The screening of phyto-pesticides for potential adverse effects on human health

By

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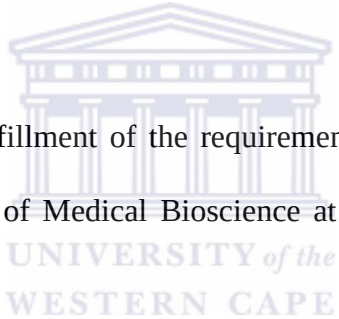

Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Medical Bioscience at the University of the Western Cape

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Background to Intellectual Property

1) Part of the data presented in this thesis forms the background intellectual property for the project titled "**Indigenous Botanical Adjuvant Technologies (iBATECH)"** funded by the **Technology Innovation Agency/Innovation Fund** on behalf of the **South African Department of Science and Technology**.

2) Elements of the data in this thesis forms an integral part of the support information that received provisional patent registration from the **Companies and Intellectual Property Registration Office (CIPRO)** of the **South African Department of Trade and Industry**.

Abstract

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Key words: Synthetic pesticides, Phyto-pesticides, Grey-mould, Cytotoxicity, Environmental toxicity, Immunotoxicity, Reproductive toxicity

Pesticides are designed to control or eliminate pests such as insects, rodents, weeds, bacteria, and fungi. They are used at a global scale for agricultural produce. Although pesticides play a significant role in increasing food production and eliminating diseases, exposure to pesticides may be harmful to non-target organisms. As a result concern over safety and resistance to pesticides has increased and there is pressure to reduce use and search for more environmentally and toxicologically safe and efficacious pesticides. Most pesticides currently in use are synthetic; therefore an alternative to synthetic pesticides is the use of naturally occurring products/ botanicals with pesticidal properties.

Two plants indigenous to South African with pesticidal properties were chosen for this study. *Dicerothamnus rhinocerotis* (*D. rhinocerotis*) and *Galenia africana* (*G. africana*) have potential antifungal properties thus, may have potential use on agricultural produce

as fungicides. *Galenia africana* and *D. rhinocerotis* extracts inhibit growth of *B. cinerea* (a fungal pathogen) at concentrations greater than 31.25 mg/ml and 125mg/ml respectively. A major consideration in approving pesticides for use is whether they pose an unreasonable risk to humans and to the environment. Toxicity studies are required to determine the safety of the plant extracts.

The purpose of this study was to evaluate potential toxicity of ethanol extracts of *D. rhinocerotis* and *G. africana*, which is important when designing practices to reduce or eliminate excess exposure to them. Natural plant products with pesticidal properties could provide an alternative to synthetic pesticides and may thus effectively reduce resistance levels.

This first objective of this study was to assess the cytotoxicity of *D. rhinocerotis* and *G. africana* on human cell cultures. Human whole blood and the human breast adenocarcinoma cell line (MCF-7) were treated with varying concentrations of the plant extracts and cytotoxicity determined. Cytotoxicity was measured using several biomarkers. Inhibiotory concentration for a 50% effect (IC_{50}) and no observable effect level (NOEL) values were obtained for *D. rhinocerotis* and *G. africana*. The results showed that extracts of *D. rhinocerotis* and *G. africana* had cytotoxic effects on the cell cultures.

The second objective of this study was to determine the ecotoxicity of *D. rhinocerotis* and *G. africana*. A series of acute toxicity tests, with effective concentration for a 50%

effect (EC_{50}) and lethal concentration for a 50% effect (LC_{50}) as biomarkers, were conducted to estimate the potential environmental effect of the two plants. The tests were carried out using *Vibrio fischeri*, *Selenastrum capricornutum*, *Daphnia pulex*, and *Poecilia reticulata* as bioindicators. Results obtained showed that *G. africana* had higher toxicity units than *D. rhinocerotis*, thus showing that *G. africana* is more toxic to the aquatic species used as compared to *D. rhinocerotis*.

The third objective of this study was to investigate the immunomodulatory effects of the two plant extracts. This was achieved by using mouse spleen cell cultures. Exposure of pesticides to the immune system may result in alteration of the normal immune functions. The cytokines IFN-γ and IL-4 were used as biomarkers to determine the T-cell activity of the immune system when exposed to the two botanical products. The results obtained showed that both *D. rhinocerotis* and *G. africana* decrease of the cytokines interferon-gamma (IFN-γ) and interleukin-4 (IL-4), thus may have immunotoxic effects.

The fourth objective was to investigate the hepatotoxicity of the two plant extracts.
Injury to the liver was investigated using a range of clinical biochemical tests that monitor liver enzyme activity and metabolic activity. Primary liver cell cultures were exposed to the plant products in question, after which the biochemical tests were carried out. The enzymes that were monitored were alanine aminotrasferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH). The results obtained showed that both of *D. rhinocerotis* and *G. africana* may have

effects on the liver, as shown by the increased levels of enzymes released from cells upon exposure to plant extracts.

The final objective of this study was to investigate the effect of the two plants on the male reproductive system. Injury to the male reproductive system was investigated using testicular cell cultures. Primary cell cultures were stimulated with luteinizing-hormone (LH) and exposed to the plant extracts. LH results in the production of testosterone, thus testosterone was used as a biomarker for assessing reproductive toxicity. The results obtained showed that both of *D. rhinocerotis* and *G. africana* have effects on the male reproductive system, as shown by the decreased testosterone secretion.

Botanicals provide a simple, inexpensive and environmentally friendly (non-pollution and lesser toxicological concerns) alternative for pest control. However, motivation for the commercial use of botanicals as pesticides requires validating the efficacy of the plant as a pesticide, and also assessing its effects on human health and the environment. An important component of this evaluation involves toxicity studies, which enables cautions of dangerous practices and toxic effects of the plants to be issued.

Declaration

I declare that “The screening of phyto-pesticides for potential adverse effects on human health” is my own work, that it has not been submitted for any other degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: _____ Date: _____

Signed: _____

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Contents

Appendix 1: E. J. Pool, J. A. Klaasen and Y. P. Shoko. The immunotoxicity of *Dicerothamnus rhinocerotis* and *Galenia africana*. African Journal of Biotechnology Vol. 8 (16), pp. 3846-3850, 18 August, 2009.

Appendix 2: E. J. Pool, J. A. Klaasen and Y. P. Shoko. The environmental toxicity of *Dicerothamnus rhinocerotis* and *Galenia africana*. African Journal of Biotechnology Vol. 8 (18), pp. 4465-4468, 15 September, 2009

List of abbreviations

ALT - Alanine aminotransferase

ANOVA - Analysis of variance

AST - Aspartate aminotransferase

BSA - Bovine serum albumin

CO_2 - Carbon dioxide

DMSO - Dimethyl sulfoxide

DPBS -Dulbecco's phosphate buffered saline

EC_{50} - Median effective concentration

EDCs - Endocrine disrupting chemicals

ELISA - Enzyme linked immunosorbant assay

g - Grams

HRP - Avidin Horseradish Peroxidase

IC_{50} - 50% inhibitory concentration

IFN-γ - Interferon-gamma

IgE - Class E immunoglobulin

IgG - Class G immunoglobulin

IgM - Class M immunoglobulin

IL-10 - Interleukin-10

IL-12 - Interleukin-12

IL-13 - Interleukin-13

IL-4 - Interleukin-4

IL-5 - Interleukin-5

IL-6	- Interleukin-6
ISO	- International Organisation for standardization
IU/L	-International Units per liter
LD_{50}	- Lethal dose 50
LDH	- Lactate dehydrogenase
LH	- Luteinizing hormone
mg/ml	- milligrams per milliliter
ml	-milliliter
mm	- millimeters
mU/ml	- milliunits per milliliter
NAD	- Nicotinamide adenine dinucleotide
NaOH	- Sodium hydroxide
NK cells	- Natural killer cells
nm	- nanometers
NOEL	- No observable effect level
^{o}C	- Degrees centigrade
OECD	- Organisation for Economic Cooperation and Development
PBS	- Phosphate buffered saline
pg/ml	- picograms per milliliter
SGOT	- serum glutamate oxaloacetate transaminase
SGPT	- serum glutamate pyruvate transaminase
Tc cells	- Cytotoxic T cells
TDS	- Testicular dysgenesis syndrome

Th cells	- T helper cells
TMB	- 3.3', 5.5'-tetramethylbenzidine
US EPA	- United States Environmental Protection Agency
v/v	- Volume to volume
w/v	- Weight to volume
WBC	- Whole blood cultures
xg	- gravitational force
XTT	- 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5carboxanilide
µg/ml	- Micrograms per milliliter
µl	- microliters
µl/well	- microliters per well

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Chapter 1

Introduction: The use of botanical products as pesticides

Synthetic pesticides are designed to control or eliminate pests such as insects, rodents, weeds, bacteria, and fungi (Arias-Estervez *et al*., 2008). They are used at a global scale for agricultural produce. Although pesticides play a significant role in increasing food production and eliminating diseases, exposure to pesticides can be harmful to humans and thus need to be investigated for potential adverse effects on human health (Hojo *et al*., 2006)

An alternative to synthetic pesticides is the exploitation of naturally occurring products with pesticidal properties (Isman, 2006). Historically humans have been known to use phyto-products (botanical products) for food, medicine, poisons, fragrances and pesticides (Isman, 2006, Regnault-Roger *et al*., 2008 and Stewart, 2008). Plants with potential pesticidal properties may be used in order to try and minimize or eradicate the adverse effects that result from use of synthetic pesticides (Isman, 2006 and Regnault-Roger *et al*., 2008).

It is therefore of utmost importance to manage the risks of potential botanical pesticides and to provide safety information of the substances. As a result human health and the environment will be better protected.

1.1 Study objective

The aim of this study was to analyze two South African plant species, with potential pesticidal properties, for their potential adverse effect on the environment and human health. *In vivo* and *in vitro* cell culture assays were used to monitor potential toxic effects of the plants. Environmental toxicity was assessed using species representative of the aquatic environment. Mammalian systemic toxicology was investigated using primary cell cultures representative of several physiological systems, such as reproductive, immune and hepatic systems. One of the major outcomes envisaged by this project was the development of a battery of rapid assays that can be used to monitor the effects of products on the mammalian physiological systems. The safety of the plant-based pesticides was assessed using the data generated from the toxicology results.

1.2 Study rationale

Plant extracts with pesticidal properties are natural alternatives to synthetic pesticides. However before these plant extracts can be used for commercial crops, toxicological assessments of these new products must be done to prevent adverse effects to the environment and consumer health.

1.3 Pesticides

Pests have been identified as a global problem that results in agricultural production constraints. In order to minimize damage caused by pests, most farmers mainly rely on the use of synthetic pesticides for pest control (Arias-Estervez *et al*., 2008). Due to the intensive cultivation of agricultural produce, so as to meet demand for both quantity and quality, pesticides are applied in high dosages and frequencies. As a result of increasing pesticide use, consumers are becoming increasingly aware of the potential adverse effects on non-target organisms, including humans (Carbone *et al*., 2007; Caserta *et al*., 2007; Kodavanti *et al*., 2008). Many synthetic pesticides are also becoming ineffective due to the development of resistance (Zhu *et al*., 1996; Zhu *et al*., 2000). This has thus resulted in pressure to reduce the use of synthetic pesticides (Isman, 2006).

Approximately less than 0.1% of the pesticides applied to crops reach their target pest, while the rest enters the environment thus reaching non-target organisms (Pimentel *et* al., 1986). Exposure to some of these chemicals, e.g. chlorinated insecticides, may lead to alteration of endocrine balance thus disrupting development and function of endocrine systems in both humans and animals (Caserta *et al*., 2007; Kodavanti *et al*., 2008). They achieve this by disrupting normal endocrine feedback mechanisms either by mimicking a hormone or by blocking hormonal effects (Caserta *et al*., 2007). These substances may consequently lead to adverse health effects including immunologic damage, birth defects, thyroid cancer, breast cancer, testicular cancer and harm to the developing nervous system (Caserta *et al*., 2007).

An alternative to synthetic pesticides is the exploitation of naturally occurring products with pesticidal properties (Isman, 2006). Historically humans have used plants (botanical products) for food, medicine, poisons, fragrances and pesticides (Isman, 2006; Regnault-Roger *et al*., 2008; Stewart, 2008). The use of plant derivatives as pesticides dates back at least two millennia in ancient China, Egypt, Greece and India (Isman, 2006; Regnault-Roger et al., 2008). Plants with potential pesticidal properties may be used in order to try and minimize or eradicate the adverse effects that result from use of synthetic pesticides (Isman, 2006; Regnault-Roger *et al*., 2008).

The active chemical compounds present in the plants are known as secondary metabolites, and are usually multifunctional (Lewinsohn and Gijzen, 2009) (Figure.1.1). Although the mechanisms of action are often not yet well understood, they combine a wide range of toxic potencies thus reducing the chance of resistance by pests (Regnault-Roger, 1997). Another advantage of using botanicals as pesticides is that their residues are non-persistent in the environment because they are readily degraded by light, oxygen and microorganisms (Philogene *et al*., 2005). Botanicals thus are attractive alternatives to synthetic pesticides because they are said to pose little threat to human health and to the environment (Regnault-Roger, 1997; Philogene *et al*., 2005; Isman, 2006).

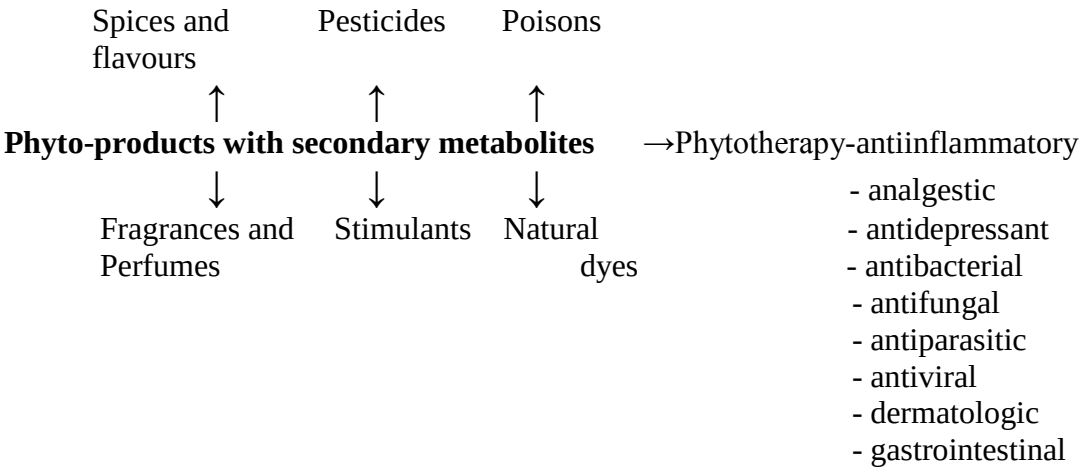

Figure 1.1 Biological properties of botanical products (Isman, 2006; Regnault-Roger *et al*., 2008; Lewinsohn and Gijzen, 2009).

In spite of the scale of scientific data documenting the activity of plant products on pests, only a handful of these products are currently being used commercially on agricultural products because of increasingly stringent regulatory requirements (Isman, 2006). Some of these include neem oil, pyrethrum and *Eucalyptus* species essential oils (Isman, 2006).

Neem oil, obtained from cold-pressing the seeds of the Indian neem tree *Azadirachta indica*, is an effective fungicide and insecticide (Isman, 2006). Pyrethrum is an oleoresin extract from the pyrethrum daisy, *Tanacetum cinerariaefolium* (Isman, 2006). It is extracted from the dried flowers of the plant and causes neurotoxicity of insects, thus attributing to its effectiveness as a pesticide. The genus *Eucalyptus*, represented by over 700 species, comprises of aromatic plants whose essential oils have been used since antiquity as antimicrobial and insecticidal agents (Dorman and Deans, 2000; Batish *et al*.,

2008). Crude eucalyptus oil was first registered as an insecticide in the US in 1949 (Batish *et al*., 2008).

1.4 *Dicerothamnus rhinocerotis*

Dicerothamnus rhinocerotis (L.f.) (=*Elytropappus rhinocerotis* (L.f.)), commonly known in vernacular as "renosterbos" is a shrub belonging to the family Asteraceae and is native to Southern Africa (Van Wyk *et al*., 1997). It is a bush shrub of about 1-2 meters in height, with small grayish-green leaves and tiny flower heads which are almost inconspicuous, figure 1.2 (Levyns, 1935). The distinctive veld type renosterveld consists of a typical assemblage of shrubs, grasses and geophytes, of which *Dicerothamnus rhinocerotis* (*D. rhinocerotis*) is a member (Levyns, 1935; Van Wyk *et al*., 1997).

Figure 1.2 Picture showing the bush shrub *D. rhinocerotis* in its natural habitat (Picture credits: Dr JA Klaasen, University of the Western Cape).

The older branches of the bush have many thin twigs that are covered with triangular leaves (Levyns, 1935; Van Wyk et al., 1997). Small and inconspicuous flower heads (capitula) are produced toward the ends of the twigs and many people cannot discern when the bush is in flower (Levyns, 1935; Van Wyk et al., 1997). Shoot growth occurs in summer, flowering in early winter and seeds are shed in late winter (Levyns, 1935; Van Wyk et al., 1997).

D. rhinocerotis grows in very arid areas and this is attributed to its extremely deep tap root, which allows it access to ground water (Levyns, 1935; Van Wyk et al., 1997). Although *D. rhinocerotis* is reported to be unpalatable to livestock, it has its own specialist browser. The small flightless locust *Lentla ontusifrons* is indigenous to the Eastern Cape (South Africa) and only eats *D. rhinocerotis* (Levyns, 1935; Van Wyk et al., 1997).

Infusions of the young branches of *D. rhinocerotis* prepared in brandy or wine are a traditional Cape (South Africa) medicine for indigestion, dyspepsia, ulcers and stomach cancer (Watt *et al*., 1962). *D. rhinocerotis* is also taken as tonic to improve lack of appetite and for colic, wind and diarrhea (Cillie', 1992). There are some reports that claim that *D. rhinocerotis* was a popular remedy during the 1918 influenza epidemic because it stimulates perspiration (Watt *et al*., 1962). The medicinal properties of *D. rhinocerotis* may be due to rhinocerotinoic acid, a labdane diterpenoid which has been shown to have anti-inflammatory properties (Dekker *et al*., 1988; Van Wyk *et al*., 1997)

In a study by Knowles (2005) extracts of the medicinal plant *D. rhinocerotis*, were shown to exhibit some antifungal properties against *Botrytis cinerea* (*B. cinerea). B. cinerea* is a fungal pathogen that causes grey/gray mould on a large number of economically important agricultural and horticultural crops such as fruits, flowers and green tissue (Jarvis, 1997).

1.5 *Galenia africana*

Galenia africana, commonly known in vernacular as kraalbos or geelbos, is a perennial shrub belonging to the family Aizoaceae (Van der Lugt *et al*., 1992). *G. africana* used to grow mainly in the Namaqualand region of South Africa, but has recently become more widespread in the Western and Southern Karoo (Kellerman *et al.*, 1988). The bush is a perennial aromatic, woody sub-shrub that grows to 0.5-1 m in height (Van der Lugt *et al*., 1992). Its hairless green leaves are oppositely arranged with small yellow flowers at the end of the twigs, figure 1.3 (De Kock, 1928; Kellerman *et al* 1988).

Figure 1.3 Picture showing the bush shrub *G. africana* during flowering (Picture credits: Dr JA Klaasen, University of the Western Cape).

G. africana is an active invader that is abundantly distributed in areas around kraals, road sides, old land and trampled veld (De Kock, 1928; Kellerman *et al*., 1988). According to farmers, if the plant is green, it is palatable and non-poisonous, but if it is yellow and dry it is non-palatable and poisonous (Le Roux *et al*., 1994). They claim that during the summer months *G. africana* is highly poisonous. This is when the plant is dry, with yellow leaves that are highly toxic (Kellerman *et al*., 1988; Le Roux *et al*., 1994).

During severe drought and in poor-conditions animals are forced to graze this plant. Ingestion of the plant is associated with liver damage and severe ascites; a condition referred to as ‘waterpens’ or ‘water belly’, in sheep and goats (De Kock, 1928; Kellerman *et al* 1988). This results in severe abdominal distension, weight loss and animals become

recumbent and die (Van der Lugt *et al*., 1992). The hepatic lesions were compatible with cyanotic induration of the liver, possibly resulting from congestive heart failure (Van der Lugt *et al*., 1992). Depending on the stage of the disease, the liver can either be smaller than normal or enlarged, the color may range from a grayish-blue to a yellowish-brown and the morphology of the liver can be un-altered or distorted by nodular hyperplasia, atrophy and/ or hypertrophy of certain parts (Kellerman *et al.,* 1988). The marked liver lesions have lead researchers to believe that the plant is hepatotoxic due to the presence of an unidentified toxin (Van der Lugt *et al*., 1992).

The Hottentots, a South African indigenous tribe, chewed the plant to relieve toothache (Watt *et al*., 1962). The plant is also used for treatment of venereal diseases and prepared as a decoction for skin diseases and for the relief of inflammation of the eyes (Watt *et al*., 1962). An ointment, made by frying the herb used as a dressing for wounds, especially wounds on the legs of women (Watt *et al*., 1962). Recently *G.africana* has been shown to exhibit antimycobacterial activity against *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* (Mativandlela *et al*., 2008).

Extracts of *G. africana* exhibit antifungal properties against several fungal species, including *Botrytis cinerea* (Knowles, 2005; Vries *et al*., 2005). *B. cinerea* is a fungal pathogen that causes grey mould on a large number of economically important agricultural and horticultural crops, including fruits, flowers and/or green tissue (Jarvis, 1997). Grey mould causes pre-and post harvest fruit decay, therefore causes a problem in the agriculture industry of South Africa (Knowles, 2005). Due to the fact that *B. cinerea*

affects a wide range of products it may cause serious economic losses (Schoonbeek *et al*., 2001).

Due to the antifungal properties of *D. rhinocerotis* and *G. africana* they may have potential use on agricultural produce as fungicides. Toxicity studies are therefore required to determine safety of the plant extracts (Hojo *et al*., 2006). This study thus investigates the toxicity of *G. africana* and *D. rhinocerotis* for a variety of physiological systems and the environment. All physiological systems, including the immune system, the reproductive system and the liver can be adversely affected by toxins (Hojo *et al*., 2006 and Caserta *et al*., 2007).

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Chapter 2

Effects of pesticides on physiological systems

2.1 Introduction

Exposure of pesticides to the body may result in alteration of the normal physiological functions. Pesticides can have a general effect on all cells or they can attack specific organ/physiological systems. Some of the physiological systems that are prone to attack by pesticides include the immune system, the reproductive system and the hepatic (liver) system (Colosio *et al*., 1999).

2.2 The Immune system

The immune system is a, well regulated organ system that involves interrelated reactions which protect an organism from invasion by foreign substances (Mims *et al*., 2004; Storni *et al*., 2005). Its complex organization engages the cooperation and interaction of various cells, cell products, tissues and organs that collectively protect the body (Ladics, 2007). The complexity and unique nature of the immune system may contribute to it being the target organ for various toxic substances, therefore resulting in multiple potential target sites and pathological effects (Colosio *et al*., 1999). It is of utmost importance to manage the risks of xenobiotics and to provide safety information of the substances (Hojo *et al*., 2006). As a result human health and wildlife will be better protected.

The immune system fights pathogens using two methods, namely the innate/ nonspecific immunity and the adaptive/specific immunity (Mims *et al*., 2004). Immunity is the ability to resist damage by identifying and destroying foreign substances and organisms (Mims *et al*., 2004).

2.2.1 Organs of the immune system

The immune system comprises various organs including the bone marrow, thymus spleen, lymph nodes and blood (Caren, 1981; Linnermeyer, 1993). These organs are responsible for production, maturation, storage and transportation of the cells of the immune system, figure 2.1 (Caren, 1981; Linnermeyer, 1993).

The bone marrow

Postnatally all cells of the immune system derive from the bone marrow and form via the process of hematopoiesis (Caren, 1981). During this process the stem cells differentiate into either mature cells of the immune system or into precursors that migrate from the bone marrow to continue their maturation elsewhere (Caren, 1981; Linnermeyer, 1993). In addition to immune system cells, the bone marrow also produces red blood cells and platelets.

The thymus

Immature thymocytes migrate from the bone marrow and complete their maturation and proliferation in the thymus, thus so-called T-lymphocytes or T-cells (Caren, 1981). Immature thymocytes are taken into the cortex of the thymus where a series of molecular events take place allowing the cells to recognize certain antigens. Some of the cells

recognize self-components and these are eliminated via apoptosis. Those that live proceed to the medulla and eventually into the blood stream, where they act upon foreign agents.

Besides T-cells, the thymus also has scattered dendritic cells (DCs), epithelial cells and macrophages (Seely *et al*., 2000). T-cells leave via the blood stream and migrate to lymph nodes, the spleen and other lymphatic tissues and organs.

The spleen

The spleen, which is the largest single mass of lymphoid tissue in the body, serves as an immunologic filter of blood. Immune cells present in the spleen include B-lymphocytes or B-cells, T-cells, macrophages DCs, natural killer cells (NK cells) and red blood cells (Linnemeyer, 1993; Seely *et al*., 2000). As blood flows through the spleen B- and T-cells carry out immune functions and spleen macrophages destroy blood-borne pathogens via phagocytosis (Seely *et al*., 2000).The spleen also stores about a third of the body's platelet supply, aids in hematopoiesis prenatally and functions in the destruction of old blood cells.

Lymph nodes

Lymph nodes are small rounded/bean shaped masses of lymphatic tissue located throughout the body along lymphatic vessels and serve as an immunologic filter of lymph (Linnermeyer, 1993). They filter via an internal honeycomb of reticular connective tissue filled with lymphocytes that collect and destroy bacteria and viruses. When the body is

fighting an infection, lymphocytes multiply rapidly and produce a characteristic swelling of the lymph nodes. The nodes thus drain fluid from most bodily tissue and trap foreign substances which are consequently destroyed by macrophages. Immune cells present in the lymph nodes include T-cells, B-cells, DCs and macrophages.

2.2.2 Overview of the cells of the immune system

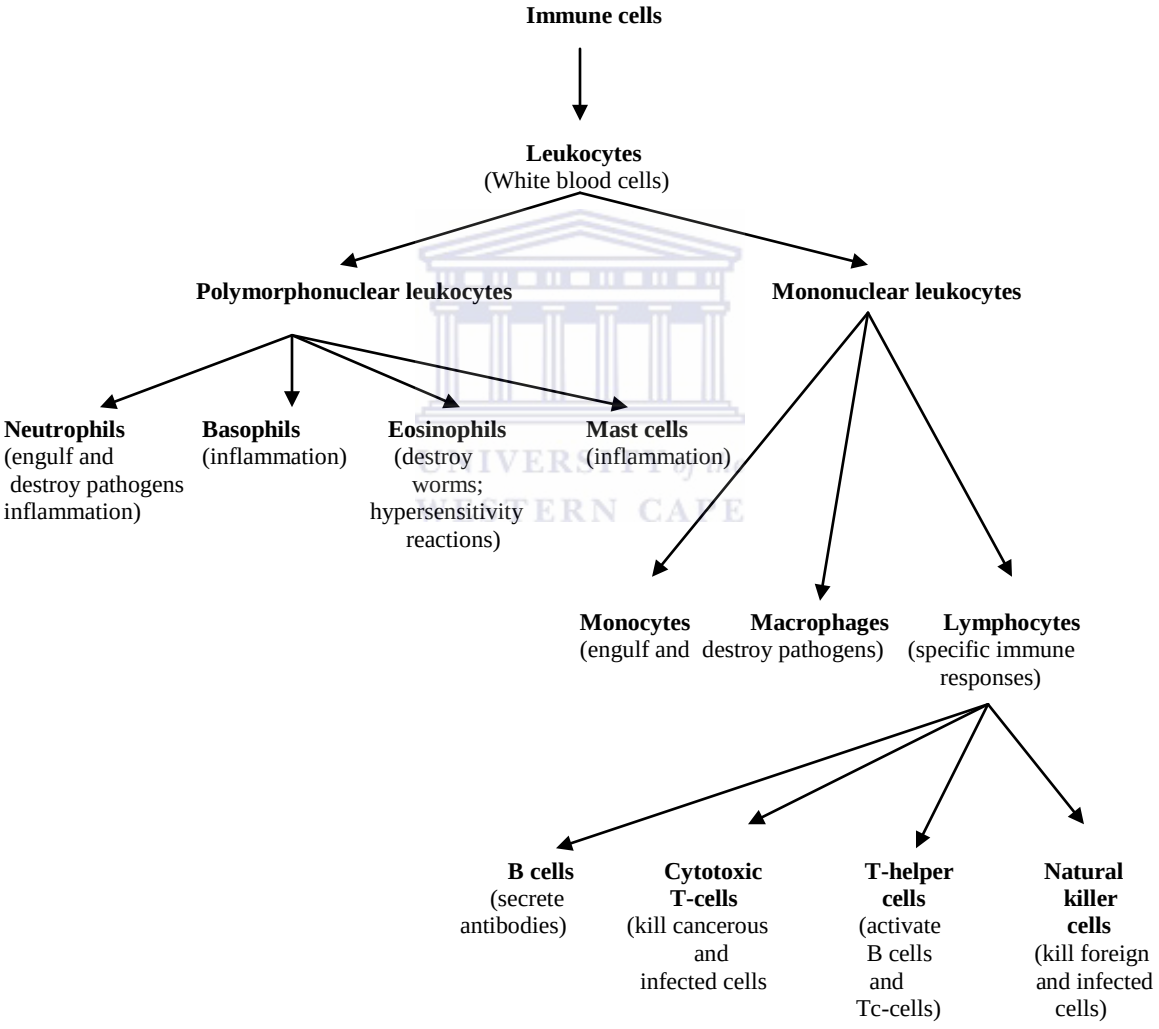

Figure 2.1 Overview of the cells of the immune system and their functions (Mims *et al*., 2004; Storni *et al*., 2005).

2.2.3 Innate immunity

Innate immunity is the inborn mechanism that protects against infection independent of previous exposure i.e. the body recognizes and destroys foreign substances but the response to them is the same each time the body is exposed to them (Bols *et al*., 2001). It makes up the first line of defense against foreign substances. The response includes the barrier systems, chemical mediators and phagocytic cells (Mims *et al*., 2004; Storni *et al*., 2005).

The barrier systems act at the body surfaces, thus an infectious agent must overcome to gain access to the body (Seely *et al*., 2000; Mims *et al*., 2004; Storni *et al*., 2005). These include the skin and mucous membranes, all of which are exposed to the environment (Seely *et al*., 2000; Mims *et al*., 2004; Storni *et al*., 2005).

Chemical mediators act directly against pathogens or activate other mechanisms which lead to destruction of foreign substances (Seely *et al*., 2000; Mims *et al*., 2004). They are found on the surface of immune cells and epithelium lining areas in direct contact with the outside environment (Seely *et al*., 2000; Mims *et al*., 2004; Storni *et al*., 2005). Chemical mediators such as lysozyme, sebum and mucous, kill pathogens or prevent their entry into cells (Seely *et al*., 2000; Mims *et al*., 2004; Storni *et al*., 2005). Other chemical mediators such as histamine, prostaglandins and luekotrines promote inflammation (Seely *et al*., 2000; Mims *et al*., 2004; Storni *et al*., 2005). Complement chemical mediators enhance phagocytosis via the production of critical factors (i.e., opsonins) and attracting

cells to the site of inflammation (i.e., chemoattractants) (Mims et al., 2004; Storni *et al*., 2005).

2.2.4 Adaptive immunity

Adaptive immunity includes the capacity to recognize, respond to, and remember specific substances (Mims *et al*., 2004; Storni *et al*., 2005). Adaptive immunity historically has been divided into two subsets, specifically the humoral and cell mediated immune responses (Mims *et al*., 2004; Storni *et al*., 2005). These are mediated and maintained by B-cells and T-cells respectively.

The adaptive response is initiated by lymphocytes (B and T-cells) encountering foreign antigens. A variety of cell–cell interactions then generate humoral mediators, including immunoglobulins or antibodies and cytokines that modulate the response (Mims *et al*., 2004; Storni *et al*., 2005). These responses result in protection against infectious agents and destruction of targets by antibody dependent cellular cytotoxicity and complement mediated lysis by enhancing phagocytosis through opsonisation (Ladics, 2007).

The adaptive immune system is dependent on various lymphocyte subpopulations, including T cells, B cells and natural killer (NK) cells (Fleisher *et al*., 2000). T cells make up approximately 75% of the lymphocyte population in the circulation, while B cells make up 10-15% of the remaining population (Fleisher *et al*., 2000). T-cells include T-helper (Th-) and T-cytotoxic (Tc-) cells (Mims *et al*., 2004 and Storni *et al*., 2005). Th-cells are further subdivided into Th1 and Th2 cells. Th1 cells produce cytokines, mainly

interferon gamma (IFNγ), which will promote cell mediated immunity (Mims *et al*., 2004 and Storni *et al*., 2005). On the other hand Th2 cells produce cytokines, such as IL-4, IL-6, IL-10 and IL-13, which promote antibody production and humoral immunity (Mims *et al*., 2004 and Storni *et al*., 2005). Upon activation of B-cells by Th2 cytokines, B cells proliferate and differentiate into antibody-secreting cells (Seely *et al*., 2000; Mims *et al*., 2004; Storni *et al*., 2005). However B-cells can also produce antibodies independent of Th2 cytokines, but by binding the antigen and directly transmitting signals to the cells interior, leading to antibody production (Storni *et al*., 2005).

Table 2.1 Overview of the immune system (Mims *et al*., 2004; Storni *et al*., 2005)

Innate Immunity	**Adaptive Immunity**
Response is antigen-independent	Response is antigen-dependent
There is immediate maximal response	There is a lag time between exposure and maximal response
Not antigen-specific	Antigen-specific
Exposure results in no immunologic memory	Exposure results in immunologic memory

2.2.5 Immunotoxicity

Immunotoxicology, a hybrid science of immunology and toxicology, studies interactions of xenobiotics with the immune system. The complexity and unique nature of the immune system may contribute to it being the target organ for various toxic substances, therefore resulting in multiple potential target sites and pathological effects. Immunotoxicology is a science discipline that has evolved gradually since its beginning and is of increasing importance because it appears to contribute to the development of several diseases including cancers, autoimmune disorders and allergies (Langezaal *et al*., 2002). Immunotoxicity can thus be defined as the adverse effects of xenobiotics, including pesticides, on the immune system (Carfi *et al*., 2007).

Immunotoxicity results in toxicant-induced injury to part of the immune system thus affecting immune functions and may result in immunostimulation, immunosuppression, hypersensitivity and autoimmunity (Descotes, 2004; Van Wijk *et al*., 2006). Each of these categories is associated with potential adverse effects associated with significant morbidity. Pesticide exposure to the immune system may result in alteration of the normal immune functions, therefore may result in immunostimulation, immunosuppression, hypersensitivity and/ or autoimmunity.

Immunosuppression

Immunosuppression directly affects the function of immune organs and/or cells as indicated by a decrease in lymphoid organ weight and/or decrease in immune cell numbers (van Wijk *et al*., 2006; De Jong *et al*., 2007). This may result in reduced

immune function expressed as decreased resistance to infection (van Wijk *et al*., 2006; De Jong *et al*., 2007). Immunosuppression has also been associated with increased susceptibility to the development of tumors (Trizo *et al*., 1988; Descotes, 2005). For example, exposure to the organophosphorous compound pentachlorophenol may lead to decreased lymphocyte proliferative responses to mitogens, resulting in immunosupression (Colosio *et al*., 1993).

Immunostimulation

Immunostimulation is the escalation in activity of the immune system organs and/or cells, which may result in amplified responses (De Jong *et al*., 2007). This may lead to exacerbated or lengthened immune responses against infection or a reduction in the capacity of the immune system to respond to external threats (van Wijk *et al*., 2006 and De Jong *et al*., 2007). A study showed an increase in lymphocyte proliferative response to mitogens and an increase in the mitogen induced IL-2 production, upon exposure to dithiocarbamates in pesticides (Colosio *et al*., 1996).

Hypersensitivity

The most common immunotoxic effect of xenobiotics, especially pharmaceuticals, is hypersensitivity (Descotes, 2005). Hypersensitivity reactions may be immune-mediated as well as non-immune mediated, thus the general term ‘hypersensitivity’, instead of allergy is recommended (Descotes, 2004). Hypersensitivity is an increase in the specific responses to the xenobiotic itself resulting in exacerbated immune responses with tissue damage (De Jong *et al*., 2007).

Hypersensitivity reactions have been classified using four mechanism based types of reactions (Table 2.2).

Table 2.2. The Gell-Coombs classification of hypersensitivity reactions (Rajan, 2003)

	Immune mediated hypersensitivity reactions	**Effector mechanism**
Type I	Immediate hypersensitivity or anaphylaxis	IgE mediated mast cell activation
Type II	Cytolytic or cytotoxic reactions	Complement activation by IgM and/or IgG antibodies
Type III	Immune complex reactions	Vasculitis induced by IgM or IgG antibody-antigen immune complexes
Type IV	Delayed type hypersensitivity	Infiltration of T-cells and/or activation of macrophages leading to contact dermatitis

Autoimmunity

Apart from sensitization or irritation due to the xenobiotic or its metabolites, as is the case with hypersensitivity, xenobiotic-induced autoimmune reactions may also occur. Autoimmunity results in reactions to auto-antigens initiated by elevated immune reactivity, immune stimulation or reactions to altered self antigens after contact of xenobiotic with tissue (De Jong *et al*., 2007).

Immunotoxic responses can thus be grouped into 3 categories according to their action on the immune system:

1) **Activation** of the immune system (immunostimulation and auto-immune processes);
2) **Sensitization** of immune responses (hypersensitivity reactions) and
3) **Impairment** of immune response mechanisms (immunosuppression) (Langezaal *et al*., 2002).

Table 2.3. Pesticides that have been shown to affect the immune system

Pesticide	**Effect on immune response**	**Reference**
Dichloro-Diphenyl-Trichloroethane (DDT)	-increased susceptibility to infection -increased IgM serum concentration	Hermanowicz *et al*., 1984 Kashaip, 1986
Chlordane	-increased thymocytes -increased B-cells -depressed antibody-dependant cell-mediated toxicity	Menconi *et al*., 1988
2,4-Dichlorophenoxyacetic acid and 4-Chloro-2-methylphenoxyacetic acid (phenoxy herbicides)	-reduction in CD4, CD8 and natural killer cells -reduction in lymphocyte proliferative response to mitogens	Faustini *et al*., 1996
Organotin compounds	-impairment of neutrophil function	Colosio *et al*., 1990

It is therefore of utmost importance to manage the risks of xenobiotics, including all forms of pesticides, and to provide safety information of the substances. If this is implemented human health and the environment will be better protected. *In vivo* models have been employed to test for the effects of xenobiotics. However because *in vivo* tests are very expensive, require a high number of animals and raise ethical concern, alternative methods are being developed (Carfi *et al*., 2007). Development of *in vitro* tests provides rapid inexpensive means of generating more comprehensive toxicological profiles for xenobiotics (Carfi *et al*., 2007). Another aim for *in vitro* studies is to reduce, or even replace the use of laboratory animals employed in scientific studies (Carfi *et al*., 2007). There are several parameters for the detection of immune alterations; therefore several tests have been developed. Tests include *in vitro* assays which consist of proliferation assays and assays that quantify cytokine concentrations using both human and rodent cells. *In vitro* studies are however not an accurate representative of an *in vivo* immune response, but on balance the practical advantages of this approach make it more attractive for immunotoxicity screening purposes.

2.3 The Liver

The liver is the largest gland in the body (Seely *et al*., 2000). The human liver has four lobes traditionally designated as left, right, quadate and caudate (Seely *et al*., 2000). It lies in the upper right quadrant of the abdomen just below the diaphragm (Seely *et al*., 2000). The liver plays major roles in synthetic, metabolic and excretory functions (Giannini *et al*., 2005; Field *et al*., 2008).

Supply of blood to the liver is from two different sources; oxygen rich blood from the hepatic artery and nutrient rich blood from the hepatic portal vein (Seely *et al*., 2000). The portal blood drains from the mesenteric, gastric, spleinic and pancreatic veins and travels to the liver (Malarkey *et al*., 2005).The portal vein supplies approximately 70% of the blood flow and 40% of the oxygen; while the hepatic artery supplies about 30% of the blood flow and 60% of the oxygen (Burt *et al*., 2002). The liver therefore receives up to 25% of the body's cardiac output at any given time (Burt *et al*., 2002).

2.3.1 Cells of the liver

The normal liver has at least 15 different cell types (Table 2.4) (Bloom *et al*., 1975; Burt and Day 2002). Hepatocytes make up 60% of the total cells and 80% of the volume of the liver (Malarkey *et al*., 2005). About 20% of the remaining cells comprise the sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, and biliary epithelium (Malarkey *et al*., 2005).

Table 2.4 Cells found in the liver (Bloom *et al*., 1975, Burt and Day, 2002)

Hepatocytes
Biliary epithelia
Endothelial cells
Sinusoidal cells
Lymphatic cells
Kupffer cells
Hepatic stellate cells (fat-storing cells)
Lymphocytes (Pit cells)
Progenitor cells
Oval cell—rodent models
Hepatoblasts—humans
Fibroblasts
Smooth muscle cells (blood vessels)
Mesothelial cells
Nerves (unmyelinated)
Neuroendocrine cells
erythrocytes

2.3.2 Functions of the liver

The basic unit of the liver referred to as the 'lobule' is a hexagonal micoanatomic compartment of about 1mm - 2mm (Malarkey *et al*., 2005; Guzman, 2008). The lobule consists of hepatocytes surrounding a central terminal hepatic vein, with portal tracts around the periphery (Saxena *et al*., 1999). The portal tract is made up of three anatomical structures, the hepatic artery and portal vein that supply the blood to the lobule and the bile duct that drains from the lobule (Guzman, 2008).

The functional or metabolic micro-compartment of the liver, the 'acinus', divides hepatocytes into three zones on the basis of distance from the supplying vessels (Giannini *et al*., 2005; Guzman, 2008). Zone 1 hepatocytes are the closest to the blood supply in the portal tracts, while zone 3 hepatocytes are the furthest, house the most mitochondria and are the most susceptible to ischemia (Giannini *et al*., 2005; Guzman, 2008).

Synthetic functions of the liver include the production and secretion of bile, which plays a role in digestion by neutralizing and diluting stomach acid and emulsifying fats (Seely *et al*., 2000; Giannini *et al*., 2005). The liver is also involved in the synthesis of many blood proteins, including albumins, fibrinogen, globulins, heparin, and clotting factors, which are released into the blood stream (Seely *et al*., 2000; Giannini *et al*., 2005).

The liver is responsible for processing nutrient rich blood and breaking down nutrients and waste products, therefore playing a role in metabolism and detoxification (Giannini *et al*., 2005; Field *et al*., 2008). The liver plays several roles in carbohydrate metabolism, including gluconeogenesis (the synthesis of glucose from certain amino acids, lactate or

glycerol), glycogenolysis (the breakdown of glycogen into glucose) and glycogenesis (the formation of glycogen from glucose) (Seely *et al*., 2000; Giannini *et al*., 2005, Field et al., 2008). In lipid metabolism, the liver is responsible for cholesterol synthesis and triglyceride production (lipogenesis) (Seely *et al*., 2000; Giannini *et al*., 2005; Field *et al*., 2008). The liver is also responsible for the mainstay of protein metabolism. Waste products of metabolism are detoxified through processes such as amino acid deamination, which results in the production of urea (Giannini *et al*., 2005).

Hepatic phagocytic cells (Kupffer cells) make up 15% of the cells of the liver and are derived from circulating monocytes (MacSween *et al*., 2002; Malarkey *et al*., 2005). They lie along the sinusoid wall of the liver and phagocytize old and damaged red and white blood cells (Seely *et al*., 2000; Giannini *et al*., 2005). They are also involved in the degradation of some bacteria and other debris that may enter the liver through circulation (Seely *et al*., 2000; Giannini *et al*., 2005).

Besides glucose (in the form of glycogen), the liver also stores several vitamins and minerals (Seely *et al*., 2000). These include vitamin A, vitamin D, vitamin B12, iron and copper (Seely *et al*., 2000). In the first trimester of gestation, the liver is the main site of red blood cell production (Seely *et al*., 2000).

The liver is therefore a complex organ that carries out various functions. As a result of direct contact with toxicants, there is an increased likelihood of hepatic injury or hepatotoxicity (Ncibi *et al*., 2007). Toxicants, such as pesticides, should be studied and ways to protect the liver from adverse effects of toxins should be investigated.

2.3.3 Hepatotoxicity

The complexity of the liver results in it being a target organ for various toxic substances that may lead to pathological effects (Ncibi *et al*., 2007). Hepatotoxicity ranks as one of the most frequent causes of post-commercialisation regulatory decisions to withdraw products and is the most common cause of acute liver failure (Andrade *et al*., 2007). It is therefore of utmost importance to manage the risks of xenobiotics, including all forms of pesticides, and to provide safety information of the substances. If this is implemented human health and the environment will be better protected.

Injury to the liver may be investigated and monitored in standard toxicity studies by using a range of parameters (Ncibi *et al*., 2007). These parameters include the liver's relative weight and the concentrations of enzymes, proteins and lipids (Ncibi *et al*., 2007; Field *et al*., 2008). The markers commonly used to determine hepatotoxicity are discussed below (Giannini *et al*., 2005).

Hepatocyte integrity

The liver contains many enzymes and two of them are commonly used to test for hepatocyte integrity (Giannini *et al*., 2005; Field *et al*., 2008). Aspartate aminotransferase (AST), also known as serum glutamate oxaloacetate transaminase (SGOT), and Alanine aminotransferase (ALT), also known as serum glutamate pyruvate transaminase (SGPT), are enzymes produced in the liver and are released by hepatocytes upon damage (Giannini *et al*., 2005; Field *et al*., 2008). Hepatocyte damage is a key process in the pathogenesis of various liver diseases (Xu *et al*., 2002; Giannini *et al*., 2005).

Aspartate aminotransferase levels are highly concentrated in the liver, but are also diffusely present in the heart, skeletal muscle, kidneys, brain, small gut epithelial cells and red blood (Giannini *et al*., 2005; Field *et al*., 2008). In the hepatocytes, AST is both cytosolic (20% of total activity) and mitochondrial (80% of total activity) (Giannini *et al*., 2005). AST catalyses the reversible transfer of the α-amino groups from aspartate to the α-keto group of ketoglutaric acid to generate oxalacetic acid, which is important for the citric acid cycle (Giannini *et al*., 2005; Field *et al*., 2008). The normal concentration range for AST in the blood stream, at a given time, is between 5 IU/L (international units per liter) and 43 IU/L (Giannini *et al*., 2005). The half-life of AST in the circulation is about 17 hours for total AST and about 87 hours for mitochondrial AST (Dufour *et al*., 2000). Higher concentrations of AST are found in zone 3 of the hepatic acinus, therefore damage to this zone, whether ischemic or toxic, may result in greater alteration of AST (Giannini *et al*., 2005). In the case of injury, AST levels usually peak before those of ALT because of the enzymes peculiar intralobular distribution (Dufour *et al*., 1988; Singer *et al*., 1995; Seely *et al*., 2000)

Alanine aminotransferase catalyses the transfer of the α-amino groups from alanine to the α-keto group of ketoglutaric acid to generate pyruvic acid, which is also an important contributor to the citric acid cycle (Giannini *et al*., 2005). In the liver, ALT is localized solely in the cellular cytoplasm (Giannini *et al*., 2005). Unlike AST, ALT levels are highly concentrated in the liver but low concentrations are present in the skeletal muscles and kidneys (Giannini *et al*., 2005). As a result an increase in serum levels of ALT, rather than AST, is more specific for liver damage (Giannini *et al*., 2005). The normal

concentration range for ALT in the blood stream is between 5 IU/L (international units per liter) and 60 IU/L. The half-life of ALT, in the circulation is, about 47 hours (Dufour *et al*., 2000).

Cholestasis

Injury to the liver may also present with partial or complete blockage of the bile ducts, a condition known as cholestasis (Giannini *et al*., 2005). Alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), and bilirubin are elevated as a result of bile duct blockage (Giannini *et al*., 2005).

Alkaline phospatase is an enzyme that catalyses the hydrolysis of phosphate ethers and is also responsible for the transportation of metabolites across cell membranes (Giannini *et al*., 2005; Field *et al*., 2008). Alkaline phosphatase is found in the liver, bones, the kidneys, the placenta, intestines and in leucocytes (Fishman, 1990; Dufour *et al*., 2000). Hepatic ALP is found on the bile duct epithelia and cholestasis results in increased ALP synthesis and release (Schlaeger *et al*., 1982; Moss, 1997; Field *et al*., 2008). The half life of ALP, in the circulation, is about one week (Dufour *et al*., 2000).

γ-glutamyl transpeptidase (GGT) is a microsomal enzyme that is found in the liver (hepatocytes and biliary epithelial cells), the kidneys (renal tubules), the pancreas and the intestines (Giannini *et al.*, 2005). γ-glutamyl transpeptidase catalyses transfer of a γ-glutamyl group between peptides (Field *et al*., 2008). Liver diseases, regardless of cause, may result in altered GGT serum levels. Due to its lack of specificity but high sensitivity

for liver disease, GGT may be useful in indentifying causes of altered ALP levels (Dufour *et al*., 2000).

Bilirubin is a breakdown product of normal hemoglobin catabolism within the reticuloendothelial system (Giannini *et al*., 2005; Field *et al*., 2008). Bilirubin, loosely bound to albumin in plasma to solubilise it, is transported to the liver where it is taken up by the hepatocytes (Giannini *et al*., 2005; Field *et al*., 2008). Here it is esterified with glucuronic acid, and then excreted into bile as water-soluble bilirubin diglucuronide (Fevery *et al*., 1986; Berk *et al*., 1994; Field *et al*., 2008). Elevated serum concentration of conjugated bilirubin may result from augmented bilirubin production, decrease in hepatic uptake or both (Giannini *et al*., 2005). It may also increase as a result of hemolysis or hereditary deficiencies (e.g. Gilbert's syndrome) (Giannini *et al*., 2005; Field *et al*., 2008). An increase in conjugated bilirubin implies regurgitation of bilirubin from the hepatocytes back into the plasma (Giannini *et al*., 2005; Field *et al*., 2008). This may be as a result of obstruction of bile outflow, cancer, cholestasis, or drug toxins (Giannini *et al*., 2005; Field *et al*., 2008). Conjugated bilirubin increase as a result of drug toxins is accompanied by an increase in ALP levels (Giannini *et al*., 2005).

Liver functioning mass

"A change in serum albumin level or prothrombin time may be associated with a decrease in liver functioning mass" (Giannini *et al*., 2005). Albumin is produced by the hepatocytes and its synthesis tends to decrease in end-stage liver disease (Giannini *et al*., 2005). Prothrombin time depends on the levels of clotting factors I, II, V, VII and X,

which are produced in the liver. Thus change in prothrombin time depends on synthesis of liver derived coagulation factors (Giannini *et al*., 2005).

Neither albumin nor prothrombin time are specific for liver injury, but when it is certain that the cause of alteration is injury to the liver tissue, then serum albumin levels and prothrombin time are useful tests for monitoring liver function (Giannini *et al*., 2005).

2.4 The Reproductive system

The reproductive system is a system of organs which work together for the purpose of procreation (i.e. to conceive offspring). Unlike most organ systems of the body which show little differences between sexes, the reproductive system displays distinct anatomical and physiological dimorphisms. The reproductive system controls the development of the structural and functional differences between males and females.

2.4.1 The male reproductive system

The male reproductive system consists of the testes (singular, testis), a series of ducts, accessory glands, and supporting structures including the scrotum and the penis (Seely *et al*., 2000). The ducts include the epididymides (singular, epididymis), the vas deferens and urethra (Seely *et al*., 2000). Accessory glands include the seminal vesicles, prostate gland and bulbourethral glands (Seely *et al*., 2000).

The testes could be regarded as both exocrine and endocrine glands. Spermatozoa form a major part of the exocrine secretions and testosterone is the major endocrine product of the testes (Seely *et al*., 2000). About 80% of the testicular mass consists of coiled seminiferous tubules; while the remaining 20% consists of Leydig/interstitial cells and Sertoli/sustentacular cells (Seely *et al*., 2000; Sikka *et al*., 2008).

Sertoli cells or “nurse cells” form a lining within the seminiferous tubules which envelopes the developing sperm during spermatogenesis (Seely *et al*., 2000; Sikka *et al*., 2008). During puberty the Sertoli cells differentiate and form the blood-testes barrier, by

virtue of tight junctions, which is important for the establishment of normal spermatogenesis (Seely *et al*., 2000; Sikka *et al*., 2008). Sertoli cells, under the influence of follicle stimulating hormone, provide nourishment for the developing sperm cells, release the hormone inhibin which aids in regulation of sperm production and secrete fluid for transport of sperm to the epididymis (Seely *et al*., 2000; Sikka *et al*., 2008). Sertoli cells also destroy defective sperm cells (Seely *et al*., 2000; Sikka *et al*., 2008).

Leydig cells are endocrine cells that are formed during the eighth week of human embryonic development (Seely *et al*., 2000, Sikka *et al*., 2008). Luteinizing hormone stimulates production of the hormone testosterone by Leydig cells (Seely *et al*., 2000; Sikka *et al*., 2008). Testosterone supports spermatogenesis and promotes development and maintenance of sex organs and secondary sexual characteristics (Anderson *et al*., 2006; Eacker *et al*., 2008; Sikka *et al*., 2008).

In mammals, sperm cells do not develop normally at normal body temperature therefore are housed in the testes which are located outside the body where the temperature is lower (Seely *et al*., 2000). The ductus deference from each testis leads into the pelvis, where they join the ducts of the seminal vesicles to form the ampullae. Extensions of the ampullae, called the ejaculatory ducts, pass through the prostate gland and empty into the urethra. The urethra in turn exits the pelvis and passes through the penis to the outside of the body (Seely *et al*., 2000).

2.4.1.1 The hypothalamus-pituitary-gonadal axis

Primary hormonal mechanisms that regulate reproductive functions involve the hypothalamus, the pituitary gland and the gonads (Seely *et al*., 2000; Anderson *et al*., 2006). Neurons in the hypothalamus secrete gonadotrophin-releasing hormone (GnRH), which in turn binds to receptors in the anterior pituitary gland and stimulates secretion luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Anderson *et al*., 2006; Seely *et al*., 2000).

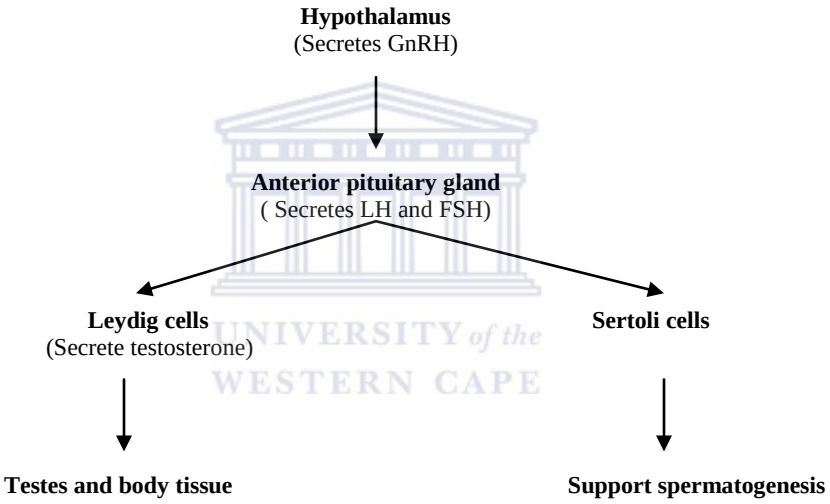

Figure 2.5 Overview of the hypothalamus-pituitary-gonadal axis (Seely *et al*., 2000; Anderson *et al*., 2006).

2.4.1.2 Testosterone biosynthesis

Testosterone is a steroid hormone and its synthesis is a highly regulated process (Anderson *et al*., 2006; Eacker *et al*., 2008; Sikka *et al*., 2008). In the male reproductive system testosterone synthesis occurs in the Leydig cells upon their stimulation by LH. Luteinizing hormone binds to the G-protein-coupled LH/chorionic gonadotropin receptor

(LHCGR= high affinity receptor) (Dufau, 1997; Ascoli *et al*., 2002). In response to LH, there is activation of adenylate cyclase that results in an increase in intracellular concentrations of adenosine 3′5′ -cyclic monophosphate (cAMP) (Eacker *et al*., 2008; Ascoli *et al*., 2002)

cAMP stimulates transport of cholesterol to the inner mitochondrial membrane, via the action of steroidogenic acute regulatory protein (StAR) (Bose *et al*., 1998). The cholesterol is then metabolized to pregnenolone by the cytochrome P450 enzyme CYP11A (Midzak *et al*., 2008). Pregnenolone is the metabolized to testosterone by enzymes of the smooth endoplasmic reticulum; shown in Fig 2.6 (Midzak *et al*., 2008).

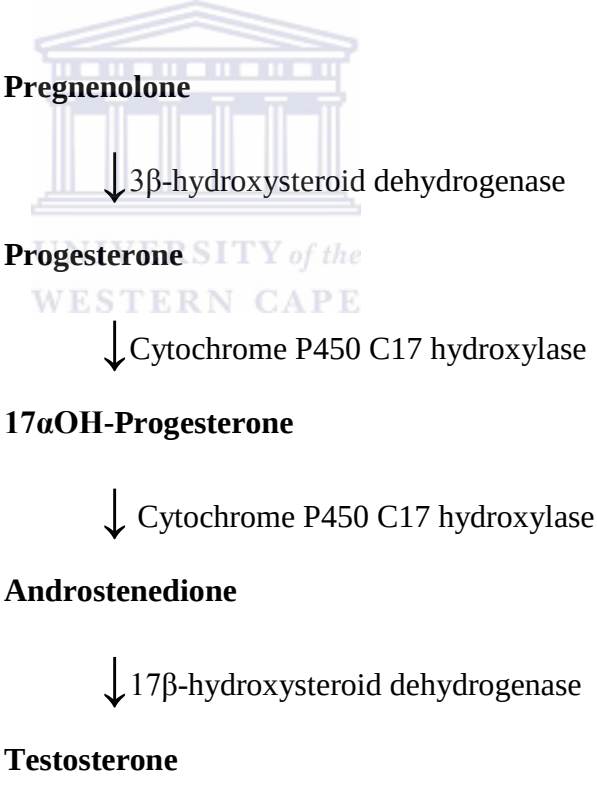

Figure 2.6 Overview of the metabolism of pregnenolone to testosterone (Midzak *et al*., 2008).

2.4.1.3 Pesticides and the male reproductive system

Humans and animals may come into contact with agricultural chemicals via air, water, food and during fetal development. Over the years there has been increasing evidence documenting the effects of agricultural chemicals, such as pesticides, on the hypothalamus-pituitary-gonadal axis of non-target organisms. According to the USA Environmental Protection Agency (USA-EPA), 'an exogenous substance that interferes with the production, release, transport, metabolism, binding, action or elimination of the natural hormone in the body responsible for the maintenance of homeostasis and the regulation of development processes', is known as an endocrine disruptor (Kavlock *et al*., 1996).

Various pesticides have been implicated in male reproductive toxicity as a result of hormonal alterations. These pesticides result in a detrimental effect on sperm concentration, motility, mobility and morphology, due to impairment of spermatogenesis, and may impact reproductive success of an organism (Whorton *et al*., 1977).

Dichlorodiphenyltrichloroethane (DDT), the most documented organochlorine pesticide, was widely used in the US, but was banned in 1972 (Bhatia *et al*., 2005; Prins, 2005). DDT is however still being used in developing countries worldwide for eradication of malaria (Bhatia *et al*., 2005; Prins, 2005). DDT functions as an exogenous oestrogen therefore is an endocrine disruptor (Bulger *et al*., 1983).

In mammals DDT has been found to reduce sperm counts, Leydig cell development, alter Sertoli cell function and cause negative feedback inhibition of the fetal pituitary gland (Sonnenschein *et al*., 1998; Sharpe *et al*., 1993). p, p'dichlorodiphenyldichloroethylene (DDE), a breakdown product of DDT, acts as an androgen receptor antagonist and inhibits the action of the hormone testosterone (Kelce *et al*., 1995; Danzo 1997). *In utero* exposure to DDT and DDE results in adverse effects on both the male and female reproductive systems. These include abnormal development of ovarian tissue, reduced penis size, reduced testosterone levels, hypospadiasis (abnormal opening of the urethra), cryptochodism (failure of one or both testicles to descend), abnormal sperm and low sperm density (Fry *et al*., 1981; Subramanian *et al*., 1987; Facemire *et al*., 1995; Guillette *et al*., 1996; Gray *et al*., 2001). DDT and DDE thus act as both an eostrogen agonist and an androgen antagonist.

Other pesticides that adversely affect the human male reproductive system include 1, 2-Dibromo-3-chloropropane (DBCP) (a soil fumigant and nematocide), chlordecone (a fungicide) and carbaryl (an insecticide) (Srinivasa *et al*., 2005). Exposure to DCPB results in decreased sperm count and decreased libido (Srinivasa *et al*., 2005; Sikka *et al*., 2008). Exposure to chlodecone and carbaryl also results in low sperm count (Srinivasa *et al*., 2005).

Due to the fact that pesticides can adversely affect the reproductive system, it is important to screen for such effects when new pesticides are being evaluated for commercial use.

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Chapter 3

The cytotoxicity of *Dicerothamnus rhinocerotis* and *Galenia africana* using cell cultures

3.1 Abstract

Resistance of pests to commonly used synthetic pesticides has increased greatly over the years. An alternative to synthetic pesticides is the exploitation of naturally occurring products with pesticidal properties. *Dicerothamnus rhinocerotis* and *Galenia africana*, two plants native to South Africa, exhibit antifungal activity against the fungal pathogen *Botrytis cinerea*. This fungal pathogen causes grey mould on a large number of economically important agricultural and horticultural products. A major consideration in approving pesticides for use is whether they pose an unreasonable risk to humans and to wildlife. This study focuses on the cytotoxicity of *Dicerothamnus rhinocerotis* and *Galenia africana* extracts on human cell cultures. Human whole blood and the human breast adenocarcinoma cell line (MCF-7) were treated with varying concentrations of the plant extracts after which cytotoxicity was determined. Cytotoxicity was measured using several biomarkers, including Lactate dehydrogenase (LDH), 2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and total protein concentration.. Inhibiting concentration for a 50% effect (IC_{50}) and no observable effect level (NOEL) values were obtained for *Galenia africana* and *Dicerothamnus rhinocerotis*.

3.2 Introduction

Substances used to prevent or destroy pests are known as pesticides (Arias-Estervez *et al*., 2008). Over the years resistance to pesticides by target species has greatly increased (Zhu *et al*., 1996). Residues of the pesticides may also pose a health risk to consumers. This has resulted in pressure to reduce the use of synthetic pesticides (Isman, 2006). An emerging alternative to synthetic pesticides is the exploitation of naturally occurring products with pesticidal properties (Isman, 2006; Regnault-Roger *et al*., 2008; Stewart, 2008). Historically humans have used plants for several things including food, medicine, poisons, fragrances and pesticides (Isman, 2006; Regnault-Roger *et al*., 2008; Stewart, 2008).

Galenia africana, commonly known in vernacular as kraalbos or geelbos, is a perennial shrub belonging to the family Aizoaceae and is native to South Africa (Kellerman *et al.*, 1988; Van der Lugt *et* al., 1992). Historically the plant has been used for medicinal uses such as to relieve toothache, treatment of venereal diseases and skin diseases and for the relief of inflammation of the eyes (Watt and Breyer-Brandwijk, 1962).

During severe drought and in poor-conditions animals are forced to graze this plant. Ingestion of the plant is associated with liver damage and severe ascites, a condition referred to as ‘waterpens’ or ‘water belly’, in sheep and goats (De Kock, 1928; Kellerman *et al*.,1988). The marked liver lesions have lead researchers to believe that the plant is hepatotoxic due to the presence of a toxin (Van der Lugt *et al*, 1992).

Dicerothamnus rhinocerotis (L.f.) (=*Elytropappus rhinocerotis* (L.f.)), commonly known in vernacular as “renosterbos” is a shrub belonging to the family Asteraceae and is native to Southern Africa (Van Wyk *et al*., 1997). Infusions of the young branches prepared in brandy or wine are a traditional Cape (South Africa) medicine for indigestion, dyspepsia, ulcers and stomach cancer (Watt *et a*l., 1962). *Dicerothamnus rhinocerotis* (*D. rhinocerotis*) is also said to be taken as tonic to improve lack of appetite and for colic, wind and diarrhoea (Cillie’, 1992). The medicinal properties of *D. rhinocerotis* may be due to rhinocerotinoic acid, a labdane diterpenoid which has been shown to have anti-inflammatory properties (Van Wyk *et al*., 1997; Dekker *et al*., 1988)

Extracts of *G. africana and D. rhinocerotis* exhibit antifungal activity against *Botrytis cinerea* (Knowles, 2005). *Botrytis cinerea* is a fungal pathogen that causes grey mould rot, on a large number of economically important agricultural and horticultural crops (Jarvis, 1997). Fractionation of ethanol extracts of *G. africana* using silica gel column chromatography and thin-layer chromatography yielded several fractions with antifungal activity (Vries *et al*., 2005). One of the fractions inhibited the growth of fourteen fungal isolates (Vries *et al*., 2005).

Due to the antifungal properties of the two plants they may have potential use on agricultural produce as fungicides. Toxicity studies are required to determine safety of the plant extracts. The toxicity of a substance is its capacity to cause injury to a cell or living system. Toxicity represents the kind and extent of damage that can be done by a particular substance. Therefore the aim of this study is to determine the cytotoxicity of *G.*

africana and *D. rhinocerotis*. Cytotoxicity in this study was measured using several biomarkers, including Lactate dehydrogenase (LDH), 2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and total protein concentration.

LDH is a stable cytoplasmic enzyme found in most cells. It is rapidly released from cells upon damage to the plasma membrane. The enzyme activity assay is based on the conversion of lactate to pyruvate in the presence of LDH, with parallel reduction of NAD (Decker and Lohmann-Matthes, 1988; Sepp *et al*., 1996).

XTT is a yellow tetrazolium salt that forms an orange formazan after reduction by metabolically active cells. This conversion only occurs in viable cells, thus its use in determining cell viability.

3.3 Materials and methods

3.3.1 Reagents

All reagents, solvents and biomolecules used in this study were purchased from either Roche (South Africa), Merck (Germany) or Sigma Chemical Company (St Louis, USA) unless stated otherwise. All reagents were of analytical grade.

3.3.2 Plant extractions

Two medicinal plant species, namely *G. africana* and *D. rhinocerotis,* used in traditional medicine practices in South Africa were obtained and prepared for analysis. Extracts (20% (w/v)) were prepared in 94.4% ethanol by Parceval (Pty) Ltd Pharmaceuticals (South Africa). Leaves of the plants were milled (sieve size~ 2mm-3mm) after which the leaves were mixed with 94.4% ethanol at 20 g leaves per 100 ml ethanol. The mixture was shaken for brief periods during an overnight extraction. The extracts were then pressed, to separate the tincture and the milled leaves. Filtration was carried out to clean the tincture from plant debris and the extract was stored at 20 ^{o}C. For study purposes the extracts were air dried and resuspended in DMSO to obtain 50% (w/v) (500 mg/ml) extract.

3.3.3 Cytotoxicity testing using whole blood culture (WBC) assays

Peripheral blood samples were obtained from consenting healthy males between the ages of 25 and 30. Criteria for exclusion included infection and use of pharmaceuticals three weeks prior to blood collection. The subjects were informed about the aims and methodology of the study. Blood samples were collected by venous puncture into heparinised vacuum tubes (Beckon Dickison Vacutainer Systems, UK). For cytotoxicity evaluation, whole blood cultures were set up in Nunc 96-well round bottom microculture plates (Serving life science, Denmark).

Serial dilutions of the plant extracts were prepared, suspended in DMSO, and transferred to the wells of the culture plate (2 µl extract/well). Control wells received the DMSO (1%) vehicle only. Each dilution was plated in triplicate and the experiment repeated three times. Whole blood was diluted 1:10 (v/v) in RPMI 1640 cell culture medium and 200 µl/well of the diluted blood was added to the cell culture plate wells containing the samples. The final sample to blood ratio was 1:100 (v/v). After tapping the plate to mix solutions, the cultures were incubated at 37 ^{o}C in a 5% CO_2 environment for twenty-four hours. The culture supernatants were then harvested and lactate dehydrogenase (LDH) activity of the supernatants was determined using a chromogenic cytotoxicity detection kit.

Cytotoxicity assessment of WBC via LDH leakage assay

Cytotoxicity induced by the extracts from the two study plans was assessed by LDH leakage into the whole blood culture medium. Release of intracellular LDH was determined using the chromogenic Cytotoxicity Detection Kit (BioVision, USA).

Cell free supernatants (10 µl) were harvested from the whole blood cultures and transferred into 96 well-plates. Lactate dehydrogenase reaction mixture was freshly prepared according to manufacturer's instructions. The LDH reaction mixture (100 µl) was then added to cell free supernatants and mixture was incubated for 15 minutes, at room temperature. Total LDH for the whole blood culture was determined by lysing the cells (200 µl of 10% whole blood in RPMI 1640, with 2 µl of TritonX-100 detergent). The detergent causes the immediate lysis of the blood cells. The cell lysate was used as the 100% LDH standard. Various dilutions of the standard were also assayed. Absorbances at 492 nm were recorded at time-zero and after 15 minutes using a plate reader (Thermo electron corporation, SA). Values obtained for the standards were used to construct a standard curve. Lactate dehydrogenase release was expressed as a percentage of total cellular LDH, to reflect the cytotoxic potential of the plant-products.

3.3.4 Cytotoxicity testing using MCF-7cell culture assays

MCF-7 cells (American Type Culture Collection, USA) were grown in cell culture flasks and routinely maintained in full medium in a humidified chamber at 37 °C in 5% CO_2. The full medium consisted of RPMI 1640 culture medium supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic mix (Sigma, USA). Medium was replaced every 48 hours.

To prepare cells for plating, the cultures were washed with Dulbecco's phosphate buffered saline (DPBS) (BioWhittaker, USA) after which they were trypsinized (BioWhittaker, USA) for 5-10 minutes. Cells were collected in 10 ml tubes and centrifuged at 1000 Xg for 10 minutes. The supernatants were discarded and the pellets were resuspended in full medium. Cells were seeded in Nunc 48 well cell culture plates (Serving life science, Denmark) at 10^6cells per well and incubated for 48 hours at 37 °C in 5% CO_2. The plant extracts of *G. africana* and *D. rhinocerotis* were added to cultures, at a starting concentration of 5000 μg/ml and a serial dilution carried out. Each dilution was done in triplicate wells and the experiment repeated three times. Plates were incubated for a further 96 hours after which cytotoxicity assessment was performed. Cytotoxicity was measured using the LDH chromogenic Cytotoxicity Detection Kit, the XTT chromogenic Cell Proliferation Kit II (Roche, South Africa) and protein concentration.

Protein determination via Bradford assay

The total protein concentration of cell cultures were determined according to the method of Bradford (1976), using Bio-Rad reagent (Bio-Rad Laboratories, Munchen) and bovine serum albumin (BSA) as a standard. Cultures were washed four times with saline. Cells were then hydrolysed with 100 µl per well of 1M NaOH for thirty minutes at room temperature. The hydrosylate was collected. The hydrosylate or BSA standards (10 µl/well) were added to a 96 well storage plate. Bradford reagent (100 µl) was then added to all wells of the plate. The contents of the wells were mixed and absorbances were measured with a plate reader (Thermo electron corporation, SA) at 620 nm and results recorded.

Cell proliferation and viability assessment via XTT assay

Quantification of viable cells, in the presence of the two samples, was determined by the use of the chromogenic Cell Proliferation Kit II (XTT).

The assay was conducted according to the manufactures instructions (Roche, South Africa). Warm XTT labeling mixture (provided in the kit and prepared according to manufacturer's instructions) was added to cell cultures. The plate was then incubated at 37 ^{o}C and 5% CO_2 for four hours. The formazan dye formed is soluble and is directly quantified using a plate reader (Thermo electron corporation, SA). Absorbances were measured at 492 nm and results recorded.

Total cellular LDH assay

The LDH assay was performed on the cell layer that remained in the culture plate after harvesting the supernatant. Cells were lysed by adding 100 µl per well of 1% (v/v) Tween20 in phosphate buffered saline (PBS). The plate was incubated for thirty minutes at room temperature after which the lysate was collected. A 10 µl aliquot of lysate was mixed with warm LDH-reaction mixture (provided in the kit). The mixture was incubated in the dark for 30 minutes, at room temperature. Absorbances at 492 nm were recorded at time-zero and after 30 minutes using a plate reader (Thermo electron corporation, SA), after which cytotoxicity was calculated.

3.3.5 Statistical analysis

All experiments were performed three times in triplicate. Data was analysed using one-way ANOVA ($P<0.001$) and polyniminal regression analysis. Regression analysis was used to determine dose response equations and the IC_{50} and NOEL were determined by solving the equations.

The inhibiting concentration for a 50% effect (IC_{50}) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular substance is required to inhibit a given biological process by half. The no observed effect level (NOEL) is the dosage level immediately below the lowest dosage level eliciting any type of toxicological response in the same study (Sevem and Ballard, 1990).

3.4 Results

3.4.1 Cytotoxicity assessment of *D. rhinocerotis* and *G. africana* using whole blood culture (WBC) assays

From the LDH assay performed a standard curve was plotted from the optical density readings obtained for the various concentrations of the standard (figure 3.1). The percentage cytotoxicity of the two plant extracts was then calculated using the standard curve.

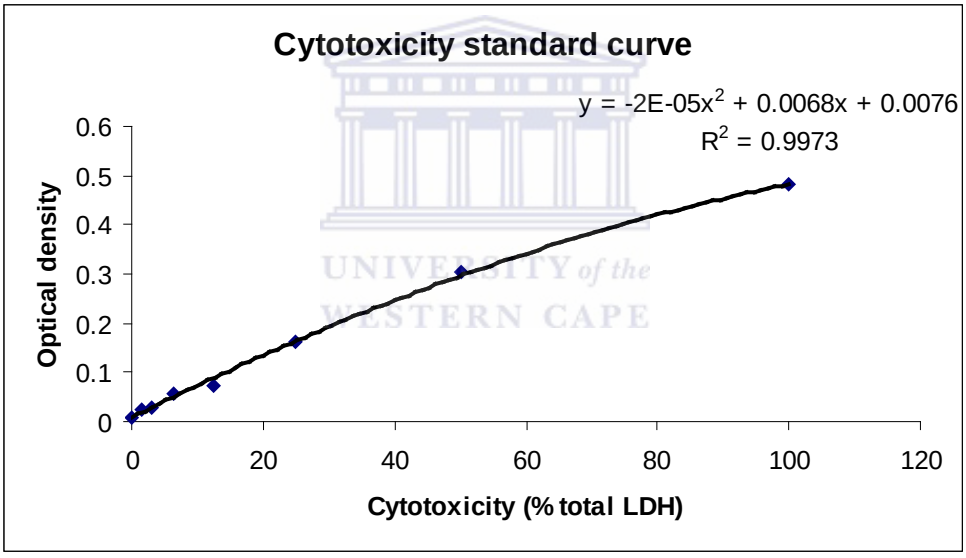

Figure 3.1 Standard curve plotted for LDH assay. Curve shows relationship between percentage LDH and optical density.

The curve above, figure 3.1, shows that there is a good polynominal relationship between percentage total LDH and optical density (R^2=0.9973). The percentage LDH of the whole blood cultures after incubation with various *G. africana* and *D. rhinocerotis* dilutions were extrapolated using the standard curve above. *G. africana* showed no toxicity at

concentrations below 625 μg/ml, while *D. rhinocerotis* showed no toxicity at concentration 1250 μg/ml and below ($p<0.001$).

Dose response curves were then plotted for *D. rhinocerotis* and *G. africana*. IC_{50} (50% inhibitory concentration) and NOEL (No Observable Effect Level) values, for the two extracts, were determined using dose response curves plotted (figures 3.2 and 3.3). The results were expressed as the effects of the individual extracts vs. the control, untreated cells.

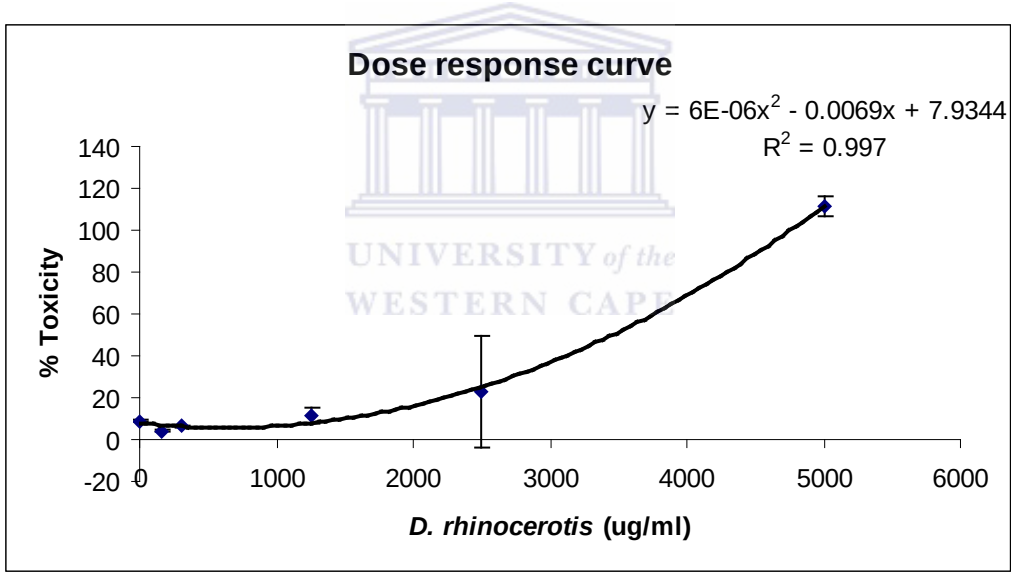

Figure 3.2 LDH dose response curve after treatment of WBC with *D. rhinocerotis*. Curve shows correlation between concentration of *D. rhinocerotis* and percentage cytotoxicity.

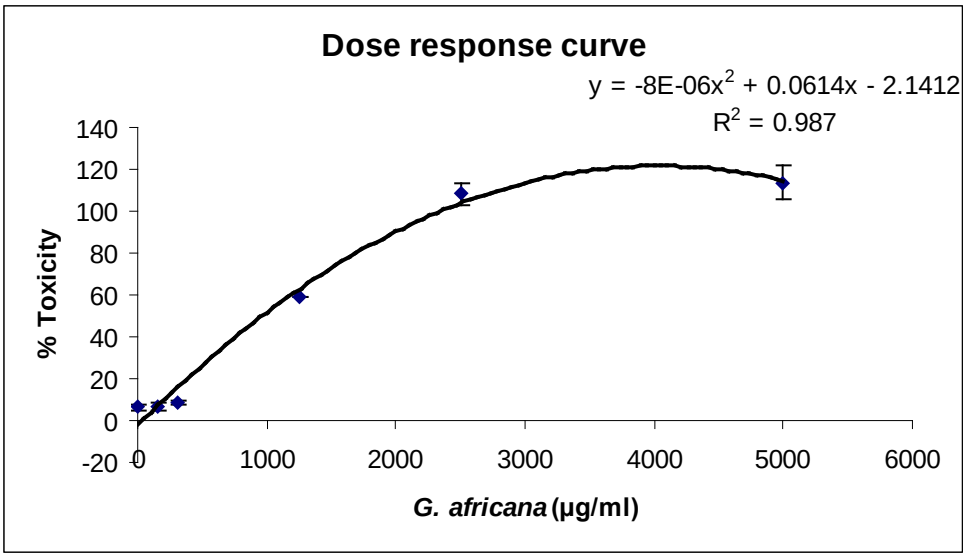

Figure 3.3 LDH dose response curve after treatment of WBC with *G. africana*. Curve shows correlation between concentration of *G. africana* and percentage cytotoxicity.

The IC_{50} and NOEL values for the two extracts are listed in table 3.1 below. For the LDH assay performed IC_{50} is the concentration of the plant extracts that result in 50% cytotoxicity and NOEL is the concentration that does not cause cytotoxicity i.e. 0% toxicity.

Table 3.1 IC_{50} and NOEL values for *D. rhinocerotis* and *G. africana*, calculated from dose response curves 3.2 and 3.3.

	IC50 values (µg/ml)	**NOEL values (µg/ml)**
G. africana	975	36
D. rhinocerotis	3285	0

The IC_{50} and NOEL values obtained for the two plant extracts are shown in the table above.

3.4.2 Cytotoxicity assessment of *D. rhinocerotis* and *G. africana* using MCF-7 cell culture assays

Protein determination via Bradford assay

A standard curve was plotted from the optical density readings obtained from the standards used in the Bradford assay (figure 3.4). This curve was used to determine the amount of protein present in the cell culture hydrolysate after exposure to the plant products.

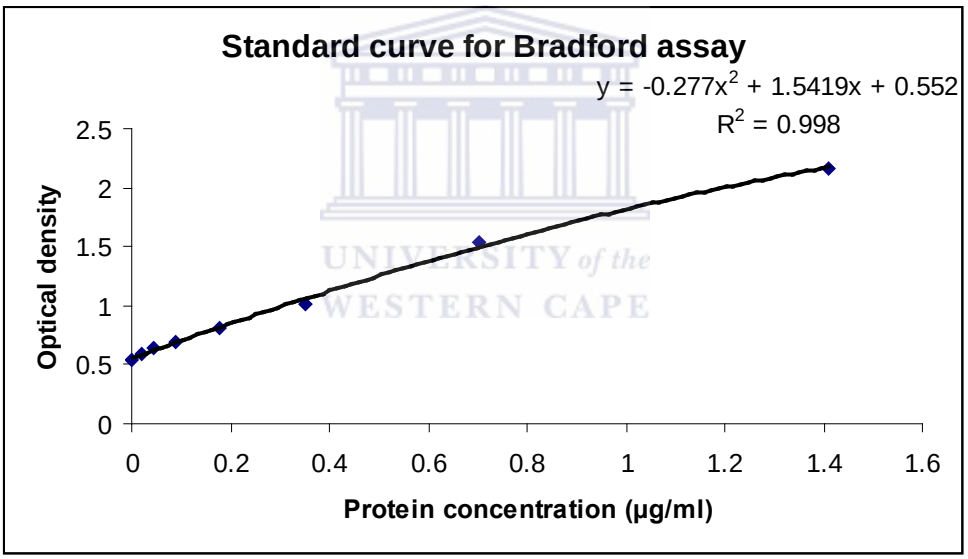

Figure 3.4 Standard curve plotted for the Bradford assay using MCF-7 cells. The curve shows the relationship between protein concentration of the standard and the optical density.

The curve above, figure 3.4, shows a polynominal relationship between protein concentration and optical density (R^2=0.998). The amount of protein present in the cell

cultures after incubation with *G. africana* and *D. rhinocerotis* was calculated using the standard curve, figure 3.4. *G. africana* decreased protein concentration at 250 μg/ml and above, while *D. rhinocerotis* decreased protein concentration at 1000 μg/ml and above. These results show a decrease in the number of cells present in the cultures after exposure to the plant extracts. This therefore depicts a cytotoxic effect of the plant extracts on the on the cells.

IC_{50} and NOEL values were then calculated using the dose response curves plotted for the two extracts (figures 3.5 and 3.6). The results were expressed as the effects of the individual extracts vs. the control, untreated cells. The NOEL values for the Bradford assay are obtained when protein concentration is 100% and IC_{50} values are obtained when protein concentration is 50%.

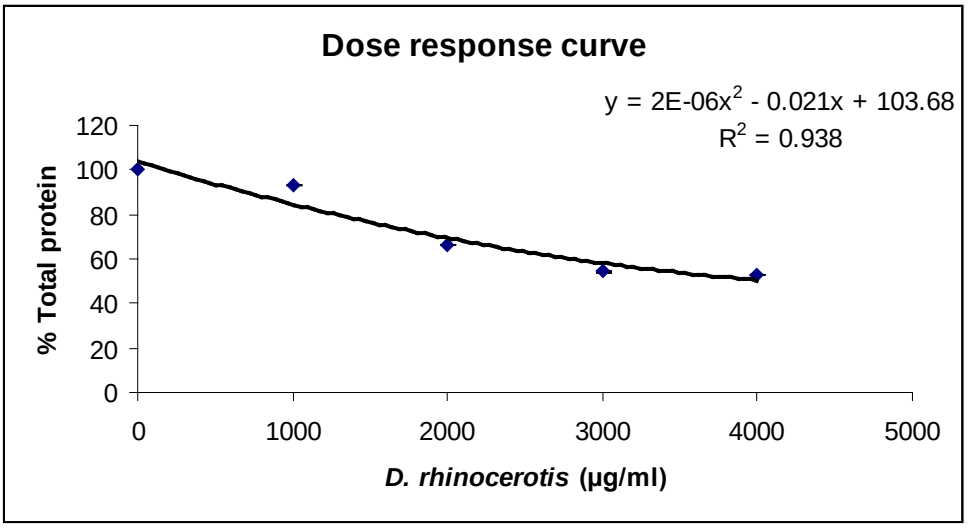

Figure 3.5 Bradford assay dose response curve after treatment of MCF-7 cells with *D. rhinocerotis*. Curve depicts a relationship between sample concentration and protein concentration.

Using figure 3.5 the IC_{50} and NOEL values obtained for *D. rhinocerotis* are approximately 4395 µg/ml and 144 µg/ml, respectively.

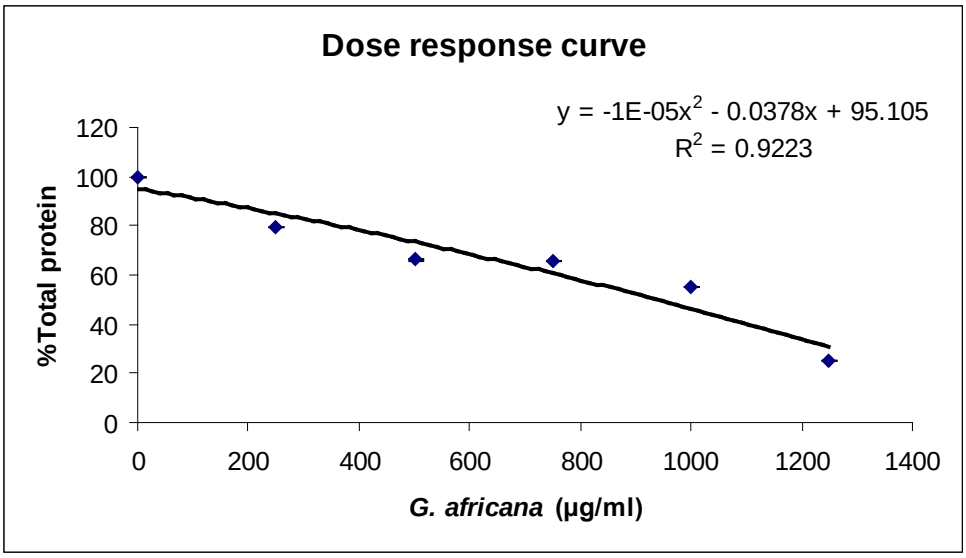

Figure 3.6 Bradford dose response curve after treatment of MCF-7 cells with *G. africana*. Curve depicts a relationship between sample concentration and protein concentration.

Using figure 6, the IC_{50} and NOEL values obtained for *G. africana* are approximately 926 µg/ml and 0 µg/ml, respectively.

Table 3.2 IC_{50} and NOEL values for *D. rhinocerotis* and *G. africana*, calculated from dose response curves 3.5 and 3.6.

	IC50 values (µg/ml)	**NOEL values (µg/ml)**
G. africana	926	0
D. rhinocerotis	4395	144

Cell proliferation and viability assessment via XTT assay

Dose response curves (figures 3.7 and 3.8) were plotted for the two plant extracts, from which IC_{50} and NOEL values were calculated. The NOEL values for the XTT assay are obtained when cell proliferation and viability is 100% and IC_{50} values are obtained when cell proliferation and viability is 50%. The results were expressed as the effects of the individual extracts vs. the control, untreated cells.

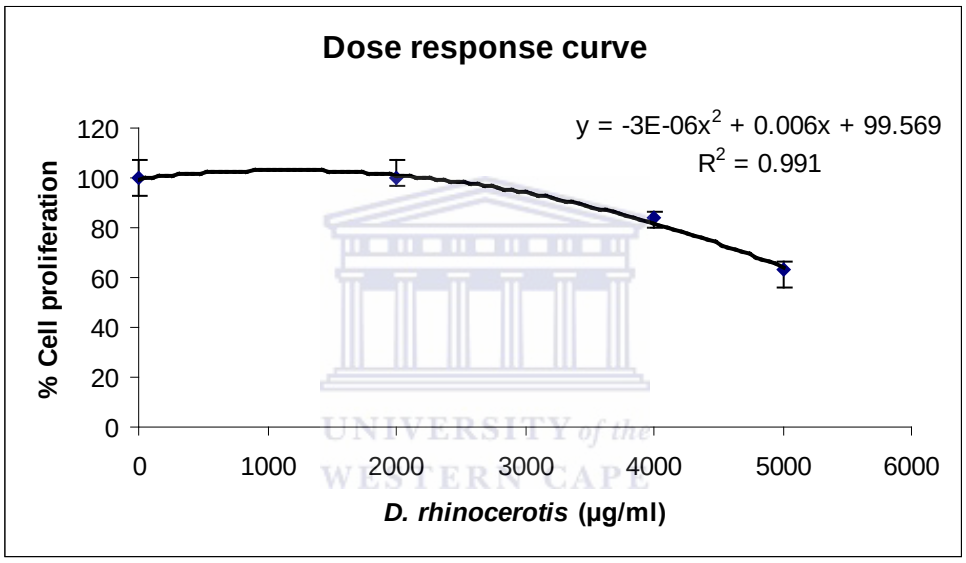

Figure 3.7 XTT dose response curve after treatment of MCF-7 cells with *D. rhinocerotis.* Curve shows a relationship between sample concentration and cell viability.

Using the dose response curve, figure 3.7, the IC_{50} and NOEL values obtained for *D. rhinocerotis* are approximately 5186 µg/ml and 132 µg/ml, respectively.

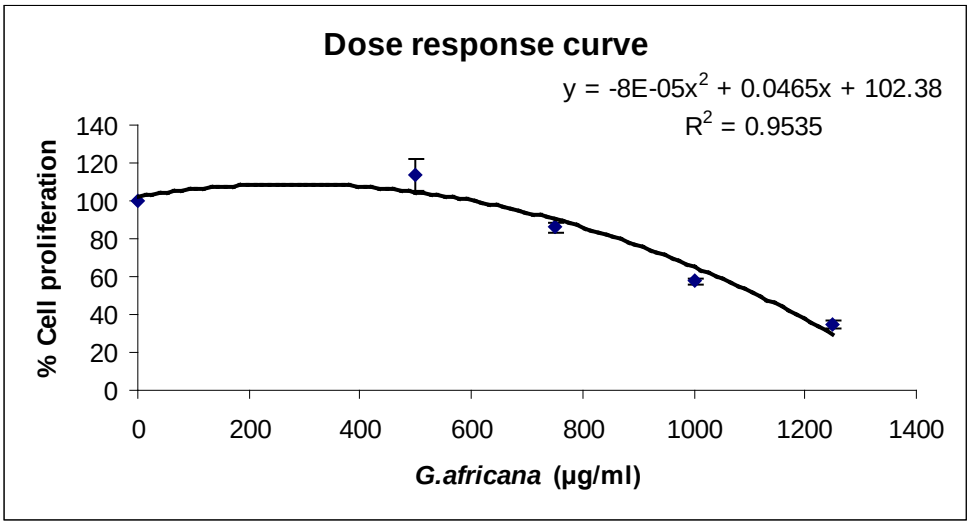

Figure 3.8 XTT assay dose response curves after treatment of MCF-7 cells with *G. africana*. Curve shows a relationship between sample concentration and cell viability.

From the dose response curve, figure 3.8, the IC_{50} and NOEL values obtained for *G. africana* are approximately 1085 µg/ml and 618 µg/ml, respectively.

Table 3.3 IC_{50} and NOEL values for *D. rhinocerotis* and *G. africana*, calculated from dose response curves 3.7 and 3.8.

	IC50 values (µg/ml)	**NOEL values (µg/ml)**
G. africana	1085	618
D. rhinocerotis	5186	132

Cytotoxicity assessment using cellular LDH leakage assay

Dose response curves were plotted for *D. rhinocerotis* and *G. africana.* IC_{50} and NOEL values were then calculated using the curves (figures 3.9 and 3.10). The NOEL values for the LDH assay, performed on the cell pellets, are obtained when the amount of LDH is 0% and IC_{50} values when LDH concentration is 50%. The results were expressed as the effects of the individual extracts vs. the control, untreated cells.

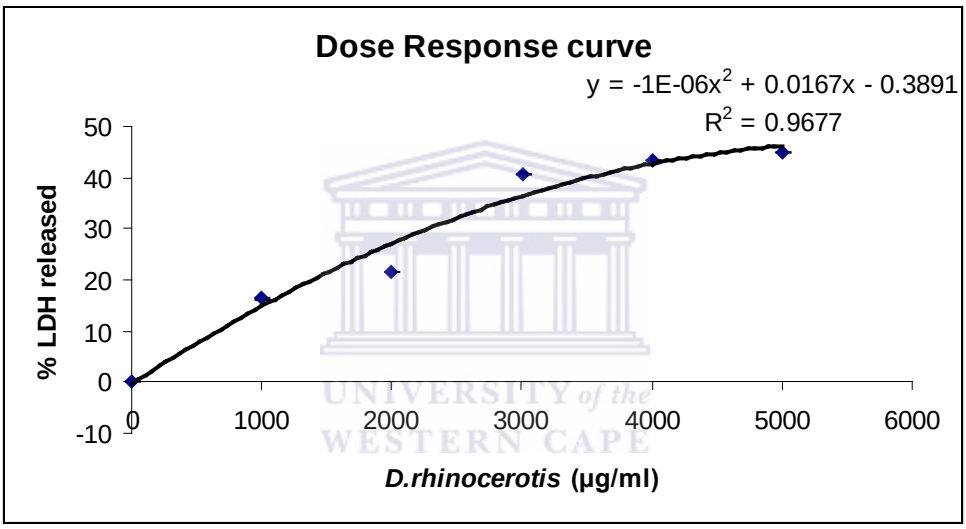

Figure 3.9 LDH dose response curve after treatment of MCF-7 cells with *D. rhinocerotis*. Curve depicts a relationship between sample concentration and amount of LDH.

From the dose response curve, figure 3.9, the IC_{50} and NOEL values obtained for *D. rhinocerotis* are approximately 5073 µg/ml and 49 µg/ml, respectively.

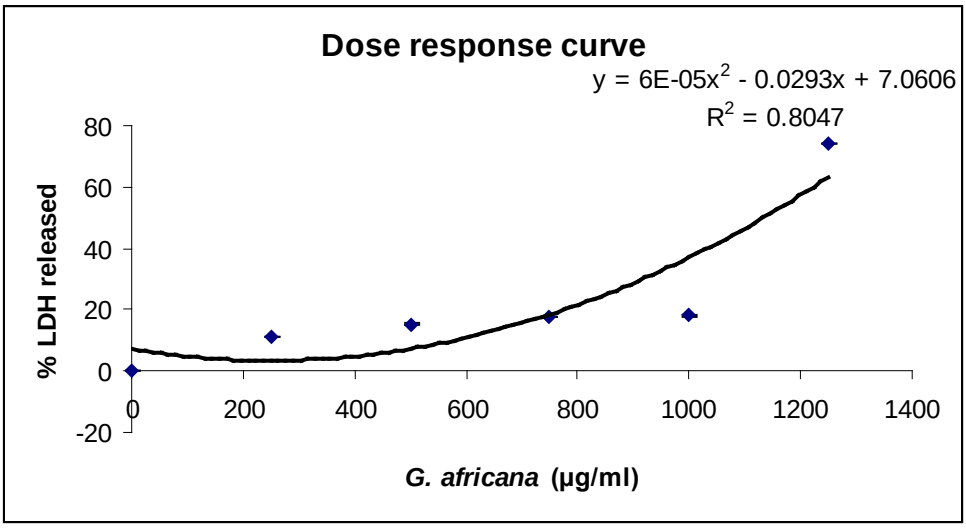

Figure 3.10 LDH dose response curve after treatment of MCF-7 cells with *G. africana*. Curve depicts a relationship between sample concentration and amount of LDH.

From the dose response curve, figure 3.10, the IC_{50} and NOEL values obtained for *G. africana* are approximately 1414 µg/ml and 0 µg/ml, respectively

Table 3.4 IC_{50} and NOEL values for *D. rhinocerotis* and *G. africana*, calculated from dose response curves 3.9 and 3.10.

	IC50 values (µg/ml)	**NOEL values (µg/ml)**
G. africana	1414	0
D. rhinocerotis	5073	49

3.5 Discussion and conclusion

A major consideration in approving pesticides for use is whether they pose an unreasonable risk to humans and wildlife. This study focused on cytotoxicity assessment of *D. rhinocerotis* and G. *africana* plant extracts on different human cell cultures. Cytotoxicity studies were carried out to establish a working concentration range, of the plant samples, for use on organ cultures. As a result fewer animals are used in trying to establish a non-cytotoxic working concentration range, therefore reducing costs and saving time.

The results obtained in this study show that *G. africana* had lower ($P<0.001$) IC_{50} values, for all assays performed, as compared to *D. rhinocerotis*. The lower the IC_{50} the more toxic the substance, therefore *G. africana* is more toxic than *D. rhinocerotis*. Different IC_{50} and NOEL values were obtained from this study. In a cytotoxicity study done using vero cells (mammalian kidney cell line) *G. africana* had an IC_{50} value of 118.2 µg/ml (Mativandlela *et al*., 2008). The different IC_{50} and NOEL concentration ranges may be attributed to the differing sensitivities of the different cell lines used.

G. africana and *D. rhinocerotis* extracts inhibit fungal growth at concentrations greater than 31.25 mg/ml and 125 mg/ml respectively (Knowles, 2005). These values are higher than the IC_{50} and NOEL values obtained in this current study. This therefore implies that if whole plant extracts are used as pesticides at concentrations in the range 31.25 mg/ml and 125 mg/ml they may result in adverse effects on human health, however more detailed research is required to validate finding.

D. rhinocerotis and G. africana extracts are more effective against the grey mould causing fungi, *B. cinerea*, when used in combination with synthetic fungicides (Vries, 2008). Complete inhibition of the fungi *B. cinerea* was observed for *D. rhinocerotis and G. africana* concentrations greater than at 0.95 mg/ml, when used in combination with kresoxim-methyl (a synthetic pesticide currently used on fungi) (Knowles, 2005; Vries, 2008). Cytotoxicity studies can therefore be carried out for the combination of kresoxim-methyl and the botanical extracts at these low concentrations.

The properties of whole plant extracts are a result of the several chemical compounds they contain (Lewinsohn and Gijzen, 2008). The active chemical compounds present in the plants are known as secondary metabolites (Lewinsohn and Gijzen, 2009). It may thus be useful to study the composition of the plant extracts to identify the compound(s) with pesticidal activity and then evaluate the individual active substances for cytotoxicity.

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Chapter 4

Environmental toxicity of *Dicerothamnus rhinocerotis* and *Galenia africana*

4.1 Abstract

Over the years the use of pesticides has greatly increased. This in turn has lead to concern about the adverse effects that the pesticides may have on non-target organisms in the environment. Due to increasing awareness there is great pressure to reduce the use of synthetic pesticides. An alternative to the use of synthetic pesticides is the exploitation of natural botanical products with pesticidal potential. *Dicerothamnus rhinocerotis* and *Galenia africana* are plants species, indigenous to South Africa, with fungicidal properties against a fungal pathogen that causes grey-mould rot on a wide range of agricultural produce. In this study a series of acute toxicity tests were conducted to establish the potential effects of *Dicerothamnus rhinocerotis* and *Galenia africana* on aquatic wildlife. The acute toxicities of the plants were determined using the species *Vibrio fischeri*, *Selenastrum capricornutum*, *Daphnia pulex*, and *Poecilia reticulata* as bio-indicators. Based on lethal concentration for a 50% effect (LC_{50}) and effective concentration for a 50% effect (EC_{50}), results obtained showed that *Galenia africana* had higher toxicity units than *Dicerothamnus rhinocerotis*, thus showing that *Galenia africana* is more toxic to the aquatic species, used in this study, as compared to *Dicerothamnus rhinocerotis*.

4.2 Introduction

Pesticides are used at a global scale for pest control thus allowing the maintenance of agricultural produce and contribute to economic growth and stability (Arias-Estevez *et al*., 2008). The wide use of pesticides has however become a pervasive threat to natural ecosystems and human health (Carbone *et al*., 2007; Caserta *et al*., 2007; Kodavanti *et al*., 2008). An emerging alternative to synthetic pesticides is the study and exploitation of naturally occurring products with pesticidal properties (Isman, 2006; Regnault-Roger *et al*., 2008). In order to evaluate the effects of new pesticides there is need for toxicological data on organisms representative of the various ecosystems.

Dicerothamnus rhinocerotis (L.f.) (=*Elytropappus rhinocerotis* (L.f.), popularly known as rhenoster bush ("renosterbos" in vernacular) or rhinoceros bush, is a bush shrub of about 1-2 meters in height, with small grayish-green leaves and tiny flower heads which are almost inconspicuous (Levyns, 1935). D. *rhinocerotis* is of the family Asteraceae and is native to Southern Africa (Van Wyk *et al*., 1997).

Galenia africana, commonly known in vernacular as kraalbos or geelbos, is a perennial shrub belonging to the family Aizoaceae (Van der Lugt *et al*, 1992). *G. africana* was most common in the Namaqualand region of South Africa but has recently become more widespread in the Western and Southern Karoo (Kellerman *et al*., 1988). The bush is a perennial aromatic, woody sub-shrub that grows to 0.5-1 m in height (Van der Lugt *et al*, 1992). Its green leaves are oppositely arranged with small yellow flowers at the end of the twigs (De Kock, 1928; Kellerman *et al*., 1988).

Extracts of D. *rhinocerotis* and *G. africana* exhibit antifungal properties against *Botrytis cinerea* (Knowels, 2005). *Botrytis cinerea* is a fungal pathogen that causes grey mould/ gray mold rot, on a large number of economically important agricultural and horticultural crops such as fruits, flowers and green tissue (Jarvis, 1997).

Due to the antifungal properties of the two plants they may have potential use on agricultural produce as fungicides. Ecotoxicity studies are required to determine safety of the plant extracts before they can be used commercially. In this study the acute toxicity of *D. rhinocerotis* and *G. africana* was determined by using the aquatic species *Vibrio fischeri, Selenastrum capricornutum, Daphnia pulex,* and *Poecilia reticulata* as bio-indicators. Toxicity of a substance usually is expressed as the effective concentration of the material that would produce a specified effect in 50% of a large population of a test species. Lethal concentration for a 50% effect (LC_{50}) and effective concentration for a 50% effect (EC_{50}) levels were obtained from this study and could be used to set maximum allowed environmental levels of these plant extracts.

4.3 Materials and methods

4.3.1 Plant extractions

Two medicinal plant species, *G. africana* and *D. rhinocerotis*, used in traditional medicine practices in South Africa were obtained and prepared for analysis. Extracts (20% (w/v)) of the two study plants were prepared in 94.4% ethanol by Parceval (Pty) Ltd Pharmaceuticals (South Africa). Leaves of the plants were milled (sieve size~ 2mm - 3mm) after which the leaves were mixed with 94.4% ethanol at 20 g leaves per 100 ml ethanol. The mixture was shaken for brief periods during overnight extraction. The extract was then pressed to separate the tincture and the milled leaves. Filtration was carried out to clean tincture from plant debris and the extracts were then stored at 20 ^{o}C. The extracts were then air dried and resuspended in DMSO to obtain 50% (w/v) extract. For this study the extracts were further diluted to 1mg/ml using deionised water, which was used as the highest concentration.

4.3.2 Test species

The test species used in this study are important components of many aquatic communities and therefore can be adversely affected by aquatic pollutants. Lyophilised *Vibrio fischeri* luminiscent bacteria (NRRL B-11177) and *Selenastrum capricornutum*, (Printz algae beads, CCAP 27814, Cambridge UK) were used in this study. 24 hour old *Daphnia pulex* (water flea) and *Poecilia reticulata* (guppy fish, also known as the million fish) at 7-21 day post-hatch were obtained from in-house cultures at Golder Associates Research laboratories and were also used for this study.

4.3.3 General test conditions

All toxicity tests were conducted in appropriate environmentally controlled rooms using standard techniques. The tests were conducted at Golder Associates Research Laboratories.

4.3.4 *Vibrio fischeri* bioluminescent test

The methods employed for the toxicity testing of *G. africana* and *D. rhinocerotis* on *Vibrio fischeri* followed those outlined by the International Organisation for Standardisation (ISO) 11348-3, 1998. Test chambers used were polystyrene cuvettes for Luminoskan TL luminometer (Hygiene Monitoring System). *Vibrio fischeri* were exposed at dilutions of plant extracts for 15 and 30 minutes. Test endpoints included percentage growth inhibition relative to control and EC_{50} values. Two replicates were carried out for each of the samples and statistical analysis was done using Bio Orbit software.

4.3.5 *Selenastrum capricornutum* growth inhibition test

The methods carried out for the toxicity testing of *G. africana* and *D. rhinocerotis* on *Selenastrum capricornutum* followed the Organisation for Economic Cooperation and Development (OECD) guideline 201, 1984. Test chambers used were 10 cm path length long cells. Exposure period was 72 hours. Test endpoints included percentage growth inhibition relative to control and EC_{50} values. Two replicates were carried out for each of the samples and statistical analysis was done using regression analyses.

4.3.6 *Daphnia pulex* acute toxicity test

The methods used for the toxicity screening of *G. africana* and *D. rhinocerotis* on Daphnia *pulex* followed those outlined by the United States Environmental Protection Agency (US EPA),1993 (600/4-90/027F). Test chambers used were 50 ml disposable polystyrene cups. Exposure periods were 24 and 48 hours. Test endpoints included percentage mortality and LC_{50} values. Four replicates, each with five test organisms per chamber, were used to test each sample. Statistical analysis was carried out using probit software/TSK.

4.3.7 *Poecilia reticulata* acute toxicity test

The methods employed for the toxicity testing of *G. africana* and *D. rhinocerotis* on *Poecilia reticulata* was according to that outlined by the US EPA, 1996 (712-C-96-118). Test chambers used were 250 ml disposable polystyrene cups. Exposure period was 96hours. Test endpoints included percentage mortality and LC_{50} values. Two replicates, each with five test organisms per chamber, were used to test each sample. Statistical analysis was carried out using probit software/TSK.

4.4 Results

The term EC_{50} (effective concentration for a 50% effect) refers to the concentration of a drug which induces a response halfway between the baseline and maximum. It is often used as a measure of the potency of a substance, therefore the lower the EC_{50} value the more potent the substance. In this study the EC_{50} values for the two plant extracts were obtained using *Selenastrum capricornutum* and *Vibrio fischeri*. The table below (Table 4.1) thus shows the concentrations of the two plant extracts where 50% of the population exhibits a response.

Table 4.1 EC_{50} values obtained for *D. rhinocerotis* and *G. africana* when using *Selenastrum capricornutum* and *Vibrio fischeri*

Test	**Exposure period**	**EC_{50} values for *D. rhinocerotis* (µg/ml)**	**EC_{50} values for *G. africana* (µg/ml)**
Vibrio fischeri bioluminiscent test	15 minutes	110	1
Vibrio fischeri bioluminiscent test	30 minutes	100	0.7
Selenastrum capricornutum test	72 hours	390	100

Lethal concentration for a 50%effect (LC_{50}) is the concentration of a substance which kills 50% of test animals exposed in a given time. In this study the LC_{50} values for the two plant extracts were obtained using *Poecilia reticulata* and *Daphnia pulex*. The table below (Table 4.2) thus shows the concentrations of the two plant extracts which kills 50% of the population of test animals.

Table 4.2 LC_{50} values obtained for *D. rhinocerotis* and *G. africana* when using *Poecilia reticulata* and *Daphnia pulex*

Test	**Exposure period**	**LC_{50} values for *D. rhinocerotis* (µg/ml)**	**LC_{50} values for *G. africana* (µg/ml)**
Daphnia pulex acute toxicity test	24 hours	340	40
Daphnia pulex acute toxicity test	48 hours	240	30
Poecilia reticulata test	96 hours	210	20

The results in table 4.1 and 4.2 show that *D. rhinocerotis* has higher EC_{50} and LC_{50} values as compared to *G. africana*, thus showing that *G. africana* is more toxic to the aquatic species as compared to *D. rhinocerotis*.

From the above results toxicity units were then calculated for each of the plant extracts. For each test performed the toxicity unit was calculated as 100% (full strength effluent expressed as percentage) divided by the EC_{50} or LC_{50} values. Toxicity units were used as a measure of acute toxicity, table 4.3.

Table 4.3 Toxicity units and the measure of toxicity (Classification scheme obtained from Golder Associates Research laboratories, South Africa.)

Toxicity Unit	**Conclusion**
<1	Limited to Not Acutely Toxic
1-2	Negligibly Acute Toxic
2-10	Mildly Acute Toxic
10-100	Acutely Toxic
>100	Highly Acutely Toxic

The toxicity units obtained when the different species were exposed to *D. rhinocerotis* and *G. africana* are shown in table 4.4 below.

Table 4.4 Toxicity units obtained for *D. rhinocerotis and G. africana*

Test species	**Toxicity units for *D. rhinocerotis***	**Toxicity units for *G. Africana***
Vibrio fischeri	10	1429
Selenastrum capricornutum	2.6	10
Daphnia pulex	4.2	33
Poecilia reticulata	5	50

G. africana had higher toxicity units than *D. rhinocerotis*, thus showing that *G. africana* is more toxic to the aquatic species as compared to *D. rhinocerotis*. *D. rhinocerotis* is mildly acutely toxic to all the species used in this study. On the other hand *G. africana* is mildly toxic to *Selenastrum capricornutum*, acutely toxic to *Daphnia pulex* and *Poecilia reticulata*, and highly acutely toxic to *Vibrio fischeri*.

4.5 Discussion and Conclusion

A major consideration in approving pesticides for use is whether they pose an unreasonable risk to humans and wildlife. In order to evaluate environmentally safe levels of chemicals, there is need for a set of toxicological data on organisms representative of the various ecosystems. Such data is often unavailable or inadequate. The aim of this study was to determine the ecological risks associated with using extracts of *D. rhinocerotis* and *G. africana*. A set of toxicological data for organisms representative of the aquatic environment (bacteria, algae, insect and fish) was obtained.

The study showed that *D. rhinocerotis* has higher EC_{50} and LC_{50} values compared to *G. africana*. From the toxicity units calculated *D. rhinocerotis* showed mild acute toxicity to all the species used in this study while *G. africana* ranged from mildly toxic to highly acutely toxic. The set of toxicological data on organisms found in the aquatic environment (bacteria, algae, insect and fish) obtained in this study therefore shows that *G. africana* is more toxic to the aquatic species used as compared to *D. rhinocerotis*. Several commercially available plant based pesticides have been shown to present with aquatic toxicity (Stark, 2001; Mondal *et al*., 2007). Examples are the neem based pesticides Neemix, Nimbecidine and NeemGold. *Daphnia pulex* has an LC_{50} value of 0.68 ppm (0.68 µg/ml) when exposed to Neemix; while the fresh water loach *Lepidocephalichthys guntea* has LC_{50} values of 0.0135 mg/l and 0.0527 mg/l when exposed to Nimbecidine and Neem gold (Stark, 2001; Mondal *et al*., 2007).

Galenia africana and *D. rhinocerotis* extracts showed inhibition of fungal growth at concentrations greater than 31.25 mg/ml and 125 mg/ml respectively (Knowles, 2005). This showed that the two plant extracts are effective as fungicides. From the results obtained in this current study, it can be concluded that at the concentrations 31.25 mg/ml (*G. africana*) and 125 mg/ml (*D. rhinocerotis*), the extracts may be toxic to the aquatic environment.

In this current study both *G. africana* and *D. rhinocerotis* extracts showed effectiveness against the bacteria *Vibrio fischeri*. Further studies may be conducted with different bacteria to ascertain if the plant extracts may be used as disinfectants.

Studies have shown that *D. rhinocerotis and G. africana* extracts are more effective against the grey mould causing fungi, *B. cinerea*, when used in combination with synthetic fungicides (Vries, 2008). Complete inhibition of the fungi *B. cinerea* was observed for *D. rhinocerotis and G. africana* concentrations greater than at 0.95 mg/ml, when used in combination with kresoxim-methyl (a synthetic pesticide currently used on fungi) (Knowles, 2005 and Vries, 2008). Ecotoxicity studies should therefore be carried out for the combination of kresoxim-methyl and the botanical extracts at these low concentrations.

Once a pesticide is introduced into the environment it is influenced by many processes which determine its ultimate fate. Pesticides thus not only affect aquatic environments, but other environments are also affected. Further studies on other environments are

therefore necessary for risk assessment of the plant extracts if they are to be used as pesticides.

4.6 References

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Chapter 5

The immunotoxicity of *Dicerothamnus rhinocerotis* and *Galenia africana*

5.1 Abstract

Increased use of pesticides has resulted in increased concern about the adverse effects on non-target organisms, including humans. Due to increasing awareness there is great pressure to reduce the use of synthetic pesticides. An alternative to the use of synthetic pesticides is the exploitation of natural botanical products with pesticidal potential. *Dicerothamnus rhinocerotis* and *Galenia africana* exhibit antifungal properties against *Botrytis cinerea*. This paper describes the immunotoxicity of extracts of *Dicerothamnus rhinocerotis* and *Galenia africana* on mouse spleenocytes. Spleen cell cultures were prepared and exposed to varying concentrations of *Dicerothamnus rhinocerotis* and *Galenia africana*. Control cultures were exposed to the DMSO vehicle only. Results obtained showed that both *Dicerothamnus rhinocerotis* and *Galenia africana* may result in immunotoxic effects due to the decrease in concentrations of cytokines interlukin-4 (IL-4) and interferon-gamma (IFN-γ). The cytokine inhibition was concentration dependent.

5.2 Introduction

A pesticide is defined as any substance(s) used for destroying or mitigating unwanted pests. Unwanted pests include species of plants or animals that cause harm during food production, processing, storage, transport and/ or marketing (Clementi *et al*., 2008). The use of pesticides has increased, over recent years due to greater demand for good quality food products. Increased use of pesticides has resulted in increased concern about the adverse effects on non-target organisms, including humans.

Due to increasing awareness there is great pressure to reduce the use of synthetic pesticides. An alternative to the use of synthetic pesticides is the exploitation of natural botanical products with pesticidal properties. *Dicerothamnus rhinocerotis* (=*Elytropappus rhinocerotis*) (*D. rhinocerotis*) and *Galenia africana* (*G. africana*) exhibit antifungal properties against *Botrytis cinerea* (Knowles, 2005). *Botrytis cinerea* is a fungal pathogen that causes grey mould rot, on a large number of economically important agricultural and horticultural crops (Jarvis, 1997).

D. rhinocerotis, popularly known as rhenoster bush ("renosterbos" in vernacular) or rhinoceros bush, is a bush shrub with small grayish-green leaves and tiny flower heads (Levyns, 1935). Infusions of the young branches prepared in brandy or wine are a traditional Cape (South Africa) medicine for indigestion, dyspepsia, ulcers and stomach cancer (Watt and Breyer- Brandwijk, 1962). It is also said to be taken as tonic to improve lack of appetite, for colic and wind diarrhoea (Cillie', 1992).

G. africana is a plant that is indigenous to the Namaqualand region of South Africa (Kellerman *et al.*, 1988). The Hottentots, an indigenous tribe, chewed the plant to relieve toothache. The plant was also used in the treatment of venereal diseases and prepared as a decoction for skin diseases and for the relief of inflammation of the eyes (Watt and Breyer-Brandwijk, 1962). An ointment, made by frying the herb was used as a dressing for wounds, especially wounds on the legs of women (Watt and Breyer-Brandwijk, 1962).

Due to the antifungal properties of the two plants they may have potential use on agricultural produce as fungicides. Exposure of pesticides to the immune system may result in alteration of the normal immune functions. This kind of activity is known as immunotoxicity (Colossio *et al*., 1999). Toxicity studies are required to determine safety of the plant extracts. Toxins can have a general effect on all cells or they can attack specific organ/physiological systems. One of the physiological systems prone to attack by toxins is the immune system (Colossio *et al*., 1999).

The immune system is a well-regulated organ system that involves interrelated reactions, which protect an organism from invasion by foreign substances (Ladics, 2007). The immune system fights pathogens using two methods, namely the innate immunity and the acquired/adaptive immunity (Seely *et al*., 2000; Storni *et al*., 2005). Due to its complexity, the immune system is a target organ for various toxic substances, including pesticides, therefore resulting in multiple potential target sites and pathological effects (Colosio *et al*., 1999).

Immunotoxicity results in toxicant-induced injury to part of the immune system thus affecting immune functions and may result in immunostimulation, immunosuppression, hypersensitivity and autoimmunity (Descotes, 2004; Van Wijk *et al*., 2006). Each of these categories is associated with potential adverse effects associated with significant morbidity. Immunotoxicity is thus an important aspect of the safety evaluation of drugs, chemicals and foods.

The aim of this study was to assess the toxicity of *D. rhinocerotis* and *G. africana* for the acquired immune pathway using *in vitro* assays. Acquired immunity is regulated by cytokines secreted by the Th1 and Th2 lymphocytes.

T-helper (Th) lymphocytes are divided into Th1 and Th2 subsets according to cytokine production profile which also correlate with their function (Mosmann *et al*., 1986). Th1 cells produce the cytokines interleukin (IL)-2 and Interferon (IFN)-γ which aid with defenses against intracellular pathogens and promote cell mediated immunity, whereas Th2 produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 which are responsible for defenses against extracellular pathogens and promote humoral immunity (Mosmann and Sad, 1996). Th1 and Th2 cytokines are mutually inhibitory for the functions of the reciprocal phenotype (Storni *et al*., 2005).

IFN-γ is a cytokine predominantly produced by Th1 cells (Mims *et al*., 2004; Seely *et al*., 2000; Storni *et al*., 2005). IFN-γ aids with defenses against intracellular pathogens and promotes cell mediated immunity ((Mims *et al*., 2004; Seely *et al*., 2000; Storni *et al*.,

2005). IFN-γ also antagonizes IL-4 leading to inhibition of Th2 cell proliferation (Storni *et al*., 2005).

IL-4 is a pleiotropic cytokine that is mainly produced by Th2 cells, basophiles and mast cells (Mims *et al*., 2004; Storni *et al*., 2005; Parker *et al*., 2006). IL-4 stimulates proliferation and differentiation of B-cells, thus inducing antibody production (Storni *et al*., 2005). IL-4 plays a key role in the induction of IgE isotype switching in B-cells (Snapper *et al*., 1987). IgE is involved in immediate allergic reactions mediated by IgE-dependant mast cells. Dysregulation of IL-4 thus results in development of uncontrolled allergic inflammation (Snapper *et al*., 1987). IL-4 also results in inhibition of Th1 cell proliferation (Fukao *et al*., 2000; Storni *et al*., 2005).

5.3 Materials and methods

5.3.1 Reagents

All reagents, solvents and biomolecules used in this study were purchased from Roche (South Africa), Merck (Germany) or Sigma Chemical Company (St Louis, USA) unless stated otherwise. All other reagents were of analytical grade.

5.3.2 Plant extractions

Two medicinal plant species, *G. africana* and *D. rhinocerotis*, used in traditional medicine practices in South Africa were obtained and prepared for analysis. Extracts (20% (w/v)) were prepared in 94.4% ethanol by Parceval (Pty) Ltd Pharmaceuticals (South Africa). Leaves of the plants were milled (sieve size~ 2mm-3mm) after which the leaves were mixed with 94.4% ethanol at 20 g leaves per 100 ml ethanol. The mixture was shaken for brief periods during the overnight extraction. The extracts were then pressed, to separate the tincture and the milled leaves. Filtration was carried out to clean tincture from plant debris and the extract was stored at 20 ^{o}C. For study purposes the extracts were air dried. The dried extracts were resuspended in DMSO to obtain 50% (w/v) (500 mg/ml) extract.

5.3.3 Spleen cell cultures

Male BALB/c mice were purchased from the University of Cape Town animal facility (South Africa). They were sacrificed via cervical dislocation (ethical clearance obtained) which the spleens were dissected out. The spleens were then disrupted via passage through a sterile steel mesh. Spleen cells were suspended in full medium, which consisted

of RPMI 1640 supplemented with 1% antibiotic/mycotic mix (Sigma, USA) and 1% serum replacement medium. The spleen cell suspension was washed and centrifuged at 1000 Xg for 10 minutes, after which the cell pellets were resuspended in fresh medium to a concentration of 2.5 x 10^6 cells/ml.

Assays to screen toxicity of the plant extracts were conducted in 96 well culture plates (Nunc, Denmark). A serial dilution range of the extracts, in DMSO, were applied to wells of the plate (2 µl/well). Control wells contained the DMSO vehicle only. Six replicates of each concentration were prepared. One set of wells (three replicates of each extract concentration) then received 200 µl of the cell suspension (unstimulated cultures). The other set of replicates received 200 µl of cell suspension to which 16 µg/ml phytohaemmagglutinin (PHA) from *Phaseolus vulgaris* (Sigma, USA) was added. The content of the wells were mixed by tapping the side of the plate, after which the plate was incubated for 48 hours in an incubator at 37 °C flushed with 5% CO_2.

After the 48 hour culture period the supernatants were harvested and cytokine concentrations in the culture medium determined. IFN-γ and IL-4 were the cytokines assessed. This was done using commercially available ELISA kits in accordance with the manufacturer's instructions (eBioscience, USA).

5.3.4 Cytokine ELISAs

Nunc 96 well microtiter ELISA plates (Serving Life Science, Denmark) were used for all ELISA protocols. Cytokine (IL-4 and IFN-γ) concentrations were determined using ELISAs. The ELISAs were carried out according to the manufacturer's instructions. The ELISA plates were coated with 100 µl/well of capturing antibody (purified anti-mouse IL-4 or IFN-γ) diluted appropriately in coating buffer and incubated overnight at 37 ^{o}C. The plates were washed 5 times with wash buffer (autoclaved PBS, 0.05% Tween-20 and distilled water), after which the non-specific binding sites were blocked with assay diluent. Cell free supernatants were then added to the plate. The assay was standardized using 2-fold serial dilutions of recombinant mouse IL-4 (500 pg/ml) and IFN-γ (2000 pg/ml) respectively. The plates were then sealed and incubated for 2 hours at room temperature. After 5 washings, with wash buffer, 100µl of detection antibody (Biotin-conjugate anti-mouse IL-4 and IFN-γ) was added to each well and the plate was incubated for 1hour at room temperature. The plate was again washed as before, after which Avidin- Horseredish peroxidase (HRP) (100 µl) was added to all wells in order to detect the bound cytokine. This was incubated for 30 minutes after which the plate was washed 7times. Substrate solution (1X TMB) was then added to every well (100 µl/well) and incubated for approximately 15 minutes. The reaction was then stopped with 50 ul/well of stop solution. Absorbances were read on a plate reader (Thermo electron corporation, SA) at 450 nm.

5.3.5 Statistical analysis

All experiments were performed three times in triplicate and data were compared using the statistical program SigmaStat (Systat Software, Inc., Point Richmond, CA). One-way ANOVA ($P<0.001$) was conducted to test for significant interaction between study plant extracts and cells. Multiple comparisons were performed using Dunnett's test to compare against the control group.

5.4 Results

5.4.1 Determination of IL-4 concentration

From the IL-4 ELISA performed a standard curve (figure 5.1) was plotted using the optical density readings obtained for the serial-dilution of the standard. The IL-4 concentration of the two plant extracts was then calculated using the standard curve.

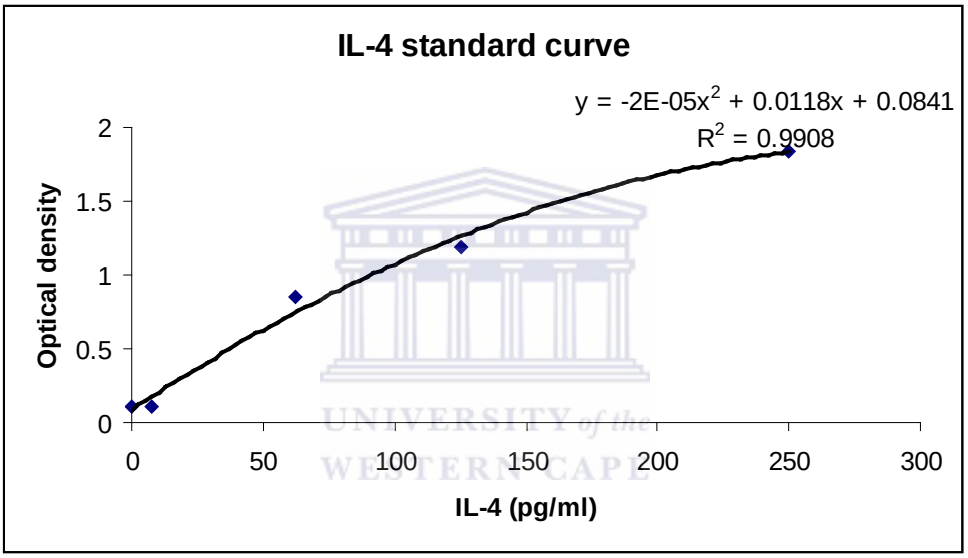

Figure 5.1 Standard curve plotted for IL-4 ELISA. Curve shows relationship between IL-4 concentration and optical density.

The curve above, figure.5.1, shows a good correlation between IL-4 concentration and optical density (R^2=0.9908). The IL-4 concentration of the spleen-cell cultures after incubation with various *D. rhinocerotis* and *G. africana* dilutions were extrapolated using the standard curve above. The results are expressed as the effects of the individual extracts vs. the control, untreated cells.

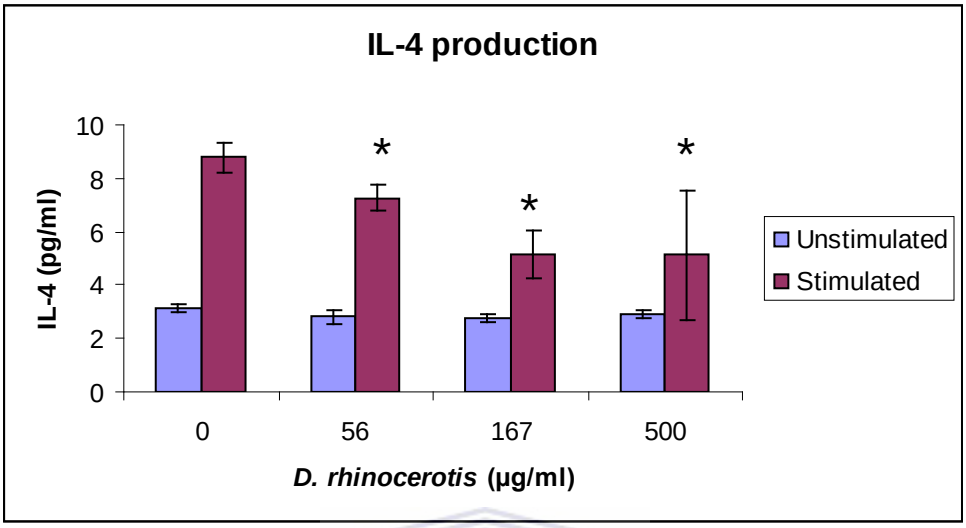

Figure 5.2 IL-4 produced by spleenocytes in the presence of *D. rhinocerotis* (***** significantly different from zero control).

The results calculated from the standard curve (figure.5.1) showed that *D. rhinocerotis* had no significant effect on IL-4 production under unstimulated conditions (figure.5.2). However under stimulated conditions *D. rhinocerotis*, at concentrations above 56 µg/ml, significantly ($P<0.001$) decreases IL-4 production (figure.5.2).

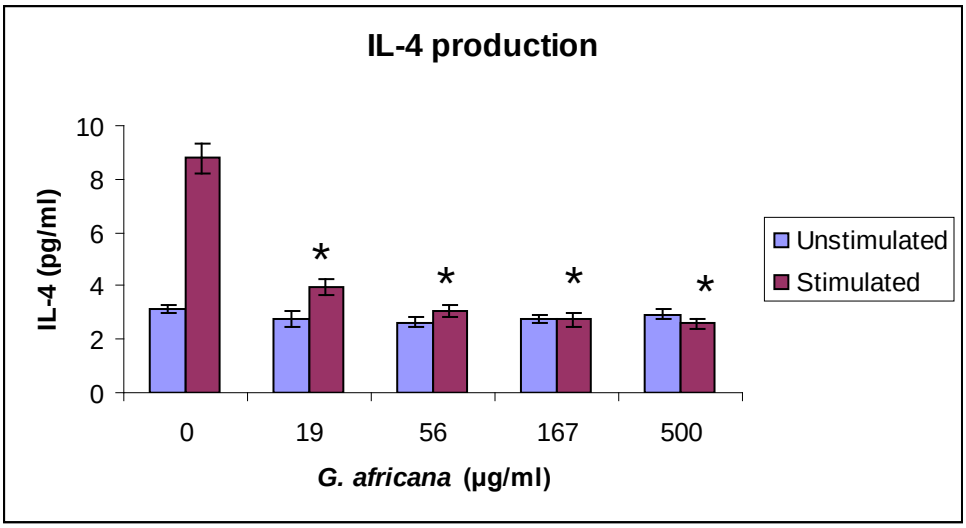

Figure 5.3 IL-4 produced by spleenocytes in the presence of *G. africana*

(***** significantly different from zero control).

G. africana, at concentrations above 19 µg/ml, significantly (P<0.001) decreases IL-4 production under stimulated conditions. Under unstimulated conditions, there is no change in production of IL-4 by the spleenocytes.

5.4.2 Determination of IFN-γ concentration

From the IFN-γ ELISA performed a standard curve (figure 5.4) was plotted from the optical density readings obtained for the serial-dilution of the standard. The IFN-γ concentration of the two plant extracts was then calculated using the standard curve.

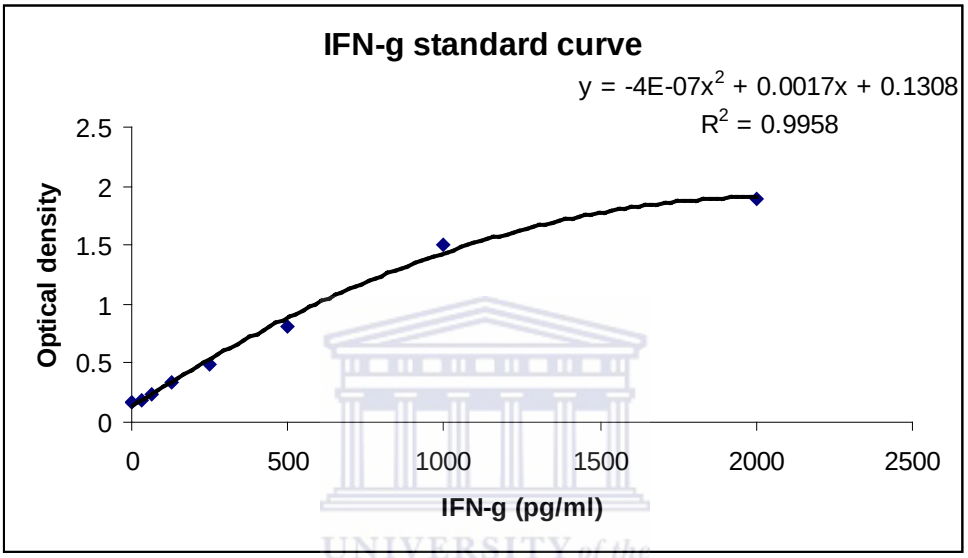

Figure 5.4 Standard curve plotted for IFN-γ ELISA. Curve shows relationship between IFN-γ concentration and optical density.

The curve above, figure.5.4, shows a good correlation between IFN-γ concentration and optical density (R^2=0.9958). The IFN-γ concentration of the spleen-cell cultures after incubation with various *G. africana* and *D. rhinocerotis* dilutions were extrapolated using the standard curve above. The results are expressed as the effects of the individual extracts vs. the control, untreated cells.

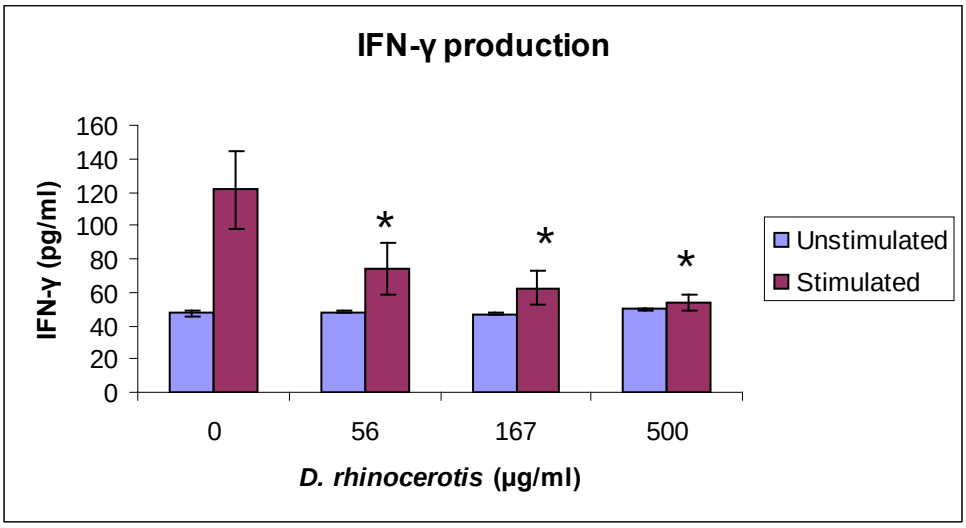

Figure 5.5 IFN-γ produced by spleenocytes in the presence of *D. rhinocerotis* (***** significantly different from zero control).

The results calculated from the standard curve (figure.5.4) showed that *D. rhinocerotis* significantly ($P<0.001$) decreased IFN-γ production, at all concentrations, under stimulated conditions (figure.5.5). However under unstimulated conditions *D. rhinocerotis* had no significant effect on IFN-γ production (figure.5.5).

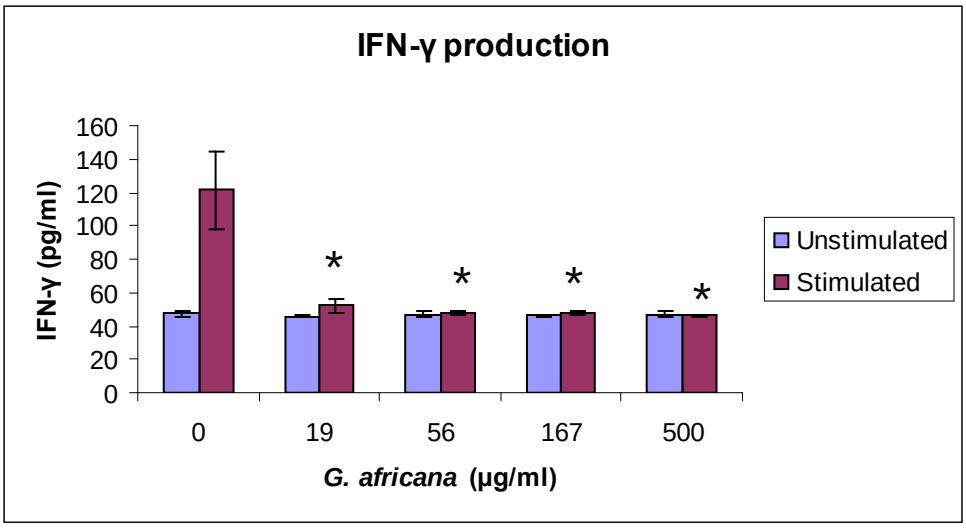

Figure 5.6 IFN-γ produced by spleenocytes in the presence of *G. africana* (***** significantly different from zero control).

G. africana significantly decreased IFN-γ production, at all concentrations, under stimulated conditions (figure.5.6). However under unstimulated conditions *G. africana* had no significant effect on IFN-γ production (figure.5.6).

The results in figures 5.1 to figure.5.6 are summarised in the table below.

Table 5.1 The effect of *D. rhinocerotis* and *G. africana* on cytokine production by spleenocytes stimulated with PHA

	Unstimulated cells	**Stimulated cells**
D. rhinocerotis	No effect on IL-4	Decreased IL-4
	No effect on IFN-γ	Decreased IFN-γ
G. africana	No effect on IL-4	Decreased IL-4
	No effect on IFN-γ	Decreased IFN-γ

5.5 Discussion and Conclusion

Exposure to pesticides may have various effects on the immune system, ranging from slight modulation of immune functions to development of clinical immune diseases (Colosio *et al*., 1999). For example exposure to the organophosphorous compound pentachlorophenol results in decreased lymphocyte proliferative responses to mitogens (Colosio, 1993). It is therefore a necessity to evaluate the immunotoxic effects of pesticides before putting them to use.

The results in this study showed that *D. rhinocerotis* had no significant effect on IL-4 production under unstimulated conditions. However, under stimulated conditions *D. rhinocerotis* decreases IL-4 production. IL-4 is a cytokine that is mainly produced by Th2 cells (Storni *et* al., 2005). This therefore implies that exposure to *D. rhinocerotis* may result in impairment of the Th2 response. Th2 cells are required to mount effective humoral and cell-mediated responses that are required to fight extracellular microbes and parasites. An impairment of these responses may thus result in increased susceptibility to extracellular microbes (O'Garra *et al*., 2000).

This study also shows that *D. rhinocerotis* significantly decreased IFN-γ production. IFN-γ is a cytokine that is predominantly produced by the Th1 cells (Storni *et al*., 2005). Th1 cells are required to mount an effective cell-mediated response that is required to fight intracellular pathogens, viruses and cancers. An impairment of these responses may thus result in increased susceptibility to intracellular pathogens, viruses and cancers (Storni *et al*., 2005, Seely *et al*., 2000).

Exposure to *G. africana* results in lowered IL-4 levels. This implies that exposure to *G. africana* may result in impairment of the Th2 response, which in turn may lead to increased susceptibility to extracellular microbes (O'Garra *et al*., 2000).

G. africana also decreased IFN-γ production. IFN-γ is a cytokine that is predominantly produced by the Th1 cells (Storni *et al*., 2005). Exposure to *G. africana* may therefore result in impairment of the Th1 response, which may in turn lead to increased susceptibility of intracellular pathogens. Previous studies, done by us, showed that *D. rhinocerotis* and *G. africana* are not cytotoxic at these low concentrations, indicating that specific immune functions are impaired at concentrations that do not necessarily cause cell death.

The properties of whole plant extracts can either be from a single chemical compound or from a combination of compounds. The active chemical compounds present in the plants are known as secondary metabolites (Lewinsohn and Gijzen, 2008). It may thus be useful to study the composition of the plant extracts to identify the compound(s) with pesticidal activity. The compound(s) may then be isolated be and evaluated for use as pesticides.

Studies have shown that *D. rhinocerotis and G. africana* extracts are more effective against the grey mould causing fungi, *B. cinerea*, when used in combination with synthetic fungicides (Vries, 2008). The study plants, *D. rhinocerotis and G. africana*, completely inhibited growth of *B. cinerea* (a fungal pathogen) at concentrations greater than at 0.95mg/ml, when used in combination with kresoxim-methyl (a synthetic pesticide currently used on fungi) (Knowles, 2005 and Vries, 2008). Immunotoxicity

studies should therefore be carried out for the combination of kresoxim-methyl and the botanical extracts at these low concentrations.

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Chapter 6

The hepatotoxixity of *Dicerothamnus rhinocerotis* and *Galenia africana*

6.1 Abstract

Pesticides are used at a global scale for agricultural produce to control pests that adversely affect the quality and quantity of products. It is well known that use of these substances may result in injury to non-target organisms, including humans. Botanicals have been in use for a long time for pest control, therefore may be used as an alternative to synthetic pesticides. When considering pesticides for commercial use, it is imperative to know their effect on non-target organisms. *Dicerothamnus rhinocerotis* and *Galenia africana*, two plants native to South Africa, exhibit antifungal activity against fungal pathogens that lead to spoilage of agricultural produce. This study focused on the hepatotoxicity testing of *Dicerothamnus rhinocerotis* and *Galenia africana* extracts using liver cell cultures. Mouse liver cell cultures were treated with varying concentrations of the plant extracts after which hepatotoxicity was determined. Hepatotoxicity was measured using several biomarkers including lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and aspartate aminotransferase (AST). Results obtained showed that *Dicerothamnus rhinocerotis* and *Galenia africana* may have toxic effects on the liver.

6.2 Introduction

Substances used to prevent or destroy pests are known as pesticides (Arias-Estervez *et al*., 2008). Over the years resistance to pesticides by target species, and adverse effects on non-target organisms by pesticides has greatly increased (Carbone *et al*., 2007; Caserta *et al*., 2007; Kodavanti *et al*., 2008; Zhu *et al*., 1996). This has resulted in pressure to reduce the use of synthetic pesticides (Isman, 2006).

An emerging alternative to synthetic pesticides is the exploitation of naturally occurring products with pesticidal properties (Isman, 2006; Regnault-Roger *et al*., 2008; Stewart, 2008). Botanicals are attractive alternatives to synthetic pesticides because they are said to pose little threat to human health and to the environment (Isman, 2006). The use of plant derivatives as pesticides dates back at least two millennia in ancient China, Egypt, Greece and India (Isman, 2006 and Regnault-Roger *et al*., 2008). In spite of the scale of scientific data documenting the activity of plant products on pests, only a handful of these products are currently being used commercially on agricultural products because of increasingly stringent regulatory requirements (Isman, 2006). Some of these include neem oil, essential oils, pyrethrum and rotenone (Isman, 2006).

Galenia africana (*G. africana*), commonly known in vernacular as “kraalbos” or “geelbos”, is a perennial shrub belonging to the family Aizoaceae and is native to South Africa (Kellerman *et al*., 1988; Van der Lugt *et al*., 1992). Historically the plant has been used medicinally to relieve toothache, treatment of venereal diseases and skin diseases and for the relief of inflammation of the eyes (Watt and Breyer-Brandwijk, 1962).

During severe drought and in poor-conditions animals are forced to graze *G. africana*. Ingestion of the plant is associated with liver damage and severe ascites, a condition referred to as 'waterpens' or 'water belly', in sheep and goats (De Kock, 1928; Kellerman *et al*., 1988). The marked liver lesions have lead researchers to believe that the plant is hepatotoxic due to the presence of a toxin (Van der Lugt, 1992).

Dicerothamnus rhinocerotis (L.f.) (=*Elytropappus rhinocerotis* (L.f.)), commonly known in vernacular as "renosterbos" is a shrub belonging to the Daisy family (Asteraceae) and is native to Southern Africa (Van Wyk *et al*., 1997). *Dicerothamnus rhinocerotis* (*D. rhinocerotis* is an erect grey-green shrub that grows up to 2m tall (Levyns, 1935).

Extracts of *G. africana and D. rhinocerotis* exhibit antifungal activity against several fungi, including *Botrytis cinerea* (Knowles, 2005; Vries 2008). *Botrytis cinerea* is a fungal pathogen that causes grey mould rot on a large number of economically important agricultural and horticultural crops (Jarvis, 1997).

Due to the antifungal properties of the two plants they may have potential use on agricultural produce as fungicides. Toxicity studies are required to determine safety of the plant extracts. The toxicity of a substance is its capacity to cause injury to a cell or living system. Therefore the aim of this study was to determine injury to the liver as a result of exposure to *G. africana* and *D. rhinocerotis*.

The liver is a complex organ that plays major roles in synthetic, metabolic and excretory functions (Giannini *et al*., 2005; Field *et al*., 2008). As a result of direct contact with toxicants, there is an increased likelihood of hepatic injury or hepatotoxicity (Ncibi *et al*., 2008). Toxicants, such as pesticides, should be studied and ways to protect the liver from adverse effects of toxins should be investigated.

Injury to the liver/ hepatotoxicity may be investigated and monitored in standard toxicity studies by a range of tests (Ncibi *et al*., 2008). Several parameters, including the liver's relative weight and serum biochemical tests (enzymes, proteins, lipids) may be used (Field *et al*., 2008; Ncibi *et al*., 2008). However, these conventional liver tests measure only a small proportion of the organ's many functions (Field *et al*., 2008). In this study hepatotoxicity was measured using several enzymes biomarkers, including lactate dehydrogenase, aspartate aminotransferase and alkaline phosphatase.

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme present in all cells. When the cell membrane is intact, LDH is contained within the cytoplasm. However upon cell damage/injury, the cell membrane loses its integrity and LDH leaches to the extracellular compartment through holes in the membrane. The amount of LDH released is therefore proportional to the extent of damage caused to the cells. The enzyme activity assay is based on the conversion of lactate to pyruvate in the presence of LDH, with parallel reduction of NAD (Decker and Lohmann-Matthes, 1988; Sepp *et al*., 1996).

Aspartate aminotransferase/ serum glutamate oxaloacetate transaminase (AST/ SGOT), is an enzyme that catalyses the reversible transfer of the α-amino groups from aspartate to the α-keto group of ketoglutaric acid to generate oxalacetic acid, shown below (Giannini *et al*., 2005; Field *et al*., 2008).

The principle of the assay used in this study is based on the principle of the reaction shown above. Oxalacetate reacts with a diazonium salt thus producing a colour complex that is measured photometically. Therefore, the AST enzyme activity is proportional to the absorbance reading.

L-Aspartate + 2-Oxoglutarate ⟶ Oxalacetate + L-Glutamate

Alkaline phosphatase (ALP) is an enzyme found in the liver that catalyses the hydrolysis of phosphate ethers and is also responsible for the transportation of metabolites across cell membranes (Giannini *et al*., 2005; Field *et al*., 2008). Hepatic ALP is found on the bile duct epithelia and cholestasis results in increased ALP synthesis and release (Schlaeger *et al*., 1982; Moss., 1997; Field *et al*., 2008). Alkaline phosphatase is an enzyme that is determined by measuring the rate of hydrolysis of phosphate esters. The assay used in this study involved the hydrolysis of p-nirophenly phosphate (p-Npp); shown below.

p-Npp + H_2O ⟶ p-Nitrophenol + H_3PO_4

p-Npp is colourless but p-Nitrophenol has a strong absorbance at 405 nm, thus absorbance readings at 405 nm are proportional to enzyme activity.

6.3 Materials and Methods

6.3.1 Reagents

All reagents, solvents and biomolecules used in this study were purchased from either Roche (South Africa), Merck (Germany) or Sigma Chemical Company (St Louis, USA) unless stated otherwise. All reagents were of analytical grade.

6.3.2 Plant extractions

Galenia africana and *D. rhinocerotis* were obtained from Namaqualand, South Africa, in April 2008 and prepared for analysis. Extracts (20% (w/v)) were prepared in 94.4% ethanol by Parceval (Pty) Ltd Pharmaceuticals (South Africa). Leaves of the plants were milled (sieve size~ 2mm - 3mm) after which the leaves were mixed with 94.4% ethanol at 20 g leaves per 100 ml ethanol. The mixture was shaken for brief periods during an overnight extraction. The extracts were then pressed, to separate the tincture and the milled leaves. Filtration was carried out to clean the tincture from plant debris and the extract was stored at 20 ^{o}C. For study purposes the extracts were air dried and resuspended in DMSO to obtain 50% (w/v) (500 mg/ml) extract.

6.3.3 Liver cell cultures

Male BALB/c mice were purchased from the University of Cape Town animal facility (South Africa). They were sacrificed via cervical dislocation after which the livers were dissected out and immediately placed into full medium (RPMI 1640 supplemented with 1% antibiotic/mycotic mix (Sigma, USA) and 10% fetal bovine serum). For consistency the piece of liver used weighed between 0.4 g and 0.6 g.

The livers were then disrupted via passage through a sterile steel mesh. Liver cells were suspended in full medium and the cell suspension was washed and centrifuged at 3000 Xg for 3 minutes. The cell suspension was washed four times after which the cell pellets were resuspended in fresh medium.

A serial dilution range of the plant extracts, in DMSO, were applied to wells of 96 well cell culture plates (Nunc, Denmark). Negative control wells contained the DMSO (1% final non-toxic volume) vehicle only and the positive control cells contained 1% triton X100 detergent. Three replicates of each concentration were prepared. The liver cell suspension was added to the wells containing the samples. The final sample to cell ratio was 1:100 (v/v). The plates were incubated overnight in an incubator at 37°C flushed with 5% CO_2. After incubation the medium was harvested and cell damage was monitored by measuring several enzymes including LDH, AST and ALP in the culture medium.

6.3.4 Determination of LDH release

Cytotoxicity induced by the two plant samples was assessed by LDH leakage into the culture medium. Supernatants were harvested and release of intracellular LDH was determined using the chromogenic Cytotoxicity Detection Kit (LDH) (Roche Applied Science, Penzberg, Germany).

The LDH assay was carried out according to the manufactures instruction. Briefly, a 10 µl aliquot of the spent culture medium was mixed with warm LDH-reaction mixture (provided in the kit). The mixture was incubated for 30 minutes, at room temperature. Absorbances at 492 nm were recorded at time-zero and after 30 minutes using plate reader (Thermo electron corporation, SA), after which cytotoxicity was calculated.

6.3.5 Determination of AST and ALP release

Hepatotoxicity induced by the two plant samples was assessed by measuring AST, ALT and ALP in the culture medium. AST, ALT and ALP were assayed using commercially available diagnostic kits (BioQuant, San Diego, CA). All enzyme assays were carried out in 96-well microtitre plates (Serving Life Science, Denmark).

ALP assay

10 µl aliquots of the harvested culture medium were added to the wells of microtitre plates. Warm (room temperature) working reagent, prepared according to manufactures

instructions, was added to the plates. The plates were read immediately at 405 nm using a plate reader (Thermo electron corporation, SA).

AST assay

AST substrate was added to the wells of microtitre plates and incubated at 37 °C for 5 minutes. 20 µl aliquots of the spent culture medium were then added to the plates. The assay was standardized using 2-fold serial dilutions of lyophilized serum with AST (20 µl/well). The plates were then incubated for a further 10 minutes, after which AST color reagent (0.25% w/v diazonium salt) was added. After 10 minutes the reaction was stopped with 0.1N HCL. Absorbances were read at 510 nm and the results were used to calculate AST concentration.

6.3.6 Statistical analysis

All experiments were performed three times in triplicate and data were compared using the statistical program SigmaStat (Systat Software, Inc., Point Richmond, CA). One-way ANOVA ($P<0.001$) was conducted to test for significant interaction between study plant extracts and cells. Multiple comparisons were performed using Dunnett's test to compare against the control group.

6.4 Results

6.4.1 Determination of LDH concentration

The LDH assay performed did not have a calibrator; therefore no standard curve was plotted. The percentage LDH in the supernatant of the liver cell cultures after incubation with *D. rhinocerotis* and *G. africana* were calculated as percentages with respect to the control, figure 6.1. The results are expressed as the effects of the individual extracts vs. the control (untreated cells).

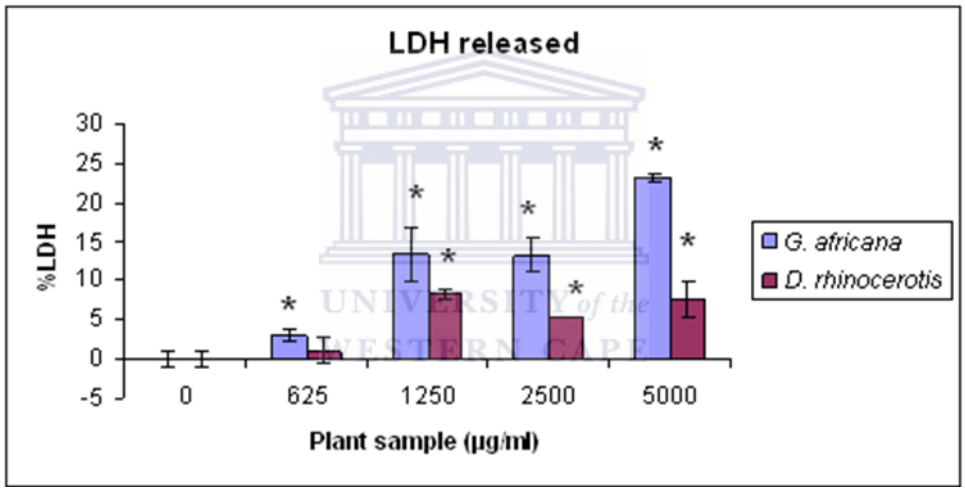

Figure 6.1 LDH released by liver cells in the presence of *D. rhinocerotis* and *G. africana* (***** significantly different from zero control).

The results showed that there is a significant increase in LDH concentration in the presence of *D. rhinocerotis* and *G. africana*, at concentrations above 625 µg/µl and 500 µg/µl, respectively, when compared with the zero control (Figure 6.1).

6.4.2 Determination of AST concentration

From the AST assay performed, a standard curve, figure 6.2, was plotted using the optical density readings obtained for the serial-dilution of the standard. The AST concentration of the liver cell cultures after incubation with *D. rhinocerotis* and *G. africana* were extrapolated using the standard curve and are shown in figure 6.3. The results are expressed as the effects of the individual extracts vs. the control, untreated cells.

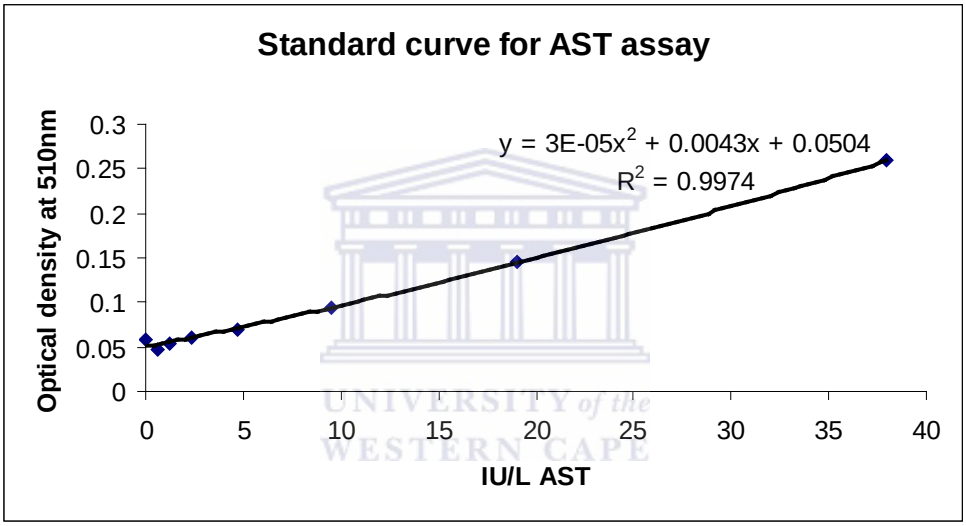

Figure 6.2 Standard curve plotted for the AST assay using liver cells. The curve shows the relationship between AST concentration of the standard and the optical density.

The curve above, figure 6.2, shows a polynominal relationship between AST concentration and optical density (R^2=0.9974). The amount of AST present in the cell cultures after incubation with *G. africana* and *D. rhinocerotis* was calculated using the standard curve.

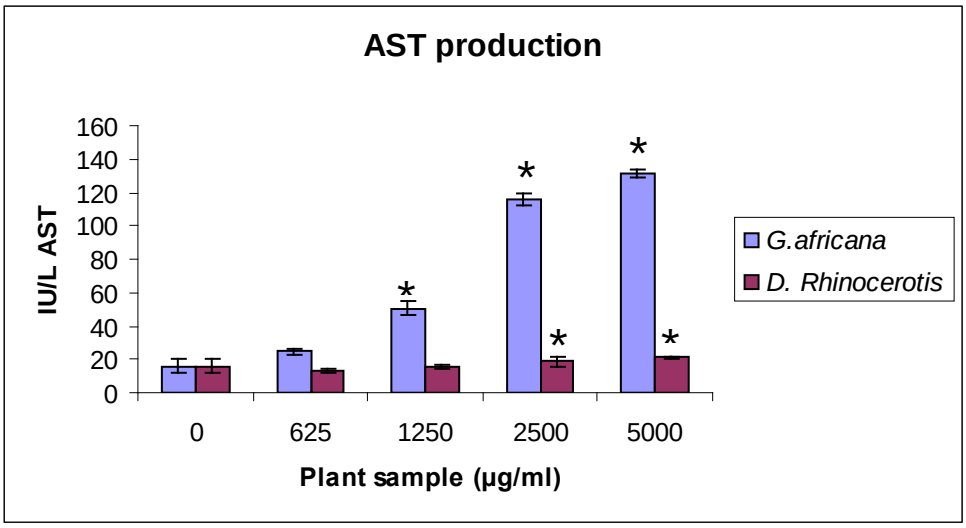

Figure 6.3 AST released by liver cells in the presence of *D. rhinocerotis* and *G. africana* (* significantly different from zero control).

The results showed that there is a significant ($P<0.001$) increase in AST concentration in the presence of *D. rhinocerotis* and *G. africana*, at concentrations above 2500 µg/ml and 1250 µg/ml, respectively, when compared with the zero control (Figure 6.3).

6.4.3 Determination of ALP concentration

The ALP assay performed did not have a calibrator; therefore no standard curve was plotted. The ALP concentration of the liver cell cultures, after incubation with *D. rhinocerotis* and *G. africana*, was expressed using the optical density readings obtained and are shown in figure 6.4. The results were expressed as the effects of the individual extracts vs. the control, untreated cells.

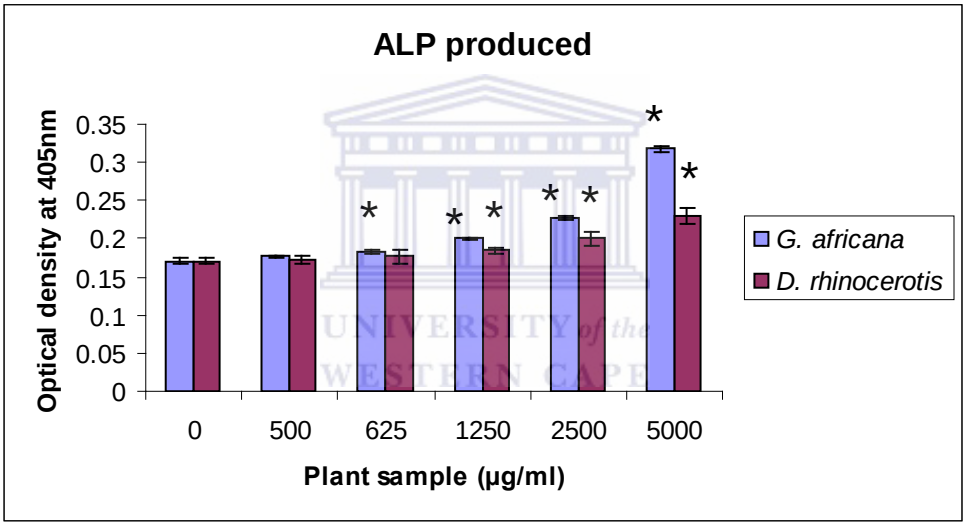

Figure 6.4 ALP produced by liver cells in the presence of *D. rhinocerotis* and *G. africana* (***** significantly different from zero control).

The results showed that *D. rhinocerotis* and *G. africana* at concentrations 1250 µg/ml and 625 µg/ml, respectively, and above, significantly ($P<0.001$) increase ALP concentration in the culture supernatants (Figure 6.4).

6.5 Discussion and conclusion

The complexity of the liver results in it being a target organ for various toxic substances that may lead to pathological effects (Ncibi *et al*., 2007). Exposure to pesticides may therefore have various effects on the liver. For example exposure to the organophosphorous insecticide diazinon affects the mitochondrial membrane transport in rat liver and it disturbs the cytochrome P450 system in human liver (Sams *et al*., 2000). It is therefore a necessity to evaluate the effects of potential pesticides on the liver before putting them to use.

Damage to the structural integrity of the liver is reflected by an increase in the hepatospecific enzymes ALP and AST from the liver. The results showed that *D. rhinocerotis* and *G. africana* at above 2500 µg/ml and 1250 µg/ml, respectively, increase AST concentrations in culture medium. AST levels are highly concentrated in the liver's hepatocytes and in the case of injury AST leaches from the cells thus resulting in a peak in AST concentration (Giannini *et al*., 2005). This therefore implies that exposure to *D. rhinocerotis* and *G. africana*, at the above concentrations, may result in injury to hepatocytes.

The study also showed that *D. rhinocerotis* and *G. africana* at above 1250 µg/ml and 625 µg/ml, respectively, increase ALP concentration in culture supernatant. Hepatic ALP is found on the bile duct epithelia and cholestasis results in increased ALP synthesis and release (Schlaeger *et al*., 1982; Moss., 1997; Field *et al*., 2008). This therefore implies

that exposure to *D. rhinocerotis* and *G. africana*, at the above concentrations, may result in cholestasis i.e. partial or complete blockage of the bile ducts.

In this study the increase of LDH levels in culture supernatants was used as an indicator of cell damage. Both *D. rhinocerotis* and *G. africana* show no cytotoxicity and hepatotoxicity at concentrations below 500 µg/ml. *D. rhinocerotis* and *G. africana* extracts showed inhibition of fungal growth at concentrations greater than 31.25 mg/ml and 125 mg/ml respectively (Knowles, 2005). These values are higher than the values obtained in this current study. Therefore if whole plant extracts of *D. rhinocerotis* and *G. africana* are used as pesticides they may result in injury to the liver. However, this study was carried out *in vitro*, meaning the samples were added directly to the cells. Effects of chemicals *in vivo* depend on the actual concentration that reaches the target organ. *In vivo* tests may thus be required to confirm effects of the actual concentration of the sample that reaches the liver.

The properties of a plant are dependent upon the presence of chemical compounds known as secondary metabolites (Lewinsohn and Gijzen, 2009). Therefore, the action of whole plant extracts can either be from a single chemical compound or from a combination of compounds. It may thus be useful to study the composition of *D. rhinocerotis* and *G. africana* to identify the compound(s) with pesticidal activity. The compound(s) may then be isolated and evaluated for use as pesticides.

Previous studies have shown that *D. rhinocerotis and G. africana* extracts are more effective against the grey mould causing fungi, *B. cinerea*, when used in combination with synthetic fungicides (Vries, 2008). Complete inhibition of *B. cinerea* was observed for *D. rhinocerotis and G. africana* at 0.95 mg/ml, when used in combination with kresoxim-methyl (a synthetic pesticide currently used on fungi) (Knowles, 2005 and Vries, 2008). Liver toxicity studies can therefore be carried out for the combination of kresoxim-methyl and the botanical extracts at these low concentrations.

Hepatotoxicity ranks as one of the most frequent causes of post-commercialisation regulatory decisions for withdrawing products from the market (Andrade *et al*., 2007). When considering pesticides for commercial use, it is imperative to know their effect on non-target organisms. It is therefore of utmost importance to manage the risks of xenobiotics, including all forms of pesticides, and to provide safety information of the substances. This study shows that *D. rhinocerotis* and G. *africana*, at concentrations used as pesticides, pose risk of hepatotoxicity *in vitro*. However it needs to be established if these concentrations will actually reach the liver under *in vivo* conditions, as the body is protected by several systems such as the skin and gut epithelium which may provide limited or no entry for the hepatotoxin in the extract.

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Chapter 7

The effects of *Dicerothamnus rhinocerotis* and *Galenia africana* on sterodogenesis in the testes

7.1 Abstract

Adverse effects of pesticides on non-target organisms first received widespread attention in the 1960s. Exposure to pesticides is one of the contributing causes of male reproductive toxicity. Botanical based products have been in use for a long time for pest control and may therefore serve as attractive alternatives to synthetic pesticides. The plants *Dicerothamnus rhinocerotis* and *Galenia africana* exhibit antifungal activity against fungal pathogens that lead to spoilage of agricultural produce. This study focused on investigating the effects of *Dicerothamnus rhinocerotis* and *Galenia africana* extracts on testosterone production. This was achieved by using mouse testes cell cultures, which were treated with varying concentrations of the plant extracts. Testosterone concentrations were measured and results obtained showed that *Dicerothamnus rhinocerotis* and *Galenia africana* decrease testosterone production.

7.2 Introduction

Pest control is an ongoing problem and over the years we have depended on the use of pesticides to eliminate unwanted pests. Adverse effects of pesticides to non-target organisms first received widespread attention in the 1960s and since then evidence of their effects has increased. As a result of increasing awareness, there is great pressure to reduce the use of synthetic pesticides (Isman, 2006).

Botanical products, with pesticidal properties, are attractive alternatives to synthetic pesticides because they are said to pose little threat to human health and to the environment (Isman, 2006; Regnault-Roger *et al*., 2008; Stewart and Stewart, 2008). In spite of the scale of scientific data documenting the activity of plant products on pests, only a handful of these products are currently being used commercially on agricultural products because of increasingly stringent regulatory requirements (Isman, 2006).

Galenia africana (*G. africana*) is a perennial shrub that is native to South Africa (Kellerman *et al*., 1988; Van der Lugt *et al*., 1992). It is used medicinally to relieve toothache, treatment of venereal diseases and skin diseases and for the relief of inflammation of the eyes (Watt *et al*., 1962).

Dicerothamnus rhinocerotis (L.f.) (=*Elytropappus rhinocerotis* (L.f)) is a shrub belonging to the Daisy family (Asteraceae) and is native to Southern Africa (Van Wyk *et al*., 1997). *D. rhinocerotis* is used as a traditional medicine for indigestion, dyspepsia, ulcers and stomach cancer (Watt *et al*., 1962). It is also used as a tonic to improve lack of

appetite and for colic, wind and diarrhea (Cillie', 1992). The medicinal properties of *D. rhinocerotis* are attributed to rhinocerotinoic acid, which has anti-inflammatory properties (Van Wyk *et al*., 1997; Dekker *et al*., 1988)

Extracts of *G. africana and D. rhinocerotis* exhibit antifungal activity against several fungi, including *Botrytis cinerea* (Knowles, 2005; Vries *et* al., 2005). *Botrytis cinerea* is a fungal pathogen that causes grey mould rot, on a large number of economically important agricultural and horticultural crops (Jarvis, 1997).

The male reproductive system consists of the testes, a series of ducts, accessory glands, and supporting structures including the scrotum and the penis, which work together for the purpose of reproduction (Seely *et al*., 2000). Reproductive toxicity refers to the adverse effects of a substance on any aspect of the reproductive cycle, which involves a broad range of targets and mechanisms such as direct effects on Leydig and Sertoli cells, spermatogenesis, sperm maturation as well as endocrine disruption (Sullivan, 1992; Mantovani *et al*., 2005). The aim of this study was to evaluate the reproductive toxicity of *D. rhinocerotis* and *G. africana* by investigating their direct effect on Leydig cells using testosterone as a biomarker.

Testosterone is a steroid hormone that is produced by the Leydig cells (Anderson *et* al., 2006; Eacker *et* al., 2008; Sikka *et al*., 2008). Testosterone synthesis is a highly regulated process that occurs upon stimulation by luteinizing hormone (LH). In response to LH, there is an increase in intracellular concentrations of 3′,5′-cyclic adenosine

monophosphate (cAMP) (Ascoli *et al*., 2002; Eacker *et al*., 2008). cAMP stimulates transport of cholesterol to the inner mitochondrial membrane, where it is metabolized to pregnenolone by the cytochrome P450 enzyme CYP11A (Midzak *et al*., 2008). Pregnenolone is then metabolized to testosterone by enzymes of the smooth endoplasmic reticulum (Midzak *et al*., 2008).

Due to the antifungal properties of the two plants they may have potential use on agricultural produce as fungicides. Toxicity studies are required to determine safety of the plant extracts. The toxicity of a substance is its capacity to cause injury to a cell or living system. Therefore the aim of this study was to investigate the male reproductive toxicity resulting from exposure to *D. rhinocerotis* and *G. africana*.

7.3 Materials and methods

7.3.1 Reagents

All reagents, solvents and biomolecules used in this study were purchased from Roche (South Africa), Merck (Germany) or Sigma Chemical Company (St Louis, USA) unless stated otherwise. All other reagents were of analytical grade.

7.3.2 Plant extractions

Two medicinal plant species, *G. africana* and *D. rhinocerotis*, used in traditional medicine practices in South Africa were obtained and prepared for analysis. Extracts (20% (w/v)) were prepared in 94.4% ethanol by Parceval (Pty) Ltd Pharmaceuticals (South Africa). Leaves of the plants were milled (sieve size~ 2mm -3mm) after which the leaves were mixed with 94.4% ethanol at 20 g leaves per 100 ml ethanol. The mixture was shaken for brief periods during the overnight extraction. The extracts were then pressed, to separate the tincture and the milled leaves. Filtration was carried out to clean the tincture from plant debris and the extract was stored at 20 ^{o}C. For study purposes the extracts were air dried. The dried extracts were resuspended in DMSO to obtain 50% (w/v) (500 mg/ml) extract.

7.3.3 Testes cell cultures

Male BALB/c mice were purchased from the University of Cape Town animal facility (South Africa). They were sacrificed by cervical dislocation (ethical clearance obtained) after which the testes were dissected out. The testes were then finely minced with a scissors. Testicular cells were suspended in medium, which consisted of modified RPMI supplemented with 1% antibiotic/mycotic mix (Sigma, USA) and 1% (v/v) glutamax (Sigma, USA) and 0.2% bovine serum albumin. The cell suspension was incubated in an incubator flushed with 5% CO_2 at 37 °C for one hour. The cell suspension was centrifuged at 1000 Xg for 10 minutes, after which the cell pellet was resuspended in fresh medium and incubated for a further thirty minutes. The cell suspension was centrifuged again at 1000xg for 10min, and the cell pellets resuspended in fresh medium to a concentration of 1 x 10^6 cells/ml.

Assays to screen toxicity of the plant extracts were conducted in 96-well culture plates (Nunc, Denmark). A serial dilution range of the extracts, in DMSO, were applied to wells of the plate (2µl/well). Control wells contained the DMSO vehicle only. Six replicates of each concentration were prepared. One set of wells (three replicates at each extract concentration) then received 200 µl of the cell suspension (unstimulated cultures). The other set of replicates received 200 µl of cell suspension to which 100 mU/ml of luteinizing hormone (LH) (Sigma, USA) was added. The content of the wells were mixed by tapping the side of the plate, after which the plate was incubated for four hours in an incubator flushed with 5% CO_2 at 37 °C. After the 4 hour culture period, cell free

supernatants were harvested and lactate dehyrogenase (LDH) and testosterone concentrations in the culture medium determined.

7.3.4 Determination of LDH release

Cytotoxicity induced by the two plant samples was assessed by LDH leakage into the culture medium. Supernatants were harvested and release of intracellular LDH was determined using the chromogenic Cytotoxicity Detection Kit (LDH) (Roche Applied Science, Penzberg, Germany).

The LDH assay was carried out according to the manufactures instruction. Briefly, a 10 µl aliquot of the supernatant was mixed with warm LDH-reaction mixture (provided in the kit). The mixture was incubated for 30 minutes, at room temperature. Absorbances at 492 nm were recorded at time-zero and after 30 minutes using an ELISA reader (Thermo electron corporation, SA), after which cytotoxicity was calculated.

7.3.5 Testosterone ELISA

The amount of testosterone produced by the Leydig cells was measured using a commercially available ELISA kit (DRG diagnostics, Germany). The ELISA was carried out according to the manufacturer's instructions. The ELISA was carried out using precoated 96-well microtitre plates, which were provided in the kit. The plates were coated with mouse monoclonal anti-testosterone antibody. The supernatants, harvested from the cell culture, were added to the plate (25 µl/well). Testosterone conjugated to

horse radish peroxidise (200 µl/well) was also added (the testosterone in the sample supernatants compete with the testosterone-enzyme conjugate for binding to the coated antibody). The assay was standardized using different dilutions of testosterone, provided in the kit. The plates were incubated for 1 hour at room temperature. After incubation the plates were washed 3 times, thus washing off the unbound conjugate, with wash buffer (provided in the kit). Substrate solution (1X tetramethylbenzidine (TMB)) was then added to every well (200 µl/well) and incubated for approximately 15 minutes at room temperature. The reaction was then stopped with 100 µl/well of stop solution (0.5M sulphuric acid). Absorbances were read on a plate reader (Thermo electron corporation, SA) at 450 nm.

7.3.6 Statistical analysis

All experiments were performed three times in triplicate and data were compared using the statistical program SigmaStat (Systat Software, Inc., Point Richmond, CA). One-way ANOVA ($P<0.001$) was conducted to test for significant interaction between study plant extracts and cells. Multiple comparisons were performed using Dunnett's test to compare against the control group.

7.4 Results

7.4.1 Determination of LDH concentration

The LDH assay performed did not have a calibrator; therefore no standard curve was plotted. The percentage LDH in the supernatant of the liver cell cultures after incubation with *D. rhinocerotis* and *G. africana* were calculated as percentages with respect to total cellular LDH, figure7.1. The results are expressed as the effects of the individual extracts vs. the control.

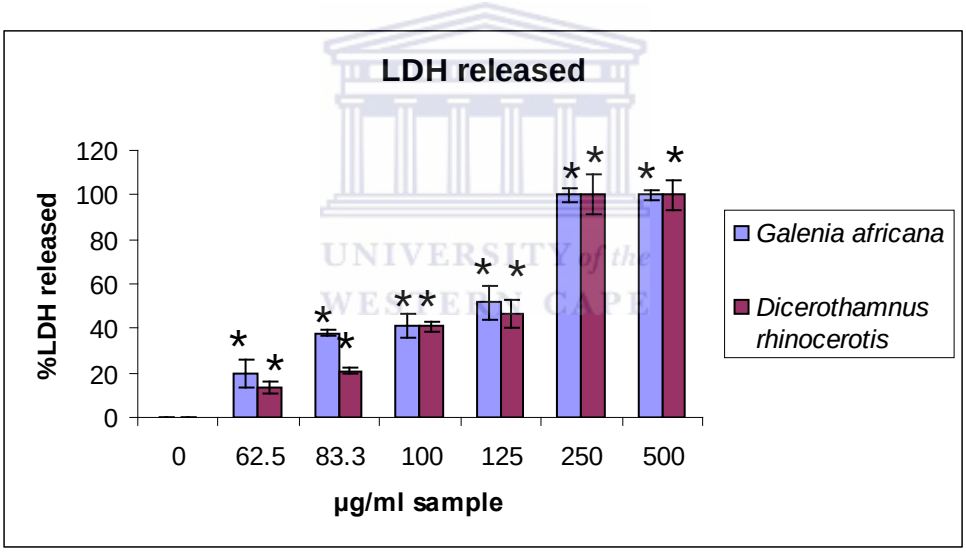

Figure 7.1 LDH released by cells in the presence of *D. rhinocerotis* and *G. africana* (***** significantly different from zero control).

The results showed that there is a significant ($P<0.001$) increase in LDH concentration in the presence of *D. rhinocerotis* and *G. africana* at all concentrations used, when compared with the zero control (Figure 7.1).

7.4.2 Determination of testosterone concentration

From the testosterone ELISA performed a standard curve (figure 7.2) was plotted using the optical density readings obtained for the serial-dilution of the standard. The testosterone concentration cultures treated with the two plant extracts was then calculated using the standard curve.

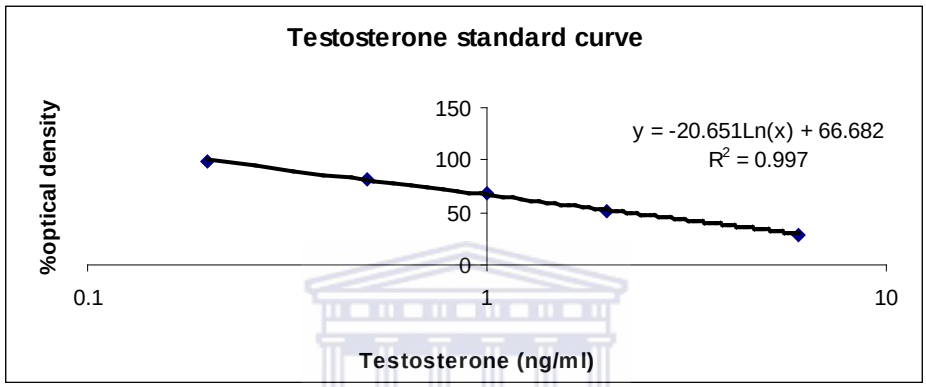

Figure 7.2 Standard curve plotted for testosterone ELISA. Curve shows relationship between testosterone concentration optical densities.

The curve above, figure 7.2, shows a good correlation between testosterone concentration and optical density (R^2=0.997). The testosterone concentration of the cell cultures after incubation with various *D. rhinocerotis* and *G. africana* dilutions were extrapolated using the standard curve above. The results are expressed as the effects of the individual extracts vs. the control, (shown in figures 7.3 and 7.4).

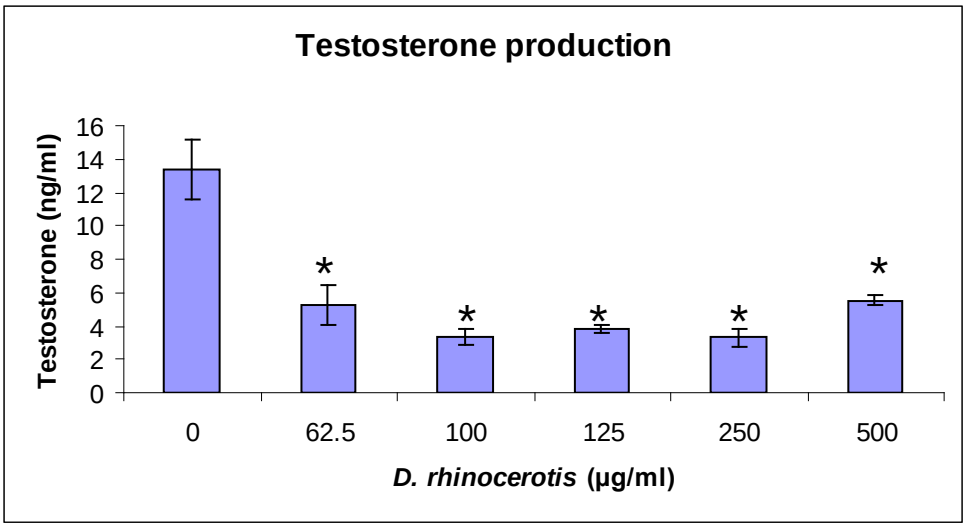

Figure 7.3 Testosterone produced, by cells stimulated with LH, in the presence of *D. rhinocerotis* (***** significantly different from zero control).

The results calculated from the standard curve (figure 7.2) showed that *D. rhinocerotis* significantly ($P<0.001$) decreases testosterone production, at all concentrations used in this study, under stimulated conditions (figure 7.3).

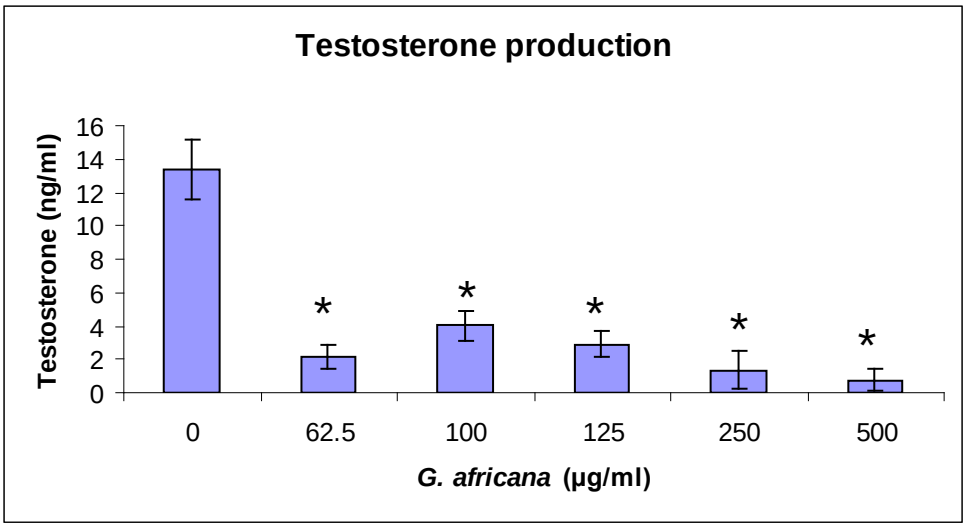

Figure 7.4 Testosterone produced, by cells stimulated with LH, in the presence of *G. africana* (***** significantly different from zero control).

The results calculated from the standard curve (figure7.2) showed that *G. africana* significantly ($p<0.001$) decreases testosterone production at all concentrations used in the study, under stimulated conditions (figure 7.4).

7.5 Discussion and conclusion

Animal studies performed using pesticides such as dicarboximide fungicides and chlorinated insecticides have shown that these chemicals alter the androgen-oestrogen balance, which can lead to alterations in normal embryonic programming and sexual development (Srinivasa *et al*., 2005; Sikka *et al*., 2008). The alterations are all symptoms of the testicular dysgenesis syndrome (TDS) and include hypospadias (incomplete fusion of the urethral folds that form the penis), cryptorchidism (undescended testis), testicular cancer and low sperm count and quality (Fry *et al*., 1981; Subramanian *et al*., 1987; Facemire *et al*., 1995; Guillette *et al*., 1996; Gray *et al*., 2001). Therefore when considering pesticides for commercial use, it is important to know their effect on non-target organisms.

In this study, male reproductive toxicity is reflected by a decrease in the levels of the hormone testosterone. The results showed that both *D. rhinocerotis* and *G. africana*, at 62.5μg/ml and above, decrease testosterone synthesis by cell cultures. This therefore implies that *in vitro* exposure to *D. rhinocerotis* and *G. africana*, at concentrations used in this, may result in male reproductive toxicity. Commercially available plant based pesticides have been shown to present with reproductive toxicity. An example is Neem oil, derived from the traditional plant *Azadirachta indica* (Upadhyay *et al*., 1993). When male rats were exposed to the neem oil they presented with reduced testicular size and blocked spermatogenesis without affecting testosterone production (Upadhyay *et al*., 1993). This study was done *in vitro*, meaning the samples were added directly to the testicular cells, therefore is more sensitive than *in vivo* tests. Effects of a product, *in vivo*,

depend on absorption of toxins into blood stream and the eventual concentration reaching the target organ. Organs also have their own barrier systems that may also affect the final concentration reaching the cells within the organ. *In vitro* tests therefore require *in vivo* tests to confirm effects of the actual concentration of sample that reaches the target organ.

In this study the increase of LDH levels was considered as an indicator of cell damage as a result of exposure to the botanical products. Both *D. rhinocerotis* and *G. africana* show cytotoxicity at all concentrations used in this study.

The properties of a plant are dependent upon the presence of chemical compounds known as secondary metabolites (Lewinsohn and Gijzen, 2009). Therefore, the action of whole plant extracts can either be from a single chemical compound or from a combination of compounds. It may thus be useful to study the composition of *D. rhinocerotis* and *G. africana* to identify the compound(s) with pesticidal activity. The compound(s) may then be isolated be and evaluated for use as pesticides.

Previous studies have shown that *D. rhinocerotis and G. africana* extracts are more effective against the grey mould causing fungi, *B. cinerea*, when used in combination with synthetic fungicides (Vries, 2008). Complete inhibition of the fungi *B. cinerea* was observed for *D. rhinocerotis and G. africana* concentrations greater than at 0.95mg/ml, when used in combination with kresoxim-methyl (a synthetic pesticide currently used on fungi) (Knowles, 2005 and Vries, 2008).

As a result of increasing evidence of the deterioration of male fertility, the importance of reproductive health cannot be overlooked. Impaired fertility due to exposure to xenobiotics, including pesticides, is resulting in increasing costly practices of assisted reproduction technologies (Mantovani *et al*., 2005). It is therefore of utmost importance to manage the risks of xenobiotics, including all forms of pesticides, and to provide safety information for these substances.

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Chapter 8

Conclusion

Pests are the largest competitors of agricultural produce and reduce global crop production by up to 50%. To prevent losses of such magnitude, large quantities of pesticides are used to get rid of pests. The majority of pesticides currently on the commercial market are based on synthetic chemicals. However these synthetic chemical compounds have major toxicological implications such as health risks for humans, increased environmental pollution and increased risk of pesticide resistance which enhances pest resurgence due to development of resistance (Zhu *et al*., 1996; Zhu *et al*., 2000; Isman, 2006).

Less than 0.1% of the pesticides applied to crops reach their target pest, while the rest enters the environment thus reaching non-target organisms (Pimentel *et* al., 1986). Exposure to some of these chemicals may lead to disruption of normal physiological processes and consequently lead to adverse health effects including immunologic damage, birth defects, thyroid cancer, breast cancer, testicular cancer and harm to the developing nervous system. Examples of these pesticides include the organochlorines dichloro-diphenyl-trichloroethane (DDT), chlordane and lindane which may result in immunologic damage, birth defects, reproductive system defects and cancers (Hermanowicz *et al*., 1984; Menconi *et al*., 1988; Kelce *et al*., 1995; Danzo 1997). Carbamate and other organophosphate pesticides such as carbaryl, carbofuran, malathion and parathion are carcinogenic, neurotoxic, immunotoxic and may also affect the respiratory system (Srinivasa *et al*., 2005; Sikka *et al*., 2008).

As a result of the negative effects that may result from pesticide use, the US environmental protection agency (US EPA) requires that before registering and selling or distributing a pesticide the US EPA must first ensure that the pesticide, when used according to label directions, can be used with a reasonable certainty of no harm to human health and without posing unreasonable risks to the environment. As a result the US EPA requires more than 100 different scientific studies and tests for potential substances to be used as pesticides.

Efforts are being made, at a global scale, to replace synthetic pesticides with safer alternatives. The use of botanical pesticides poses lower risks, as compared to synthetic pesticides, thus may result in the reduced use of synthetic pesticides while maintaining both the quality and quantity of agricultural produce (Isman, 2006). However, motivation for the commercial use of botanicals as pesticides requires validating the efficacy of the plant as a pesticide, and also assessing its effects on human health and the environment. An important component of this evaluation involves toxicity studies, both *in vitro* and *in vivo*, which enables cautions of dangerous practices and toxic effects of the plants to be issued. Not much research has been done on *D. rhinocerotis* and *G. africana*, but based on this study and other studies conducted to date; the results obtained in evaluating pesticidal and toxicological properties of the plants show that they are promising candidates for consideration as botanical pesticides. These preliminary results can be seen as ground work for further studies on *in vivo* toxicological activity, and mechanism of action experiments. Current regulatory assessments, in South Africa, for potential effects on non-target organisms are performed by adequate routine toxicity studies.

D. rhinocerotis and *G.* a*fricana* are effective against the fungal pathogen *B. cinerea*. *B. cinerea* causes grey mould disease on commercially important horticultural produce in South Africa. Grey mould causes pre- and post harvest decay of up to 235 plant species, including fruit, vegetables and flowers (Jarvis, 1997). This poses a problem to the South African agricultural industry. Large amounts of fungicides are therefore used in order to contain the fungal pathogen and produceexport quality agricultural producucts, which is one of South Africa's main export sectors. Pesticide regulatory policies are aimed at reducing the amount of chemicals applied to agricultural produce. Environmentally friendly alternative procedures for pest control are thus high priority in efforts to protect the environment.

Plants contain a virtually untapped reservoir of pesticides, and South Africa is in a good position to develop and utilize botanicals as pesticides because the country has a rich floral biodiversity. Due to increased consumer request for organically grown produce in developed countries, the use of botanical pesticides may create opportunities for economic growth. The benefits of natural pesticides can also be realized in developing countries, where farmers may not be able to afford synthetic insecticides and the traditional use of plants and plant derivatives for protection of stored products is long established. Even where synthetic insecticides are affordable to growers (e.g., through government subsidies), limited literacy and a lack of protective equipment result in thousands of accidental poisonings annually. It would therefore be worthwhile to invest more time and resources into further investigating botanicals for use as pesticides.

In vivo studies are expensive, lengthy, require lots of animals and raise important ethical concern. For these reasons there is a move towards alternative, efficient methods to screen for potential adverse effects posed by products. *In vitro* tests capable of predicting the toxicity of products are thus developed at different institutes worldwide. Aside from being time and cost effective, i*n vitro* tests are also advantageous in that they are very sensitive.Samples are added directly to cells of an organ and can therefore detect low level toxicity. One of the outcomes envisaged by this study was the development of a battery of rapid *in vitro* assays to monitor the effects of products, such as pesticides, on various physiological systems. This aim was met, as shown by the results, therefore use of these assays will be money well spent as they provide a ground work for further studies.

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Full Length Research Paper

The immunotoxicity of *Dicerothamnus rhinocerotis* and *Galenia africana*

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Increased use of pesticides has resulted in increased concern about the adverse effects on non-target organisms, including humans. Due to increasing awareness there is great pressure to reduce the use of synthetic pesticides. An alternative to the use of synthetic pesticides is the exploitation of natural botanical products with pesticidal potential. *D. rhinocerotis* and *G. africana* exhibit antifungal properties against *Botrytis cinerea*. This paper describes the immunotoxicity of extracts of *D. rhinocerotis* and *G. africana* on mouse spleenocytes. Spleen cell cultures were prepared and exposed to varying concentrations of *D. rhinocerotis* and *G. africana*. Control cultures were exposed to the DMSO vehicle only. Results obtained showed that both *D. rhinocerotis* and *G. africana* have immunomodulatory effects. Exposure of cell cultures to both extracts resulted in a decrease in both IL-4 and IFN-γ. The cytokine inhibition was concentration dependent.

Key words: Pesticides, *Galenia africana*, *Dicerothamnus rhinocerotis*, immunotoxicity, cytokines.

INTRODUCTION

A pesticide may be defined as any substance(s) used for destroying or mitigating unwanted pests. Unwanted pests include species of plants or animals that cause harm during food production, processing, storage, transport and/or marketing (Clementi et al., 2008). The use of pesticides has increased, over recent years due to greater demand for good quality food products. Increased use of pesticides has resulted in increased concern about the adverse effects on non-target organisms, including humans.

Due to increasing awareness there is great pressure to reduce the use of synthetic pesticides (Isman, 2006). An alternative to the use of synthetic pesticides is the exploitation of natural botanical products with pesticidal potential (Isman, 2006). *Galenia africana* (*G. africana*) and *Dicerothamnus rhinocerotis* (= *Elytropappus rhinocerotis*) (*D. rhinocerotis*) exhibit antifungal properties against *Botrytis cinerea* (Knowles, 2005). *B. cinerea* is a fungal pathogen that causes grey mould rot, on a large number of economically important agricultural and horticultural crops (Jarvis, 1997).

D. rhinocerotis, popularly known as rhenoster bush ("renosterbos" in vernacular) or rhinoceros bush, is a bush shrub with small grayish-green leaves and tiny flower heads (Levyns, 1935). Infusions of the young branches prepared in brandy or wine are a traditional Cape (South Africa) medicine for indigestion, dyspepsia, ulcers and stomach cancer (Watt and Breyer-Brandwijk, 1962). It is also said to be taken as tonic to improve lack of appetite, for colic and wind diarrhoea (Cillie', 1992). The medicinal properties of *D. rhinocerotis* may be due to rhinocerotinoic acid, a labdane diterpenoid with anti-inflammatory activity (Dekker et al., 1988)

G. africana is a plant that is indigenous to the Namaqualand region of South Africa (Kellerman et al., 1988). The Hottentots, an indigenous tribe, chewed the plant to relieve toothache. The plant was also used in the treatment of venereal diseases and prepared as a decoction for skin diseases and for the relief of inflammation of the eyes (Watt and Breyer-Brandwijk, 1962).

Animals are forced to graze this plant during droughts and in poor-conditions. Ingestion of the plant is associated with liver damage and severe ascites, a condition referred to as 'waterpens' or 'water belly', in sheep and goats (De Kock, 1928; Kellerman et al 1988). The mar-

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Abbreviations: IL-4, Inteluekin-4 ; **IFN-γ**, interferon-gamma.

ked liver lesions have lead researchers to believe that the plant is hepatotoxic due to the presence of a toxin (Van der Lugt et al., 1992).

Due to the antifungal properties of the two plants they may have potential use on agricultural produce as fungicides. Exposure of pesticides to the immune system may result in alteration of the normal immune functions. This kind of activity is known as immunotoxicity (Colossio et al., 1999). Toxicity studies are required to determine safety of the plant extracts. Toxins can have a general effect on all cells or they can attack specific organ/ physiological systems. One of the physiological systems prone to attack by toxins is the immune system (Colosio et al., 1999).

The immune system is a well-regulated organ system that involves interrelated reactions, which protect an organism from invasion by foreign substances (Ladics, 2007). The immune system fights pathogens using two methods, namely the innate immunity and the acquired/ adaptive immunity (Seely et al., 2000; Storni et al., 2005). Due to its complexity, the immune system is a target for various toxic substances, including pesticides, therefore resulting in multiple potential target sites and pathological effects (Colosio et al., 1999).

Immunotoxicity results in toxicant-induced injury to part of the immune system thus affecting immune functions and may result in immunostimulation, immunosuppression, hypersensitivity and autoimmunity (Descotes, 2004; Van Wijk et al., 2006). Each of these categories is associated with potential adverse effects associated with significant morbidity.

The aim of this study was to assess the toxicity of *D. rhinocerotis* and *G. africana* for the acquired immune pathway using *in vitro* assays. Acquired immunity is regulated by cytokines secreted by the Th1 and Th2 lymphocytes.

T-helper (Th) lymphocytes are divided into Th1 and Th2 subsets according to cytokine production profile which also correlate with their function (Mosmann et al., 1986). Th1 cells produce the cytokines interleukin (IL)-2 and Interferon (IFN)-γ which aid with defenses against intracellular pathogens and promote cell mediated immunity, whereas Th2 produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 which are responsible for defenses against extracellular pathogens and promote humoral immunity (Mosmann and Sad, 1996). Th1 and Th2 cytokines are mutually inhibitory for the functions of the reciprocal phenotype (Storni et al., 2005).

IFN-γ is a cytokine predominantly produced by Th1 cells (Mims et al., 2004; Seely et al., 2000; Storni et al., 2005). IFN-γ aids with defenses against intracellular pathogens and promotes cell mediated immunity ((Mims et al., 2004; Seely et al., 2000; Storni et al., 2005). IFN-γ also antagonizes IL-4 leading to inhibition of Th2 cell proliferation (Storni *et al*., 2005). On the other hand IL-4 is a cytokine that is mainly produced by Th2 cells (Mims et al., 2004; Storni et al., 2005). IL-4 stimulates proliferation and differentiation of B-cells, thus inducing antibody production (Storni et al., 2005). IL-4 also results in inhibition of Th1 cell proliferation (Fukao et al., 2000; Storni et al., 2005).

MATERIALS AND METHODS

Reagents

All reagents, solvents and biomolecules used in this study were purchased from Roche (South Africa), Merck (Germany) or Sigma Chemical Company (St Louis, USA) unless stated otherwise. All other reagents were of analytical grade.

Plant extractions

Two medicinal plant species, *G. africana* and *D. rhinocerotis,* used in traditional medicine practices in South Africa were obtained and prepared for analysis. Extracts (20%, w/v) were prepared in 94.4% ethanol by Parceval (Pty) Ltd Pharmaceuticals (South Africa). Leaves of the plants were milled (sieve size~ 2-3 mm) after which the leaves were mixed with 94.4% ethanol at 20 g leaves per 100 ml ethanol. The mixture was shaken for brief periods during the overnight extraction. The extracts were then pressed, to separate the tincture and the milled leaves. Filtration was carried out to clean tincture from plant debris and the extract was stored at 20°C. For study purposes the extracts were air dried. The dried extracts were resuspended in DMSO to obtain 50% extract.

Spleen cell cultures

Male BALB/c mice were purchased from UCT animal facility (South Africa). They were sacrificed via cervical dislocation after which the spleens were dissected out. The spleens were then disrupted via passage through a sterile steel mesh. Spleen cells were suspended in full medium, which consisted of RPMI 1640 supplemented with 1% antibiotic/mycotic mix (Sigma, USA) and 1% serum replacement factor. The spleen cell suspension was washed and centrifuged at 1000 x g for 10 min, after which the cell pellets were resuspended in fresh medium to a concentration of 2.5 x 10^6 cells/ml.

Assays to screen toxicity of the plant extracts were conducted in 96-well culture plates (Nunc, Denmark). A serial dilution range of the extracts, in DMSO, were applied to wells of the plate. Control wells contained the DMSO vehicle only. Six replicates of each concentration were prepared. One set of wells (three replicates of each extract concentration) then received the cell suspension (unstimulated cultures). The other set of replicates received the cell suspension and 16 µg/ml phytohemmagglutinin (PHA) from *Phaseolus vulgaris* (Sigma, USA) was added. The plate was incubated for 48hours in an incubator at 37°C flushed with 5% CO_2.

After the 48hour culture period the supernatants were harvested and cytokine concentrations in the culture medium determined. IFN-γ and IL-4 were the cytokines assessed. This was done using commercially available ELISA kits in accordance with the manufacturer's instructions (eBioscience, USA).

Cytokine ELISAs

Nunc 96 well microtiter ELISA plates (Serving life science, Denmark) were used for all ELISA protocols. Cytokine (IL-4 and IFN-γ) concentrations were determined using ELISAs. The ELISAs were carried out according to the manufacturer's instructions. The ELISA plates were coated with capturing antibody (purified anti-mouse IL-4 or IFN-γ) diluted appropriately in coating buffer and incubated over-

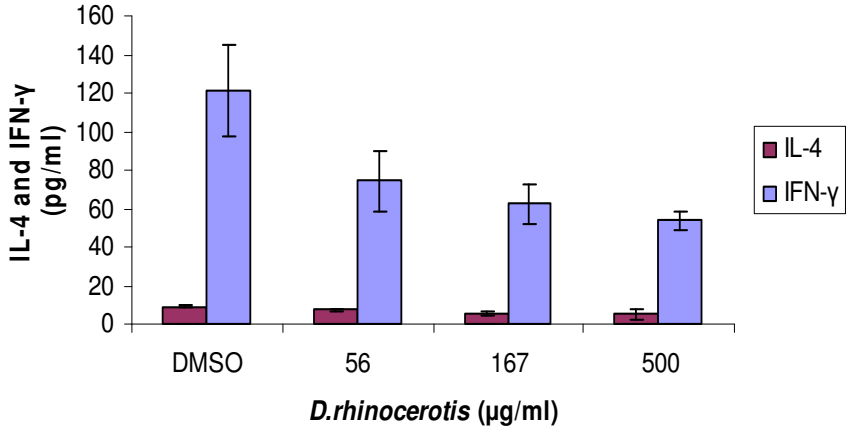

Figure 1. IL-4 and IFN-γ produced by spleenocytes, under stimulated conditions, in the presence of *D. rhinocerotis*.

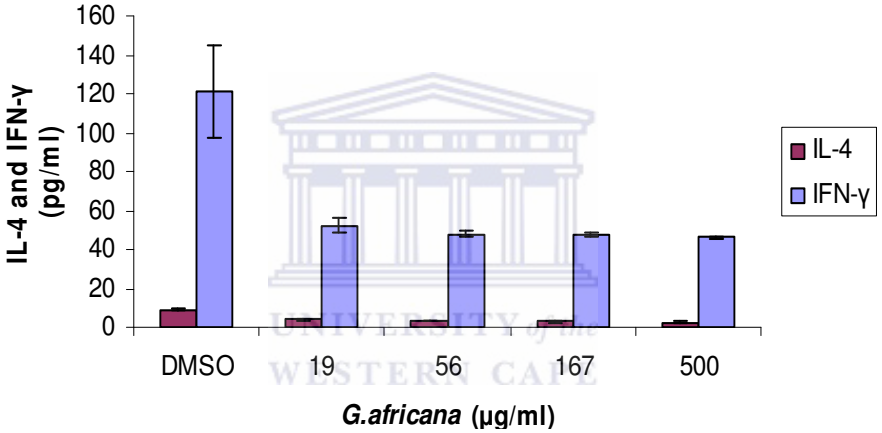

Figure 2. IL-4 and IFN-γ produced by spleenocytes, under stimulated conditions, in the presence of *G. Africana*.

night at 37°C. The plates were washed 5 times with wash buffer (autoclaved PBS, 0.05% Tween-20 and distilled water), after which the non-specific binding sites were blocked with assay diluent. Cell free supernatants were then added to the plate. The assay was standardized using 2-fold serial dilutions of recombinant mouse IL-4 (500pg/ml) and IFN-γ (2000 pg/ml) respectively. The plates were then sealed and incubated for 2 h at room temperature. After 5 washings, with wash buffer, detection antibody (Biotin-conjugate anti-mouse IL-4 and IFN-γ) was added to each well and the plate was incubated for 1 h at room temperature. Avidin- Horseredish peroxidase (HRP) was added to all wells in order to detect the bound cytokine. This was incubated for 30 min after which the plate was washed 7 times. Substrate solution (1X TMB) was then added to every well and incubated for approximately 15 min. The reaction was then stopped with stop solution. Absorbances were read on a plate reader at 450 nm.

Statistical analysis

All experiments were performed three times in triplicate and data was statistically analysed via one-way ANOVA (P<0.001) and regression analysis.

RESULTS

From the IL-4 and IFN- γ ELISAs performed, standard curves were plotted using the optical density readings obtained for the serial-dilution of the standards. The IL-4 and IFN-γ concentrations of the spleen-cell cultures after incubation with *D. rhinocerotis* and *G. africana* were extrapolated using the standard curves and are shown in Figures 1 and 2. The results are expressed as the effects of the individual extracts vs. the control, untreated cells.

The results showed that *D. rhinocerotis* had no significant (P<0.001) effect on both IL-4 and IFN-γ production under unstimulated conditions. However under stimulated conditions *D. rhinocerotis*, at concentrations above 56 µg/ml, significantly decreases IL-4 production (Figure 1). *D. rhinocerotis also* significantly (P<0.001) decreased IFN-γ production, at all concentrations, under stimulated conditions (Figure 1).

G. africana, at concentrations above 19 µg/ml, significantly (P<0.001) decreases IL-4 production under stimu-

lated conditions (Figure 2). It also significantly (P< 0.001) decreased IFN-γ production, at all concentrations, under stimulated conditions (Figure 2). However under unstimulated conditions *D. rhinocerotis* had no significant effect on IFN-γ production.

DISCUSSION

Exposure to pesticides may have various effects on the immune system, ranging from slight modulation of immune functions to development of clinical immune diseases (Colosio et al., 1999). For example exposure to the organophosphorous compound pentachlorophenol results in decreased lymphocyte proliferative responses to mitogens (Colosio et al., 1993). It is therefore a necessity to evaluate the immunotoxic effects of pesticides before putting them to use.

The results in this study showed that *D. rhinocerotis* had no significant effect on IL-4 production under unstimulated conditions. However under stimulated conditions *D. rhinocerotis*, decreases IL-4 production. IL-4 is a cytokine that is mainly produced by Th2 cells (Storni et al., 2005). This therefore implies that exposure to *D. rhinocerotis* may result in impairment of the Th2 response. Th2 cells are required to mount effective humoral and cell-mediated responses that are required to fight extracellular microbes and parasites. An impairment of these responses may thus result in increased susceptibility to extracellular microbes (O'Garra and Arai 2000).

This study also shows that both *D. rhinocerotis* and *G. africana* significantly decreased IFN-γ production. IFN-γ is a cytokine that is predominantly produced by the Th1 cells (Storni et al., 2005). Th1 cells are required to mount an effective cell-mediated response that is required to fight intracellular pathogens, viruses and cancers. An impairment of these responses may thus result in increased susceptibility to intracellular pathogens, viruses and cancers (Storni et al., 2005, Seely et al., 2000). This therefore implies that exposure to *D. rhinocerotis* or *G. africana* may result in impairment of the Th1 response, which may in turn lead to increased susceptibility of intracellular pathogens. Previous studies, done by us, showed that *D. rhinocerotis* and *G. africana* are not cytotoxic at the low concentrations used in this current study, indicating that specific immune functions are impaired at concentrations that do not necessarily cause cell death.

Exposure to *G. africana* also results in lowered IL-4 levels. This implies that exposure to *G. africana* may result in impairment of the Th2 response, which inturn may lead to increased susceptibility to extracellular microbes (O'Garra and Arai 2000).

The properties of whole plant extracts can either be from a single chemical compound or from a combination of compounds. The active chemical compounds present in the plants are known as secondary metabolites (Lewinsohn and Gijzen, 2008). It may thus be useful to study the composition of the plant extracts to identify the compound(s) with pesticidal activity. The compound(s) may then be isolated be and evaluated for use as pesticides.

Studies have shown that *D. rhinocerotis and G. africana* extracts are more effective against the grey mould causing fungi, *B. cinerea*, when used in combination with synthetic fungicides (Vries, 2007). Complete inhibition of the fungi *B. cinerea* was observed for *D. rhinocerotis and G. africana* concentrations greater than at 0.95 mg/ml, when used in combination with kresoxim-methyl (a synthetic pesticide currently used on fungi) (Knowles, 2005; Vries, 2007). Immunotoxicity studies can therefore be carried out for the combination of kresoxim-methyl and the botanical extracts at these low concentrations.

A major consideration in approving pesticides for commercial use is whether they pose an unreasonable risk to humans and to the environment. In order to evaluate safe levels of chemicals toxicological data is required. The aim of this study was to determine the immunological risks associated with using extracts of *D. rhinocerotis* and G. *africana*, and toxicological data was acquired.

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Full Length Research Paper

The environmental toxicity of *Dicerothamnus rhinocerotis* and *Galenia africana*

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Over the years the use of pesticides has greatly increased. This in turn has lead to concern about the adverse effects that the pesticides may have on non-target organisms in the environment. Due to increasing awareness there is great pressure to reduce the use of synthetic pesticides. An alternative to the use of synthetic pesticides is the exploitation of natural botanical products with pesticidal potential. *Dicerothamnus rhinocerotis* and *Galenia africana* are plants, indigenous to South Africa, with fungicidal properties against a fungal pathogen that causes grey-mould rot on a wide range of agricultural produce. In this study a series of acute toxicity tests were conducted to estimate the potential environmental effect of *D. rhinocerotis* and *G. africana*. The acute toxicities of the plants were determined using the species *Daphnia pulex*, *Selenastrum capricornutum*, *Vibrio fischeri* and *Poecilia reticulata* as bio-indicators. Results obtained showed that *G. africana* had higher toxicity units than *D. rhinocerotis*, thus showing that *G. africana* is more toxic to the aquatic environment compared to *D. rhinocerotis*.

Key words: Pesticides, *Galenia africana*, *Dicerothamnus rhinocerotis*, acute toxicity.

INTRODUCTION

Pesticides are used at a global scale for pest control thus allowing the maintenance of agricultural produce and contribute to economic growth and stability (Arias-Estevez et al., 2008). The wide use of pesticides has however become a pervasive threat to natural ecosystems. There is increasing awareness of potential environmental and health problems associated with pesticide use (Carbone et al., 2007; Caserta et al., 2007; Kodavanti et al., 2008). This has resulted in pressure to reduce the use of synthetic pesticides. An emerging alternative to synthetic pesticides is the study and exploitation of naturally occurring products with pesticidal properties (Isman, 2006 and Regnault-Roger and Philogene, 2008). In order to evaluate the effects of new pesticides, there is need for toxicological data on organisms' representative of the various ecosystems.

Dicerothamnus rhinocerotis (L.f.) (= *Elytropappus rhinocerotis* (L.f.), popularly known as rhenoster bush ("renosterbos" in vernacular) or rhinoceros bush, is a bush shrub of about 1 - 2 m in height, with small grayish-green leaves and tiny flower heads which are almost inconspicuous (Levyns, 1935). *D. rhinocerotis* is of the family asteraceae and is native to Southern Africa (Van et al., 1997).

Galenia africana, commonly known in vernacular as "kraalbos" or "geelbos", is a perennial shrub belonging to the family aizoaceae (Van der Lugt et al., 1992). *G. africana* was most common in the Namaqualand region of South Africa but has recently become more widespread in the western and southern Karoo (Kellerman et al., 1988). *G. africana* is an aromatic, woody sub-shrub that grows to 0.5 - 1 m in height (Van der Lugt et al., 1992). Its green leaves are oppositely arranged with small yellow flowers at the end of the twigs (Kock, 1928; Kellerman et al., 1988).

Whole plant extracts of D. *rhinocerotis* and *G. africana* exhibit antifungal properties against *Botrytis cinerea*. *B. cinerea* is a fungal pathogen that causes grey mould/gray mold rot, on a large number of economically important agricultural and horticultural crops such as fruits, flowers and green tissue (Jarvis, 1997).

Due to the antifungal properties of the two plants they may have potential use on agricultural produce as fungi-

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Table 1. EC_{50} values obtained for *D. rhinocerotis* and *G. africana* when using *S. capricornutum* and *V. fischeri.*

Test	Exposure period	EC_{50} values for *D. rhinocerotis* (µg/ml)	EC_{50} values for *G. africana* (µg/ml)
V. fischeri bioluminiscent test	15 min	110	1
V. fischeri bioluminiscent test	30 min	100	0.7
S. capricornutum test	72 h	390	100

cides. Ecotoxicity studies are required to determine safety of the plant extracts before they can be used commercially. In this study the acute toxicity of *D. rhinocerotis* and *G. africana* was determined by using several aquatic species. LD_{50} and EC_{50} levels obtained from this study will be used to set maximum allowed environmental levels of these plant extracts.

MATERIALS AND METHODS

Plant extractions

Two medicinal plant species, *G. africana* and *D. rhinocerotis,* used in traditional medicine practices in South Africa were obtained and prepared for analysis. 20% (w/v) extracts were prepared in 94.4% ethanol by Parceval (Pty) Ltd pharmaceuticals (South Africa). Leaves of the plants were milled (sieve size~ 2 - 3 mm) after which the leaves were mixed with 94.4% ethanol at 20 g leaves per 100 ml ethanol. The mixture was shaken for brief periods during overnight extraction. The extracts were then pressed to separate the tincture and the milled leaves. Filtration was carried out to clean tincture from plant debris and the extracts were then stored at 20°C. The extracts were then air dried and resuspended in DMSO to obtain 50% (w/v) extract. For this study the extracts were further diluted to 1 mg/ml using deionised water, which was used as the highest concentration.

Test species

The test species used in this study are important components of many aquatic communities and therefore can be adversely affected by aquatic pollutants. *Poecilia reticulata* (guppy fish, also known as the million fish) at 7 - 21 day post-hatch and 24 h old *Daphnia pulex* (water flea) were obtained from in-house cultures at Golder Associates Research Laboratories. *Selenastrum capricornutum*, (Printz algae beads, CCAP 27814, Cambridge UK) and lyophilised *Vibrio fischeri* luminiscent bacteria (NRRL B-11177) were also used for this study.

General test conditions

All toxicity tests were conducted in appropriate environmentally controlled rooms using standard techniques. The tests were conducted at Golder Associates Research Laboratories.

V. fischeri bioluminiscent test

The methods employed for the toxicity testing of *G. africana* and *D. rhinocerotis* on *Vibrio fischeri* followed those outlined in EN ISO (International Organisation for Standardisation) 11348-3, 1998. Test chambers used were polystyrene cuvettes for Luminoskan TL luminometer (Hygiene Monitoring System). *V. fischeri* were exposed at dilutions of plant extracts for 15 and 30 min. Test endpoints included percentage growth inhibition relative to control and EC_{50} values. Two replicates were carried out for each of the samples and statistical analysis was done using bio orbit software.

Selenastrum capricornutum growth inhibition test

The methods carried out for the toxicity testing of *G. africana* and *D. rhinocerotis* on *S. capricornutum* followed the Organisation for Economic Cooperation and Development (OECD) guideline 201, 1984. Test chambers used were 10 cm path length long cells. Exposure period was 72 h. Test endpoints included percentage growth inhibition relative to control and EC_{50} values. Two replicates were carried out for each of the samples and statistical analysis was done using regression analyses.

Daphnia pulex acute toxicity test

The methods used for the toxicity screening of *G. africana* and *D. rhinocerotis* on D. *pulex* followed those outlined by the United States Environmental Protection Agency (US EPA),1993 (600/4-90/027F). Test chambers used were 50 ml disposable polystyrene cups. Exposure periods were 24 and 48 h. Test endpoints included percentage mortality and LC_{50} values. 4 replicates, each with 5 test organisms per chamber, were used to test each sample. Statistical analysis was carried out using probit software/TSK.

Poecilia reticulata acute toxicity test

The methods employed for the toxicity testing of *G. africana* and *D. rhinocerotis* on *P. reticulata* was according to that outlined by the US EPA, 1996 (712-C-96-118). Test chambers used were 250 ml disposable polystyrene cups. Exposure period was 96 h. Test endpoints included percentage mortality and LC_{50} values. 2 replicates, each with 5 test organisms per chamber, were used to test each sample. Statistical analysis was carried out using probit software/ TSK.

RESULTS

The term EC_{50} refers to the concentration of a drug which induces a response halfway between the baseline and maximum. It is often used as a measure of the potency of a substance, therefore the lower the EC_{50} value the more potent the substance. In this study the EC_{50} values for the 2 plant extracts were obtained using *S. capricornutum* and *V. fischeri*. Table 1 shows the concentrations of the 2 plant extracts where 50% of the population exhibits a response.

Table 2. LC_{50} values obtained for *D. rhinocerotis* and *G. africana* when using *P. reticulata* and *D. pulex.*

Test	Exposure period (h)	LC_{50} values for *D. rhinocerotis* (ug/ml)	LC_{50} values for *G. africana* (ug/ml)
D. pulex acute toxicity test	24	340	40
D. pulex acute toxicity test	48	240	30
P. reticulata test	96	210	20

Table 3. Toxicity units and the measure of toxicity.

Toxicity unit	Conclusion
< 1	Limited to not acutely toxic
1 - 2	Negligibly acute toxic
2 - 10	Mildly acute toxic
10 - 100	Acutely toxic
> 100	Highly acutely toxic

Table 4. Toxicity units obtained for *D. rhinocerotis and G. africana.*

Test species	Toxicity units for *D. rhinocerotis*	Toxicity units for *G. africana*
V. fischeri	10	1429
S. capricornutum	2.6	10
D. pulex	4.2	33
P. reticulata	5	50

LC_{50} is the concentration of a substance which kills 50% of test animals exposed in a given time. In this study the LC_{50} values for the 2 plant extracts were obtained using *P. reticulata* and *D. pulex*. Table 2 thus shows the concentrations of the 2 plant extracts which kills 50% of the population of test animals.

The results in Tables 1 and 2 show that *D. rhinocerotis* has higher EC_{50} and LC_{50} values as compared to *G. africana*, thus showing that *G. africana* is more toxic to the aquatic species as compared to *D. rhinocerotis*.

From the above results toxicity units were then calculated for each of the plant extracts. For each test performed the toxicity unit was calculated as 100% (full strength effluent expressed as percentage) divided by the EC_{50} or LC_{50} values. Toxicity units were used as a measure of acute toxicity, Table 3.

The toxicity units obtained when the different species were exposed to *D. rhinocerotis* and *G. africana* are shown in Table 4. *G. africana* had higher toxicity units than *D. rhinocerotis*, thus showing that *G. africana* is more toxic to the aquatic species as compared to *D. rhinocerotis*. *D. rhinocerotis* is mildly acutely toxic to all the species used in this study. On the other hand *G. africana* is mildly toxic to *S. capricornutum*, acutely toxic to *D. pulex* and *P. reticulata* and highly acutely toxic to *V. fischeri*.

DISCUSSION

A major consideration in approving pesticides for commercial use is whether they pose an unreasonable risk to humans and to the environment. In order to evaluate environmentally safe levels of chemicals toxicological data on organisms, representative of the various ecosystems are required. Such data is often unavailable or inadequate. The aim of this study was to determine the ecological risks associated with using extracts of *D. rhinocerotis* and G. *africana*. A set of toxicological data for organisms' representative of the aquatic environment (algae, water flea, fish and bacteria) was obtained.

The study showed that *D. rhinocerotis* has higher EC_{50} and LC_{50} values compared to *G. africana*. From the toxicity units calculated, *D. rhinocerotis* showed mild acute toxicity to all the species used in this study while *G. africana* ranged from mildly toxic to highly acutely toxic. The set of toxicological data on organisms representative of the aquatic environment (algae, water flea, fish and bacteria) obtained in this study therefore shows that *G. africana* is more toxic to the aquatic environment as compared to *D. rhinocerotis*.

G. africana and *D. rhinocerotis* extracts showed inhibition of fungal growth at concentrations greater than 31.25 and 125 mg/ml, respectively (Knowles, 2005). This showed that the 2 plant extracts are effective as fungicides. From the results obtained in this current study, it can be concluded that at concentrations greater than 31.25 mg/ml (*G. africana*) and 125 mg/ml (*D. rhinocerotis*), the extracts will be toxic to the aquatic environment.

In this current study both *G. africana* and *D. rhinocerotis* extracts showed effectiveness against the bacteria *V. fischeri*. Further studies may be conducted with different bacteria to ascertain if the plant extracts may be used as disinfectants.

Studies have shown that *D. rhinocerotis and G. africana* extracts are more effective against the grey mould causing fungi, *B. cinerea*, when used in combination with synthetic fungicides (Vries, 2007). Complete inhibition of the fungi *B. cinerea* was observed for *D. rhinocerotis and G. africana* concentrations greater than at 0.95 mg/ml, when used in combination with kresoxim-methyl (a synthetic pesticide currently used on fungi) (Knowles, 2005; Vries, 2007). Ecotoxicity studies can therefore be carried out for the combination of kresoxim-

methyl and the botanical extracts at these low concentrations.

Once a pesticide is introduced into the environment it is influenced by many processes which determine its ultimate fate. Pesticides thus not only affect aquatic environments, but other environments are also affected. Further studies on other environments are therefore necessary for risk assessment of the plant extracts if they are to be used as pesticides.

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