measured in whole blood samples of control and experimental groups. Significance in relation to control and experimental group - *P≤ 0.05, **P≤0.01. Panels with no stars have no significantly different data - P≥0.05.





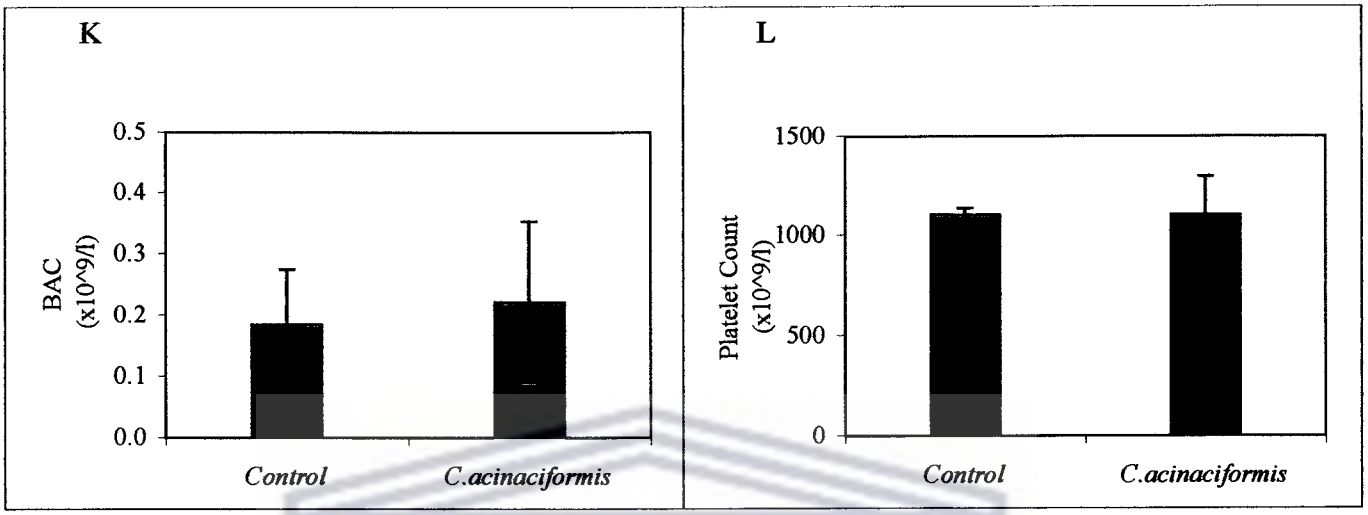


Fig. 6A-L: WBC parameters and platelets measured in whole blood samples of control and experimental groups. Significance in relation to control and experimental group - *P≤ 0.05, **P≤0.01. Panels with no stars have no significantly different data - P≥0.05.

The hematological results show that there was not much noticeable variation in the red blood cell and the white blood cell parameters measured with the exception of the RDW and the Neutrophil count respectively. The RDW, a red blood cell parameter, and the Neutrophil count, a white blood cell parameter, was significantly higher in the control groups receiving water in comparison with the experimental rat group that was fed the *C.acinaciformis* filtrate. Significance occurred at the minimum level of P≤ 0.05.

2.12.6 Animal histology

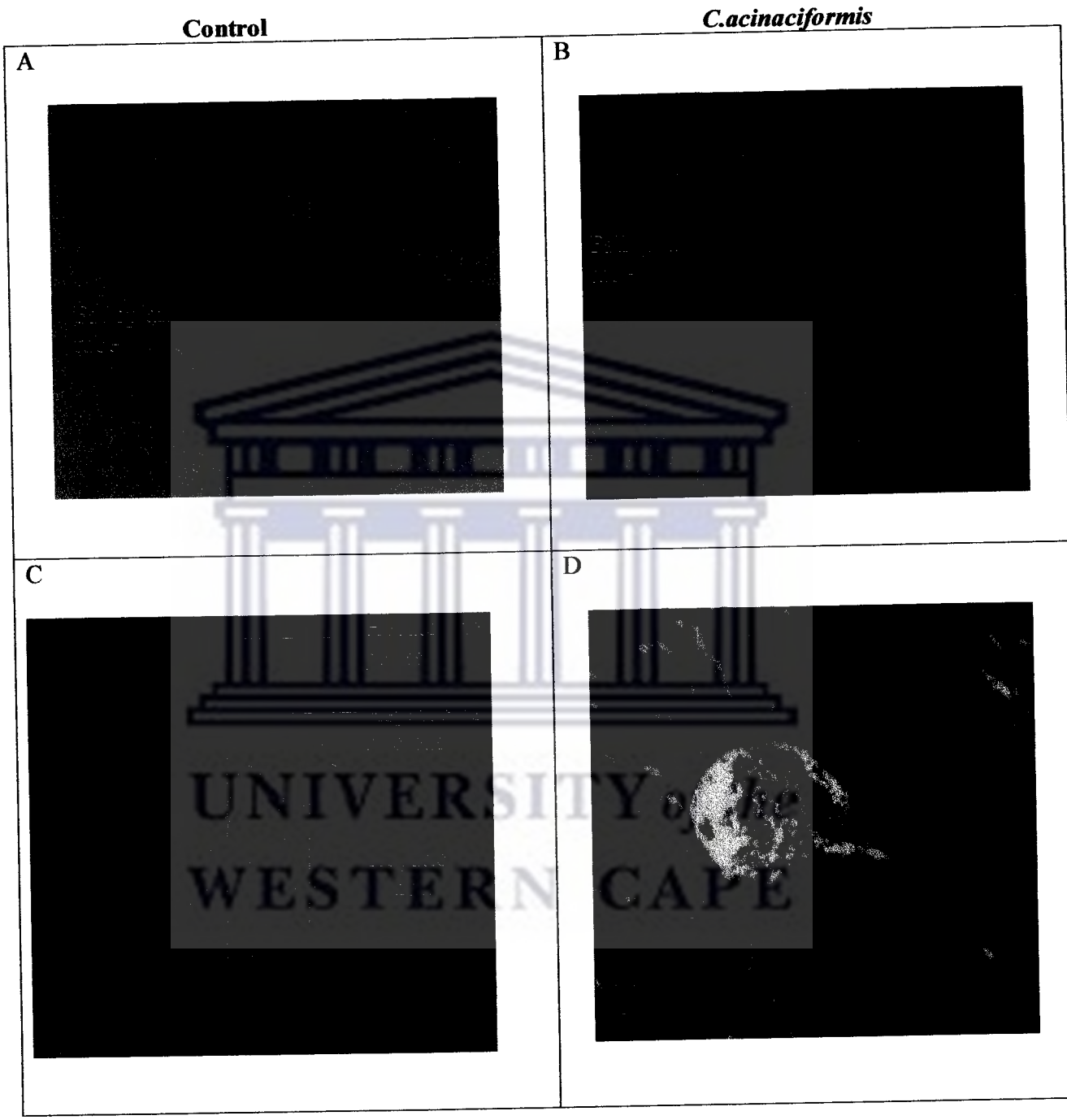


Fig. 7 (A) Ovarian cross section section of control group (40x magnification); (B) Ovarian cross section of experimental group (40x magnification); (C) Liver cross section of control group (100x magnification); (D) Liver cross section of experimental group (100x magnification.

The ovarian and liver sections were compared to slides and textbook pictured of normal and healthy organs to determine whether the herbal medicine had any negative affect on the tissues most sensitive to toxic alterations within the system. Comparison showed no significant differences for both the ovaries and the livers of the experimental and control groups.

2.13 Discussion

The section of the leave of *C.acinaciformis* clearly shows various plant organs. Of interest is the gland/cell above the vascular bundle (Fig. 1B). The colour of the gland may be an indication of the tannin content of the leaves. The leaves of *Carpobrotus* species are known to have high tannin content. Tannins have different effects on biological system because they are potential metal ion chelators, protein precipitating agents and biological antioxidants. They even cause side effects in animals and man (Van der Watt & Pretorius 2001). It obstructs TLC-separation of plant extracts and may even mask the effect of other biologically active compounds. With the removal of tannins from extracts, the plant may show more effective antimicrobial properties. The leaves also contain flavanoids, which is well known for their antibacterial activity (Van der Watt & Pretorius 2001).

All the elements tested have some relation to reproductive health. Different plants have varying elemental needs and therefore will not absorb elements in similar concentrations or ratios. There are a number of factors that determine the availability of essential elements. Some of these are the soil pH, chelators (compounds that increase solubility by electron donation), the make-up of the soil and other elements (Williams 1963).

The alkaline soil from which the plant was collected played a role in nutrient availability. It is known that alkalinity decreases the availability of nutrients (Moore *et al* 1995). Only certain elements, e.g. Ca^{2+} are readily available in lime soils (Fig. 2A). Ca^{2+} is a very important component in the cell wall. K^+ was excessively higher in the plant in comparison with the soil (Fig. 2B). K^+ is known as the quality nutrient because it is involved in many functions of the plant and as a result is normally absorbed in high concentration by plants (Williams 1963).

Na^+ levels were extremely low in soil yet very high in the plant sample (Fig.2C) *Carpobrotus* is a succulent plant. The high Na^+ levels could perhaps influence the water-influx of the plant by creating a gradient for successful water absorption (osmosis).

Na^+ is not an essential element but it is known to have an effect on water relations in plants. Mg^{2+} is more readily available in acidic soils, which possibly explains the lower concentration in plant sample (Fig. 2D). This accounts for Cu^{2+} (Fig. 2E) as well, which was higher in the plant sample (Williams 1963). P is easily absorbed in alkaline soils but the plants do not require this element in high concentrations (Fig. 2F).

In order to assess the anti-infective potency of *C.acinaciformis,* both the fresh and dried leaf extracts were tested against the four selected pathogens. Only the highest concentrations of both fresh and dried sample inhibited *M. smegmatis* (Table 1, Fig. 3A). The dried sample of *C.acinaciformis* that was tested was more potent than the fresh sample (Table 1, Fig. 3B-C). It was most effective against *S.aureus*, gram-positive bacterium. This follows the trend already set by previous papers (Chariandy *et al.* 1999). Previous studies have shown that gram-negative bacteria are more resistant against antibiotic in comparison with gram-positive bacteria (Paz et al 1995; Vlietnick et al, 1995; Kudi et al. 1999; Palombo & Semble 2001; Van der Watt & Pretorius 2001). This phenomenon may possibly be due to the difference in the cell wall structures of these two pathogens. The cell wall of gram-negative bacteria acts as a coarse sieve, preventing the entrance of any harmful substances (Kaiser 2002). The antibiotic can only effectively destroy the bacteria by preventing the synthesis of peptidoglycan (makes up a portion of the cell wall) causing the cell to rupture (Kaiser 2002). Although Carpobrotus spp. are known for their antimicrobiaal activity, *C.acinaciformis* did not perform optimally against the selected pathogens. The tannin content may be influencing or affecting the antimicrobial activity of the flavanoids in the plant. Masking of bio-active compounds is a common occurrence and in such cases separation of these compounds is necessary for assessment.

It is important to perform in *vivo* studies of the herbal extract to investigate its effects at a cellular level. No significant differences were apparent in the mass of the animal groups over the experimental period (Fig. 4A). Urine volumes were significantly higher ($P<0.05$) during day 15 and day 30 for the experimental group (Fig. 4B).

Metabolic studies showed that the experimental group ate significantly more ($P<0.05$) food in comparison with the control group during day 45 (Fig. 4C). This could again be explained by the fact that the experimental group weighed more then the control group and therefore needed to consume more food to supplement its energy supplies. Water intake was not significantly different (Fig. 4D). The urine pH for day 15, day 30 and day 45 were significantly higher for the control group (Fig. 4E). Significance ranges from $P<0.05$ to $P<0.00001$. Even though both the control and experimental groups had alkaline urine, it is possible that the consumption of the medicine may cause a decrease in then alkaline pH of the urine. During days 15 and 30 (Fig. 4F) stool had a significant difference of $P<0.01$ and $P<0.05$ respectively. A correlation can be noted between the amount of food consumed, the water intake and weight of the feaces excreted.

The blood parameters measured included the RBC, WBC and the platelet count. Two blood parameters showed significant differences. RDW and neutrophils were higher in control group as to the experimental group (Fig. 5G and Fig. 6B). Significant levels were $P<0.0.5$ and $P<0.01$ respectively. Neutrophils constitute 40-70% of circulating leucocytes. Their primary function is in the inflammatory response to tissues damage by ingesting and destroying injured tissue as well as invading microorganisms. They are particularly attracted by the presence of bacteria. Since the neutrophil count was higher in the control group it could possibly be that it had a slight bacterial infection at the time of blood collection.

The surface epithelium covering the ovaries consists of a single, focally pseudostratified layer of modified peritoneal cells. These cells can either be flat, cuboidal or columnar. Studies have shown that various medicinal plants produce secondary metabolites that may have an affect on reproduction in man and various mammals (Butenandt and Jacobi 1933, Lightfoot *et al.* 1967, Sakomato *et al.* 1988 and Telefo *et al.* 1988). The ovaries were selected for its sensitivity to toxins and to investigate the medicinal remedy's effect on reproductive health. The ovary of the control and experimental groups showed no vital differences after comparison with normal and abnormal ovarian sections (Fig. 7A and Fig. 7B).

Livers are tissues that are extremely sensitive to toxicological results. The functional unit of the liver is the hepatic lobule. The control group and the experimental group clearly depict the hepatocyte (Fig. 7C and Fig.7D). No vital variations were noted in the cross sections of the liver when it was compared to both existing liver slides and pictures in anatomical textbooks.

Considering the fact that the *C.acinaciformis* showed anti-infective activity, though minimal, together with the safety profile demonstrated in the study, one could suggest that it can serve as potential antimicrobial agent. The reason that it did not show any phenomenal antimicrobial properties can be attributed to the fact that the presence of tannins in the plant may have masked the activity of the flavanoids (known for its antimicrobial activity). Further studies need to be embarked on to investigate the full potential of this plant.

Chapter 4

Summary

WHO defined a medicinal plant as a plant that consists of either one or more plant parts with therapeutic properties that can be used in the development of drugs (Sofowora 1982). A great number of medicinal plants have been used over the years for their healing properties (Cowan 1999). Plant-derived remedies have been part of traditional healthcare in most parts of the world for centuries (Palombo & Semble 2001; Salie 1998).

Indiscriminate use of antibiotics has led to resistance of many bacterial strains and this compels us to a search for alternative sources due to the constant need for new effective therapeutic agents that are safe to use (Martini & Eloff 1999; Salie 1998; Salie *et al.* 1996).

The Cape Fynbos covers a nutrient poor area of approximately 90,000 km^2. It consists of a species richness of more than 8,600 (Salie *et al* 1996; Salie 1998; Elmqvist 1999). Considering this great diversity in plant species, it is clear that the Cape Fynbos represents a potential source of new anitmicrobial agents. This had led to the study of two potential Cape Fynbos species known as *C.capensis* and *C.acinaciformis.*

Elemental analyses of *C.capensis* showed that K^+ (6.21mg/g) was the highest element in the plant and Ca^{2+} (10.79mg/g) the highest element in the soil. The fresh and dried rhizome extract of *C.capensis* was tested against *C.albicans, S.aureus, P. aeruginosa* and *M.smegmatis*. *S.aureus* It is clear from the research performed *C.capensis* was most effective against *Staphylococcus aureus. M.smegmatis* was also successfully inhibited but it was less effective against *Candida albicans*. The rhizomes of a number of studied *Cissampelos* species are rich in bisbenzyltetrahydroisoquinoline-type alkaloids. Therefore the possibility exists that it can be used as a potential therapeutic agent against *Staphylococcus aureus, Mycobacterium smegmatis* and *C. albicans.*

Metabolic parameters also showed significance for the mass of the animal groups, their food consumption, water intake, urine excretion and the mass of stool excreted. Blood results showed no significant differences. There were no obvious differences between the ovary and the liver of the experimental and control group.

Cross sections of the leaf of C.acinaciformis showed an interesting gland/cell next to its vascular bundle. Its may be indicative of a high tannin content in the leaves. The plant and soil samples that were collected and analyzed indicated that Ca^{2+} was the highest element in the soil and Na^{+} was the highest element present in the leaf of *C.acinaciformis*. *C.acinaciformis* was less effective than *C.capensis* in its antimicrobial affect against the four pathogens. *Staphylococcus aureus* was most easily inhibited by the leave extract. Slight activity was noticed against *M.smegmatis*. Certain metabolic measurements showed significance at a minimum level of $P< 0.05$. The RDW and neutrophils of the control group were significantly higher ($P<0.05$ and $P<0.01$ respectively) than the experimental group. In conclusion it was noted that *C.acinaciformis* showed antibacterial properties against gram- positive and gram-negative bacteria, though it was minimal for the latter. Therefore it may possibly be used as a potential therapeutic agent against selected pathogens.

From the study it is apparent that *C.capensis* and *C.acinaciformis* have anti-infective value against a number harmful pathogens. The parameters investigated to assess the safety profile of both herbs indicated no apparent negative results. It will however be favourable to embark on further scientific experimentation to fully ensure its safety profile and to assess its mode of action. Isolation of active compounds are necessary to allow for a better understanding of which compounds are most active and the mechanism by which the medicinal plants inhibit the growth of harmful pathogens.

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