SCREENING OF FOUR PLANTS COMMONLY USED IN ETHNOVETERINARY MEDICINE FOR ANTIMICROBIAL, ANTIPROTOZOAL AND ANTI-OXIDANT ACTIVITY

A dissertation submitted in partial fulfillment of the
requirements for the degree of
Masters in Science

by

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2004

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ACKNOWLEDGEMENT

I would like to thank Prof Swan for the opportunity to do my masters degree in his department, and under his tutelage. More importantly I would like to express my thanks for his decision in appointing me as a lecturer in his department while completing these studies.

Prof Kobus Eloff from whom I learned to interpret the data, and more importantly how to write it up as a scientific paper.

Dr Erik Zweygarth, without whom I would have never been able to carry out the anti-protozoal and anti-rickettsial screening.

Dr Luseba Jean-Marě: Thanks for your help during the collecting of the Aloe from Proef - plaas, and for the use of the OVI herbarium for the storage and identification of the plant material.

Havana Chicoto, for trying and eventually succeeding in teaching me the finer details of the polyphenol assay, and especially for his enthusiasm for running my sample on the trolox equivalent anti-oxidant capacity method. Without this, I would have never known that *Rhoicissus tridentata* was such an active plant.

Lastly and definitely not easily forgotten. Many thanks go out to Dr David Katerere for going that extra distance, which was above and beyond the call of his duties. It was his tireless enthusiasm and unending optimism that inspired me to carry out this project further than I initially intended. Through endless discussions and friendly fights, have I come to realise how much fun science can really be. Thanks for proving that I have made the right step in my life, by attempting to become a career researcher.

ABSTRACT

Urginea sanguinea, *Aloe marlothii*, *Elephantorrhiza elephantina* and *Rhoicissus tridentata* are all plants utilized for the management of tick borne diseases in the Madikwe area of North-west province. These plants, in certain concoctions, are believed to be effective against "*seme*", "*gala*" and "*Bolwetsi jwa mothlapo o moshibidu*" which we have assumed to represent heartwater, gallsickness and redwater from circumstantial epidemiological data available.

To obtain a representative extract, which would be indicative of the general activity of the plant, only acetone or methanol extracts were tested for the presence of antimicrobial, antiparasitic or anti-oxidant activity within that specific plant. Activity in all cases made use of either an *in vitro* biological assay or more specific chemical tests, which were validated in all cases.

Ehrlichia ruminantium, *Babesia caballi* and *Theileria equi*, all grown in specific cell cultures, were used as a model for evaluating the efficacy against the common protozoan and rickettsial diseases caused by these organisms in livestock. *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli*, four human nosocomial infectious agents, were used as an indicator for the presence of antibacterial activity against these common animal bacterial pathogens.

Diphenyl-picrylhydrazyl and the trolox equivalent anti-oxidant chemical assays were used to determine anti-oxidant activity, which although not curative, may aid in the recovery from an infection by stimulating the immune system.

The activities demonstrated among the various plants and organisms were not consistent. *E. elephantina* extracts were the most effective, with activity demonstrable in all biological and chemical screening assays. Although *R. tridentata* demonstrated poor activity (> 100 µg/ml) against the tick-borne parasites, the plant extract did demonstrate significant anti-oxidant activity. *U. sanguinea* extracts showed good activity in both the antibacterial and anti-rickettsial assays (EC_{50} = 44.49 ng/ml), which may be due to the presence of the toxic

bufadienolides present within the plant. *A. marlothii* possessed significant anti-rickettsial activity (EC_{50} = 111.4 µg/ml) and to a lesser degree antibacterial activity.

The results of the study support the use of these plants against heartwater, gallsickness and redwater, which gives credence for the traditional use against "*Seme*, *Gala*, and *Bolwetsi jwa mothlapo o moshibidu*". Further studies are required to isolate and determine the structure of the active compounds of these plants as well as to confirm the safety and efficacy of the extracts against disease conditions in livestock.

OPSOMMING

Urginea sanguinea, *Aloe marlothii*, *Elephantorrhiza elephantina* and *Rhoicissus tridentata* word tradisioneel gebruik vir die bekamping van siektes deur bosluise oorgedra in die Madikwe gebied van die Noordwes provinsie. Ekstrakte van hierdie species word gebruik teen "*seme*", "*gala*" en "*Bolwetsi jwa mothlapo o moshibidu*" wat waarskynlik op hartwater, galsiekte and rooiwater dui volgens die beskikbare epidemiologiese data.

Asetoon en metanol ekstrakte is gebruik vir die bepaling van antimikrobiese, antiparasitiese en antioksidant aktiwiteite in verskillende species deur gevalideerde *in vitro* metodes.

Selkulture van *Ehrlichia ruminantium*, *Babesia caballi* en *Theileria equi*, , is in 'n model gebruik om die doeltreffendheid van ekstrakte teen algemene siektes deur protozoa en ricketsias te bepaal. Vier algemene menslike nosokomiale patogene *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli*, is gebruik om antibakteriese aktiwiteit van ekstrakte te bepaal.

Difeniel-pikrielhidrasiel en die trolox ekwivalente anti-oksidant essajeermetode is gebruik om anti-oksidantaktiwiteit te bepaal. Antioksidante mag herstel na infeksies bespoedig deur stimulering van die immuunstelsel.

Daar was 'n groot verskil in die aktiwiteite tussen die verskillende ekstrakte en organismes. *E. elephantina* ekstrakte was die mees doeltreffende met die biologiese and chemiese bepalings. *R. tridentata* het sterk anti-oksidantaktiwiteit gehad, maar het lae aktiwiteit (> 100 µg/ml) teen bosluis-oorgedraagde parasiete gehad. *U. sanguinea* ekstrakte was aktief in beide die antibakteriese en anti-riketsiale bepalings (EC_{50} = 44.49 ng/ml), wat moontlik toegeskryf kan word aan die giftige bufadienoliede teenwoordig in hierdie species. *A. marlothii* ekstrakte het betekenisbolle anti-riketsiale aktiwiteit (EC_{50} = 111.4 µg/ml) maar slegs geringe antibakteriese aktiwiteit gehad.

Hierdie resultate bevestig die moontlike waarde van hierdie species teen hartwater, galsiekte and rooiwater, en ondersteun die tradisionele etnoveterinêre gebruik teen "*Seme*, *Gala*, and *Bolwetsi jwa mothlapo o moshibidu*". Verdere studies word benodig om die

aktiewe verbindings te isoleer en te karakteriseer en om die veiligheid en doeltreffendheid van ekstrakte teen hierdie siektes in vee te bevestig.

PAPERS PREPARED FROM THIS DISSERTATION

Naidoo V, Katerere D, Swan GE, Eloff JN, Pretreatment of *Urginea sanguinea* bulbs, used in ethnoveterinary medicine, influences chemical composition and biological activity, *Pharmaceutical biology*. (Accepted, not published)

CONFERENCE CONTRIBUTIONS FROM THIS DISSERTATION

Indigenous Plant Use Forum (IPUF), Rustenberg (South Africa), 2003
Paper: Naidoo V, Katerere DR Eloff JN, Swan GE, Extraction of Bioactive Compounds from fresh and dried parts of *Urginea sanguinea* used in ethnoveterinary medicine: Effects of freezing on extraction

University of Pretoria, Faculty of Veterinary Science, Faculty day, 2003
Paper: Naidoo V, Eloff JN, Swan GE, Extraction of bioactive compounds from fresh and dried parts of *Urginea sanguinea* used in ethnoveterinary medicine: Biological activity

Onderstepoort Veterinary Institute African Ethnoveterinary Conference, Dikulolo (South Africa), 2004
Paper: Naidoo V, Bioassays

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TABLE OF ABBREVIATIONS

Am	*Aloe marlothii*
ANOVA	Analysis of variance
CEF	Chloroform (5): Ethyl acetate (4): Formic acid (1)
Cl	Chloroform
D	*U. sanguinea* bulbs Fresh dried
DIC	Disseminated intravascular coagulation
DMSO	Di-methyl-sulphoxide
DNA	Deoxyribose nucleic acid
DPPH	Diphenyl-picrylhydrazyl
EA	Ethyl acetate,
EC	Effective concentration
Ee	*Elephatorrhiza elephantina*
EH:	Ethyl acetate (2): Hexane (1)
Elisa	Enzyme linked immuno-sorbent assay
EMW	Ethyl acetate (10): Methanol (1.35): Water (1)
F	*U. sanguinea* bulbs Fresh
H	Hexane
HE	Hexane (2): Ethyl acetate (1)
HW	Heartwater
INT	p-iodonitrotetrazolium violet
MH	Mueller Hinton broth
MIC	Minimal inhibitory concentration
MPC	Mean parasailed cells
Mvym	Modified Vega y Martinez phosphate-buffered saline solution
MW	Methanol/water
OVI	Onderstepoort Veterinary Institute
PPC	Percentage parasitized cells
PPI	Percentage parasitic inhibition
RNA	Ribose nucleic acid
Rt	*Rhoicissus tridentata*

T	*U. sanguinea* bulbs Thawed
TD	*U. sanguinea* bulbs Thawed Dried
TEAC	Trolox equivalent anti-oxidant
TLC	Thin layer chromatography
UV	Ultraviolet light
Us	*Urginea sanguinea*

CHAPTER I

INTRODUCTION

South Africa has a long tradition in the use of herbal remedies for the management and treatment of disease both in humans and livestock[103]. Traditional medicines are based on knowledge passed down from generation to generation and are largely dependent on the use of plants. This type of complementary medicine has become deeply entrenched in the beliefs and daily lives of the people involved.

In certain parts of the country, specifically the Madikwe region of the Northwest province, traditional use of plants and plant parts to treat a wide variety of cattle infections have been documented[93]. Three stock disease conditions of particular importance treated traditionally are "*gala*, *seme* and *bolwetsi jwa mothlapo o moshibidu*". These conditions appear to resemble gallsickness, redwater and heartwater, respectively.

These three diseases represent a major economic burden to the country[96], with the large-scale loss of either production or animal life. They also constitute a major threat to the sustainability of farming. The effective treatment of these diseases and the confirmation of the efficacy of traditional medicines is of utmost importance to small scale livestock farmers. We therefore decided to determine the biological activity of four plants known to be used for the treatment of "*gala*, *seme* and *bolwetsi jwa mothlapo o moshibidu*", under controlled laboratory conditions. The study intended to examine the potential activity of extracts of these plants and depending on activity, develop an alternate new therapeutic modality. Plants or plant parts showing no activity could be eliminated as possible treatments by small scale farmers.

1.1. HYPOTHESIS

Ethnoveterinary use of *U. sanguinea*, *E. elephantina*, *A. marlothii* and *R. tridentata* used for the treatment of "*seme, gala* and *Bolwetsi jwa mothlapo o moshibidu*" in cattle are effective against parasites causing babesiosis, anaplasmosis and heartwater or have inhibitory effects on *S. aureus*, *E. faecalis*, *P. aeruginosa, E. coli* and selected free radicals.

1.2. JUSTIFICATION OF THE STUDY

The results of this study will contribute towards the growing database of knowledge on ethnoveterinary medicines and help to advocate the safe and effective use of traditional herbal remedies. Ineffective remedies will be identified and excluded as treatment modalities in future.

The use of plants for treatment of animal disease by stock owners in developed communities, although rooted in the traditional method of treatment, is also largely due to the inability to afford veterinary consultation or the use of registered pharmaceutical products. It is hoped that the ratification of their use will allow farmers to use the best crude drugs suited to their needs.

It is believed that the screening of these drugs will also add to the ever increasing scientific database of medicinal plants, not only in South Africa but also globally. Any plant showing substantial activity will be studied further including the isolation and elucidation of the active component(s). The ever-increasing threat of antimicrobial and antiparasitic resistance necessitates the screening for and discovery of new compounds.

1.3. OBJECTIVES OF THE STUDY

To evaluate the efficacy of *Elephantorrhiza elephantina*, *Rhoicissus tridentata*, *Urginea sanguinea* and *Aloe marlothii*, which are used in ethnoveterinary medicines in South Africa, against *Babesia caballi*, *Theileria equi*, *Ehrlichia ruminantium*, *Escherichia coli*,

Pseudomonas aeruginosa, *Staphylococcus aureus* and *Enterococcus faecalis* under *in vitro* conditions.

As a secondary objective the plants will also be tested for their anti-oxidant activity, because compounds with high anti-oxidant activity may enhance the immune system of the infected animal and promote control of infective agents.

CHAPTER 2
LITERATURE REVIEW

2.1. PLANTS: A THERAPEUTIC GOLDMINE

Therapies of plant origin have been the backbone of human medicine for a number of millennia[23,34,35,78,82,93]. Similarly, there exists a history of plant-derived remedies being used in the management of disease in animals[35]. Whether rational or merely due to superstition, this practice has become deeply entrenched in many cultures, and farming systems, as the knowledge gets passed down from generation to generation. In South Africa these herbal medicines are being used widely in both animal and man[103].

Generally indigenous African medicinal healing systems can be divided into two broad categories[99]. The one category includes those conditions, which are treated without religious interventions and with known remedies, normally of plant origin. These medicines hold the key to the potential discovery of new beneficial compounds. The second category includes more seriously perceived conditions, ranging from serious accidents to chronic disease. These conditions are believed to be of supernatural origin and treatment therefore involves some sort of divination or communication with ancestral spirits, to appease the entity responsible for the condition[99].

With the history of utilization in both Africa and the rest of the world, it is not surprising that the higher plants continue to provide mankind with new remedies, and in some cases are important sources of old remedies. Van Wyk *et al* reported that natural products represent more than 50% of all drugs in clinical use in the world[103]. Well known plant-derived medicines include digoxin from *Digitalis spp.*, quinine from *Cinchona spp.*, morphine and codeine from *Papaver somniferum*, atropine from *Atropa belladona*, and pilocarpine from *Pilocarpus jaborandi*[82]. More recently new anticancer drugs such as taxol from *Taxus spp.* (*T. brevifolia* and *T. bacata*) and vincristine from *Catharanthus roseus* have been developed. The discovery of both taxol and vincristine has demonstrated the value of plants as an important source of new molecules[103].

Numerous surveys have been conducted throughout southern Africa and have documented plants, which are used in the treatment of human conditions[14,24,68,102,103]This database is continually expanding. Likewise, the use of plants in animals is also being documented throughout southern Africa. Surveys have been conducted in the Northwest Province[93], the Eastern Cape Province[25,67], Lesotho and Kwa-Zulu Natal[47]. In these surveys, it was shown that, certain communal farmers use herbal remedies on an extensive basis. In some instances it appears that farmers prefer these traditional methods of treatment to the modern "western" methods. In a study conducted in the Alice district of the Eastern Cape Province, by Dold *et al.*[25], it was shown that two thirds of the community believed that traditional medicines were more effective than "western" medications for certain diseases, notably redwater and heartwater.

With problems like the emergence of antibiotic resistance, plant medicines are being re-examine as a potential resource of new compounds[23]. Currently plants, which have been documented as traditional medicines, are being examined in the hope of finding new or improved medication. This includes research on the antimicrobial, anthelmintic, antifungal and anti-inflammatory activity of plant extracts, as well as on other aspects of systemic pharmacology[42,53,70,81,87].

Despite certain herbal remedies being used on an extensive basis to treat various disease conditions in animals by the rural populations of South Africa[93], there is very little research documenting their efficacy and safety. Since their use lacks proper scientific investigation, many of these treatments may actually not be effective or safe.

2.2. IMPORTANCE AND TREATMENT OF TICK BORNE DISEASES

Anaplasmosis, heartwater and babesiosis are very important veterinary tick-borne diseases in South Africa[96] resulting in both high mortality and severe financial losses[13]. Another important tick-borne disease, East Coast Fever, although eradicated locally it is still of major economic significance in other parts of Africa.

2.2.1. Babesiosis

Babesia is a protozoan parasite belonging to the phylum Apiclomplexa; class *Aconoidasida*; order Piroplasmorina; and the family Babesiidae. It is the cause of babesiosis; a common and economically important disease of both the tropical and sub-tropical parts of the world[96] **(**Figure 2-1**).** In South Africa babesiosis is also known as red water. The economic impact of the disease, although not quantifiable, is assumed to be in the millions of Rands due to a combination of direct losses (death), the long convalescence periods induced, as well as incidental costs such as vaccinations, treatment and veterinary fees[96].

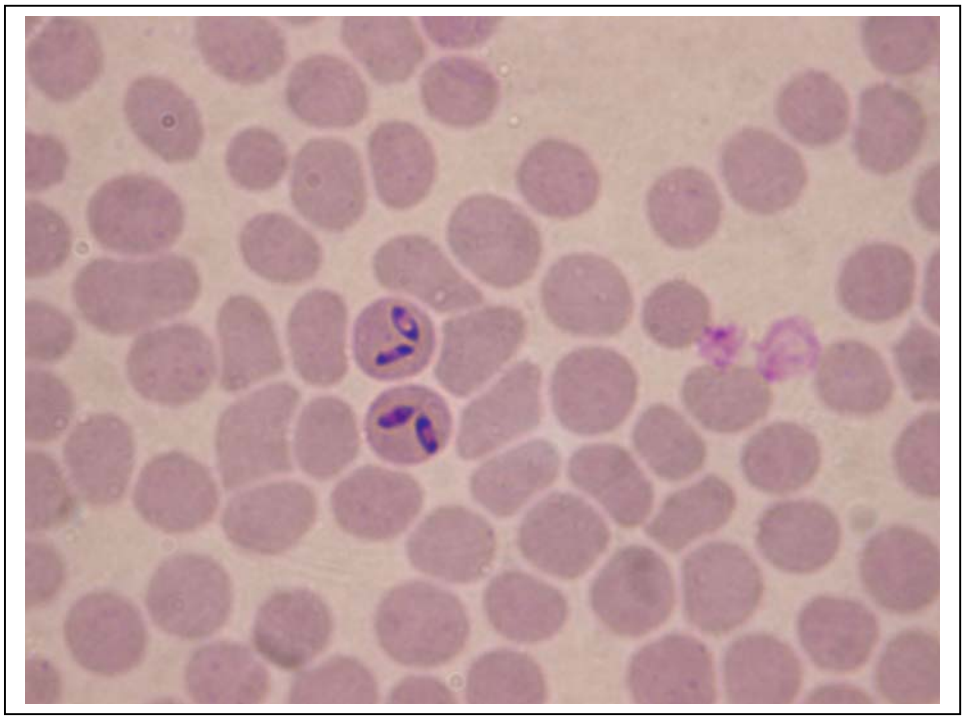


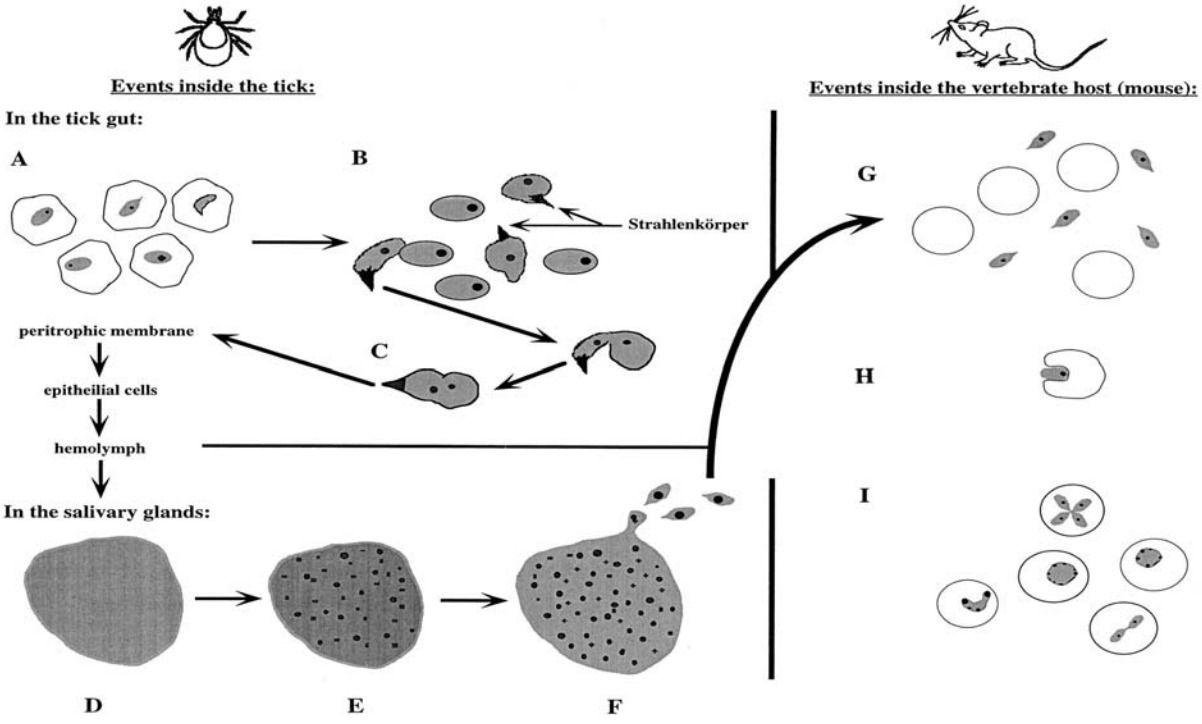
Figure 2-1: *Babesia caballi* within equine erythrocytes

(Photograph at 1000x magnification under oil)

Babesiosis is a tick-borne disease affecting a wide range of vertebrate hosts. Species infected range from companion to production animals and may sometimes include man and diverse wildlife species, such as the jackass penguin[39]. The parasitic distribution and the hosts infected are, however, dependant on the distribution of the particular vector species, which varies according to the micro-environmental conditions needed by the host's tick[95].

Four subspecies of babesia have been identified in cattle viz. *Babesia bigemina*, *B. bovis*, *B. occultans* and a fourth yet unnamed. Of this *B. bigemina* and *B. bovis* are the most common. The other domestic species are infected by different babesia species, as the parasite tends to be very host specific.

Babesia is an intra-erythrocytic parasite that induces a moderate to severe haemolysis and subsequent anaemia (Figure 2-2). The exact pathogenesis is unknown although certain parasitic proteolytic enzymes have been implicated. These enzymes could possibly destroy erythrocytes, disturb blood coagulation or even trigger specific macrophage degranulation pathways[96].



Events in the tick begin with the parasites still visible in consumed erythrocytes (A). The released gametes begin to fuse (B). The formed zygote then goes on to infect and move through other tissues within the tick (C) to the salivary glands. Once a parasite has infected the salivary acini, a undifferentiated sporoblast is formed (D). After the tick begins to feed, the specialized organelles of the future sporozoites form (E). Finally, mature sporozoites bud off of the sporoblast (F). As the tick feeds on a vertebrate host, these sporozoites are inoculated into the host (G). Sporozoites (or merozoites) contact a host erythrocytic and begin the process of infection by invagination (H). The parasites become trophozoites and can divide by binary fission within the host erythrocyte, creating the various ring forms and crosses seen on stained blood smears (I).

Figure 2-2: Life cycle of *Babesia spp*. (Homer *et al*[46]).

If the resultant haemolysis is severe, death results due to a hypoxic hypoxia. In less severe cases, one sees a more moderate anaemia with induced ischaemic changes. Other pathogenic effects include disseminated intravascular coagulation (DIC), which can result in the precipitation of the condition known as cerebral babesiosis, for *B. bovis*[15].

A presumptive diagnosis of babesiosis may be made from the presenting clinical signs, especially pyrexia and pallor of the oral, conjunctival or vulval mucous membranes. A large number of possible differential diagnoses exist. As such the diagnosis needs to be confirmed by means of stained thin blood smear evaluated under a light microscope for the presence of the babesial parasite (Figure 2-1). Considering that babesiosis cannot be conclusively diagnosed without the blood examination for the presence of the organism it is not possible for rural farmers to confirm the diagnose this condition.

A number of drugs are available on the South African market. These include the diamidines, such as diminazene and imidocarb; and dyes, such as euflavine and trypan blue. All are known to possess a low therapeutic index and to have severe side effects. For this reason newer and safer drugs need to be discovered. This study thus attempts to evaluate ethnoveterinary medicines as a source of new therapeutic compounds. Ethnoveterinary medicines shown to have antibabesial activity may also serve as an indicator of potential antimalarial activity, a very important human disease in Africa[20].

2.2.2. Cowdriosis

Ehrlichia ruminantium (*Cowdria ruminantium*) is a rickettsial parasite belonging to the class *Proteobacteria*, order Rickettsiales, family Rickettsiaceae, tribe Ehrlichia, and genus Cowdria[104]. It is the cause of cowdriosis; a major production animal disease in South Africa and Africa as a continent, whose importance is surpassed only by East coast fever. In endemic areas of South Africa, it is believed that the resultant mortalities may exceed those of both Babesia and Anaplasma by a factor of three[13].

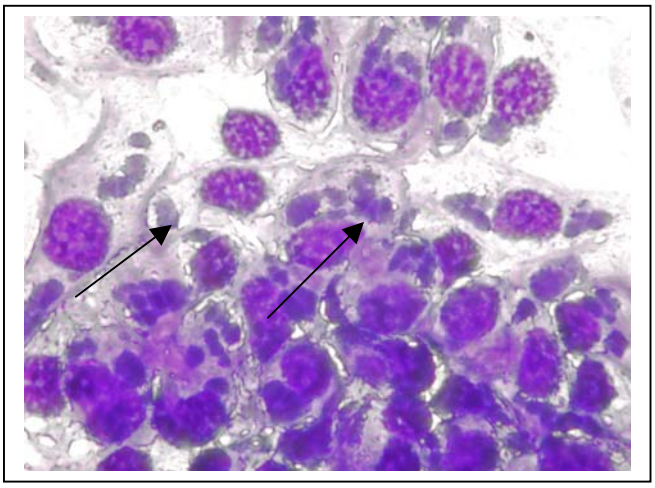


Figure 2-3: Pure *E. ruminantium* cultures, with large proliferating colonies

(300x magnification)

In South Africa cowdriosis is also known as heartwater (HW). Heartwater is a tick borne disease affecting both domestic and wild ruminants. Parasitic distribution, as with babesiosis, is dependant on the microenvironment required by the vector tick, *Amblyoma spp* (Bont tick)[104]. Although the parasite is not host specific, there are definite differences in host susceptibility[26].

The pathogenesis of HW is not completely understood. The parasite gains entry into the circulation during the feeding cycle of an infected tick, after a suitable incubation period in the invertebrate host. After a period of multiplication within the host the parasite disseminates to all endothelial cells, but especially the neural endothelial cells. Once established therein they replicate by binary fission.

This results in an increase in endothelial permeability that causes an effusion of a serous exudate into all body cavities. Throughout this period endothelial cell integrity remains unchanged, thereby suggesting that endothelial death is not linked to the fluid effusions. Endotoxin release has been implicated as a cause of the effects seen[13].

A diagnosis of HW may be suspected from the characteristic neurological clinical signs such as ataxia, chewing movements, circling, aggression, convulsions and death, and the presence of the specific vector on the animal. A confirmatory diagnosis of heartwater is extremely difficult in the live animal as the clinical signs, although suggestive are not

pathognomic[15]. In the past, corrugated brain squash smears were made from the live animal as a diagnostic aid. This method is no longer practiced due to its invasiveness[92].

A post mortem examination is the only current means of making a confirmatory diagnosis by examining infected brain smears under a light microscope for the presence of rickettsial colonies within the endothelial cells[80].

The rickettsial parasites are susceptible to antibiotic therapy[13]. The commonly used drugs are oxytetracycline, doxycycline and the sulphonamides.

2.2.3. Anaplasmosis

Anaplasma spp are rickettsial parasites belonging to the genus Anaplasma, family *Anaplasmataceae*, order Rickettsiales and is the cause of is anaplasmosis; a worldwide cause of cattle mortality[16] (Figure 2-4). In South Africa the disease is widespread with an estimated 99% of the cattle population being susceptible to infection[27].

In South Africa, the disease is also known as gallsickness, and is caused by either *A. marginale* or *A. centrale*. Gallsickness is a tick borne disease for which the exact vector parasite is unknown. Experimentally, five species of ticks are capable of transmitting the disease: *Boophilus microplus*, *Rhipicephalus evertsi evertsi*, *R. simus*, *Hyalomma marginatum rufipes* and *B. decoloratus*[96]. In a Zimbabwean study it was shown that mechanical vectors, such as the biting flies, might also transmit the parasite. This was believed to be of secondary importance due to the poor survival of the organism[58].

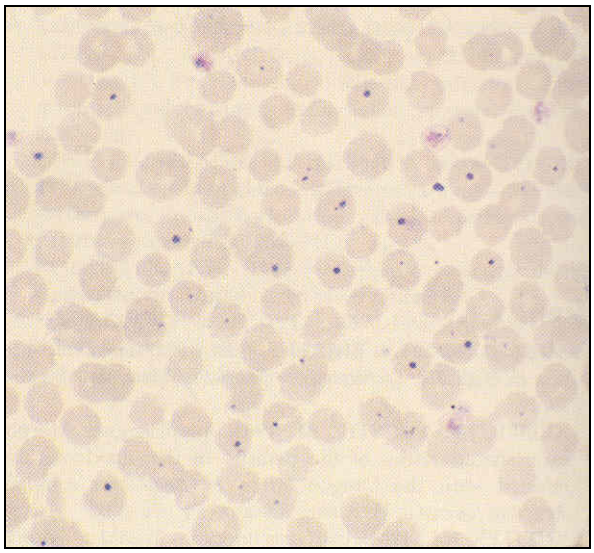


Figure 2-4: Bovine erythrocytes infected with *Anaplasm spp*.

(Photographed at 300X magnification, Penzhorn B, 2004)

The pathogenesis of this parasitic disease is not completely understood. It multiplies within erythrocytes by means of binary fission, but is not directly responsible for the clinically significant anemia seen. It is believed that the parasite induces physical and chemical changes in the parasitized erythrocytes. With an altered erythrocytic antigenic variation the cells become foreign to the reticuloendothelial system and are removed/destroyed. The altered erythrocytes may also induce a humeral response that aids in the removal of the antigenic altered cells.

A presumptive diagnosis of anaplasmosis may be made from the presenting clinical signs of a temperature reaction, anorexia, rumen stasis and icterus[16]. The diagnosis needs to be confirmed, as with the other blood borne parasites, by means of a thin blood smear[27] (Figure 2-4). Card agglutination and enzyme linked immuno-sorbent assay (ELISA), as well as indirect immunofluorescence tests are available to detect carrier animals within a herd[59].

2.2.4. Theileriosis

Theileria is a protozoan parasite belonging to the phylum Apicomplexa, class Aconoidasida, order Piroplasmorina, and the family *Theileriidae*[96]. It is the cause of theileriosis[46], a disease of major economic importance around the world, which resulted in

an estimated loss of US$168 million, in 1989[98]. Untreated the disease is invariably fatal and has a mortality as high as 60% in the indigenous or zebu breeds.

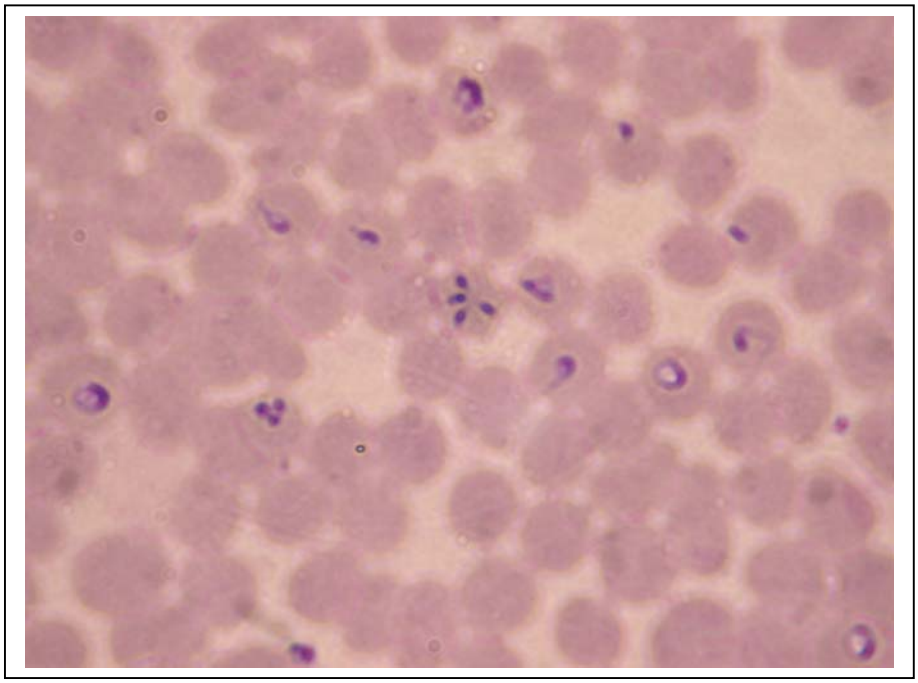


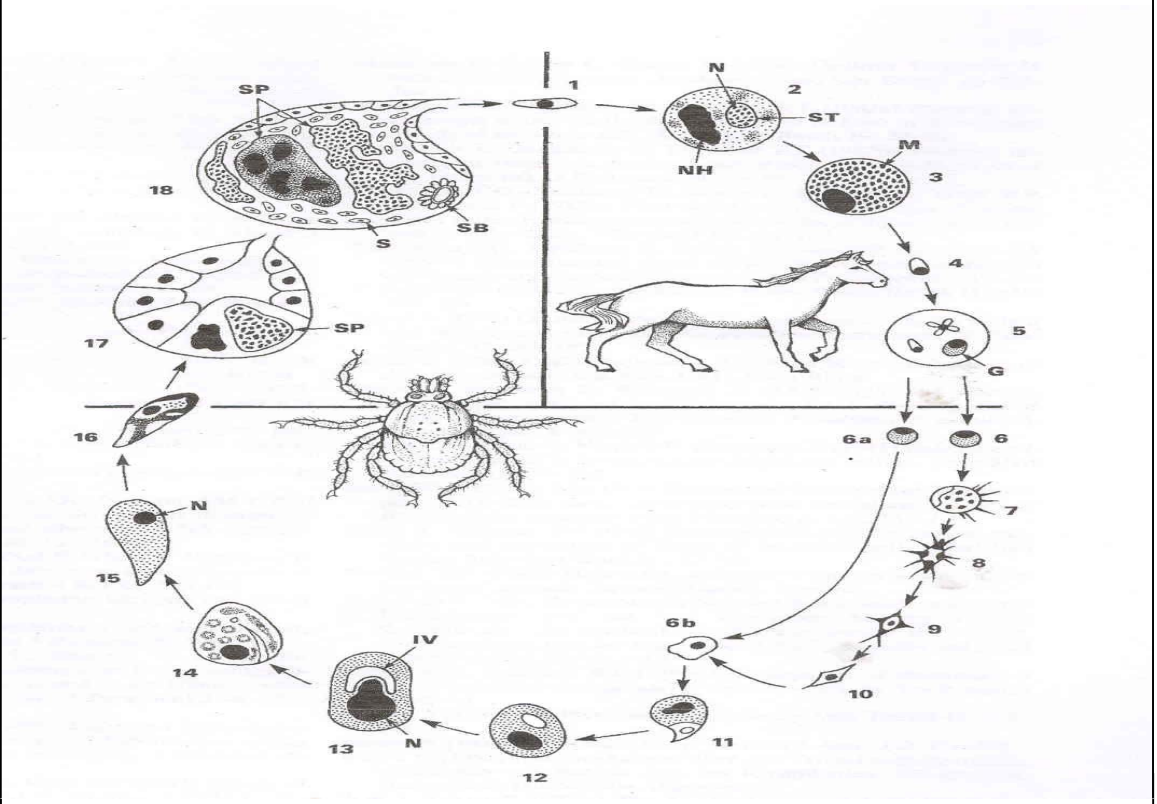
Figure 2-5: *T. equi* cell culture under 1000x magnification

(1000x magnification under oil)

Theileriosis is a tick borne-disease, transmitted by *Rhipicephalus appendiculatus*, and is locally known, in South Africa, as East Coast Fever (ECF) and corridor disease. Susceptible animals include all cattle species and certain wild bovidae[88]. *Theileria parva parva* causes ECF, while *Theileria parva lawrensii* causes corridor disease.

In South Africa the parasites involved are *T. parva lawrensi*, *T. terautragi*, and *T. mutans*. *T. parva parva* was eradicated in the early half of the last century in South Africa[96]. Both ECF and corridor diseases are still controlled diseases in cattle in South Africa[1].

The pathogenesis of theileriosis is fairly well understood. Theileria, like all other apicomplexa, are obligate intracellular parasites (Figure 2-6). The parasite gains entry into the circulation during the feeding cycle of an infected tick, after a suitable incubation period in the invertebrate host. The sporozoites enter the hosts' lymphocytes[88] and undergo repeated schizogony, where they also induce lymphocytic proliferation. At a later stage microschizonts develop which are released as small merozoites, which subsequently infect red cells.



Sporozoite injected in by tick (1), development of schizont within lymphocyte (2-3), free merozoite released from infected lymphocytes (4), entry and development within erythrocytes (5), development of parasite within the intestine of the infected tick after engorgment (6-11), development of parasite within the salivary gland of the tick after migration from the gut (12-17), mature sporozoites ready to be released on the next parasitic feeding (18)

Figure 2-6: Life cycle of *T. equi* (Mehlhorn and Schein[71]).

A diagnosis of ECF is based on the clinical signs of lymphadenopathy, pyrexia and loss of production due to wasting, and the presence of the vector in the area. The diagnosis needs to be confirmed by demonstrating the schizonts in the lymphocytes and the piroplasms in the red blood cells (Figure 2-5), on thin blood smears or by fine needle aspirates of the lymphoid tissue[60].

There are several drugs available for the treatment of theileriosis. They include drugs such as parvaquone, buparvaquone and the halofuginones. Tetracyclines are also effective when combined with vaccination programmes. However, according to current South African legislation, animals diagnosed with the disease may only be placed under quarantine.

Treatment is not allowed, as treated animal become carriers and serve as a source of infection to the ticks[60,74].

2.3. ETHNOVETERINARY TREATMENT OF ANAPLASMOSIS, BABESIOSIS AND HEARTWATER

2.3.1. Plants

Seventeen plants have been documented as being effective in the treatment of "semê, *gala* and *bolwetsi jwa mothlapo o moshibidu*", as used by the indigenous people in the Madikwe district of the North West Province (Figure 2-7)[93]. These are disease conditions of animals that are most likely indicative of heartwater, anaplasmosis and babesiosis respectively, which are known to occur in the region. Van der Merwe[93] not only documented the condition for treatment, but also the plant or plant parts being used, the local methods of preparation and in most cases the method of application.

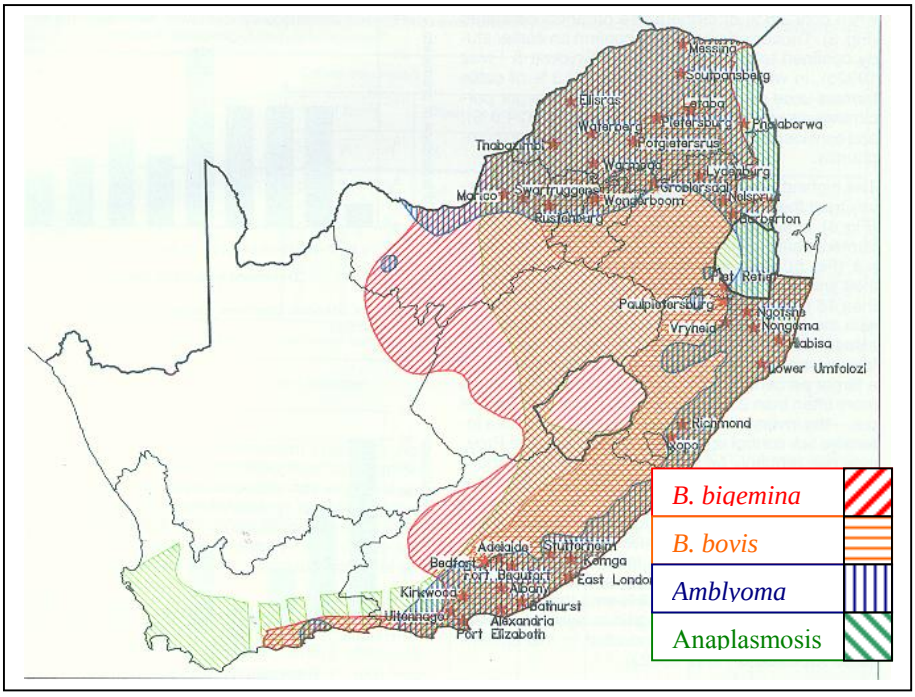


Figure 2-7: Diagram illustrating the distribution of redwater and gallsickness in South Africa (adapted from du Plessis *et al*[27])

"Semê" is used to describe a disease condition, which is a major cause of morbidity and mortality in goats, but is less common in cattle. Animals appear to show nervous signs without distinction of ataxia caused by weakness or without neurological origin.

These clinical signs are similar to those seen with cowdriosis (heartwater parasite), which is characterised by signs such as ataxia, chewing movements and convulsions[16,96]. Mortality is the usual outcome of the disease, with goats being more susceptible to infection than cattle.

Plants, the plant part utilised and their method of preparation used for the treatment of "*Seme*" are listed in Table 2-1[93].

Table 2-1: List of plants, plant parts and their method of use for treatment of "*Semê*" (Adapted from van der Merwe[93]).

Plant species	**Tswana/ Sotho names**	**Plant part used**	**Preparation**
Elephantorrhiza elephantina	mosetlhane	Root-stock	Decoction
Hypoxis rigidula	tsuku-ya-poo	Corm	Decoction
Senna italica	sebete	Roots	Decoction
Senna italica and *Urginea sanguinea* (in combination)	sebete sekaname	Roots Bulbs	Infusion or decoction
Rhoicissus tridentata	ntagaraga	Tubers	Decoction
Schkuhria pinnata	santhloko / lefero	Aerial plant parts	Infusion or decoction

"Gala" is used broadly to describe any condition in animals that results in poor appetite, lethargy and weights loss without any obvious cause. It is sometimes used to describe a disease accompanied by constipation. These clinical signs are similar to those arising from infections with bovine anaplasmosis, which is characterised by poor appetite, rumen stasis and dryness of the faeces[16].

Plants, the plant part utilised and their method of preparation is listed in Table 2-2[93].

Table 2-2: List of plants, plant parts and their method of use for "*Gala*" (adapted from van der Merwe[93]).

Plant species	**Tswana/ Sotho Names**	**Plant part used**	**Preparation**
Rhus lancea	moshabele	Roots	Decoction
Aloe marlothii	mokgopa	Leaves	Crushed fresh or dried leaves were dosed to animals in either feed or water
Senna italica	sebete	Roots	Infusion or Decoction
Urginea sanguinea and *Senna italica* (used in combination)	sekename sebete	Bulb Roots	Infusion

"***Bolwetsi jwa mothlapo o moshibidu"*** is used broadly to describe conditions in cattle that cause a reddish discolouration of the urine. Bovine babesiosis is characterised by anaemia, haemoglobinaemia, icterus and haemoglobinuria (reddish discolouration of the urine)[15].

Plants, the plant part utilised and their method of preparation are listed in Table 2-3[93].

Table 2-3: List of plants, plant parts and their method of use for "*Bolwetsi jwa mothlapo o moshibidu*"(adapted from van der Merwe[93]).

Plant species	**Tswana/ Sotho names**	**Plant part used**	**Preparation**
Asparagus laricinus	lesitwane	Tubers	Decoction
Asparagus suaveolens	lesitwane	Tubers	Infusion
Ozoroa paniculosa	monokane	Bulb	Infusion or Decoction
A. laricinus and *Urginea sanguinea* (in combination)	lesitwane sekename	Roots	
Rhoicissus tridentata	ntagaraga	Tubers	Decoction

2.3.2. Pharmacological and chemical evaluations of ethnoveterinary plants

Aaku *et al*[5] studied the effects of crude extracts from *Elephantorrhiza elephantina* collected in Botswana. They concluded that *E. elephantina* showed antimicrobial activity, without providing any quantitative data. Although never confirmed, it has been stated that *E. elephantina* has a high tannin content[103].

Lin *et al*[61] investigated the antimicrobial and anti-inflammatory effects of *R. tridentata* extracts, from plants collected in South Africa. No antimicrobial activity was present, although the plant did show significant anti-inflammatory activity. The plant material was extracted in both water and methanol, and tested against a wide spectrum of bacteria (including *Escherichia coli*) using the disc diffusion method. Lin *et al*[61] also demonstrated that *R. tridentata* had significant anti-tumour activity. Opoku *et al*[77] showed the plant to possess anti-oxidant activity equivalent to that of vitamin E *in vitro* and to contain abundant phenolic compounds.

Majinda *et al*[65] studied *Urginea sanguinea* collected from Kgale, Botswana. The crude extracts (extracted in cold water) demonstrated weak activity against *Bacillus subtillus* and *S. aureus* using the disc diffusion method. *U. sanguinea* is also a known toxic plant in South Africa that contains cardiac glycosides, specifically, bufadienolides[50,52].

In a study in Botswana to determine the value of certain trees as an alternate source of food it was found that *Rhus lancea*, contained 5.07 % m/m of condensed tannins, as determined by a butanol-HCl method[9]. Tannins are known to have non-selective anti-bacterial activity *in vitro*, due to their ability to kill living cytoplasm and to precipitate proteins[11,21,43].

Ozoroa paniculosa was included in a study conducted in Namibia to determine the polyphenolic, condensed tannin contents and protein precipitating capacity of about twenty browse plant species[91]. The condensed tannin content of this browse plants was 50.9 %. It was also found that the condensed tannin content correlated negatively with the *in vitro* dry matter digestibility. It was concluded that the high tannin content may affect the ability of the *in vitro* tests to successfully determine the activity of other constitutes in the crude extracts.

In a survey conducted in the Northern province it was discovered that the ash of *Aloe marlothii* was mixed in with maize to aid in the storage process[6]. In an attempt to determine the efficacy of this process, it was found that the ash inhibited the oviposition of adult weevils as well causing their death possibly due to the presence of a substance/s in the plant. McGaw *et al* [70]demonstrated that *A. marlothii* had no antibacterial activity, when extracted in hexane, ethanol and water and tested using the disc diffusion method.

Although some of the above documented plants have been tested for their antibacterial activity, as related to their use in people, they have not been tested for their anti-rickettsial or anti-babesial activity. On the other hand, the three conditions mentioned; "*seme*, *gala* and *Bolwetsi jwa mothlapo o moshibidu*" may not necessarily represent heartwater, gallsickness or redwater, but may only represent general ailments affecting these animals i.e. the results seen in the animals treated may be due to a bacterial infection responding to the inherent antibacterial activity of the herbal preparation used. It should also be noted that rickettsial infections also respond well to certain antimicrobials and therefore plants with an inherent antibacterial activity may also be active against rickettsia. Furthermore in most cases agar diffusion methods have been used to determine antibacterial activity. This method has been criticised lately and been shown to be unreliable for plant extracts[33].

2.3.3. Collective activity of plant constituents

Failure of a plant extract to demonstrate *in vitro* activity during general screening may not necessarily imply that the plant has no inherent medicinal value[19]. It appears that a synergism may exist between the plant constituents and that plants do not contain a single active ingredient. Pillipson[78], showed that certain plants contain a number of minor constituents that work in synergy when the plant is used as a crude preparation. When these compounds are isolated their activity may not be sufficient to reflect the true medicinal value of the plant.

2.4. PLANTS SELECTED IN THE STUDY

E. elephantina, *R. tridentata*, *U. sanguinea* and *A. marlothii* were selected from the data of van der Merwe[93] because they were all reported to possess apparent antibacterial or

anti-parasitic activity. From the original list of 17 plants documented by van der Merwe for the treatment of "*Seme*, *gala*, and *Bolwetsi jwa mothlapo o moshibidu*" only plants that were being used medicinally by other communities in South Africa for the treatment of human infections were selected. As indigenous medicinal plants knowledge systems are generally passed down from generation to generation, it is believed that by the process of trial and error, their use becomes more rational in the treatment of ailments[97], thereby increasing the possibility of having high biological activity.

2.4.1. Aloe marlothii (Berger)

Family: Asphodelaceae

Tswana name: mokgopa

English Name: Mountain or Bergalwyn Aloe

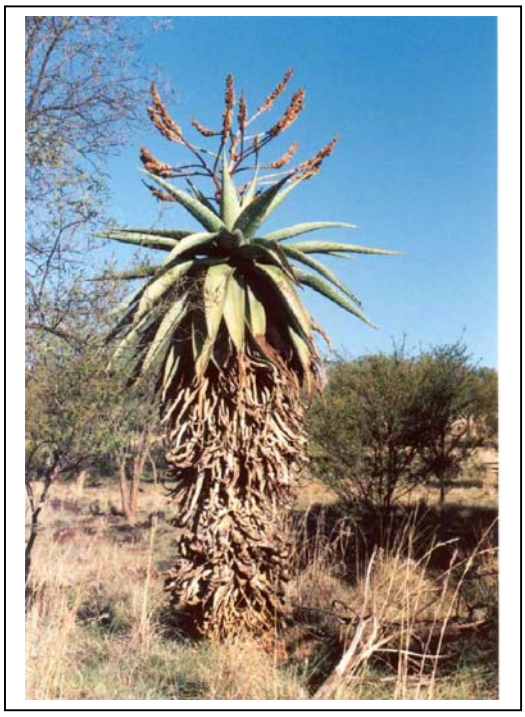


Figure 2-8: *A. marlothii* with flowers

(Kaalplaas Onderstepoort, 2002)

A. marlothii is a typical member of the aloe family, with tough spiked leaves, unpalatable juice and brilliant yellow-orange flowers pointed back towards the main axis. The plant possesses a shallow fibrous root system and can grow up to six metres tall. The tall stem is covered with old leaf bases and large rosettes of spiny leaves. The plant is commonly distributed in the northern half of South Africa (old Transvaal) and the northern parts of Kwa-Zulu Natal[22] (Figure 2-8).

The leaves are used to treat and prevent "*gala*" and to treat helminthiasis, diarrhoea, constipation, general ailments, retained placentas, dystocia, maggot wound infestations and to reduce tick burdens[93]. Both fresh and dried leaves are used in different preparations. Most commonly plant material is mixed with water and dosed to the animals. There was no mention as to whether the gel or outer husk was separated prior to use[93].

2.4.2. Rhoicissus tridentata (Wild & Drum)

Family: Vitaceae

Tswana name: ntagaraga

English Name: Wild grape

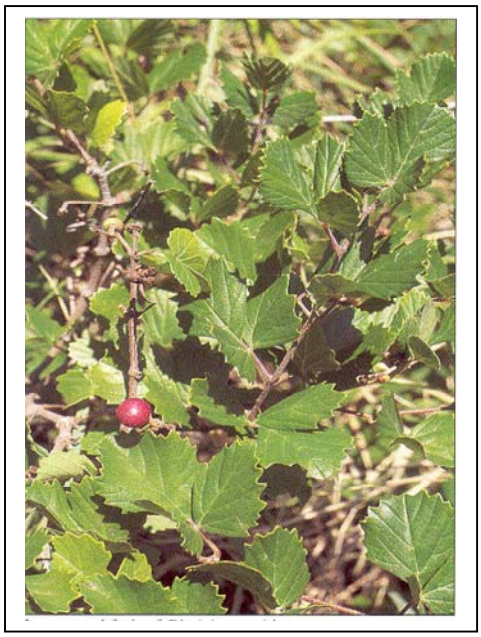


Figure 2-9: *R. tridentata* with fruit

(Van Wyk et al [103])

R. tridentata is shrubby creeper with branches spreading outwards from a thick woody base[47]. The dark green glossy leaves have three leaflets, each wedge shaped, with serrated margins, from which the plants' name has been derived (Figure 2-9). The plant also has deeply red tubers attached to the taproot system (Figure 2-10). The plant is part of the family Vitaceae, which includes the commercial grape cultivars.

The tubers are used to treat "*gala*", "*seme*", helminthiasis, general ailments and cows that have aborted[93]. The tubers are crushed and boiled in water for a few minutes to form a decoction, which is dosed once cooled. The decoction is notably red in colour.

Figure 2-10: *R. tridentata* tubers

(Van Wyk et al [103])

2.4.3. Urginea sanguinea (Schinz)

Family: Hyacinthaceae

Tswana name: sekaname

Local name: Transvaal slangkop

Urginea sanguinea is a common invader, distributed throughout South Africa[52]. The plant has a deep red, pear-shaped onion like bulb, which is buried just under the surface (Figure 2-11). The entire plant is toxic to animals, with the flowers containing the most toxic principles. The toxic principle in the plant was identified as the bufadienolide transvaalin ($C_{36}H_52O_3$)[52].

Figure 2-11: *U. sanguinea* bulb and florets

(Photographed, Botha CJ, 2002)

Bulbs are used either alone or in combinations to treat general ailments, general intestinal problems, helminthiasis, to clean the blood, "*gala*", "*seme*", "*bolwetsi jwa mothlapo moshibidu*", sores and retained placenta[93]. A spoonful of the powdered plant is mixed with warm water and dosed. For the treatment of "*bolwetsi jwa mothlapo moshibidu*" it is mixed with *A. laricinus* tubers prior to dosing. Currently, these plant extracts are a known cause of human poisonings, so it is assumed that animal mortality may also result from their use[63].

2.4.4. Elephantorrhiza elephantina (Skeels)

Family: Fabaceae

Tswana name: Mosetlhane

English name: Eland's seed or Elephant's root

Figure 2-12: *E. elephantina* in seed

The plant has several unbranched, annual stems of nearly one metre in height, growing from an underground rhizome[47]. The finely divided leaves have numerous small, narrow leaflets. Clusters of small, cream-coloured flowers are produced along the lower half of the aerial stem, giving rise to the seed pods. (Figure 2-12 and Figure 2-13)

Rhizomes are used to treat diarrhoea, "*bolwetsi jwa mothlapo moshibidu*", coughing and pneumonia[93]. For the treatment of diarrhoea and "*bolwetsi jwa mothlapo moshibidu*" the crushed rhizomes are mixed with water and allowed to stand for a few hours to obtain an infusion. Alternately the crushed rhizome is boiled in water to obtain a decoction. The brownish-red liquid is dosed once or twice a day for as long as needed.

Figure 2-13: *E. elephantina* rhizomes

2.5. ANTIOXIDANT ACTIVITY

The body is constantly exposed to the negative and sometimes lethal effects of oxidants during normal physiological processes. On a daily basis, up to 5% of inhaled oxygen may be converted to reactive oxygen species (ROS). These ROS have the ability to bind to cellular structures, such as deoxyribose nucleic acid (DNA), ribose nucleic acid (RNA), proteins and the cell membrane. The damage is cumulative and may be the trigger for diseases such as artheriosclerosis, cancer and even Parkinson's disease in man[86]. Two processes, which produce free radicals *in vivo*, have been identified and named the Fenton reaction and the Haber-Weiss reaction (Figure 2-14)[44].

Fe^{3+}/Cu^{2+}	+	O_2	→	Fe^{2+}/Cu^{+}	+	O_2				**metal reduction**
Fe^{2+}/Cu^{+}	+	H_2O_2	→	Fe^{3+}/Cu^{2+}	+	OH	+	OH		**Fenton Reaction**
O_2	+	H_2O_2	→	O_2	+	OH	+	OH		**Haber-Weis Reaction**

Figure 2-14: The Fenton and Haber Weiss equation

The body does, however, have dedicated protective mechanisms, which neutralize these products. This could be either by enzymatic breakdown (glutathione peroxidase system), or the active scavenging, of ROS, by anti-oxidants[86]. For the proper functioning of the latter systems anti-oxidants need to be supplied via normal dietary intake. This includes compounds such as vitamin E, A, C, carotenoids and the polyphenols i.e. phytogenic compounds abundant within fruit and vegetables[37].

Anti-oxidants play an important role in animal health. Conventional antioxidants have been shown to improve animal performance during conditions characterised by increased tissue oxidant levels such as stress, injury and infection[76].

In addition to this ability to neutralize harmful oxidative reactants, the anti-oxidants may also be protective against neoplastic growth and proliferation, via other mechanisms. These include:

- **Immune stimulation**: The anti-oxidants enhance the production and activity of cytotoxic immune cells by promoting the release of chemotactic factors. This allows for the accumulation of leukocytes at sites of proliferation, where they are able to destroy cancerous cells[89].

- **Genetic mechanism**: They appear to enhance certain suppressor cancer genes, while simultaneously inhibiting the oncogenes, expressed in certain cancers cell types[89].

- **Angiogenesis inhibition**: They inhibit tumour angiogenesis. Without a proper blood supply proliferation into tumour masses is not possible[36,89]

- **Enzyme inhibition**: Currently it appears that the polyphenols are microsomal enzyme inhibitors. Phenolsulphotransferase is one such enzyme, which is occasionally involved in lethal synthesis i.e. activates compounds which inflict DNA damage, leading to carcinogenicity or mutogenicity[38].

- **Inhibit cell membrane pumps:** They influence the functioning of the P-glycoprotein pump (P-gp). These extrusion pumps actively exclude xenobiotics and drugs; including the cytostatic drugs from within cells i.e. they play an important

role in cancer cell drug resistance[94]. With inhibition, one has an increased biophasic availability, and increased cancer cell death.

Anti-oxidants are thus very important in the management of disease and disease conditions. With anti-oxidants being so abundant in many plants, they could have an important influence on animal health. It is thus possible that medicinal plants may contribute to the control of disease not only by affecting a specific pathogen but also by aiding in the clearance of the pathogen by being an immune stimulator.

R. tridentata is a member of the Vitaceae family[103]. Other members of this family include the common grape cultivars. Since this family and particularly the grape seed is known to posses polyphenolic compounds and to posses high anti-oxidant activity it was decided that it would be interesting to include anti-oxidant screening as part of the *in vitro* analysis of extracts.

2.6. CONCLUSION

Plants are an important component in our therapeutic arsenal in the fight against disease and disease conditions. They have and will most likely continue to serve as an important source of new therapeutic molecules. It is hoped that the documented ethnoveterinary plant archives would be an asset from which further discoveries are made.

Although currently lagging behind its human counterpart, research into ethnoveterinary medicine is gaining in importance. These plants are being used in the management of a number of different animal disease conditions, including the economically important tick-borne diseases. This has largely has been surmised by detailed epidemiological surveys in their specific communities of use. Three important diseases described include "*gala*", "*seme*" and "*bolwetsi jwa mothlapo o moshibidu*" that are believed to represent gallsickness, heartwater and redwater, respectively. However, although these conditions are tick-borne illnesses, non-specific bacterial infections may also be involved or contribute to the disease condition.

Bacterial infections *inter alia* result in impaired animal production. Infections could result from either a single or a combination of etiological agents. This is largely dependant on factors such as animal condition, nutrition and environmental contamination or the presence of predisposing factors such as injury and impaired immune functioning[15,96].

Infected animals generally require medical treatment. Unfortunately, with the majority of infections, the presenting clinical signs tend to be non-specific. Also, at times animals may also be infected by a protozoal or rickettsial infections, which also tend to manifest with this similar non-specific diagnostic picture[15]. In small-scale farming it is difficult for the layperson to recognise these infections since a sound theoretical knowledge of the condition and availability of diagnostic tools are lacking.

It is thus easy for a layperson to mistake a bacterial infection for a blood borne parasitic infection and *vice versa*. Thus, when plants are used medicinally for conditions presumed due to protozoal or rickettsial infections the results gained are due to treatment of an underlying bacterial infection.

For the above reason four ethnoveterinary utilized plants, *A. marlothii*, *R. tridentata*, *U. sanguinea* and *E. elephantina* will be evaluated for antiprotozoal and anti-rickettsial activity as well as antibacterial activity. As a secondary objective, the plant extracts will also be tested for anti-oxidant activity as this may aid the innate immune system in the clearance of infectious antigens.

CHAPTER 3
MATERIALS AND METHODS

3.1. INTRODUCTION

Validated *in vitro* methods were used to screen crude extracts of the *U. sanguinea*, *E. elephantina*, *A. marlothii* and *R. tridentata* for antibacterial, antirickettsial antiprotozoal and anti-oxidant activity, which served as an indicator of the *in vivo* efficacy of the extract, against anaplasmosis, babesiosis, heartwater, oxidative stress and non-specific bacterial infections[31,105,106]

3.2. PREPARATION OF PLANT MATERIAL AND EXTRACTION

Extraction of plant active compounds was carried out only once. Dried extracts were stored until required and only reconstituted, in acetone, prior to the experimentation. Stability of the stored material was not tested as part of this research. It was assumed that dried material remained stable as it has been showed that material stored for 80 years retain activity[51]. Extracts from dried plant material had the same minimum inhibitory concentration (MIC) as fresh samples although some differences in the thin layer chromatography (TLC) results were seen.

3.2.1. Plant collection

Approximately 0.5 kg of the plant was collected were possible. *Aloe marlothii* was collected on Kaalplaas (Onderstepoort, Pretoria). *Urginea. sanguinea* was obtained from the Section of Toxicology at the Onderstepoort Veterinary Institute (OVI) from their freezer store and toxicology garden. *E. elephantina* was collected from a vacant plot in Erasmia, in the west of Pretoria.

R. tridentata was purchased from an indigenous plant nursery (Patryshoek, Pretoria) due to an inability to find the plant in a natural habitat near Pretoria. The plant came with an extensive history and had been grown from seed approximately five years previously. It was kept in a 10-litre sized container in the shade of a full-grown tree.

3.2.2. Plant storage and identification

A specimen from *A. marlothii* (Naidoo 1), *E. elephantina* (Naidoo 2) and *R. tridentata* (Naidoo 3) was dried in a plant press and deposited in the herbarium of the Onderstepoort Veterinary Institute.

The identity of *E. elephantina* and *A. marlothii* was confirmed by the National Botanical Institute, *U. sanguinea* by the OVI and Prof CJ Botha of the Faculty of Veterinary Science and *R. tridentata* by a horticulturist from the nursery where the plant was purchased. The latter was confirmed by Prof TW Naudé of the Faculty of Veterinary Science.

3.2.3. Preparation of plant material

Urginea sanguinea

A large proportion of work done to date was on dried leaves, bark and roots since extraction from fleshy bulbs is more complicated. It was decided to determine whether freezing could aid in the extraction process. Fresh and frozen bulbs, together with the dried leaves of *U. sanguinea,* were tested. The bulbs were analysed in four different treatments. The freshly harvested bulbs were extracted as either fresh material (F) or after oven drying (FD). The frozen bulbs (kept at -3ºC) were extracted after overnight thawing at room temperature (T), or after thawing and oven drying (TD). The leaves were extracted after being dried (L) at room temperature.

The scales of the bulbs were separated and dried at 37° C for several days to a constant mass (same mass as measured two days apart) prior to being ground.

Aloe marlothii

The leaves were cut in half and dried to a constant mass in an oven at 37 ºC. The outer husk and inner succulent, gel-like content, were not separated.

Elephantorrhiza elephantina

The leaves and rhizomes were separated, and dried to a constant mass at room temperature.

Rhoicissus tridentata

The plant was separated into root bark, tuber, stem bark and leaves. All fractions were dried at room temperature to a constant mass. The fibrous component of the roots and stems were discarded.

All dried material was stored at room temperature until required. The material was ground prior to extraction. *E. elephantina* rhizomes were ground in a hammer mill due to the extreme toughness of the dried material. All other material was ground in a commercial blender unit (pulsematic, Osterizer).

3.2.4. Extraction procedures

Dried material was used during the extraction process, except for the *U. sanguinea* bulbous material tested fresh. Extraction of the milled plant material was by means of maceration in acetone. Eloff found that for broad screening acetone was best and least toxic to organisms in subsequent bioassays[31]. Acetone was also the only extractant that showed no evidence of cytotoxicity when added to rapidly proliferating cell cultures (E. Zweygarth, OVI, Per. Com 2002).

The plant material was soaked in a 5:1 (v/m) ratio of acetone and shaken for 30 minutes on a shaker platform. The supernatant was collected and filtered. Extraction was repeated for an additional two times.

Extracts were dried in a rotary evaporator under vacuum, weighed and stored in dry solid form until required. The three sequential extracts were kept separate until their individual mass was determined.

The mass of the individual extracts were used to calculate the efficiency of extraction for each of the three sequential acetone extractions, by dividing the actual quantity extracted by the total amount extracted i.e. if the first extract removed 8 mg from a total of 10 mg, its relative percentage extractable would be 80%. The percentage yield was also calculated for each plant or plant part, by dividing the total mass extracted by the mass of the plant material used i.e. if a total of 10 mg from extracted from 500 mg of plant material, its percentage yield would be 2%.

In addition to the acetone extraction, some *U. sanguinea* and *A. marlothii* plant fractions chosen randomly were subsequently extracted in methanol (only once) to investigate the presence of additional antibacterial activity.

3.3. ANALYSIS AND CHEMICAL COMPLEXITY OF PLANT EXTRACTS

For the chemical analysis of extracts, plant material was separated by TLC on normal phase silica gel thin layer chromatography plates. The eluents used, as listed below, are part of the Standard Operating Procedure within the Phytomedicine research program. Thin layer chromatography relies on the capillary action of the eluents to separate simple extracts on normal or reverse phase silica gel plates[90].

3.3.1. Preparation of TLC plates

Silica thin layer chromatography plates (Merck® or Alugram®, 60) were used in all cases. One hundred micrograms (in 10 µl of acetone) of each extract was spotted onto the TLC plates and developed in a variety of solvent mixtures. These systems separate components over a wide range of polarities. The plates were placed in pre-saturated glass tanks lined with filter paper. All the mobile phases were of technical grade (Merck). Once developed, chromatograms were dried at room temperature[90].

Solvent system (mobile phases) utilised included:
ethyl acetate: methanol: water (EMW) (10: 1.35: 1)
chloroform: ethyl acetate: formic acid (CEF) (5: 4: 1)
hexane: ethyl acetate (HE) (2: 1)
ethyl acetate: hexane (EH) (2: 1)

3.3.2. Visualization of separated compounds

After development the chromatograms were examined under ultraviolet (UV) light (254 and 360 nm, Camac Universal UV lamp TL-600). Flourescent bands were marked with a solid pencil line at the 254 nm wavelength and a broken pencil line at the 360 nm wavelength. All chromatograms were subsequently sprayed with freshly prepared spray reagents. The plates were heated at 100º C until the colour bands were visible.

Two chromatograms of *U. sanguinea* and *A. marlothii* extracts, developed in EMW, were sprayed with either a 5% anisaldehyde (in 5% H_2SO_4 in ethanol) or the 0.35% vanillin spray reagent (0.1 g vanillin, 28 ml methanol and 1 ml sulphuric acid)(Sigma)[90]. Since more bands were visible after using the vanillin spray reagent, it was chosen as the spray-reagent for all subsequent chromatograms.

3.3.3. Retardation factor

The position of the bands on the TLC plate was noted by calculating the retardation factor (R_f) i.e. the distance compound traveled divided by distance the solvent had traveled from the origin.

3.3.4. Chemical composition

In an attempt to characterise the anti-oxidant activity of *E. elephantina* and *R. tridentata* samples, extracts were separated by TLC, with catechin (Sigma) as a standard, and eluted in EMW and CEF. Catechin was utilised as *R. tridentata* was known to contain polyphenols[72]. The plant is also a part of the family Vitaceae which is known to contain catechin and the proanthocyandins.

To confirm the presence of transvaalin in the *U. sanguinea* fresh extract the sample was analysed with the Lieberman colour indicator test[90]. This test is specific for cholesteroles and esters, steroids and the triterpene glycosides. The *U. sanguinea* fresh extract was reconstituted in 1 ml ethyl acetate. A 1 ml stock solution of acetic anhydride and sulphuric acid (1:50 v/v) was prepared and added to the ethyl acetate sample. The sample was incubated at room temperature, until a colour change was evident. If the triterpene glycosides (bufadienolides) are present, the sample should change from a transient red to a brownish-green colour[62].

3.4. EXPERIMENTAL DESIGN FOR ANTIBACTERIAL AND ANTIPARASITIC ACTIVITY

For antimicrobial and antiprotozoal effect extracts were tested using the following experimental design. No positive control was used for the antitheilerial assay.

Group 1: Test group: Consist of the organisms plus different concentrations of the extract (This group was used to determine if the extracts are effective).

Group 2: Positive control: Organism plus a known anti-microbial or anti-protozoal drug (This was used to ensure that the organisms utilised are susceptible to common chemotherapeutics and are not a resistant strain).

Group 3: Pure culture: Only the organism (This ensured that the organism was growing properly under the defined laboratory conditions. This was necessary to distinguish poor growth from inhibition of growth).

Group 4: Negative control: Organisms plus the pure extraction solvent (This was necessary to confirm that the extraction solvent has no inhibitory action of its' own).

3.5. EVALUATION OF ANTIBACTERIAL ACTIVITY

For the determination of antibacterial activity it is necessary to actively grow the bacterial cultures with the plant extract for which numerous different techniques have been described[82]. In the current study bioautography and microplate dilutions assays were used.

3.5.1. Bioautography

All extracts were initially studied by thin layer chromatography and bioautography according to the Standard Operating Procedures of the Phytomedicine program, Department of Paraclinical Sciences, University of Pretoria. The aim was to fingerprint the sample and to determine which component/s were active[12,45].

The principle of the assay is based on the spraying of an actively growing bacterial suspension onto developed chromatograms. The method relies on the direct inhibition or killing of the bacteria on contact with the active band. Unlike older methods this system is not dependant on the polarity of the active component as no agar is utilised[12,45].

3.5.1.1. Bioautography Spray Method

Bacterial cultures of *Staphylococcus aureus* and *Escherichia coli* in Mueller Hinton (MH)(Merck) broth were prepared for use one day prior to the experiment. TLC plates were developed in the required solvent system, marked under UV light and allowed to dry overnight. The bacterial species were chosen as they grew better and represented activity against one Gram-positive and one Gram-negative organism.

On the day of the experiment, chromatograms were sprayed with a dense bacterial suspension prepared from the overnight cultures. Thereafter the bioautogram was incubated at 100% relative humidity and 38°C for c. 24 hours.

The following day iodonitrotetrazolium chloride (INT)(Sigma), made up to a 2% m/v solution in water, was sprayed onto the bioautograms and thereafter incubated for a further one hour. Inhibition of growth was indicated by clear zones on the bioautogram[12,45,70]. Bacterial growth was detected by the reduction of the colourless INT to a red-coloured formazan, as INT is a tetrazolium salt, which is reduced to formazan by biologically active bacteria.

3.5.2. Microdilution antibacterial assays

The test organisms used in the quantification of antibacterial activity included two Gram-positive bacteria, *Enterococcus faecalis* (ATCC 29219) and *Staphylococcus aureus* (ATCC 29213), and two Gram-negative species *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922). The microplate method of Eloff was used[30]. This method is more efficient than the old system of agar disc diffusion as it is less affected by the polarity of the active compound. The method allowed for the calculation of a minimum inhibitory concentration (MIC) for active plant extracts against each bacterial species.

A two-fold serial dilution of plant extract, made to a 20 mg/ml stock solution, was prepared in 96-well microtitre plates and bacterial culture added to each well (Figure 3-1 illustrates the dilutions present in each well after the second dilution). The presence of bacterial growth was detected by the addition of INT as with the bioautograms. If the solution in the well remained clear after addition of the indicator, bacterial growth was inhibited by that particular concentration of plant extract.

The antibiotic neomycin (Sigma) was included as a reference standard in each assay, and pure acetone was used as the negative control.

Used for the plant extracts | Used for the controls

	1	2	3	4	5	6	7	8	9	10	11	12
A	5											
B	2.5											
C	1.25											
D	0.625											
E	0.31											
F	0.16											
G	0.08											
H	0.04											

Figure 3-1: Illustration of the microtitre plate, made up of 12 columns (1-12) and 8 rows (A-H), with the concentration of the plant extract in mg/ml present in each well after dilution

3.5.2.1. Preparation of bacterial cultures

Bacterial cultures were prepared for use one day prior to the experiment. On the day of the experiment, bacterial cultures were diluted at 1/100 with MH broth, to ensure a constant bacterial population growth. This was necessary to ensure that the resultant MIC was a true reflection of antibacterial activity of the extractant and not merely due to differences in microbial population size[85].

3.5.2.2. Preparation of extracts of positive and negative controls

Plant extracts were made up to a concentration of 20 mg/ml of extract in acetone (Merck). In cases where dried extracts failed to dissolve in acetone, di-methylsulphoxide (DMSO)(Merck) was included in the formulation (1: 4 v/v ratio). Once solubilised in the DMSO, acetone was added to make up the final volume. DMSO was previously tested by the laboratory and was shown to have no significant antibacterial activity at the concentrations tested[51]. Neomycin was freshly made up to a stock solution of 500 µg/ml.

3.5.2.3. Preparation of microplate

One hundred micro-litres of the sterile water was added to all wells prior to the addition of 100 µl of extract, to well A (Figure 3-1). After proper mixing 100 µl of the mixture was removed from well A and added to well B. The process was repeated until well H was reached. This brought the final volume in each well to 100 µl (after discarding 100 µl from last well) i.e. the first of the two-fold serial dilutions[66]. Each row (1 to 12) contained a different extract.

The last three wells (10 to 12) for all experiments were used for the neomycin stock solution (+ve control), pure bacterial culture (growth control) and the 100% acetone extraction medium.

The bacterial culture (100 µl) was added to each well, to bring the final volume to 200 µl i.e. the second of the two-fold serial dilutions. One plate was used per bacterial species. This was to prevent cross contamination during the initial stage of culture inoculation and the incubation period.

3.5.2.4. Determination of MIC

The microplates were incubated overnight at 37°C, prior to the addition of the 0.2 % m/v INT solution into each well. Plates were thereafter incubated for a further 10-30 min following addition of INT. Growth was indicated by a red colour change and read visually. The MIC was determined as the minimum concentration at which growth was inhibited i.e. no colour change was visible[30].

3.5.3. Significance of Antibacterial Activity

According to the criteria of the Journal of Phytomedicine (instruction to authors), crude plant activity, using the common bacterial species, will only be considered for publication if the activity determined was below 100 ug/ml[4]. This criteria was not followed as it was decided that the initial quantification of the probable minimum inhibitory concentration was more significant than imparting significance to the value calculated. However, any

plant that failed to inhibit activity in the first well (well A) was considered non-active or ineffective.

It was decided arbitrarily that plant activity would only be considered significant if the activity was reproducible: This could be either be on subsequent replication at different time periods and within one serial dilution concentration (most important); or by demonstrating similar results between the fractions being tested in the case of *U. sanguinea* (within one serial dilution concentration), i.e. between Fresh and Fresh dried; or Thawed and Thawed dried; or Fresh and Thawed; or Fresh dried and Thawed dried; or thirdly through corroborating evidence in other screening tests, such as bioautography. If only one of the latter two criteria were met plants would be seen as possessing significant activity but that the MIC results would still need confirmation.

3.6. EVALUATION OF ANTIBABESIAL ACTIVITY

3.6.1. Introduction

Babesia is an intracellular blood borne parasite. As such it has adapted to the intracellular environment where they are able to actively divide[56]. For this reason a defined medium and conditions are required for their *in vitro* growth and culture. Without this proper growth is not possible. The test drugs, diminazene aceturate and imidocarb diproprionate, were tested initially to validate a method to best quantify activity. Plant extracts were only tested thereafter.

3.6.2. Reconstitution of plant extracts

Plant acetone extracts were assigned a number from one to ten. Numbers were assigned randomly by selecting numbers from an open box. This allowed the experiment to be conducted as a blind study, to rule out researcher bias during the cell counting. Extracts were reconstituted, in all cases, in di-methyl-sulphoxide (DMSO) (Merck) and acetone at a ratio of 1:4 (v/v) to a final concentration of 50 mg /ml and stored at 3 ºC until utilised. Prior to each experiment plant extracts were diluted to 10 mg/ml. Only ten plant acetone extracts were reconstituted due to the lack of sufficient material. This included, four samples for *R. tridentata*, three samples for *U. sanguinea* bulbs, two samples for *E.*

elephantina, and one sample for *A. marlothii*. *U. sanguinea* leaves and *U. sanguinea* thawed dried bulbs were not tested as available extracts were used in earlier studies.

The reconstituted extracts were stored in a refrigerator for the duration of the experiment. The controls included a negative control of the DMSO: acetone diluent and positive controls of diminazene aceturate (Sigma) and imidocarb diproprionate (Sigma). Only acetone and DMSO were used as they were deemed safe compounds. In previous studies the protozology section of the Onderstepoort Veterinary institute (OVI) had shown that acetone and water were non-lethal to cell cultures (E. Zweygarth, 2002, Per com). DMSO was however, used for the first time, for solubilizing purposes. For this reason the negative control consisted of DMSO: acetone at a ration of 1:4.

3.6.3. Babesia caballi *cell cultures*

The system made use of *Babesia caballi* grown in erythrocyte cell cultures, developed by OVI[106]. The use of *B. bigemini* or *B. bovis* is not currently possible due to their poor growth *in vitro*. Any extract showing marked activity may at a later stage be tested on bovine *Babesia spp*[108]. From a therapeutic point of view, both *B. caballi* and *B. bovis* respond to similar drug treatment. Thus if a sample shows significant activity against *B. caballi* the results would most likely be a fair reflection of activity against *B. bovis*.

All cell cultures were grown and infected by the Section of Protozology (OVI) for the duration of the experiment. The culture systems made use of stabilates frozen in liquid nitrogen, designated stock 502. The parasites were isolated from a naturally infected horse at the National Yearlings Sale in South Africa in March 2002[109]. The stock, stored at the OVI, was thawed when required.

Equine blood was used to supply the cells for the culture medium. Blood was collected from an uninfected donor animal kept under tick free conditions, at the OVI stables, by means of venopuncture into sterile vacuum tubes containing EDTA as anticoagulant. (Vacutainer, Becton Dickinson, Meylan France).

The blood was washed four times by centrifugation (650G; 10 minutes at room temperature) and re-suspended in a modified Vega y Martinez phosphate-buffered saline

solution (mVYM)[107]. Normal mVYM consists of $CaCl_2 \cdot 2H_20$, KCL, KH_2PO_4, $MgSO_4 \cdot 7H_20$, $NaHPO_4 \cdot 7H_20$, NaCl and dextrose. Adenine, guanosine, penicillin and streptomycin, which is also present in the normal solution was omitted when constituting the modified medium.

After each wash the white blood cell layer was removed. After the fourth and final wash the horse red blood cells were re-suspended in the mVYM medium at a concentration of 10% (v/v) and stored at 4ºC until used.

3.6.4. Culture medium

A modified HL-1 medium (BioWhittaker, Walkersville, MD, USA) as described recently for the culture initiation of *T. equi* (*Babesia equi*), was used[109]. It was supplemented with 20 % horse serum, 2 mM L-glutamine, 0.2 mM hypoxanthine, 1 mM L-cysteine hydrochloride, 0.02 mM 2,9-dimethyl-4, 7-diphenyl-1, 10-phenanthrolinedisulphonic acid disodium salt (bathocuproine sulphonate, BCS; Serva Feinbiochemica, Heidelberg, Germany), 100 IU/ml penicillin and 100 µg/ml streptomycin. The medium was buffered with 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and 2.2 gl l $NaHCO_3$.

3.6.5. In vitro *assay*

Suspensions of continuously growing *B. caballi* cultures[109] were harvested and used in the *in vitro* assays. Aliquots of 1 ml (250 µl of infected erythrocytes in 750 µl of culture medium) were pipetted into each well of the 24 well culture plate (Corning, Bibby Sterilin, Staffordshire, UK). For twenty-four hours (-24 h) before the start of the experiment the cultures were incubated at 37 °C in a humidified 5% CO_2-in-air atmosphere, within the culture plate, to allow the cultures to establish themselves prior to the addition of the extract/drug.

At the start of the experiment (0 h) the extract (20 mg/ml) or drug was added into each well, together with new culture medium and uninfected erythrocytes. The final culture was made up of 250 µl infected cell culture suspension, 75 µl uninfected pelleted erythrocytes, 10 µl of the extract/drug and 665 µl of the culture medium i.e the initial 750 µl of culture medium was replaced with culture medium plus extract or drug. The final concentration of

the extract or drug in each well was 100 µg/ml, after this second dilution (diluted by the culture medium). The cultures were thereafter incubated for 24 h prior to a change in the culture medium.

At 24 h, 700 µl of culture supernatant was removed and replaced with 693 µl of fresh medium and 7 µl of the extract/drug. Initially cultures were grown for 48 h without a change in the culture medium. This was, however, accompanied by a high degree of parasite mortality as a result of the exhaustion of the available nutrients. For this reason the culture medium was changed at 24 h and included 7 µl of extract/drug to approximately replace the extract/drug removed.

At 48 h Giemsa-stained thin smears were prepared from the culture wells. At this stage 750 µl of the culture suspension was removed from each well and replaced with 75 µl uninfected erythrocytes and 675 µl fresh culture medium. At 72 h 700 µl of medium was replaced with culture medium. After 96 h, Giemsa stained thin smears were prepared and the experiment terminated. All experiments were repeated 10 days apart. This thus allowed the parasites to grow with the extract/drug being withdrawn.

All plant extracts were tested at a final concentration of 100 µg/ml. The positive controls diminazene aceturate and imidocarb diproprionate were tested at the following concentrations: 10, 1, 0.1, 0.01 and 0.001 µg/ml.

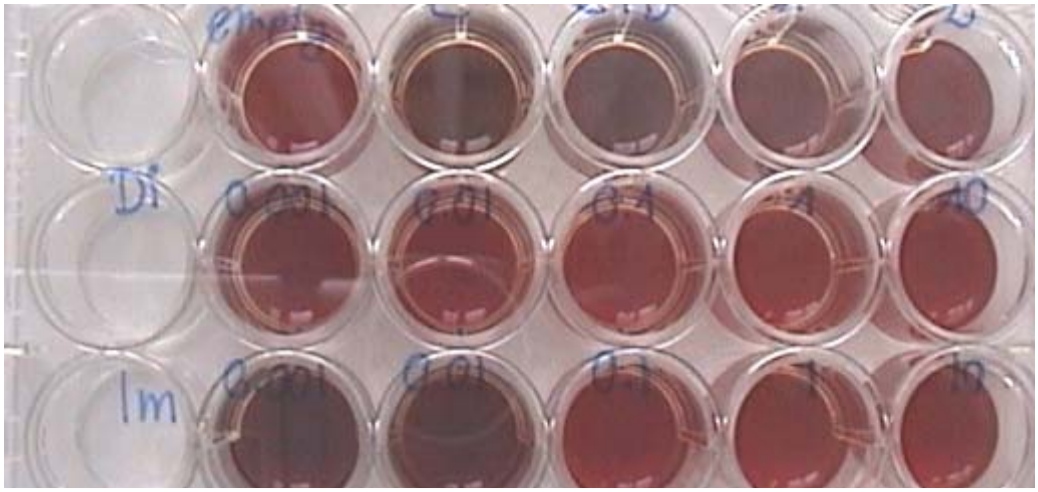
3.6.6. Measurement of antibabesial effect

3.6.6.1. Visual colour indicator

Growth was determined by a visual evaluation of a colour change within the culture wells. Mishra *et al*[73] determined that the colour of the culture cells was an indirect method of determining the exoantigen (Babesia) concentration. At maximal protozoal growth the colour of the culture changed from a bright red to a dark coffee colour. For a comparison one well always consisted of a pure culture without the extract or drug, to indicate the colour change associated with maximum growth.

The chocolate colour seen is due to a decrease in the available oxygen tension within each well. With the resultant decreased tension, high levels of carbon dioxide are available for binding with haemoglobin, and shows up as a dark chocolate brown/blue colour. When parasite growth is inhibited, free oxygen is available for binding making the haemoglobin appear bright red.

Figure 3-2 illustrates the colour changes one could expect: **A** is the pure blood. For an extract to have good activity, it should display a colour similar to that for fresh blood, as is seen with diminazene (D), and imidocarb (F). For poor activity, samples would be a chocolate brown/blue colour as seen with for the pure culture (B) and negative control (C).



Pure blood (A), Pure culture (B), DMSO: Acetone Diluent (Negative control) (C) , Diminazene at 10 µg/ml (D), Diminazene at 0.0001 µg/ml (E), Imidocarb diproprionate at 10 µg/ml (F), Imidocarb at 0.001 µg/ml (G)

Figure 3-2: Colour changes expected with the culture medium with *B. caballi*

3.6.6.2. Effects of a lower initial parasitic load

The method of quantification based on the visual colour change was regarded as being very subjective as the parasitic load responsible for the colour change was unknown. It was felt that at a fairly high parasitic load a drug that could only marginally affect the cell parasitaemia would not show a colour change i.e. the resultant reduced parasitic load would still be at a level at which all available oxygen is utilised.

For this reason, a second modified assay was included to determine the effect of a lower starting parasitic yield and to determine if an error was present in the methodology i.e. if the initial parasitic load was substantially lower, a colour change might be visible once the plant extract inhibited protozoal activity. A smaller initial parasitic load was introduced into the culture flasks. Instead of the usual 250 µl of infected cells, only 50 µl of infected cells were introduced into the culture. The experiment made use of the same extracts used in the fractionation assay (see 3.6.7).

3.6.6.3. Qualitative quantification of anti-babesial activity

A second method of quantification was also utilised. Diff quick (Kyron, SA) stained thin smears prepared from the infected cell cultures were evaluated under a light microscope to determine the mean number of parasitized cells (MPC). The MPC was determined by manually counting a total of one hundred cells in each of five microscopic fields. The number of infected cells was recorded and a mean parasitaemia determined.

Counting made use of a battlement technique in only the tapered edges, with the fields chosen at random[28]. Since the parasitized erythrocytes have a higher molecular mass they tend to be dragged to the edges of the smears i.e. the tapered edge.

The counting was made using a Zeiss light microscope (Carl Zeiss, West Germany) with a non-adjustable light source and a blue filter under 1000x magnification, with an oil immersion lens. To prevent bias during the counting process only one hundred cells were counted within each field (Usually 200-300 hundred cells per field). Each field was divided into four quadrants and numbered 1 to 4 anti-clockwise (Figure 3-3).

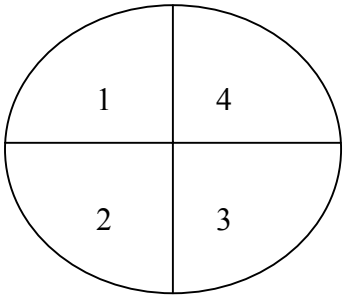


Figure 3-3: The division of fields of cell culture smears into quadrants prior to quantification of antibabesial activity

A table of five hundred random numbers, from 1 to 4, were generated by Excel (Microsoft, Office 2000). The table of random numbers was used to determine the quadrant in which counting was started. Occasionally 2 quadrants had to be counted to ensure that at least 100 cells were included. Once started, counting continued from either top to bottom or *vice versa* depending on field involved.

3.6.6.4. Evaluation of the results

For the drugs/extracts the percentage parasitized cells (PPC) was determined by dividing the percentage of the MPC of the extracts or drugs to the MPC of the pure culture.

PPC values below 100 % were statistically evaluated using Excel (Microsoft Office 2000) for significance with a 95 % confidence level. For both the plant extracts and drugs the cell counts per field from the five fields were compared between the control, diluent, and extract using Analysis of Variance (ANOVA).

The cell counts per field from the five fields of the pure culture and negative control was compared using the t-test, to determine whether the diluent had any inherent inhibitory activity on the growth of the parasites.

3.6.6.5. Determination of the effective concentration for the control drugs

The percentage inhibition was determined for the different drug concentrations

(100 % - PPC). Semi-log graphs were plotted, using natural logarithms (ln) i.e. the graphs were standard dose response curves with the percentage inhibition as the dependant variable.

The linear portion of the graph was analysed by linear regression to obtain a best-fit line. The equation for the best-fit line ($y = ax + c$), as determined by Excel, was used to determine the ln dose inhibiting 50% (ED_{50}) and 90% (ED_{90}) of the organism. The effective concentration for diminazene and imidocarb was thereafter calculated by using the equations obtained for the best-fit line

3.6.7. Fractionation assays

The *E. elephantina* rhizomes extracts were further evaluated by fractionation based on polarity to determine the probable polarity of the active component(s). The dried acetone extracts were dissolved in 50 % methanol-water. After adding ethylacetate the two phases were separated in a separating funnel. The methanol water phase was then partitioned into chloroform and finally a hexane phase. This process yielded a hexane, chloroform, ethylacetate and methanol-water fraction (Figure 3-4). Babesial cultures were then testes against each of the fractions as described in section 3.6.3.

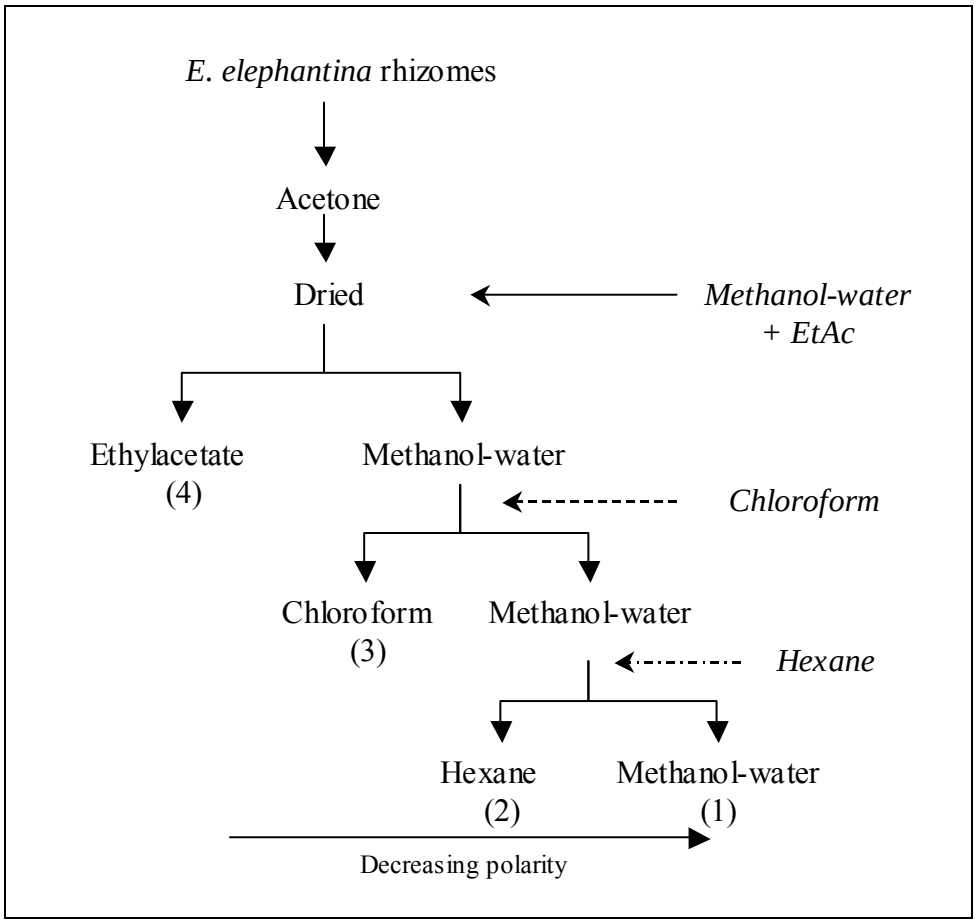


Figure 3-4: Illustration of process of fractionation, using four solvents ranging from very polar to non-polar

3.7. EVALUATION OF ANTI-THEILERIAL ACTIVITY

3.7.1. Introduction

The bioassay made use of *Theileria equi* grown in erythrocyte cell cultures, developed by OVI[106]. Cattle theileria species were not used as *T. equi* cultures were already available. Any extract showing marked activity against *T. equi* at a later stage would be tested against bovine *Theileria spp*[108]. Furthermore therapeutically both *T. equi* and the cattle *Theileria spp* respond to the same drug treatment, parvoquine and halofuginone. Therefore samples that show significant activity against *T. equi*, would most likely indicate activity against other theileria species.

The aim of the experiment was to determine the effect of these plant extracts only on the erythrocytic stages of parasitic infections. The effect against the lymphocytic stages in the parasitic life cycle was not be tested.

3.7.2. Preparation of plant extracts

All extracts were prepared using the same procedure as for the antibabesial screening.

3.7.3. Theileria equi cultures

Cell cultures were grown and infected within the Section of Protozology (OVI). The same material and methods, as used for the babesial assays, were employed. The experiment made use of stabilates frozen in liquid nitrogen and stored at the OVI. The organism (SW African Stock) was thawed when required.

3.7.4. In vitro *assay*

The culture method as employed for the determination of antibabesial activity was utilized for the antitheilerial screening.

3.7.5. Measurement of antitheilerial effect

The same method of evaluation as employed for the antibabesial assay was used for the antitheilerial assay.

3.8. EVALUATION OF ANTIRICKETTSIAL ACTIVITY

3.8.1. Introduction

The assay made use of *Ehrlichia ruminantium*. No model for *Anaplasma spp*. was available. Plants with possibly activity against the *Anaplasma spp* were tested using *E.*

ruminantium. This was considered relevant, as both *Anaplasma spp* and *E. ruminantium* are rickettsial parasites which respond to similar therapy, such as oxytetracycline.

The bench model developed by the OVI was used[105]. This model uses bovine aortic endothelial cell cultures (BA 886) infected with the *Ehrlichia ruminantium* (Welgevonden stock). Oxytetracycline was used as the positive control.

3.8.2. Preparation of plant extracts

The plant extracts, which were used for the protozoan assays, were also utilised for the rickettsial assays as testing occurred concurrently. Oxytetracycline and the plant extracts were reconstituted in all cases in di-methylsulphoxide (DMSO) and acetone at a ratio of 1:4 (v/v). The plant extract were diluted to a final concentration of 50 mg /ml. Prior to each experiment the re-constituted plant extracts were diluted with sterile water to 10 mg/ml. (first of two dilutions). The oxytetracycline control was tested at the following concentrations: 10, 1, 0.1, 0.01 and 0.001 µg/ml. The negative control samples were tested as a pure DMSO: acetone diluent at same ratio of 1:4.

3.8.3. Ehrlichia ruminantium *cultures*

The BA 886 cell lines were used as host cells for *E. ruminantium*[105]. Cells were cultured as monolayers at 37°C in a humidified atmosphere of 5% CO_2 in air on the floor of 25 cm^2 culture flasks. Endothelial cell lines were used at passage 100 to 125.

3.8.3.1. Rickettsial culture medium

Cells were propagated in a medium consisting of Dulbecco's modified Eagle's nutrient mixture, Ham F-12 (DME/F-12)(Sigma, St. Louis, MO, USA; D 0547) containing 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and 1.2 g/l sodium bicarbonate[105]. The medium was supplemented with 10 % (v/v) heat-inactivated foetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. This medium was used for both infected and uninfected cell cultures.

3.8.3.2. *In vitro* assay

The endothelial cell cultures, heavily infected with *E. ruminantium*, were harvested by scraping off the cell monolayer into 5 ml of fresh rickettsial culture medium. The cell suspension was centrifuged (800 x g for 10 min at room temperature). Two hundred µl of the supernatant, containing *E. ruminantium* elementary bodies, together with 2 ml of the culture medium was distributed into 25 cm² culture flasks containing the established BA 886 cells cultures (grown for 24 hours) adhered to the floor of the flask, (Figure 3-5). Cultures were incubated for approximately 3 hours, where the medium was changed with 5 ml of fresh medium and 10 µl of the respective extract or control. The final concentration of the extract in each plate was 100 µg/ml (The extract diluted in the culture medium). Cultures were thereafter incubated for 48 h at 37 °C.

Figure 3-5: A culture flask with an actively growing *E. ruminantium* culture in endothelial cells

3.8.4. Measurement of antitheilerial activity

After 48 h, the supernatant medium was removed from within the flask, and the attached cell monolayer air-dried and fixed in methanol. Culture flasks were stained with diff quick (Kyron, SA) prior to evaluation. The mean parasitized cells (MPC) was determined by manually counting a total of one hundred cells in each of five microscopic fields, using a Zeiss light microscope, with a non-adjustable light source and a blue filter under 300x magnification.

Fields were chosen at random. The counting process as represented in Figure 3-6 started in zone A, C, E, G and I. Once started counting continued, towards the opposite side of the flask, until one hundred cells were counted in each of the five zones.

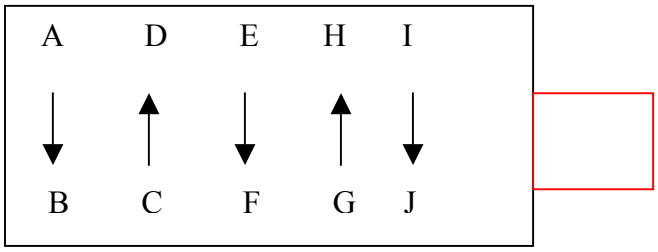


Figure 3-6: Cell count method used

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3.8.4.1. Evaluation of the results

The same method of evaluation was employed as for the antibabesial assay.

3.8.4.2. Determination of the effective concentrations of tetracycline and the plant extracts

The same method of evaluation was employed as for the antibabesial assay.

3.9. ANTI-OXIDANT ACTIVITY

3.9.1. Introduction

Numerous methods are available for determining the presence and quantification of the degree of anti-oxidant activity present in herbal extracts. Most of these methods make use of a colour reaction and indicator to assess the degree of anti-oxidant activity. The Phytomedicine program uses the diphenyl-picrylhydrazyl (DPPH) and trolox equivalent anti-oxidant capacity assay (TEAC).

The DPPH assay is a qualitative indicator of free radical scavenging activity. DPPH is reduced from a stable free radical that is purple in colour to diphenylpicryl hydrazine that is yellow, in the presence of an anti-oxidant (Figure 3-7). The visual colour change is observed on the chromatograms. This technique shows the number of anti-oxidant compounds separated by TLC and also gives an indication of the polarity of the separated compounds.

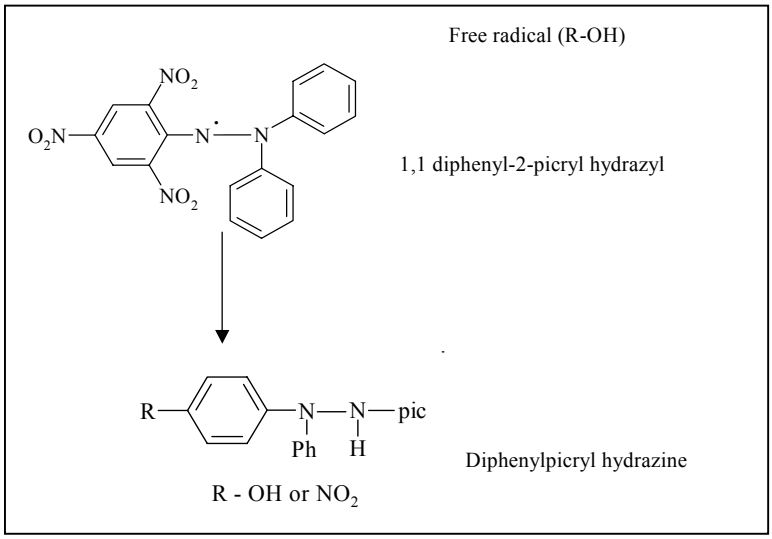


Figure 3-7: Reaction of DPPH with hydroxyl groups of free radical (R-OH) to produce 2-(4-hydroxyphenyl)-2-phenyl-1-picryl hydrazine and R-NO2, 2-(4 nitrophenyl)-2phenyl-1-picrylhydrazine

The TEAC assay makes use of a preformed blue/green ABTS [2,2'-azinonis-(3-ethyl-benzothiazoline-6-sulfonic acid)](Sigma) radical cation. The blue/green $ABTS^{+-}$ chromophore radical is produced through the reaction between ABTS and potassium persulfate[83].

In the presence of an anti-oxidant the $ABTS^{+}$-radical changes from blue/green to colourless depending on the degree of the reaction. The reaction is dependent on a multitude of factors e.g. time scale, anti-oxidant capacity of the compound, its concentration and the duration of the reaction. The extent of discolouration as measured by spectrophotometry, is expressed as the percentage of inhibition of the $ABTS^{+}$-radical. The result was compared to the percentage inhibition of the standard trolox, a synthetic water-soluble vitamin E analogue.

3.9.2. DPPH Assay

The purple DPPH reagent (Sigma, SA) was made up to a concentration of 0.2% (m/v) in methanol. Chromatograms, eluted in CEF and EMW, were sprayed with the DPPH-radical, as described by Braca *et al*[17] and monitored for a visual colour change over 30 minutes.

The CEF and EMW eluent systems were chosen as they were fairly polar and since anti-oxidant substances are usually polar in nature.

3.9.3. Trolox equivalent anti-oxidant capacity (TEAC)

Pure ABTS (192 mg) was mixed with 50 ml of sterile water to make up a 7 mM stock solution. The $ABTS^{+}$-radical was thereafter produced by reacting the pure ABTS stock solution with 33 mg of potassium sulphate 12 to 16 hours before use. The preformed radical was stored at 4 ºC until needed.

The plant extracts were made up in water, to a concentration of 1, 0.5, 0.25, 0.125 and 0.0625 mg/ml prior to the experiment. Trolox (Sigma,SA) was made up to a concentration of 0.5, 0.25 and 0.125 mg/ml in ethanol.

Prior to the quantification of activity, the pre-formed $ABTS^{+}$-radical was diluted in ethanol (Merck) to an absorbance of 0.70 ($\pm$0.02), at a wavelength of 734 nm (Beckman spectrophotometer blanked with ethanol).

Once the plant extract/trolox (1 µl) was combined with the $ABTS^{+}$-radical (1 ml), the absorbancy at 734 nm was measured at minute intervals for a total of six minutes. The experiment was terminated after six minutes. In order to produce an acceptable dose response curve for analysis results were examined after the first minute. Under conditions where the reaction was perceived to proceed too rapidly, the experiment was stopped and results discarded. For very potent compounds complete reduction of the ABTS radical may occur within the first minute, which would preclude a proper evaluation over the six minute period. The subsequent dilution was thereafter tested. The process was continued until two appropriate dilutions, which were active for the entire six-minute period, were found.

Once the correct concentrations were determined the test was repeated four times. The two samples, which differed by a minimum 0.02 of absorbency units, were used to determine the new mean absorbency i.e. after the anti-oxidant neutralization/reduction of the $ABTS^{+}$-radical. This mean absorbency was utilised to determine the percentage change in absorbency, for each minute, by comparing this new absorbency to the initial absorbency of the $ABTS^{+}$- radical.

Percentage change in absorbancy =

$$\frac{\text{Initial absorbancy of } ABTS^{+}\text{- radical (734nm) - New mean absorbancy of } ABTS^{+}\text{- radical}}{\text{Initial absorbency of } ABTS^{+}\text{-radical (734 nm) x 100}}$$

Figure 3-8: Equation used to determine the % change in absorption for each of the concentration of plant extract or the trolox standard

This allowed for proper graphical representation of the degree of anti-oxidant activity. The curves were plotted for each period (minute) with the dependant variable being the percentage change in absorbency and the independent variable being the different concentrations at which the extract was analysed ($y = ax + c$). For the mathematical comparison of anti-oxidant activity the slope (a) of the extract curve was divided by the slope (a) of trolox curve to obtain the TEAC value. If a sample has equivalent activity to trolox its TEAC value would be 1 and if the extract is more active, the TEAC value would be greater than 1.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. EXTRACTION EFFICACY FROM THE SELECTED PLANTS

The efficiency of extraction of the 3 sequential acetone extractions and percentage yield of plant material from *A. marlothii*, *R. tridentata*, *E. elephantina* and *U. sanguinea* are listed in Table 4-1 and Table 4-2. The percentage yield ranged from 0.4 % for dried *U. sanguinea* bulbs to 7.8 % for the *E. elephantina* rhizomes.

In all cases the largest percentage relative yield was obtained after the first extraction with a decrease in the extraction efficiency for each subsequent extraction. For all samples the efficiency of extraction of the third extraction process was below 20 % of the total yield. If a forth extraction was included it is believed that the extracted mass would be negligible.

Table 4-1: Efficiency of extraction for each of the three subsequent extractions with acetone extraction solvent and the percentage yield based on original mass of plant material.

Sample	Efficiency of extraction			Percentage yield
	1	2	3	
Am leaves	45.97	40.84	13.19	1
Rt leaves	57.38	28.53	14.09	4.27
Rt stem bark	77.73	14.67	7.60	3.38
Rt tubers	65.89	23.28	10.83	6.02
Rt root bark	54.66	26.25	19.09	2.69
Ee rhizomes	86.21	11.98	1.81	7.88
Ee leaves	64.60	29.27	6.13	5.48
Us Leaves	65.63	18.75	15.62	2.03

Am: *A. marlothii* (Am); *E. elephantorrhiza* (Ee); *R. tridentata* (Rt); *Urginea sanguinea* (Us)

Table 4-2: Efficiency of extraction for each of the three subsequent extractions with acetone extraction solvent and the percentage yield for the various *U. sanguinea* bulb preparations

U. Sanguinea bulb preparations	Efficiency of extraction			Percentage Yield
	1	2	3	
Fresh (F)	75.58	18.60	5.81	0.69
Thawed (T)	85.19	11.04	3.76	0.82
Fresh Dried (FD)	56.03	30.65	13.32	0.40
Thawed Dried (TD)	82.97	13.86	3.17	0.69

U. sanguinea fresh bubs (F), thawed bulbs (T), fresh dried bulbs (FD) and thawed dried bulbs (TD)

When the extractability from the different plant leaves was compared, the *A. marlothii* leaves yielded only 1% of extractable mass. This was considerably lower than that for the other leaf material: *U. sanguinea* 2%, *R. tridentata* 4.27% and *E. elephantina* 5.48 %. The quantity extractable from these leaves were considerably lower than the quantity extracted from members of the Combretaceae with thinner leaves e.g. the yield from *A. marlothii* leaves was up to twelve times lower[32]. It would thus appear that leaves of shrubbery are more difficult to extract than those from trees.

When the extractability of the underground storage structures were compared, the tuber of *R. tridentata* had the lowest value at 6 % and *E. elephantina* rhizomes had the highest value at 8%. Both these structures had a much higher percentage yield than the *U. sanguinea* bulbs (Table 4-2). When comparing the different barks of *R. tridentata*, the root bark had a poorer extractability than the stem bark. This extractability was midway to that for the leaves and the tubers. From this data it would appear that the greatest yields are obtainable from the rhizomes, tubers, leaves and lastly from bulbs.

4.1.1. Effects of freezing and drying on the extraction efficiency from U. sanguinea *bulbs*

Extractability of *U. sanguinea* bulbs, including the effect of drying and freezing are given in Table 4-2. The largest yield was obtained from the frozen bulbs (0.82%). Minocha *et al*[72] showed that the extraction of cellular polyamines and ions from plants may be

increased by first freezing the samples, prior to extraction. They ascribed the increased extractability to low temperature-induced damage to the cell membrane, an effect that occurs when freezing is carried out at low temperatures over a period of time[100]. It is thus plausible that a similar process was contributory to the increased extractability seen. For this reason it is assumed that the cryogenically induced cellular damage allowed more intracellular components to be extracted.

In investigating the percentage extracted with each subsequent extraction, extractability was highest for the first extraction in all samples. The mass extracted with the first extraction was higher for both thawed samples than for the fresh material yielding 80% of all material extracted (Table 4-2). Thus freezing not only increases the yield extracted, but also appeared to enhance the efficacy of a single extraction.

Drying substantially decreased the rate of extraction for all dried samples in comparison to either the fresh or thawed bulbs samples. With the fresh dried bulbs it decreased the yield by 42% and the frozen dried bulbs by 16%. It appears that the procedure of drying bulbs before extraction, as is usually done, substantially decreases the yield of compound extracted by acetone. Differences between dried and fresh material could be due high the water content within the fresh bulbs (c. 50%). With extraction at a 5:1 (m:v) ratio, one is more likely extracting with 80% aqueous acetone instead of 100% acetone. It is, however, unlikely that this would have had a major effect on the results.

The fresh material was also slimy and a large proportion of the extracted material would be mucilage. If mucilage does not react with the vanillin spray reagent the lower number of compounds visible after TLC may be due to lower quantity of separated material on the chromatograph. It is not clear whether the effects of drying are an artifact in the drying process, or whether mucilage present in fresh material had an effect on extractability of compounds.

Intra-species variation can also not be ruled out (numerous bulbs were utilized). This was, however, considered non-significant as all material had originated from the same field, although harvested at different times. Since all plants were exposed to similar environmental conditions natural phenotypic variation is mostly likely small.

The extractability from the bulbs differed markedly form the other plant storage parts as well as from the different bulbous fractions of *U sanguinea* (Table 4-2,), which demonstrated poor extractability (< 0.82 % in all cases). This would further illustrate that bulbous material is more difficult to extract. It should however be kept in mind that the *U. sanguinea* is composed of sheaths, while *R tridentata* is a solid tuber and *E. elephantina* a solid rhizomes. Although there is no general information available on the physiological differences between bulbs, tubers and rhizomes, it is possible that a difference in moisture content and/or constituents may have had an influence on the final extraction concentration.

4.2. COMPLEXITY OF THE CHROMATOGRAMS

Thin layer chromatography (TLC) was used to fingerprint the plant extracts. This allowed for a comparison of the R_f values and thus aided in the identification of biologically active bands on the chromatograms, used for bioautography. The Rf value can however provide corroborative evidence as to the identity of a compound. If the identity of the compound is suspected but not proven, a pure standard need to be run simultaneously i.e. it is generally not possible to further evaluate compounds/bands on a TLC without the availability of pure standards. If two compounds have the same R_f values in several solvent systems they are most likely, although not necessarily, the same compounds.

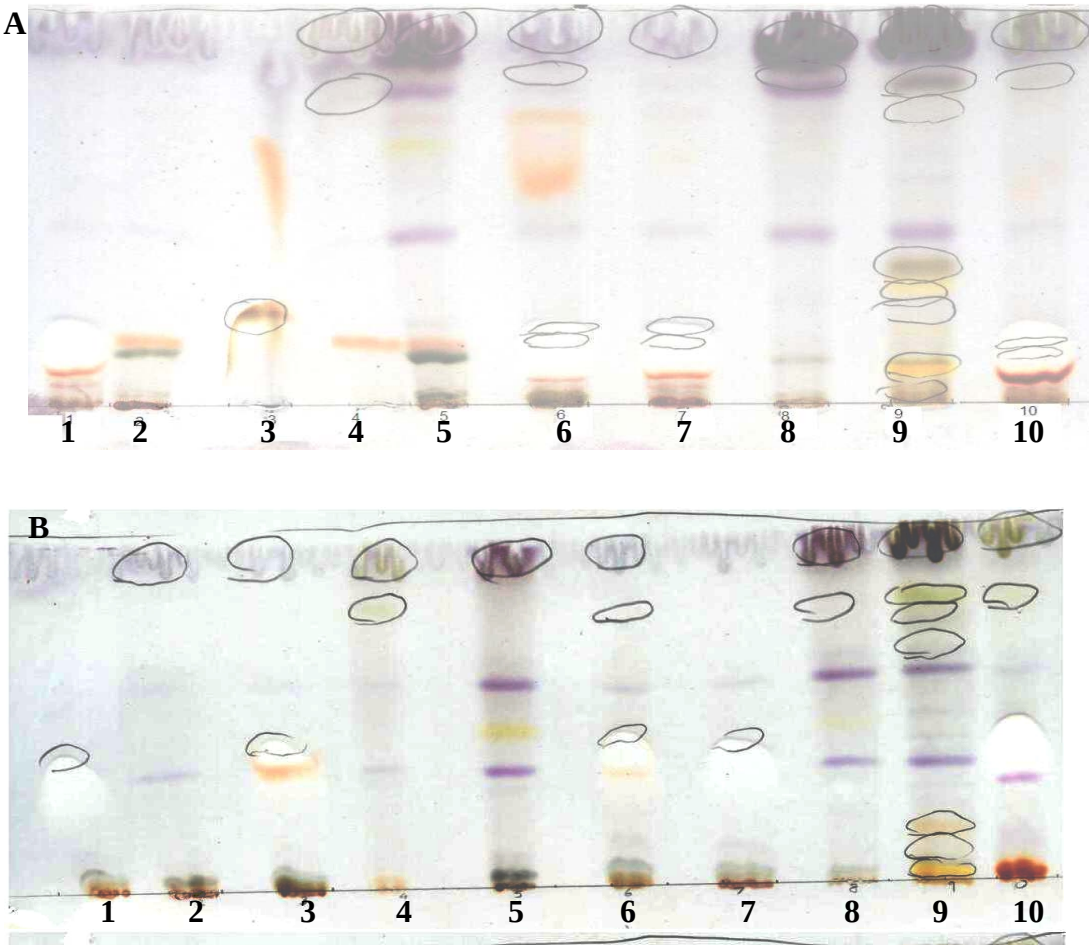
Two spray reagents, vanillin and anisaldehyde were available for the visualization of compounds on the developed chromatograms. Two TLC plates spotted with extracts from *U. sanguinea* and *A. marlothii*, developed in EMW, were sprayed with either a vanillin or anisaldehyde spray reagent. For the majority of the extracts, the vanillin spray reagent was either equal or superior to the anisaldehyde spray reagent in that more bands were visible. The vanillin spray reagent was thus chosen for the subsequent visualization of compounds, for all the other solvent systems and plants extracts.

Table 4-3: Comparison of the number of TLC bands visible of plant extracts using EMW with either the vanillin and anisaldehyde spray reagents

Extract	Anisaldehyde	Vanillin
Am Leaves acetone	6	10
US Defrosted Bulbs acetone	4	4
US Defrosted Dried acetone	7	9
US Fresh Bulbs acetone	1	4
US Fresh Bulbs methanol	1	3
US Fresh Dried acetone	5	6
US Fresh Dried methanol	2	3
US Frozen Dried methanol	2	3
Us Leaves acetone	4	4
Us Leaves methanol	4	4

U. sanguinea (Us); *A. marlothii* (Am)

From the solvent systems used CEF and EMW were more efficient in their ability to separate compounds than HE. Both systems also appeared to be equally efficient in their ability to separate out the compounds (Figure 4-1). Since normal phase silica allows only the less polar compounds to be eluted, due their ability to retain polar substances and since EMW and CEF are the more polar eluent systems, it would appear that the majority compounds of the plant are of intermediate polarity i.e. they are sufficiently non-polar to be eluted on normal phase silica but while still polar enough to be eluted by the more polar solvent systems. This is as expected as the acetone extraction solvent is broad spectrum in its ability to remove compounds of the different polarity.



U. sanguinea F methanol (1); *U. sanguinea* F acetone (2); *U. sanguinea* T acetone (3); *U. sanguinea* Leaves acetone (4); *U. sanguinea* TD acetone (5); *U. sanguinea* FD methanol (6); *U. sanguinea* TD methanol (7); *U. sanguinea* FD acetone (8); *A. marlothii* acetone (9); *U. sanguinea* Leaves methanol (1)

Figure 4-1: Chromatograms for *A. marlothii* and *U. sanguinea* eluted in EMW (A) and CEF (B)

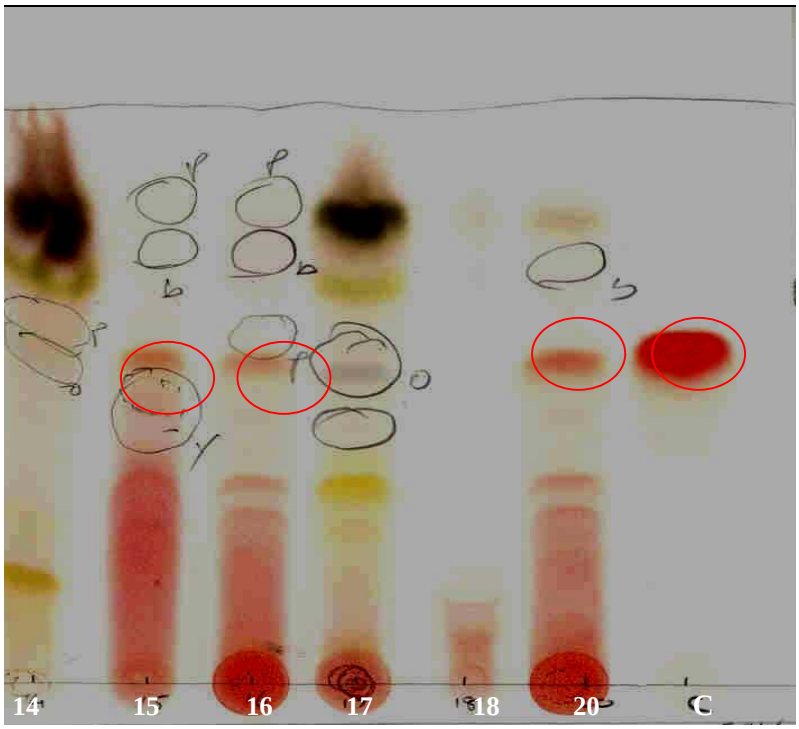
For the *U. sanguinea* bulbs there was more than two times the number of bands from dried material than from fresh material for both the fresh and thawed bulbs (Table 4-4). Extracts from fresh bulbs did however contain bands with the same R_f values as for the extracts from the dried bulbs. There was little difference in the number of bands seen under UV light.

Since TLC is not accurate as a qualitative tool, it may be possible that "inert" compounds such as mucilages may be extractable from fresh material. These compounds may be insoluble upon drying and explain the lower yield from dried bulbs.

Table 4-4: Total number of bands seen for the various *U. sanguinea* bulb preparations, with the different eluents systems using vanillin spray reagent

Extract	**Number of bands Seen**				
	EMW	CEF	HE	E H	Total
Fresh	4	3	2	1	10
Thawed	4	3	2	1	10
Fresh dried	6	5	5	6	22
Thawed dried	9	5	5	6	25

Chloroform: Ethyl Acetate: Formic Acid (CEF); Hexane: Ethyl Acetate (HE); Ethyl Acetate: Methanol: Water (EMW); Ethyl Acetate: Hexane (EH)



E. elephantina leaves acetone (14); *E. elephantina* roots acetone (15); *R. tridentata* bark acetone (16); *R. tridentata* leaves acetone (17); *R. tridentata* bulbs acetone (18); *R. tridentata* stem acetone (20), catechin (C)

Figure 4-2: Chromatogram of *R. tridentata* and *E. elephantina* eluted in EMW with the pure catechin standard

All *R. tridentata* extracts eluted poorly form the starting zone, as seen by the large quantity of the visible red material that failed to elute (Figure 4-2). This could be either the result of overloading the thin layer plates or due to the presence of very polar compounds. Normal phase silica allows for the movement of the less polar compounds[90]. For the grape seed, this has been described as the condensed tannins[84]. It is deduced that *E. elephantina* rhizomes had a high tannin content due to their prominent red colour. The presence of catechin indicates that the plant may also contain the condensed tannins.

The condensed tannins are made up of the catechins and their oiligomers (Figure 4-3). These compounds tend to impart a red colour to the plant and plant extract[90]. The oligomers are also large molecules which results in the poor elution from the starting zone. Since both the plants are of the same family it is plausible that the *R. tridentata* also contains the proanthocyanidins[84]. Further evaluation of extracts requires separation by HPLC or the isolation of specific oligomers. This would not only aid in the understanding of the plants medicinal activity, but may also confirm the plants taxonomy from a chemical classification.

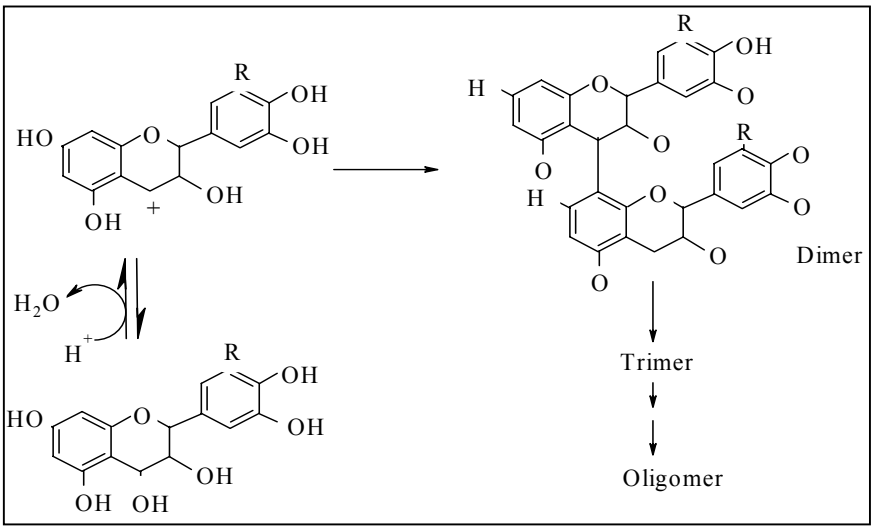


Figure 4-3: Chemical structures in the formation of the dimers and oligomers, which are also known as the proanthocyanidins

4.3. ANTIBACTERIAL ACTIVITY

4.3.1. Bioautography

Chromatograms developed in CEF and EMW were used for the bioautography as the HE eluent system demonstrated poor separation during the initial chromatographic analysis. All plant extracts had activity against *S. aureus* (Figure 4-4 to Figure 4-7) while only *A. marlothii* and *U. sanguinea* leaves were active against *E. coli* (Figure 4-8). There was, however, a difference in the activity between the different plant parts extracted.

Except for the *U. sanguinea* leaves, no apparent antibacterial activity was observed for plant methanol extracts. In the chloroform-ethyl acetate-formic acid (CEF) eluent (Figure 4-6) more active chromatographic bands (n=3) were observed in *U. sanguinea* leaves after methanol extraction than after acetone extraction (n=1) on the same bioautogram. This indicates a more polar nature of the active compound.

From the extracts eluted in EMW and sprayed with *S. aureus*, the thawed dried *U. sanguinea* (A) and *A. marlothii* (D) acetone extracts, both had one active band while the fresh dried acetone extract showed two active bands (B & C) (Figure 4-4). Both the *U. sanguinea* fresh dried and thawed dried samples had a common active band (A & B). *U. sanguinea* and *A. marlothii* had band A and band D in common respectively. Although the R_f values suggest that these two plants may have a similar compound, the results are not conclusive.

All *R. tridentata* and *E. elephantina* acetone extracts eluted in EMW showed activity against *S. aureus*. The bands were broad, and seemed to coalesce (B, C and E, F) (Figure 4-5). Since these compound were surmised to be condensed tannins the activity demonstrated by these plant extracts may represent non-selective anti-bacterial activity[11,21,43].

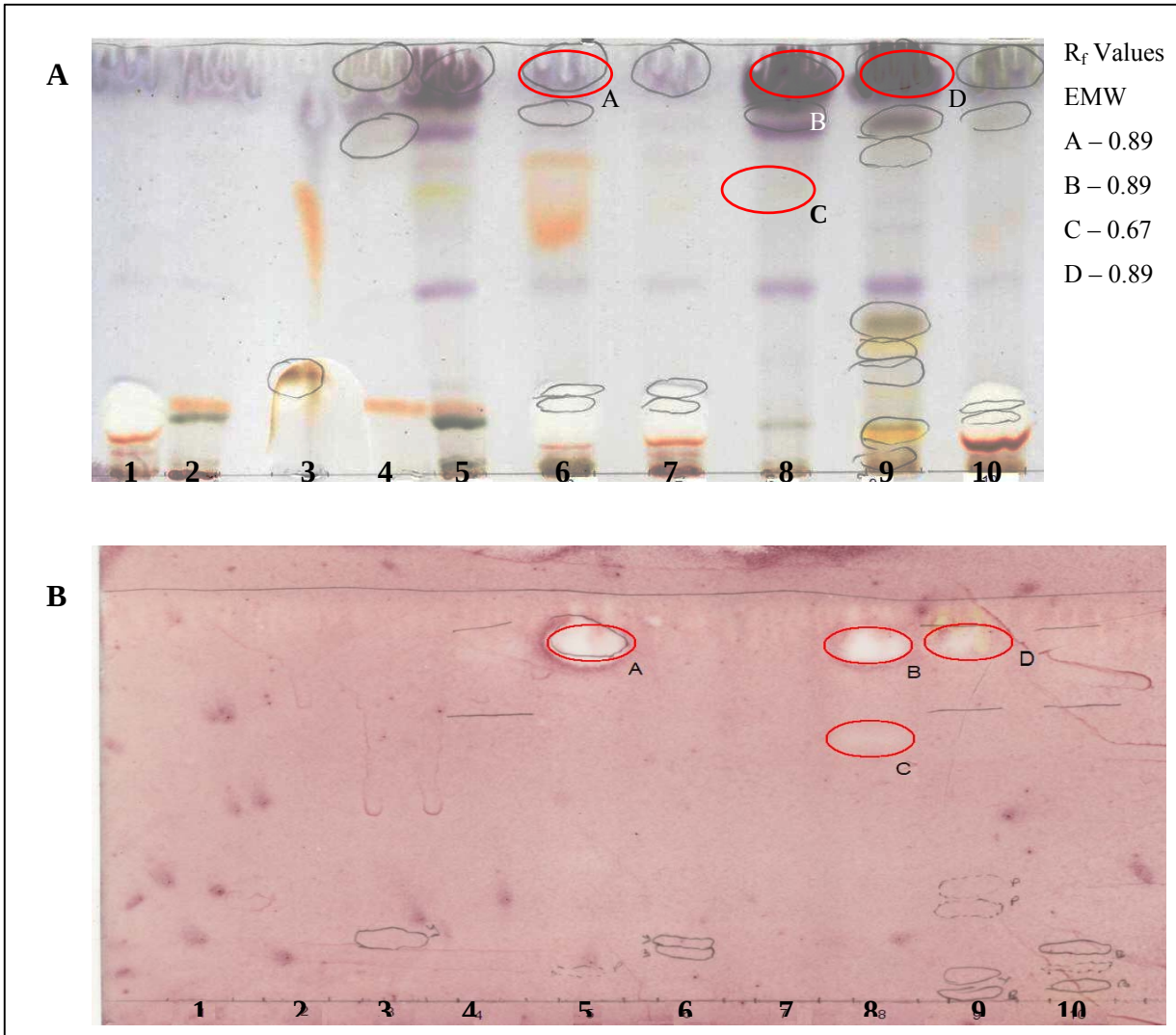
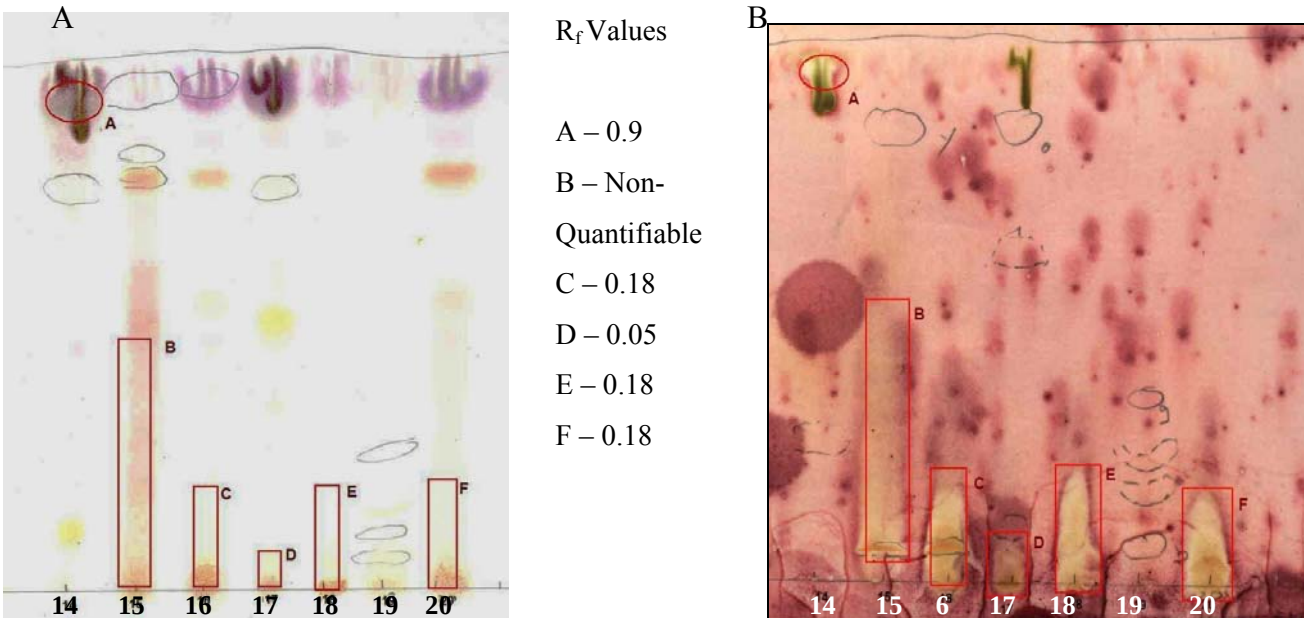


Figure 4-4: Chromatograms of *A. marlothii* and *U. sanguinea* eluted with EMW and sprayed with vanillin (A) and with *S. aureus* (B)

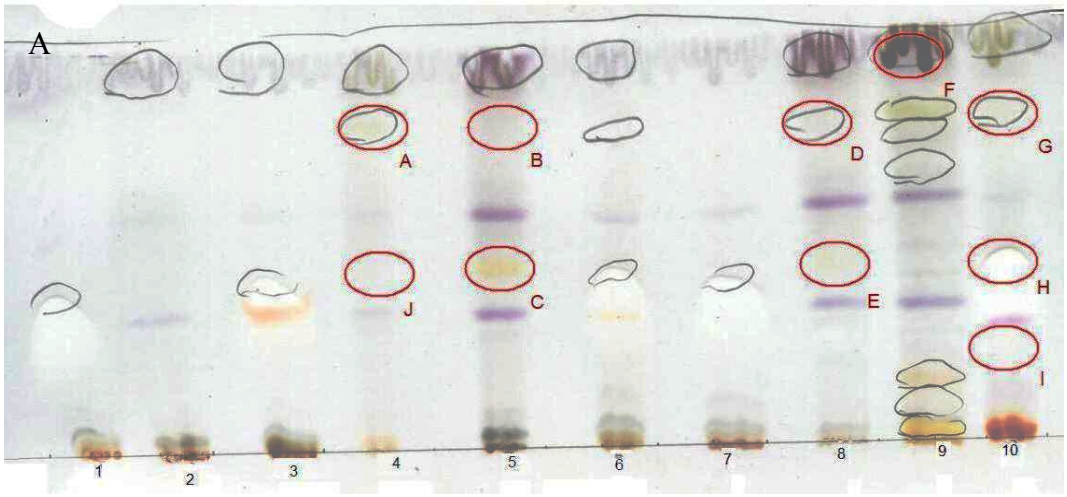


E. elephantina leaves acetone (14); *E. elephantina* roots acetone (15); *R. tridentata* bark acetone (16); *R. tridentata* leaves acetone (17); *R. tridentata* bulbs acetone (18); *R. tridentata* stem acetone (20)

Figure 4-5: Chromatograms of *E. elephantina* and *R. tridentata* eluted in EMW and sprayed with vanillin (A) and *S. aureus* (B)

From the extracts eluted in CEF (Figure 4-6), *U. sanguinea* leaves, in acetone showed one active spot (Band A). A possible second spot may also be present (Band J) which was not very clear on the bioautogram. In contrast the methanol fraction from leaves, had three active bands (G, H and I). When comparing R_f values, in addition to the two similar bands (A, G and J, H) band I was the additional band. The *E. elephantina* and *A. marlothii* leaves each showed one active band (Band A-Figure 4-7 and band F-Figure 4-6).

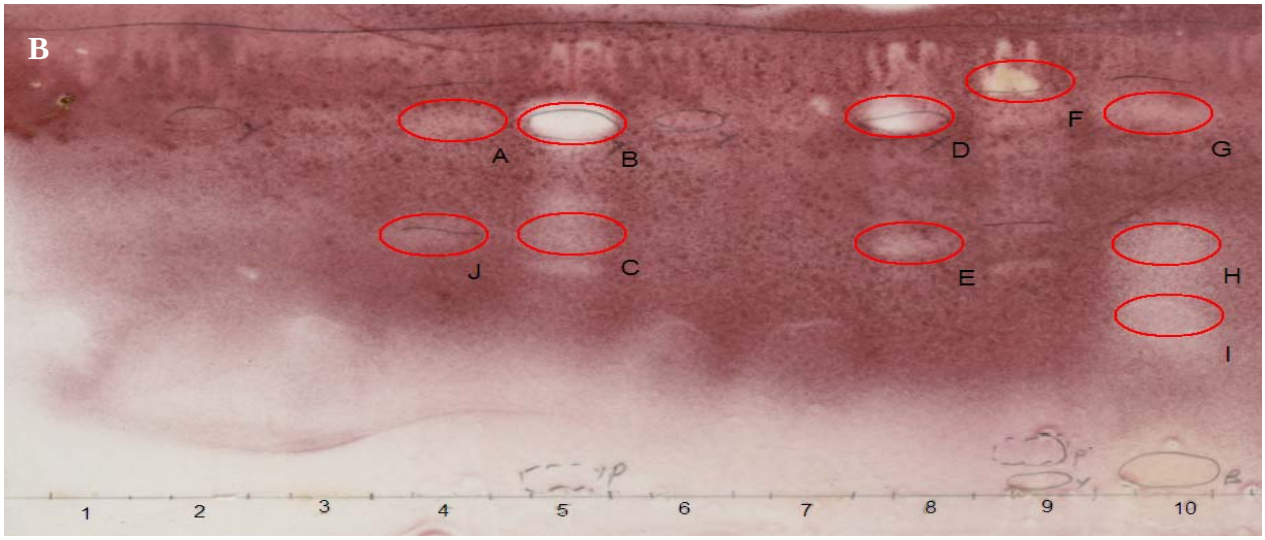
Both the thawed dried and fresh dried *U. sanguinea* bulbs had two active bands (B,C & D,E), with the same R_f values. The reason for two bands being visible in CEF and not EMW is unknown as the same stock solution was spotted onto the chromatograms, prior to elution. In all cases it was only the dried *U. sanguinea* material that showed activity.



R_f Values

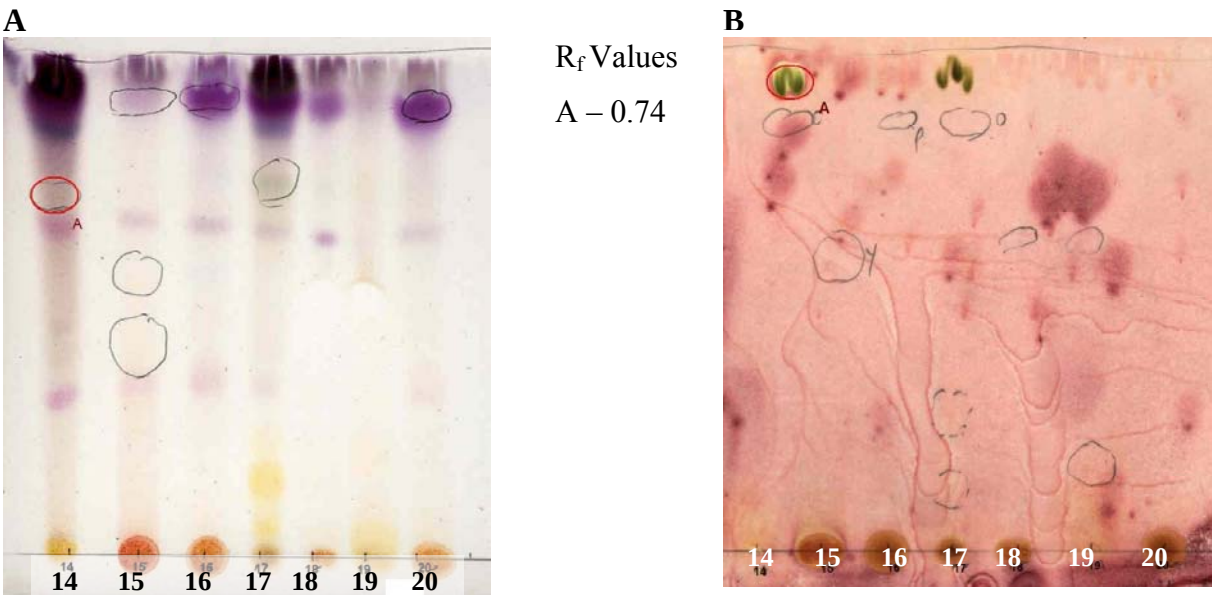
CEF

A - 0.75
B – 0.75
C – 0.5
D – 0.75
E – 0.5
F - 0.93
G - 0.75
H - 0.51
I - 0.31
J – 0.5



U. sanguinea F methanol (1); *U. sanguinea* F acetone (2); *U. sanguinea* T acetone (3); *U. sanguinea* Leaves acetone (4); *U. sanguinea* TD acetone (5); *U. sanguinea* FD methanol (6); *U. sanguinea* TD methanol (7); *U. sanguinea* FD acetone (8); *A. marlothii* acetone (9); *U. sanguinea* Leaves methanol (10)

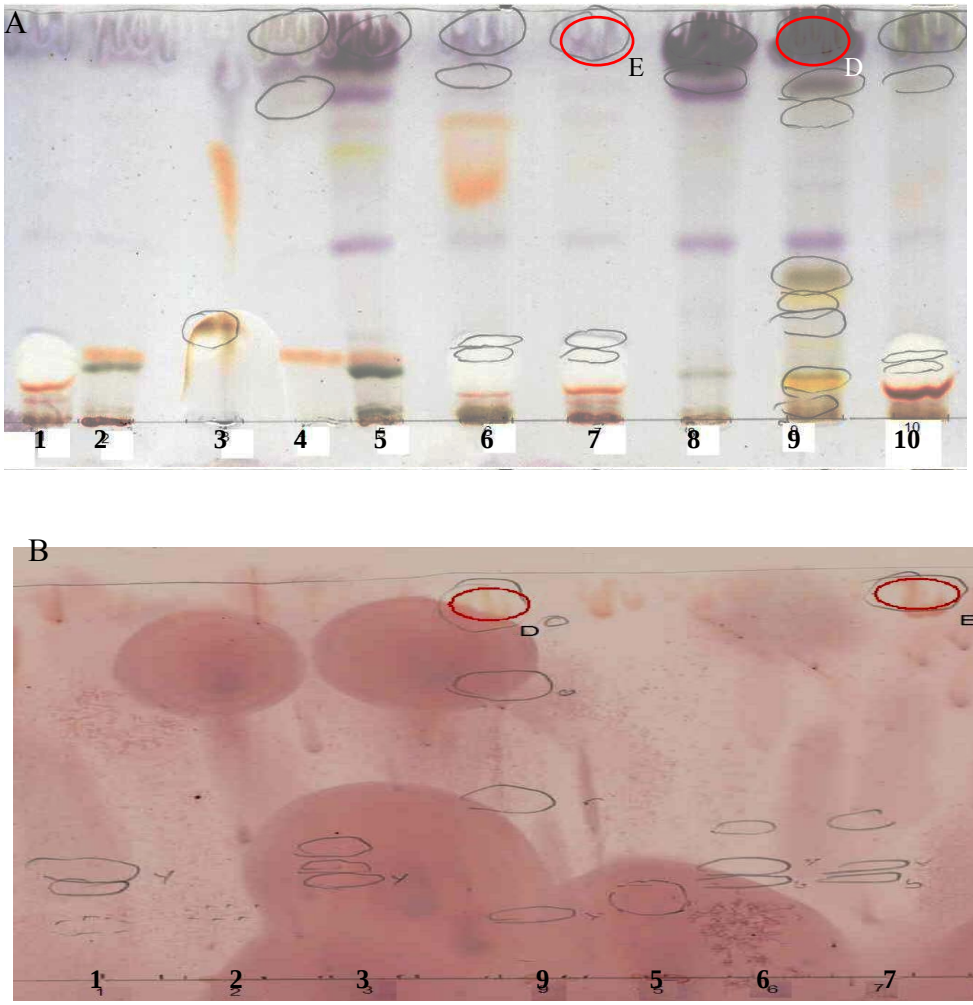
Figure 4-6: Chromatograms of *A. marlothii* and *U. sanguinea* eluted in CEF and sprayed with vanillin (A) and *S. aureus* (B)



E. elephantina leaves acetone (14); *E. elephantina* roots acetone (15); *R. tridentata* bark acetone (16); *R. tridentata* leaves acetone (17); *R. tridentata* bulbs acetone (18); *R. tridentata* stem acetone (2)

Figure 4-7: Chromatograms for *E. elephantina* and *R. tridentata* eluted in CEF and sprayed with vanillin (A) and *S. aureus* (B)

From all the plant extracts, only the *A. marlothii* acetone extract (band D) and the thawed dried *U. sanguinea* methanol extract (band E) eluted in EMW demonstrated activity against *E. coli* (Figure 4-8). The same band appeared to be active against *S. aureus* and *E. coli* for the *A. marlothii* extract. Both *R. tridentata* and *E. elephantina* showed no apparent activity against *E. coli*. However, it appears that the time period of culture was critical. When the plates were initially sprayed with the p-iodonitrotetrazolium (INT) violet and examined 0.5 and 1 hour thereafter, inhibition zones were present with a distribution similar to that of *S. aureus*. However when left to grow for the full 4 hour period no inhibition zones were visible. The plant could perhaps be mildly bacteriostatic, as bacterial growth was marginally inhibited, at the concentrations present on the chromatogram i.e. when allowed to proliferate for an additional three hours, bacterial colonies where probably able to reach a sufficient colony size, where the biological conversion of the INT became possible (INT is converted by only actively growing organisms to red formazan).



R_f Values
EMW
D – 0.89
E – 0.89

U. sanguinea F methanol (1); *U. sanguinea* F acetone (2); *U. sanguinea* T acetone (3); *U. sanguinea* TD acetone (5); *U. sanguinea* FD methanol (6); *U. sanguinea* TD methanol (7); *U. sanguinea* FD acetone (8); *A. marlothii* acetone (9)

Figure 4-8: Chromatograms of *A. marlothii* and *U. sanguinea* eluted in EMW and sprayed with vanillin (A) and *E. coli* (B)

When comparing the two eluent systems, EMW separated more active bands for the *R. tridentata* and *E. elephantina*, while CEF allowed for the better separation with *U. sanguinea* and *A. marlothii*. This would support the theory that the active compounds in *R. tridentata* and *E. elephantina* are the condensed tannins, as these compounds are known to be more water-soluble.

4.3.2. Minimal Inhibitory Concentrations

Each extract was tested in duplicate one day apart. The same re-constituted extract was used in each case and was refrigerated in between.

As expected the negative control grew well in all wells. The pure acetone negative control showed no inhibitory activity even at the high concentration, equivalent to 25 % acetone. The inhibitory activity of methanol was not tested as acetone was the only dilution solvent. The methanol extraction solvent was evaporated after the extraction process.

The MIC results obtained for neomycin and extracts are listed in the tables below (Table 4-5 and Table 4-6). The MIC values for neomycin were reproducible for each of the bacterial specie cultures when run one day apart. A difference was, however evident for the *P. aeruginosa* strain used for the culture with *U. sanguinea* and *A. marlothii* to that used for *R. tridentata* and *E. elephantina*. It is unknown why this particular strain had an MIC of around 125 µg/ml. According to Prescott *et al*[79], *P. aeruginosa* is considered resistant when its MIC is above 8 µg/ml. It is possible that during the process of subculture, one had favoured for the growth of resistant bacteria. Other factors such as error during the procedure were considered less likely as the MIC result were repeatable in both cases.

No reproducible antibacterial activity against *E. coli*, *E. faecalis*, *P. aeruginosa* and *S. aureus* was observed for most plant extracts (n=23) (Table 4-5 and Table 4-6). Replication was not attempted for a third time due to the limited availability of the extracts with the result material was conserved for the other biological screening assays.

For *U. sanguinea* the antibacterial activity, demonstrable in the microdilution assay against *S. aureus,* confirmed the results obtained by bio-autography (Table 4-7). The MICs for the fresh dried and thawed dried was reproducibly determined as 1.25 mg/ml. (Mean MIC of 1,25 mg/ml.) The anti-staphylococcal activity supported the results described by Majinda *et al*[65].

For *E. elephantina* the leaf extracts were repeatably active against *S. aureus* and *E. coli*, with the greatest activity against *E coli*. The rhizomes were only active against *S. aureus* (Table 4-6). There appears to be a difference in the activity of the different plant parts. The results obtained from the microtitre plate method supported the antimicrobial activity determined by Aaku *et al*[5].

One would have expected the rhizomes to have the greater activity, as these are the plants storage organ and the constituents would require a "preservative" as is the case with the sulphur compounds found in the bulbs of the *Alum* species[57]. This plant would presumably make use of an alternate protection system; possibly the anti-oxidant compounds (condensed tannins). However, if the bulbs were to have contained a high concentration of the tannins, as stated in the introduction, one would have expected a greater degree of antibacterial activity.

For *R. tridentata* the activity of the different plant parts varied. The leaves showed the best results against *E. coli*, while the root bark, was most active against *S. aureus*. Similar activity was seen for the other bacterial species. All results were reproducible. The results seen may have been due to the presence of polyphenols in this plant, which may possibly be condensed tannins[77]. With the general activity against living cytoplasm one would expect to see antibacterial activity.

Since no activity was, however, demonstrated against *E. coli* on the bioautograms, it is unknown why tannins would kill only *S. aureus*. The speculation that the results are due to the presence of tannins with general activity against living cells does not hold. The difference in the sensitivity of the *S. aureus* as compared to *E. coli* may have resulted due to a difference in the structure of the respective bacterial cell walls. The additional lipopolysaccharide layer of the Gram-negative cell wall may make the bacterium less sensitive to the protein precipitating effects of the tannins, which are more polar in nature[79].

Table 4-5: MIC values for *U. sanguinea* and *A. marlothii*

Sample (mg/ml)	E coli		*E. faecalis*		*P. aeruginosa*		*S. aureus*	
	1	2	1	2	1	2	1	2
Am leaves acetone	0.625	> 5	0.625	> 5	1.25	> 5	5	> 5
Am leaves methanol	> 5	> 5	2.5	2.5	> 5	> 5	> 5	>5
Us Fresh acetone	> 5	> 5	> 5	> 5	> 5	1.25	> 5	> 5
Us Fresh Dried acetone	5	0.625	> 5	> 5	> 5	> 5	> 5	1.25
Us Fresh Dried methanol	> 5	1.25	> 5	> 5	> 5	1.25	> 5	2.5
Us Fresh methanol	> 5	> 5	> 5	> 5	5	1.25	> 5	> 5
Us leaves acetone	> 5	> 5	> 5	> 5	> 5	1.25	> 5	> 5
Us leaves methanol	0.3125	> 5	0.625	> 5	0.3125	> 5	1.25	0.625
Us Thawed acetone	> 5	> 5	5	2.5	> 5	0.3125	> 5	> 5
Us Thawed dried acetone	> 5	1.25	> 5	0.625	> 5	0.625	> 5	1.25
Us Thawed dried methanol	> 5	0.625	> 5	> 5	> 5	> 5	> 5	> 5
Neomycin (µg/ml)	<1	<1	<1	<1	62.5	125	<1	<1

Urginea sanguinea (Us); A. marlothii (Am)

Table 4-6: MIC values for *E. elephantina* and *R. tridentata*

Sample (mg/ml)	*E. coli*		*E. faecalis*		*P. aeruginosa*		*S. aureus*	
	1	2	1	2	1	2	1	2
Ee leaves acetone	0.625	0.625	2.5	0.625	> 5	2.5	1.25	0.625
Ee roots acetone	> 5	> 5	> 5	> 5	> 5	> 5	0.625	0.625
Rt bark acetone	> 5	> 5	> 5	1.25	> 5	> 5	0.3125	0.625
Rt bulbs acetone	1.25	1.25	> 5	1.25	> 5	1.25	0.625	2.5
Rt leaves acetone	0.625	0.625	1.25	> 5	2.5	1.25	0.625	1.25
Rt stem acetone	1.25	> 5	> 5	> 5	> 5	> 5	0.625	> 5
Neomycin (µg/ml)	<1	<1	4	4	<1	<1	<1	<1

E. elephantina (Ee); *R. tridentata (Rt)*

Despite the variability in the MIC results a number of tentative conclusions may be made. The stability of the extracts may have been contributory. It should, however, be noted that in most cases the best results were produced by the second assay, which was a day after

the product was formulated. It is unknown as to whether a breakdown product could be more active.

Another possible reason could be the subjective nature of reading the results from the microtitre plates. Ideally one should use an automated plate reader. Unfortunately the presence of natural plant pigments, leads to ineffective and inaccurate results[30]. Thus for the confirmation of results, visual evaluation of the degree of colour change was necessary. (The plates were read by the same person to minimize the degree of variation.)

Table 4-7: The MIC for the various *U. sanguinea* samples cultured with *S. aureus*

Sample	MIC
Fresh bulbs	> 5 mg/ml
Thawed bulbs	> 5 mg/ml
Fresh dried bulbs	1.25 mg/ml
Thawed then dried bulbs	1.25 mg/ml
Dried leaves	> 5 mg/ml
Pure acetone solvent	No Inhibition
Neomycin	< 1 µg/ml

Certain *U. sanguinea* extracts, which previously showed no activity on the bioautogram, had now demonstrated activity in the microdilution assays e.g. *U. sanguinea* fresh dried acetone extract against *E. coli*. A plausible explanation would be additive or synergistic activity being present i.e. activity is due to the combination of secondary plant metabolites[78]. When separated out on the chromatograms, they would no longer demonstrate substantial activity either due to being stand-alone bands or because the concentrations at which they separated out, were too low for biological activity to be seen.

For the *U. sanguinea* bulbous material, the samples treated by freezing, demonstrated the best overall activity. The MIC was as low as 0.625 mg/ml against *E. faecalis* and *P. aeruginosa* for the thawed dried-material and 0.313 mg/ml against *P. aeruginosa* (MIC results were non-confirmable).

Only the *U. sanguinea* thawed dried methanol extracts, which corresponded well to the bioautography results, were considered significantly active against *E. coli*, although the MIC value of 0.625 mg/ml was non-reproducible. At this stage it is unknown why the drying process enhanced the biological activity of the compounds. Activity may be due to the breakdown of an endogenous substance. It was also shown that freezing enhanced extractable mass. If this also increased the overall relative quantity of active components being extracted, it would be most likely result in increased activity.

For *U. sanguinea* the leaf methanol extracts were the most active, with activity demonstrable against all bacterial species. Synergism could explain the greater activity seen, as the additional compound appears to have increased overall activity. As discussed under the bioautography results, the methanol extract had three active bands against *S. aureus* and one band against *E. coli*. The leaf MIC although not reproducible, still confirmed the bioautogram results for both the *E. coli* and *S aureus*. Activity could be as low as 0.3 mg/ml. Considering that both Gram-positives and Gram-negatives organism showed similar activity, the mean MIC may be around 0.5 mg/ml.

For *A. marlothii* the acetone extracts showed poor activity against *S. aureus* and good activity against both *E. coli* and *E. faecalis*. The results were not reproducible on the microdilution, but the plant did show activity against both *E. coli* and *S. aureus* on the bioautograms. The results were contrary to those demonstrated by McGaw *et al* (2000), which showed the plant to be completely ineffective. The difference in the results seen, may have been due to a difference in the plant constituents due to geographic differences, but it could have also resulted due to difference in the methodology used i.e. disc diffusion versus microtitre plate method.

In hindsight it would have been better to determine MIC values in triplicate on the two days, but limited material made this difficult. This was the first time that such variation was obtained in the phytomedicinal laboratory. The following factors are suspected as being contributory to the poor repeatability: variation in environmental temperature, fluctuations in oven temperature (not monitored), difference in sub-culture growth and natural error variation.

In comparison to antibacterial activity found in other plants antibacterial activity was low. It was suggested that values above 1 mg/ml of crude plant extracts should not be considered active (JN Eloff, phytomedicinal program, 2004 Per, comm.)

4.4. ANTIBABESIAL ACTIVITY

The *in vitro* model for evaluating the efficacy of plant extracts using *B. caballi* cultures worked well. A positive bright red colour change in the blood cultures indicative of antibabesial activity was observed as concentrations of 1 µg/ml for diminazene and imidocarb. Below this concentration cultures had changed to a brown/blue colour. However after quantifying the percentage parasitized red blood cells (PPC) (Table 4-8) parasitic death was present at concentration as low as 0.001 µg/ml.

Table 4-8 The percentage parasitized erythrocytes in wells treated with diminazene and imidocarb following initial culture and subculture

Concentration	Diminazene	Imidocarb	Diminazene subculture	Imidocarb subculture
10 µg/ml	0.00	10.34	0	0
1 µg/ml	10.34	6.90	0	0
0.1 µg/ml	24.14	27.59	0	0
0.01 µg/ml	68.97	141.38	7	89
0.001 µg/ml	48.28	110.34	25	79

When correlating the PPC to the visual colour change it appears that a PPC as high as 27 % could still result in a brown/blue colour change. Thus it would appear that a lower parasitic load is needed to demonstrate activity for the visual evaluation of activity.

The subculture results were used as an indicator of latent drug activity i.e. could the drug still interfere with parasitic division after withdrawal**.** The subculture results for diminazene showed no parasites at concentrations of 0.1 µg/ml and higher. At 0.01 µg/ml

and 0.001 µg/ml the PPC was 7 % and 25 % respectively, for diminazene. A similar pattern was evident with the imidocarb, but the PPC was much higher. Although the initial cultures were inhibited to a concentration of 0.01 µg/ml, the subculture results show that the drugs was effective as low as 0.001 µg/ml, as the result of possible residual activity. The diluent had no inhibitory activity on the babesial infected cells.

Diminazene and probably imidocarb have been shown to bind to the minor groove on DNA. This induced intercalation of the DNA results in the subsequent destruction of the parasite. At a higher concentration the large-scale DNA destruction resulting in immediate parasitic death occurs[3]. At lower concentrations, it is possible that the effects on the DNA only become lethal when the parasite enters the stage of DNA replication as part of binary fission i.e. during the subculture, when the parasites should be able to recover with drug withdrawal, the parasites die.

No parasites were observed in the culture thin smears, made from the highest concentration in the diminazene and imidocarb wells, even after complete scanning of the slides. Jacobson *et al*[48] showed that the parasites are removed from the circulation, without a drop in the haematocrit. It was believed that parasites are removed from the circulation by the spleen. Since the cultures were in closed system, it could be possible that the drug induces intracellular parasitic death with subsequent autolysis and disappearance of the parasites, as the destroyed parasite is no longer able to protect itself in the harsh intracellular environment. Another supposition would be lyses of the infected cells after introduction of the drugs. This would result in only non-infected cells being available for smears. This could not be corroborated in this study, as the tissue culture wells were not evaluated for the degree of erythrocytic breakdown.

From the data obtained the dose effective against 50% and 90 % of the parasites for both drugs were calculated in Microsoft Excel ($y = 12.729x + 6.8966$ for diminazene and $y = 20.217x - 37.931$ for imidocarb). The correlation coefficient was 90 % in both cases. (Figure 4-9). EC_{50} and EC_{90} values for diminazene and imidocarb were 0.08 and 0.55 µg/ml, and 0.03 and 0.68 µg/ml, respectively (Table 4-9). No data for the effective concentration on these drugs against *B. caballi* is apparently available. The effective concentration for diminazene against *B. bovis* was previously reported as 2.5 µg/ml[101]. It would appear that either *B. caballi* is either more sensitive to the lethal effect of this drugs, or that this test system is more sensitive.

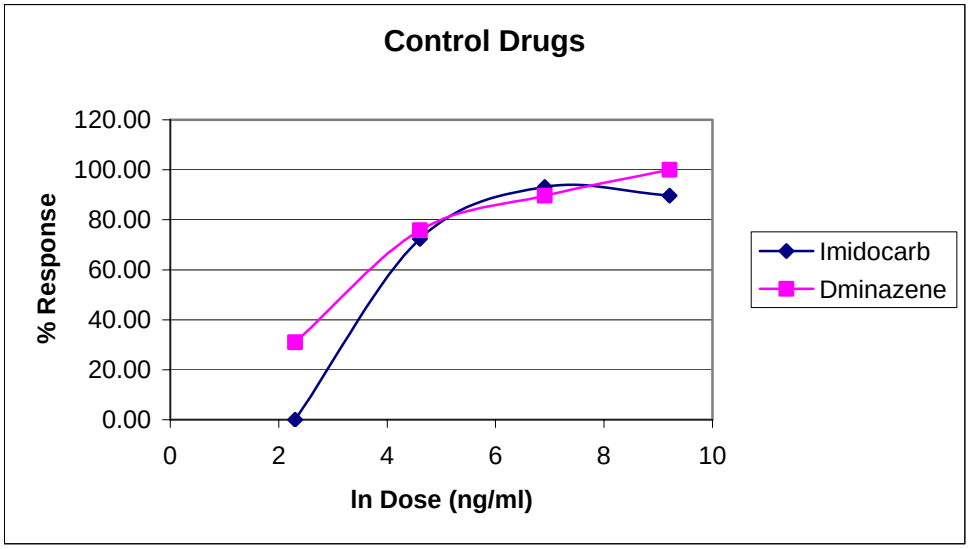


Figure 4-9: Semi-logarithmic dose response curve for diminazene and imidocarb against *B. caballi*

Table 4-9: Calculated Effective concentrations for diminazene aceturate and imidocarb diproprionate against *B. caballi*

Drug	Effective Conc. (µg/ml)	
	EC_{50}	EC_{90}
Imidocarb	0.08	0.55
Diminazene	0.03	0.68

4.4.1. Antibabesial activity of plant extracts

From the visual colour change all samples had failed to demonstrate any significant antibabesial activity i.e. all samples had the same colour as the control and diluent.

A number of the plant extracts had a PPC of below 100% for the initial culture, which was not reproducible in the repeat culture and *vice versa*. The PPC values (Table 4-10) were reproducibly below 100% for the *E. elephantina* rhizome, *A. marlothii* leaves, *R. tridentata* leaves, *R. tridentata* root bark, and *U. sanguinea* fresh dried bulbs extracts.

Table 4-10: Percentage *B. caballi* parasitized cells following exposure to plants extracts for both the initial and repeat cultures and subcultures

Sample	Babesia initial culture	Babesia initial subculture	Babesia repeat culture	Babesia repeat subculture
Diluent	132.26	130.77	97.96	102.27
Am leaves	77.42	92.31	51.02†	127.27
Ee leaves	167.74	173.08	71.43	68.18
Ee rhizomes	51.61†	88.46	24.49†	27.27
Rt leaves	83.87	119.23	28.57†	54.55
Rt root bark	67.74	119.23	57.14†	122.73
Rt stem bark	112.90	126.92	85.71	93.18
Rt tubers	132.26	126.92	93.88	79.55
Us fresh dried bulbs	67.74	134.62	55.10†	88.64
Us thawed bulbs	122.58	126.92	102.04	93.18
Us fresh bulbs	96.77	130.77	118.37	106.82

A. marlothii (Am; *E. elephantina* (Ee); *R. tridentata* (Rt); *U. sanguinea* (Us)

† Cells counts differed ($p < 0.05$) in ANOVA

The *E. elephantina* rhizome extract was the only extract significantly active for both experiments. The *R. tridentata* leaf extracts results were highly significant in its activity for only the second experiment. However, considering the poor repeatability it was assumed that the differences seen might have resulted due some external variables. This could include differences in environmental conditions, differences in parasitic growth rates or the quantification process.

Only the *E. elephantina* extracts subculture results were statistically analysed. The other plant extracts were not analysed as these plant extracts had failed to show activity when incubated with the cultures. No significant difference was present between both the controls and extract. It would appear that this plant did not possess any residual activity.

The active substance(s) against *B. caballi* were not identified. The activity was not considered to be due to the presence of the condensed tannins in the *E. elephantina*, even though condensed tannins are known to show non-specific activity against biological

material, such as ruminal protozoa. The absence of repeatable activity of *R. tridentata,* which also contains the condensed tannins, and the styptic effects of the tannins is the basis for this conclusion Styptics should cause the haemolysis of the erythrocytes within the cultures[8], which was not evident. It was therefore concluded that the concentration of the condensed tannins in the crude extracts, at a total concentration of 100 µg/ml, was too low for non-specific activity to be demonstrable.

If the activity was due to the effects of the tannins, it is believed that this may be as a result of a more specific mechanism of action. Recently it was shown that the hydrolysable tannins that use gallic acid as a building block, such as ellagitannin punicalagin, extracted from *Combretum molle*, are active against trypanosoma and *Plasmodium falcipirum*[10].

The apparent inefficacy of *U. sanguinea* extracts against *B. caballi* is most likely due to the fact that these parasites are not effected by the cardiac glycosides effects since protozoan parasites do not possess Na^+/K^+ ATP pumps[56].

In the initial experiment only the hexane (H), and ethyl acetate (EA) fractions had a PPC of below 100%. In the repeat experiment the hexane, chloroform (Cl) and ethyl acetate (EA) fractions were below 100%. Since only the H and EA fraction were consistent, their significance was determined using ANOVA. Both samples differed significantly from the controls.

To determine if the extracts had any residual activity, the differences between the extracts and the subculture was determined in an ANOVA. Both the H and EA fractions were significantly different from the controls. It would appear that the active compound/s do possess some residual activity i.e. when cultured without the extract they were unable to multiply at the same rate as the controls.

Since both EA, and H extracted active compounds, the active compounds may have different polarities. This is not conclusive, as the same compound could have fractionated into both solvents, although the solvents differ in polarity, as seen with certain antibiotics such as chloramphenicol[79].

The ethyl acetate sample was the most active fraction, with a PPC of 24%, which was similar to the PPC of the crude sample (25%). With fractionation it could be expected that a greater concentration of the active compound(s) will be in a particular fraction i.e. the concentration in a particular fraction should be higher than that of the crude extract. The greater concentration should also have resulted in higher activity assuming that linearity was present.

The similarity in efficacy before and after fractionation could be due to synergism or additive effects assuming that two or more active compounds were present in the crude extract. When the compounds are present in combination they are more effective and results in the low PPC. After separation this synergism or additive effect is no longer present and despite an increase in the concentration of the individual compound, they retain the same activity. This assumption could not be confirmed in the current study.

The four extracts were also tested with a lower parasitic yield of 50 µl of infected cells. Both the hexane and ethyl acetate extracts showed a positive colour reaction, giving a red colour in the respective wells. When the PPC was calculated, the ethyl acetate sample was also more active than in the original experiment using the 250 µl of infected cells (10% vs. 25%). This further illustrates the importance of the concentration of the initial inoculant when using colour change as a measure of efficacy.

4.5. ANTITHEILERIAL ACTIVITY

From the visual colour change, all samples had failed to demonstrate activity i.e. all samples had the same colour as the control and diluent. Although the diluent had a PPC of less than 100 % in both experiments, the results were not significantly different from that of the pure culture.

The confirmatory PPC values are listed in Table 4-11. A number of the plant extracts had a PPC of below 100% for the initial culture, which was not reproducible in the repeat culture and *vice versa*. Only the *A. marlothii* and *R. tridentata* leaves were reproducibly below 100%. Both these results were, however, not significant when analysed by ANOVA. This

was not unexpected as the piroplasms of *T. equi* do not respond to therapy easily. In most cases it is the lymphocytic stages which are most sensitive.

The addition of the antitheilerial assay was based on the assumption that anti-babesial effect may not indicate general protozoal activity due to the fundamental pathophysiological differences in the theilerial and babesial life cycles. Though babesia and theileria belong to the same order (Piroplasmorina); theileria has a pre-erythrocytic development within lymphocytes, which is absent in the babesia (Figure 2-2,P23) while, babesia in addition to entering the salivary glands on the tick vector, also enter other organs including the ovaries, and thus have a trans-ovarial stage. This latter step is absent in the theileria (Figure 2-6, P29).

Table 4-11: PPC of initial and repeat culture and their subcultures for *T. equi*

Sample	Theileria initial culture	Theileria initial subculture	Theileria repeat culture	Theileria repeat subculture
Diluent	83.56	90.83	81.52	79.17
Am leaves	86.30	76.15	91.30	80.21
Ee leaves	86.30	71.56	104.35	52.08
Ee rhizomes	100.00	88.07	117.39	83.33
Rt Bulbs	109.59	83.49	105.43	75.00
Rt Leaves	87.67	74.31	94.57	72.92
Rt root bark	108.22	94.50	76.09	84.38
Rt stem bark	83.56	88.07	101.09	97.92
Us fresh bulbs	102.74	76.15	86.96	93.75
Us fresh dried bulbs	95.89	72.48	104.35	83.33
Us thawed bulbs	115.07	80.73	103.26	94.79

A. marlothii (Am; *E. elephantina* (Ee); *R. tridentata* (Rt); *U. sanguinea* (Us)

4.6. ANTIRICKETTSIAL ACTIVITY

4.6.1. Initial screening

The oxytetracycline control inhibited rickettsial growth as low as 0.001 µg/ml. The PPC values are listed in Table 4-12. From the data obtained the dose effective against 50% and 90 % of the parasites for the oxytetracycline was calculated in Excel. The equation for the best-fit lines, with 95 % confidence, was y = 40.082x – 178.05 for the oxytetracycline control. (Figure 4-10). An EC_{50} and EC_{90} of 0.29 and 0.80 µg/ml were obtained respectively. The reported MIC value for Ehrlichial parasites is below 16 µg/ml. It would appear that either the *E. ruminantium* is more sensitive to the lethal effect of oxytetracycline, or that this test system is more sensitive. The diluent showed no inhibitory action in all experiments. This result was statistically confirmed using the student t-test. (Excel, Office2000)

Table 4-12: The percentage parasitized endothelial cells in flasks treated with oxytetracycline in an initial and repeat culture against *E. ruminantium*

Concentration	Initial	Repeat	Mean PPC
1 µg/ml	0	0.43	0.22
0.1 µg/ml	2.14	0.22	1.18
0.01 µg/ml	89.53	97.41	93.47
0.001 µg/ml	99.57	91.14	95.36

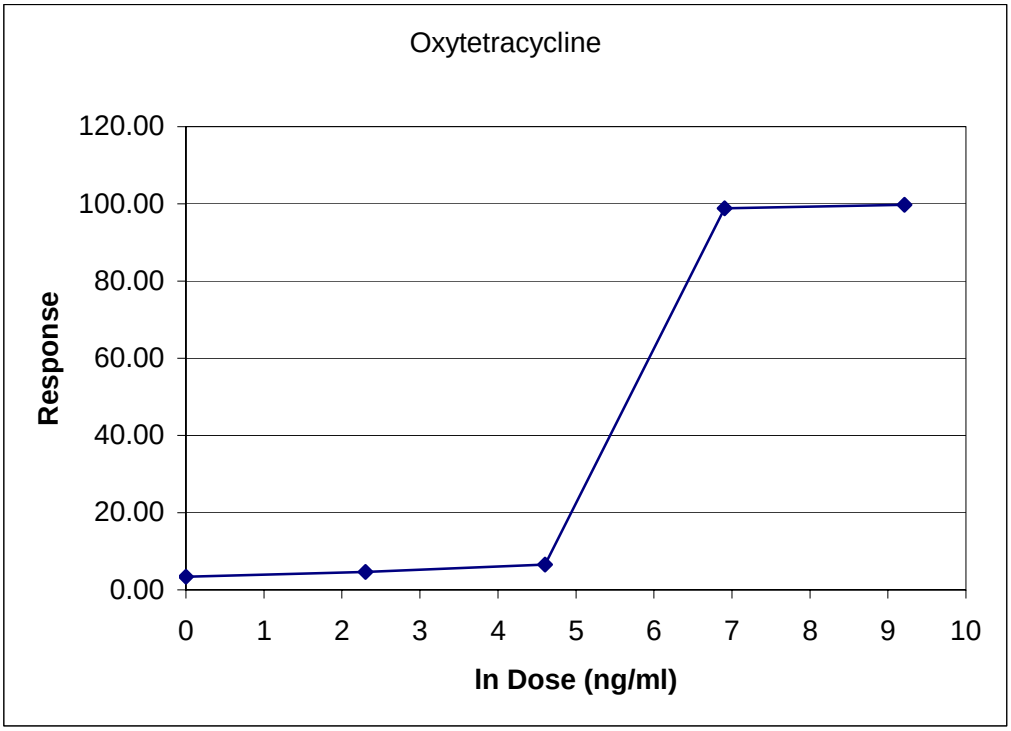


Figure 4-10: Semi-logarithmic dose response curve for Oxytetracycline against *E. ruminantium*

All plant extracts were tested at a concentration of 100 µg/ml. The PPC for these cultures are listed in Table 4-13. The results for the *U. sanguinea* are not listed, as it appeared to be completely toxic to the cell cultures i.e. the cell cultures had detached and floated free within the culture medium.

A number of the plant extracts had a PPC of below 100% for the initial culture, which was not reproducible in the repeat culture and *vice versa*. The PPC values were reproducibly below 100% for the *E. elephantina* leaves, *A. marlothii* leaves, *R. tridentata* bulbs and *R. tridentata* root bark extracts. Significance was determined for each these samples by ANOVA.

The *E. elephantina* leaves and Aloe marlothii leaves extracts were significantly active for both experiments. At 100 µg/ml the *E. elephantina* was initially active at a PPC of 77,2 % and 24,4 % in the subsequently experiment. The average of 51% was used thereafter as the mean PPC. The reason for the marked difference in cell count may be due to a counting error. The Ehrlichial colonies, within the endothelial cell, were very small and poorly visible under a light microscope and as a result it was easy to miss an infected cell. The results for *A. marlothii* was repeatable, and had a mean PPC of 19.3 µg /ml.

When comparing the infected colonies between the *E. elephantina* and the controls, it was noted that in addition to inhibiting the Ehrlichial colonies, that *E. elephantina* extract caused only small colonies to develop (Figure 4-11). It is believed that the plant had its effect by interfering with intracellular binary fission. Without a rapid rate of proliferation the parasites were unable to infect other cells in the culture flask or to proliferate into larger colonies.

Since both *E. elephantina* and *A. marlothii* have shown active bands on the bioautography, it is possible that the active component for the antibacterial and anti-rickettsial activity, are due to the same compound/s.

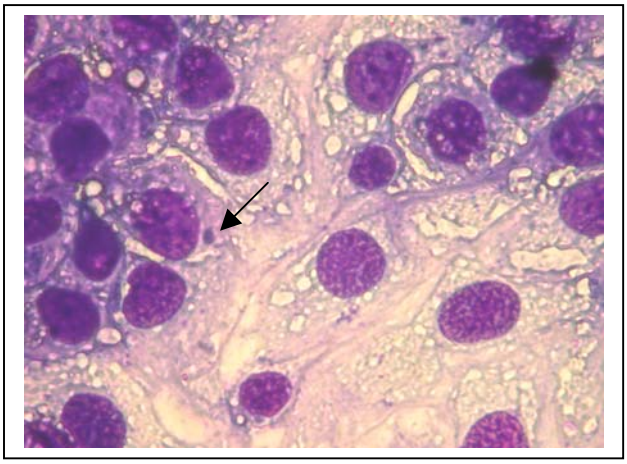


Figure 4-11: Ehrlichial cultures incubated with *E. elephantina* extracts, with arrow indicating the tiny colonies

Table 4-13: The percentage parasitized endothelial cells in flasks treated with the plant extracts in an initial and repeat culture against *E. ruminantium*

Sample	Original	Repeat	Mean PPC
Diluent	102.65	98.07	100.36
Ee leaves	85.40†	26.12†	55.76
Rt leaves	101.33	92.00	96.66
Rt bulbs	93.14	91.37	92.26
Ee rhizomes	102.43	103.16	102.80
Rt root bark	89.16	95.58	92.37
Rt stem bark	105.09	93.68	99.39
Am leaves acetone	22.79†	19.27†	21.03

A. marlothii (Am; *E. elephantina* (Ee); *R. tridentata* (Rt); *U. sanguinea* (Us)

† Cell counts differed ($P < 0,05$) in ANOVA

4.6.2. Minimal Effective Concentrations

The minimal effective concentration of samples was determined for both the *E. elephantina* leaves and the *A. marlothii* leaves. Samples were tested at a concentration of 100, 50, 25 and 10 µg/ml. The PPC results are listed Table 4-14.

Table 4-14: The percentage parasitized endothelial cells in flasks treated with plant extracts at various concentrations against *E. ruminantium*

	PPC at various drug concentration				
Conc. (µg/ml)	**Ee leaves**	**Us thawed bulbs**	**Us fresh dried bulbs**	**Us fresh bulbs**	**Am leaves**
100	56.19				20.66
50	94.86				73.23
25	98.50				103.00
10	101.28	*			102.36
1		78.11	*	*	
0.1		99.93	91.56	23.25	
0.01			0.00	99.31	

A. marlothii (Am); *E. elephantina* (Ee); *U. sanguinea* (Us)

* Least concentration at which the *U. sanguinea* extracts were toxic to the endothelial cell cultures

The effective dose against 50% and 90 % of the parasites for both drugs were calculated from the data obtained. The equation for the best-fit lines, as determined in Excel with 95 % confidence, was y = 57.229x - 188.51 for *A. marlothii* and y = 55.783x - 213.09 for *E. elephantina* (Figure 4-12). The EC_{50} and EC_{90} results are listed in the table below (Table 4-15).

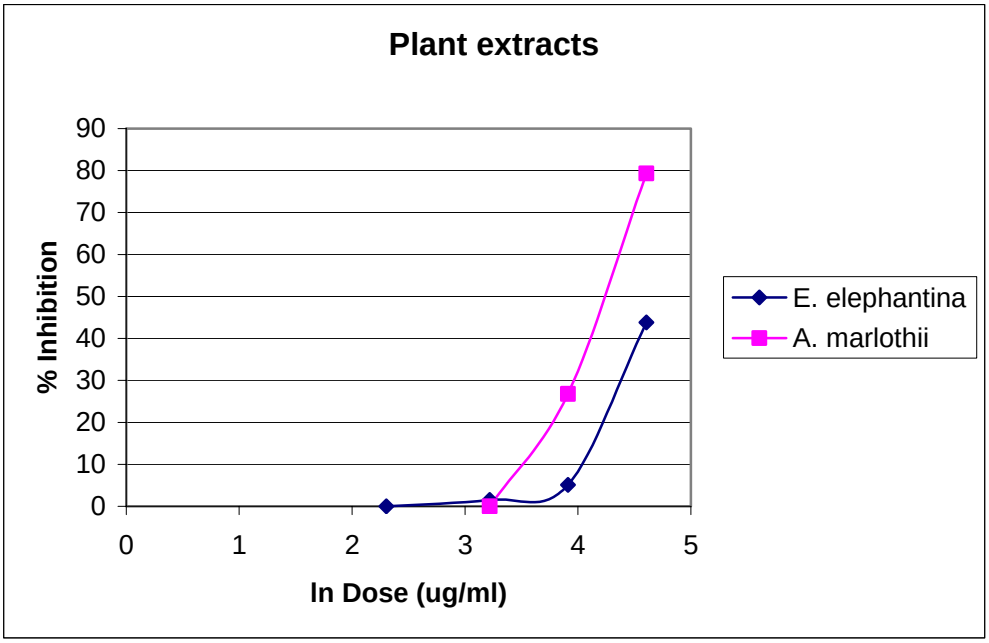


Figure 4-12: Semi-logarithmic graphs for the two effective plant extracts

Table 4-15: Effective concentrations, which suppress 50 % and 90% of *E. ruminantium*

Plant leaves	EC_{50}	EC_{90}
E. elephantina	111.398	228.92
Aloe marlothii	64.548	129.877

When comparing the semi-log graphs for both the *E. elephantina* and *A. marlothii*, it was noted that the two graphs were almost parallel. (m=55.783 and 57.229) respectively. It would appear that the two plants have a similar intra-parasitic mechanism.

Recently researchers have begun working on the Aloe family of plants, as a possible means of reducing tick yields (J Myburgh, Faculty of Veterinary Science; D van der Merwe, formally of the OVI; I Horak, Faculty of Veterinary Science; Pers. Comm., 2003). It has been noted that wild ruminants naturally feed on the Aloes plants. In areas were ectoparasites are in abundance, these animals tended to have a reduced tick burdens. For this reason it was postulated that the Aloes prevent tick infestations. It was also noted that these animals were less inflicted with heartwater. It is believed that the limited exposure to

ticks minimises animal exposure and thus the overall occurrence of heartwater. For the above results it would appear that *A. marlothii* might provide direct protection against the heartwater parasite.

4.6.3. Urginea sanguinea

Samples of fresh bulbs (F), thawed bulbs (T) and fresh dried bulbs (FD) were tested. The defrosted dried was no longer available.

As mentioned above, all sample were cytotoxic at 100 µg/ml i.e. they caused cell detachment. In the initial protocol it was decided that if any extract showed toxicity, they would not be studied further. After completing the experiment it was decided that the extract might actually still be effective although at a much lower dose than tested. Samples were subsequently retested at 50, 25, 10, 1, 0.1, 0.01 µg/ml.

Samples thawed (T) and fresh dried (FD) were tested down to 0.1 µg/ml. At 0.1 µg/ml the FD and F sample had a minimal effect on the parasitic growth. Fresh samples were tested to 0.01 µg/ml, as it was still toxic at 1 µg/ml. The fresh material appeared to be more toxic (Table 4-14). The drying process decreased the toxicity, probably as a result of poor stability of the compound. It would also appear that freezing also decreased the toxicity. This would support the results seen by Kellerman *et al*[52], who stated that the *U. sanguinea* plant was less toxic after the first frosts.

The PPC results are listed in Table 4-14. At lower concentrations, the fresh *U. sanguinea* extracts had a measurable effect on parasitic multiplication i.e. the endothelial cells were minimally affected although fairly large gaps were still evident between cells (Figure 4-13).

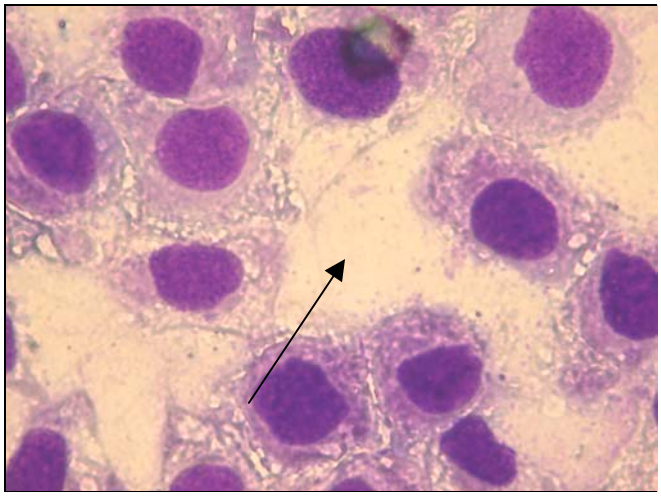


Figure 4-13: *U. sanguinea* extract showing no parasitic growth with large intracellular gaps

Semi-log graphs were plotted (ln dose versus % inhibition) (Figure 4-14) with the assumption that 1ng/ml would be the no effect concentration. The equation for the best-fit lines, as determined in Excel with 95 % confidence, was y = 33.028x - 75.35. The dose effective against 50% and 90 % of the parasites for both drugs were calculated in Excel. The EC_{50} and EC_{90} are 44.49 ng/ml and 149.36 ng/ml, respectively.

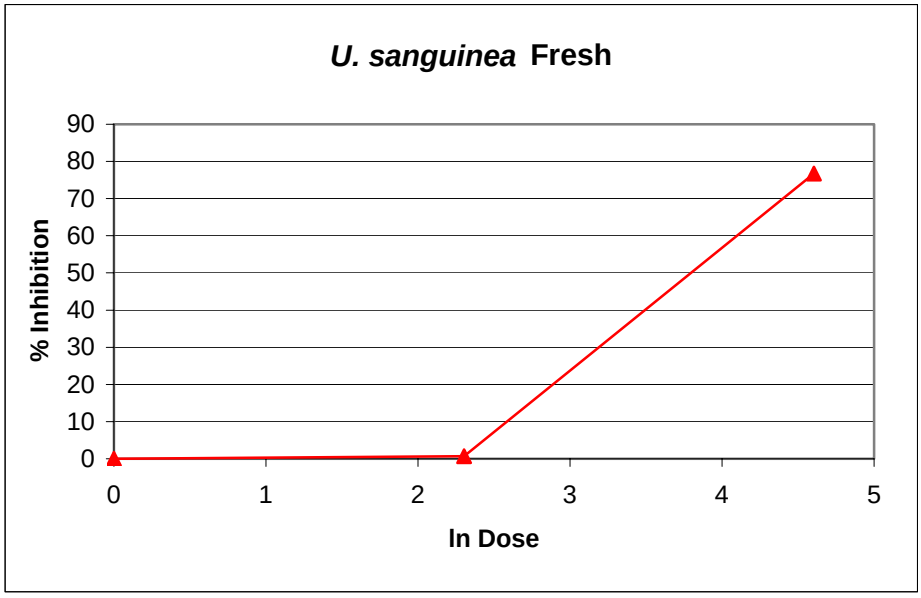


Figure 4-14: Logarithmic dose response curve for *U. sanguinea* fresh bulb extract

It is plausible that the cardiac glycoside transvaalin was responsible for the effects seen against both the rickettsial parasites and the endothelial cells. The presence of the transvaalin was suspected as extraction was with acetone, in which the compound is known to be very soluble. Its' presence was confirmed by the positive result from the Lieberman test i.e. the sample changed from a transient red to a brownish-green colour[62].

Cardiac glycosides have been shown to have effects on membrane Na^+/K^+ pumps. This results in an increase in the intracellular sodium concentration. In an attempt to stabilise the intracellular sodium concentrations, the cells activate Na^+/Ca^{2+} pumps, which exchange the intracellular sodium for calcium[7]. The resultant cell death is most likely due to this increased calcium build-up. This causes an increase in calcium concentrations within the inner mitochondrial membrane. This activates specific mitochondrial membrane calcium influx pumps. The massive calcium influx changes mitochondrial function. Instead of generating ATP, the mitochondrion starts using energy. Once the available ATP is used, apoptosis results[29]. This mechanism may explain the death of endothelial cells at higher doses, while some other mechanism could be responsible for rickettsial death at lower doses.

Although at this stage there are no published reports on the use of the cardiac glycosides as antimicrobials, there are a number of reports documenting the physiology of the rickettsial prokaryotic cell. Rickettsia appears to be the genetic ancestor of the mitochondria[41]. As such the rickettsia possess all the enzymes necessary for aerobic respiration together with the same calcium channels as the mitochondria[40]. It is thus possible that the cardiac glycosides could induce parasitic death by the same mechanism as with the mitochondria. The dose dependant effect seen may be due to increased parasitic sensitivity compared to the mammalian cell.

Currently cardiac glycosides are also being investigated as adjuncts in cancer therapy. In addition to being cytotoxic to certain *in vitro* cell lines[49,69], they appear to reduce cancer cell proliferation. The latter mechanism has been attributed to the ability of the cardiac glycosides to inhibit angiogenesis. Cancer cell lines like all other cells require proper nutrition to enable rapid proliferation. Cancer cells appear to gain access to the bodies' blood supply by either attaching to a major blood vessel in the region or by stimulating the growth of new blood vessel by the process of angiogenesis. It has been shown that without

a proper blood supply, neoplastic cells can remain dormant and never reach the stage of tumour masses[36]. Thus when treated with the cardiac glycosides, the cancer cells are unable to stimulate angiogenesis and remain dormant.

Recently it was demonstrated that endothelial cells produce self-growth factors[75]. These factors are, however, not secreted by the classical golgi pathway, but via other pathways known as non-classical protein secretion[75]. It is believed that these secretory mechanisms are coupled to the functioning of the Na^+/K^+ pump. When the pumps are inhibited by the cardiac glycosides, the secretory pathway is also inhibited and cell growth stops. The cardiac glycoside proscillaridin was tested in one experiment with other glycosides and was shown to inhibit cell cultures, at a level of 6 nMol (equivalent to approx 3,4 ng/ml), by 50%[49].

A pure compound should be much more toxic than the crude extract, which in this case was inhibitory to a concentration of 1 µg/ml. The exact concentration of transvaalin within the plant was not calculated. With mathematical extrapolation, from the known concentration of 0.05 to 0.01 % of transvaalin per wet plant mass in the usual bulb, the possible transvaalin concentration in the sample was extrapolated to be between 0.072 µg/ml (0.5%) and 0.0145 µg/ml (0.01%) respectively.

4.6.4. R. tridentata *tubers and* E. Elephantorrhiza rhizomes

No *R. tridentata* extracts and the *E. elephantina* rhizome extracts, showed any inhibitory activity on the rickettsial cell cultures. It is possible that the concentration at which the plants extracts were tested was too low for activity to be demonstrated.

For the *E. elephantina* rhizomes and *R. tridentata* root bark, it was extremely difficult to quantify the percentage parasitized cell counts as the endothelial cells of the cultures had coalesced i.e. they prevented light from penetrating. It appeared as if these cell culture exposed to these two plant extracts were proliferating at a much faster rate than the controls (Figure 4-15).

The *E. elephantina* rhizomes and *R. tridentata* root bark samples were shown to contain catechin and is believed to a have high concentration of polyphenols as determined by their potent anti-oxidant activity. These substances were shown to have good tumour

angiogenic inhibitory effects[18,64] as a result of the down regulation of endothelial vascular growth factor receptors which subsequently decreased the ability of these cells to divide.

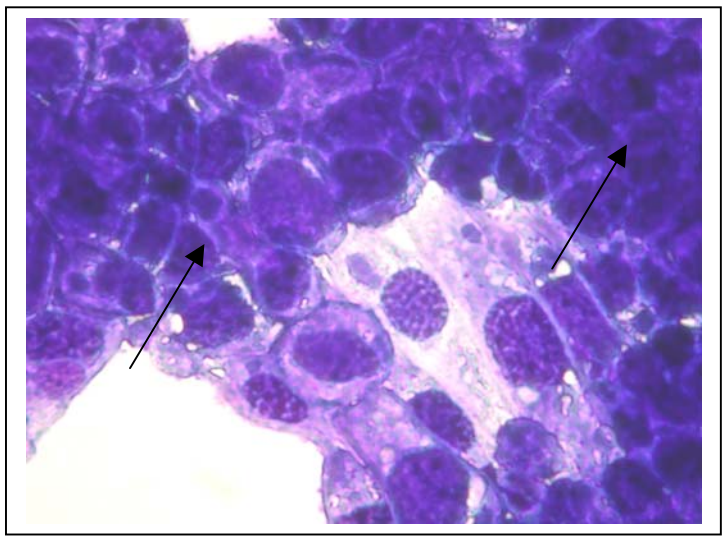


Figure 4-15: Random field from a *R. tridentata* culture, indicating the dense cell growth

The reasons for the dense cultures were not obvious, as one would have expected cell death, as was the case with *U. sanguinea*. Another explanation would be that they stimulated cell proliferation, since it is known that the topical administration of grape seed extract to wounds enhance wound healing, by stimulating growth[54,55]. The grape seed extract increased the release of growth factor from surrounding keratinocytes, by an indirect mechanism. Considering that the endothelial cells can also produce similar growth factors, it is plausible that the polyphenols in the *R. tridentata* and the *E. elephantina* rhizome extracts stimulated *in vitro* culture proliferations.

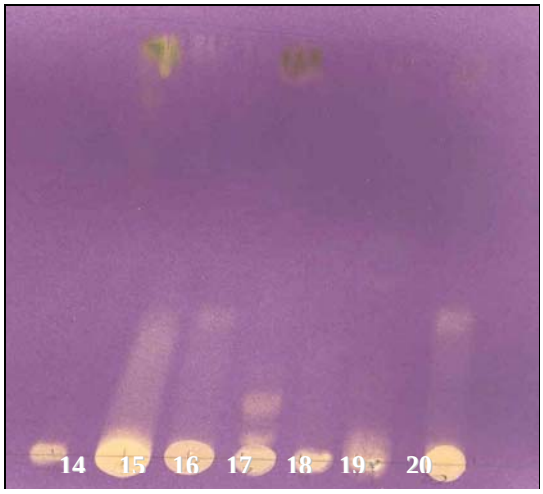
R. tridentata has been shown to be directly cytotoxic to cancer cell lines. It would appear that the extracts are selective in their cytotoxic effects, as the rapidly growing endothelial cells were not visibly affected. As proposed with the babesial screening, it is considered that the condensed tannins were not at sufficient concentration to cause non-specific cell inhibition.

4.7. ANTI-OXIDANT ACTIVITY

4.7.1. DPPH Assay

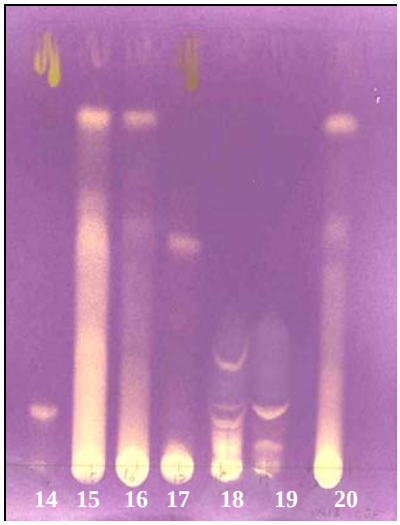
The acetone extracts of *U. sanguinea* and *A. marlothii* had no free radical scavenging activity, whereas the *A. marlothii* methanol extract had at least one active anti-oxidant band (Figure 4-16and Figure 4-17). In general plants with phenolic compounds possess high anti-oxidant activity. No phenolics have been reported to occur in *U. sanguinea*.

Both *R. tridentata* and *E. elephantina* extracts had good free radical scavenging activity, with a colour change evident within seconds of spraying with the DPPH (Figure 4-16 and Figure 4-17). The active bands separated better when eluted in the CEF. The acidic medium, more than the systems polarity, was responsible for the better separation, because a few drops of formic acid added to the EMW system led to better separation. This indicates that the compounds separated were acidic and the acid medium may have suppressed partial ionisation leading to narrow bands. The bands appeared to be the same bands, which demonstrated activity in the bioautography indicating that the antibacterial compounds also have free-radical scavenging activity.



E. elephantina leaves acetone (14); *E. elephantina* roots acetone (15); *R. tridentata* bark acetone (16); *R. tridentata* leaves acetone (17); *R. tridentata* bulbs acetone (18); *R. tridentata* stem acetone (2)

Figure 4-16: Chromatogram developed in EMW and sprayed with DPPH, with the clear zones indicating the zones of anti-oxidant activity



E. elephantina leaves acetone (14); *E. elephantina* roots acetone (15); *R. tridentata* bark acetone (16); *R. tridentata* leaves acetone (17); *R. tridentata* bulbs acetone (18); *R. tridentata* stem acetone (2)

Figure 4-17: Chromatogram developed in CEF and sprayed with DPPH, with the clear zones indicating the zones of anti-oxidant activity

4.7.2. TEAC Assay

All extracts of *R. tridentata* and the *E. elephantina* rhizomes showed the best activity on the DPPH assay and were selected for further analysis and quantification of activity with the TEAC method.

The absorbancy results were plotted and the mean change in anti-oxidant activity was determined from the gradient of each plant extract. An example of these graphs is illustrated in Figure 4-18, which was plotted for the sixth minute.

All *R. tridentata* extracts, besides the leaves, showed good activity according to their Trolox equivalence (Table 4-16). The leaves showed activity of only 25 % to that of trolox, while the other samples were all more active, ranging from 130 % for the tubers to as high as 248 % for the stem bark. This activity was extremely high, considering that this was a crude plant extract. When extracting the condensed tannins an 80 % aqueous acetone solution is generally recommended for maximal extractability[2]. Thus with the use of acetone one would expect a high degree of extractability if antioxidants were present.

Commercial grape seed extract is available as an anti-oxidant supplement. In comparison grape seed has only about 1.5 the activity of trolox (H Chickoto, phytomedicinal laboratory, pers. Comm., 2003). The activity of *R. tridentata* was compared to grape seed, as both species are part of the Vitaceae family. *R. tridentata* is also known locally as the wild grape.

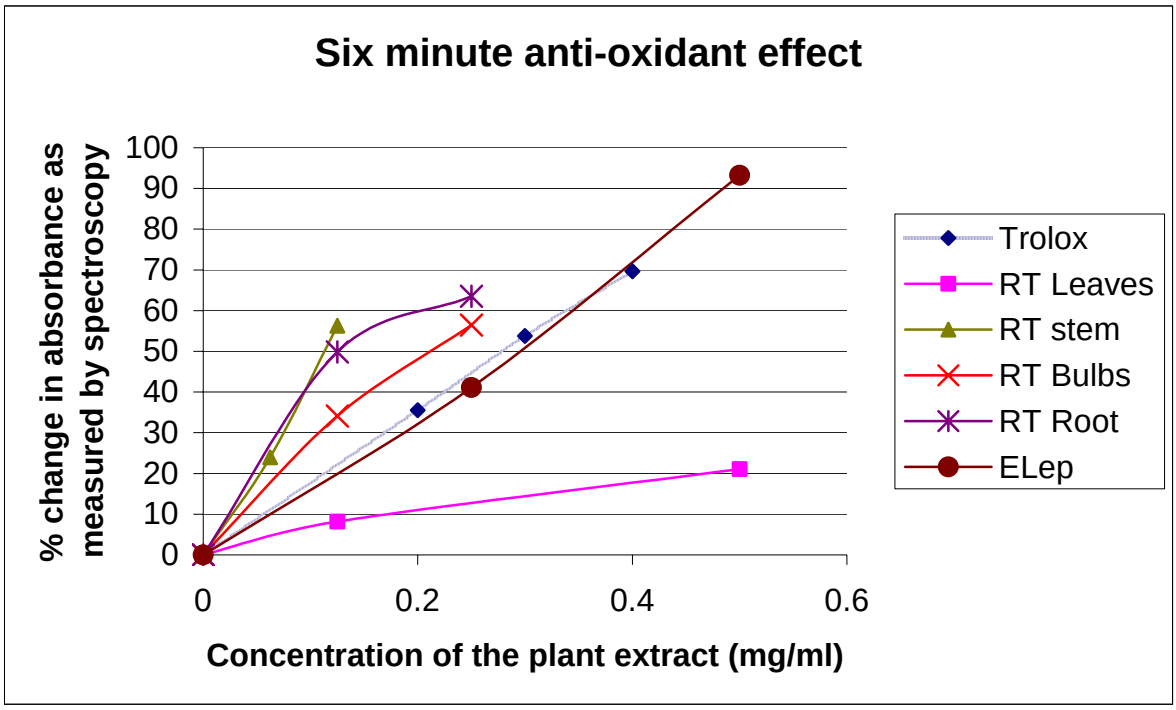


Figure 4-18: Illustration of the % change in absorbency at the sixth minute for all samples. The graphs to the left of trolox are more active and vice versa

Tubers are a plants' nutrient "warehouse", where nutrient can be stored for extended periods of time. Considering that most stored nutrients are susceptible to oxidative damage, one would expect good anti-oxidant activity in these organs, as was indicated by the TEAC. The high anti-oxidant activity found in the stem bark on the other hand was unexpected. The secondary antioxidant metabolite could be synthesised in the bark, and transported to the tubers for storage.

R. tridentata was previously studied for anti-oxidant activity by Opoku *et al*[77]. Methanol Soxhlet extracts, were tested using a variety of methods including a DPPH method, adapted for spectroscopic analysis. They concluded that the plant had activity equivalent to that of vitamin E, and that activity was due to the presence of polyphenols. In the current study it appeared that acetone was a better solvent in extracting anti-oxidant components than methanol, and that the TEAC method could be a more sensitive method of quantifying anti-oxidant activity.

The *E. elephantina* rhizomes had anti-oxidant activity equivalent to that of trolox. Once again it appeared that these storage organs have an inbuilt protective mechanism against oxidative injury. In studies on onion bulbs, it was determined that the sulphur content contributed to the shelf-life during storage[57,63] If other underground storage devices can use anti-bacterial compounds to enhance storage, it is plausible that these plants use anti-oxidants for a similar purpose.

Table 4-16: Comparison of the TEAC value between *R. tridentata* and *E. elephantina*

Minute	*Rhoicissus tridentata*				*Elephatorrhiza elephantina*
	Leaves	Tubers	Root bark	Stem bark	Rhizome
1	0.15	1.04	1.33	1.98	0.93
2	0.17	1.10	1.42	2.17	0.99
3	0.19	1.18	1.50	2.22	1.01
4	0.22	1.24	1.55	2.32	1.02
5	0.22	1.29	1.56	2.38	1.03
6	0.25	1.33	1.61	2.48	1.03

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1. EXTRACTION: EFFECTS OF FREEZING

Although the freezing process allowed a greater mass to be extracted, there was little difference in the number of bands seen when comparing thawed and fresh bulbs. More than two times the number of bands was seen when comparing extracts of dried bulbs with fresh bulbs. Only the dried bulb extract material inhibited *S. aureus* at an MIC of 1.25 mg/ml. Freezing did not affect the *in vitro* antibacterial activity of the bulbs and from the bio-autography data the R_f values of the antibacterial compounds were similar. Differences between dried and fresh material could be due high the water content within the fresh bulbs (c. 50%). It is, however, unlikely that this would have had a major effect on the results.

It is not clear whether the chromatographic profile of the dried bulbs of *U. sanguinea* is an artifact in the drying process, or whether mucilage present in fresh material had an effect on extractability of compounds. Based on the current study it appears that freezing significantly increases extractable mass. This would be of interest in the commercial herbal industry where maximum yield is important.

5.2. CHEMICAL COMPLEXITY

Both *R. tridentata* and *U. sanguinea* extracts had catechin present. From the red colour on the chromatograms, the high anti-oxidant activity and the poor elution of spotted material, it would appear that the proanthocyanidins are a major secondary plant metabolite within these plants.

5.3. ANTIBACTERIAL ACTIVITY

The results for the antibacterial micriotitre assay were not reproducible for a number of the plant extracts (n=23). For this reason, the antimicrobial activity of these plant extracts will need confirmation.

However if one is to look at the overall activity (the highest activity for a particular plant for any of samples), the following antimicrobial activity may be present. The *U. sanguinea* bulbs tend towards a more Gram-negative spectrum, with the highest activity against *P. aeruginosa*. The *U. sanguinea* leaves in general appear more active than the bulbs, with a lowest inhibitory concentration of 0.31 mg/ml being seen for both the Gram-negative organisms and a MIC of 0.63 mg/ml for both Gram-positive organisms. Considering that the difference is limited to one dilution factor, this difference is probably not significant, indicating that the leaves have a broad spectrum in activity. More importantly the compounds involved have a greater extractability with methanol than acetone, and on bioautography one had a greater number of active bands. It is possible that this increase is due to a synergism between these compounds.

For the *R. tridentata*, the tubers and leaves demonstrated the best results. The plant was most effective against *E. coli*, and had weak activity against *P. aeruginosa* and *S. aureus*. Considering that the same bands which were active during anti-oxidant screening were also antibacterial, the active compounds could possible be condensed tannins.

A. marlothii extracts showed greater activity against the *E. coli* and *E. faecalis*. It would appear that the plant was more active against the enteric pathogens.

E. elephantina extracts was most active against the Gram-negative organisms. For the rhizomes, bands that were antibacterial were also anti-oxidant. It is possible that the active compounds are the condensed tannins, as the plant was shown to possess catechin. For the leaves, the active compound had no anti-oxidant activity. The activity of the different plant parts are thus due to the presence of different active compounds as they had different R_f values.

5.4. ANTIBABESIAL ACTIVITY

Only *E. elephantina* rhizome extracts demonstrated significant activity against *B. caballi.* There also appears to be more than one active compound. The same compound fractionated into both the ethylacetate and hexane fractions or alternately there may be more than one compound present. Based on the activity before and after fractionation, there may be synergistic effects.

The EC_{50} for diminazene was 0,68 µg/ml and 0.55 µg/ml for imidocarb. At this stage, the EC_{50} values for the plant extracts could not be determined due to a shortage in uninfected blood.

The colour method utilised in this experiment was not a sensitive method of demonstrating activity for plant extracts. For the method to be of value, the initial parasitic load would need to be reduced. Preliminary indications are that 50 ul of infected cells would be sufficient.

5.5. ANTITHEILERIAL ACTIVITY

None of the plant extracts demonstrated significant activity against the *T. equi* erythrocytic piroplasms. Since the assay for the lymphocytic stage is currently not available, further testing is needed to determine if *in vitro* activity against both of the intracellular stages is absent.

5.6. ANTIRICKETTSIAL ACTIVITY

The *E. elephantina* and *Aloe marlothii* leaf extracts demonstrated good activity against the rickettsia with an EC_{90} of 228.92 and 129.877 µg/ml respectively. The activity correlated well with the antibacterial screening, where both plant extracts were active.

The *R. tridentata* root bark and the *E. elephantina* rhizomes appeared to induce culture proliferation. This could be an important safety issue as the increased cytogenic activity could be indicative of inherent anaplasia.

The *U. sanguinea* extract inhibited the growth of the cell culture at the higher concentrations, and was only effective against the parasites at the lower concentration. The bufadienolides may thus be a possible adjunct to cancer therapy, as is the case with the cardenolides. The cardiac glycosides may possibly also be an alternative in the management of rickettsial infections, in the ever need to avoid the over utilisation of antibiotics?

5.7. ANTI-OXIDANT ACTIVITY

The *R. tridentata* and *E. elephantina* species both demonstrated considerable anti-oxidant activity with the *R. tridentata* stem bark demonstrating activity twice of that for vitamin E. The *R. tridentata* was previously tested and was shown to have equivalent activity to vitamin E. It would appear that acetone is a better extraction solvent especially when using the TEAC method.

5.8. GENERAL CONCLUSION

From the results achieved there appears to be a rationale for the use of these plants to combat tick borne diseases, as certain extracts of *U. sanguinea*, *E. elephantina*, *A. marlothii* and *R. tridentata* had an inhibitory effect on the parasites causing babesiosis, anaplasmosis and heartwater; selected bacterial organisms as well as free radical scavenging activity. It would thus appear that the use of the selected plant extracts for the treatment of animal infections has merit, and should be studied further. Because traditional healers have mainly water available as an extractant, the use of water extracts in the field may not be beneficial unless a saponin-rich plant is used as part of the preparation. The saponins in *U. sanguinea* may aid the solubilization of non-polar compounds in water.

If the three diseases mentioned "*seme, gala and Bolwetsi jwa mothlapo o moshibidu*", are actually redwater, gallsickness and heartwater respectively, the *E. elephantina* would be the most effective plant as it demonstrated good activity against both the rickettsia and the protozoa. This would add merit to its traditional use in the management of "*seme*".

A. marlothii demonstrated good activity against the rickettsial parasite. With both *E. ruminantium* and *Anaplasma spp.* being rickettsial parasite, this would add merit to the use of the plant in the management of *"gala"*.

R. tridentata although very active as an anti-oxidant had no activity against the blood borne parasites. Although less effective in the control of the *in vitro* infections, the *in vivo* immune boosting effect of the plant still needs to be examined.

U. sanguinea demonstrated good activity against the rickettsial parasites, but was also toxic to the endothelial cell cultures at higher doses. The plant had no effect against *"seme"*, for which it appears to be clinically utilised. Due to the lethal effect of the transvaalin, it is suggested that this plant be avoided, as the toxic and active components may be the same compound.

GLOSSARY

ANOVA	Analysis of variance. This was the statistical method employed when deducing significance from the cell count data, from the cell cultures.
Battlement technique	Method of counting red blood cells. Described by Duncan and Prusser[28].
Bulb	The true bulb is composed of five parts: The basal plate with the roots; the fleshy sheaths, tunic, the shoot and lateral bulbs
Decoction	Decoctions are prepared by placing the plant material (usually Macerated) into water and boiling the plant material for a variable length of time. The extract is then used.
Diff Quick	Stain commonly used for the staining of cells for evaluation under a light microscope. Diff quick is a combination of an acidic and a basic stain[28].
EC	Effective concentration. This is the concentration at which activity was demonstrable. At this stage it is not known whether the activity seen is due to a static or a cidal effect
Extract	Extract are prepared by extracting the active principle of the crude drug with a suitable solvent. For this study, acetone and methanol was used.
Infusion	Infusions are made by pouring hot or cold water onto plant material (usually macerated) and letting it stand for a variable length of time. The extract is then used.
Maceration	This involves soaking the plant material in a suitable solvent, filtering and concentrating the extract. The advantage of this method is that it uses cold solvent, which reduces decomposition, but it takes longer and uses greater volumes of solvent.
MPC	The mean parasitized cells, is the mean cells parasitaemia calculated from the five fields, of each one hundred cells, counted.

PPC	The percentage parasitaemia, calculated by dividing the MPC of the sample with the MPC of the pure culture. This was used to compare results between the different extracts.
Rhizome	Are underground horizontal storage structure
Term	Definition
Tuber	Tubers differ from bulbs by not having a basal plate
Antimicrobial	Any substance of natural, semisynthetic, or synthetic origin that kills or inhibits the growth of micro-organisms.
Antibacterial	Any substance of natural, semisynthetic, or synthetic origin that kills or inhibits the growth of bacteria.
Micro-organisms	Microscopic organisms, which include bacteria, rickettsia, protozoa, and the helminths.

REFERENCES

1 Animal Diseases Act, (Act 35 of 1984) of South Africa

2 2003 Tannins: Chemical Analysis. Available online at http://www.ansci.cornell.edu/plants/toxicagents/tannnins/chem_an1.html

3 2004 DNA-Drug Interaction. Available online at: http://www.biophysik.physik.unimuenchen.de/singlemol/projects/dnadrug.html

4 2004 Phytomedicine. Available online at http://www.urbanfischer.de/journal/frame_template.htm?/journals/phytomed/phytomed.htm :

5 Aaku E, Office M, Dharani SP, Majinda RRT, and Motswaiedi MS 1998 Chemical and antimicrobial studies on *Elephantorrhiza elephantina*, *Fitoterapia*, 69: 464-465.

6 Achiano KA, Giliomee JH, and Pringle KL 1999 The use of ash from *Aloe marlothii* Berger for the control of maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), in stored maize, *African Entomology*, 7: 169-172.

7 Adams HR 2001 Digitalis and Vasodilator Drugs, In: Adams HR (eds), *Veterinary Pharmacology and Therapeutics* (8'th edn), Iowa State University Press, Iowa: 453-481.

8 Adams HR 2001 Haemostatic and Anticoagulant Drugs, In: Adams HR (eds), *Veterinary Pharmacology and Therapeutics* (8'th edn), Iowa State University Press, Iowa: 571-585.

9 Aganga AA, Mosase KW, Pell AN, Mackie RI, Mueller HI, and Ndlovu LR 1996 Tannin content, nutritive value and dry matter digestibility of *Lonchocarpus capassa*, *Zizyphus mucronata*, *Sclerocarya birrea*, *Kirkia acuminata* and *Rhus lancea* seeds, Special issue: Tannins: analysis and biological effects in ruminant feeds, Matopos Conference Center, Bulawayo, Zimbabwe, 11-14 October, 1999. *Animal Feed Science and Technology*, 2001, 91: 107-113.

10 Asres K, Bucar F, Knauder E, Yardley V, Kendrick H, and Croft SL 2001 *In vitro* antiprotozoal activity of extract and compounds from the stem bark of *Combretum molle*, *Phytotherapy Research*, 15: 613-617.

11 Baumann M and Muller W 1993 The effects of tannin containing plants on pathogenic microorganisms *in vitro*, *Tierarztliche Umschau*, 48: 738-741.

12 Begue WJ and Kline RM 1972 The use of tetrazolium salts in bioautographic procedures, *Journal of Chromatography*, 64: 182-184.

13 Bezuidenhout JD, Prozesky L, du Plessis JL and van Ster SR 1994 Heartwater, In: JAW Coetzer, GR Thomson, RC Tustin, and NPJ Kriek. (eds), *Infectious Diseases of Livestock*, Oxford university press, Cape town: 351-370.

14 Bhat RB and Jacobs TV 1995 Traditional herbal medicine in Transkei, *Journal of Ethnopharmacology*, 48: 7-12.

15 Blood DC and Radostits OM 1989 Diseases caused by Protozoa, *Veterinary Medicine* (7'th edn), Bailliere Tindall, London: 984-1015.

16 Blood DC and Radostits OM 1989 Diseases caused by Rickettsia, *Veterinary Medicine* (7'th edn), Bailliere Tindall, London: 963-972.

17 Braca A, Sortino C, Poloiti M, and Mendez J 2002 Antioxidant activity of flavenoids from *Licania licaniaeflora*, *Journal of Ethnopharmacology*, 79: 379.

18 Cao Y, Cao R and Brakenhielm E 2002 Antiangiogenic mechanisms of diet-derived polyphenols, *The Journal of Nutritional Biochemistry*, 13: 380-390.

19 Chen ZP, Cai Y, and Phillipson JD 1994 Studies on the anti-tumour, anti-bacterial, and wound-healing properties of dragon's blood, *Planta Medica*, 60: 541-545.

20 Chulabhorn M 2004 Malaria: integrated approaches for prevention and treatment, *Acta Tropica*, 89: 265-269.

21 Chung KT, Lu Z, and Chou MW 1998 Mechanisms of inhibition of tannic acid and related compounds on the growth of intestinal bacteria, *Food and Chemical Toxicology*, 36: 1053-1060.

22 Court D 1981 Succulent Flora of Southern Africa, AA Balkema, Cape Town.

23 Cowan MM 1999 Plant products as antimicrobial agents, *Clinical microbiology reviews*, October: 564-582.

24 Dold AP and Cocks ML 2000 The medicinal use of some weeds, problem and alien plants in the Grahamstown and Peddie districts of the Eastern Cape, South Africa, *South African Journal of Science*, 96: 467-473.

25 Dold AP and Cocks ML 2001 Traditional veterinary medicine in the Alice district of the Eastern Cape Province, South Africa, *South African Journal of Science*, 97: 375-379.

26 Donkin EF, Stewart CG, Macgregor RG, Els Hc, and Boyazoguku PA 1992 Resistance of indigenous and cross-bred goats to heartwater (*Cowdria ruminantium*), *Recent Advances in Goat Production*. (Proceedings of the fifth International Conference on Goats): 1716-1719

27 du Plessis Jl, de Waal DT, and Stoltsz WH 1994 A survey of the incidence and importance of the tick-borne diseases heartwater, redwater and anaplasmosis in the heartwater-endemic regions of South Africa, *Onderstepoort Journal of Veterinary Research*, 61: 295-301.

28 Duncan JR, Prasse KW, and Mahaffey EA 1994 *Veterinary Laboratory Medicine: Clinical Pathology*, Ames: Iowa State University Press, Iowa.

29 Duncan MR 1999 Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signaling and cell death, *Journal of Physiology*, 516: 1-17.

30 Eloff JN 1998 A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria, *Planta Medica*, 64: 711-713.

31 Eloff JN 1998 Which extractant should be used for the screening and isolation of antimicrobial components from plants?, *Journal of Ethnopharmacology*, 60: 1-8.

32 Eloff JN 1999 It is possible to use herbarium specimens to screen for antibacterial components in some plants, *Journal of Ethnopharmacology*, 67: 355-360.

33 Eloff JN 2003 Antibacterial assays of plant extracts by agar disc diffussion assay may give misleading results. *South African Journal of Botany* (Proceedings of the 29'th annual congress of the South African Association of Botanists) 69: 229

34 FAO 1991 *Traditional veterinary medicine in Napal*, FAO/APHCA Publications, 10: 1.

35 FAO 1991 *Traditional veterinary medicine in Sri Lanka*, FAO/APHCA Publications, 12: 1.

36 Folkman J 2000 Tumour Angiogenesis, In: Holland JF and Frei III, E. (eds), *Cancer Medicine* (5'th edn), B.C. Decker Inc., London.

37 Garcia AM, de Pascual ST, Santos CB, and Rivas CRJ 2003 Evaluation of the Antioxidant Properties of Fruits, *Food Chemistry*, 1-5.

38 Ghazali RA and Waring RH 9-10-1999 The effects of flavonoids on human phenolsulphotransferases: Potential in drug metabolism and chemoprevention, *Life Sciences*, 65: 1625-1632.

39 Graczyk TK, Brossy JJ, Sanders ML, Dubey JP, Plos A, and Stoskopf MK 1996 Immunological survey of babesiosis (*Babesia peircei*) and toxoplasmosis in Jackass penguins in South Africa, *Parasite*, 3: 313-319.

40 Gray MA, Burger G, and Lang BF 2001 The Origin and Evolution of the Early Mitochodria, *Genome Biology*, 2: 1018.1-1018.5.

41 Gray MW 1998 Rickettsia, Typhus, and the Mitochondria Connection, *Nature*, 396: 109-110.

42 Grierson DS and Afolayan AJ 1999 Antibacterial activity of some indigenous plants used for the treatment of wounds in the Eastern Cape, South Africa, *Journal of Ethnopharmacology*, 66: 103-106.

43 Guerin B and Hart B 1993 Techniques for the *in vitro* study of acaricides, *Revue Francaise d'Allergologie at d' Immunologis Clinique*, 33: 33-39.

44 Halliwell B and Gutteridge JMC 1985 *Free radicals in biology and medicine*, Oxford University Press, Oxford.

45 Hamburger MO and Cordell GA 1987 A direct bioautographic TLC assay for compounds possessing antibacterial activity, *Journal of Natural Products*, 50: 19-22.

46 Homer MJ, Aguilar DI, Telford SR III, Krause PJ and Persing DH 2000 Babesiosis, *Clinical microbiology reviews*, 13: 451-469.

47 Hutchings A, Scott AH, and Cunningham A 1996 *Zulu Medicinal Plants: an Inventory*, University of Natal Press, Pietermaritzburg.

48 Jacobson LS, Reyers F, Berry WL, and Viljoen E. 2003 Changes in haematocrit after treatment of uncomplicated canine babesiosis: a comparison between diminazene and trypan blue and an evaluation of the influence of parasitaemia, *Journal of the South African Veterinary Association*, 67: 77-82.

49 Johansson S, Claeson P, Bohlin L, Larsson R, Gullbo J and Lindholm, P 2001 Cytotoxicity of digitoxin and related cardiac glycosides in human tumor cells, *Anticancer Drugs*, 12: 475-483.

50 Joubert JPJ and Schultz RA 1982 The treatment of *Urginea sanguinea* Schinz poisoning in sheep with activated charcoal and potassium chloride, *Journal of the South African Veterinary Association*, 53: 25-28.

51 Katerere D R and Eloff J N 2004 *Va*riation in the chemical composition, antibacterial and anti-oxidant activity of fresh and dried Acacia leaf extracts, *South African Journal of Botany*, *70*: 303-305.

52 Kellerman TS, Coetzer JAW and Naudë TW 1988 *Plant Poisoning and Mycotoxicoses of Livestock in Southern Africa*, Oxford University Press, Cape Town.

53 Kelmanson JE, Jager AK and van Staden J 2000 Zulu medicinal plants with antibacterial activity, *Journal of Ethnopharmacology*, 69: 241-246.

54 Khanna SR, Sashwati B, Debasis BM and Sen CK 7-1-2001 Upregulation of oxidant-induced VEGF expression in cultured keratinocytes by a grape seed proanthocyanidin extract, *Free Radical Biology and Medicine*, 31: 38-42.

55 Khanna S, Venojarvi M, Roy S, Sharma N, Trikha P, Bagchi D, Bagchi M and Sen, CK 10-15-2002 Dermal wound healing properties of redox-active grape seed proanthocyanidins, *Free Radical Biology and Medicine*, 33: 1089-1096.

56 Kirk K 2001 Membrane Transport in the Malaria-Infected Erythrocyte, *Physiological Review*, 81: 495-528.

57 Lancaster J, Brash D, Bycroft B, Shaw J and Farrant J 2001 Does Sulphur Supply to the Bulbs Affect Storage of Onions, *Act Horticulture* 555: 115.

58 Lawrence JA 1977 The mechanical transmission of Anaplasma under Rhodesian conditions, *Rhodesian Veterinary Journal*, 8: 74-76.

59 Lawrence JA, Whiteland AP, Kafuwa PT, and Njuguna LM 1996 Improving the specificity of indirect immunofluorescence for the serological diagnosis of bovine anaplasmosis, *Onderstepoort Journal of Veterinary Research*, 63: 53-55.

60 Lawrence JA, de Vos AJ, and Irvin AD 1994 East Coast Fever, In: Coetzer JAW, Thompson GR, and Tustin RC (eds), *Infectious Diseases of Livestock* (1'st edn), Oxford University Press, Cape Town: 309-325.

61 Lin J, Opoku AR, Geheeb KM, Hutchings AD, Terblanche SE, Jager AK, Staden J van, and Van Staden J 1999 Preliminary screening of some traditional Zulu medicinal plants for anti-inflammatory and anti-microbial activities, *Journal of Ethnopharmacology*, 68: 267-274.

62 Louw PGJ 1952 Transvaalin, a cardiac glycoside isolated from *Urginea burkei*, Bkr. (Transvaal Slangkop), *Onderstepoort Journal of Veterinary Research*, 25: 123-131.

63 Louw CAM, Regnier TJC, and Korsten L. 2002 Medicinal bulbous plants of South Africa and their traditional relevance in the control of infectious diseases, *Journal of Ethnopharmacology*, 82: 147-154.

64 Maiti TK, Chatterjee J, and Dasgupta S 2003 Effect of green tea polyphenols on angiogenesis induced by an angiogenin-like protein, *Biochemical and Biophysical Research Communications*, 308: 64-67.

65 Majinda RRT, Waigh RD, and Waterman PG 1997 Bufadienolides and other constituents of *Urginea sanguinea*, *Planta Medica*, 63: 188-190.

66 Martini ND 1998 The isolation of antimicrobial components from *Combretum Erthrophyllum* [burch.] sond. *MSc Dissertation*, University of Pretoria,

67 Masika PJ, Sonandi A and van Averbeke W 1997 Perceived causes, diagnosis and treatment of babesiosis and anaplasmosis in cattle by livestock farmers in communal areas of the central Eastern Cape Province, South Africa, *Journal of the South African Veterinary Association*, 68: 40-44.

68 Mavi S and Huntley BJ 1999 Medicinal plants and their uses in Zimbabwe, *Botanical diversity in southern Africa*, 3: 169-174

69 McConkey DJ, Ozel H Z, Lin Y, Nutt LK and Newman RA 2003 Cardiac glycosides stimulate Ca2+ increases and apoptosis in androgen-independent, metastatic human prostate adenocarcinoma cells, *Cancer Research*, 60: 3807-3812.

70 McGaw LJ, Jager AK and van Staden J 2000 Antibacterial, anthelmintic and anti-amoebic activity in South African medicinal plants, *Journal of Ethnopharmacology*, 72: 247-263.

71 Mehlhorn H, Schein E 1998 Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998, *Parasitology Research*, 84: 467-475

72 Minocha R, Shortle WC, Long SL and Minocha SC 1994 A rapid and reliable procedure for extraction of cellular polyamines and inorganic ions from plant tissue, *Journal of Plant Growth Regulation*, 13: 187-193.

73 Mishra AK, Clabaugh G, and Kakoma I 1992 Human serum for *in vitro* cultivation of *Babesia bovis*, *Veterinary Parasitology*, 45: 153-156.

74 Muraguri GR, Kiari HK, and McHardy N 1999 Treatment of East Coast Fever: a comparsion of parvaquone and buparvaquone, *Veterinary Parasitology*, 87: 25-37.

75 Nickel W 2003 The Mystery of Non-Classical Protein Secretion, *European Journal of Biochemistry*, 270: 2109-2119.

76 Nockels CF 1996 Antioxidants improve cattle immunity following stress, *Animal Feed Science and Technology*, 62: 59-68.

77 Opoku AR, Maseko NF and Terblanche S E 2002 The *In vitro* Antioxidant Activity of Some Traditional Zulu Medicinal Plants, *Phytotherapy Research*, 16: S51-S56.

78 Phillipson JD 2001 Phytochemistry and medicinal plants, *Phytochemistry*, 56: 237-243.

79 Prescott JF, Baggot JD, and Walker RD 2000 *Antimicrobial Therapy in Veterinary Medicine*, Iowa State University Press, Iowa State.

80 Prozesky L 1987 The pathology of heartwater. A review, *Onderstepoort Journal of Veterinary Research*, 54: 281-286.

81 Rabe T and van Staden J 1997 Antibacterial activity of South African plants used for medicinal purposes, *Journal of Ethnopharmacology*, 56: 81-87.

82 Rates SMK 2001 Plants as source of drugs, *Toxicon*, 39: 603-613.

83 Re-Roberta P, Proteggente N, Pannala A, Yang A, and Rice-Evans C 1999 Antioxidant activity applying an improved ABTS radical cation decolorization assay, *Free Radical Biology and Medicine*, 26: 1231-1237.

84 Ricardo da Silva JM, Rigaud R, Cheynier V, Cheminat A, and Moutounet M 1991 Procyanidin dimers and trimers from grape seeds, *Phytochemistry*, 30: 1259-1264.

85 Sahm DF and Washington JA 1991 Antimicrobial susceptibility tests: dilution methods, In: WJ Hausler Jr, KL Herrmann, HD Isenberg, and HJ Shadomy (eds), *Manual of Clinical Microbiology* (5'th edn) Library of Congress Cataloging in Publication data, Massachusetts: 1105-1116.

86 Setiadi DH, Chass GA, Torday LL, Varro A, and Papp J G 2003 Vitamin E models. Can the anti-oxidant and pro-oxidant dichotomy of α-tocopherol be related to ionic

ring closing and radical ring opening redox reactions, *Journal of Molecular Structure*, 620: 93-106.

87 Shale TL, Stirk WA and van Staden J 1999 Screening of medicinal plants used in Lesotho for anti-bacterial and anti-inflammatory activity, *Journal of Ethnopharmacology*, 67: 347-354.

88 Shaw MK 1996 The Same but Different: the Biology of Theileria Sporozoite Entry into Bovine Cells, *International Journal for Parasitology*, **27**: 457-474.

89 Shklar G. 1998 Mechanisms of cancer inhibition by anti-oxidant nutrients, *Oral Oncology*, 34: 24-29.

90 Stahl E 1969 *Thin Layer Chromatography*, Springer-verlag, Berlin, Heidenberg, New York.

91 Strydom PJ and Smith WA 1994 Effect of inherent polyphenolics on the nutritional value of Namibian browse species, *Dinteria*, No. 24, 19-27; 33 ref.: 19-27.

92 Synge BA 1978 Brain biopsy for the diagnosis of heartwater, *Tropical Animal Health and Production*, 10: 45-48.

93 van der Merwe D 2000 Use of ethnoveterinary medicinal plants in cattle by Setswana-speaking people in the Madikwe area of the North West Province. *MSc Dissertation*, University of Pretoria, 1-132

94 van Tellingen O 2001 The Importance of Drug-Transporting P-glycoproteins in Toxicology, *Toxicology Letters*, 1: 31-41.

95 de Vos AJ 1999 Epidemiology and control of bovine babesiosis in South Africa, *Wellcome Haemotropic Diseases Symposium*, 50: 357-362

96 de Vos AJ and Potgieter FT 1994, In: JAW Coetzer, GR Thomson, RC Tustin, and NPJ Kriek (eds), *Infectious Diseases of Livestock*, Oxford Univesity Press, Cape Town: 278-294.

97 Wijesekera ROB 2000 Plant-derived medicines and their role in global health, In: Wijesekera ROB (eds), *The medicinal plant industry*, CRC Press, New York City: 1-18.

98 Wilkie GM, Brown CGD, Kirvar E, Thomas M, Williamson SM, Bell-Sakyi LJ and Sparagano, O. 1998 Chemoprophylaxis of *Theileria annulata* and *Theileria parva* infections of calves with buparvaquone, *Veterinary Parasitology*, 78: 1-12.

99 Williamson EM, Okpako DT, and Evans FJ 1996 *Selection, Preparation and Pharmacological Evaluation of Plant Material*, John Wiley and Sons Ltd, West Sussex: 15-23.

100 Wolfe J and Bryant G 2001 Cellular cryobiology: thermodynamics and mechanical effects, *International Journal of Refrigeration*, 24: 450.

101 Wu J, Yu J, and Wang Z 2002 *In vitro* cultivation of Chinese strains of *Babesia spp*, *Parasitology Research*, *88*: S41-S42

102 Wyk B van and Gerika N 2000 *People's Plants*, Briza Publication, Pretoria.

103 Wyk B van, Oudtshoon B van, and Gerika N 1997 *Medicinal plants of South Africa*, Briza Publications, Pretoria.

104 Yunker CE 1996 Heartwater in sheep and goats: a review, *Onderstepoort Journal of Veterinary Research*, 63: 159-170.

105 Zweygarth E and Josemans AI 2001 A chemically defined medium for the growth of *Cowdria ruminantium*, *Onderstepoort Journal of Veterinary Research*, 68: 37-40.

106 Zweygarth E, Just MC, Niekerk C van, Waal DT de, Van Niekerk C, De Waal DT, Preston PM, and Yin Hong 1996 *In vitro* cultivation of *Babesia equi* and its applications, *Proceedings of the European Union International Symposium on ticks and tickborne diseases*, September 2 6, 1996, Xi'an, China, 29: 60S.

107 Zweygarth E, Just MC and de Waal DT 1995 Continuous *in vitro* cultivation of erythrocytic stages of *Babesia equi*, *Parasitology Research*, 81: 355-358.

108 Zweygarth E and Lopez-Rebollar LM 2000 Continuous *in vitro* cultivation of *Babesia gibsoni*, *Parasitology Research*, 86: 905-907.

109 Zweygarth E, Lopez-Rebollar LM, Nurton J, and Guthrie AJ 2002 Culture, isolation and propagation of *Babesia caballi* from naturally infected horses, *Parasitology Research*, 88: 460-462.