In Vitro Tests for Immunomodulatory Effects of Medicinal Plants used in the treatment of Malaria in South Africa

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A project submitted to the Department of Pharmacology, University of Cape Town in fulfilment of the requirements for the degree

MASTER OF SCIENCE (MEDICINE)

SUPERVISORS
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Declaration

I, ..., hereby declare that the work on which this thesis is based is my own original work and that neither the work nor any part of it has been, is being or is to be submitted for another degree in this or any other University.

I give the University the right to reproduce any portion of this thesis for the purpose of research.

Signed:.....

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To my late cousin Mfundo your departure was unexpected but you will always be remembered and I will keep the promise I made to you.

Abstract

Using the ethno-medicinal data approach, nine South African plants used traditionally in the treatment of malaria were collected and evaluated for cytotoxic and lymphocyte-proliferating effects. These included *Acokanthera oppositofolia, Zanthoxylum capense, Glycyrrhiza glabra, Harpephyllum caffrum, Lippia javanica, Pentanisia prunneloides, Psidium guajava, Typha capensis* and *Cannabis sativa.* The cytotoxic effect of the aqueous, methanol and dichloromethane extracts of these plants was evaluated *in vitro* in Rat-1 fibroblasts. There was no observable difference in cytotoxic activity between cold- and hot-water extracts of all the plants investigated. Cell proliferation greater than 80 % was observed for aqueous extracts of *A. oppositofolia, H. caffrum, L. javanica, P. prunneloides, P. guajava* and *T. capensis* which would suggest that the decoctions or infusions prepared from these plants are considered safe for consumption. However, high cytotoxic effect was exhibited by dichloromethane extracts of *Z. capense, L. javanica, C. sativa* and *P. guajava* at 100 μg/ml. These findings suggest that dichloromethane extracted compounds that are not normally extractable by traditional methods.

Cold- and hot-water extracts of *Z. capense, G. glabra and C. sativa* were evaluated for mitogenic activity in human peripheral blood lymphocytes. The effect of these extracts on proliferative responsiveness of lymphocytes to phytohemagglutinin (PHA) was also determined. The results obtained indicate that both cold- and hot-water extracts of *Z. capense* stimulate lymphocytes at 1 μg/ml. The cold-water extract of *Z. capense* stimulates lymphocytes by a factor of 1.37 ± 0.1 at 1 μg/ml. The hot-water extract of *Z. capense* stimulates lymphocytes by a factor of 1.28 ± 0.17 at 1 μg/ml. Cold- and hot-water extracts of *G. glabra* and *C. sativa* do not exert lymphocyte stimulating or suppressant effect at the concentrations tested.

Solid phase extraction of the hot-water extract of *Z. capense* produced two fractions, designated F2 and F3. F2 increased PHA-induced lymphocyte proliferation at 100 μg/ml. HPLC analysis of F2 suggests that it is composed of two peaks that absorb in similar ultraviolet (UV) spectra. F2 migrated as three spots on thin layer chromatographic (TLC) analysis for coumarins. Separation of these spots, individual testing for lymphocyte proliferating activity and structural identification of active compounds might aid in understanding the activity at a chemical level.

This study demonstrates the capacity of the cold- and hot-water extracts of *Z. capense* to enhance lymphocyte proliferation in the absence of PHA. It also shows that solid phase extraction of the hot-water extract of *Z. capense* extracted more active fractions, which augment PHA-induced lymphocyte proliferation.

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Abbreviations

PHA-Phytohaemagglutinin

TLC-Thin Layer Chromatography

FCS-Fetal Calf Serum

PBS-Phosphate Buffered Saline

LDH-Lactate Dehydrogenase

DMSO-Dimethyl sulfoxide

CO_2-Carbon dioxide

LMW-Low molecular weight

HMW-High molecular weight

HPLC-High pressure liquid chromatography

RPMI-Roswell Park Memorial Institute

1. INTRODUCTION

1.1. Malaria

Malaria is one of the deadliest diseases in the tropics. It affects 200-300 million people worldwide and kills 1-2 million people every year, mostly in Sub-Saharan Africa (Ghosh A et al, 2000). *Plasmodium falciparum* is the most prevalent and virulent of the malaria parasites responsible for the disease in humans (Winstanley PA. et al, 2000). Malaria control in the past three decades has been dominated by the increasing problem of parasite resistance to existing antimalarials. In Africa, there is widespread resistance to chloroquine, and resistance to the second-line drug pyrimethamine-sulphadoxine (Fansidar) is increasing (Hastings IM. et al, 2000).

Traditional medicines have been used throughout the world to treat many tropical diseases, including malaria (Wilcox M et al, 2001). Two antimalarial compounds derived from natural sources include quinine from *Cinchona* spp., and artermisinin from *Artemisia annua* (Wilcox M et al, 2001). A number of plants used traditionally in the treatment of malaria were screened in the Department of Pharmacology, University of Cape Town, for *in vitro* antimalarial activity using chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*. Most of these plants were not active against the strains tested (Matsabisa MG, 2001). The symptoms of malaria include fever, headaches and pain therefore the same plants used in the treatment of malaria could be used in the treatment of ailments that may be unrelated to malaria. The fact that these plants were not active against malaria parasites *in vitro* could be that these extracts relieve the symptoms of malaria or else they need to be metabolised to active constituents.

These extracts could also be acting on the immune system of the individual to help combat malaria. Immunomodulating agents from plants have been reported to act primarily on cellular rather than humoral immune responses and to restore the immunocompetency of the impaired host without hyper stimulating the normals (Sairam M et al, 1997). These agents augment macrophage chemotaxis, phagocytosis and promote interaction with other immunoregulatory lymphoid cells. It was in the interest of this study to investigate the effects that these plants might have on the human immune system (using human blood lymphocytes).

1.2. The Use of Natural Products in Immunotherapy

In recent years the concept of immunotherapy to develop and improve defence mechanisms has developed from a theoretical concept into a very promising field of drug therapy (Tyler V et al, 1988). A protein-bound glucan extracted from edible mushroom *Coriolus versicolor* is used in clinical oncology in Japan under the name Krestin or PSK (Werner GH et al, 1996). Krestin has been shown in various experimental models to exert significant antitumor activities mediated through stimulation of macrophages, monocytes, Natural Killer (NK) cells and various T lymphocyte populations; it also enhances resistance of laboratory animals to bacterial, fungal and viral infections and exerts a chemo-protective effect against carcinogenesis (Werner GH et al, 1996). Immunostimulating activities have also been reported for polysaccharides extracted from *Echinacea purpurea* and *Viscum album* (Werner GH et al, 1996). These plants are currently administered, generally by the oral route, to young children and to elderly individuals to increase their natural resistance to bacterial or viral infections of the upper respiratory tract; their widespread use in some areas gives indirect indication of their efficacy in this context.

The root of *Astragalus membranaceus* is widely used throughout China as a food supplement and a medicinal plant. When the biological effects of this plant were assessed it was found that aqueous extracts augmented spontaneous [^{3}H] thymidine incorporation in the mononuclear cells 2-fold (Sun Y et al, 1983). The herb also increased lymphocyte stimulation induced by the mitogens phytohaemagglutinin (PHA), concanavalin A, and pokeweed mitogen (PWM) (Sun Y et al, 1983). The unique norlignan diglucoside, hypoxoside, first isolated in the 1980's from the bulbous South African plant, *Hypoxis hemerocallidea* is one of the best studied phytochemicals from an African plant (Dagne E, 1999). When the anticancer properties of this plant were assessed on cancer patients it was found that the plant's phytosterols also enhanced mitogen-induced proliferation of lymphocytes. *In vitro* tests on human peripheral blood lymphocytes were also carried out using purified sterols and sterolins. Lymphocyte proliferating effects of phytosterols were further confirmed by *in vivo* studies on healthy volunteers (Bouic P, 1997).

1.3. Aims and Objectives

The main aim of this project was to perform *in vitro* tests to investigate immunomodulatory effects of selected medicinal plants used in the treatment of malaria in South Africa. The objectives set out for this study were:

i. Collection of plants used traditionally in the treatment of malaria in South Africa (KwaZulu-Natal and Mpumalanga provinces)
ii. Extraction of plant material
iii. Screening of plant extracts for cytotoxicity against Rat-1-fibroblasts
iv. Performing *in vitro* lymphocyte proliferation assays

v. Performing bioassay-guided fractionation of active extracts using thin layer chromatography (TLC), solid phase extraction and high-pressure liquid chromatography (HPLC)

2. METHODOLOGY

2.1. Plant Collection

Different approaches of plant collection are used when searching for novel drugs from plants. These include random and targeted plant selection programs. In random plant selection programs, plants are collected from a given region and screened without regard to their taxonomic affinities, ethno-botanical context, or other intrinsic qualities (Balick MJ et al, 1996). An ethno-botanical survey is an example of a targeted plant selection program in which plants used traditionally by indigenous people are chosen for the study (Balick MJ et al, 1996).

2.1.1. Ethno-botanical Survey

In South Africa malaria occurs in the KwaZulu-Natal, Mpumalanga and Northern provinces. This indicates the importance of imported malaria or failure of malaria control in neighbouring countries (Sharp BL et al, 1996). To carry out the search for plants used in the treatment of malaria, healers from the KwaZulu-Natal and Mpumalanga provinces were consulted. Figures 2.2 and 2.3 on p. 6 and 7, respectively, show the distribution of malaria in these provinces. Areas visited in the Mpumalanga province included Nelspruit, Bushbuckridge and Acornhoek. Areas visited in the KwaZulu Natal province included Nongoma, Mtubatuba, Hluhluwe, Nqutu and Greytown. Since these areas were of low malaria risk our visits were based on the assumption that healers from these areas would probably have had some experience with malaria. Interviews were conducted with a number of traditional healers from these two provinces. The main objectives were to determine the knowledge the healers had about malaria and also to find out the form of treatment they used in treating malaria.

Northern Province and Mpumalanga Malaria Risk

The map below details the Malaria risk in the Northern Province and Mpumalanga. The numbers in the map refer to game reserves.

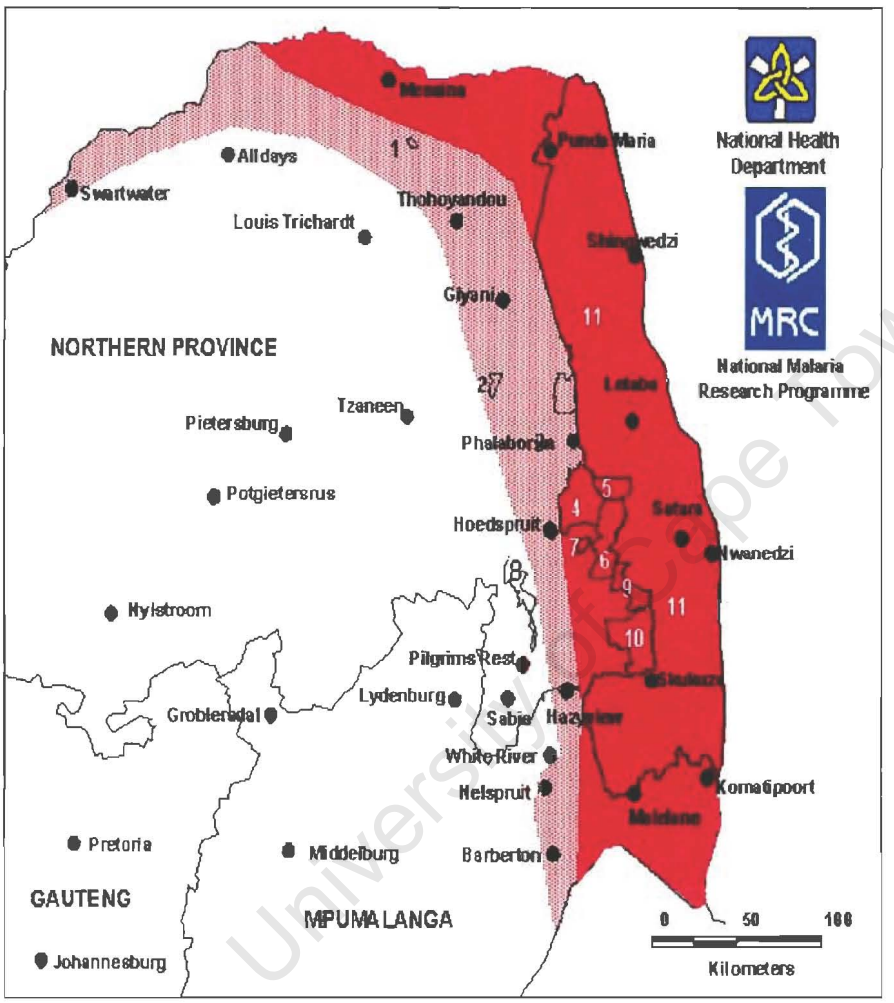


Figure 2.1. Distribution of malaria in the Mpumalanga and Northern provinces (http://www.malaria.org.za/malaria-Risk/Cases/cases.html)

Acornhoek is about +/- 20 km from Pilgrim's Rest and Bushbuckridge is +/- 50 km north of Sabie

KwaZulu-Natal Malaria Risk

The map below details the Malaria risk in KwaZulu-Natal. The numbers in the map refer to game reserves.

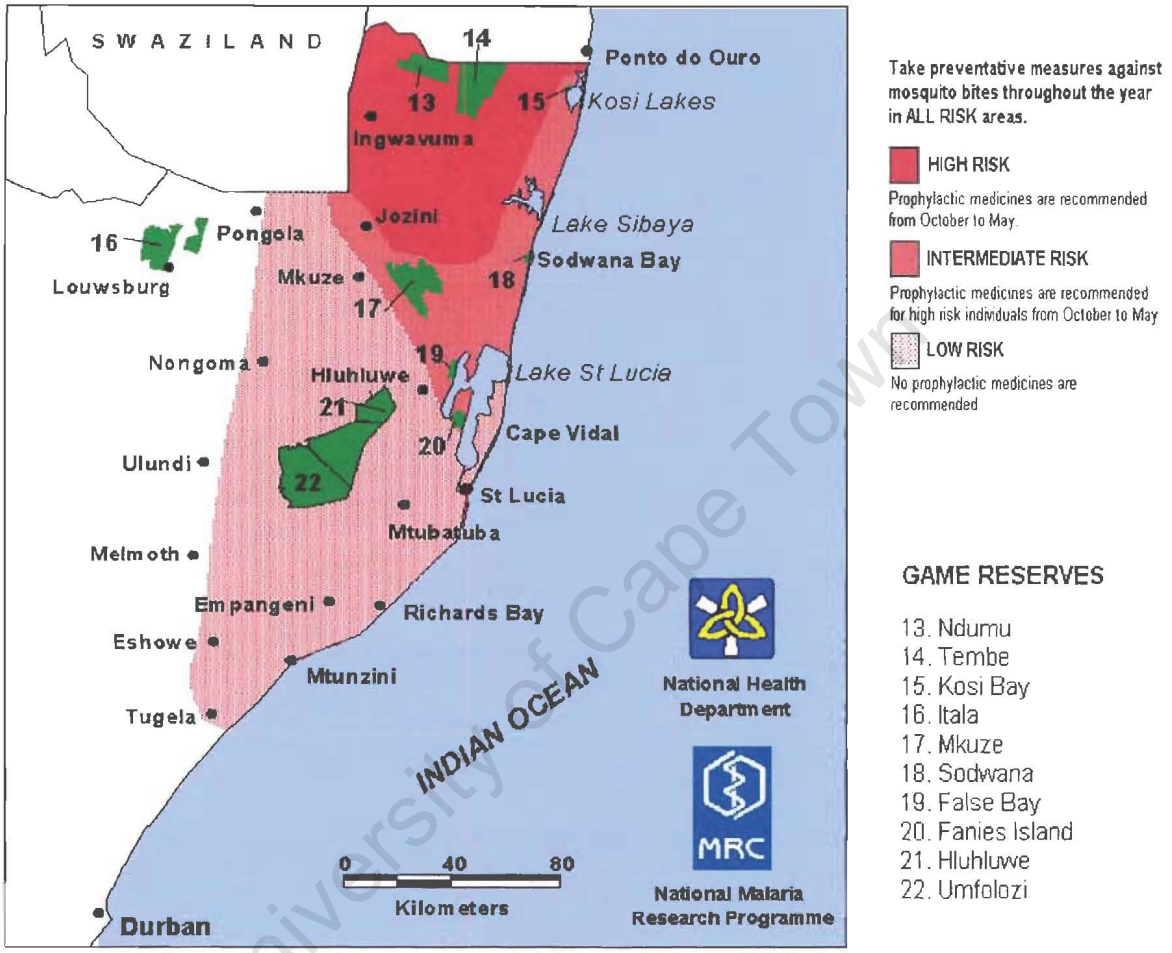


Figure 2.2. Distribution of malaria in KwaZulu-Natal
(http://www.malaria.org.za/malaria-Risk/Cases/cases.html)
Nqutu is +/- 100 km northeast of Mtubatuba and Greytown is +/- 100 km east of Tugela

2.2. Plant Extractions

To extract compounds with different polarities, extractions were carried out in the following sequence: cold and hot water, dichloromethane (Saarchem, South Africa (SA)), cold and hot methanol (AnalaR,

Saarchem, SA). For cold-water extractions, air-dried plant samples (100 g / 200 ml water) were weighed and transferred to conical flasks. The plant samples were left to extract on a 1086K shaker (Labcon) overnight. On completion, the cold aqueous extracts were filtered and the plant residues were loaded into separate soxhlet apparatus for the hot water extraction. This was done for an hour. For dichloromethane extractions, air-dried plant samples (100 g / 200 ml dichloromethane (Saarchem, SA)) were weighed and transferred to conical flasks. The plant samples were left to extract on a 1086K shaker (Labcon) overnight. The dichloromethane extracts were filtered and the plant residues were air-dried to prepare them for the methanol extraction. The air-dried plant residues were extracted with cold methanol (AnalaR, Saarchem, SA) and then left to extract on a 1086K shaker (Labcon) overnight. After these extractions, the methanol extracts were filtered and the plant residues were loaded into separate soxhlet apparatus to carry out hot methanol (AnalaR, Saarchem, SA) extractions.

2.2.1. Concentration of Extracts: Freeze-drying and Rotary Evaporation

Freeze-drying is the dehydration of solvent in a sample. Freeze-drying is used for long-term storage stability of food products, other biological materials (microbial cultures, enzymes, vaccines, blood fractions and pharmaceuticals) (Flink JM et al, 1983). To freeze-dry, aqueous extracts were dispensed into round bottom flasks that were frozen on the freezing roller of the freeze-drier (Model 10-030) (Virtis) until the ice cracked. The temperature of the freeze-drier was at -30 °C and the atmospheric pressure was set at 2000 millittor. The frozen samples were transferred to freeze-drier chambers and freeze-drying was carried on until the extracts were completely dry. To evaporate organic extracts, extracts were dispensed into round-bottom flasks. Extracts were evaporated by

means of reduced pressure and boiling temperature between 30-40 °C on a Buchi evaporator. When most of the solvent had evaporated from the plant material, the remaining syrupy extract was removed by means of a pipette from the flask and then transferred to a glass vial. Extracts were left to dry in a fume- cupboard.

2.3. Cytotoxicity Screening of Plants

2.3.1. Fibroblast Cell Culture

Frozen Rat-1 fibroblasts (Department of Medical Biochemistry, University of Cape Town (UCT)) were thawed and dispensed into a sterile eppendorf tube. The Rat-1 fibroblast was the cell-line of choice since it has been used to evaluate *in vitro* cytotoxicity of cancer drugs in the Department of Medical Biochemistry (UCT). The fibroblasts were first washed with 0.5 ml, 1 ml followed by 2 ml of medium (RPMI-1640, Bio Whitaker) with mixing for 2 minutes during the washes. The eppendorf tube was then filled up with medium (RPMI-1640, Bio Whitaker) and fibroblasts were centrifuged at 1800 rpm for 10 minutes (Universal 16, Hettich).

The supernatant was discarded and fibroblasts were washed for the second time with 20 ml of medium (RPMI-1640, Bio Whitaker) making sure that they were fully suspended. The fibroblasts were centrifuged for another 10 minutes (Universal 16, Hettich). The supernatant was discarded and fibroblasts were resuspended in 4 ml of complete medium (RPMI-1640 supplemented with 15 % fetal calf serum (Highveld Biological, South Africa) and 80 mg/ml gentamicin (Intramed)).

2.3.1.1. Cell Counting

Fibroblasts were diluted 1:1 with counting fluids trypan blue (Sigma) and crystal violet (Sigma) and counted in a haemocytometer on a light microscope (Diavert, Leitz). Each square in a haemocytometer (Sigma) with cover slip in place represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to 1 ml, the subsequent cell concentration per ml (and the total number of cells) will be determined using the following calculations. Cells per ml = the average count per square x dilution factor x 10^4 (count 10 squares) (Sigma Biosciences (Cell Culture Catalogue), 1997). Crystal violet gives an indication of how many cells are available in the suspension; therefore, all the cells on the chosen haemocytometer square are counted. Trypan blue gives an indication of cell viability. Dead cells retain the dye and viable cells do not. Percentage cell viability is determined by substituting counted cells in this equation (Sigma Biosciences (Cell Culture Catalogue), 1997):

$$\text{Cell viability (\%)} = \frac{\text{total viable cells (unstained)}}{\text{Total cells (stained and unstained)}}$$

The total number of fibroblasts was determined using the above equation and 1 x 10^6 fibroblasts were seeded into 75cm^2 flasks (Corning) and 13 ml of complete medium (RPMI-1640 with 15 % fetal calf serum and 80 mg/ml gentamicin (Intramed)) was added. The fibroblasts were incubated at 37°C for 48 hours in an Autoflow CO_2 water-jacketed incubator (Nuaire).

2.3.1.2. Change of Medium

The fibroblast culture was examined for signs of contamination and deterioration. Half the medium in the flask was discarded and the other half left in the flask. This was necessary to preserve growth factors released by growing fibroblasts. Fresh warm medium was added and the flask (Corning) was returned to the incubator (RPMI-1640 with 15 % FCS and 80 mg/ml gentamicin (Intramed)).

2.3.1.3. Subculture

When cells have reached confluency, or when cell density exceeds the capacity of the flask, either the frequency of the medium changing must increase or cells must be divided. The usual practice in sub-culturing an adherent cell line involves removal of the medium and dissociation of the cells in the monolayer with trypsin (Freshney RI et al, 1994). Medium was withdrawn from the flask and then discarded. The fibroblast monolayer was rinsed with warm phosphate buffered saline (PBS) (Oxoid) that was then discarded. This step was necessary to remove traces of serum that would inhibit the action of trypsin.

Three ml of 0.25 % trypsin (Highveld Biological, SA) was mixed with 3 ml of 0.1 % EDTA (Highveld Biological, SA). This mixture was added to the sides of the flask opposite the fibroblast monolayer. The flask was overturned to ensure lifting of the fibroblast monolayer. The flask was returned to the incubator for 2 minutes. Six ml of complete medium was added to the flask, fibroblasts were resuspended and then transferred to a centrifuge tube. The fibroblasts were centrifuged at 1800 rpm (Universal 16, Hettich) for 10 minutes. The supernatant was discarded and fibroblasts were resuspended in 2 ml of complete medium (RPMI-1640 supplemented with 15 % fetal calf serum).

The fibroblasts were counted in a haemocytometer with crystal violet (Sigma). 1 x 10^6 fibroblasts were seeded into new flasks (Corning) until they were confluent. Confluent fibroblasts were used in plant cytotoxicity assays.

2.3.2. Cytotoxicity Assays

Quantification of plasma membrane damage has been used as a measure of cell death that would indicate toxicity of the tested compound. Widely used standard methods are based on the uptake or exclusion of vital dyes like trypan blue, eosin Y, nigrosine, propidium iodide or ethidium bromide (Boehringer-Mannheim (Cytotoxicity Detection Kit Manual), 1994). Dead and viable cells are discriminated by differential staining and counted using a light or fluorescence microscope. These methods are problematic since they do not allow the processing of large sample numbers and do not account for dead cells that may have lysed. Thus, the actual rate of cell death in long-term cultures can be underestimated.

Measurement of cytoplasmic enzyme activity released by damaged cells (enzyme release assays) has been described for alkaline and acid phosphatase, glutamate-oxaloacetate transaminase, and glutamate pyruvate transaminase and arginosuccinate lyase. However, their use has been hampered by the low amount of those enzymes present in many cells and by the elaborate kinetic assays required to quantitate most enzyme activities (Boehringer-Mannheim (Cytotoxicity Detection Kit Manual), 1994). In contrast to the above-mentioned cytoplasmic enzymes, lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the culture supernatant upon damage of the plasma membrane. With the use of the Cytotoxicity Detection Kit (Boehringer Mannheim, Germany), LDH activity can easily

be measured in culture supernatants by a single measurement at one time point.

2.3.2.1. Determination of Optimal Target Cell Concentration for Rat-1 fibroblasts

Different cell types may contain different amounts of LDH. Therefore, the optimum cell concentration for a specific cell type should be determined in a preliminary experiment. In general, this cell concentration in which the difference between the low and high control is at a maximum should be used for the subsequent assay. With most cell lines the optimal target cell concentration is between 0.5 - 2 x 10^4 cells/well in 200 µl (=0.25-1 x 10^5 cells/ml) (Boehringer-Mannheim (Cytotoxicity Detection Kit Manual), 1994). Rat-1 fibroblasts were trypsinised and counted with crystal violet (Sigma). Fibroblast density was adjusted to 2 x 10^6 cells/ml and two fold serial dilutions were carried out. Fibroblast density ranged from 2 x 10^5 cells/well- 3.125 x 10^3 cells/well. The 96-well plate (Costar, Sterilab) was filled up with 100 µl of medium (RPMI –1640, Bio Whitaker) supplemented with either 1 or 3 % fetal calf serum (Highveld Biological, SA).

Table 2.1, p. 15 shows the plate set-up for the assay. The controls were: low control- for determination of spontaneous LDH release from cells; background control-blank that measures presence of LDH in the medium; high control-for measurement of maximal LDH release from cells. Fibroblasts were incubated for 24 hours at 37°C in an Autoflow CO_2 water-jacketed incubator (Nuaire). Thirty minutes prior to stopping the culture to determine LDH activity, 100 µl of Triton X-100 (Merck) was added (2 % in assay medium final concentration in well 1 %). Triton X-100 (Merck) lysed the fibroblasts thus providing maximal LDH release. To determine LDH activity the 96-well plate was centrifuged at 250-x g

(Heraeus, Sepatech) for 10 minutes and the supernatant (100 µl per test well) was harvested and transferred onto clear optical 96-well plates (Costar-Sterilab). Hundred µl of reaction mixture made up of 250 µl of bottle 1 (catalyst) and 11.25 ml of bottle 2 (dye solution) (Boehringer-Mannheim) was added to the wells and the colour reaction was allowed to develop in the dark for 30 minutes. The absorbance of the samples was measured at 492 nm and reference wavelength at 600 nm using an ELISA-plate reader (SLT-Spectra).

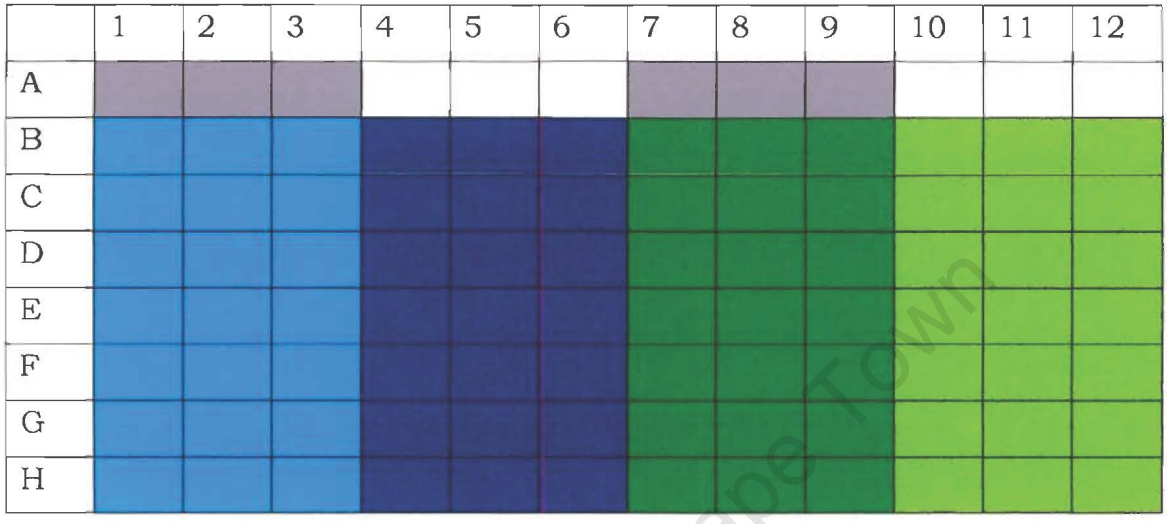
2.3.2.2. Screening of Plant Extracts

Thirty-four plant extracts from the 9 selected plants were screened for cytotoxicity against Rat-1 fibroblasts. Confluent fibroblasts from continuous culture were trypsinised and centrifuged at 1800 rpm for 10 minutes (Universal 16, Hettich), and then resuspended in 1 % RPMI-1640 (RPMI-1640 supplemented with 1 % fetal calf serum). Fibroblasts were counted in a haemocytometer with crystal violet (Sigma) and adjusted to a density of 5 x 10^5 cells/ml in RPMI-1640 supplemented with 1 % fetal calf serum and plated onto a 96-well plate (Costar, Sterilab). Fibroblasts were incubated for 24 hours at 37°C on an Autoflow CO_2 water-jacketed incubator (Nuaire) for good attachment. Old medium was aspirated from fibroblasts and replaced with 100 µl of fresh medium 1 % RPMI-1640 (RPMI-1640 supplemented with 1 % fetal calf serum).

2.3.2.2.1. Preparation of Extracts and Controls

Two mg aqueous plant extracts were dissolved in 1ml PBS (Oxoid) and filter-sterilised with 0.45 µm filters (Millipore). Two mg methanol extracts were dissolved in 200 µl of methanol (Merck) and made up to 1 ml with RPMI-1640 medium i.e. 20 % methanol concentration.

Two mg dichloromethane extracts were dissolved in 200 µl of dimethyl-sulfoxide (DMSO) (Merck) and made up to 1 ml with (RPMI-1640, Bio-Whitaker) medium i.e. 20 % DMSO concentration



	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Key:

Background control (Medium supplemented with 1 and 3 % fetal calf serum (FCS)) for Rows 1-3 and Rows 7-9 respectively

Low control for Rat-1 fibroblasts in medium with 1 % FCS

High control for Rat-1 fibroblasts in medium with 1 % FCS

Low control for Rat-1 fibroblasts in medium with 3 % FCS

High control for Rat-1 fibroblasts in medium with 3 % FCS

Table 2.1. The plate set-up for optimal target cell concentration determination

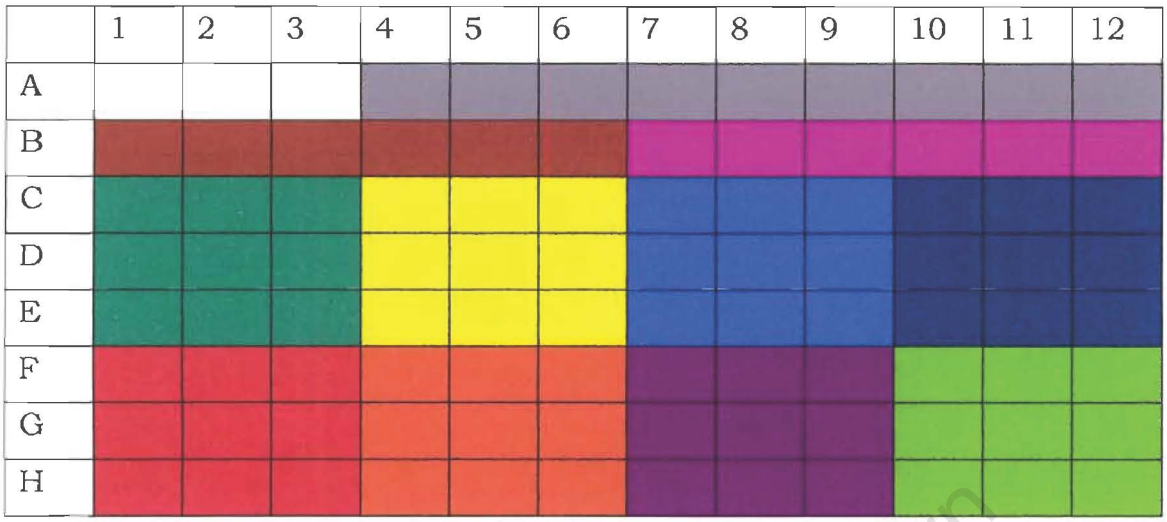
2.3.2.2.2. Plate Set-up

To measure LDH activity from the plant extracts (substance control 1), 100 µl of extracts were added to the fibroblasts at a concentration of 100 µg/ml.

To determine plant cytotoxicity, 100 µl of extracts at concentrations of 1, 10 and 100 µg/ml were added to the test wells i.e. 1/2000, 1/200 and 1/20 dilutions i.e. 1%, 0.1% and 0.01% of the organic solvents. Solvents used to dissolve extracts were also assayed to determine their effect on LDH release from cells. The solvents included PBS, methanol and DMSO. For simplicity, cold and hot water extracts were given the designations WC and WH respectively. Methanol and dichloromethane extracts were given designations M and D, respectively.

A similar plate set-up as the one in table 2.2, p.17 was used for the remaining aqueous extracts, methanol and dichloromethane extracts. The plates were incubated for 48 hours at 37°C in an Autoflow CO_2 water-jacketed incubator (Nuaire). Thirty minutes before analysis of LDH activity, 100 µl of Triton X-100 (Merck) (2 % in assay medium) was added to determine high control. Plates were taken out and centrifuged at 250-x g (Heraeus, Sepatech) for 10 minutes. Hundred µl of supernatant from each test well was harvested and transferred onto clear optical 96-well plates (Costar, Sterilab). LDH activity was determined as explained in section 2.3.2.1, p. 13-14 and absorbance was measured on an Elisa plate reader (SLT-Spectra) at 492 nm with reference wavelength at 600 nm. To determine percentage cytotoxicity, the average absorbance triplicate values were calculated and from these the absorbance value obtained in the background control was subtracted. The following equation was used to calculate cytoxicity (Boehringer-Mannheim (Cytotoxicity Detection Kit), 1994):

$$\text{Cytotoxicity (\%)} = \frac{\text{experimental value-low control}}{\text{High control- low control}} \times 100\%$$



	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Key:

PBS control and background control

Low control and High control

Cold and hot water extracts of *A. oppositofolia*

Cold and hot water extracts of *Z. capense*

Cold and hot water extracts of *G. glabra*

Cold and hot water extracts of *H.caffrum*

Table 2.2. Typical plate set-up for determination of cytotoxicity by aqueous plant extracts

2.4. Lymphocyte Proliferation Assays

Twenty ml of fresh heparinised blood was collected from a healthy individual. Ten ml of ficoll (Sigma) was dispensed into two 50 ml centrifuge tubes and 10 ml of fresh blood was layered on top of the ficoll. Blood was centrifuged at 1800 rpm for 30 minutes (Universal 16, Hettich). The plasma layer was removed with a sterile pipette and the mononuclear cell layer was transferred into a clean centrifuge tube.

Lymphocytes were washed with PBS (Oxoid) and centrifuged at 1800 rpm for 10 minutes (Universal 16, Hettich). The supernatant was discarded and lymphocytes were washed again with PBS and centrifuged for 5 minutes. This step was repeated and lymphocytes were resuspended in RPMI-1640 with Glutamax-1 (Life Technologies) supplemented with 10 % human AB serum (Groote Schuur Hospital, SA) and 80mg/ml gentamicin (Intramed). Lymphocytes were counted in a haemocytometer with trypan blue (Sigma) and density was adjusted to 1 x 10^6 cells/ml in complete medium (RPMI-1640/10 % AB serum/gentamicin (80 mg/ml).

2.4.1. Screening of Plant extracts for Lymphocyte Proliferation

2.4.1.1. Preparation of Extracts and Controls

Two mg of the cold (WC) and hot (WH) water extracts of *Z. capense, G. glabra* and *C. sativa* dissolved in RPMI-1640 medium with Glutamax-1 (Life Technologies) to make up a stock of 2 mg/ml. Extracts were centrifuged at 13 000 rpm for 5 minutes (Biofuge 13, Heraeus, Sepatech). The supernatants were recovered and filter-sterilised with 0.45 µm filters (Millipore). One in ten dilutions of plant extracts were carried out. The mitogen, phytohaemagglutinin (PHA) (Murex Diagnostics) was prepared by 20 in 200 dilution of the stock (5.75 U/ml) to give 0.0575 U/ml before addition to the lymphocytes. The final concentration of the mitogen per well was 0.00575 U/ml (per well volume of 200 µl). This concentration was shown to give maximum stimulation (data not shown).

2.4.1.2. Plate set-up

Hundred µl of lymphocytes at a density of 1 x 10^5 per well were added to each test well. The plate set-up is shown in table 2.3, p. 20. The final volume per well was 200 µl in (RPMI-1640/10 AB serum/gentamicin (80 mg/ml). A similar plate set-up was used for aqueous extracts of *C.*

sativa. The lymphocytes were left to incubate for 72 hours at 37°C in an Autoflow CO_2 water-jacketed incubator (Nuaire).

2.4.1.3. Pulsing and Harvesting of Lymphocytes

After the 72-hour incubation, the lymphocytes were pulse-labelled with 20 µl of [^{3}H] thymidine (1µCi) (Amersham). Eighteen hours later the lymphocytes were harvested onto filter papers using an automated cell harvester (Model CH3 H/W, Insel, England). The harvester aspirates cells and lyses them to extract DNA and transfers it onto a filter paper and unincorporated [^{3}H] thymidine is washed out. The filter papers were oven-dried after/which filter circles were punched out and transferred to scintillation vials filled with 3 ml of scintillation fluid (Zinsser Analytic). The samples were counted on liquid scintillation analyser (TriCarb-2100 TR, Packard).

2.5. Bioassay-guided Fractionation of *Z. capense*

2.5.1. Fractionation according to Molecular Weight

Fractionation according to molecular weight was done so as to determine whether the compound(s) responsible for lymphocyte proliferation, were of high or low molecular weight. One mg of hot-water extract of *Z. capense* was dissolved in 1ml of water and spun at 13 000 rpm for 5 minutes (Biofuge 13, Heraeus, Sepatech) to remove particulate matter. The supernatant was loaded into a size–exclusion filter (Millipore) and centrifuged at 30 000 rpm for 30 minutes. Essentially, compounds with a molecular weight lower than 10 000 MW pass through the filter and collect into the centrifuge tube. Compounds with a molecular weight higher than 10 000 MW are retained in the filter and can be recovered by washing the filter with water.

Fractions recovered from this separation were concentrated by freeze-drying. These fractions were assayed for lymphocyte proliferating activity as described in section 2.4, pages 17-19.

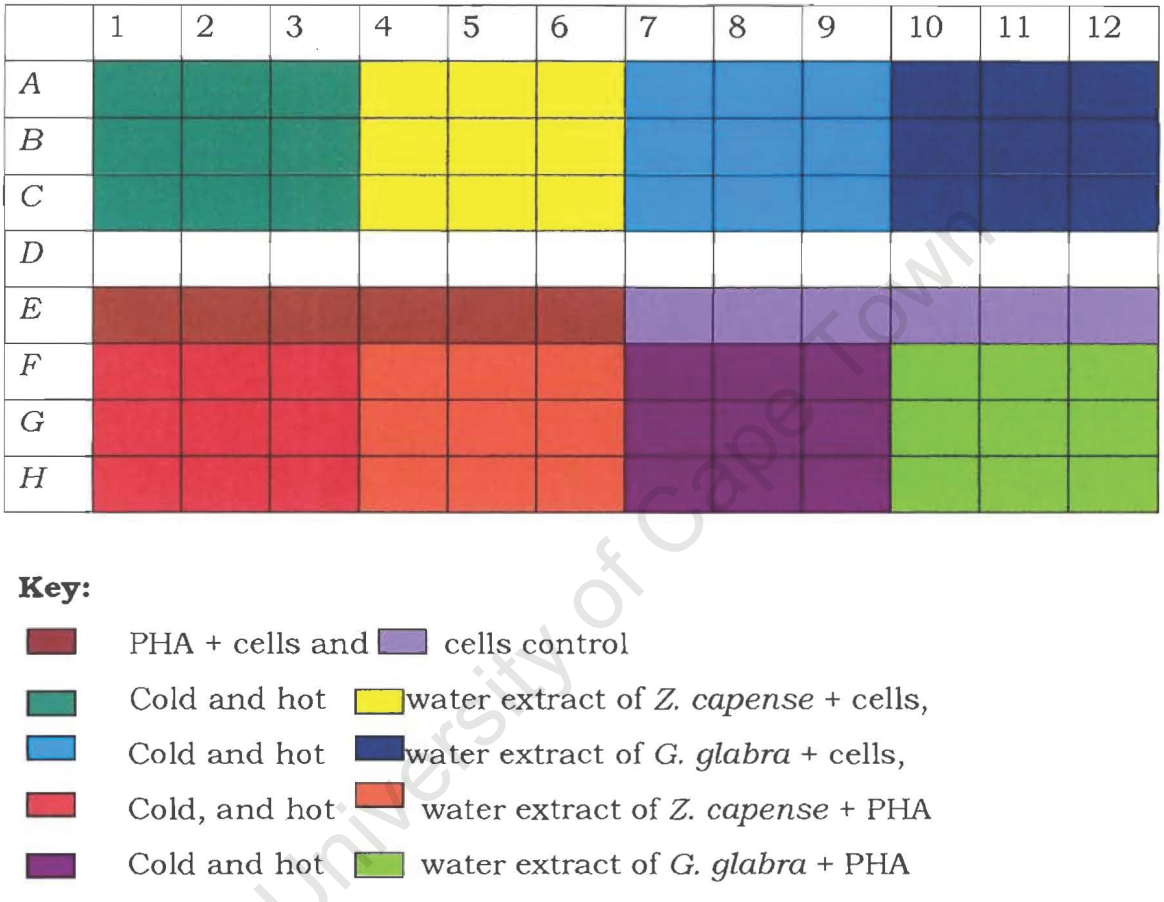


Table 2.3. Plate set-up for cold and hot water extracts of *Z. capense* and *G. glabra* to evaluate lymphocyte proliferating activity.

2.5.2. Solid-Phase Extraction of the hot-water extract of *Z. capense*

Solid-phase extraction (SPE) utilises selective retention for rapid sample preparation. An SPE cartridge consists of a bed of large particulate

adsorbent held between two fritted discs in a disposable tube. In addition, for the rapid purification of small volumes of the sample matrix, the extraction profiles and analytes from a range of SPE cartridges with differing retention mechanisms may classify active metabolites by their different chemistries for prioritisation with regards to isolation work (Macherey-Nagel (Solid Phase Extraction Application Guide). One hundred and twenty mg of plant sample dissolved in 12 ml of water. This sample was centrifuged at 13 000 rpm for 5 minutes (Biofuge 13. Heraeus, Sepatech) to remove particulate matter.

The cartridge (SPE Cartridge (SAX-Ion exchanger, sorbent-SAX, mass 10 g, reservoir volume 70 ml), IST Technologies, UK) was preconditioned by rinsing with 25 ml of methanol followed by 25 ml of water. Six ml (60 mg/ml) of sample was applied on top of the cartridge. The first sample (percolate) was filtered through under light vacuum using a Vac Master (IST Technologies, UK). A series of solvent wash steps were carried out in the order of increasing ionic strength. The wash steps were done in the following order: H_2O, 20 % methanol/80 % H_2O, 1 M, 0.5 M, 1 M and 5 M NaCl followed by 2 M HCl. The washes yielded 8 fractions, which were concentrated by freeze-drying. Since the stimulatory activity was observed in aqueous extracts of *Z. capense* it was therefore decided that we concentrate on the first two fractions (F2 and F3). These fractions were evaluated for lymphocyte proliferating activity as described in section 2.4, pages 17-19.

2.5.3. Phytochemical Analysis

Separation and purification of plant constituents is mainly carried out using one or other, or a combination, of four chromatographic techniques: paper chromatography (PC), thin layer chromatography (TLC), gas liquid chromatography (GLC) and high performance liquid

chromatography (HPLC) (Harborne JB, 1998). The choice of technique depends largely on the solubility properties and volatilities of the compounds to be separated. TLC is the method of choice for separating all lipid-soluble components, i.e. the lipids, steroids, carotenoids, simple quinones and chlorophylls (Harborne JB, 1998). Less volatile constituents can be separated by HPLC, a method that combines column efficiency with speed analysis. Colorimetric phytochemistry was used to investigate the presence of saponins, quinones, tannins, anthraquinones and flavonoids, and thin layer chromatography was used to screen plant extracts for alkaloids and coumarins. High-pressure liquid chromatography (HPLC) was also used to characterise the compounds available in the extract.

2.5.3.1. Colorimetric Phytochemistry and TLC analysis

Saponins

A plant decoction of the hot-water extract (1mg in 10 ml water) of *Z. capense* was boiled for 2-3 minutes and shaken vigorously for 10 seconds in a test tube. This was allowed to settle for 10 minutes and the height of any persistent foam was measured and expressed in mm (Ikhiri K et al, 1992).

Quinones

One mg of the hot water-extract of *Z. capense* was moistened with 10 % HCl solution. This was allowed to stand in diethyl ether-chloroform mixture (3:1 40 ml). The filtrate was treated with 1ml of 10 % NaOH. A red colour was a positive identification for quinones (Ikhiri K et al, 1992).

Tannins

One mg of the hot-water extract of *Z. capense* was boiled in water, cooled and filtered. Few drops of 5 % ferric chloride solution were added to the

filtrate. A bluish-black precipitate indicated the presence of tannins (Ikhiri K et al, 1992).

Anthraquinones

One mg of the hot-water extract of *Z. capense* was shaken in a test tube with 15 % ferric chloride solution and 5 ml HCl. The solution was immersed in a water bath for 10 minutes. This was filtered and cooled. Extraction with 10 ml chloroform was done. The chloroform layer was separated and washed with water. This was shaken with 5 ml of ammonia solution. A rose pink to cherry-red colour in the ammoniacal layer was a positive indication for anthraquinones (Ikhiri K et al, 1992).

Flavonoids

One mg of the hot-water extract of *Z. capense* was boiled for 2-5 minutes in a water bath. This was filtered and to 3 ml of the filtrate; 3 ml of acid alcohol [EtOH: H_2O: HCl 1:1:1], solid magnesium and a few drops of t-amyl alcohol were added. The appearance of a rose-orange or violet colour was a positive indication for flavonoids (Ikhiri K et al, 1992).

Alkaloids

Two hundred µg each of the hot-water extract of *Z. capense* and its solid phase fraction F2 was moistened with 1 ml of 10 % ammonia solution and extracted with 10 ml of methanol and then concentrated to dryness. For TLC analysis, 10 µl of dried extracts (reconstituted in methanol) were spotted onto an aluminium silica gel plate (Merck). For plate development a solvent composition of benzene (BDH) and ethanol (Merck) (9:1) was used. The spots were analysed by spraying with Dragendorff reagent (Appendix, p.81) (Harborne JB, 1998).

Coumarins

Fifty µg each of the hot water extracts *Z. capense* and its solid phase fraction (F2) was dissolved in 100 µl of DMSO (Merck). For TLC analysis, 10 µl of each extract was spotted onto an aluminium silica gel plate (Merck). Chloroform (Merck) was used as the solvent for plate development. To intensify the spots, plates were sprayed with 10 % potassium hydroxide in methanol (Harborne JB, 1998).

2.5.3.2. High Performance Liquid Chromatography (HPLC)

HPLC is a technique that separates mixtures on columns filled with small particles (typically 10 µm or less in diameter) by elution with a liquid under high pressure (Gill R, 1986). The HPLC machine was a Shimadzu (South Africa) equipped with two LC-10AS pumps, a degasser, SIL-10A auto injector and photodiode array detector (SPD-M10A) connected to a flexi 486 DX12-50 desktop PC running software, via a Shimadzu CMB-10A communication bus module. A normal phase (NH_2, 5 µm, 4.6 mm x 250 mm, Waters Spherisorb, SN 98011458, Supelco Inc) column was used with a C18 guard column. The mobile phase was composed of two buffers: Buffer A, containing, acetonitrile/water/ethyl alcohol/acetic acid/0.83 M sodium acetate (800/127/68/2/3) and buffer B (400/400/68/53/79) pH 3.6 (Liscovitch M et al, 1985). The crude hot-water extract of *Z. capense* and solid phase fraction (F2) were prepared by dissolving 100 µg and 1 mg of the two extracts respectively, in 1 ml (50/50 buffer A and B). The extracts were centrifuged at 13 000 rpm for 5 minutes (Biofuge 13, Heraeus, Sepatech) to remove particulate matter. The supernatants were dispensed into HPLC vials. Extracts were injected at 10 µg/ml for the crude hot-water extract and 100 µg/ml for F2. For optimal separation of the fractions an isocratic elution was applied with buffer A (pump B) and buffer B (pump A). The flow rate was 1 ml/min over 60 minutes. The eluates were detected at 274 nm.

3. RESULTS

3.1. Healer's perception about malaria

Most healers in the areas visited understood what malaria was and how to differentiate it from other forms of diseases. The healers could tell if a person showed malaria symptoms. These included shivers, weakness, joint pains and fever. Healers without prior exposure to malaria did not know how to treat the disease, however they indicated that they made referrals to other healers who are more knowledgeable about the disease. One healer from the Mpumalanga province (Mr. Mathaba) had been infected by the malaria parasite before and had used some of his own medicinal concotions to clear the infection. The healers often use the prescribed malaria drugs if and when they are available and would advise their patients to use them as well.

3.2. Plant Selection

Acokanthera oppositofolia, *Zanthoxylum capense*, *Glycyrrhiza glabra*, *Lippia javanica*, *Pentanisia prunelloides* and *Typha capensis* were amongst the plants that were mentioned by healers in the previous survey of antimalarial plants. Healers that were visited for this study mentioned none of these plants. The above-mentioned plants were purchased from the Durban muti market (KwaZulu-Natal). *Psidium guajava* and *Cannabis sativa* were selected on the basis of information gathered from the literature (Gericke N, 1997, Hutchings A, 1996). These two plants were collected from the Western Cape.

3.3. Plant Extractions

Table 3.1 below gives a list of medicinal uses of the plants studied according to *Medicinal Plants of South Africa* by Van Wyk, BE, 1997 and Zulu Medicinal plants by Anne Hutchings A, 1996. Also included are the parts used for the extractions.

Plant Name	Medicinal Uses	Plant parts used	References
A. oppositofolia	Headaches, snakebite, colds, toothache, anthrax and tapeworm	Leaves	Van Wyk BE, 1997, Hutchings A, 1996
Z. capense	Flatulent colic, stomach ache, fever, toothache, mouthwash and epilepsy	Bark	Van Wyk BE, 1997, Hutchings A, 1996
G. glabra	Coughs and stomach ulcers	Rhizomes	Bisset NG, 1994
H. caffrum	Blood purifier or emetics and skin problem treatment	Bark	Van Wyk BE, 1997, Hutchings A, 1996
L. javanica	Coughs, colds, fever, influenza, measles, malaria, headaches and bronchitis	Leaves and Twigs	Van Wyk BE, 1997, Hutchings A, 1996
P. prunelloides	Heartburn, vomiting, fever, tuberculosis, chest pain, and snakebite	Rhizome	Van Wyk BE, 1997, Hutchings A, 1996
P. guajava	Diarrhoea, diabetes, fever, cough, ulcers, boils, wounds and malaria	Leaves	Van Wyk BE, 1997, Hutchings A, 1996
T. capensis	Venereal diseases, diarrhoea, dysentery	Rhizomes	Van Wyk BE, 1997, Hutchings A, 1996
C. sativa	Snakebite, malaria, blood poisoning and glaucoma	Leaves and Twigs	Van Wyk BE, 1997, Hutchings A, 1996

Table 3.1. List of medicinal uses of plants studied and parts used for extractions

Plant Sample	Cold Water	Hot Water	Dichloromethane	Methanol
	% Yield	% Yield	% Yield	% Yield
A. oppositofolia	4.70	2.60	3.60	4.62
Z. capense	3.40	1.80	2.05	7.02
G. glabra	9.40	1.90	9.4	7.50
H. caffrum	1.37	1.80	0.30	1.65
L. javanica	4.50	2.90	1.35	1.80
P. prunelloides	1.20	1.80	2.94	2.80
P. guajava	5.60	1.60	3.90	7.15
T. capensis	6.10	2.50	Nd*	7.96
C. sativa	1.50	1.40	4.50	3.56

Nd- not determinable/ no extract recovered

Table 3.2. Percentage Yields for the plant extractions. This table shows that each and every plant is chemically diverse hence the different yields for the different extraction methods.

3.4. Determination of Optimal Target Cell Concentration for Rat-1 fibroblasts for use in LDH assay

To find out which media composition would be suitable for Rat-1 fibroblasts and sensitive for the LDH assay, fibroblasts were grown in two different compositions of RPMI medium, one supplemented with 1 % fetal calf serum and another composition supplemented with 3 % fetal calf serum. RPMI medium supplemented with 1 % fetal calf serum was the suitable composition. The concentration in which the difference between the high control and low control was maximal (optimal target cell concentration) was determined in the medium supplemented with 1% fetal calf serum. The optimal concentration was 5 x 10^5 cells/ ml (0.5 x 10^5 cells/well. This concentration was used in the cytotoxicity assays.

Number of Cells Per well	Low Control Reading 1	Low Control Reading 2	Low Control Reading 3	High Control Reading 1	High Control Reading 2	High Control Reading 3
3.125 x 10^3	0.115	0.121	0.163	0.142	0.174	0.119
6.25 x 10^3	0.310	0.357	0.168	0.447	0.383	0.330
1.25 x 10^4	0.777	0.831	0.854	0.685	0.625	1.001
2.5 x 10^4	1.016	1.092	------	1.442	1.371	2.037
5 x 10^4	0.816	0.939	------	2.689	2.621	3.265
1 x 10^5	0.793	1.009	------	3.399	3.528	2.691
2 x 10^5	1.132	1.613	------	3.319	2.351	------

Low Control- spontaneous LDH release, High Control- Maximal LDH release- Absorbance measured at 492 nm. Comparison made between low and high control. Results analysed using Prism Software and are shown in figure 3.1 (A) below
(----) indicates missing values from experimental errors.

Table 3.3. Determination of Optimal Target Cell Concentration for Rat-1 fibroblasts using RPMI-1640 with 1 % fetal calf serum

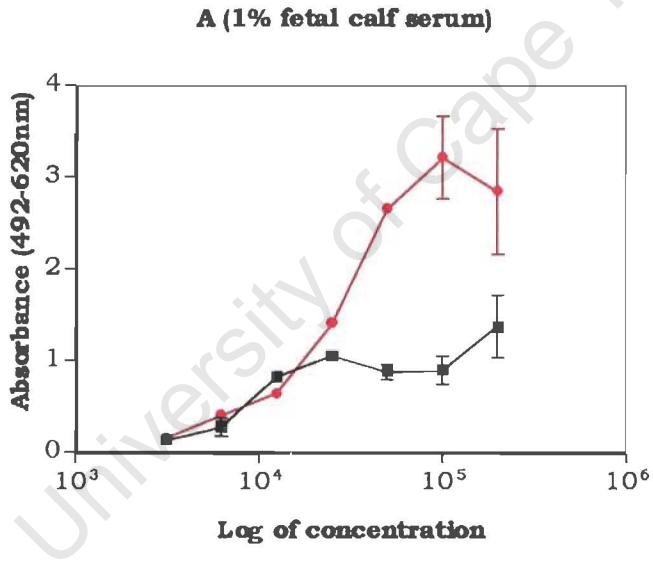


Figure 3.1A. Determination of optimal target cell concentration for Rat-1 fibroblasts using fibroblasts grown in RPMI supplemented with 1 % fetal calf serum. Each point represents the mean of 3 readings and the error bars are standard deviations (SD) of the mean. From this figure it can be seen that RPMI supplemented with 1 % fetal calf serum did not affect lactate dehydrogenase activity. Therefore, the concentration in which the difference between spontaneous LDH release (low control (■)) and maximal LDH release (high control (●) is at a maximum (optimal target cell concentration) could be determined (Boehringer-Mannheim Cytotoxicity Detection Kit, 1994). The optimal target cell concentration for Rat-1 fibroblasts was 0.5 x 10^5 cells/well. This cell concentration was used in the subsequent cytotoxicity assays.

Number of Cells Per well	Low Control Reading 1	Low Control Reading 2	Low Control Reading 3	High Control Reading 1	High Control Reading 2	High Control Reading 3
3.125 x 10^3	0.345	0.350	0.374	0.355	0.359	0.335
6.25 x 10^3	0.456	0.475	0.411	0.520	0.562	0.478
1.25 x 10^4	1.112	1.106	0.880	1.196	1.094	0.966
2.5 x 10^4	2.000	1.937	1.831	1.793	1.862	1.749
5 x 10^4	2.427	2.439	2.565	2.446	2.429	2.425
1 x 10^5	3.415	3.417	3.362	3.346	3.336	3.525
2 x 10^5	3.322	3.359	3.255	3.248	3.274	3.298

Absorbance was measured at 492nm and values were plotted using Prism Software and shown in figure 3.1 (B) below

Table 3.4. Determination of Optimal Target Cell Concentration Rat 1 fibroblasts using RPMI-1640 with 3 % fetal calf serum

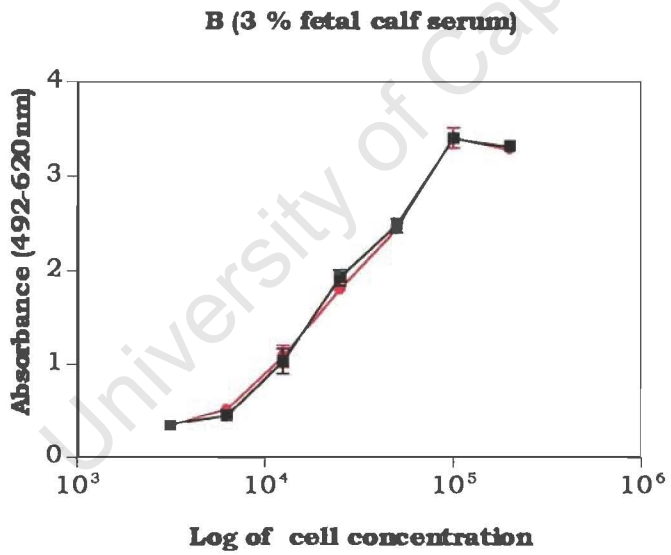


Figure. 3.1B. Determination of optimal target cell concentration for Rat-1 fibroblasts using cells grown in RPMI supplemented with 3 % fetal calf serum. Each point represents the mean of 3 readings and the error bars are standard deviations (SD) of the mean. The high concentration of serum in RPMI supplemented with 3 % fetal calf serum produced high background LDH readings from the low controls. There was no difference between the low (■) and high (●) control. Therefore, the optimal target cell concentration for the Rat-1 fibroblasts could not be determined.

3.5. Cytotoxicity Assays

Thirty-four extracts from 9 selected plants were evaluated for cytotoxicity against Rat-1 fibroblasts. Cell density was set at 5 x 10^5 cells/ml in RPMI supplemented with 1 % fetal calf serum and 80mg/ml gentamicin (Intramed). Extracts were tested at concentrations 1, 10 and 100 µg/ml. Controls included: background control (measurement of LDH contained in the medium), low control (spontaneous LDH release from cells), and high control (maximal LDH release from cells caused by cell burst due to addition of Triton-X-100. Percentage cytotoxicity was determined using the following equation:

$$\text{Cytotoxicity (\%)} = \frac{\text{experimental value - low control}}{\text{high control - low control}} \times 100\%$$

Figures 3.2-3.5, p. 32, 34, 36 and 38 (and corresponding tables) show the effects of cold (WC) and hot (WH) water extracts of *A. oppositofolia*, *Z. capense*, *G. glabra*, *H. caffrum*, *L. javanica*, *P. prunneloides*, *P. guajava*, *T. capensis* and *C. sativa* on Rat-1 fibroblasts *in vitro*. The cold- and hot-water extracts of the plants mentioned above exhibited the least cytotoxic effect (≥ 80 % cell proliferation) at all tested concentrations. In figures 3.6-3.9, p. 40, 42, 44, 46, the dichloromethane extracts of *Z. capense*, *L. javanica*, *P. guajava* and *C. sativa* exhibited a pronounced cytotoxic effect at 100 µg/ml when compared to 1 and 10 µg/ml. Methanol extracts of *Z. capense* and *G. glabra* exhibited dose-dependent cytotoxic effects to the fibroblasts. None of the solvents used to dissolve organic extracts affected LDH release from fibroblasts. Therefore, the concentrations of solvents used to dissolve extracts were considered non-toxic. None of the extracts investigated produced background LDH (data not shown).

Sample	Concentration (µg/ml)	Reading 1	Reading 2	Reading 3	Percentage Cytotoxicity Mean ± SD
Low Control	NA	0.827	NA	NA	NA
High Control	NA	3.04	NA	NA	NA
A. oppositofolia WC	1	0.936 (4.92)	0.810 (-0.77)	0.907 (3.60)	2.58 ± 2.97
	10	0.995 (7.59)	0.767 (-2.71)	0.883 (2.53)	2.49 ± 5.15
	100	1.106 (12.60)	0.899 (3.25)	0.990 (7.36)	7.73 ± 4.68
A. oppositofolia WH	1	0.747 (-3.61)	0.653 (-7.86)	0.785 (-1.89)	-4.45 ± 3.07
	10	0.945 (5.33)	0.841 (0.63)	0.912 (3.84)	3.26 ± 2.40
	100	0.943 (5.24)	1.010 (8.27)	0.879 (2.35)	5.28 ± 2.96
Z. capense WC	1	0.800 (-1.22)	0.796 (-1.40)	0.871 (1.98)	-0.21 ± 1.90
	10	0.875 (2.17)	0.870 (1.94)	0.778 (-2.21)	0.63 ± 2.46
	100	0.703 (-5.60)	0.767 (-2.71)	0.730 (-4.38)	-4.23 ± 1.45
Z. capense WH	1	0.826 (-0.04)	0.855 (1.26)	0.805 (-0.99)	0.07 ± 1.13
	10	0.743 (-3.79)	0.910 (3.75)	0.797 (-1.35)	-0.46 ± 3.84
	100	0.806 (-0.95)	0.775 (-2.35)	0.790 (-1.67)	-1.65 ± 0.70

Absorbance readings reflecting LDH release was measured at 492 nm and blank readings (reading from wells with RPMI medium alone) subtracted from each test value. Low Control- spontaneous LDH release from Rat-1 fibroblasts, High Control- maximal LDH release from fibroblasts. Plant extracts were tested at concentrations 1, 10 and 100 µg/ml. Percentage cytotoxicity (in parentheses) was calculated using the values above and data were analysed using Prism Software. The cytotoxic effects of these plants are shown in figure 3.2, page 32. NA- Not available

Table 3.5. Cytotoxic effects of the cold (WC) and hot (WH) water extracts of *A. oppositofolia* and *Z. capense* on Rat-1 fibroblasts

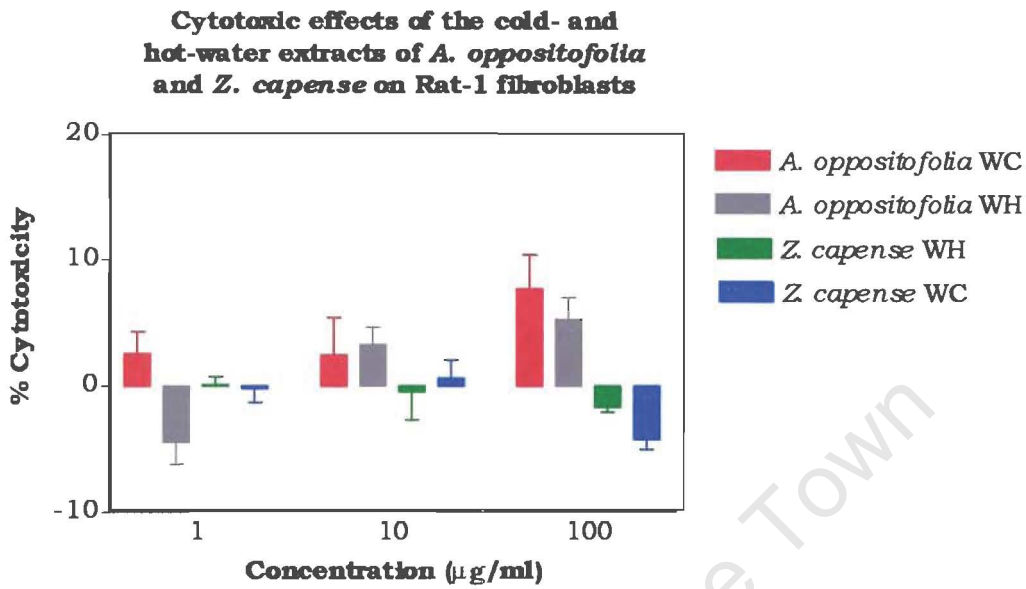


Figure 3.2. Cytotoxic effects of the cold (WC) and hot (WH) water extracts of *A. oppositofolia* and *Z. capense* on Rat-1 fibroblasts. Each point represents the mean of 3 tests and the error bars are standard deviations (SD) of the mean. Both the cold- and hot-water extracts of *A. oppositofolia* exhibited a cytotoxic effect on Rat-1 fibroblasts at the highest concentrations tested (100 µg/ml). Neither extract of *Z. capense* was cytotoxic to the fibroblasts.

Sample	Concentration (µg/ml)	Reading 1	Reading 2	Reading 3	Percentage Cytotoxicity Mean ± SD
Low Control	0.827	NA	NA	NA	NA
High Control	3.04	NA	NA	NA	NA
G. glabra WC	1	0.797 (-13.50)	0.882 (2.48)	0.862 (1.58)	0.90 ± 2.00
	10	0.967 (6.32)	0.894 (3.02)	0.996 (7.63)	5.65 ± 2.37
	100	0.888 (2.75)	0.849 (0.99)	0.807 (-0.90)	0.94 ± 1.82
G. glabra WH	1	0.874 (2.12)	0.808 (-0.85)	0.772 (-2.48)	-0.40 ± 2.33
	10	0.885 (2.62)	0.749 (-3.52)	0.766 (-2.75)	-1.21 ± 3.34
	100	0.814 (-0.58)	0.799 (-1.26)	0.767 (-2.71)	-1.51 ± 1.08
H. caffrum WC	1	0.933 (4.79)	0.816 (-0.49)	0.832 (0.22)	1.50 ± 2.86
	10	0.986 (7.18)	0.899 (3.25)	0.874 (2.12)	4.18 ± 2.65
	100	0.966 (6.28)	0.913 (3.88)	0.942 (5.19)	5.11 ± 1.20
H. caffrum WH	1	0.820 (-0.31)	0891 (2.89)	0.891 (2.89)	1.82 ± 1.84
	10	1.034 (9.35)	0.897 (3.16)	0.902 (3.39)	5.30 ± 3.51
	100	0.888 (2.75)	0.973 (6.59)	0.951 (5.60)	4.98 ± 1.99

Absorbance readings reflecting LDH release was measured at 492 nm and blank readings (reading from wells with RPMI medium alone) subtracted from each test value. Low Control- spontaneous LDH release from Rat-1 fibroblasts, High Control- maximal LDH release from fibroblasts. Plant extracts were tested at concentrations 1, 10 and 100 µg/ml. Percentage cytotoxicity (in parentheses) was calculated using the values above and data were analysed using Prism Software. The cytotoxic effects of these plants are shown in figure 3.3, page 34. NA- Not available

Table 3.6. Cytotoxic effects of the cold (WC) and hot (WH) water extracts of *G. glabra* and *H. caffrum* on Rat-1 fibroblasts

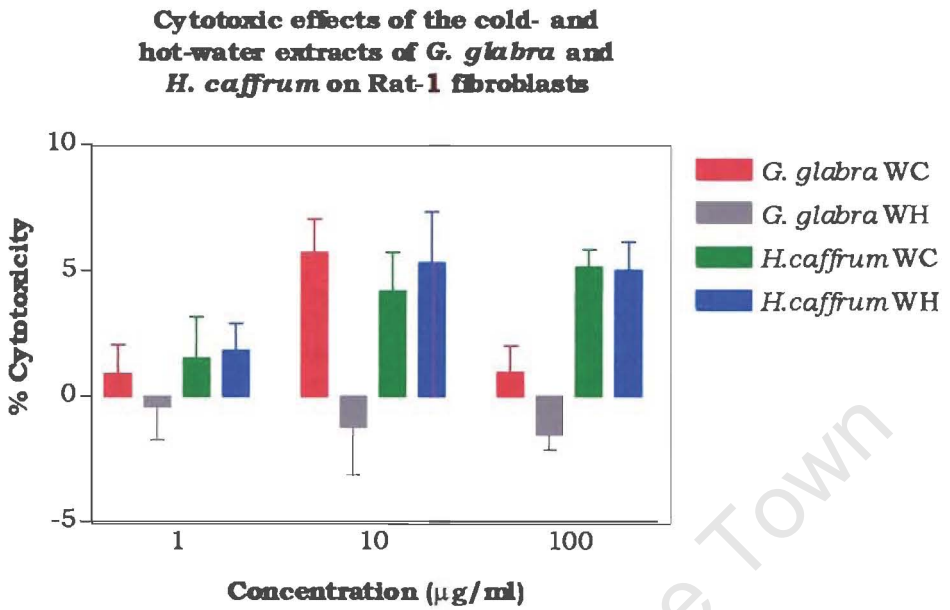


Figure 3.3. Cytotoxic effects of the cold (WC) and hot (WH) water extracts of *G. glabra* and *H. caffrum* on Rat-1 fibroblasts. Each point represents the mean of 3 tests and the error bars are standard deviations (SD) of the mean. The hot-water extract of *G. glabra* was not cytotoxic to the fibroblasts at all concentrations tested. Both the cold- and hot-water extracts of *H. caffrum* exhibited a cytotoxic effect at all concentrations tested.

Sample	Concentration (µg/ml)	Reading 1	Reading 2	Reading 3	% Toxicity (Mean& SD)
Low Control	NA	0.635	NA	NA	NA
High Control	NA	2.852	NA	NA	NA
L. javanica WC	1	0.661 (1.17)	0.751 (5.23)	0.654 (0.85)	2.41 ± 2.44
	10	0.707 (3.24)	0.750 (5.18)	0.692 (2.57)	3.66 ± 1.35
	100	0.705 (3.15)	0.892 (11.59)	0.708 (3.29)	6.01 ± 4.83
L. javanica WH	1	0.772 (6.18)	0.708 (3.29)	0.788 (6.90)	5.45 ± 1.91
	10	0.711 (3.43)	0.729 (4.24)	0.707 (3.24)	3.63 ± 0.53
	100	0.825 (8.57)	0.741 (4.78)	0.790 (6.99)	6.78 ± 1.90
P. prunelloides WC	1	0.674 (1.76)	0.858 (10.06)	0.660 (1.12)	4.31 ± 4.98
	10	0.734 (4.46)	0.713 (3.52)	0.735 (4.51)	4.16 ± 0.55
	100	0.853 (9.83)	0.816 (8.16)	0.828 (8.70)	8.89 ± 0.85
P. prunelloides WH	1	0.719 (3.79)	0.646 (0.49)	0.795 (7.21)	3.83 ± 3.36
	10	0.853 (9.83)	0.717 (3.69)	0.800 (7.44)	6.98 ± 3.09
	100	0.863 (10.28)	0.802 (7.53)	0.894 (11.68)	9.83 ± 2.11

Absorbance readings reflecting LDH release was measured at 492 nm and blank readings (reading from wells with RPMI medium alone) subtracted from each test value. Low Control- spontaneous LDH release from Rat-1 fibroblasts, High Control- maximal LDH release from fibroblasts. Plant extracts were tested at concentrations 1, 10 and 100 µg/ml. Percentage cytotoxicity (in parentheses) was calculated using the values above and data were analysed using Prism Software. The cytotoxic effects of these plants are shown in figure 3.4, page 36. NA- Not available

Table 3.7. Cytotoxic effects of the cold (WC) and hot (WH) water extracts of *L. javanica* and *P. prunelloides* on Rat-1 fibroblasts

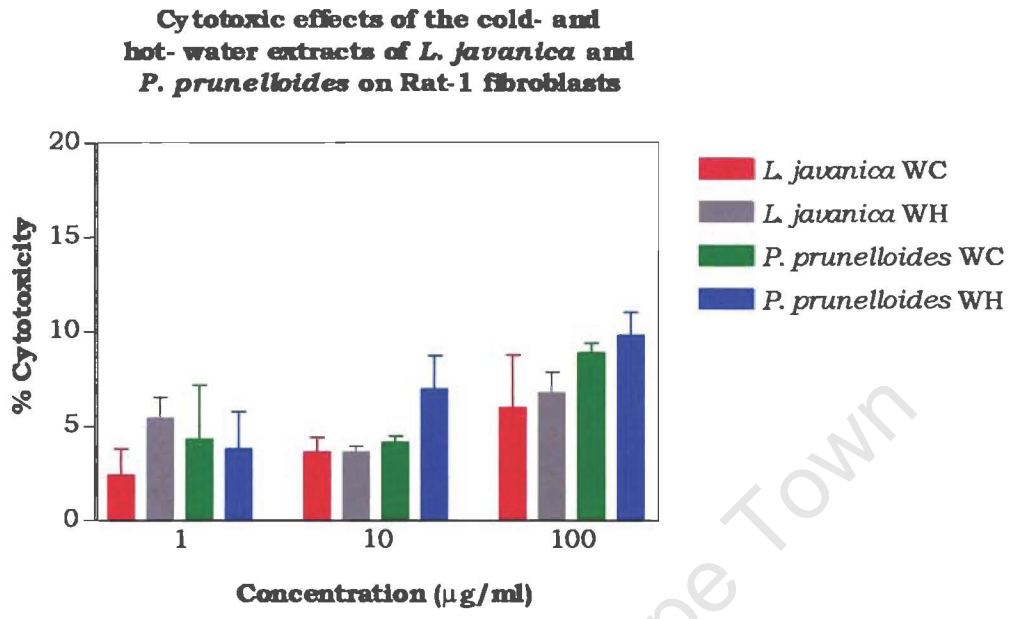


Figure 3.4. Cytotoxic effects of the cold (WC) and hot (WH) water extracts of *L. javanica* and *P. prunelloides* on Rat-1 fibroblasts. Each point represents the mean of 3 tests and the error bars are standard deviations (SD) of the mean. Both the cold- and hot-water extracts of *L. javanica* and *P. prunelloides* exhibited a cytotoxic effect at all concentrations tested.

Sample	Concentration (µg/ml)	Reading 1	Reading 2	Reading 3	Percentage Cytotoxicity Mean ± SD
Low Control	NA	0.635	NA	NA	NA
High Control	NA	2.852	NA	NA	NA
P. guajava WC	1	0.821 (8.36)	0.832 (8.88)	0.761 (5.68)	7.65 ± 1.72
	10	0.794 (7.17)	0.779 (6.49)	0.827 (8.66)	7.44 ± 1.11
	100	1.078 (19.98)	0.975 (15.33)	0.995 (16.23)	17.18 ± 2.46
P. guajava WH	1	0.698 (2.84)	0.709 (3.30)	0.940 (13.75)	6.63 ± 6.17
	10	0.818 (8.25)	0.803 (7.56)	0.783 (6.67)	7.49 ± 0.79
	100	1.061 (19.21)	1.112 (21.51)	1.044 (18.44)	19.72 ± 1.59
T. capensis WC	1	0.674 (1.76)	0.648 (0.58)	0.587 (-2.16)	0.06 ± 2.01
	10	0.689 (2.43)	0.676 (1.85)	0.739 (4.69)	2.99 ± 1.50
	100	0.683 (2.16)	0.683 (2.16)	0.679 (1.98)	2.1 ± 0.10
T. capensis WH	1	0.650 (0.67)	0.655 (0.90)	0.721 (3.88)	1.81 ± 1.79
	10	0.727 (4.15)	0.742 (4.82)	0.672 (1.67)	3.54 ± 1.66
	100	0.692 (2.57)	0.703 (3.06)	0.667 (1.44)	2.35 ± 0.83
Low Control	NA	1.454	NA	NA	NA
High Control	NA	2.36	NA	NA	NA
C. sativa WC	1	1.501 (5.18)	1.521 (7.39)	1.493 (4.30)	5.62 ± 1.59
	10	1.615 (17.77)	1.593 (15.34)	1.568 (12.58)	15.23 ± 2.57
	100	1.519 (7.17)	1.577 (13.57)	1.399 (-6.07)	4.89 ± 10.01
C. sativa WH	1	1.398 (-6.18)	1.488 (3.75)	1.222 (-25.60)	-9.34 ± 14.92
	10	1.436 (-1.98)	1.443 (-1.21)	1.467 (1.43)	-0.58 ± 1.78
	100	1.586 (14.56)	1.484 (3.31)	1.538 (3.56)	8.87 ± 5.62

(Same conditions as in table 3.7, p.35 apply)

Table 3.8. Cytotoxic effects of the cold (WC) and hot (WH) water extracts of *P. guajava*, *T. capensis* and *C. sativa* on Rat-1 fibroblasts

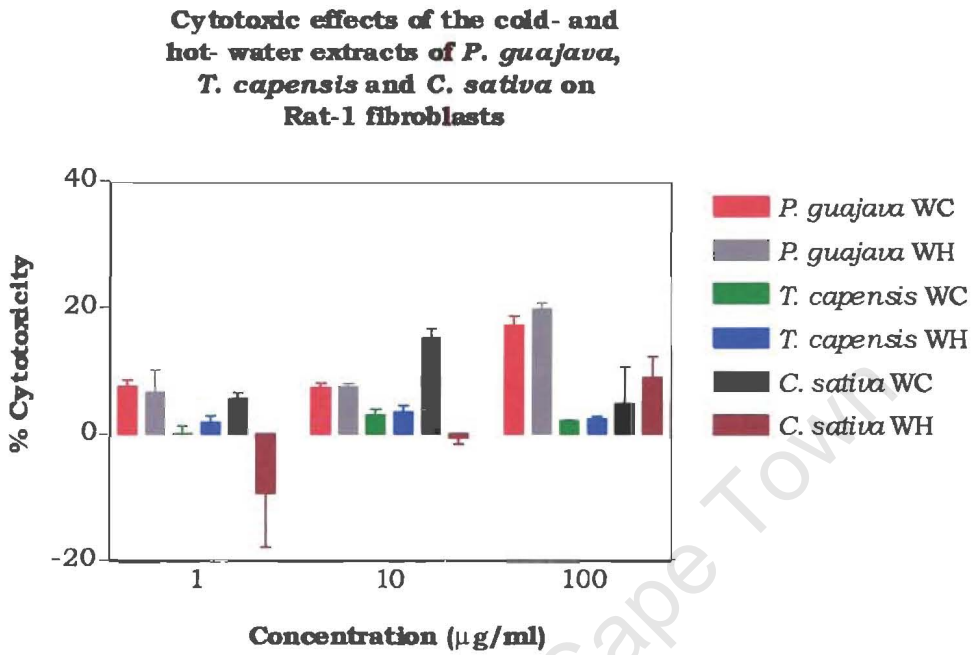


Figure 3.5. Cytotoxic effects of the cold (WC) and hot (WH) water extracts of *P. guajava*, *T. capensis* and *C. sativa* on Rat-1 fibroblasts. Each point represents the mean of 3 tests and the error bars are standard deviations (SD) of the mean. Both the cold- and hot-water extracts of P *guajava* exhibited a cytotoxic effect at all concentrations tested. Both the cold- and hot-water extracts of *T. capensis* and *C. sativa* failed to show a consistent cytotoxic effect at all concentrations tested.

Sample	Concentration (µg/ml)	Reading 1	Reading 2	Reading 3	Percentage Cytotoxicity (Mean& SD)
Low Control	NA	1.454	NA	NA	NA
High Control	NA	2.360	NA	NA	NA
A.oppositofolia M	1	1.291 (-17.99)	1.472 (1.98)	1.432 (-2.43)	-6.14 ± 10.49
	10	1.588 (14.79)	1.487 (3.64)	1.460 (0.66)	6.36 ± 7.45
	100	1.477 (2.54)	1.490 (3.97)	1.231 (-24.61)	-6.03 ± 16.10
A.oppositofolia D	1	1.359 (-10.48)	1.415 (-4.30)	1.406 (-5.29)	-6.69 ± 3.32
	10	1.388 (-7.28)	1.288 (-18.32)	1.272 (-20.08)	-15.22 ± 6.94
	100	1.589 (14.90)	1.595 (15.56)	1.894 (48.56)	26.34 ± 19.24
Z. capense M	1	1.529 (8.28)	1.426 (-3.09)	1.512 (6.40)	3.86 ± 6.09
	10	1.584 (14.34)	1.655 (22.18)	1.532 (8.61)	15.04 ± 6.81
	100	1.755 (33.22)	1.688 (25.82)	2.090 (70.2)	43.08 ± 23.77
Z. capense D	1	1.365 (-9.82)	1.447 (-0.77)	1.440 (-1.54)	-4.04 ± 5.01
	10	1.537 (9.16)	1.550 (10.59)	1.567 (12.47)	10.74 ± 1.66
	100	2.224 (84.98)	2.243 (87.08)	2.383 (102.53)	91.53 ± 9.58

Absorbance readings reflecting LDH release was measured at 492 nm and blank readings (reading from wells with RPMI medium alone) subtracted from each test value. Low Control- spontaneous LDH release from Rat-1 fibroblasts, High Control- maximal LDH release from fibroblasts. Plant extracts were tested at concentrations 1, 10 and 100 µg/ml. Percentage cytotoxicity (in parentheses) was calculated using the values above and data were analysed using Prism Software. The cytotoxic effects of these plants are shown in figure 3.6, page 40. NA- Not available

Table 3.9. Cytotoxic effects of methanol (M) and dichloromethane (D) extracts of *A. oppositofolia* and *Z. capense* on Rat-1 fibroblasts

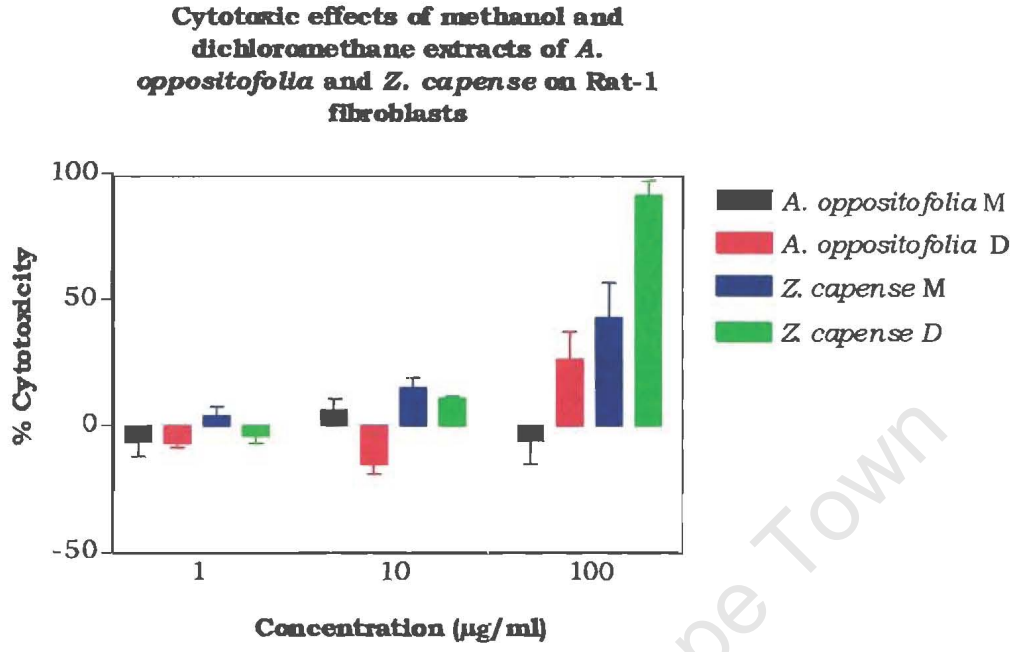


Figure 3.6. Cytotoxic effects of methanol (M) and dichloromethane (D) extracts of *A. oppositofolia* and *Z. capense* on Rat-1 fibroblasts. Each point represents the mean of 3 tests and the error bars are standard deviations (SD) of the mean. The dichloromethane extract of *A. oppositofolia* was slightly cytotoxic at 100 µg/ml, but not at 1 and 10 µg/ml. Both dichloromethane and methanol extracts of *Z. capense* exhibited a dose-dependent cytotoxic effect. The cytotoxic effect was most pronounced for both extracts of *Z. capense* at 100 µg/ml.

Sample	Concentration (µg/ml)	Reading 1	Reading 2	Reading 3	Percentage Cytotoxicity (Mean ± SD)
Low Control	NA	1.400	NA	NA	NA
High Control	NA	2.440	NA	NA	NA
G.glabra M	1	1.468 (6.54)	1.479 (7.59)	1.252 (-14.23)	-0.03 ± 12.30
	10	1.873 (45.48)	1.969 (54.71)	1.848 (43.07)	47.75 ± 6.14
	100	3.032 (156.92)	2.965 (150.48)	2.978 (151.73)	153.04 ± 3.41
G.glabra D	1	1.521 (11.63)	1.490 (8.65)	1.600 (19.23)	13.17 ± 5.45
	10	1.483 (7.98)	1.357 (-4.13)	1.387 (-1.25)	0.86 ± 6.32
	100	1.504 (10.00)	1.475 (7.21)	1.350 (-4.80)	4.13 ± 7.86
H. caffrum M	1	1.482 (7.88)	1.544 (13.84)	1.543 (13.75)	11.82 ± 3.41
	10	1.553 (14.71)	1.538 (13.27)	1.464 (6.51)	11.49 ± 4.37
	100	1.631 (22.21)	1.662 (25.19)	1.538 (13.27)	20.22 ± 6.20
L. javanica M	1	1.452 (5.00)	1.362 (-3.65)	NA	0.67 ± 6.11
	10	1.337 (-6.05)	1.357 (-4.13)	NA	-5.09 ± 1.35
	100	1.610 (20.19)	1.530 (12.50)	1.331 (-6.63)	8.68 ± 13.80
L. javanica D	1	1.044 (-34.23)	1.224 (-16.92)	1.420 (1.92)	-16.41 ± 18.00
	10	1.641 (23.17)	1.367 (-3.17)	1.480 (7.69)	9.23 ± 13.20
	100	3.090 (162.5)	2.994 (153.27)	3.067 (160.29)	158.68 ± 4.82

Absorbance readings reflecting LDH release was measured at 492 nm and blank readings (reading from wells with RPMI medium alone) subtracted from each test value. Low Control- spontaneous LDH release from Rat-1 fibroblasts, High Control- maximal LDH release from fibroblasts. Plant extracts were tested at concentrations 1, 10 and 100 µg/ml. Percentage cytotoxicity (in parentheses) was calculated using the values above and data were analysed using Prism Software. The cytotoxic effects of these plants are shown in figure 3.7, page 42. NA- Not available

Table 3.10. Cytotoxic effects of methanol (M) and dichloromethane (D) extracts of *G. glabra*, *H. caffrum* and *L. javanica* on Rat-1 fibroblasts.

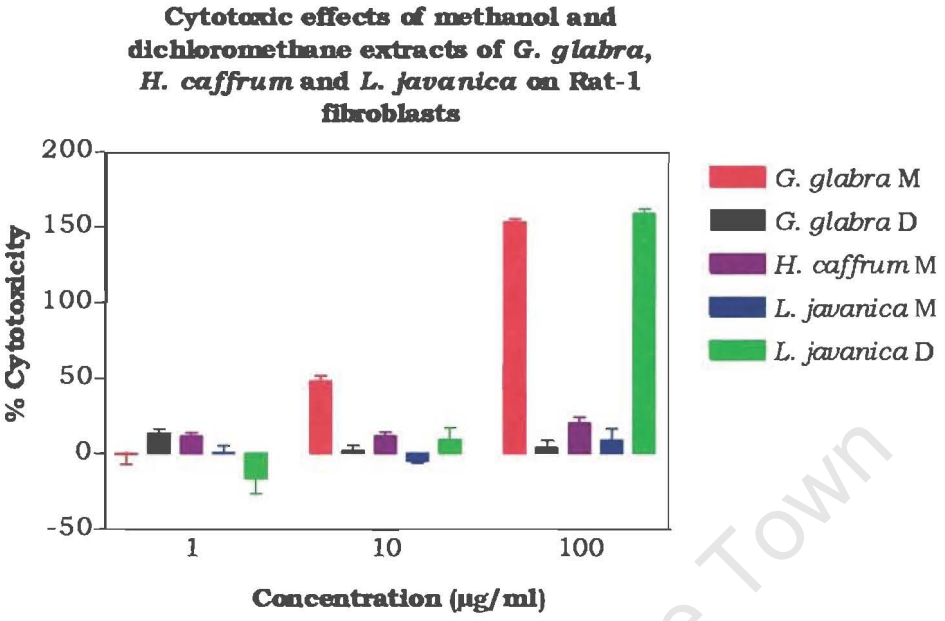


Figure 3.7. Cytotoxic effects of methanol and dichloromethane extracts of *G. glabra*, *H. caffrum* and *L. javanica* on Rat-1 fibroblasts. Each point represents the mean of 3 tests and the error bars are standard deviations (SD) of the mean. The methanol extract of *G. glabra* and dichloromethane extract of *L. javanica* expressed cytotoxicity at 10 and 100 µg/ml, but not at 1 µg/ml.

Sample	Concentration (µg/ml)	Reading 1	Reading 2	Reading 3	%Toxicity (Mean& SD)
Low Control	NA	1.400	NA	NA	NA
High Control	NA	2.440	NA	NA	NA
P. prunelloides M	1	1.561 (15.48)	1.570 (16.34)	1.433 (3.17)	11.66 ± 7.36
	10	1.689 (27.78)	1.557 (15.09)	1.518 (11.34)	18.07 ± 8.61
	100	1.677 (26.63)	1.600 (19.23)	1.536 (16.92)	20.92± 5.07
Low Control	NA	1.410	NA	NA	NA
High Control	NA	2.580	NA	NA	NA
P. prunelloides D	1	1.454 (3.76)	1.440 (2.56)	1.068 (-29.23)	-7.63 ± 18.70
	10	1.371 (-3.33)	1.287 (-10.51)	1.239 (-14.61)	-9.48 ± 5.71
	100	1.843 (37.00)	1.597 (15.98)	1.545 (11.53)	21.50 ± 13.60
P. guajava M	1	1.386 (-2.05)	1.496 (7.35)	1.432 (1.88)	2.39 ± 4.72
	10	1.485 (6.41)	1.451 (3.50)	1.522 (9.57)	6.49 ± 3.03
	100	1.496 (7.35)	1.476 (5.64)	1.443 (2.82)	5.27 ± 2.28
P. guajava D	1	1.378 (-2.73)	1.388 (-1.88)	1.378 (-2.73)	-2.42 ± 0.53
	10	1.366 (-3.76)	1.339 (-6.06)	1.373 (-3.16)	-4.32 ± 1.53
	100	2.246 (86.83)	2.069 (56.32)	1.994 (49.91	64.32 ± 19.72

Absorbance readings reflecting LDH release was measured at 492 nm and blank readings (reading from wells with RPMI medium alone) subtracted from each test value. Low Control- spontaneous LDH release from Rat-1 fibroblasts, High Control- maximal LDH release from fibroblasts. Plant extracts were tested at concentrations 1, 10 and 100 µg/ml. Percentage cytotoxicity (in parentheses) was calculated using the values above and data were analysed using Prism Software. The cytotoxic effects of these plants are shown in figure 3.8, page 44. NA-Not available

Table 3.11. Cytotoxic effects of methanol (M) and dichloromethane (D) extracts of *P. prunelloides* and *P. guajava* on Rat-1 fibroblasts

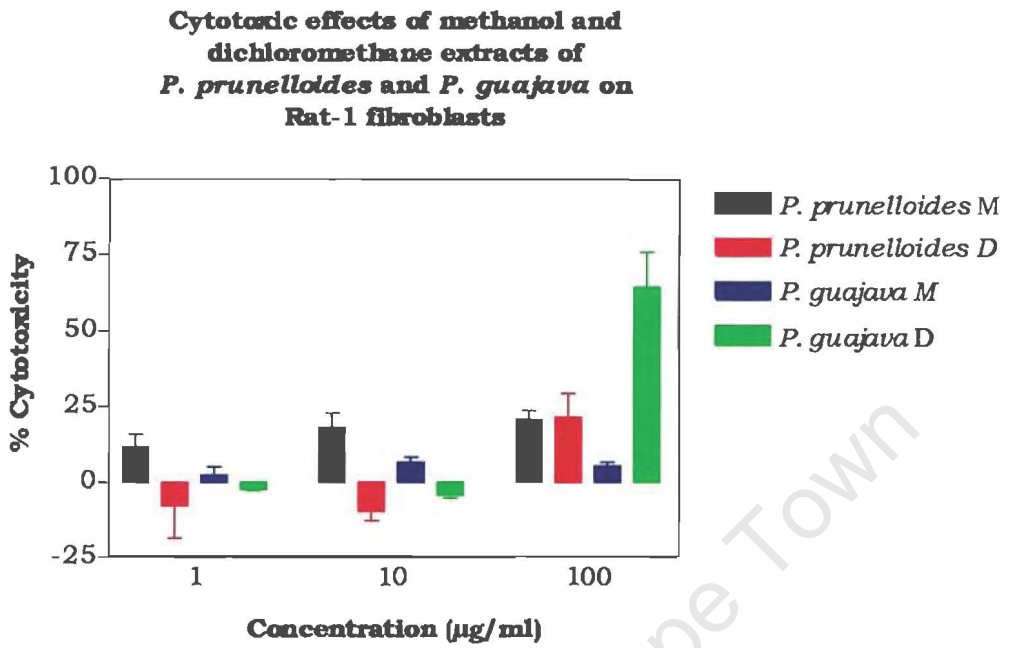


Figure 3.8. Cytotoxic effects of methanol (M) and dichloromethane (C) extracts of *P. prunelloides* and *P. guajava* on Rat-1 fibroblasts. Each point represents the mean of 3 tests and the error bars are standard deviations (SD) of the mean. The dichloromethane extract of *P. prunelloides* showed significant cytotoxicity at 100 µg/ml but not at 1 and 10 µg/ml. The dichloromethane extract of *P. guajava* exhibited a pronounced cytotoxic effect at 100 µg/ml but not at 1 and 10 µg/ml.

Sample	Concentration (µg/ml)	Reading 1	Reading 2	Reading 3	% Toxicity (Mean& SD)
Low Control	NA	1.410	NA	NA	NA
High Control	NA	2.580	NA	NA	NA
T.capensis M	1	1.328 (-7.00)	1.385 (-2.13)	1.211 (-17.00)	-8.71 ± 7.58
	10	1.471 (5.21)	1.345 (-5.55)	1.296 (-9.74)	-3.36 ± 7.71
	100	1.376 (-2.90)	1.372 (-3.24)	1.207 (-17.35)	-7.83 ± 8.24
C. sativa M	1	1.337 (-6.24)	1.473 (5.38)	1.494 (7.18)	2.10 ± 7.28
	10	1.309 (-8.63)	1.368 (-3.58)	1.368 (-3.58)	-5.26 ± 2.91
	100	1.884 (40.51)	1.998 (50.25)	1.795 (32.90)	41.22 ± 8.69
C. sativa D	1	1.356 (-4.61)	1.458 (4.10)	1.347 (-5.38)	-1.96 ± 5.26
	10	1.046 (-31.11)	1.262 (-12.64)	1.172 (-20.34)	-21.36 ± 9.27
	100	2.746 (114.18)	2.795 (118.37)	2.825 (120.94)	117.83 ± 3.41

Absorbance readings reflecting LDH release was measured at 492 nm and blank readings (reading from wells with RPMI medium alone) subtracted from each test value. Low Control- spontaneous LDH release from Rat-1 fibroblasts, High Control- maximal LDH release from fibroblasts. Plant extracts were tested at concentrations 1, 10 and 100 µg/ml. Percentage cytotoxicity (in parentheses) was calculated using the values above and data were analysed using Prism Software. The cytotoxic effects of these plants are shown in figure 3.9, page 46. NA-Not available

Table 3.12. Cytotoxic effects of methanol (M) and dichloromethane (D) extracts of *T. capensis* and *C. sativa* on Rat-1 fibroblasts

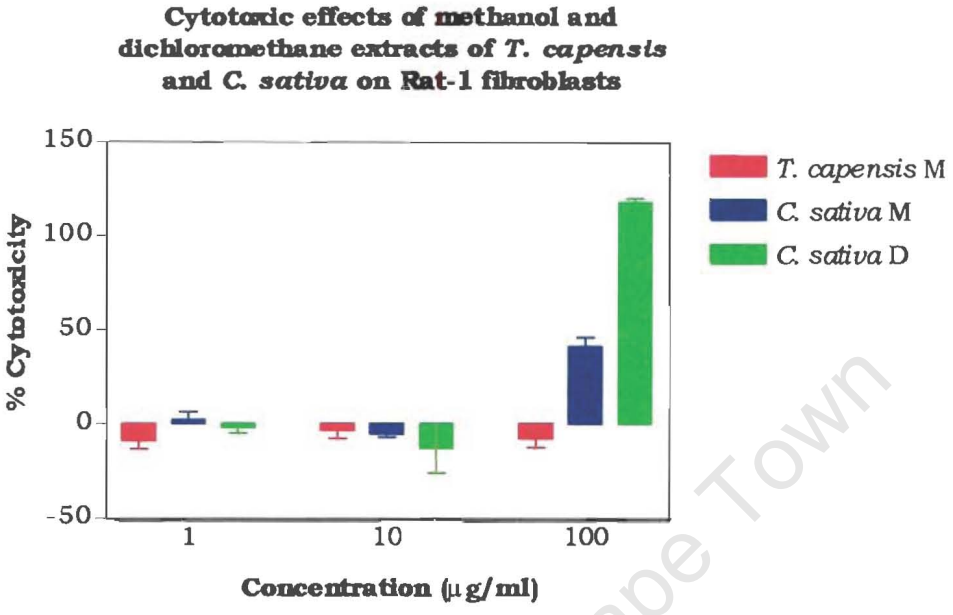


Figure 3.9. Cytotoxic effects of methanol (M) and dichloromethane (D) extracts of *T. capensis* and *C. sativa* on Rat-1 fibroblasts. Each point represents the mean of 3 tests and the error bars are standard deviations (SD) of the mean. The methanol extract of *T.capensis* was not cytotoxic at all concentrations tested. Both methanol and dichloromethane extracts of *C. sativa* exhibited pronounced cytotoxic effect at 100 µg/ml but not at 1 and 10 µg/ml.

3.6. Lymphocyte Proliferation Assays

Cold- and hot-water extracts of *Z. capense*, *G. glabra* and *C. sativa* were assayed for lymphocyte proliferating activity. Two independent experiments were carried out for all the extracts and the means of these experiments were calculated and standard deviations determined. The results of proliferation assays were expressed as relative lymphocyte stimulation, which was calculated by substituting the experimental values in the following equation:

$$\text{Relative Lymphocyte Stimulation} = \frac{\text{DPM in the presence of test extract}}{\text{DPM in the absence of test extract}}$$

Disintegrations per minute (DPM) in the presence of extract would be the DPM value of lymphocytes inoculated with different concentrations of the extract. DPM in the absence of extract would be either the negative control (lymphocytes only) or positive control (lymphocytes with PHA). There was no difference in stimulating activity between the cold- and hot-water extracts of *Z. capense*. The cold-water extract of *Z. capense* stimulated lymphocytes by a factor of 1.37 ± 0.1 at 1 µg/ml. The hot-water extract of *Z. capense* stimulated lymphocytes by a factor of 1.28 ± 0.17 at 1 µg/ml (figure 3.10 (A-B), p. 51). However, this stimulatory effect was lost at 100 µg/ml for both extracts of *Z. capense*. Neither extract of *Z. capense* had an effect on PHA-induced lymphocyte proliferation. The cold- and hot-water extracts of *G. glabra* neither stimulated nor suppressed lymphocytes including when the lymphocytes were stimulated with PHA (figure 3.11 (A-D), p. 54). The hot-water extract of *C. sativa* failed to produce a significant stimulatory effect on non-stimulated and PHA-stimulated lymphocytes (figure 3.12 (A-D), p. 57).

Both low- and high-molecular weight (LMW and HMW) fractions of *Z. capense* (figure 3.13 (A-D), p. 60) failed to produce a stimulatory or suppressive effect on non-stimulated and PHA-stimulated lymphocytes. F2 increased PHA-induced lymphocyte proliferation at 100 μg/ml (Figure 3.14 (A-D), p.63).

Cells Control Data Set 1	*Z. capense* WC 1µg/ml	*Z. capense* WC 10µg/ml	*Z. capense* WC 100µg/ml	Cells Control Data Set 2	*Z. capense* WC 1µg/ml	*Z. capense* WC 10µg/ml	Z. capense WC 100µg/ml
309	701	441	419	404	509	586	391
413	526	385	302	427	685	530	371
355	517	412	341	363	654	429	441
441	598	403	406	308			
669	487	408	359	482			
				561			
Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
437	566	410	366	425	616	515	401
PHA Control Data Set 1	*Z. capense* WC + PHA 1µg/ml	*Z. capense* WC + PHA 10µg/ml	*Z. capense* WC + PHA 100µg/ml	PHA Control Data Set 2	*Z. capense* WC + PHA 1µg/ml	*Z. capense* WC + PHA 10µg/ml	*Z. capense* WC + PHA 100µg/ml
194044	155688	150050	131444	124186	172839	111948	77240
130524	133417	131173	104001	127890	109071	102862	101997
147772	119335	122834	108890	132031	179108	181629	90218
147149	117302	110905	111997	105875			
161290	154045	133014	99047	175211			
				91629			
Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
156156	135958	129595	111076	126137	153673	132146	89818

[^{3}H] (tritiated) thymidine incorporation was measured in a scintillation counter. Readings were expressed as disintegrations per minute (DPM). Cell control-measures the amount of tritiated thymidine incorporated into lymphocytes and PHA control-measures the amount of tritiated thymidine incorporated into PHA-stimulated cells. To determine relative lymphocyte stimulation by extracts on stimulated and non-stimulated lymphocytes, the dpm value for lymphocytes inoculated with extract was divided by the cell control and to determine the effect the extract had on lymphocyte stimulation, the dpm value for extract + PHA + lymphocytes was divided by the dpm value for extract + lymphocytes.

Table 3.13. Effect of the cold-water (WC) extract of *Z. capense* on lymphocytes *in vitro*. Values are expressed in disintegrations per minute (DPM)

Cells Control Data Set 1	*Z. capense* WH 1µg/ml	*Z. capense* WH 10µg/ml	*Z. capense* WH 100µg/ml	Cells Control Data Set 2	*Z. capense* WH 1µg/ml	*Z. capense* WH 10µg/ml	Z. capense WH 100µg/ml
309	648	362	329	404	530	584	386
413	395	382	406	427	522	525	271
355	560	353	371	363	739	888	307
441	470	302	280	308			
669	466	474	490	482			
				561			
Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
437	508	375	375	425	597	666	322
PHA Control Data Set 1	*Z. capense* WH + PHA 1µg/ml	*Z. capense* WH + PHA 10µg/ml	*Z. capense* WH + PHA 100µg/ml	PHA Control Data Set 2	*Z. capense* WH + PHA 1µg/ml	*Z. capense* WH + PHA 10µg/ml	*Z. capense* WH + PHA 100µg/ml
194044	145675	155926	118425	124186	135847	138887	83081
130524	149882	116819	91649	127890	130206	79559	97132
147772	146044	115918	74512	132031	158432	116501	115350
147149	166156	115940	77095	175211			
161290	194409	177023	131980	105875			
				91629			
Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
156156	160433	136325	98732	128036	141495	111649	98521

[^{3}H] (tritiated) thymidine incorporation was measured in a scintillation counter. Readings were expressed as disintegrations per minute (DPM). Cell control-measures the amount of tritiated thymidine incorporated into lymphocytes and PHA control-measures the amount of tritiated thymidine incorporated into PHA-stimulated cells. To determine relative lymphocyte stimulation by extracts on stimulated and non-stimulated lymphocytes, the dpm value for lymphocytes inoculated with extract was divided by the cell control and to determine the effect the extract had on lymphocyte stimulation, the dpm value for extract + PHA + lymphocytes was divided by the dpm value for extract + lymphocytes.

Table 3.14. Effect of the hot-water (WH) extract of *Z. capense* on lymphocytes *in vitro*. Values are expressed in disintegrations per minute (DPM)

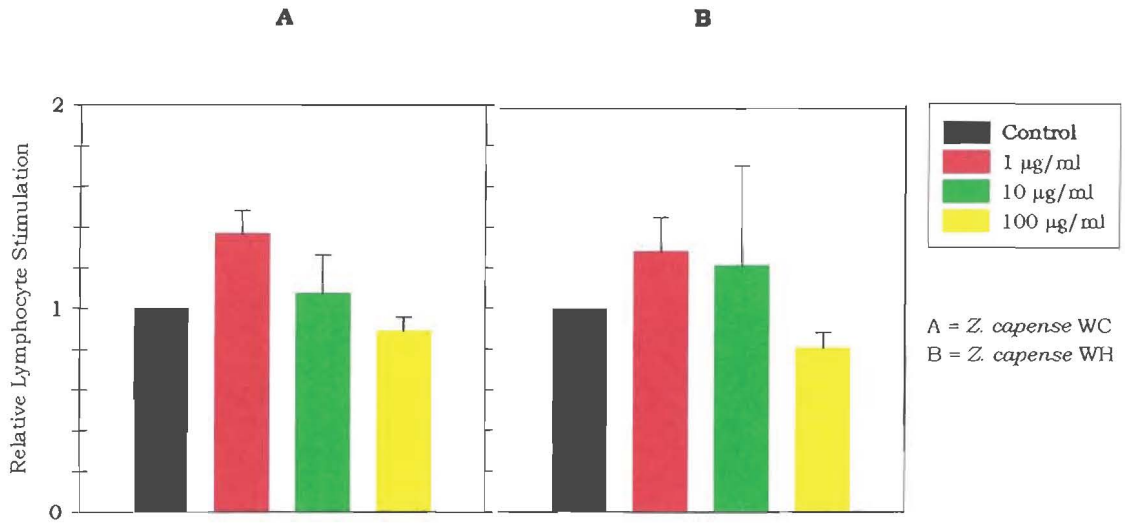


Figure 3.10 (A-B). Effects of the cold (WC) and hot (WH) water extracts of *Z. capense* on lymphocytes *in vitro*. Relative lymphocyte stimulation was calculated relative to the cells control value. Each point represents the mean of two experiments and the error bars are standard deviations of the mean. Both extracts stimulated lymphocytes at 1 µg/ml. However, this stimulatory effect was lost at 100 µg/ml for both extracts.

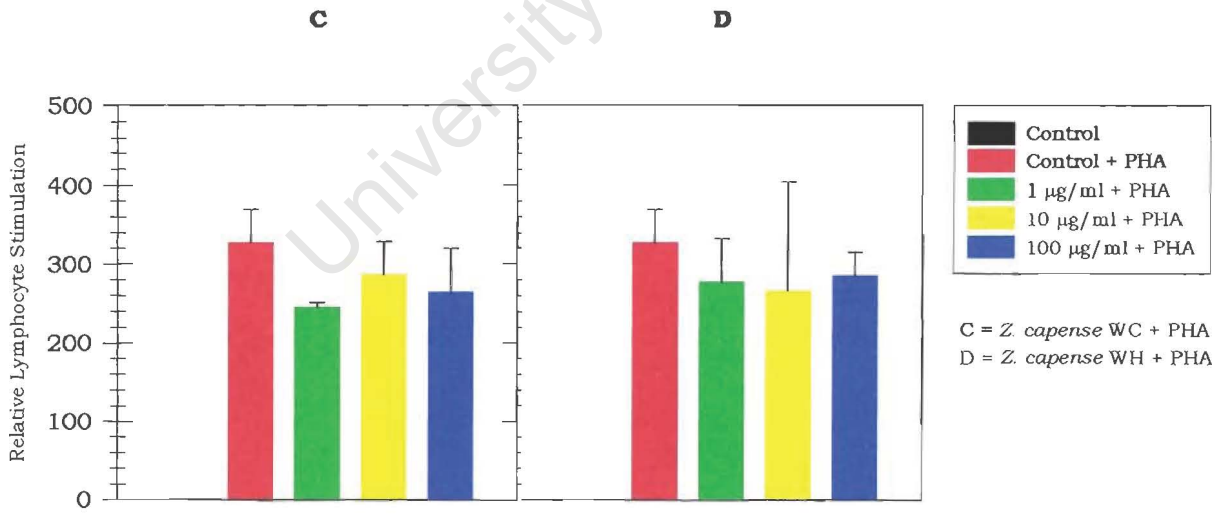


Figure. 3.10 (C-D). Effects of cold (WC) and hot (WH) water extracts of *Z. capense* on PHA-stimulated lymphocytes *in vitro*. Neither extract of *Z. capense* had an effect on PHA-induced lymphocyte proliferation.

Cells Control Data Set 1	*G. glabra* WC 1µg/ml	*G. glabra* WC 10µg/ml	*G. glabra* WC 100µg/ml	Cells Control Data Set 2	*G. glabra* WC 1µg/ml	*G. glabra* WC 10µg/ml	*G. glabra* WC 100µg/ml
597	391	542	515	2028	1324	1696	2899
903	448	542	638	2187	1759	1864	2012
728	359	868	-----	2232	1676	2118	2667
1119				2715	2365	2162	2262
787				1884	3238	3178	2152
517				1939	6569	5688	19049
Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
776	399	651	577	2164	2822	2784	5173
PHA Control Data Set 1	***G. glabra* WC + PHA 1µg/ml**	***G. glabra* WC + PHA 10µg/ml**	***G. glabra* WC + PHA 100µg/ml**	**PHA Control Data Set 2**	***G. glabra* WC + PHA 1µg/ml**	***G. glabra* WC + PHA 10µg/ml**	***G. glabra* WC + PHA 100µg/ml**
43623	48334	64607	41012	286062	337111	326232	330028
47442	61481	43754	44823	250978	303749	327169	312742
47626	59535	44723	111.34	316678	284168	299095	190104
				309395	290871	223563	131165
				317467	278881	295967	275265
				335207	312094	245134	140260
Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
46230	56450	51028	28648	302631	301146	286193	210261

[^{3}H] (tritiated) thymidine incorporation was measured in a scintillation counter. Readings were expressed as disintegrations per minute (DPM). Cell control-measures the amount of tritiated thymidine incorporated into lymphocytes and PHA control-measures the amount of tritiated thymidine incorporated into PHA-stimulated cells. To determine relative lymphocyte stimulation by extracts on stimulated and non-stimulated lymphocytes, the dpm value for lymphocytes inoculated with extract was divided by the cell control and to determine the effect the extract had on lymphocyte stimulation, the dpm value for extract + PHA + lymphocytes was divided by the dpm value for extract + lymphocytes.

Table 3.15. Effect of the cold-water (WC) extract of *G. glabra* on lymphocytes *in vitro*. Values are expressed in disintegrations per minute (DPM)

Cells Control Data Set 1	*G. glabra* WH 1µg/ml	*G. glabra* WH 10µg/ml	*G. glabra* WH 100µg/ml	Cells Control Data Set 2	*G. glabra* WH 1µg/ml	*G. glabra* WH 10µg/ml	*G. glabra* WH 100µg/ml
597	445	339	561	2028	1061	2093	1344
903	493	425	486	2187	2112	1378	1238
728	696	515	669	2232	2389	1530	762
1119				2715	1846	2326	978
787				1884	1905	2373	1041
517				1939	1326	2670	1403
Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
776	545	426	572	2164	1773	2062	1128
PHA Control Data Set 1	*G. glabra* WH + PHA 1µg/ml	*G. glabra* WH+ PHA 10µg/ml	*G. glabra* WH + PHA 100µg/ml	PHA Control Data Set 2	*G. glabra* WH + PHA 1µg/ml	*G. glabra* WH + PHA 10µg/ml	*G. glabra* WH + PHA 100µg/ml
43623	49920	57115	49732	286062	301370	129026	190185
47442	35906	57779	72423	250978	202884	274541	250291
47626	58798	66674	43015	316678	311069	293564	264424
				317467	333866	336790	124544
				335207	617311	486	9442
				309395	3602	5938	20535
Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
46230	48208	60523	55056	302631	295017	173391	143237

[^{3}H] (tritiated) thymidine incorporation was measured in a scintillation counter. Readings were expressed as disintegrations per minute (DPM). Cell control-measures the amount of tritiated thymidine incorporated into lymphocytes and PHA control-measures the amount of tritiated thymidine incorporated into PHA-stimulated cells. To determine relative lymphocyte stimulation by extracts on stimulated and non-stimulated lymphocytes, the dpm value for lymphocytes inoculated with extract was divided by the cell control and to determine the effect the extract had on lymphocyte stimulation, the dpm value for extract + PHA + lymphocytes was divided by the dpm value for extract + lymphocytes.

Table 3.16. Effect of the hot-water (WH) extract of *G. glabra* on lymphocytes *in vitro*. Values are expressed in disintegrations per minute (DPM)

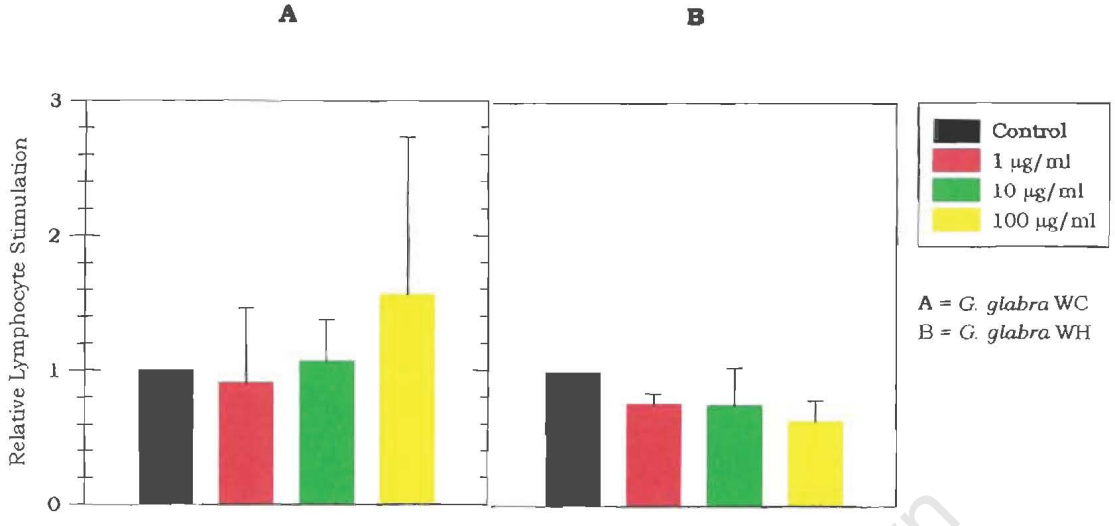


Figure 3.11 (A-B). Effects of the cold (WC) and hot (WH) water extracts of *G. glabra* on lymphocytes *in vitro*. Values are counted relative to the DPM value of cell control. Each point represents the mean of two experiments and the error bars are standard deviations (SD) of the mean. Neither extract of *G. glabra* had a stimulatory or suppressive effect on lymphocytes.

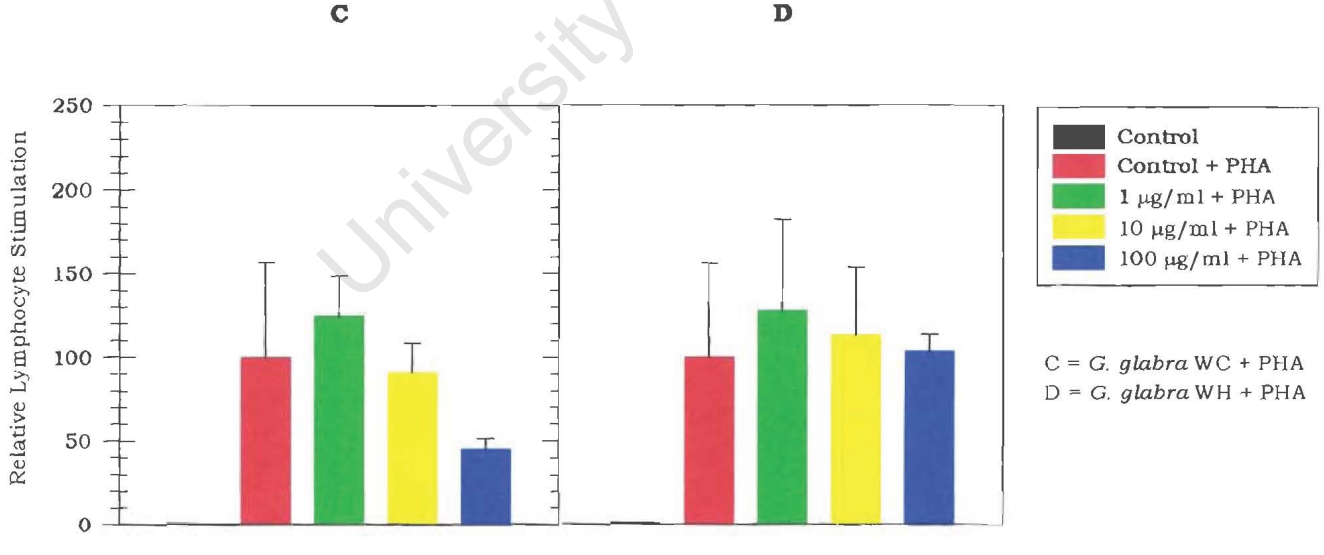


Figure 3.11 (C-D). Effects of the cold (WC) and hot (WH) water extracts of *G. glabra* on PHA-stimulated lymphocytes *in vitro*. Each bar represents the mean of two experiments and the error bars are standard deviations of the mean. Neither extract of *G. glabra* had a stimulatory or suppressive effect on PHA-induced lymphocyte proliferation

Cells Control Data Set 1	*C. sativa* WC 1µg/ml	*C. sativa* WC 10µg/ml	*C. sativa* WC 100µg/ml	Cells Control Data Set 2	*C. sativa* WC 1µg/ml	*C. sativa* WC 10µg/ml	*C. sativa* WC 100µg/ml
653	600	544	56	1593	1545	3166	1360
1169	694	601	101	1495	1383	1404	1879
639	777	681	71	1135	2264	3200	2048
				942	1476	2602	2541
				1407	1143	3218	2099
				4061	2704	4347	550
Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
820	690	609	76	1772	1753	2989	1746
PHA Control Data Set 1	*C. sativa* WC + PHA 1µg/ml	*C. sativa* WC + PHA 10µg/ml	*C. sativa* WC + PHA 100µg/ml	PHA Control Data Set 2	*C. sativa* WC + PHA 1µg/ml	*C. sativa* WC + PHA 10µg/ml	*C. sativa* WC + PHA 100µg/ml
47545	59331	74615	86410	301212	263874	363591	235717
43135	80539	57783	52600	265959	290611	333306	320456
48165	84505	57845	58129	310495	314758	290719	296103
				298278	293953	279170	280086
				366139	320266	346680	322503
Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
46282	74791	63414	65713	307010	296692	315611	298750

[^{3}H] (tritiated) thymidine incorporation was measured in a scintillation counter. Readings were expressed as disintegrations per minute (DPM). Cell control-measures the amount of tritiated thymidine incorporated into lymphocytes and PHA control-measures the amount of tritiated thymidine incorporated into PHA-stimulated cells. To determine relative lymphocyte stimulation by extracts on stimulated and non-stimulated lymphocytes, the dpm value for lymphocytes inoculated with extract was divided by the cell control and to determine the effect the extract had on lymphocyte stimulation, the dpm value for extract + PHA + lymphocytes was divided by the dpm value for extract + lymphocytes.

Table 3.17. Effect of the cold-water (WC) extract of *C. sativa* on lymphocytes *in vitro*. Values are expressed in disintegrations per minute (DPM)

Cells Control Data Set 1	*C. sativa* WH 1µg/ml	*C. sativa* WH 10µg/ml	*C. sativa* WH 100µg/ml	Cells Control Data Set 2	*C. sativa* WH 1µg/ml	*C. sativa* WH 10µg/ml	*C. sativa* WH 100µg/ml
653	1003	834	981	1593	2519	1968	4638
1169	518	2159	747	1495	1366	1324	2440
639	522	3004	907	1135	2557	1422	2344
				942	2814	1251	3870
				1407	1499	1545	5541
				4061	1669	1880	2440
Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
820	681	1999	878	1772	2071	1565	3538
PHA Control Data Set 1	*C. sativa* WH 1µg/ml	*C. sativa* WH 10µg/ml	*C. sativa* WH 100µg/ml	PHA Control Data Set 2	*C. sativa* WH 1µg/ml	*C. sativa* WH 10µg/ml	*C. sativa* WH 100µg/ml
47545	60206	69299	54620	301212	316833	275637	282088
43135	90998	66401	45926	265959	348682	302832	288176
48165	48841	64497	960	310495	303618	313281	324493
				298278	315491	276009	296190
				366139	295969	283144	288082
				299975	322898	293880	345020
Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
46282	66681	66732	33835	307010	317249	290797	304141

[^{3}H] (tritiated) thymidine incorporation was measured in a scintillation counter. Readings were expressed as disintegrations per minute (DPM). Cell control-measures the amount of tritiated thymidine incorporated into lymphocytes and PHA control-measures the amount of tritiated thymidine incorporated into PHA-stimulated cells. To determine relative lymphocyte stimulation by extracts on stimulated and non-stimulated lymphocytes, the dpm value for lymphocytes inoculated with extract was divided by the cell control and to determine the effect the extract had on lymphocyte stimulation, the dpm value for extract + PHA + lymphocytes was divided by the dpm value for extract + lymphocytes.

Table 3.18. Effect of the hot-water (WH) extract of *C. sativa* on lymphocytes *in vitro*. Values are expressed in disintegrations per minute (DPM)

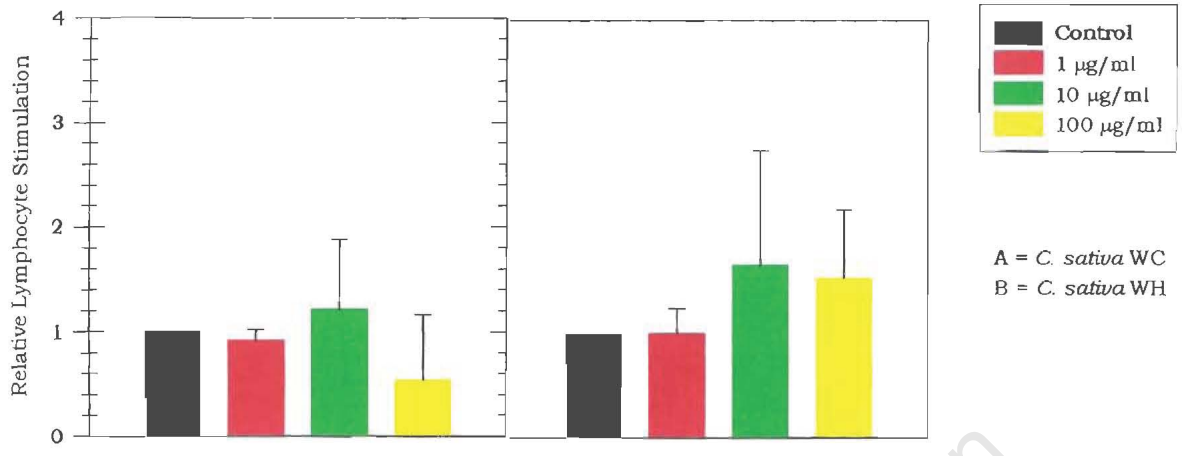


Figure 3.12 (A-B). Effects of the cold (WC) and hot (WH) water extracts of *C. sativa* on lymphocytes *in vitro*. Values are counted relative to the DPM value of cell control. Each point represents the mean of two experiments and the error bars are standard deviations (SD) of the mean. The hot-water extract of *C. sativa* failed to produce a significant stimulatory effect on lymphocytes

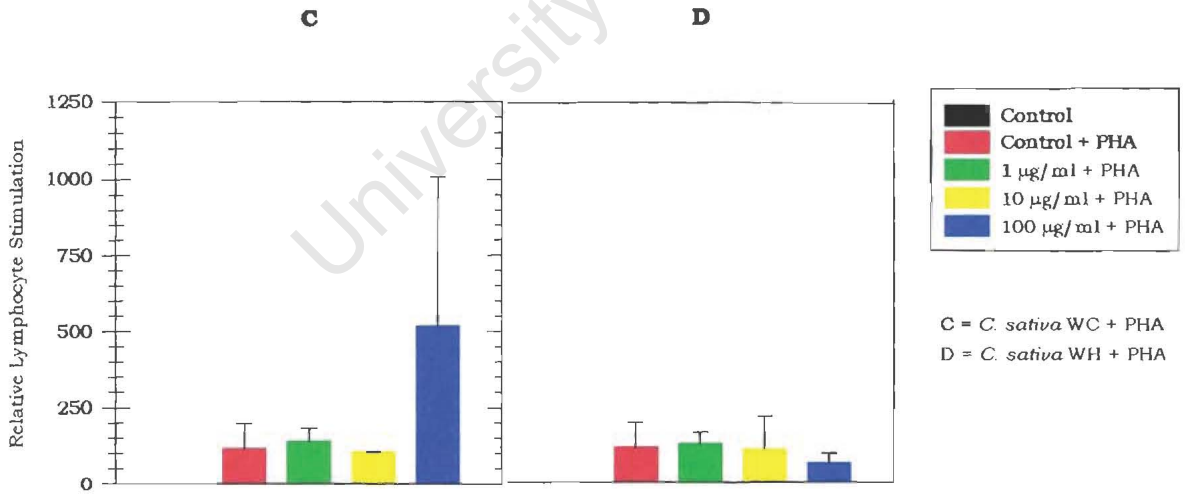


Figure 3.12 (C-D). Effects of cold (WC) and hot (WH) water extracts of *C. sativa* on PHA-stimulated lymphocytes *in vitro*. Values are counted relative to the DPM value of cell control. Each bar represents the mean of two experiments and the error barsarestandardeviations (SD) of the mean. Neither extract of *C. sativa* produced a stimulatory or suppressive effect on PHA-induced lymphocyte proliferation.

Cells Control Data Set 1	*Z. capense* LMW 1µg/ml	*Z. capense* LMW 10µg/ml	*Z. capense* LMW 100µg/ml	Cells Control Data Set 2	*Z. capense* LMW 1µg/ml	*Z. capense* LMW 10µg/ml	*Z. capense* LMW 100µg/ml
797	746	622	384	620	760	464	273
762	581	454	659	886	670	550	270
795	594	759	623	890	692	664	526
817				594	971	564	516
533				656	710	617	511
596							
Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
717	640	612	555	730	761	572	419
PHA Control Data Set 1	*Z. capense* LMW + PHA 1µg/ml	*Z. capense* LMW + PHA 10µg/ml	*Z. capense* LMW + PHA 100µg/ml	PHA Control Data Set 2	*Z. capense* LMW + PHA 1µg/ml	*Z. capense* LMW + PHA 10µg/ml	*Z. capense* LMW + PHA 100µg/ml
91553	70569	70259	92151	81771	116169	91101	55670
93453	67431	78464	81350	136083	74442	82119	98235
82756	82736	74850	61029	112013	134314	119009	64584
79025				70142	173611	116818	98277
82195				115077	179592	128928	65663
71444							
Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
83404	73578	74524	78176	103017	135626	107595	76486

[^{3}H] (tritiated) thymidine incorporation was measured in a scintillation counter. Readings were expressed as disintegrations per minute (DPM). Cell control-measures the amount of tritiated thymidine incorporated into lymphocytes and PHA control-measures the amount of tritiated thymidine incorporated into PHA-stimulated cells. To determine relative lymphocyte stimulation by extracts on stimulated and non-stimulated lymphocytes, the dpm value for lymphocytes inoculated with extract was divided by the cell control and to determine the effect the extract had on lymphocyte stimulation, the dpm value for extract + PHA + lymphocytes was divided by the dpm value for extract + lymphocytes.

Table 3.19. Effect of the low-molecular weight (LMW) fraction of hot-water extract of *Z. capense* on lymphocytes *in vitro*. Values are expressed in disintegrations per minute (DPM).

Cells Control Data Set 1	*Z. capense* HMW 1µg/ml	*Z. capense* HMW 10µg/ml	*Z. capense* HMW 100µg/ml	Cells Control Data Set 2	*Z. capense* HMW 1µg/ml	*Z. capense* HMW 10µg/ml	Z. capense HMW 100µg/ml
797	627	562	599	620	618	388	536
762	522	614	570	886	620	850	465
795	615	685	649	890	685	721	595
817				594	630	539	495
533				656	678	707	503
596							
Average 717	**Average** 588	**Average** 620	**Average** 606	**Average** 730	**Average** 646	**Average** 639	**Average** 519
PHA Control Data Set 1	*Z. capense* HMW + PHA 1µg/ml	*Z. capense* HMW + PHA 10µg/ml	*Z. capense* HMW + PHA 100µg/ml	PHA Control Data Set 2	*Z. capense* HMW + PHA 1µg/ml	*Z. capense* HMW + PHA 10µg/ml	*Z. capense* HMW + PHA 100µg/ml
91553	91482	65059	98523	81771	185892	112458	99631
93453	82666	69279	60720	136083	110186	105576	98301
82756	116374	62171	87374	112013	162764	120434	85082
79025				70142	73171	112012	113297
82195				115077	167707	86888	120786
71444							
Average 83404	**Average** 96840	**Average** 65503	**Average** 82209	**Average** 103017	**Average** 139944	**Average** 107473	**Average** 103419

[^{3}H] (tritiated) thymidine incorporation was measured in a scintillation counter. Readings were expressed as disintegrations per minute (DPM). Cell control-measures the amount of tritiated thymidine incorporated into lymphocytes and PHA control-measures the amount of tritiated thymidine incorporated into PHA-stimulated cells. To determine relative lymphocyte stimulation by extracts on stimulated and non-stimulated lymphocytes, the dpm value for lymphocytes inoculated with extract was divided by the cell control and to determine the effect the extract had on lymphocyte stimulation, the dpm value for extract + PHA + lymphocytes was divided by the dpm value for extract + lymphocytes.

Table 3. 20. Effect of the high-molecular weight (HMW) fraction of the hot-water extract of *Z. capense* on lymphocytes *in vitro*. Values are expressed in disintegrations per minute (DPM)

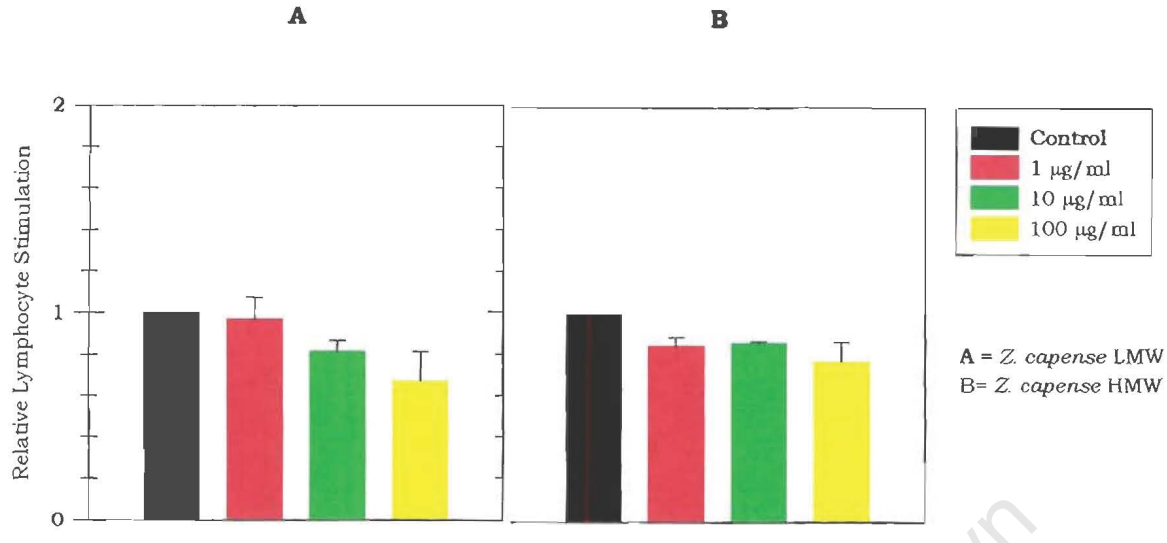


Figure 3.13 (A-B). Effects of the low (LMW) and high (HMW) molecular weight fractions of the hot-water extract of *Z. capense* on lymphocytes *in vitro*. Each bar represents the mean of two experiments and the error bars are standard deviations (SD) of the mean No significant dose-dependent suppression or stimulation was observed.

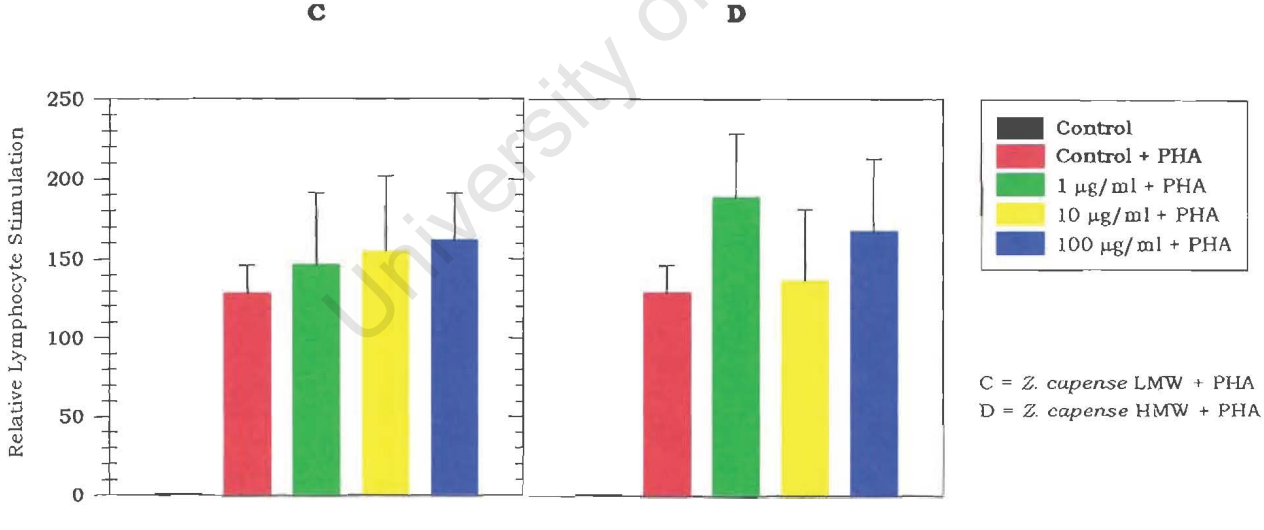


Figure. 3.13 (C-D). Effects of the low (LMW) and high (HMW) molecular weight fractions of the hot water extract of *Z. capense* on PHA-stimulated lymphocytes *in vitro*. Values are counted relative to the DPM value of cell control. Each bar represents the mean of 2 experiments and the error bars are standard deviations (SD) of the mean. Both fractions failed to produce significant stimulation of PHA-induced lymphocyte proliferation.

Sample Concentration (µg/ml)	Data Set 1	Data Set 2	Data Set 1 (+ PHA)	Data Set 2 (+ PHA)
Cells Control (0)	1063	1389	167934	133625
	898	1656	186907	120566
	1479	1904	188118	154563
	2118	1639		98830
Average	1390	1647	180986	126896
F2 (0.1)	734	1204	275584	143774
	1470	1130	230140	149887
	911	1528	210774	111442
	3808	1728	157284	118483
Average	1731	1397	218445	130896
F2 (0.5)	752	708	225606	121371
	1152	1095	218942	184131
	754	907	244942	96328
	3395	1576	215066	88609
Average	1513	1071	226139	122610
F2 (1)	542	1370	246339	194918
	631	1353	163281	146093
	1553	1666	175508	158572
	3437	746	187893	131300
Average	1541	1277	193255	157721
F2 (10)	442	1294	261555	179000
	1760	1627	212775	208256
	1429	1397	183209	175002
	6819	802	220831	212050
Average	2612	1280	219592	193577
F2 (100)	1111	1610	277074	282235
		737	198948	241603
		1674	273589	253980
		1307	280113	188792
Average	1111	1332	257431	241652

[^{3}H] (tritiated) thymidine incorporation was measured in a scintillation counter. Readings were expressed as disintegrations per minute (DPM). Cell control-measures the amount of tritiated thymidine incorporated into lymphocytes and PHA control-measures the amount of tritiated thymidine incorporated into PHA-stimulated cells. To determine relative lymphocyte stimulation by extracts on stimulated and non-stimulated lymphocytes, the dpm value for lymphocytes inoculated with extract was divided by the cell control and to determine the effect the extract had on lymphocyte stimulation, the dpm value for extract + PHA + lymphocytes was divided by the dpm value for extract + lymphocytes.

Table 3.21. Effect of the solid phase fraction of the hot-water extract of *Z. capense* (F2) on lymphocytes *in vitro*. Values are expressed in disintegrations per minute (DPM)

Sample Concentration (µg/ml)	Data Set 1	Data Set 2	Data Set 1 (+ PHA)	Data Set 2 (+ PHA)
Cells Control (0)	1064	1389	167934	133625
	898	1656	186907	120566
	1479	1904	188118	154563
	2118	1639		98830
Average	1390	1647	180986	126896
F3 (0.1)	1354	882	144275	230736
	1237	810	162160	210801
	1163	938	233045	100770
	988	1295	236279	137895
Average	1185	981	193940	170050
F3 (0.5)	946	1218	221050	179166
	1005	893	161084	114647
	672	675	232558	112279
	517	702	293715	744
Average	785	872	227102	101709
F3 (1)	917	928	166189	118069
	688	634	139458	112164
	713	714	178414	86865
	2262	805	1257	160723
Average	1145	770	121329	119455
F3 (10)	1353	966	239378	176134
	1000	1092	160783	214696
	1386	1064	182233	147719
	1650	5918	379198	221325
Average	1347	2260	240398	189968
F3 (100)	1022	823	245358	166080
	1513	1037	190775	188889
	1021	1132	243442	198011
	2598	1024	218971	192492
Average	1539	1004	224637	186400

[^{3}H] (tritiated) thymidine incorporation was measured in a scintillation counter. Readings were expressed as disintegrations per minute (DPM). Cell control-measures the amount of tritiated thymidine incorporated into lymphocytes and PHA control-measures the amount of tritiated thymidine incorporated into PHA-stimulated cells. To determine relative lymphocyte stimulation by extracts on stimulated and non-stimulated lymphocytes, the dpm value for lymphocytes inoculated with extract was divided by the cell control and to determine the effect the extract had on lymphocyte stimulation, the dpm value for extract + PHA + lymphocytes was divided by the dpm value for extract + lymphocytes.

Table 3.22. Effect of the solid phase fraction of the hot-water extract of *Z. capense* (F3) on lymphocytes *in vitro*. Values are expressed in disintegrations per minute (DPM)

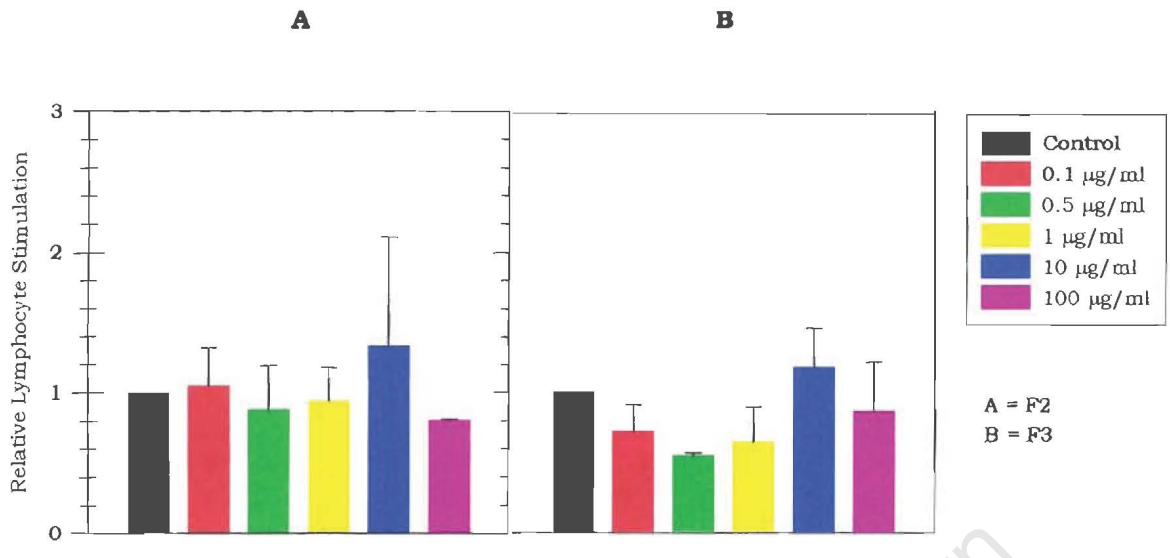


Figure. 3. 14 (A-B). Effects of the solid phase fractions of the hot-water extract of *Z. capense* (F2 and F3) on lymphocytes *in vitro*. Each point represents the mean of two experiments and the error bars are standard deviations of the mean. Neither fraction had a stimulatory or suppressive effect on lymphocytes

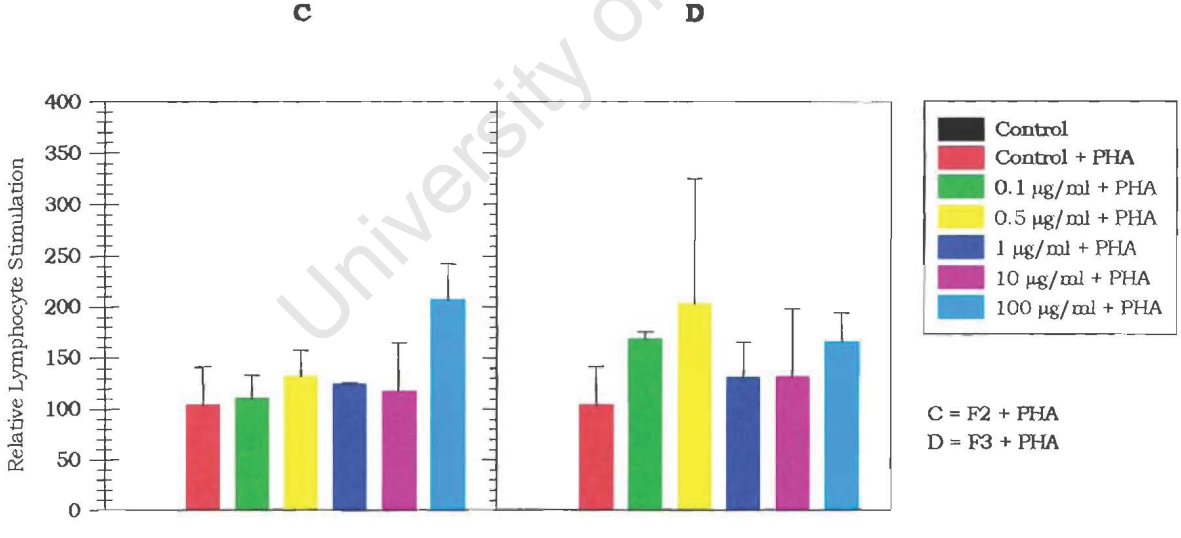


Figure 3.14 (C-D). Effects of solid phase fractions of the hot water extract of *Z. capense* (F2 and F3) on PHA-stimulated lymphocytes *in vitro*. Each point represents the mean of two experiments and the error bars are standard deviations of the mean. The stimulatory effect fraction 2 at 100 µg/ml was higher compared to that of control (PHA+ lymphocytes).

3.7. Phytochemical Analysis

3.7.1. Colorimetric Phytochemistry and TLC Analysis

The hot-water extract of *Z. capense* and its solid phase fraction (F2) were tested for the presence of various classes of natural products by TLC analysis and spraying with specific colour reagents (Dragendorff reagent for alkaloids and 10 % potassium hydroxide in methanol for coumarins) and viewing under ultra-violet (UV) light. The following classes of compounds were found:

i. The hot-water extract of *Z. capense* was positive for tannins. The tannins gave a strong colour reaction upon addition of ferric chloride.

ii. TLC analysis for the presence of coumarins revealed the presence of 3 coloured spots (yellow (Rf value-0.07), orange (Rf value-0.4) and blue (Rf value-0.57). These were viewed under UV after intensifying the spots with 10 % potassium hydroxide in methanol.

iii. TLC analysis for the presence of alkaloids: one dark-orange spot was observed in the hot-water extract of *Z.capense*. Spraying with Dragendorff reagent intensified the spot. No alkaloids were detected in F2.

A summary of compounds found in the samples is listed in table 3.23, page 65.

Compound Class	*Z. capense* (WH)	Fraction 2
Alkaloids	+	-
Anthraquinones	-	-
Coumarins	+	+
Flavonoids	-	-
Saponins	-	-
Tannins	+	-
Quinones	-	-

Key: WH-hot water extract, + or - (Positive or negative for specific class of compound)

Table 3.23. Phytochemical screening of hot water extract of *Z. capense* and solid phase fraction (F2)

3.7.2. HPLC Analysis

Figures 3.15 (a-b), p. 66-67 explain the chromatograms of the hot-water extract of *Z. capense* and its solid-phase fraction (F2). The crude hot-water extract of *Z. capense* appeared to have a wide diversity of compounds. There were two groups that were most abundant. The first group of compounds eluted early (in the first four minutes). The second group of compounds eluted much later (19.832 and 21.183 min) (figure 3.15 (a) p. 66). There seemed to be a number of compounds eluting between these two groups. F2 appeared to have the same group of compounds found in the chromatogram for the crude hot water extract (figure. 3.15b, page 67). The peaks from the chromatogram of F2 have similar absorption spectra as shown by the spectrum indices (figure 3.16 b-c, page 68). The second group of compounds available in the crude hot water-extract was not available in F2. TLC analysis of this fraction showed that it contained three coumarins (table 3.23 p.65).

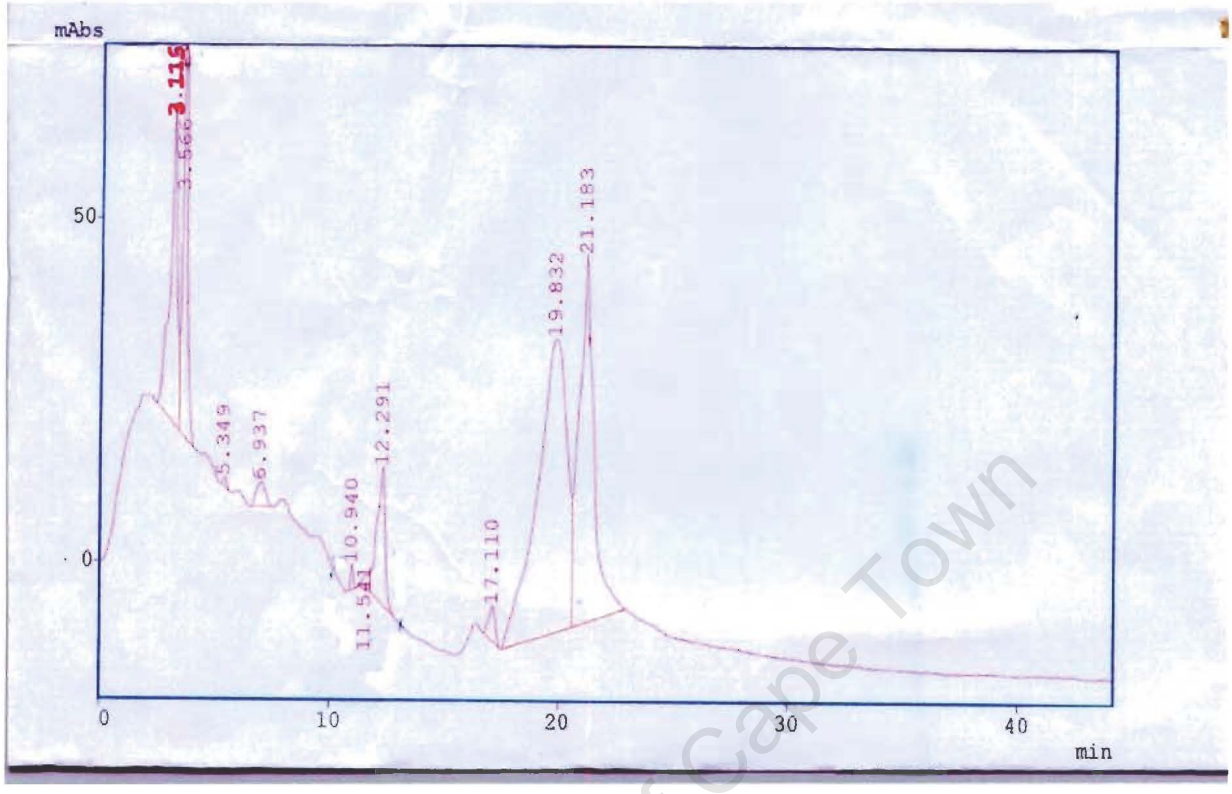
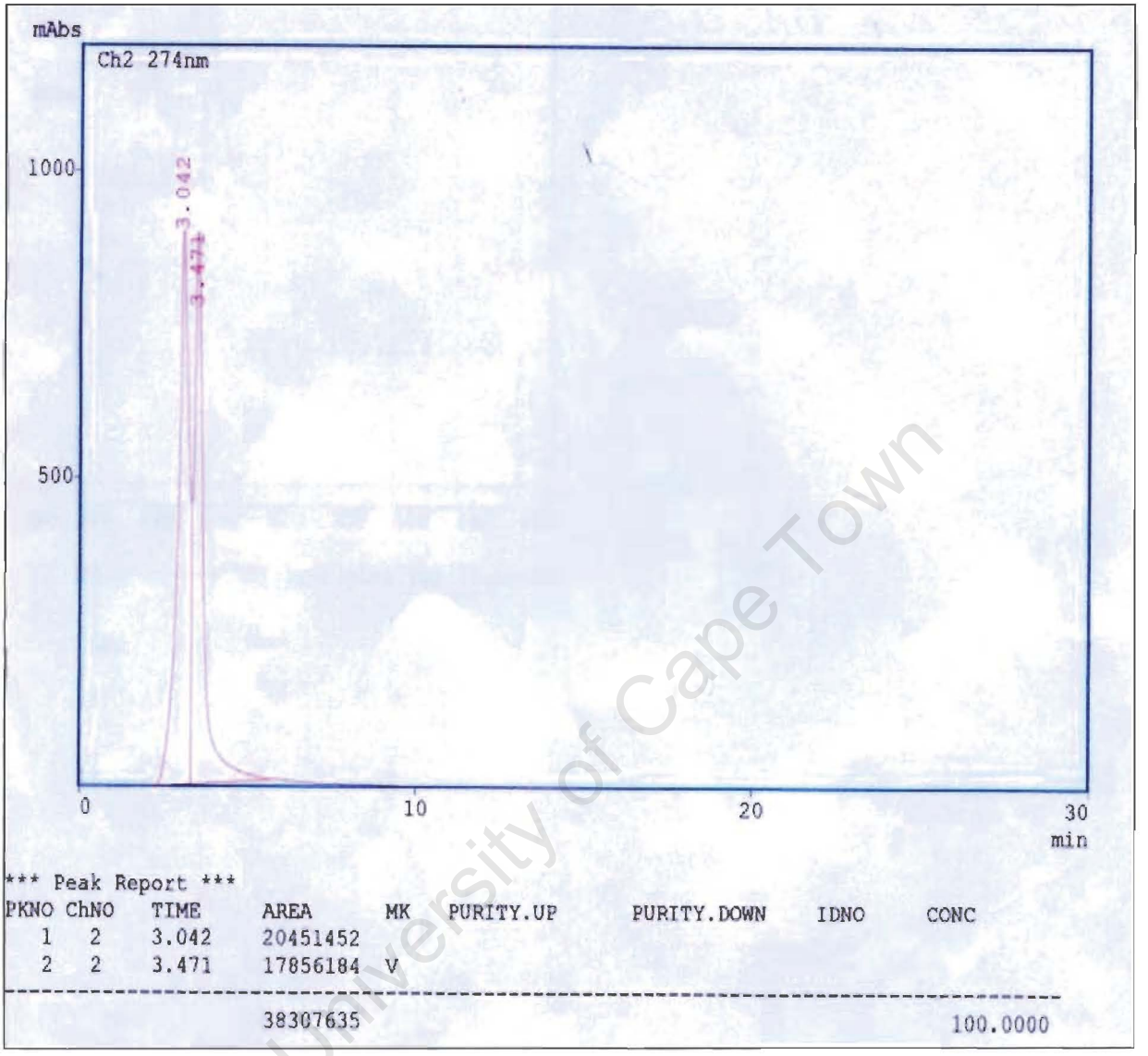


Figure 3.15 (a). Chromatogram of the hot-water extract of *Z. capense*. There are two groups of compounds eluting from the column, the first group elutes during the first 4 minutes of the run (3.115 and 3.556 minutes) and the second group elutes after 10 minutes.



*** Peak Report ***

PKNO	ChNO	TIME	AREA	MK	PURITY.UP	PURITY.DOWN	IDNO	CONC
1	2	3.042	20451452					
2	2	3.471	17856184	V				
			38307635					100.0000

Figure 3.15 (b). Chromatogram of solid phase fraction of the hot-water extract of *Z. capense* (F2). This chromatogram shows only two peaks that absorb under UV, the same peaks that are found in the chromatogram of the hot-water extract of *Z. capense*.

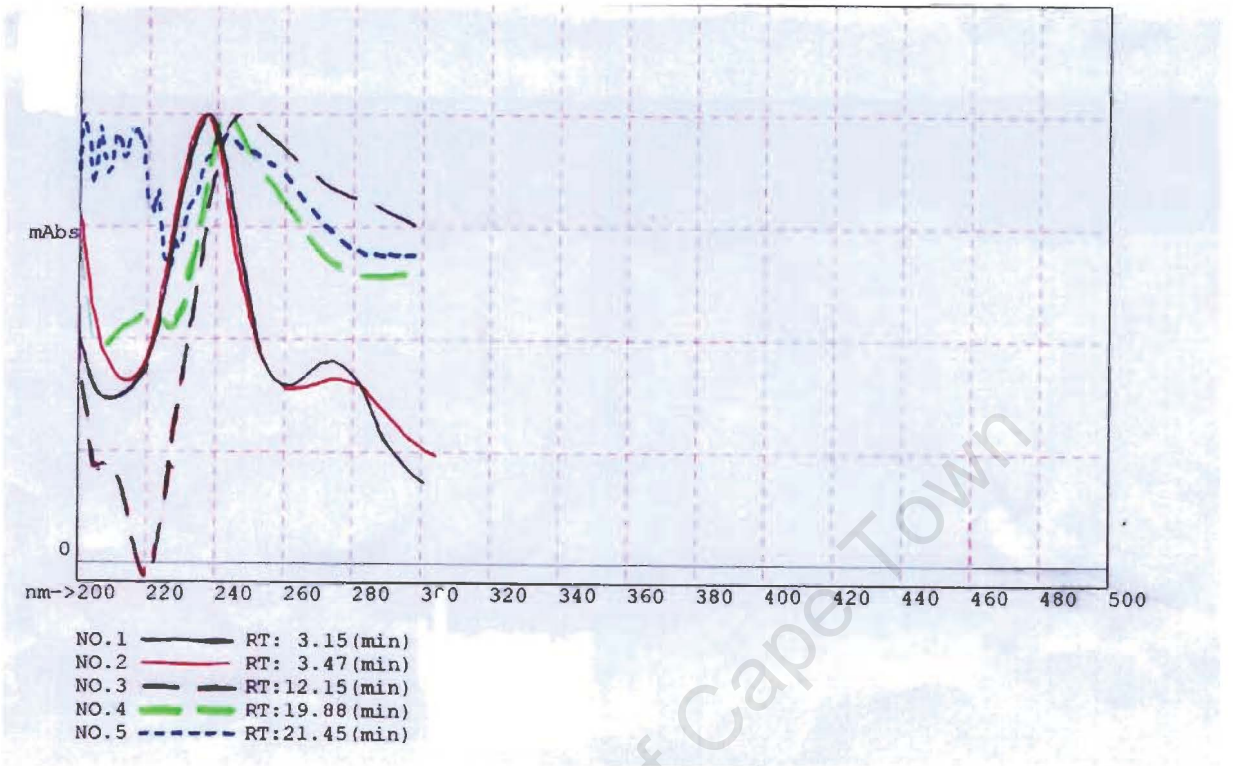


Figure. 3.16(a). Spectrum index of the hot-water extract of *Z. capense*. This figure shows that the crude hot-water extract contains a variety of compounds with different absorption spectra. The first two peaks (NO.1 and NO.2) have similar absorption spectra.

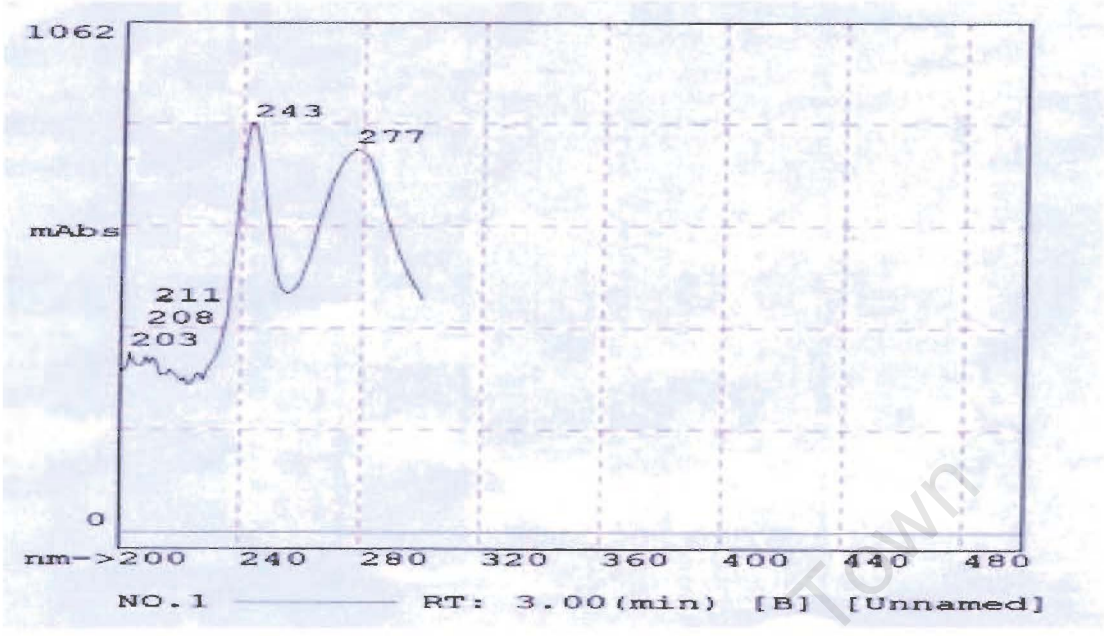


Figure 3.16 (b). Spectrum index of fraction 2 (peak number 1 on chromatogram, figure 3.15 (b), page 67)

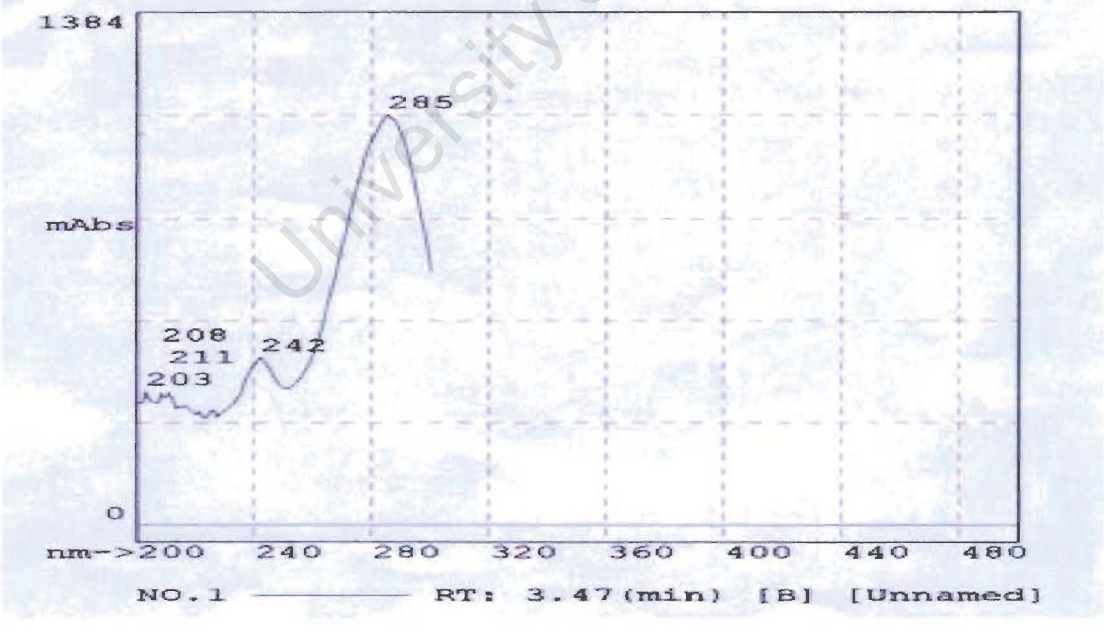


Figure 3.16 (c). Spectrum index of fraction 2 (peak number 2 on chromatogram, figure 3.15 (b), page 67)

3.8. Summary of Results

3.8.1. Cytotoxicity

The cytotoxicity results show that:

1. Both the cold- and hot-water extracts of *A. oppositofolia* exhibited a cytotoxic effect on Rat-1 fibroblasts at the highest concentration tested (100 µg/ml) but not at 1 and 10 µg/ml (figure. 3.2, p. 32). The cold- and hot-water extracts of *Z. capense* did not produce a cytotoxic effect on the fibroblasts at any concentration tested.

2. The cold-water extract of *G. glabra* failed to show a consistent cytotoxic effect at concentrations tested whereas the hot-water extract of *G. glabra* produced no cytotoxic effect. The cold- and hot-water extracts of *H. caffrum* exhibited a cytotoxic effect at all concentrations tested (figure. 3.3, p. 34)

3. The cold- and hot-water extracts of *L. javanica* and *P. prunelloides* exhibited a cytotoxic effect at all concentrations tested (figure. 3.4, p. 36).

4. Cold- and hot-water extracts of *P. guajava* exhibited a cytotoxic effect at all concentrations tested. Cold- and hot-water extracts of *T. capensis* and *C. sativa* failed to show a consistent cytotoxic effect on the fibroblasts (figure. 3.5, p. 38).

5. The dichloromethane extract of *A. oppositofolia* was found to be slightly cytotoxic to the fibroblasts at the highest concentration (100 µg/ml), but not at 1 and 10 µg/ml. Both dichloromethane and methanol extracts of *Z. capense* exhibited a dose-dependent

cytotoxic effect. The cytotoxic effect was most pronounced for both extracts at 100 μg/ml (figure. 3.6, p. 40).

6. The methanol extract of *G. glabra* exhibited a dose-dependent cytotoxic effect. This cytotoxic effect was most pronounced at 100 μg/ml. A dose-dependent cytotoxic effect was observed in the dichloromethane extract of *L. javanica.* This cytotoxic effect was most pronounced at 100 μg/ml (figure. 3.7, p.42).

7. The methanol extract of *P. prunelloides* exhibited a cytotoxic effect at all concentrations tested whereas the dichloromethane extract exhibited cytotoxicity only at 100 μg/ml. The dichloromethane extract of *P. guajava* exhibited a cytotoxic effect at 100 μg/ml (figure. 3.8, p. 44).

8. The methanol extract of *T. capensis* produced no cytotoxic effect on fibroblasts. Both the methanol and dichloromethane extracts of *C. sativa* exhibited a pronounced cytotoxic effect at 100 μg/ml but not at 1 and 10 μg/ml (figure. 3.9, p. 46)

3.8.2. Lymphocyte Proliferation

1. Cold- and hot-water extracts of *G. glabra* did not produce a stimulatory or suppressive effect on lymphocytes *in vitro,* even upon stimulation with PHA (figure. 3.11, p. 54). A similar response was observed with the both aqueous extracts of *C. sativa* (figure. 3.12, p. 57).

2. Cold-water extract of *Z. capense* increased lymphocyte proliferation by a factor of 1.37 ± 0.1 at 1 μg/ml but not at 100 μg/ml. The hot-

water extract of *Z. capense* increased lymphocyte proliferation by a factor of 1.28 ± 0.17 at 1 μg/ml, but not at 100 μg/ml. Neither extract of *Z. capense* had an effect on PHA-induced lymphocyte proliferation (figure. 3.10, p. 51).

3. Low- and high-molecular weight fractions of the hot-water extract of *Z. capense* produced no stimulatory or suppressive effect on either non-stimulated or PHA-stimulated lymphocytes at the concentrations tested (figure. 3.13, p. 60)

4. The two fractions recovered from solid phase extraction of the hot-water extract of *Z. capense*, F2 and F3 produced no stimulatory or suppressive effect on non-stimulated lymphocytes at the concentrations tested. However, F2 increased PHA-induced lymphocyte proliferation at 100 μg/ml when compared to the positive control (PHA + lymphocytes) (figure. 3.14, p. 63).

3.8.3. Phytochemical Analysis

3.8.3.1. Colorimetric and TLC analysis

Colorimetric analysis of the hot-water extract of *Z. capense* and the solid phase fraction showed that:

1. The hot-water extract of *Z. capense* contained tannins. No tannins were detected from the solid phase fraction (F2).

2. Coumarins were detected in both the hot-water extract of *Z. capense* and F2. The Rf values were 0.07, 0.4, and 0.57.

3. Alkaloids were detected in the hot-water extract of *Z. capense*. No alkaloids were detected in F2.

3.8.3.2. HPLC Analysis

The results of the analysis of the hot-water extract of *Z. capense* and F2 were:

1. The hot-water extract of *Z. capense* had two groups of compounds eluting at different times. The first group eluted in the first 4 minutes and the second group after 10 minutes of the run (figure. 3.15 (a), p. 66). Only two compounds were found in F2 (figure.3.15 (b), page 67).

2. The absorption spectra for the hot-water extract of *Z. capense* showed that it contained a mixture of compounds absorbing at different wavelengths (figure. 3.16 (a), page 68). The absorption spectra for the first two peaks were similar and could also be visualised in the spectra indices of F2 (figure. 3.16 (b-c), page 69).

4. DISCUSSION

Seven plants previously screened for antimalarial activity in the Department of Pharmacology, UCT, were investigated to determine whether they have immunomodulatory properties. These plants included *A. oppositofolia, Z. capense, G. glabra, H. caffrum, L. javanica, P. prunelloides* and *T. capensis*. Two other plants, *P. guajava* and *C. sativa*, were selected on the basis of information gathered from literature (Van Wyk BE, 1997, Hutchings A, 1996). All the plants were extracted in cold and hot water, methanol and dichloromethane. Extracts were evaluated for cytotoxicity against the Rat-1 fibroblast cell-line.

Cold- and hot-water extracts of *A. oppositofolia, H. caffrum, L. javanica, P. prunelloides, P. guajava* and *T. capensis* were cytotoxic at all the concentrations tested (1- 100µg/ml). The cytotoxic effect ranged from 0-20 % with most cytotoxicity noted at 100 µg/ml. It might be argued that the low cytotoxic effect justifies the common use in traditional practice. On the other hand, dichloromethane extracts of *Z. capense, L. javanica, P. guajava* and *C. sativa* exhibited high cytotoxicity at the highest concentration tested (100 µg/ml), compared with 1 and 10 µg/ml (figures. 3.6-3.9, p.40, 42, 44 and 46). These findings suggest that compounds extracted with dichloromethane might not normally be extractable by traditional methods. Cold- and hot-water extracts of *G.glabra, C. sativa* and *Z. capense* are not cytotoxic to fibroblasts. Therefore, these extracts were investigated for their lymphocyte proliferating effects. Glycyrrhizic acid from *G. glabra* has been shown to stimulate T- and B-lymphocytes by inducing T–lymphocytes responses to PHA, Con A and to T-independent B-lymphocyte stimulation by lipopolysaccharide S (LPS) (Chavali SR et al, 1987).

Neither extract of *G. glabra* stimulated or suppressed lymphocyte proliferation, even in the presence of PHA (figure.3.11, p.54). Our findings are in contrast to the results of Chavali SR et al, 1987 on the immunomodulatory properties of glycyrrhizic acid from *G. glabra.* It is possible that both extracts of *G. glabra* tested in our system had low concentrations of glycyrrhizic acid, or that compounds antagonising the effects of glycyrrhizic acid were present in the aqueous extracts of *G. glabra.*

There was no significant difference in lymphocyte-stimulating activity between the cold- and hot-water extracts of *Z. capense.* Both extracts exhibited a stimulatory effect at the lowest concentration tested (1 μg/ml). However, the stimulatory effect of both extracts was lost at 100 μg/ml. Neither extract of *Z. capense* produced a stimulatory or a suppressive effect on PHA-induced lymphocyte proliferation at the concentrations tested. Further studies were carried out with the hot water extract of *Z. capense* since it showed promising lymphocyte proliferating effect compared to the aqueous extracts of *G. glabra* and *C. sativa.*

To determine whether the active component(s) producing the stimulatory effect were of low- or high-molecular weight, the hot-water extract of *Z. capense* was separated on a Millipore filter with a cut-off of 10 000 MW. Neither fraction of the hot-water extract of *Z. capense* had an effect on lymphocyte proliferation, even in the presence of PHA. This loss of stimulatory effect from the fractions concerned suggests that compounds within the hot-water extract of *Z. capense* act in synergy to produce the stimulatory effect. Following a bioassay-guided fractionation approach, the hot-water extract of *Z. capense* was subjected to solid phase extraction using solvents with increasing ionic strengths.

Two fractions recovered from the solid phase extraction, designated F2 and F3, were investigated for lymphocyte proliferating effects. F2 increased PHA-induced lymphocyte proliferation at 100 µg/ml (figure 3.14 (B-D), p. 63). Samples of the crude hot water extract of *Z. capense* and F2 (10 and 100 µg/ml, respectively) were injected on a normal phase column. The HPLC chromatograms are shown in figures. 3.15a and 3.15b, p.66 and 67. These chromatographic profiles were compared to determine the differences between the crude hot-water extract of *Z. capense* and F2. The chromatographic profiles revealed that F2 is purer when compared with the crude hot-water extract of *Z. capense*. On analysis for coumarins, both the crude hot-water extract of *Z. capense* and F2 migrated as three spots on a TLC plate in chloroform (results not shown). The crude hot-water extract of *Z. capense* also tested positive for alkaloids and tannins, although none were detected in F2 (table 3.23, p.65). The presence of alkaloids and tannins may account for the differences observed in the chromatographic profiles of the crude hot-water extract of *Z. capense* and F2.

Investigation of the effects that the active plant fractions might have on other cells of the immune system such as macrophages and mixed lymphocyte preparations may help in understanding the role played by the active plant extracts. *Z. capense* is a chemically diverse plant. A number of compounds have been reported from this plant that include tannins, lignans and alkaloids. Isolation of these classes of compounds, and testing them for biological activity using a variety of methods, may help verify the use of the plant for the conditions for which it is used in traditional practice. Separation of the coumarins found in this study, and individual testing for immunomodulatory effects, and structural identification would help in understanding their medicinal activity at a chemical level.

5. CONCLUSION

The crude cold- and hot-water extracts of *Z. capense* exhibit lymphocyte proliferating activity at low concentrations in the absence of a stimulant. Fractions 2 and 3 (F2 and F3) increase PHA-induced lymphocyte proliferation in a manner that becomes more pronounced for F2 at the highest concentration used in this study. The differences in the way the cold- and hot-water extracts of *Z. capense* and F2 affect lymphocyte stimulation perhaps indicate various modes of action. These findings raise the need for further investigations, in particular of the possible action of the extracts in interfering with cell signalling and cytokine production.

6. REFERENCES

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7. APPENDIX

Solutions

Crystal Violet

For 500 ml

Citric acid	9.61 g
Crystal violet	0.50 g
Double distilled water	500 ml

Preparation

475 ml of water + 9.61 g of citric acid + 0.5 g of crystal violet. Adjust pH to 2.26-2.30 using 1N sodium hydroxide and fill up to 500 ml with distilled water. Filter with Nalgene 0.22 µm filters and dispense into clean storage bottles.

Dragendorff Reagent

Solution A

Basic bismuth nitrate	1.7 g (in 80 ml water/ 20 ml acetic acid)

Solution B

Potassium iodide	40 g in 100 ml water

Preparation

Mix reagents together as follows: 5 ml A + 5 ml B + 20 ml acetic acid + 70 ml water. Spray plates, orange spots develop. Spots intensify if sprayed later with HCl, or 50 % water-phosphoric acid. Good for phenols.

Ferric Chloride

5 %

Ferric Chloride	5 g / 100 ml of water

15 %

Ferric Chloride	15 g / 100 ml of water

Hydrochloric Acid

10 % Hydrochloric acid solution

Hydrochloric Acid	15.15 ml
Water	35.85 ml

Phosphate buffered saline (PBS)

PBS tablets	10
Distilled Water	1L

Sodium bicarbonate (5 %)

For 1L	
Sodium bicarbonate	50 g
Deionized water up to	1L

Sodium chloride (0.1, 0.5, 1, 5 M NaCl)

For 200 ml	
0.1 M	
Sodium chloride	1.169 g
Distilled water up to	200 ml
0.5 M	
Sodium chloride	5.84 g
Distilled water up to	200ml
1M	
Sodium chloride	11.69 g
Distilled water up to	200 ml
5M	
Sodium chloride	58.44 g
Distilled water up to	200 ml

Trypan Blue

Trypan Blue Solution (Sigma) 0.4%	500 µl
PBS	500 µl