Immune modulatory effect of *Dichrostachys cinerea, Carpobrotus dimidiatus, Capparis tomentosa* and *Leonotis leonurus*

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REFERENCE DECLARATION IN RESPECT OF A MASTER'S DISSERTATION

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DEDICATION

I dedicate this work to:

My late mother, Mrs. P. Hurinanthan, and father Mr. K. P. Hurinanthan, my friends and teachers, for their inspiration and guidance, and

to **GOD** who makes all things possible.

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Immunomodulatory effect of *Dichrostachys cinerea, Carpobrotus dimidiatus, Capparis tomentosa* and *Leonotis leonurus*

ABSTRACT

Dichrostachys cinerea, Carpobrotus dimidiatus, Capparis tomentosa and *Leonotis leonurus* are all plants that are indigenous to South Africa. These plants are used in traditional medicine to treat various ailments. However, there is little or no scientific data to justify these traditional uses. Furthermore, it is difficult to reconcile traditional knowledge with scientific evidence because of the overwhelming targeting of signal-responsive systems by plant defensive compounds, multiple sites of action and the connectedness of the signaling pathways, which provide many cures and have pleiotropic effects. In order to evaluate the action spectrum of these plants, and validate its widespread use, this research evaluated the antibacterial, antioxidant, anti-inflammatory, anti-mosquito and immunomodulatory properties of these plants.

Antimicrobial activity of the extract was determined by evaluating the bactericidal and fungicidal action using the agar disc diffusion assay. Anti-oxidative properties of the extracts were tested using the DPPH photometric assay. Anti-inflammatory properties were carried out using the 5-lipoxygenase assay. The larvicidal, repellency and insecticidal assay was determined against *A.arabiensis*. The safe use of these plant extracts was determined by evaluating toxicity, a brine shrimp lethality assay and an *in vitro* cell culture system using human myelogenous leukemia cell line. Potential carcinogenic activity was evaluated using the Ames Salmonella Mutagenecity assay. The immunomodulatory activity of the extracts on human peripheral blood mononuclear cells

was evaluated on freshly harvested lymphocytes using the MTT assay. Cytokine response was evaluated by measuring the secretion of interferon-gamma and interleukin-10. Elucidation of the B cells, T cells, activated T cells, CD 4^+, CD 8^+ and NK cells was performed by flow cytometry.

The extracts showed anti-microbial activity against *Escherichia coli, Pseudomonas aeruginosa, Klebsiella oxytoca, Salmonella typhimurium, Serratia marcescens, Bacillus cereus* and *Tricoderm sp.* The highest activity was shown by methanolic and aqueous extracts of *L. leonurus* leaves followed by methanolic and aqueous extracts of *D. cinerea*. Extracts of *C. tomentosa* and *D.cinerea* demonstrated a higher degree of free radical scavenging than rutin, which was used as a standard indicating that these plants have strong antioxidant properties. None of the plants showed significant anti-inflammatory activity when compared to NDGA. In the anti-mosquito assays, the extracts showed strong repellency and insecticidal activity. *L. leonurus* extracts demonstrated the highest insecticidal and repellency activity against the mosquito, and was also found to cause 'knockdown' and mortality. The extracts display no toxicity, cytotoxicity and mutagenicity. The immunological studies for immune modulation showed that the methanol extracts of these plants induce a Th1- predominant immune response because they significantly suppressed the secretion of IL-10 and augment IFN-γ production, which are hallmarks used to indicate a stimulation of the innate immune response.

This study also provides new information, with respect to the potential use of these plants in producing a mosquito repellent and an immunostimulant.

TABLE OF CONTENTS

		Page
AUTHORS DECLARATION		i
DEDICATION		ii
ACKNOWLEDGEMENTS		iii
ABSTRACT		iv
TABLE OF CONTENTS		vi
ABBREVIATIONS		x
LIST OF TABLES		xii
LIST OF FIGURES		xiv
CHAPTER ONE: INTRODUCTION		
1.1	**Background and aim of the study**	1
CHAPTER TWO: LITERATURE REVIEW		
2.1	**Overview of the plants**	
2.1.1	*Dichrostachys cinerea*	3
2.1.2	*Carpobrotus dimidiatus*	6
2.1.3	*Capparis tomentosa*	8
2.1.4	*Leonotis leonurus*	10
2.2	**Scientific Rationale**	
2.2.1	Antibacterial activity	14
2.2.2	Antifungal activity	15
2.2.3	Anti-oxidant activity	16
2.2.4	Anti-inflammatory activity	17
2.2.5	Anti- mosquito activity	18
2.2.6	Safety of traditional medicines	20
2.3	**Immunomodulating properties of plants**	
2.3.1	Immuno-modulation and phytochemicals	23
2.3.2	Stimulation of the immune system	24
2.3.3	Cytokines	26

2.3.4 Lymphocytes surface markers 30
2.3.5 CD 4 Cells 30
2.3.6 The role of CD 4 cells in the immune system 31

CHAPTER THREE: METHODOLOGY

3.1 Collection and preparation of plant material 32
3.2 Sample preparation 33
3.3 Biological Screening
3.3.1 Antimicrobial activity 35
3.3.2 Antioxidant activity 37
3.3.3 Anti-inflammatory activity 38
3.3.4 Anti-Mosquito activity 40
3.4 Evaluation of safety of plants
3.4.1 Ames Mutagenicity Test 45
3.4.2 Brine Shrimp Lethality Assay 47
3.4.3 Cytotoxicity 48
3.5 Immuno-modulation
3.5.1 Crude extracts and chemicals 51
3.5.2 Lymphocyte isolation 52
3.5.3 Lymphocyte maintenance 54

3.6 Optimization Studies

3.6.1 Optimization of standards and controls 56

3.6.2 Evaluation of the effect of plant extracts on PBMC 57

3.6.3 Lymphocyte proliferation assay 57

3.6.4 Cytokine response of PBMC exposed to plant extracts 58

3.6.5 Effect of plant extracts on T-cell sub-population 59

CHAPTER FOUR: RESULTS

4.1 Biological Activity

4.1.1 Yield and description 61

4.1.2 Antimicrobial activites of the extracts of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* 65

4.1.3 Antioxidant activity of the extracts of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* 72

4.1.4 Anti-inflammatory activity of the extracts of *D. cinerea, C.dimidiatus, C. tomentosa* and *L. leonurus* 74

4.1.5 Anti-mosquito properties

4.1.5.1 Larvacidal activity 75

4.1.5.2 Repellency activity 77

4.1.5.3 Insecticidal activity 79

4.2 Safety Assays

4.2.1 Ames Mutagenicity Test 82

4.2.2 Brine Shrimp Lethality Assay 86

4.2.3 Cytotoxicity

4.2.3.1 Effect of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* extracts on K 562 cells 86

4.3 Immmunomodulatory activities of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus*

4.3.1 Optimum concentration of the controls Con A, LPS, PHA, CSA

and DMSO 88
4.3.2 Morphological effect of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* (250 µg/ml) on lymphocytes
4.3.2.1 Methanolic plant extracts 89
4.3.2.2 Aqueous plant extracts 90
4.3.3 Effect of *D. cinerea, C. dimidiatus, C.tomentosa* and *L. leonurus* on PBMC
4.3.3.1 Cell viability activity *of D.cinerea* 91
4.3.3.2 Cell viability activity of *C. dimidiatus* 92
4.3.3.3 Cell viability activity of *C.tomentosa* 93
4.3.3.4 Cell viability activity of *L. leonurus* 94
4.3.3.5 Cell viability activity of *L. leonurus flower* 95
4.3.4 Immunomodulatory activity evaluated by the lymphocyte secretion of IFN- γ and IL-10 96
4.3.5 Effect of plant extracts on T-cell sub-population 97

CHAPTER FIVE: DISCUSSION
5.1 ***D. cinerea*** 99
5.2 ***C. dimidiatus*** 105
5.3 ***C. tomentosa*** 108
5.4 ***L. leonurus*** 111

CHAPTER SIX: CONCLUSION 115
CHAPTER SEVEN: REFERENCES 117
CHAPTER EIGHT: APPENDICES
Appendix One 137
Appendix Two 144
Appendix Three 155

ABBREVIATIONS

A	:	Larvae that reached the adult stage
BCRFL		Epstein-Barr virus protein
BSL	:	Brine Shrimp lethality
CD	:	Cluster of differentiation
Con A	:	Concanavalin A
COX	:	Cyclo-oxygenase
CSA	:	Cyclosporine A
CSIF		Cytokine Synthesis Inhibitory Factor
DBT	:	Durban University of Technology Culture Collection
DEET	:	N,N-Diethyl-meta-toluamide
DMEM		Dulbecco's Modified Eagle's Medium
DMSO	:	Dimethyl sulphoxide
DPPH	:	2,2-Diphenyl-1-Picrylhydrazyl
ELISA	:	Enzyme-Linked ImmunoSorbent Assay
FCS		Foetal Calf Serum
GM-CSF		granulocyte monocyte colony stimulating factor
HBSAg	:	surface antigen of the Hepatitis-B-Virus
HBsAg		Hepatitis B surface antigen
HCV	:	Hepatitis C virus
IC_{50}	:	concentration of inhibitor necessary for 50% inhibition
IFN- γ	:	Interferon gamma
IL-10	:	Interleukin 10
K562	:	Human erythroleukemia cell line
L	:	Larvae
LC_{50}	:	The concentration of a chemical in the environment that kills 50% of the test animals in a given time is the LC50 value.
LOX	:	Lipooxygenase
LPS	:	Lipo-polysaccharide
MHC		Major histocompatibility complex
MIC	:	Minimum inhibitory concentration
MRC	:	Medical Research Council
MTT	:	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NaN_3	:	Sodium azide
NDGA	:	Nordihydroguaiaretic Acid
NEAA		Non- Essential Amino acids
NK cells	:	Natural killer cells
NRC	:	National Research Council
NSAIDs	:	Non steroidal anti-inflammatory drugs
P	:	Larvae that reached the pupae stage
PBMC	:	Peripheral Blood Mononuclear Cell
PBS	:	Phosphate buffer saline
PHA	:	Phytohaemagglutinin
ROS	:	Reactive oxygen species
SDA	:	Sabouraud dextrose agar
Tc cell	:	Cytotoxic T cells
Th cell	:	T helper cell
TNF		Tumor necrosis factor
WHO	:	World Health Organization

LIST OF TABLES

Page

Table 1: Nomenclature and characteristics of *Dichrostachys cinerea, Carpobrotus dimidiatus, Capparis tomentosa* and *Leonotis leonurus* 13

Table 2: Functions of the cytokines 27

Table 3: Cell type and their specific human clusters of differentiation molecules 30

Table 4: Details of plants used in this study 32

Table 5: Flourochromes used for each monoclonal antibodies 60

Table 6: Anti-bacterial activity of the methanolic and aqueous extracts of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus.* 69

Table 7: The minimum inhibitory concentrations of the methanolic and aqueous extracts of *D. cinerea, C. dimidiatus, C. tomentosa* and. *L. leonurus.* 70

Table 8: Antifungal activity of methanolic and aqueous extracts of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* (1000 µg/ml) against *Tricoderm* sp 71

Table 9: DPPH scavenging capacity of the methanolic and aqueous extracts of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* 73

Table 10: Larvacidal activity of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* 76

Table 11: Repellent activity of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* (1000 µg/ml) on *A. arabiensis* mosquito 78

Table 12: Insecticidal activity of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* (1000 µg/ml) on *A. arabiensis* mosquito 80

Table 13: Activity of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* against *A. arabiensis* 81

Table 14: Mutagenic response of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* to *S. typhimurium* strain T 98 84

Table 15: Mutagenic response of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* to *S. typhimurium* strain TA 100 85

Table 16: Cytokine activity (IFN-γ and IL- 10) by *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* 97

Table 17: Immunomodulatory activity evaluated by flow cytometry of PBMC treated with *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* 98

LIST OF FIGURES

		Page
Figure 1.	*Dichrostachys cinerea*	3
Figure 2.	*Carpobrotus dimidiatus*	6
Figure 3.	*Capparis tomentosa*	8
Figure 4.	*Leonotis leonurus*	10
Figure 5.	The immune system results in a specific and complex series of defensive actions throughout the body	26
Figure 6.	Schematic diagram of the Biological Assay	34
Figure 7.	Overview of anti-inflammatory activity: 5- Lipooxygenase (5-Lox) Assay.	39
Figure 8.	Repellency assay as followed by the South African Medical Research Council: Malaria Unit standard protocols	42
Figure 9.	Insecticidal assay following modified version of the WHO standard method (WHO, 1981a)	44
Figure 10.	Overview of the immuno-modulatory methodology	51
Figure 11.	Peripheral Blood Mononuclear Cell isolation	53
Figure 12.	Guava system, Guava Check (Guava Technologies,United States of America) and Guava PCA-96 (Cytosoft 2.0 software)	56
Figure 13.	*D. cinerea*	63
Figure 14.	*C. dimidiatus*	63
Figure 15.	*C. tomentosa*	64
Figure 16.	Dried flowers of *L. leonurus*	64
Figure 17.	*L. leonurus* whole plant	64

Figure 18. The anti-inflammatory activity of *L. leonurus C. dimidiatus, D. cinerea, and C. tomentosa* and NDGA using the 5-lipoxygenase assay. 74

Figure 19. Mutant revertants of *S. typhimurium* TA 98 (A) *C. tomentosa* (1000µg/ml) (B) Sodium Azide (5µg/ml) 83

Figure 20. Stock Cultures of TA 98 (A) and TA 100 (B) 83

Figure 21. Cytotoxic effect of K 562 cells exposed to the methanolic and aqueous extracts of *D. cinerea, C. dimidiatus*, C. *tomentosa* and *L. leonurus.* Activity indicated by percentage cell viability 87

Figure 22. PBMC exposed to different concentrations of Con A (20 µg/ml, 10 µg/ml, 5 µg/ml), LPS (20 µg/ml, 10 µg/ml, 5 µg/ml), PHA (250 µg/ml, 125 µg/ml), CSA (20 µg/ml, 10 µg/ml, 5 µg/ml), DMSO (10 µl, 5 µl). 89

Figure 23. Morphological effect of the methanolic extracts of *C. dimidiatus, C. tomentosa, D. cinerea* and *L. leonurus* (250 µg/ml) on lymphocytes 89

Figure 24. Morphological effect of the aqueous extracts of *C. dimidiatus, C. tomentosa, D. cinerea* and *L. leonurus* (250 µg/ml) on lymphocytes 90

Figure 25. Effect of *D. cinerea* extracts (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 24h period 91

Figure 26. Effect of *D. cinerea* extracts (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 48 h period 91

Figure 27. Effect of *C. dimidiatus* extracts (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 24h period 92

Figure 28. Effect of *C. dimidiatus* extracts (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 48 h period 92

Figure 29. Effect of *C.tomentosa* extracts (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 24h period 93

Figure 30. Effect of *C.tomentosa* extracts (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml,

1mg/ml) on PBMC over a 48 h period 93

Figure 31. Effect of *L. leonurus* extracts (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 24h period 94

Figure 32. Effect of *L. leonurus* extracts (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 48 h period 94

Figure 33. Effect of flower extract of *L. leonurus* extracts (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 24h period 95

Figure 34. Effect of flower extract of *L. leonurus* extracts (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 48 h period 95

Chapter One: Introduction

1.1 Background and aims of the study

It has been estimated that 7% of the pharmacologically active, plant derived components were discovered after the ethnomedical uses of the plants began being investigated (Farnsworth and Soejarto, 1991). Examples of successful medicines derived from natural product include most antibiotics, the acetylcholine esterase inhibitors, many anticancer agents, the immunosuppressants, (cyclosporine and rapamycin), and the antiparasitic avermectins (Harvey and Waterman, 1998).

Africa consists of thousands of plants that have been used over the years for medicinal purposes. Although there are no unique grounds upon which South Africa can claim a competitive advantage in the global plant biotechnology industry there is a combination of factors that are in South Africa's favour. These include:

- Location within the Southern Hemisphere – since growing regions in the Northern Hemisphere is affected seasonally.
- Traditional strong trade links with other African countries.
- Established as a world class agricultural producer in a wide range of products.
- A diverse climate with a range of biomes - which allow for a good selection of essential crops to be grown.
- Good quality soils – being largely uncontaminated by centuries of exploitation as is the case in the developed world.

After searching various literatures for plants that may have useful properties we selected *Dichrostachys cinerea, Carpobrotus dimidiatus, Capparis tomentosa and Leonotis leonurus* based on their importance in their culture and traditions of South African people. (Table. 1). Apart from cultural significances, these plants are more accessible and

affordable (Mander, 1998) and can contribute to new bioactive compounds that are safe and effective.

Thus the aim was to investigate the biological properties of *D. cinerea*, *C. dimidiatus, C. tomentosa* and *L. leonurus.*

The objectives were as follows:

1. To determine the bioactivity (antimicrobial, antioxidant, anti-carcinogenic, insecticidal and anti-inflammatory) of *L. leonotis , C. dimidiatus, C. tomentosa* & *D. cinerea*
2. To determine the immune modulatory potential of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* by evaluating, the lymphocyte proliferation using the MTT assay, the cytokine secretion by elisa kit, and the pattern of immunostimulation by flow cytometry.
3. To evaluate the safety and toxicity of *D. cinerea*, *C. dimidiatus, C. tomentosa* and *L. leonurus*, toxicity using the *brine* shrimp assay, cytotoxicity the MTT assay and mutagenicity by the Ames assay.

Thesis layout

This thesis is divided into five chapters. Chapter one (this chapter) is the rationale, aims objectives and outline of the thesis layout. Chapter two deals with literature review, Chapter three covers the materials and methods. This is followed by Chapter four which presents the results and discussion followed by the Chapter Five which is a synopsis of the results obtained for *D. cinerea, C. dimidiatus, C. tomentosa and L. leonurus.*

Further work in which the chemical which contributes to common activity may lead to novel drug development.

Chapter Two: Literature Review

2.1 Overview of the plants

For easy reference an overview is presented in Table 1. of *D. cinerea*, *C. dimidiatus, C. tomentosa and L. Leonurus.*

2.1.1 *Dichrostachys cinerea*

Fig. 1. *Dichrostachys cinerea* - Fertile, bright pink flowers in the upper portions of the cluster, whereas the sterile, bright yellow flowers are at the bottom of the cluster

Dichrostachys cinerea belongs to the family Fabaceae. It is commonly referred to as sickle bush or Sekelbos, Marabou-Thorn, Kalahari Christmas tree and Chinese lantern tree. In Zulu it is called as Ugagane, Umthezane and Umzilazembe (Pacific Island Ecosystem at Risk (Pier), 2005).

It is widely distributed throughout South Africa, and has now encroached upon many overgrown bushveld regions. Despite this undesirable ecological impact, it is a valuable medicinal plant (Van Wyk and Gericke, 2003).

D. cinerea is a small tree or strongly growing shrub which grows up to eight meters in height and has an untidy growth pattern with thorny branches. It forms impenetrable thickets in overgrazed bushveld (Van Wyk and Gericke, 2003). The feather like leaves show similarities with the leaves of the Acacia species. The characteristic flowers hang in clusters from branches. The curved and coiled seedpods are borne in clusters on long stalks (Pier, 2005).

The Haikum Bushmen of Namibia chews the fresh leaves to treat diarrhoea, toothaches and earache (Van Wyk and Gericke, 2003). It is also applied directly to snakebites (Van Wyk *et al.*, 2002). Extracts of the leaves and bark, as well as powdered bark are used for wound healing (Van Wyk *et al.*, 2002). Infusions of the roots are used to treat abdominal pain, coughs and pneumonia (Van Wyk and Gericke, 2003). Powdered roots are sniffed to curb nose bleeds, whilst the leaves and roots are smoked to relieve head colds, and to treat tuberculosis and for treatment of epilepsy (Van Wyk and Gericke, 2003). In Sri Lanka, it is commonly used for traditional medicinal purposes as an aphrodisiac and for eye diseases (Wijesundara, 2003). In Sudan, it is used for the treatment of wounds (Eisa *et al.*, 1999) and in Zimbabwe it is frequently used for the treatment of sexually transmitted diseases (Kambizi and Afolayan, 2001).

The phytochemicals present in the leaves are saponins, tannins, flavonoids, sterols and triterpenes (Eisa *et al.*, 1999). The large family Fabaceae is characterized by impressive phytochemical diversity. These include polyphenols, flavonoids, tannins and alkaloids. In

some genera e.g. *Genista*, *Cytisus* and *Laburnum*, the phytochemicals such as quinolizidine alkaloids, including cystisine and sparteine are common. Another group of important natural products present are isoflavonoids that are known for their oestrogenic activity, and coumarin which is used as anticoagulant (Raven *et al.*, 1999).

Furthermore other important chemicals with biological properties have been described in yet other genera in this family. *Cystisus scoparius* yields sparteine which is used for cardiac arrhythmias (Raven *et al.*, 1999). *Melilotus officinalis* has dicoumarol from which the anticoagulant drug warfarin was developed (Raven *et al.*, 1999). *Physostigma venenosum* is used on the tips of West African traditional poison arrows. It also has a cholinesterase inhibitor, physostigmine which is used as a myotic in glaucoma, in postoperative paralysis of the intestine and to counteract atropine poisoning (Manosroi *et al.*, 2004). Recent studies by Bessong *et al*. (2005) show that *Peltophorum africanum* has RNA dependent DNA polymerase activity of reverse transcriptase which could have activity against human immunodeficiency virus type 1 reverse transcriptase and integrase.

2.1.2 *Carpobrotus dimidiatus*

Fig. 2. *Carpobrotus dimidiatus*

Carpobrotus dimidiatus belongs to the family Mesembryanthemaceae and is commonly referred to as Natal sour figs (Van Wyk *et al.*, 2002). The Zulu name is ikhambi-lamabulawo, umgongozi (Malan and Notten, 2006), whilst the Khoi name is gaukum, ghaukum (Van Wyk *et al.*, 2002).

This plant occurs naturally in the sandy areas in the Western Cape, along the Cape South Coast extending towards the Eastern Cape, but is now commonly grown in many parts of South Africa (Van Wyk *et al.*, 2002). It is a robust, flat-growing, trailing perennial, rooting at nodes and forming dense mats (Fig. 2). The succulent horizontal stems curve upwards at the growing point (Malan and Notten, 2006). The smooth fleshy leaves are erect, triangular in cross section and often reddish-green in colour. The rose-purple flowers are large and fleshy and soon develop into fragrant fleshy fruit with a jelly-like, somewhat slimy, sour sweet fruit pulp, which contains a multitude of small brown seeds.

The leaf extracts and leaf pulp are used most often and fruits and stem are used occasionally (Malan and Notten, 2006). The leaf juice is astringent and mildly antiseptic. It is mixed with water and swallowed to treat diarrhoea, dysentery and stomach cramps and is used as a gargle to relieve laryngitis, sore throat and infections. It is also used as a soothing lotion for burns, bruises, scrapes, cuts, grazes and sunburn, ringworm, eczema, dermatitis, herpes, nappy rash, thrush, cold sores, cracked lips and chafing skin allergic reactions. It is also said to be effective against toothache, earache and eye infections. The leaf juice is also used to treat insect bites such as mosquitoes, ticks, and spiders. The leaf is also famous for the cure of blue bottles stings (Van Wyk, *et al.*, 2002). The leaf extracts contain malic acid, citric acid and other calcium salts which may be attributed to soothing blue bottle stings (Watt and Breyer-Brandwijk, 1962). In the Eastern Cape region it is used in controlling diabetes and diphtherias (Malan and Notten, 2006).

The tannins present as active compounds in the leaf juice, make it mildly antiseptic and highly astringent. These are associated with their ability to form complexes with proteins such as digestive enzymes and fungal or viral toxins. They also have a vasoconstrictor effect and reduce fluid loss from wounds and burns, thereby enhancing tissue regeneration (Van Wyk and Gericke, 2003).

Carpobrotus species display phytochemical profile of flavonoids, tannins, alkaloids, phytosterols and aromatic acids. The flavonoids present in *Carpobrotus* species are rutin, neohesperidin, hyperoside, catechin and ferulic acid which are shown to have biological properties such as antimicrobial, antioxidant, anti-inflammatory activity (Van der Watt and Pretorius, 2001; Springfield *et al.*, 2003).

2.1.3 *Capparis tomentosa*

Fig. 3. *Capparis tomentosa*

Capparis tomentosa belongs to the family Capparaceae commonly referred to as 'Woody Caper Bush' in English or in Afrikaans as the Wollerige Kapperbos. The Zulu names include Iqwaningi, Umabusane, Inkunzi, Ebomvu and Ukokwane. The Venda name is Gwambadzi or Mubadai. The Shona people call it Gamobor and in Swahilli it is called Mbada paka (Windadri, 2001).

It is an indigenous South African plant that grows naturally along the easternmost part of South Africa (Van Wyk *et al.*, 2002). It is a scrambling shrub, sometimes developing into a tree that can grow as high as 10 meters tall. The twigs and leaves are yellow-green in colour and are covered in soft, velvety hairs. The oblong leaves are approximately 50 × 20 mm, with a pair of sharp, hook-like thorns at the junction of the stem and leaf base. The white and pink flowers have multiple stamens as shown in Fig.3. The fruit is pink to orange in colour, round and stalked. The seeds are surrounded by fleshy, grey fruit pulp (Windadri, 2001).

It is used as a popular medicine for rheumatism, insanity, snakebite, chest pain, jaundice, malaria, headache, coughs, pneumonia, constipation, infertility and to prevent abortions. It is also used to treat leprosy, tuberculosis and gonorrhea (Van Wyk and Gericke, 2003). The roots is boiled in water and half a cup of this infusion is drunk three times per day for coughs and chest pains (Van Wyk *et al.*, 2002 ; Van Wyk and Gericke, 2003).

The phytochemicals that give the plant its unique biological activity are alkaloids, L-stachydrine, saponin glycosides, tannins, sterol, polyphenols, flavinoids and anthranoids, (Sama and Ajaiyeoba, 2006; Oluwole *et al.*, 2007)**.** Two important alkaloids, Stachydrine, and 3-hydroxy-4-methoxy-3-methly-oxindole have been isolated from *C. tomentosa*. The oxindole compound has been found to possess weak anti-spasmodic activity, substantiating the traditional use of *Capparis* species for their anti-inflammatory and anti-convulsive properties. Stachydrine has been widely researched in the treatment for rheumatism (Van Wyk *et al.*, 2002).

Biological activity of *C. zeylanica* which was found to evoke an immunostimulatory effect on the humoral immune response of sheep, and oral administration of extracts in mice evoked a delayed type hypersensitivity reaction in sheep red blood cells (Ghule *et al.*, 2006). C. *spinosa* exhibits a potent lipid lowering activity in both normal and severe hyperglycemic rats (Eddouks *et al.*, 2005).

2.1.4 *Leonotis leonurus*

Fig. 4. *Leonotus leonurus*

Leonotis leonurus belongs to the family Lamiaceae and is commonly referred to as Wild dagga (Van Wyk and Gericke, 2003). The Zulu name is Umunyane, the Sotho name is Lebake, the Xhosa name is Umfincafincane and the Shona name is Umhlahlampetu (Van Wyk *et al.*, 2002).

L. leonourus is distributed over a large part of South Africa (Van Wyk *et al.*, 2002) and it is a shrub that grows between 2 to 5 meters in height and has a substantial, woody foundation, with branches that are pale brown. The entire plant has a strong odour. The characteristically hairy leaves, located opposite each other on the stem, are elongated, narrow and toothed along the upper half. The flowers are bright orange in colour, hairy and tubular in shape. They are borne in rounded groups neatly arranged at the tips of the branches (Fig. 4). The name leonurus (meaning lion's ears) was given to the plant due to the resemblance of the flowers to lion's ears (Botanical South Africa, 2007).

There are a number of traditional uses recorded. The stems, leaves and flowers are the main parts used (Van Wyk *et al.*, 2002; Van Wyk and Gericke, 2003). The KhoiKhoi first used it as a tobacco and later introduced it to settlers as a medicine to help alleviate aliments of the chest (Malan and Notten, 2006). The Zulu, Xhosa and Khoikhoi people make a tea of the flowers for a soothing cough and cold remedy. This tea has also been used effectively for the treatment of jaundice, cardiac problems, asthma, haemorrhoids, headaches, chest aliments, bronchitis and epilepsy. It is known that the tea of leaves and flowers used to be drunk daily by older generation for water retention, obesity and digestive tract problems, intestinal worms and constipation (Malan and Notten, 2006). The plant has been smoked in order to relieve epilepsy (Van Wyk and Gericke, 2003). The leaves and roots have been used as a remedy for snakebites, insect bites, and stings. Decoctions have been applied as local dressings for boils, eczema, and ailments such as itching and muscle cramps. Herbal decoction can be used internally to treat coughs, colds, influenza, bronchitis, hypertension and headaches. Leaf infusions are used to treat hepatitis and asthma (Van Wyk *et al.*, 2002).

L. leonurus contains volatile oils as well as several unusual diterpenoids (Labdane type lactones). One such isolated diterpenoid is Marrubin which is the main diterpenoid lactone found in the European herb, *Marrubium vulgare* (White Horehound) that is traditionally used to treat acute bronchial coughs. The exact pharmacological effect, however, is yet unknown (Van Wyk *et al.*, 2002). Essential oil in the epidermal glands is very common. Some segments of the family are known to accumulate monoterpenoid glycosides (iridoid). Many species also accumulate rosmarinic acid and other derivatives of caffeic acid. Rosmarinic acid is of some pharmaceutical importance because of its non-specific complement activation and

inhibition of the biosynthesis of leukotrienes (leading to an anti inflammatory effect) as well as its antiviral activity (Raven *et al.*, 1999).

Plants from the family Lamiaceae contain various phenolic compounds such as flavonoids, phenolic diterpenes, monoterpenoid glycosides (iridoids), rosmarinic acid and other derivatives of caffeic acid (Raven *et al.*, 1999; Erdemoglu *et al.*, 2006). Essential oils from the leaf and flower of *L. leonurus* illustrate diverse phytochemicals such as limonene, (Z)-β-ocimene, terpinene, caryophyllene, humulene and germacrene (Raven *et al.*, 1999). Other members from this family that are important include *Lavandula angustifolia*, *Melissa officinalis* and *Mentha arvensis* which have a mild sedative, carminative and spasmolytic activity (Erdemoglu *et al.*, 2006).

Table 1: Nomenclature and characteristics of *Dichrostachys cinerea*, *Carpobrotus dimidiatus*, *Capparis tomentosa* and *Leonotis leonurus*

Scientific Name	**Family Name**	**Common Name**	**Zulu Name**	**Active Compounds**	**Traditional Uses**
D. cinerea	Fabaceae	Sickle bush or Sekelbos, Marabou-Thorn, Kalahari Christmas tree and Chinese lantern tree	Ugagane, Umthezane and Umzilazembe	Polyphenols (especially flavonoids and tannins)	Diarrhoea, toothaches and earache, abdomininal pain, coughs and pneumonia
C. dimidiatus	Mesembryanthemaceae	Natal Sour figs	Isikhambi-lamabulawo and umgongozi	Tannins, malic acid, hyperoside and citric acid	Dysentery laryngitis, soothing cure for blue bottle sting, ringworm, eczema, dermatitis, herpes, nappy rash, thrush, cold sores, cracked lips, chafing skin allergic reactions, earache and eye infections
C. tomentosa	Capparaceae	Woody Caper Bush	Iqwaningi, Umabusane, Inkunzi, Ebomvu and Ukokwane	Stachydrine, and 3-Hydroxy-4-methoxy-3-methly-oxindole	Anti-inflammatory , anti-convulsive, rheumatism, insanity, jaundice, malaria, headache, coughs and pneumonia
L. leonurus	Lamiaceae	Wild dagga	Umunyane	Rosmarinic acid and other derivatives of caffeic acid. Diterpenoids(Labdane type lactones)	Coughs, colds, influenza, bronchitis, hypertension jaundice, cardiac problems, asthma, hemorrhoids, headaches, chest aliments, bronchitis, epilepsy and earaches

2.2 Scientific rationale

Traditional healing is widely practiced in South Africa and about 80% of the black populations consult traditional healers. Recently, some major Medical Aid schemes have included payment for traditional healing in their cover. This necessitates the need for scientific evaluation of traditional uses of plants. As many of traditional uses of plants are based on antibacterial, antifungal, antimalarial, anti-inflammatory, antioxidant activity and immunomodulatory activity this is discussed below.

2.2.1 Antibacterial activity

In many parts of Africa the local and indigenous plants are often the only available means of treating infections (Taylor *et al.,* 2001). In South Africa many plants have been tested for antimicrobial activity (Rabe and van Staden, 1997*;* Lin *et al.,* 1999*;* Luyt *et al.,* 1999; Shale *et al.,* 1999*;* Kelmanson *et al.,* 2000 and Tetyana *et al.,* 2002). The selection of plants for evaluation in these studies was based on traditional uses documented in the literature (Watt and Breyer-Brandwijk, 1962; Gelfand *et al.,* 1985*;* Pujol, 1990 and Hutchings *et al.,* 1996). In many of these studies the screening of plant extracts was based on activity against a number of different bacteria, using the agar dilution (in broth) assay or the disc diffusion (in agar) assay (Rasoanaivo and Ratsimamanga-Urverg, 1993). Variation in reports exists due to a variety of factors. These include: the assay technique; culture medium; strain of bacteria used for testing; age of the plate; plant source; whether it is used fresh or dried, and the quantity of extract tested. In addition, there is no standardized method for expressing the results of antibacterial testing (Rios and Villar, 1988), thus making it difficult to compare results from different studies.

In some of these studies the active compounds have been isolated and identified. The antibacterial compound, muzigadial isolated from *Warburgia salutaris* is traditionally used as a topical application for sores and inflammation (Rabe and Van Staden, 2000). In the leaf extract of *Vernonia colorata*, vernodalin (Reid *et al.*, 2001) and sesquiterpene lactones (Rabe and Van Staden, 2002) were identified as antibacterial compounds. In *Erythrina lysistemon* a major antibacterial compound wighteone was found (Pillay *et al.,* 2001). In *Kigelia africana* palmitic acid was isolated (Grace *et al.,* 2002). Most of the literature of the antimicrobial properties indicates good activity against gram-positive bacteria (Rabe and Van Staden., 1997 and Kelmanson *et al.*, 2000).

2.2.2 Antifungal activity

The incidence of serious fungal infections is increasing partly due to the increasing number of immunocompromised patients due to HIV/AIDS (Espinel-Ingroff *et al.,* 1998). Many diseases, which were thought to have been eradicated from Western countries, might once again become a serious health problem. *Candida albicans* is one of the earliest opportunistic infections in HIV patients (Fan-Harvard *et al.*, 1991). Several plants are used by traditional healers in the treatment of oral candidiasis. Studies of Motsei *et al.* (2003) of aqueous extracts of *Tulbaghia violacea*, Allium *sativum*, *Polygala myrtifolia* and *Glycyrrhiza glabra* showed very good activity in treating fungal infections.

2.2.3 Anti-oxidant activity

Currently there is a great deal of interest in newer bioactive molecules from nature with health-promoting potential (Yesilada, 2005). Many plant compounds (notably phenolics) are antioxidants and function by scavenging free radicles such as hydroxyl (OH^-) and hydrogen superoxide ($H_2O_2^-$) free radicals (ROS). Antioxidants are defined as "any substance that when present at low concentration compared to that of oxidizable substrate, significantly delays or inhibits the oxidation of that substrate (Young and Woodside, 2001). Free radicals play a key role in many degenerative diseases of ageing such as cancer, cardiovascular disease, cataracts, weak immune system and brain dysfunction (Percival, 1998).

Anti-oxidants are capable of deactivating free radicals, before the radicals are able to attack cells and biological targets. Antioxidant bioactive compounds from plant sources are commercially promoted as nutraceuticals, and have been shown to reduce the incidence of diseases (Hermans *et al*., 2007). They also provide important health benefits in the form of anti-oxidant activity (Lindsey *et al*., 2002). Although there are many reports on anti-oxidant activity only those with activity higher than 70% are considered important. Extracts with inhibition values greater than 70%, and thus with high antioxidant activity, were found in the potherbs *Amaranthus* spp, *Sisymbrium thellungii* and *Urtica dioica* (Fennell *et al.*, 2004). Traditionally in South Africa, these plants are used in the preparation of 'imfino' which forms an important part of the diet. High activity (greater than 70%) was also shown by the tuberous *Colocasia esculenta* and the teas, *Galium aparine* L. and *Aspalathus linearis* (Fennell *et al.*, 2004).

2.2.4 Anti-inflammatory activity

Prostaglandins are involved in the complex process of inflammation and are partly responsible for the sensation of pain (Mantri and Witiak., 1994). Many plant derived compounds inhibit the formation of pro-inflammatory signaling molecules such as prostaglandins (made via cyclo-oxygenase pathway) or leukotrienes (made via lipo-oxygenase pathway). To evaluate the efficacy of a plant in reducing pain and inflammation, extracts can be tested for prostaglandin synthesis inhibitory activity in an *in vitro* assay (White and Glassman, 1974). This assay measures the degree of inhibition of the cyclo-oxygenase (COX) enzyme active in prostaglandin synthesis. Cyclo-oxygenase exists in two isoforms: COX-1 and COX-2 (Mantri and Witiak., 1994; Vane., 1994). Recent attention has focused on finding non steroidal anti-inflammatory drugs (NSAIDs) which selectively inhibit COX-2 with little interference of COX-1. Compounds which are selective inhibitors of COX-2 are potentially anti-inflammatory and nonulcerogenic and would therefore be of considerable interest for therapeutic use (Mantri and Witiak., 1994).

Recent studies have also looked at metabolism of arachidonic acid which is involved in inflammation. Arachidonic acid metabolism through COX and Lipo-oxygenase (LOX) pathways generates various biologically active intermediates that play an important role in inflammation, thrombosis and tumor progression. In addition to arachidonic acids conversion to prostaglandins by the COX enzymes, it may also be converted to leukotrienes by the action of 5-Lipoxygenase (Fiorucci *et al.*, 2001).

In South Africa many plants used in the treatment of pain and inflammation have been studied (Jager *et al.*, 1995; McGaw *et al*., 1997; Lindsey *et al*., 1999 and Shale *et al*., 1999). To

classify a plant as active, the minimum inhibition by aqueous extracts tested at a final concentration of 250 μg per test solution must be 59% and for methanolic extracts, 70% (Fennell *et al.*, 2004). On the basis of this minimum inhibition criterion, *Siphonochilus aethiopicus*, *Ocotea bullata* and *Eucomis autumnalis* were identified as having good activity (McGaw *et al.*, 1997; Lindsey *et al*., 1999 and Zschocke *et al.*, 2000).

Ocotea bullata is one of the top ten traditional medicinal plants used in South Africa (Mander, 1998). The stem bark is used to cure headaches, urinary disorders and stomach ailments. Extracts of the bark have good prostaglandin synthesis inhibitory activity (Jager *et al*., 1995). Two compounds sibyllenone and ocobullenone, were found to contribute to inhibitory activity of *O. bullata* bark extracts (Zschocke *et al.*, 2000). Taylor *et al.* (2001) also found significant COX-2 inhibitory activity with extracts from *Eucomis autumnalis* which is a well used plant.

2.2.5 Anti- mosquito activity

Malaria is caused by a parasite called *Plasmodium*, which is transmitted via the bites of infected mosquitoes. In the human body, these parasites multiply in the liver, and then infect red blood cells (World Health Organization, 2005).

Although many drugs are available more than two million people die from malaria each year (Prozesky *et al*., 2001) due to the fact that the *Plasmodium* parasites responsible for malaria have developed drug resistance (Peters, 1998). Traditional remedies are continually being investigated as plant derived antimalarial drugs become more sought after. Prozesky *et al.* (2001) investigated 14 South African plant species for antimalarial activity, most showed potential anti-plasmodial activity and against a chloroquine-resistant strain of *Plasmodium*

falciparum. Other plants studied include *Ajuga remota* and *Caesalpinia volkensii* (Kuria *et al*., 2001), *Morinda lucida* (Makinde *et al*, 1994), *Morinda morindoides*, *Cassia occidentalis* and *Phyllanthus niruri* (Mesia *et al.*, 2001). The most commonly used plants in Africa include neem (*Azadirachta indica*), *Hyptis* spp. (bushmint family), *Ocimum* spp. (basil family), *Corymbia* spp. (formerly *Eucalyptus* spp.) and *Daniellia oliveri* (locally known as churai), all of which were >70% effective in field-trials against *Anopheles gambiae* (Debboun *et al*., 2006).

Personal protective measures, including repellents and larvicidal inhibition are widely used to prevent the transmission of arthropod-borne diseases by minimizing the contact between humans and vectors. In contrast to vaccines and chemoprophylaxis as means of personal protection, repellents and larvicidals are convenient, inexpensive, and afford advantages in protection against a wide range of vectors (WHO, 1995). They are also the primary means of mosquito-borne disease prevention available in areas where vector control is not practical (Gupta and Rutledge, 2002).

Majority of commercial repellent products contain the chemical diethyl-3-methylbenzamide, formerly known as diethyl-m-toluamide (DEET), (McCabe *et al*., 1954). Although effective, DEET is not the ideal product, as allergic and toxic effects have been documented (Gupta and Rutledge, 2002) and its solvent characteristic can damage plastics and other synthetic materials. Because of the undesirable effects of DEET, research was actively carried out to find alternative compounds that are safer to use and equally or more effective (Robert *et al*., 1990; Schreck and Leonhardt, 1991; Dua *et al*., 1996 and Walker *et al.*, 1997). One new promising repellent is the piperidine compound (AI3-37220) which provides equal or better

protection against certain mosquitoes than that obtained with DEET (Coleman *et al*., 1994; Frances *et al*., 1996 and Walker *et al*., 1997).

2.2.6 Safety of traditional medicines

Although plants may possess pharmacological properties, they may also be toxic or mutagenic. The toxic effects of most widely used medicinal plants are not well documented in the literature although one might expect plants used in traditional medicine over a long period to be safe (Elgorashi *et al.*, 2003). Hence, an evaluation of the toxicity, cyto-toxicity and mutagenicity are important parts of all scientific studies (Schimmer *et al*., 1988; Schimmer *et al*., 1994; Higashimoto *et al.*, 1993; Kassie *et al.*, 1996; De Sa Ferrira and Ferrao Vargas, 1999).

There are many toxicity and cytotoxicity tests available to test for the safety of plant extracts or compounds. Cytotoxicity is the measurement of the ability of a chemical to damage or kill cells. Toxicity is evaluated using two different criteria, firstly by its capacity to cause injury to a living organism and secondly as any diverse effects of a chemical on a living system (Verschaeve *et al.*, 2004). The severity of toxicity produced by any chemical is directly proportional to the concentration and the exposure time. This relationship depends on the developmental stage of an organism and its physiological status (Meyer *et al.*, 1982). The 3-4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay using a tetrazolium salt has been used to develop a quantitative colorimetric assay for mammalian cell to evaluate survival and proliferation (Hanelt *et al*., 1994). The assay detects living but not dead cells. Hence by evaluating the cell survival one can deduce the LC_{50} therefore the cytotoxicity of the plant extract can be elicited.

The brine shrimp lethality bioassay is another simple bioassay for natural products and known to be rapid, reliable, inexpensive and convenient, as a general bioassay for screening of toxicity (Meyer *et al.,* 1982). The brine shrimp *Artemia salina* Leach, from the family Artemiidae is an invertebrate (crustacean) component of saline aquatic and marine ecosystems (Ayo *et al.,* 2007). The procedure determines LD_{50} values in µg/ml of plant extract. It is known that a positive correlation exists between brine shrimp lethality and cytotoxicity (Ayo *et al.,* 2007).

Many plants also have compounds that are mutagenic which are used in traditional medicine, and have been also found to cause damage to genetic material. In a study by Elgorashi *et al*. (2003) potential genotoxic effects were studied using *in vitro* bacterial and mammalian cell assays such as Ames test (tester strains TA98 and TA100), VITOTOX test, micronucleus test and comet assay. Using these tests, most of the plant species investigated caused either DNA damage or chromosomal aberrations, and only a few plants showed frame shift mutations in the *Salmonella* / microsome assay using strain TA98.

Among the plants that had mutagenic effects in the *Salmonella*/microsome assay were *Crinum macowanii*, *Chaetacme aristata*, *Plumbago auriculata*, *Catharanthus roseus* and *Ziziphus mucronata* (Elgorashi *et al*., 2003). Of the 51 plant species tested using human white blood cells, *Kigelia africana*, *Merwilla plumbea*, *Boophane disticha*, *Celtis africana*, *Crinum macowanii*, *Erythrina caffra*, *Ochna serrulata*, *Sclerocarya birrea* and *Tulbaghia violacea* showed genotoxicity in the micronucleus test, in the form of structural and numerical chromosome aberrations. *Acokanthera oblongifolia*, *Afzelia quanzensis*, *Bersama lucens*,

Ocotea bullata, *Siphonochilus aethiopicus* and *Tetradenia riparia* caused DNA damage as detected in the comet assay (Taylor *et al*., 2003).

Some plants listed below are reported to be highly toxic because they cause both DNA damage and chromosomal aberrations: *Antidesma venosum*, *Balanities maughamii*, *Catharanthus roseus*, *Catunaregam spinosa*, *Chaetacme aristata*, *Diospyros whyteana*, *Euclea divinorum*, *Gardenia volkensii*, *Heteromorpha arborescens*, *Heteromorpha trifoliata*, *Hypoxis colchicifolia*, *Ornithogalum longibracteatum*, *Plumbago auriculata*, *Prunus africana*, *Rhamnus prinoides*, *Ricinus communis*, *Spirostachys africana*, *Trichilia emetica*, *Turraea floribunda*, *Vernonia colorata* and *Ziziphus mucronata* (Taylor *et al*., 2003).

2.3 Immunodulating Properties of Plants

There is growing evidence that drugs or biological agents capable of modulating single pathways or targets are of limited value as immune related therapies and this has stimulated interest in the use of 'cocktails' of immunodrugs to restore immunostasis. As botanicals are chemically complex and diverse they could provide appropriate combination of synergistic compounds useful in drug discoverery (Patwardhan and Gautam., 2005). Many agents of synthetic and natural origin have stimulatory, suppressive or regulatory activity on the immune system (Cherng *et al*., 2008).

Immunomodulatory agents of plant and animal origin increase the immune responsiveness of the body against pathogens by activating the non-specific immune system. However there is a need to subject such medicinal plants to systematic studies to substantiate the therapeutic claims made with regard to their clinical utility (Fulzele, 2003). Recently, there is an

enthusiasm towards exploration of a novel group of compounds from natural sources that modulate the immune response of living systems and influence the disease process (Gulati, 2002).

2.3.1 Immuno-Modulation and phytochemicals

The immune system can be modulated by either immunosuppression or immunostimulation. These can be via immunodrugs which include synthetic organics and biological compounds such as cytokine and antibodies as well as microbial compounds and botanical natural products. These influence immunoregulatory cascades to bring about specific stimulatory, suppressive or regulatory effect (Patwardhan and Gautam, 2005).

Botanicals produce a diverse range of products with immunomodulating potential, including isoflavonoids, indoles, phytosterols, polysaccharides, sesquiterpenes, alkaloids, glucans and tannins (Patwardhan and Gautam, 2005). It has also been observed that triterpene, saponins and flavonoid derivatives possess a remarkable effect on the immune system (Ferreira *et al.*, 2003).

Numerous medicinal plants have been used in traditional medicine to stimulate the immune system however very few have been scientifically researched. A study conducted by Ratan *et al.* (2008) demonstrated that the alcohol extract of the roots of *Baliospermum montanum* has exhibited significant effect on phagocytosis by human neutrophils and chemotactic locomotion of neutrophils. Thus, the plant can be further explored for its phytochemical profile to identify the active constituents responsible for the above mentioned activities. In another study by Madan et *al*. (2008), *Aloe vera* extracts were administered to swiss albino

mice daily for five days and these showed an increase in the total white blood cells count and also showed an increase humoral immune response. In another study by Nworu *et al*. (2008), methanolic seed extracts of *Garcinia kolahas* showed immunomodulatory effects on both the cell mediated and humoral components. A study by Ju lee *et al.* (2007) showed that the methanolic fruit extracts of *Amorpha fruiticosa* had a rotenoid isoflavone glycoside which demonstrates increased immunomodulatory effect against T cells.

2.3.2 Stimulation of the Immune System

According to Cherng *et al*. (2008), there are three types of immunomodulation after treatment with phytochemicals:

- Type 1: enhanced lymphocyte activation and secretion of IFN- γ.
- Type 2: augmented lymphocyte activation.
- Type 3: elevated secretion of IFN- γ.

A study of phytochemicals to improve the immune function, currently receives a lot of attention.

Therefore we start with a brief overview of the immune system. The immune system protects the human body against invasion of pathogens (i.e. bacteria, viruses and parasites) and is made up of two mutually interactive systems, i.e. the innate and adaptive system shown in Fig. 5.

The innate immune response is rapid and functional, but non-specific. Epithelial barriers like the skin and the linings of the gastrointestinal tract, lungs and urinary tract are the first line of defence of the innate immune system. Leukocytes that are involved in this early innate response

are granulocytes (neutrophils), monocytes/ macrophages and natural killer (NK) cells and complement systems. These cells play an important role in phagocytosis of pathogens, free radical production (oxidative burst), cytokine production, and antigen presentation to lymphocytes.

The adaptive immune response is somewhat slower, but highly specific. This response depends on the production of antibodies by B-lymphocytes directed against specific antigens present on pathogens (called humoral immune response) and on the attack of infected body cells by cytotoxic (Tc cells) and helper T-lymphocytes cells (Th cells), called cell-mediated immune response. In addition, due to the presence of memory cells, the adaptive immune response is characterized by enhanced and fast responses after repetitive contacts with the same antigen. Ultimately, an immune response is the result of these two interacting systems. After leukocytes of the innate immune system are activated, they produce cytokines and present antigens to T- and B-lymphocytes, which in turn activates the adaptive immune system (Abbas *et al.*, 2004).

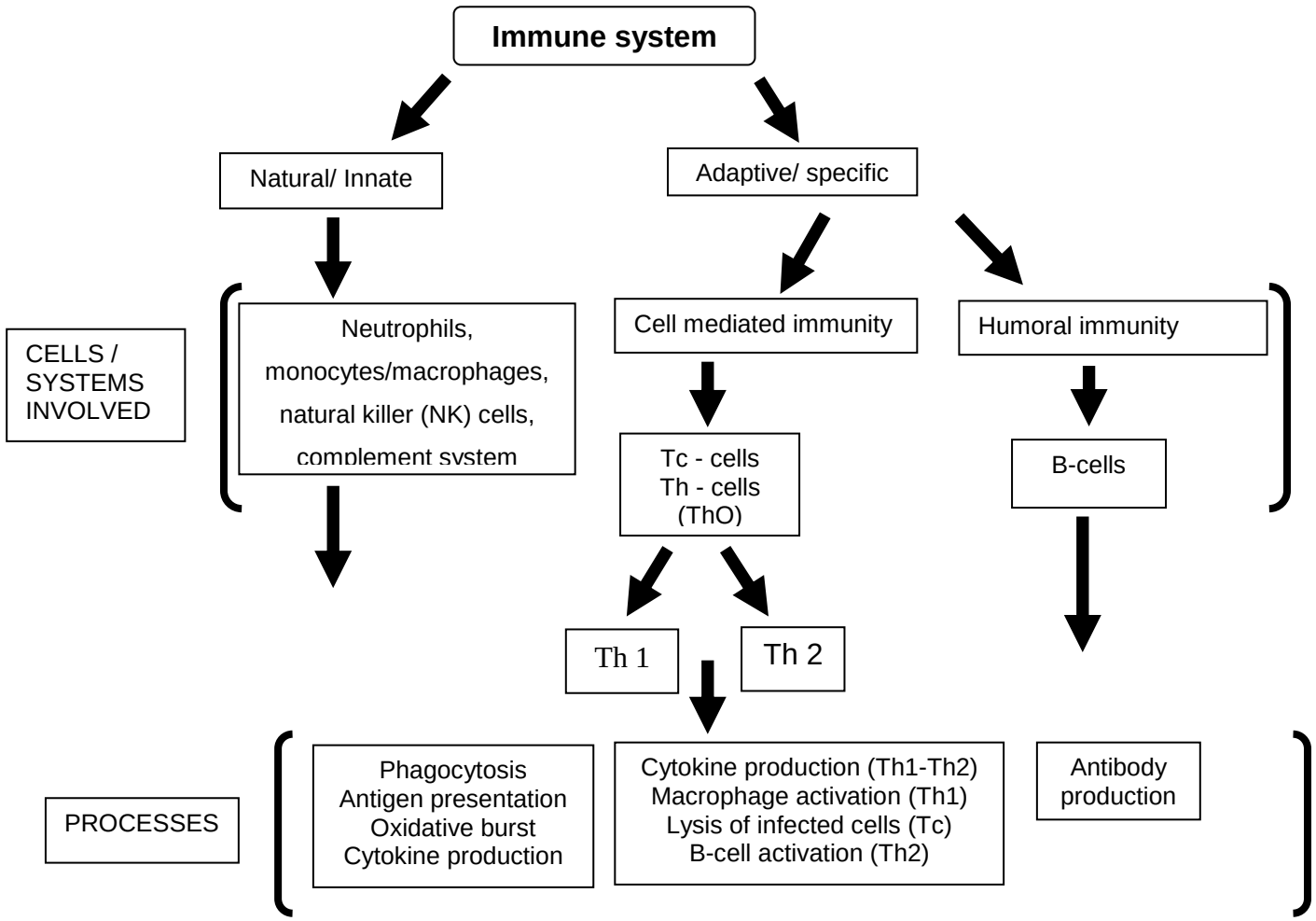


Fig. 5: The immune system results in a specific and complex series of defensive actions throughout the body (Prescott *et al.*, 1993).

Cytokines

Cytokines are signalling proteins secreted by a wide variety of cell types and used for inter-cell communication. Cytokines exert their effect primarily by binding to specific plasma membrane receptors called "cell associated differentiation antigens on target cells" (CD). Cytokines function in four different ways:

- as mediators of natural/ non-specific immunity
- as activators of effector cell mediators of mature cell activation
- as mediators of differentiation, behaviour and growth
- as mediators of immature cell growth and differentiation.

The types of cytokines produced determine whether a naïve helper T-lymphocyte (Th0) develops into a type 1 helper T-lymphocyte (Th1) or a type 2 helper T-lymphocyte (Th2). For example, interleukin (IL)-12, produced by activated macrophages, stimulates Th1 cell development. This leads to the production of typical Th1 cytokines like , IL-1β, interferon, IL-2, and tumor necrosis factor (TNF-α), which play an important role in cell-mediated immunity. In contrast, IL-4 results in the development of Th2 cells, producing IL-5, IL-6, IL-10, and IL-13, which are involved in the humoral immune response. Recently, also regulatory T-lymphocytes (defined as Th3 cells) have been described. Their role is most likely to influence the Th1/Th2 balance by inhibiting Th1 activity by producing anti-inflammatory cytokines like transforming growth factor (TGF)β and IL-10 (Kidd, 2003).

Table 2: Functions of the cytokines

Cytokine	**Cell source**	**Functions of cytokine**
IFN-γ (interferon- γ)	Tcells (T_H, T_c), NK cells	Activation of T cells, macrophages, neutrophils and NK cells; Increases Class I and II MHC molecules.
IL- 10 (interleukin- 10)	Tcells (T_H 2), B cells, macrophages, keratinocytes	Reduces the production of IFN-γ, IL-1, TNFα, and IL-6 by macrophages in combination with IL-3 and IL-4 cause's mast cell growth; in combination with IL-2 causes growth of cytotoxic T cells and differentiation of $CD8^+$ cells.

(Adapted from Prescott *et al.,* 1993)

Th1 cells (IFN-α, IL-2 and TNF) and Th2 (IL-3, IL-4, IL-5, IL-10) effector cells, this classification is based on their functional capabilities and cytokine profiles (Cherng *et al*, 2008). Th1 cells drive the cellular immunity to fight intracellular organisms, eliminate cancerous cells and stimulate delayed hypersensitivity reactions. By contrast, Th2 cells drive humoural immunity and unregulated antibody production to fight extracellular organisms. T-cell homeostasis or immunostasis requires a fine balance between Th1-Th2 response and plant extracts could exhibit stimulatory, suppressive or regulatory activity (Patwardhan and Gautam, 2005).

a. Interleukin -10 (IL-10)

Interleukin-10 originally known as Cytokine Synthesis Inhibitory Factor (CSIF) is an 18.5 kD protein that shares over 80% sequence homology with the Epstein –Barr virus protein (BCRFL). IL-10 can inhibit the synthesis of certain cytokines IL-1, IL-2, IL-6, IL-8, IL-12, GM-CSF and TNF by stimulated macrophages, NK cells and T cells co-stimulate the proliferation of B cells and their differentiation into antibody-producing cells which can express high levels of IgM, IgG and IgA antibodies (Agrawal *et al.*, 2008). It is mainly expressed in monocytes and type 2 T helper cells (T_H2), mast cells, CD4+CD25 regulatory T cells and also in a certain subset of activated T cells and B cells. It is released by cytotoxic T-cells to inhibit the actions of NK cells during the immune response to viral infection (Cherng *et al*., 2008)

b. Interferon gamma (IFN-γ)

Interferon gamma or immune interferon is a potent multifunctional type II interferon which is secreted by activated NK cells and T cells**.** IFN-γ binds to different receptors than the type I viral interferons IFN- α and IFN- β, with which little sequence homology is shared. In addition, to its anti-viral effects, IFN-γ can unregulate a number of lymphoid cell functions, including the antimicrobial and antitumor responses of macrophages, NK cells, and neutrophils. In addition IFN-γ can exert strong regulatory influences on the proliferation, differentiation, and effector responses of B cell and T cell subsets. These influences can involve IFN-γ capacity to boost MHC class I and II expression by antigen presenting cells as well as direct effects on B cells and T cells themselves. Human IFN-γ is a 35kD protein containing 143 amino acid residues. IFN-γ upregulates ICAM-1 expression on endothelial cells, and has become a primary marker for Th-1 like immune response research (Agrawal *et al.*, 2008).

IFN-γ is secreted by Th1 cells, Tc cells, dendritic cells and NK cells. IFN-γ has antiviral, immunoregulatory, and anti-tumour properties. It alters transcription in up to 30 genes producing a variety of physiological and cellular responses. IFN-γ is the hallmark cytokine of Th1 cells (whereas Th2 cells produce IL-4 and Th17 cells produce IL-17). NK cells and CD8+ cytotoxic T cells also produce IFN-γ (Cherng *et al*., 2008).

2.3.5 Lymphocytes surface markers

Lymphocytes (and other leucocytes) express a large number of different molecules on their surfaces which can be used to distinguish (mark) cell subsets. Many of these cell markers can now be identified by specific monoclonal antibodies (mAbs). A systematic nomenclature has been developed in which the term CD (cluster of differentiation) refers to groups (clusters) mAb, each binding specifically to a particular molecule (Roitt *et al.,* 2002). Monoclonal antibodies with similar characteristics are grouped together and given a CD number (Table 3). Although it is sometimes useful to define markers in this way, it is not always possible to do so. A maturation marker for one lineage is sometimes an activation marker for the same lineage. CD 10 present on immature B cells is lost on mature B cells but reappears on activation. Sometimes 'activation' markers may already be present at low density on cells but increase following activation.

Table 3: Cell type and their specific human clusters of differentiation molecules

Type of cell	**CD markers**
Stem cells	CD 34 $^{+}$, CD 31^{-}
All leukocyte groups	CD 45^{+}
Granulocyte	CD 45^{+}, CD 15^{+}
Monocyte	CD 45^{+}, CD 14^{+}
T lymphocyte	CD45^{+}, CD 3^{+}
T helper cell	CD 4 $^{+}$
B lymphocyte	CD 3^{-}, HLA-DR $^{+}$
Natural killer cell	CD 3^{-}, CD 16 $^{+,}$ CD 56 $^{+}$

(Adapted from Roitt *et al.*, 2002)

2.3.6 CD4 Cells

CD4^{+} T cells are white blood cells. They are also called T-helper/inducer cells (Rodriguez *et al.*, 2003), which organize the response of the immune system's to some microorganisms, including bacteria, fungal infections and viruses. CD4^{+} T cells are made in the spleen, lymph

nodes, and thymus gland and they circulate throughout the body in the bloodstream (Roitt *et al.*, 2002).

A CD4 is an antigen present on human T-helper lymphocytes, macrophages and dendritic cells that mediates major histocompatability complex class II (MHC II) restricted immune response (Roitt *et al.*, 2002). $CD4^+$ T cells constitute about 60% T-cells secreting a number of immunological messenger proteins which are known as cytokines. Cytokines recruit and co-ordinate other immunological processes during activation. They are activated when epitopes are presented to them in association with MHC class II molecules (Miller, 2002).

2.3.7 The Role of CD4 Cells in the Immune System

$CD4^+$ T cells play a vital role in making sure that a person's immune system is working properly. They target foreign substances that should not be in a person's blood. $CD4^+$ T cells attach themselves to these foreign substances and release various chemicals. These chemicals attract other cells called $CD8^+$ T cells, which then come along and destroy the foreign substances (Roitt *et al.*, 2002). Human CD4 cells can be separated into three subsets termed T-helper 0 (Th0), T-helper 1(Th1), and T-helper 2 (Th2). Th1 stimulates cell mediated immunity. Th2 increases antibody production. Th0 cells are precursors expressing many different types of cytokines. The difference between Th1 and Th2 cells occurs in response to antigens, or is related to the type of cytokines already present at the time of differentiation (Miller, 2002).

Chapter Three: Methodology

3.1 Collection and preparation of plant material

The leaves of *D. cinerea*, *C. dimidiatus*, *C. tomentosa* and the leaves and flowers of *L. leonurus* (± 5 kg fresh weight) were collected from the greater Durban area in KwaZulu-Natal, South Africa during 2006 and 2007. These plants were collected and identified using taxonomic keys by Professor H. Baijnath, School of Biological and Conservation Sciences, University of KwaZulu Natal. Herbarium specimens were prepared and are lodged at the Ward Herbarium as voucher specimens. The scientific name, family name, common name, and the location from which they were collected are listed in Table 4. The plants were de-leafed, washed repeatedly with distilled water until no foreign material remained (damaged leaves were removed) and dried in an oven (Memmert B. Owen Jones limited, South Africa) at 25°C for 48 h (Jeremy and Whiteman., 2003). The dried samples were milled in an industrial grinder (Retsch Gmbh, West Germany) and stored in labelled Schott bottles in a cool dark place.

Table 4: Details of plants used in this study

Scientific Name	**Family Name**	**Common Name**	**Location collected**
D. cinerea	Fabaceae	Sickle bush or Sekelbos, Marabou-Thorn, Kalahari, Christmas tree and Chinese lantern tree	Reservoir Hills (2006)
C. dimidiatus	Mesembryanthemaceae	Sour figs	Park Rynie (2007)
C. tomentosa	Capparaceae	Woody Caper Bush	Reservoir Hills (2006)
L. leonurus	Lamiaceae	Wild dagga	Shongweni (2007) Flowers : Randles Municipal Nursery (2006)

3.2 Sample Preparation

Methanolic and aqueous extract of the dried plant material were prepared according to the procedure outlined by Jeremy and Whiteman (2003) with minor modifications.

Methanolic extract: Fifty grams of the powdered plant material was weighed out, stirred in 200 ml of 80% methanol (v/v) and agitated for 24 hours. The filtrate was collected using Whatman No. 1 filter paper. Solvents were removed by evaporation using a rotoevaporator (Buchi RE) which was connected to a water bath set at a temperature of 50°C. The remaining slurry was freeze dried in freeze Dryer (Virtis Benchtop) and dried to form a powdery residue. This was used for all subsequent experiments.

Aqueous extract: Fifty grams of the powdered plant material was stirred in 200 ml of distilled water and agitated for 24 hours, before centrifuging at 8000 rpm for 10 min. The supernatant was filtered using Whatman No. 1 filter paper. This was dried in an oven (Memmert, South Africa) set at 35°C. The powdery dried crude extract, was dissolved in appropriate solvents, depending on the assay requirements. The yield from 100 g of dried powdered material was calculated as follows:

$$\text{Product yield} = \frac{\text{Amount of product}}{\text{Amount sample added}} \times 100$$

The methonolic plant extracts were diluted in either acetone, methanol or in Dimethyl sulphoxide (DMSO) and the aqueous extracts were diluted in double distilled deionised water or DMSO to give concentrations of 1000 µg/ml, 500 µg/ml, 250 µg/ml, 100 µg/ml, 10 µg/ml and 1 µg/ml.

3.3 Biological Screening

A schematic outline of the biological tests viz., anti-bacterial, anti-fungal, anti-inflammatory, anti-oxidant and anti-mosquito activity is shown in Fig. 6.

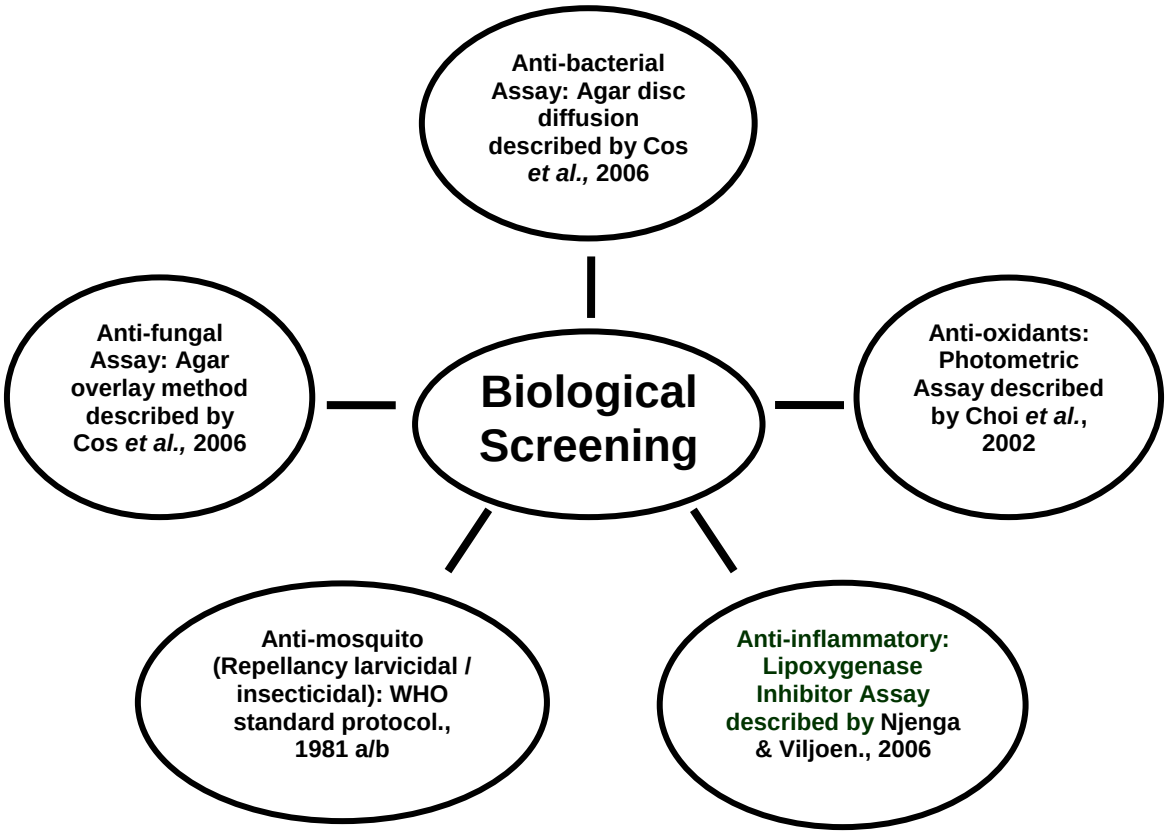


Fig. 6. Schematic diagram of the Biological Assays

3.3.1 Antimicrobial Activity

The antimicrobial activity (anti-bacterial and anti-fungal) and the minimum inhibitory concentration of the methanolic and aqueous plant extracts were carried out using the agar disc diffusion method and agar overlay method (Cos *et al.*, 2006).

Antibacterial assay

The ten bacterial strains used were based on the standard recommendations of Springfield *et al.*, (2003) and Sama and Ajaiyeoba.(2006), and were obtained from the stock collection in the Department of Biotechnology and Food Technology, Durban University of Technology (DBT*). They were: *Esherichia coli* (DBT*_L), *Pseudomonas aeruginosa* (DBT*_D), *Klebsiella oxytoca* (DBT*_AM), *Serratia marcescens* (DBT*_ZM), *Salmonella typhimurium* DBT*_AF) and *Staphylococcus epidermis* (DBT*_Q), *Staphylococcus aureus* (DBT*_E), *Bacillus cereus* (DBT*_F), *Bacillus stearothermophilus* (DBT*_Q), *and Micrococcus* spp. (DBT*_AR).

Cultures were plated out and verified. Stock cultures were stored in micro bank vials (Davies diagnostics, South Africa) using 50% glycerol. When needed they were plated out on Tryptone Soya Agar (Biolab) plates and grown in Tryptone Soya Broth (Biolab) for 24 h at 37°C. The concentrations of bacterial cells were adjusted to MacFarland Standard of 0.5 which corresponded to 10^8 cfu.ml^{-1} (Matasyoh *et al*., 2007).

A suspension (1 ml of 10^8 cfu/ml) of the test bacteria was spread on Mueller Hinton Agar plates (Biolab, Merck, South Africa). Fifty microtitres of the aqueous or methanolic extract, (1000 µg/ml, 500 µg/ml, 250 µg/ml, 100 µg/ml, 10 µg/ml, 1 µg/ml) was transferred onto sterile 9 mm discs made from Whatman No. 1 filter paper. Each concentration was tested in triplicate. 100% methanol (50µl) was used as the negative control and Ciprofloxacin 5 µg/ml (Fluka, Biochemika), was used as a positive control. The impregnated discs were dried in open sterile petri dishes in a biological safety cabinet (Labtec Bioflow II, South Africa). These were placed onto inoculated agar plates and incubated at 37°C for 24 h except for *B. stearothermophilus* which was incubated at 55°C for 24 h. The inhibition was determined from the diameter of clearing around the disks in mm. The MIC was taken as the lowest concentration that inhibited growth.

Anti-fungal assay

The aqueous and methanolic extracts were tested for antifungal activity using the paper disc diffusion method. Two yeast cultures, *Candida albicans* (DBT_AB) and *Saccharomyces cerevisiae* (DBT_R), and seven moulds *Aspergillus flavus* (DBT_AR), *Cladosporium sp.* (DBT_AS), *Fusarium verticilloides* (DBT_AT), *Geotrichum sp.* (DBT_AA), *Penicillium sp*. (DBT_AC), *Rhizopus sp.* (DBT_Y) and *Trichoderma sp.* (DBT_AU) were inoculated on Sabourand Dextrose Agar (SDA) plates (Biolab).

The yeast cultures were grown in Sabourand Dextrose Broth for 24 h at 28°C. The moulds were incubated at 28°C for 4 to 7 days in Sabourand Dextrose Agar until sporulation. The

spores were collected in 10 ml sterile distilled water, counted in a Neubauer counting chamber and the concentration adjusted to 10^6 spores/ml.

Sterile distilled water containing the fungal spores (10^6 spores/ml) was poured over the Sabourand Dextrose Agar base plates (Biolab). Fifty microlitres of each extracts (1000 μg/ml, 500 μg/ml, 250 μg/ml, 100 μg/ml, 10 μg/ml and 1 μg/m) was transferred on each of three sterile 9 mm discs made from Whatman no. 1 filter paper. 100% Methanol (50μl) served as the negative control, whilst amphotericin B, 5 μg/ml (Fluka, Biochemika), served as a positive control. Each sample was tested in triplicate. The plant extracts and ethanol impregnated discs were allowed to evaporate in an open sterile Petri dish in a biological safety cabinet with a vertical laminar flow (Labtec Bioflow II, South Africa) before the impregnated discs were placed onto the agar plates. Agar plates inoculated with the respective yeasts and fungi containing the discs with the extracts and controls respectively were incubated ± 30°C until growth was observed. The antifungal activities were recorded as the width (millimetres, diameter of the disc) of the zone of inhibition after incubation. The MIC was determined.

3.3.2 Antioxidant Activity

The anti-oxidative properties of the crude extracts was tested using the 1.1-diphenyl-2-picrylhydrazyl radical (DPPH) photometric assay described by Choi et al., 2002.

Preparation of plant material

The freeze dried aqueous and methanolic plant material (1000 μg/ml) were diluted to final concentrations of 500μg/ml, 250μg/ml, 100μg/ml, 50μg/ml, 10 μg/ml and 1μg/ml in

methanol. Rutin (Appendix1.1) found in the buckwheat plant *Fagopyrum esculentum*, was used as a comparative standard.

DPPH Photometric Assay

One millilitre of a 0.3 mM DPPH in ethanol was added to 2.5 ml of plant sample solution of different concentrations and was allowed to react at room temperature for 30 minutes. One ml methanol plus plant extract solution (2.5 ml) was used as a blank and DPPH solution and 2.5 ml ethanol was used as a negative control. The positive control was DPPH solution (1 ml) plus 2.5 ml 1mM Rutin. Each test was carried out in triplicate and results are expressed as the mean±standard deviation. The absorbance values were measured in a Varian Cary 1E UV-visible spectrophotometer at 518 nm and the average absorbance values was converted into the percentage antioxidant activity, using the following equation:

$$\text{Scavenging capacity \%} = 100 \text{ X } \frac{(\text{Abs of sample} - \text{Abs of blank})}{\text{Abs of negative control}} \times 100$$

3.3.3 Anti-inflammatory activity

Determination of the 5- lipooxygenase activity

Lipooxygenase is known to catalyse the oxidation of unsaturated fatty acids containing 1-4 diene structures. The conversion of linoleic acid to 13- hydroperoxy linoleic acid was followed spectrophotometrically by the appearance of a conjugate diene at 234 nm on a UV/visible spectrophotometer (Varion Cary 1E UV- Visible spectrophotometer). This is represented in Fig.7. Nordihydroguaiaretic acid (NDGA) and Rutin known inhibitors of soybean lipooxygenase, were used as controls. The reaction was initiated by the addition of

aliquots (50 µl) of a soybean lipooxygenase solution (prepared daily in potassium phosphate buffer pH 9.0) in a sufficient concentration to give an easily measurable initial rate of reaction to 2.0 ml of sodium linoleate (100 µM) in phosphate buffer; the enzymatic reactions were performed in absence or in presence of inhibitor and their kinetics were compared. The inhibitors were dissolved in DMSO in such a manner that an aliquot of each (30 µl) yielded a final concentration of maximum 100 ppm in each assay. The initial reaction rate was determined from the slope of the straight line portion of the curve and the percentage inhibition of the enzyme activity was calculated by comparing with the control (using 30 µl of phosphate buffer (pH 9.0) instead of 30 µl of the inhibitor solution). Each inhibitor concentration was tested in triplicate. The concentration that gave 50% inhibition (IC_{50}) was calculated from the outline of the inhibition percentages as a function of the inhibitor concentration (Njenga and Viljoen., 2006). Aqueous extracts (IC_{50} ≥100 µg/ml) were not taken in this study. All the analyses were carried out in triplicate and the results were expressed as mean (±SD). Regression analysis was used to calculate IC_{50}, defined as the concentration of inhibitor necessary for 50% inhibition of the enzyme reaction (Appendix 2.2).

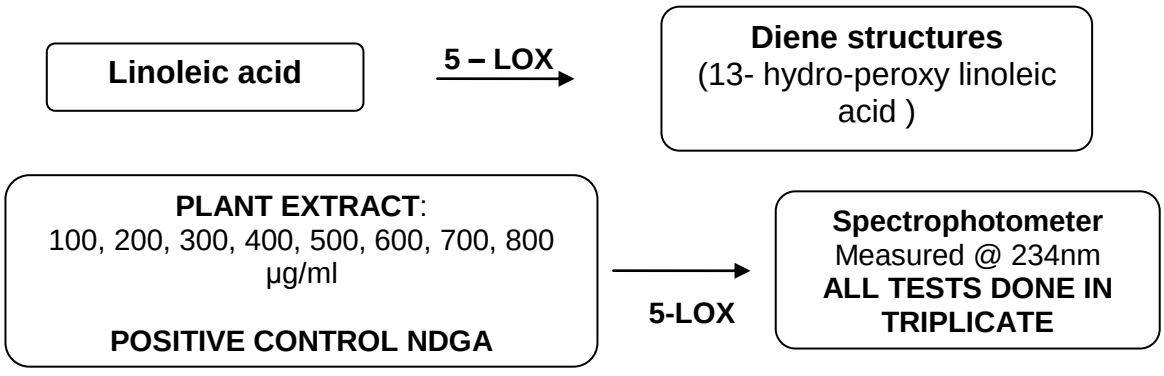


Fig. 7. Overview of anti-inflammatory activity: 5- Lipooxygenase (5-Lox) Assay.

3.4.4 Anti Mosquito Activity

These experiments were carried out at the Medical Research Council, Malaria Unit South Africa

Sample Preparation

The freeze dried aqueous and methanolic plant material was diluted to a final concentration of 1000μg/ml in distilled water or methanol respectively. The stock solution of 1000 μg/ml in DMSO was used for the larvicidal, repellency and adulticidal assays. To ensure consistency amongst results, each sample had undergone a repeat trial for each assay, whilst control assays were conducted to make certain the trial had been performed under controlled conditions.

Larvacidal assay

The larvicidal bioassay followed the WHO standard protocols (WHO., 1981 a/b) with slight modifications. One millilitre of the stock solution was added to polypropylene containers (10 cm ×10 cm) containing 0.25 litres of distilled water. Thirty 3rd instar larvae of *Anopheles arabiensis* were placed in the container. A negative control was set up in which solvent was added only instead of extract. A positive control was set up using Mostop, an organo-phosphate (used by the malaria control program as a larvicide). Each container was monitored for larval mortality.

Repellency

The rodent *Mastomys coucha* was the test animal used for the screening of extracts for repellency activity. Repellent activity was assessed by topical application of 1000 µg/ml of plant extract to the skin and subsequent exposure of the treated areas of skin to unfed female mosquitoes. The repellency analysis followed the South African Medical Research Council standard protocols and is depicted in Fig.8 below. Ethical approval for the use of *Mastomys* in these trials was obtained from the MRC's Ethics Committee for Research on Animals.

Animal preparation

For each plant extract two adult Mastomys were weighed individually, and injected intraperitoneally with sodium pentobarbital (Fig. 8b). The anaesthetised rodents were shaved on the ventral surface and 1000 µg/ml of plant extract was applied to each of two rodent's abdomens (Fig. 8c). The third animal served as a negative control and the fourth animal was a positive control 97 % DEET.

Repellency assay

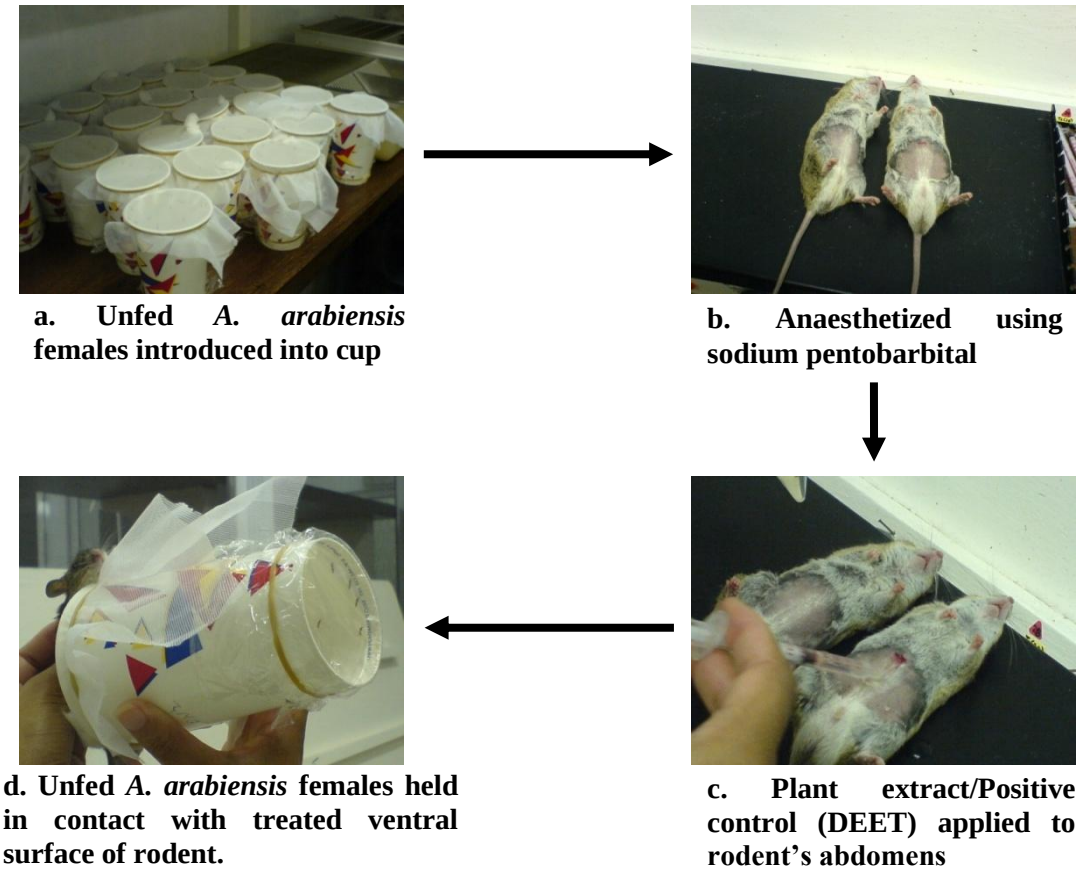
Paper cups (500ml) were modified by replacing the base of the cup with mosquito netting held in place with a rubber band and covering the mouth of the cup with transparent plastic film. Thirty unfed 4-day old *A. arabiensis* females (Fig. 8a) were introduced into the cup and held in contact with the treated ventral surface of each rodent (Fig. 8c). Mosquito activity was observed through the transparent plastic film (Fig.8d). After a period of two minutes the numbers of mosquitoes probing were recorded. The rodent was then returned to the animal

facility and allowed to recover from anaesthetic. Each rodent was monitored for 7 days for adverse reactions to the applied plant extracts (Fig. 8).

Repellency of the extracts was calculated using the formula:

$$\% \text{ mosquito repelled} = \frac{\text{number repelled}}{\text{number introduced}} \times 100$$

The following diagrams give representation of the repellency assays



a. Unfed *A. arabiensis* females introduced into cup

b. Anaesthetized using sodium pentobarbital

c. Plant extract/Positive control (DEET) applied to rodent's abdomens

d. Unfed *A. arabiensis* females held in contact with treated ventral surface of rodent.

Fig. 8: Repellency assay as followed by the South African Medical Research Council standard protocols

Insecticidal Assay

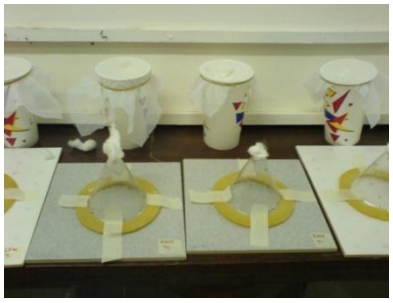
The adulticidal effect was assayed following a slightly modified version of the WHO standard method (WHO, 1981a) depicted by Fig. 9.

One millilitre each of, plant extract solution , positive control (K-Orithrine), negative control (acetone) and distilled water respectively was sprayed onto clean dry non-porous ceramic tiles using the pre-calibrated Potter's Tower. The negative controls in this experiment were acetone and distilled water. The Potter's Tower (Fig. 9a) was cleaned with acetone between each different extract application. The sprayed tiles (Fig. 9b) were air dried and assayed within 24 hours of spraying. A standard bioassay cone was fixed in place over the sprayed tile and thirty blood-fed *A. arabiensis* females 3-5 days old were introduced into the cone (Fig. 9c). The mosquitoes were then observed for knockdowns after thirty and sixty minutes of exposure (Fig. 9d). The test species were thereafter removed from the bioassay cone and transferred to a holding cage containing a glucose nutrient solution (Fig. 9e) After 24 hours the number of dead mosquitoes was recorded and percentage mortality calculated.

a. Potters Tower

b. Plant extract/ Positive control (K-Orithrine) sprayed on ceramic non-porous tiles left for 24h to dry

c. *A. arabiensis* (30) females introduced into bioassay cone



d. Observed for knockdown after 30 & 60 min of exposure

e. Transferred to holding cage containing nutrient solution overnight to check for mortality

Fig. 9: Insecticidal assay following modified version of the WHO standard method (WHO, 1981a)

3. 4 Evaluation of safety of plants

3.4.1 Ames Mutagenicity Test

The *Salmonella* mutagenicity assay was conducted according to the method described by Maron and Ames, (1983) with minor modifications. The tester strain TA 98 was obtained on disc cultures from MOLTOX^{TM}. The disk cultures described were prepared from master cultures obtained from Dr. B.N. Ames (Berkeley, California, USA)

From the frozen disc culture of the *S. typhimurium* TA 98 and TA 100 tester strain, broth cultures were made. Using a flamed bacteriological needle, one of the culture disks were aseptically removed and inoculated into a sterile 250 ml Erlenmeyer flask containing 25 ml of nutrient broth (Oxoid) and 78 µl of 8 mg/ml Ampicillin (to maintain the stability of the plasmid). The flask was incubated on a shaker (150 rpm) at 37ºC for 16 h to obtain an optical density of between 1.2 and 1.4 at 660 nm.

In a sterile test tube, 100 µl of grown culture was added to 2 ml of 0.05 mM histidine/0.05 mM biotin top agar (Appendix 1.2), vortexed and plated onto a minimal glucose agar plate. The plate was incubated at 37ºC for 48h. Well separated colonies were used from this plate for initial broth cultures.

Broth cultures of *S. typhimurium* were made by inoculating nutrient broth with master plate colonies. These cultures were incubated on a shaker (150 rpm) at 37 ºC for 24 h. The crude

plant extracts were dissolved in DMSO to obtain concentrations of 100 µg/ml, 1000 µg/ml and 10 000 µg/ml.

Sodium azide (NaN_3) is a highly mutagenic compound and was used as a positive control (Maron and Ames., 1983). NaN_3 was dissolved in DMSO to obtain concentrations of 5 µg/ml, 10 µg/ml and 20 µg/ml. Sterile distilled water was used as a negative control.

Three plates were prepared for each concentration of test compound. In a sterile test tube, 100 µl of bacterial culture, 100 µl of test compound and 2.9 ml of soft agar (Appendix 1.2) held at 45 ºC, were added. This was briefly mixed with a vortex mixer and poured onto glucose minimal agar plates (Appendix 1.2). Once the agar overlay solidified, the plates were inverted and incubated at 37ºC for 48h, after which the number of revertant colonies (i.e. histidine dependant) colonies were counted and the mutant frequency determined. The mutant frequency is expressed as the quotient of the number of revertant colonies over the number of colonies in the negative control (Maron and Ames, 1983). This can be expressed in the formula:

$$\text{Mutant Frequency} = \frac{\text{Revertant number of colonies}}{\text{No. of colonies negative control}}$$

3.4.2 Brine Shrimp Lethality Assay

The brine shrimp lethality assay was used with minor modifications (Meyer *et al*., 1982).

Sample Preparation

Ten, hundred and thousand µg/ml of plant extract were dissolved in DMSO and 50 µl was impregnated on Whatman No. 1 filter paper disks. The disks were allowed to dry in an open sterile Petri dish in a biological safety cabinet with a vertical laminar flow for one hour. Control disks were prepared using only DMSO. Three replicates of each dose and the control were tested.

Hatching the shrimp

Twenty five milligrams of class C *Artemia salina* eggs (Natures Petland, Durban, South Africa) was added to artificial salt water (23 g NaCl, 11g $MgCl_2 \cdot 6H_2O$, 4 g Na_2SO_4, 1.3 g $CaCl_2 \cdot 2H_2O$, 0.7 g KCl in 1000 ml distilled water) and kept at room temperature. The pH was adjusted to 9.0 using Na_2CO_3 to avoid risk of death to the *Artemia* larvae by decrease of pH during incubation. This was incubated in a hatching chamber at room temperature. After 24h, 15 ml of yeast solution was added to the chamber for every litre of salt water in order to feed the larvae. Forty eight hours after the eggs were incubated; the larvae were extracted by picking up the moving larvae and visibly counted.

Bioassay

Every vial with 100 µl of plant sample at different concentration (10, 100, 1000 µl/mg) contained 10 larvae of brine shrimp, including the control group, and was filled to 5 ml total volume with artificial salt water. A drop of yeast suspension (3 mg in 5 ml sea water) was

added to each vial. The vials were then incubated at 27°C for 24 h after which, dead larvae were counted and percentage death determined.

3.4.3 Cytotoxicity

Cell proliferation inhibition was assessed by the MTT assay (Hanelt *et al*., 1994).

1. Cell Line

Human chronic myelogenous leukaemia (K562) cell line was used in this study. The K562 cell line was purchased from Highveld Biological, Modderfontein, South Africa. The cells were received in an active state and incubated at 37ºC in a humidified incubator (Snjiders Hepa, United Scientific group, Cape Town, South Africa) with 5% CO_2. When the cells were 80% confluent, they were sub-cultured, and stock cultures were stored at -70ºC until required. Cell maintenance was performed according to protocols obtained from Freshney, (1987). All cell culture procedures were carried out in a laminar flow cabinet containing a UV light (Scientific Engineering INC). The unit was swabbed /sterilized with 70% ethanol (Merck, South Africa) before each use.

The cells were grown aseptically in 75 cm^2 tissue culture flasks (T 75) (Greiner, Germany) using filter sterilized (0.22 mm) 10% Complete Culture Medium (CCM), (Appendix 1.3) which comprised of Dulbecco's modified Eagle's medium (DMEM), containing 10% foetal calf serum and supplemented with antibiotics (penicillin, 10 000 U/ml, streptomycin sulphate, 10 000 U/ml and 1 mM sodium pyruvate). Cells were incubated in a humidified incubator under 5% CO_2 at 37°C and passaged weekly. All the above media and chemicals were obtained from Highveld Biological, South Africa. The cultures were incubated at 37°C in a

humidified incubator (Snjiders Hepa, United Scientific group, Cape Town, South Africa) with a 5% CO_2 atmosphere. The culture flasks were examined for colour changes and turbidity of the media on a daily basis. This determined the frequency of media changes. The culture was examined under an inverted microscope (Nikon) for cell growth.

The cells were harvested when the culture was 80% confluent and divided into two separate flasks, then more medium added to each culture flask and incubated at 37°C humidified incubator (Snjiders Hepa, United Scientific group, Cape Town South Africa) with a 5% CO_2 atmosphere.

The cell was enumerated using a haemocytometer. The cell suspension was mixed with an equal aliquot of 0.2% Trypan Blue [Biowhittaker, Wakersville (USA)] (v/v 1:1) (Appendix 1.4). This mixture was drawn across the grid by capillary action. The volume of cell suspension that occupied one primary square is $0.1mm^3$ ($1.0mm^2$, 0.1mm/ 1.0, 10^4 mL). Only the viable (translucent) cells that lay within, or that touched, the left or top boundary, were counted. The number of viable cells per ml in the original sample was calculated as follows:

Cells/ml = Average number of cells per primary square × 10^4 dilution factor

2. Storage of cells

Cells were stored according to protocols of Freshney, (1987). The cells were pelleted and washed twice with pre-warmed phosphate buffered saline, pH 7.2 (PBS) resuspended in 0.5 ml fetal calf bovine serum and cooled on ice. A 20% DMSO in DMEM (v/v 1:4) solution was prepared as the cryoprotecting agent and also placed on ice. Equal aliquots (0.5ml) of the cell suspension and the cryoprotective agent were added to a cryotube (Corning, South Africa).

The tubes were transferred to the thermos flask and kept overnight at -20°C. The cells were subsequently transferred to a –70°C bio-freezer and stored until required.

3. Regeneration of cells

Cells were regenerated as described by Freshney, (1987). Cells were removed from the –70°C biofreezer. And vial swabbed with 70% ethanol to sterilize, and rapidly thawed. The cells were then transferred to 20 ml of pre-warmed 10% CCM in 75 cm^2 tissue culture flasks and incubated at 37°C humidified incubator with a 5% CO_2 atmosphere.

4. *Effect of plant extracts on K 562 cells*

The effect of extracts was assayed using MTT assay according to protocols Mosmann (1983). The assay was carried out in 96 well, flat bottomed microtitre plates (Cellstar, Greiner, Germany) (Appendix One, Section C). Two hundred micro litres of $\pm 1.2 \times 10^6$ cells was added into each well, 20 µl of the plant (1000 µg/ ml, 100 µg/ ml, 10 µg/ ml) extracts were added to the respective wells. In the control wells 20 µl DMSO and 20 µl media only were added respectively. The plate was incubated in a 37°C humidified incubator with a 5% CO_2 atmosphere for 20 hours. Then 20 µl of MTT reagent (Sigma, St Louis, USA) was added and the plates were then incubated for a further 4 hours at 37ºC in a humidified incubator with 5% CO_2 atmosphere. Subsequently 100 µl of 100% DMSO was added to each well and the plate was incubated for an additional 1 hour. The absorbance was read at 578 nm on an ELISA plate reader (Digital Analogue Systems, Italy).

The percentage viability was determined using the formula below:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

3.5 Immuno modulation

This study was conducted at HIV Pathogenesis Programme *(HPP)* Laboratories, Doris Duke Medical Centre, University of Kwa Zulu Natal, and Nelson Mandela School of Medicine. The aim was to determine the immune modulatory effects of *D. cinerea*, *C. dimidiatus*, *C. tomentosa* and *L. leonurus.* In Fig.10, a scheme that was followed is shown.

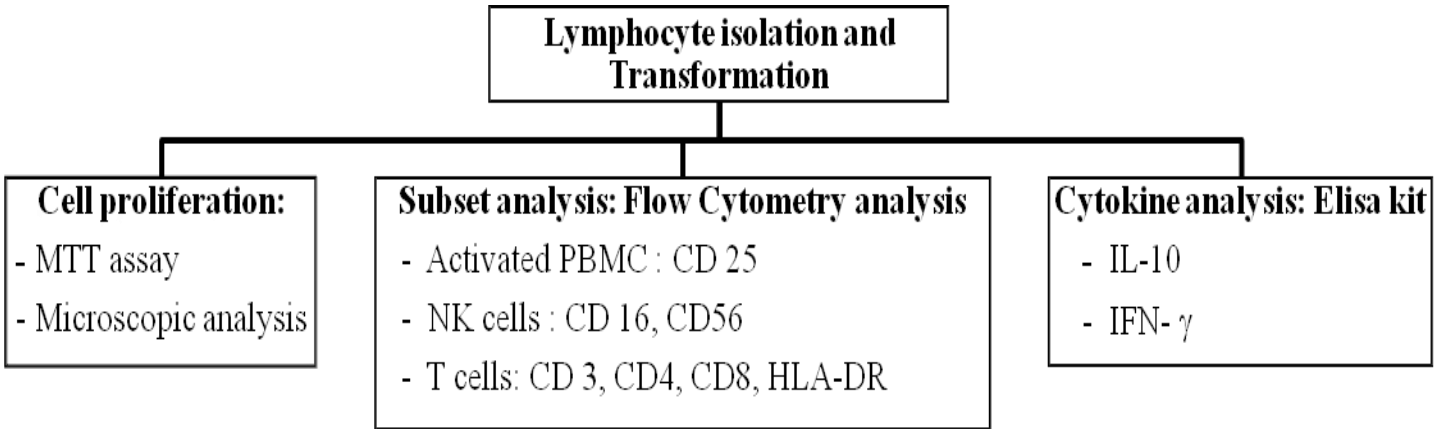


Fig. 10. Overview of the immuno-modulatory methodology

3.5.1 Crude extracts and chemicals

Deaminated heparin, Ficoll Histopaque, DMSO, phyto-mitogens of phytohaemagglutinin (PHA), and Concanavalin (Con A), Phosphate buffered saline (PBS) Pen/Strep, RPMI 1640 and Fungizone were obtained from Sigma, South Africa. The BD OptEIA Human Interferon gama (IFN-γ) and Interlukin – 10 (IL- 10) ELISA kit was purchased from B&D Systems Inc. (USA). Conjugated monoclonal antibodies (mAbs): Fluorescein isothiocyanate (FITC) Phycoerythrin (PE), Allophycocyanin (APC), Peridinin chlorophyll protein (PerCP), PerCP-Cy5.5, was purchased from Becton Dickinson (USA). The buffy coat is the fraction of an

anticoagulated blood sample after density gradient centrifugation that contains most of the white blood cells and platelets.

Reagent Buffy coats were obtained from the South African Blood bank (East Coast Region, Pinetown), Batch no 10128:-1016252, 1015175. They were negative for markers of disease namely Hepatitis B surface antigen (HBsAg) and antibodies to HIV 1 and 2, Hepatitis C (HCV) and Syphilis. In addition a nucleic acid test (ID NAT) was performed for HIV 1, HBV and HCV. The blood was handled and disposed of in accordance with established procedures SOP / HPP/01*.

*SOP / HPP/01: Standard operating procedure from HIV Pathogenesis Programme *(*HPP*)* Laboratories.

Methanolic and aqueous plant extracts were diluted in dimethylsulphoxide (DMSO) to a concentration of 1000 µg/ml and diluted in RPMI to yield test concentration (500 µg/ml, 250 µg/ml, 100 µg/ml, 10 µg/ml and 1 µg/ml).

3.5.2 Isolation of lymphocytes

The isolation of the peripheral blood mononuclear cell layer, PBMC, was conducted according to the SOP / HPP/01. Everything was kept inside the Class II Biological Safety Cabinet (Lab Aire, South Africa). All reagents were swabbed with 70% ethanol before being placed in the hood to warm to a room temperature. An autoclavable plastic bucket was filled with 2% Virkon (The Scientific Group, South Africa) solution, was placed in the hood for bio-hazardous waste.

Buffy coat was carefully mixed to equal parts of pre-warmed Phosphate buffered saline with 1% Pen/Strep and Fungizone. This was carefully layered onto equal volume of Ficoll Histopaque, Blood + PBS + Ficoll (1: 1: 1). Care was taken not to disturb the tubes as mixing could delay the sedimenation of the different cell types. This was centrifuged at 1500 rpm for 30 minutes at room temperature. After centrifugation four distinct layers were obtained as shown in Fig. 11.

The upper plasma layer was aspirated and discarded. The mononuclear cell layer, PBMC, which contained the lymphocytes, was carefully removed using a 3 ml sterile Pasteur pipette and transferred into new sterile tube (If red blood cells were present several drops of double distilled water was added to lyse the red blood cells). PBS (pH 7.2) was added immediately to prevent all the cells from lysing. These cells were washed thrice with PBS and Pen/Strep (45 ml) by centrifugation at 1500 rpm for 10 minutes, room temperature. After the third wash, all the PBS was removed and the pellet was resuspended in RPMI- 1640.

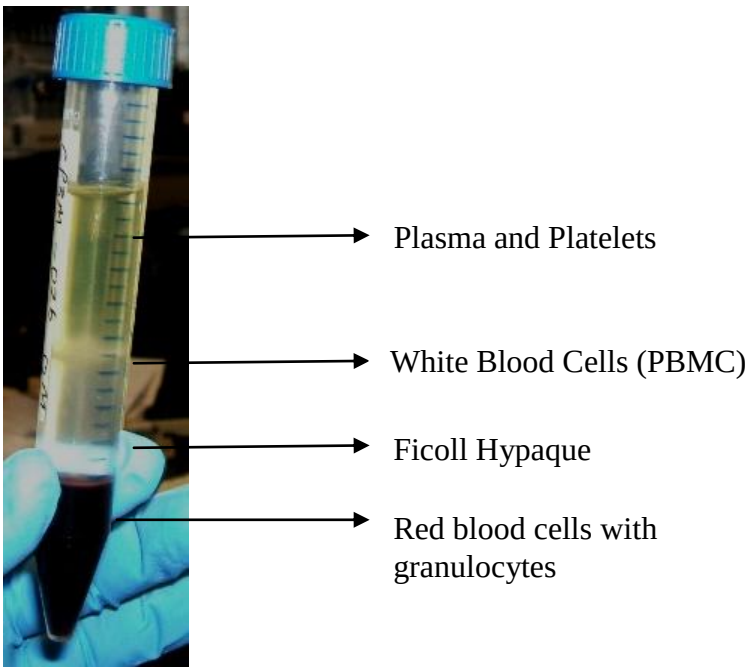


Fig. 11. Peripheral Blood Mononuclear Cell isolation

3.5.3 Lymphocyte maintenance

The PBMC were grown aseptically in 75 cm^2 tissue culture flasks (T 75) (Sigma, South Africa) in filter sterilized, R 10 medium (435 ml RPMI, 50 ml heat inactivated filtered FCS, 5 ml of L-glutamine and 5ml Penicillin/ Streptomycin/ fungizone and 5 ml HEPES buffer) and were incubated at 37°C in 5% CO_2 humidified incubator (Hera Cells 150, Heraeus). The cultures were monitored for colour changes and turbidity of the media on a daily basis to determine the frequency of media changes. The culture was examined under a microscope (Nikon) for cell growth and the cells were either stored or counted and used immediately.

a. Storage of cells

The PBMC were pelleted and washed twice with pre-warmed PBS (pH 7.2) resuspended in 0.5 ml FCS and cooled on ice. Dimethyl sulphoxide (20%) in RPMI was prepared as the cyroprotecting agent and also placed on ice. Equal aliquots (0.5ml) of the cell suspension and the cyroprotective agent were added to a cryotube. The tubes were transferred to the thermos flask (Mr Frosty) and kept overnight at -20°C. The PBMC was subsequently transferred to a –70°C bio-freezer and stored until required.

b. Regeneration of cells

PBMC was removed from the –70°C biofreezer, swabbed with 70% ethanol and rapidly thawed. The cells were then transferred to 20ml of pre-warmed 10% CCM in 75 cm^2 tissue culture flasks. The PBMC was incubated at 37°C humidified incubator with a 5% CO_2 atmosphere.

c. Cell Enumeration

The viability and the number of lymphocytes were determined using Guava system, Guava Check (Guava Technologies, United States of America) and Guava PCA-96 (Cytosoft 2.0 software).

Cell Enumeration: A Guava Check was done on a daily basis before sample analysis. The Guava Check verifies the performance of the system (Fig. 12) and to assess the accuracy and fluorescence detection using standardized fluorescent bead reagent (Whitehead Scientific). A 1:20 dilution was prepared (25µl of Guava check beads and 475µl of the Guava check diluent (Whitehead Scientific) was mixed by vortexing and this was incubated for 8 min in the dark at room temperature. Three replicates of bead suspension were acquired on Guava microcapillary cytometer. The particle counts displayed in green on the screen ranged between 45 000- 55 000. Samples were loaded, if particle counts that were outside the expected range were displayed in red on the screen, and the machine was washed and recalibrated.

A 1: 10 dilution of PBMC to counting solution (Guava ViaCount reagent) which is 20 µl of cells to 180 µl Counting solution was aliquoted into a 1.5 ml microcentrifuge tube. The tubes were capped and vortexed at medium intensity to avoid leaving cells to dry on the side of the tube which may cause erroneous results. The samples were then incubated for 8 minutes at room temperature in the dark. After incubation, the samples were analyzed using CytoSoft version 2.1.3 Software.

Fig. 12 Guava system, Guava Check (Guava Technologies, United States of America) and Guava PCA-96 (Cytosoft 2.0 software)

3.6 Effect of extract on PBMC

Initial experiments were conducted to determine the optimum dose and time of exposure of Con A), LPS and PHA: positive controls, CSA: negative control and DMSO using the MTT Assay (Appendix 1.5).

Concentrations determined above was used and the effect of the extracts of *D. cinerea, C. dimidiatus, C. tomentosa and L. leonurus* was determined using Guava system, Guava Check and MTT Assay.

3.6.1 Optimization of standards and controls

The Dose and Time Response assay was conducted by using MTT assay of Mosmann (1983). The assay was carried out in 96 well, flat–bottomed microtitre plates (Cellstar, Greiner, Germany). 200 µl of ±1.2 $\times 10^6$ of PBMC was added into each well. To this 20 µl of Con A (20 µg/ml, 10 µg/ml, 5 µg/ml), LPS (20 µg/ml, 10 µg/ml, 5 µg/ml), PHA: positive controls (250 µg/ml, 125 µg/ml), CSA: negative control (20 µg/ml, 10 µg/ml, 5 µg/ml) and DMSO:

diluent (10 µl, 5 µl) was added in to each well respectively for MTT Assay, as described earlier.

3.6.2 Evaluation of the effect of plant extract

The Dose and Time Response assay was conducted according to Mosmann (1983) with minor modifications. The assay was carried out in 96 well, flat –bottomed microtitre plates (Cellstar, Greiner, Germany). 200 µl of $\pm 1.2 \times 10^3$ of PBMC was added into each well, 20 µl of the plant extracts (1000 µg/ ml, 250 µg/ ml, 100 µg/ ml and 10 µg/ ml) were added to the respective wells. In the control well 10 µl of PHA (250 µg/ ml were added to the respective well. 20µl DMSO and 20µl RPMI- 1640 only, were added to the respective wells. The plate was incubated in a 37°C humidified incubator with a 5% CO_2 atmosphere for 1h, 6h, 18h 24h. After each incubation period the cell viability was analyzed on the Guava system, Guava Check (Guava Technologies, United States of America) and Guava PCA-96 (Cytosoft 2.0 software).

3.6.3 Lymphocyte proliferation assay

The Lymphoproliferation test was carried out using the protocol according to Mosmann, *(*1983) with minor modifications.The assay was carried out in 96 well, flat–bottomed microtitre plates (Cellstar, Greiner, Germany). 200 µl of $\pm 1.2 \times 10^3$ of PBMC was added into each well, 20 µl of the plant (1000 µg/ ml, 250 µg / ml, 100 µg/ ml and 10 µg/ ml) extracts or 20 µl, 10 µl, 5 µl of PHA, CSA, ConA and LPS or 20 µl DMSO and 20 µl RPMI- 1640 were added to the respective wells. This was incubated in a 37°C humidified incubator with a 5% CO_2 atmosphere for 20 hours. Then 20 µl of 5 mg / ml MTT reagent (Sigma, St Louis, USA) was added and the plates were incubated for a further 4 h at 37ºC in a humidified incubator

with 5% CO_2 atmosphere. Subsequently, 100 µl DMSO (100%) was added to each well and was incubated for an additional 1 hour. The absorbance was read at 578 nm on an ELISA plate reader (Digital Analogue Systems, Italy), and the % of viable cells was counted.

The percentage viability was determined using the formula below:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

3.6.4 Cytokine reponse of PBMC exposed to plant extract

The amounts of IFN-γ and IL-10 released by PBMC was carried out using an ELISA procedure [BD OptEIA ELISA kit II (Becton Dickinson, San Jose, CA, USA)].

The cultivation and treatment of human PBMC was completed as previously described in the lymphoproliferation test. After 24 h particulates were removed from the supernatants by centrifugation and the samples were stored at -70 ºC until used. The sample diluent (100 µl) was added to each well and then 100 µl/well of cytokine (IL-10 / IFN-γ**)** standard or supernatant sample was added. The plate was then covered with an adhesive strip and incubated for 2 h at room tempaerature. The samples from each well was aspirated and washed, and the process was repeated four times. The cytokine conjugate for IL–10 and IFN-γ respectively, (200 µl/ well) was added covered with new adhesive strip and incubated for 2 h at 37 ºC. The washing process was repeated four times. The substrate solution (200 µl/ well) was added and incubated for 30 minutes at room temperature. Finally the stop solution (50 µl/ well) was added and the optical density of each well determined within 30 minutes, using an ELISA reader at a test wavelength of 450 nm and a reference wavelength of 540 nm.

A standard curve was initially plotted for each interleukins. The standard for each cytokine had a straight linear shape with positive gradient. (Fig. 6 and 7, Appendix 2) that displayed a direct dose dependent relationship between the interleukin concentration and the corresponding optical densities (absorbance).

3.6.5 Effect of plant extracts on T-cell sub-population

The populations activated PBMC, activated T cells, T cells, CD 4^{+}, CD 8^{+}, total B cells and NK cells was determined using a flow cytometry (LSR 11 Sorvall RT7 Plus, Becton Dickinson, USA) according to procedure described by Cherng *et al*. (2008). All flow cytometry analysis was performed according to Cherng *et al*, (2008) with minor modification. All FITC, PE, APC, PerCP, PerCP-Cy5.5 – conjugated monoclonal antibodies (mAbs) were purchased from Becton Dickonson (San Jose, CA, USA). Optimal concentrations of mAbs were determined for each mAbs by titration. The cultivation and treatment (duplicate) of human PBMC was completed as previously described in the lymphoproliferation test. The PBMC were cultured in duplicate with the plant extracts (250 µg / ml), control (20 µl PHA, 20 µl DMSO or 20 µl RPMI- 1640). After 24 h, the numbers of NK cells (CD 3^{-}, CD 16^{+}, CD 56^{+}), activated PBMC (CD 25^{+}), T cell subset (CD 3^{+}, CD $4^{+,}$ CD 8^{+}), total T cells (CD 3^{+}). Total B cells (CD 3^{-}, HLA-DR $+^{)}$ and active T cells (CD 3^{+}, HLA-DR$+^{)}$ were determined by standard FACScan procedures with mAbs according to the manufactures protocol. In brief at the end of treatment the cultured cells (0.25- 0.5 million) were aliquoted into a FACS tube then washed with 2ml phosphate buffer, centrifuged for 5 minutes at 2000

rpm at RT, the supernatant was decanted. The cells were then stained with the exact amount of required mAbs, shown in Table 5 below:

Table 5: Flourochromes used for each monoclonal antibodies

Conjugated monoclonal antibodies (mAbs)	**Volume (µl)**
Fluorescein isothiocyanate (FITC)	10
Phycoerythrin (PE),	10
Allophycocyanin (APC),	4
Peridinin chlorphyll protein (PerCP),	4
PerCP-Cy5.5	4

The cells were then incubated for 30 minutes at room temperature in the dark. The stained cells were washed with 2ml of PBS, and then centrifuged for 5 min at 2000 rpm at room temperature; supernatant was aspirated until there was 50 µl of residual fluid left. The cell pellet was re-suspended in the residual fluid, and then 0.5 ml of 1% paraformaldehyde was added to each tube. Finally the stained PBMC was analyzed by flow cytometric analyzer (LSR 11 Sorvall RT7 Plus, Becton Dickinson, USA) with Cell Quest software (Becton Dickinson, USA). Only single events were gated. Results were analysed using Flow Jo 7.0 (USA)

Chapter Four: Results

4.1 Biological Activity

4.1.1 Yield and description

The field visit showed that the local species of *D. cinerea* (Fig. 13) is widespread in the summer rainfall area of South Africa, and flowers in the spring. *D. cinerea* was modified from, is a shrub up to 5-6 m in height, occurring on a variety of soils in wooded grassland; often forming secondary bush in degraded areas Palgrave, 2002. The bark is dark grey-brown, the stems often twisted and seamed and the branches interwined, giving a thick matted appearance. The leaves bipinnate; leaflets narrowly obovate to lanceolate, dark green, rather glossy above but dull below; petiole without a gland. The flowers are axillary, bicoloured spikes, half the spike formed by long, slender, pink, sterile staminodes and the other half a short, very compact, yellow catkin-formed by the fertile flowers, the whole flower hangs upside down on the tree, so the pink sterile section is seen above the yellow fertile section. *D. cinerea* fruits form a cluster of pods, each up to 10 × 1cm, twisted and contorted, indehiscent. The yield from the aqueous extract was 2.45g/100g and from the methanolic extract was 10.08g/100g for the leaves.

The field visit showed that the local species of *C. dimidiatus* (Fig. 14) is found along the sea shore in the sandy dunes, down to the high tide mark. *C. dimidiatus* was description from Pooley, 1998 *C. dimidiatus* stems are fleshy at first becoming wood with age, the leaves are 70-80 × 10 mm joined at the base, bright green with a tinged red, the flowers are solitary up to

60 mm diameter. Magenta flowers all year round. The yield from the aqueous extract was 2.4g/100g and from the methanolic extract was 27.5g/100g for the leaves.

The field visit showed that the local species of *C. tomentosa*, (Figure 15) a spiny scrambler, or a small tree up to 10 m in height, well branched and densely leafy. The branches are usually green becoming brown, often with pairs of sharp, downward-hooked spines which are persistent and distinctive even on old wood (Palgrave, 2002). Leaves are oblong to broadly elliptic, light green to grayish green, sometimes rust coloured, soft and velvety, the flowers are up to 3.5 cm in diameter, yellowish green, scented, the central mass of stamens white or pinkish which are up to 3.5 cm long, the fruit is rather pendulous on a stout stalk round up to 3.5 cm in diameter, pink to bright orange, with semi transparent, bluish grey to orange brown flesh (Palgrave, 2002). The yield from the aqueous extract was 3.5g/100g and from the methanolic extract was 10.25g/100g for the leaves.

The field visit showed that the local species of of *L. leonurus* (Figure 16, 17) grows in grasslands and flowers in the winter months. It's a robust shrub which grows up to 3 m, stems are velvety but woody at base, leaves are narrow about 50-100 × 10-20 mm in length, flowers are compact clusters, about 25-40 mm in diameter, bright orange in colour and velvety to texture (Pooley, 1998). The yield from the flower aqueous extract was 1.3g/100g and from the methanolic extract were 8.4 g/100g for the leaves and 1.95g/100g and 5.55g/100g for the flowers.

Figure 13: *D. cinerea*

Figure 14: *C. dimidiatus*

Figure 15: *C.tomentosa*

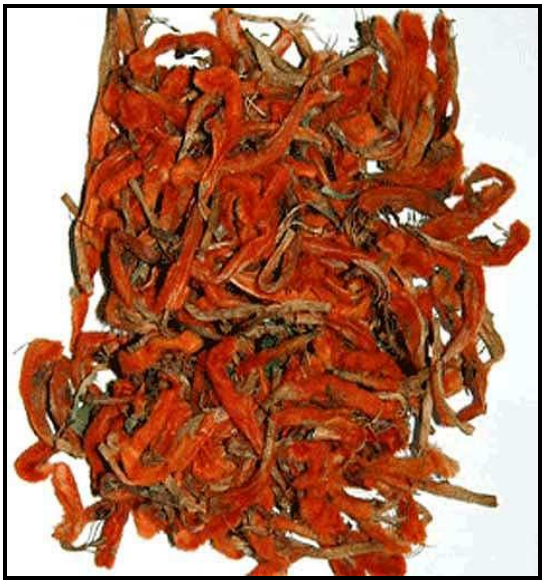


Figure 16: Dried flowers of *L. leonurus*

Figure 17: *L. leonurus* whole plant.

For all the plants, higher yields were obtained from the methanolic plant extracts.

4.1.2. Antimicrobial activites of the extracts from *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus*

The antimicrobial activity of the aqueous and methanolic extracts from *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* against *E. coli, P aeruginosa, K. oxytoca, S. typhimurium, S. marcescens, B. cereus, S. epidermis, S. aureus, B. stearothermophilus and Micrococcus sp*. showed no antibacterial activity against *S. epidermis, S. aureus, B. stearothermophilus* and *Micrococcus* sp. The plant extracts demonstrated activity aganist Gram negative bacterium: *E. coli, P. aeruginosa, K. oxytoca, S. typhimurium, S. marcescens* and Gram positive bacterium *B. cereus*. The antibacterial activity is shown as zones of inhibition of the extracts in the Table 6. The minimum inhibitory concentrations are summarized in the Table 7.

These plant extracts also showed no anti-fungal activity against *Penicillum* sp., *Rhizopus* sp., *Geotrium* sp., *Fusarium* sp., *Aspergillus niger, Cladosporium* sp., and against the yeast *Saccharomyces cerevisiae* and *Candida albicans.* However all four plants showed activity against *Tricoderm sp* and the inhibitory activity is shown Table 8.

The methanolic and aqueous leaf extract of *D. cinerea* at a concentration of 1000 µg/ml displayed antibacterial activity against five Gram negative bacteria *viz*., *E. coli, P. aeruginosa, K. oxytoca, S. typhimurium, S. marcescens* and a Gram positive bacteria *B. cereus*. For the methanolic extract the zone of inhibition for the same five Gram negative bacteria, and a Gram positive bacteria were comparable to the positive control Ciprofloxacin (50 µg/ml) (Table 6).

Aqueous leaf extracts of *D. cinerea* demonstrated good inhibition against *B. cereus* (a zone of inhibition) of 18.417 ± 0.804 mm and a MIC value of 100 µg/ml while the methanolic extracts showed a zone of inhibition of 18.333 ± 1.607 mm and a MIC value of 100 µg/ml. *D. cinerea* extract exhibited a strong inhibition for *E. coli* with the aqueous extract displayed a zone of inhibition of 5.412 ± 0.878 mm with a MIC of 100 µg/ml and the methanolic extract displayed a zone of inhibition of 4.667 ± 0.577 mm with a MIC of 10 µg/ml.

For *P. aeruginosa* the aqueous extract displayed a zone of inhibition of 6.083 ± 0.520 mm with a MIC of 100 µg/ml and the methanolic extract displayed a zone of inhibition of 7.833 ± 0.289 mm with a MIC of 10 µg/ml.

For *K. oxytoca* the aqueous extract displayed a zone of inhibition of 9.25 ± 0.661mm with a MIC value of 100 µg/ml and the methanolic extract displayed a zone of inhibition of 15.5 ± 0.5 mm with a MIC value of 10 µg/ml.

D. cinerea is moderate to strong in inhibition of *S. typhimurium* and aqueous extract displayed a zone of inhibition of 22.833 ± 1.010 mm with a MIC of 10 µg/ml and the methanolic extract displayed a zone of inhibition of 22.083 ± 1.127 mm with a MIC of 10 µg/ml comparable to the positive control.

The aqueous extract from *D. cinerea* displayed a zone of inhibition of 8.083 ± 0.382 mm with a MIC of 100 µg/ml against *S. marcescens* and the methanolic extract displayed a zone of inhibition of 8.667 ± 0.764 mm with a MIC of 100 µg/ml compared to the positive control Ciprofloxacin (50 µg/ml) 20.5 ± 1.323 mm..

The methanolic and aqueous leaf extracts of *C. dimidiatus* at a concentration of 1000 µg/ml displayed antibacterial activity against Gram negative bacteria *viz.,, E. coli, P.aeruginosa, K. oxytoca* and *S. typhimurium* and Gram positive *B. cereus*.

The methanolic and aqueous leaf extract of *C. tomentosa* at concentration of 1000 µg/ml displayed antibacterial activity against *P. aeruginosa* and *S. typhimurium.* For the methanolic extract of *C. tomentosa*, the MIC was 100 µg/ml and the zone of inhibition was 11.167 ± 1.607 mm for *P. aeruginosa* and for *S. typhimurium* the MIC was 500 µg/ml and zone of inhibition was 17.833 ± 0.804 mm. The aqueous extract of *C. tomentosa* had a MIC value of 10 µg/ml and a zone of inhibition of 14.167 ± 0.289 mm for *P. aeruginosa* and for *S. typhimurium* MIC of 250 µg/ml and a zone of inhibition is 19.583 ± 1.422 mm.

The methanolic and aqueous leaf and flower extracts of *L. leonurus* at a concentration of 1000 µg/ml displayed antibacterial activity against three Gram negative bacteria *viz*., *P. aeruginosa, K. oxytoca, S. typhimurium* and against a Gram positive bacteria *B. cereus*. The leaves and flowers of *L. leonurus* methonalic and aqueous extracts at a concentration of 1000 µg/ml extract did not inhibit *Penicillum* sp., *Rhizopus* sp., *Geotrium* sp., *Fusarium* sp., *Aspergillus niger, Cladosporium* sp., *S. cerevisiae* and *C. albicans*, however there was inhibition against a *Tricoderm* sp.

For *L. leonurus* leaf methanolic extract, the MIC was 10 µg/ml and the zone of inhibition was 14.667 ± 1.155 mm for *P. aeruginosa* and for *K. oxytoca* the MIC was 100 µg/ml and the zone of inhibition was 8.833 ± 0.764 mm. For *S. typhimurium*, the MIC was 100 µg/ml and the zone of inhibition was 17.5 ± 0.5 mm and for *B. cereus* the MIC was 100 µg/ml and the zone of inhibition was 17.833 ± 0.764 mm. With aqueous leaf extract of *L. leonurus*, the MIC

was 100 µg/ml and the zone of inhibition was 12.412 ± 0.764 mm for *P. aeruginosa* and for *K. oxytoca* the MIC was 10 µg/ml and the zone of inhibition was 7.583 ± 0.382 mm. For *S. typhimurium* the MIC was 100 µg/ml and the zone of inhibition 19.667 ± 1.041 mm and for *B. cereus* the MIC was 100 µg/ml and the zone of inhibition was 14.333 ± 2.565 mm.

In *L. leonurus* flower methanolic extract, the MIC was 10 µg/ml and the zone of inhibition was 14.167 ± 0.289 mm against *P. aeruginosa* and for *K. oxytoca*, the MIC was 100 µg/ml and zone of inhibition was 9.5 ± 0.5 mm. And for *S. typhimurium*, the MIC was 250 µg/ml and a zone of inhibition was 13.917 ± 1.010 mm. And for *B. cereus*, the MIC was 1 µg/ml and the zone of inhibition was 22.917 ± 0.764 mm. For *L. leonurus* flower aqueous extract, the MIC was 10 µg/ml for *P. aeruginosa, K. oxytoca, S. typhimurium* and *B. cereus* and the zone of inhibition was 12.167 ± 0.289 mm, 11.667 ± 0.764 mm, 5.417 ± 0.629 mm and 22.667 ± 2.565 mm respectively.

Table 6: Anti-bacterial activity of the methanolic and aqueous extracts of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus*

Plant extract (1000 µg/ml)	Bacteria Zone of Inhibition in mm									
	E.c	**P.a**	**K.o**	**S.t**	**B.c**	**S.m**	**S.e**	**S.a**	**B.s**	**M**
D. cinerea	**4.667** ± 0.577	**7.833** ± 0.289	**15.5** ± 0.5	**22.083** ± 1.127	**18.333** ± 1.607	**8.667** ± 0.764	**0**	**0**	**0**	**0**
	5.412 ± 0.878	**6.083** ± 0.520	**9.25** ± 0.661	**22.833** ± 1.010	**18.417** ± 0.804	**8.083** ± 0.382	**0**	**0**	**0**	**0**
C. dimidiatus	**4.583** ± 0.144	**10.833** ± 0.804	**8.417** ± 0.382	**23.083** ± 0.804	**15.833** ± 1.041	**0**	**0**	**0**	**0**	**0**
	5.583 ± 0.577	**13.333** ± 1.44	**8.767** ± 0.874	**23.667** ± 0.629	**19.417** ± 0.629	**0**	**0**	**0**	**0**	**0**
C. tomentosa	**0**	**11.167** ± 1.607	**0**	**17.833** ± 0.804	**0**	**0**	**0**	**0**	**0**	**0**
	0	**14.167** ± 0.289	**0**	**19.583** ± 1.422	**0**	**0**	**0**	**0**	**0**	**0**
***L. leonurus* (leaves)**	**0**	**14.667** ±1.155	**8.833** ± 0.764	**17.5** ± 0.5	**17.833** ± 1.258	0	0	0	0	0
	0	**12.412** ± 0.382	**7.583** ± 0.382	**19.667** ± 1.041	**14.333** ± 0.764	**0**	**0**	**0**	**0**	**0**
***L.leonurus* (Flower)**	**0**	**14.167** ± 0.289	**9.5** ± 0.5	**13. 917** ± 1.010	**22.917** ± 1.942	**0**	**0**	**0**	**0**	**0**
	0	**12.167** ±0. 289	**11.667** ± 0.764	**15.417** ± 0.629	**22.667** ± 2.565	**0**	**0**	**0**	**0**	**0**
Methanol (100 %)	**1.25** ± 0.25	**1.667** ± 0.577	**1.667** ± 0.144	**1.75** ± 0.25	**1.417** ± 0.144	**1.417** ± 0.382	**1.95** ± 0.25	**1.87** ± 0.144	**1.97** ± 0.382	**1.75** ± 0.25
	1.25 ± 0.25	**1.667** ± 0.577	**1.667** ± 0.144	**1.75** ± 0.25	**1.417** ± 0.144	**1.417** ± 0.382	**1.95** ± 0.25	**1.87** ± 0.144	**1.97** ± 0.382	**1.75** ± 0.25
Ciprofloxacin	**18.333** ± 0.946	**16.667** ± 2.89	**18.333** ± 0.764	**19.167** ± 1.258	**17.167** ± 1.764	**20.5** ± 1.323	**18.167** ± 1.258	**20.167** ± 1.764	**20.51** ± 1.323	**18.167** ± 1.258
	18.333 ± 0.946	**16.667** ± 2.89	**18.333** ± 0.764	**19.167** ± 1.258	**17.167** ± 0.764	**20.5** ± 1.323	**18.167** ± 1.258	**20.167** ± 1.764	**20.51** ± 1.323	**18.167** ± 1.258

Values in red indicates methanolic and blue represents aqueous extract

Values are mean± SD. (n = 6)

The antimicrobial properties are determined by measuring the zones of inhibition of microbial growth (mm). The criteria used for activity is in mm as: 10 > strong, > 10 moderate, > 5 weak, na: no activity.

Table 7: The minimum inhibitory concentrations of the methanolic and aqueous extracts of *D. cinerea*, *C. dimidiatus*, *C. tomentosa* and *L. leonurus*

Plant extract (1000 µg/ml)	**Bacteria**									
	E.c	**P.a**	**K.o**	**S.t**	**B.c**	**S.m**	**S.e**	**S.a**	**B.s**	**M**
D. cinerea	**10**	**10**	**10**	**10**	**100**	**100**	**na**	**na**	**na**	**na**
	100	**100**	**100**	**10**	**100**	**100**	**na**	**na**	**na**	**na**
C. dimidiatus	**100**	**100**	**100**	**100**	**500**	**na**	**na**	**na**	**na**	**na**
	100	**10**	**100**	**100**	**100**	**na**	**na**	**na**	**na**	**na**
C. tomentosa	**na**	**100**	**na**	**500**	**na**	**na**	**na**	**na**	**na**	**na**
	na	**10**	**na**	**250**	**na**	**na**	**na**	**na**	**na**	**na**
***L. leonurus* (leaves)**	**na**	**10**	**1**	**100**	**100**	**na**	**na**	**na**	**na**	**na**
	na	**100**	**10**	**100**	**100**	**na**	**na**	**na**	**na**	**na**
***L. leonurus* (Flower)**	**na**	**10**	**10**	**250**	**1**	**na**	**na**	**na**	**na**	**na**
	na	**10**	**10**	**10**	**10**	**na**	**na**	**na**	**na**	**na**

Values in red indicates methanolic and blue represents aqueous extract

na : No activity

Table 8: Antifungal activity of methanolic and aqueous extracts of *D. cinerea, C. dimidiatus, C. tomentosa* and . *L. leonurus* (1000 µg/ml) against *Tricoderm* sp.

Plant sample	**Zone of Inhibition (mm)**	
	Methanolic extract	**Aqueous extract**
D. cinerea (leaves)	12.33 ± 1.15	11.33 ± 2.52
C. dimidiatus (leaves)	11.33 ± 1.53	10.33 ± 4.16
C. tomentosa (leaves)	14.33 ± 2.08	12.67 ± 1.16
L. leonurus (leaves)	21.67 ± 3.51	16.33 ± 1.53
L. leonurus (flower)	11.33 ± 2.08	11.33 ± 2.51
Methanol	4.67 ± 1.53	5.33 ± 1.53
Amphotericin (50µg)	21.33 ± 3.51	22.33 ± 2.08

Values in red indicates methanolic and blue represents aqueous extract

Values are mean ± SD (n = 3)

4.1.4 Antioxidant activity of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus*

The methanolic and aqueous extracts of *D. cinerea* demonstrated excellent scavenging capability. The methanolic extract had an IC_{50} value of 4 µg/ml and the aqueous extract had an IC_{50} value of 22 µg/ml.

C. dimidiatus methanolic extract had an IC_{50} value of 240 µg/ml and the aqueous extract had an IC_{50} value of 1125 µg/ml.

C. tomentosa demonstrated excellent scavenging activity with a IC_{50} value of 6 µg/ml. The methanolic extract had an IC_{50} value of 3 µg/ml and the aqueous extract had an IC_{50} value of 12 µg/ml.

L. leonurus methanolic leaf extract had an IC_{50} value of 55 µg/ml and the aqueous extract had an IC_{50} value of 100 µg/ml. The methanolic flower extract had an IC_{50} value of 20 µg/ml and the aqueous extract had an IC_{50} value of 125 µg/ml.

The highest amount of free radical scavenging activity was found in the methanolic plant extracts compared to the aqueous extract. Compared to positive control rutin the methanolic extracts of *D. cinerea* and *C. tomentosa* showed higher degree of free radical scavenging capacity while C. *dimidiatus* and *L. leonurus* extracts displayed a moderate to low degree of free radical scavenging capacity. The free radical scavenging capacity of aqueous and methanolic extracts from *D. cinerea, C. tomentosa, C. dimidiatus* and *L. leonurus* plants is listed in Table 9 (Appendix 2.1).

Table 9: DPPH scavenging capacity of the methanolic and aqueous extracts of *D. cinerea*, *C. dimidiatus*, *C. tomentosa* and *L. leonurus*

Plant	% Radical Scavenging Capacity						
	1000 (µg/ml)	**500 (µg/ml)**	**250 (µg/ml)**	**100 (µg/ml)**	**10 (µg/ml)**	**1 (µg/ml)**	**IC_{50} (µg/ml)**
D. cinerea	97 ± 0.57	95 ± 1.52	92 ± 0.57	91 ± 0.57	18 ± 1.52	8 ± 0.57	22
	96 ± 1	94 ± 0.57	90 ± 1.15	90 ± 1	83 ± 2	11± 2	4
C. dimidiatus	83 ± 0.57	64± 4.72	60 ± 1.52	21 ± 3.78	5 ± 0.57	0	1125
	46 ± 1.15	22 ± 4.58	13 ± 3.51	4 ± 1.15	0	0	240
C. tomentosa	97 ± 0.57	96 ± 1.15	94 ± 0.57	90 ± 1.15	50 ± 1.52	25 ± 4.16	12
	93 ± 0.58	91 ± 0.58	89 ± 0.58	86 ± 1.53	76 ± 3.79	23 ± 4.58	6
L. leonurus(flowers)	95 ± 0.57	93 ± 0.57	69 ± 0.57	44 ± 1.15	5 ± 2.64	0	125
	95 ± 1.15	94 ± 0.57	93 ± 0.57	89 ± 0.57	22 ± 3.05	5 ± 0.57	20
L. leonurus (leaves)	93 ± 0.57	92 ± 1.15	89 ± 0.57	47 ± 1.52	8 ± 2.51	0	100
	92 ± 0.57	90 ± 1.15	89 ± 0.57	87± 1.15	22 ± 5.85	5 ± 1.53	55
Rutin	97 ±1.15	97 ± 1.15	92 ± 3.21	90 ± 1.52	74 ± 3.51	9 ± 1.52	6
	97 ± 1.16	97± 1.16	92 ± 3.22	90 ± 1.528	74 ± 3.512	9 ± 1.528	6

Values in red indicates methanolic and blue represents aqueous extract

Values are mean±SD (n=3)

4.1.4 Anti-inflammatory activity of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus*

Reduced IC_{50} values suggest a greater inhibitory activity against 5- LOX enzyme representing a greater anti-inflammatory activity. The positive control NDGA is a very potent anti-inflammatory compound and the IC_{50} was 2.4 µg/ml. Compared to this none of plant extracts showed any anti-inflammatory activity at any of doses tested (100 µg/ml to 6 µg/ml) and they displayed $IC_{50} > 200$. The IC_{50} are shown in Fig. 18 (Appendix 2.2)

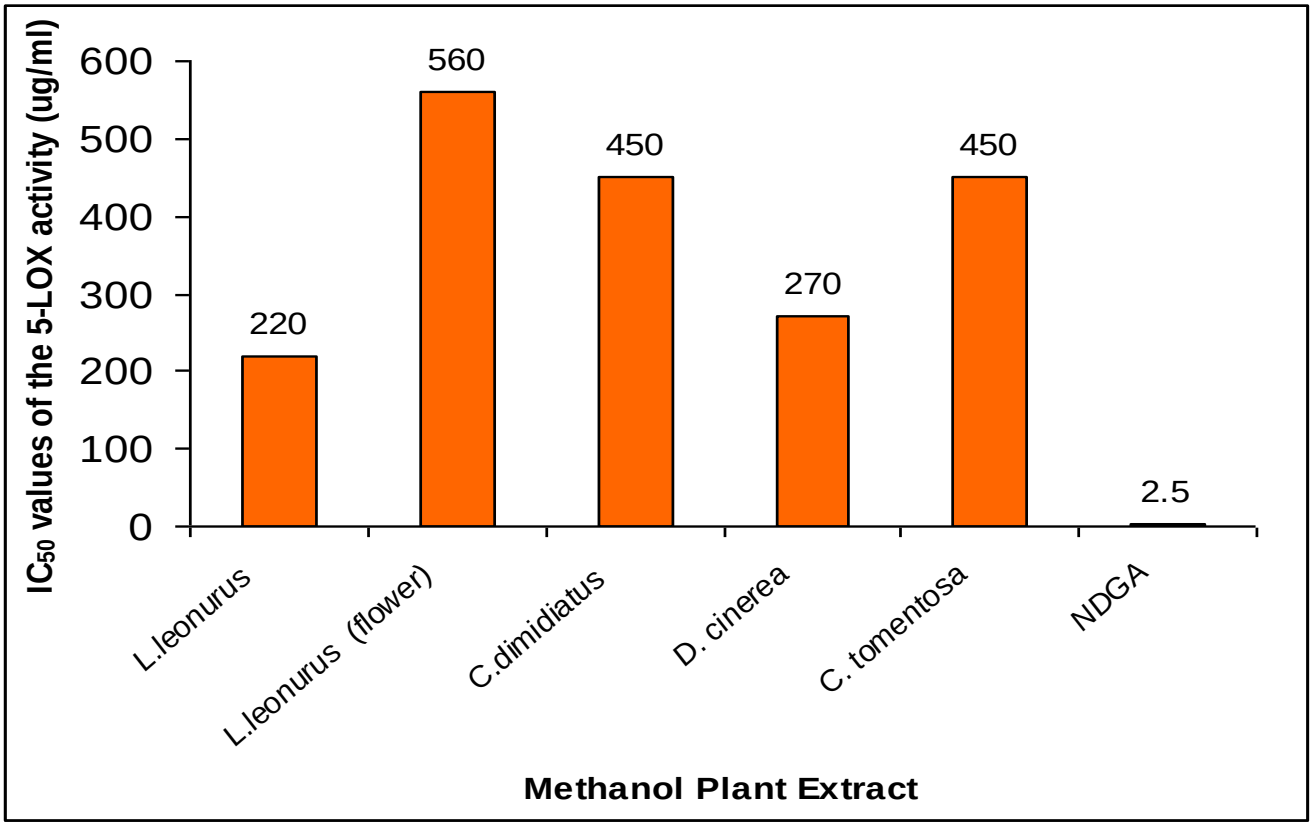


Fig 18: The anti-inflammatory activity of *L. leonurus*, *C. dimidiatus*, *D. cinerea* and *C. tomentosa* and NDGA using the 5-lipooxygenase assay

4.1.5 Anti-Mosquito activity

4.1.5.1 Larvacidal activity

The larvacidal activity of the extracts (1mg/ml) from *D. cinerea*, *C. dimidiatus*, *C. tomentosa* and *L. leonurus* (leaves and flowers), is given in Table 10. None of the plant extracts tested displayed any effect on the immature third in-star stage of *A. arabiensis* mosquitoes over the 7 day period, as compared to the positive control Mostop (an organophosphate). None of the plant extracts displayed any irregularity (inhibition or mutation) of growth pattern throughout the seven day trial. All larvae exposed to plant extract developed to pupae stages normally.

Table 10: Larvacidal activity of *D. cinerea*, *C. dimidiatus*, *C. tomentosa* and *L. leonurus*

Plant Extracts	**Mortality per day**							
	Day 0	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
D. cinerea	L	L	L	L	P	P	A	A
	L	L	L	L	P	P	A	A
C. dimidiatus	L	L	L	L	P	P	A	A
	L	L	L	L	P	P	A	A
C. tomentosa	L	L	L	L	P	P	A	A
	L	L	L	L	P	P	A	A
L. leonurus	L	L	L	L	P	P	A	A
	L	L	L	L	P	P	A	A
L. leonurus (Flowers)	L	L	L	L	P	P	A	A
	L	L	L	L	P	P	A	A
Acetone	L	L	L	L	P	P	A	A
	L	L	L	L	P	P	A	A
Distilled Water	L	L	L	L	P	P	A	A
	L	L	L	L	P	P	A	A
Mostop (Organophosphate)	L	L	L	L	P	P	A	A
	D	D	D	D	D	D	D	D

Values in red indicates methanolic and blue represents aqueous extract

L : Larvae , P: Pupae , A: adult mosquitoes , D: dead; Activity indicated by the mortality and stage of cycle of the larvae per day; **n=3**

4.1.5.2 Repellence activity

All plant extracts showed strong repellence in comparison to the two negative controls acetone and distilled water, 63.5 ± 4.95 and 41.5 ± 2.12 respectively, as shown in Table 11. The aqueous and methanolic plant extracts from *D. cinerea* showed a repellence of 99% and 97% respective but none of the extracts showed knockdowns or mortality. The aqueous extract of *D. cinerea* showed better repellence than the methanolic extracts.

In the *C. dimidiatus* aqueous and methanolic extracts 97% and 87% repellence was recorded but no knockdowns in the two minute exposure period, and also no mortality in the 24 hour period. The aqueous extracts showed greater repellency than the methanolic extract.

C. tomentosa aqueous plant extract showed 97% repellence but no knockdowns or mortality and methanolic plant extract showed 84% repellence and a 53% knockdown after two minute exposure time and 25% mortality was observed.

L. leonurus extracts showed the highest repellence against the mosquitoes, and was also found to cause mortality and 'knockdown' which is the rapidly and normally reversible paralysis of the mosquitoes. At the end of two minutes exposure period, about 50% of the mosquitoes became inactive (knockdown). Nevertheless when transferred from the experimental cones to the holding tubes only under 30% mortality was noticed. The methanolic extracts from *L. leonurus*, repelled 92% of the mosquitoes, at the end of the two minutes exposure, gave a 47% knockdown, and 32% mortality after the 24 hour period were

recorded, and aqueous plant extract showed a 97% repellency but no knockdowns and mortality.

The *L. leonurus* aqueous flower extracts showed 77% repellency after two-minute exposure time and no knockdown or mortality. The *L. leonurus* methanolic flowers extract displayed 100% repellency and 82% knockdowns after the two minute exposure period, and a mortality of 15% after 24 hours. Methanolic extract of *L. leonurus* showed a greater repellency in comparison to the aqueous extract. These results are presented in Table 11.

Table 11: Repellent activity of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* (1000 µg/ml) on *A. arabiensis* mosquito

Plant	**Aqueous extracts**	**Methanolic extracts**		
	% Repelled (2 min)	% Repelled (2 min)	% Knockdown* (2 min)	% Mortality (24 hours)
D. cinerea	98.5 ± 2.12	96.5±4.96	0	0
C. dimidiatus	97 ± 0	86.5±9.19	0	0
C.tomentosa	96.5 ± 4.95	83.5±4.95	53.5±4.94	25±2.82
L. leonurus (flowers)	76.5 ± 9.19	100±0	81.5±12.02	15±2.82
L.leonurus	97± 0	92±7.07	46.5±9.19	31.5±2.12
Acetone	63.5 ± 4.95	63.5±4.95	0	0
Distilled Water	41.5 ± 2.12	41.5±2.12	0	0
DEET	100 ± 0	100±0	0	0

* (Knockdown the rapidly and normally reversible paralysis)

Values are mean ± SD (n = 2)

4.1.5.3 Insecticidal Activity

The results of the *A. arabiensis* insecticidal assay using four different plants are shown in Table 12. *D. cinerea* aqueous plant extract showed 25% knockdown in the first half hour and 48% knockdown in the first hour and a mortality of 45% after the 24 hour period. *D. cinerea* methanolic plant extract exhibited no knockdown in the first half hour and still 8% knockdown in the first hour and a mortality of 28% after the 24 hour period.

C. dimidiatus aqueous plant extract has exhibited 12% knockdown in the first half hour and 43% knockdown in the first hour and a mortality of 42% after the 24 hour period and the methanolic plant extract has exhibited 15% knockdown in the first half hour and 15% knockdown in the first hour and a mortality of 33% after the 24 hour period.

C. tomentosa aqueous plant extract has exhibited 28% knockdown in the first half hour and 53% knockdown in the first hour and a mortality of 17% after the 24 hour period, and the methanolic plant extract has exhibited 32% knockdown in the first half hour and 50% knockdown in the first hour and a mortality of 53% after the 24 hour period.

L. leonurus aqueous flower extract has exhibited 30% knockdown in the first half hour and 67% knockdown in the first hour and a mortality of 63% after the 24 hour period, and the methanolic flower extract exhibited 23% knockdown in the first half hour, 87% knockdown in the first hour and a mortality of 28% after the 24 hour period. *L. leonurus* aqueous plant extract has exhibited 8% knockdown in the first half hour and 40% knockdown in the first hour and a mortality of 25% after the 24 hour period and the methanolic plant extract

exhibited 65% knockdown in the first half hour, 65% knockdown in the first hour and a mortality of 42% after the 24 hour period. The L. *leonurus* flower extracts showed a stronger repellence and mortality than *L. leonurus* leaves extracts.

Table 12: Insecticidal activity of *D. cinerea*, *C. dimidiatus*, *C. tomentosa* and *L. leonurus* (1000 µg/ml) on *A. arabiensis* mosquito

Plant extracts	**% Knockdown (min)***		**% Mortality (hrs)**
	30	**60**	**24**
D. cinerea	25 ± 7.07	48.34 ± 2.35	45 ± 7.07
	0±0	8.34 ± 2.35	28.33 ± 7.07
C. dimidiatus	11.67 ± 2.35	43.34 ± 9.43	41.67 ± 11.79
	15 ± 2.35	15 ± 2.35	33.34 ± 4.72
C. tomentosa	28.34 ± 2.35	53.34 ± 9.43	16.67 ± 4.72
	31.67 ± 11.79	50 ± 4.71	53.34 ± 9.34
L. leonurus (leaves)	8.34 ± 2.35	40 ± 9.43	25 ± 2.36
	65 ± 2.36	65 ± 7.07	41.67 ± 11.79
L. leonurus (flower)	23.35 ±4.72	85 ± 2.36	28.33 ± 7.07
	30 ± 4.71	66.67 ± 4.71	63.34 ± 4.72
Negative control - distilled water	1.67 ± 2.35	10 ± 0	11.67 ± 2.35
Negative control – acetone	8.34 ± 2.35	13.34 ± 9.42	10 ± 4.71
Positive control - K-orithrine	98.34 ± 2.35	100 ± 0	100 ± 0

Values in red indicates methanolic and blue represents aqueous extract

* (Knockdown the rapidly and normally reversible paralysis)

Values are mean±SD (n = 2)

Table 13: Activity of *D. cinerea*, *C. dimidiatus*, *C. tomentosa* and *L. leonurus* against *A. arabiensis*

Plant extract		**Larvicidal**	**Repellence**	**Insecticidal**
D. cinerea	aqueous	0	3	1
	methanolic	0	3	0
C.dimidiatus	aqueous	0	3	1
	methanolic	0	3	0
C. tomentosa	aqueous	0	3	1
	methanolic	0	3	1
L. leonurus (leaves)	aqueous	0	3	1
	methanolic	0	3	2
L. leonurus (flower)	aqueous	0	2	2
	methanolic	0	3	3

0. no activity **1. weak activity** **2. moderate activity** **3. strong activity**

None of the plants displayed any significant larvacidal activity but all displayed strong repellent activity against *A. arabiensis.* *C. tomentosa* showed weak insecticidal activity and *L. leonurus* showed strong activity. *L. leonurus* extracts show the highest repellence and anti-insecticidal activity.

4.2 Safety Assays

4.2.1 Ames Assay

Tables 14 and 15 shows the frequency of the plant extract tested on the *S. typhimurium* TA 98 and TA 100 strain (Fig. 19 ; 20). The mutant frequency is expressed as the quotient of the number of revertant colonies over the number of colonies in the negative control. The greater the number of revertant colonies, the greater the mutant frequency. A mutagenic potential is assumed if the mutant frequency is greater than 2. A possible mutagenic potential is assumed if the mutant frequency ranges between 1.7 and 1.9. No mutagenic potential is assumed if the mutant frequency is lower than 1.6. From the results shown in Table 14-15, none of the plant extracts up to concentration of 1000 µg/ml showed any mutagenic potential. The methanolic and aqueous plant extracts had some revertant colonies but not enough to consider it mutagenic. Sodium azide, positive control, showed a mutagenic potential, as the concentration increased so did the number of revertant colonies.

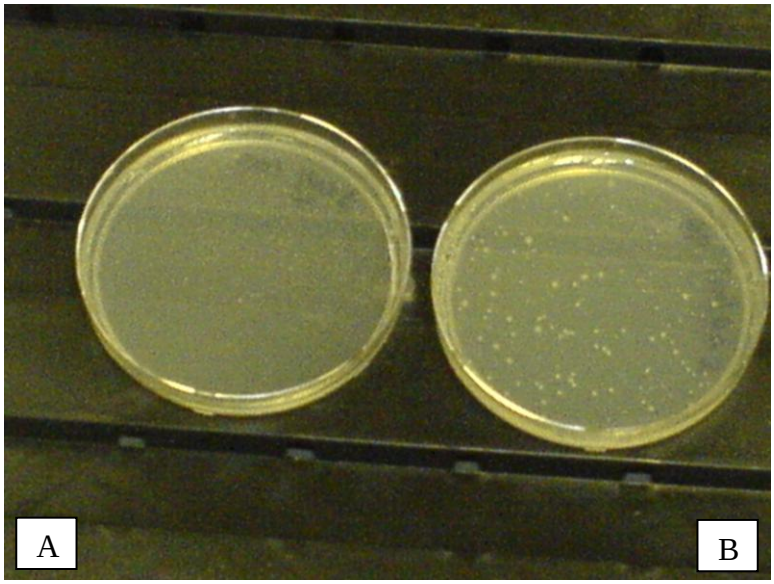


Fig. 19. Mutant revertants of *S. typhimurium* TA 98 (A) *C. tomentosa* (1000µg/ml) (B) Sodium Azide (5µg/ml)

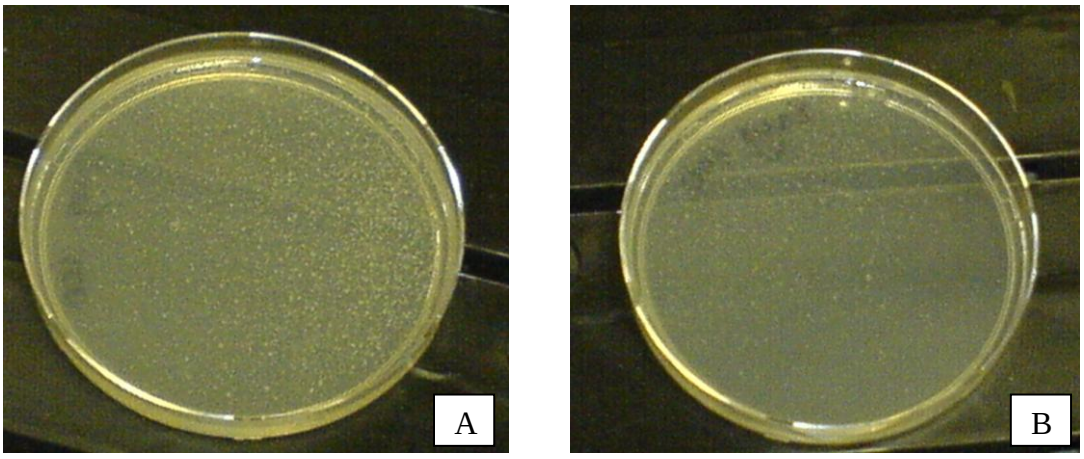


Fig. 20. Stock Cultures of TA 98 (A) and TA 100 (B)

Table 14: Mutagenic response of *D. cinerea*, *C. dimidiatus*, C. *tomentosa* and *L. leonurus* to *S. typhimurium* strain T 98

Plant Extracts	**Mutant frequency of revertants at different concentrations**				
	5µg/ml	**10µg/ml**	**20µg/ml**	**100µg/ml**	**1000µg/ml**
D. cinerea	na	0.571	na	0.678	0.713
	na	0.333	na	0.466	0.533
C. dimidiatus	na	0.821	na	0.821	0.821
	na	0.167	na	0.433	0.433
C. tomentosa	na	0.75	na	0.75	0.75
	na	0.367	na	0.533	0.633
L. leonurus (flowers)	na	0.321	na	0.714	0.714
	na	0.2	na	0.567	0.567
L.leonurus (leaves)	na	0.714	na	0.751	0.782
	na	0.467	na	0.567	0.567
Sodium Azide	1.178	2.357	5.321	na	na
	1.2	2.233	4.733	na	na

Values in red indicates methanolic and blue represents aqueous extract

n = 3

na= not applicable

Table 15: Mutagenic response of *D. cinerea*, *C. dimidiatus*, *C. tomentosa* and *L. leonurus* to *S. typhimurium* strain TA 100

Aqueous Plant Extracts	**Mutant frequency of revertants at different concentrations**				
	5µg/ml	**10µg/ml**	**20µg/ml**	**100µg/ml**	**1000µg/ml**
D. cinerea	na	0.671	na	0.676	0.678
	na	0.561	na	0.561	0.664
C. dimidiatus	na	0.559	na	0.574	0.574
	na	0.478	na	0.483	0.493
C. tomentosa	na	0.671	na	0.671	0.678
	na	0.568	na	0.578	0.664
L. leonurus (flowers)	na	0	na	0	0
	na	0	na	0	0
L. leonurus (leaves)	na	0.668	na	0.671	0.668
	na	0.483	na	0.509	0.506
Sodium Azide	1.044	2.039	3.895	na	na
	1.067	2.162	4.149	na	na

Values in red indicates methanolic and blue represents aqueous extract

n= 3

na= not applicable

0= no activity

4.2.2 Brine Shrimp Assay

For a test compound to be considered not lethal it needs to demonstrate shrimp death of less than 50% (Meyer *et al.*,1982). None of the plants displayed any level of toxicity.

The aqueous and methanolic extracts of *D. cinerea*, *C. dimidiatus*, C. *tomentosa* and *L. leonurus* (leaves and flowers) observable no bioactivity against, *Artemia* sp. at 6 h, 12 h, 18 h, 24 h and concentrations of 1000 µg/ml, 100 µg/ml, 10 µg/ml, 1 µg/ml whereas the organophosphate show 100% mortality in the first 6 h.

4.2.3 Cytotoxicity

4.2.3.1. Effect of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* extracts on K 562 cells

The effects of *D. cinerea*, *C. dimidiatus*, C. *tomentosa* and *L. leonurus* on K 562 was assessed by determining the viability of cells after exposure to extracts (Appendix 2.3). These results are shown in Fig. 21. There was a stimulatory effect the plant extracts exhibited on the cells except for *C. tomentosa* which showed a decrease in viability and this was cytotoxic at high concentrations.

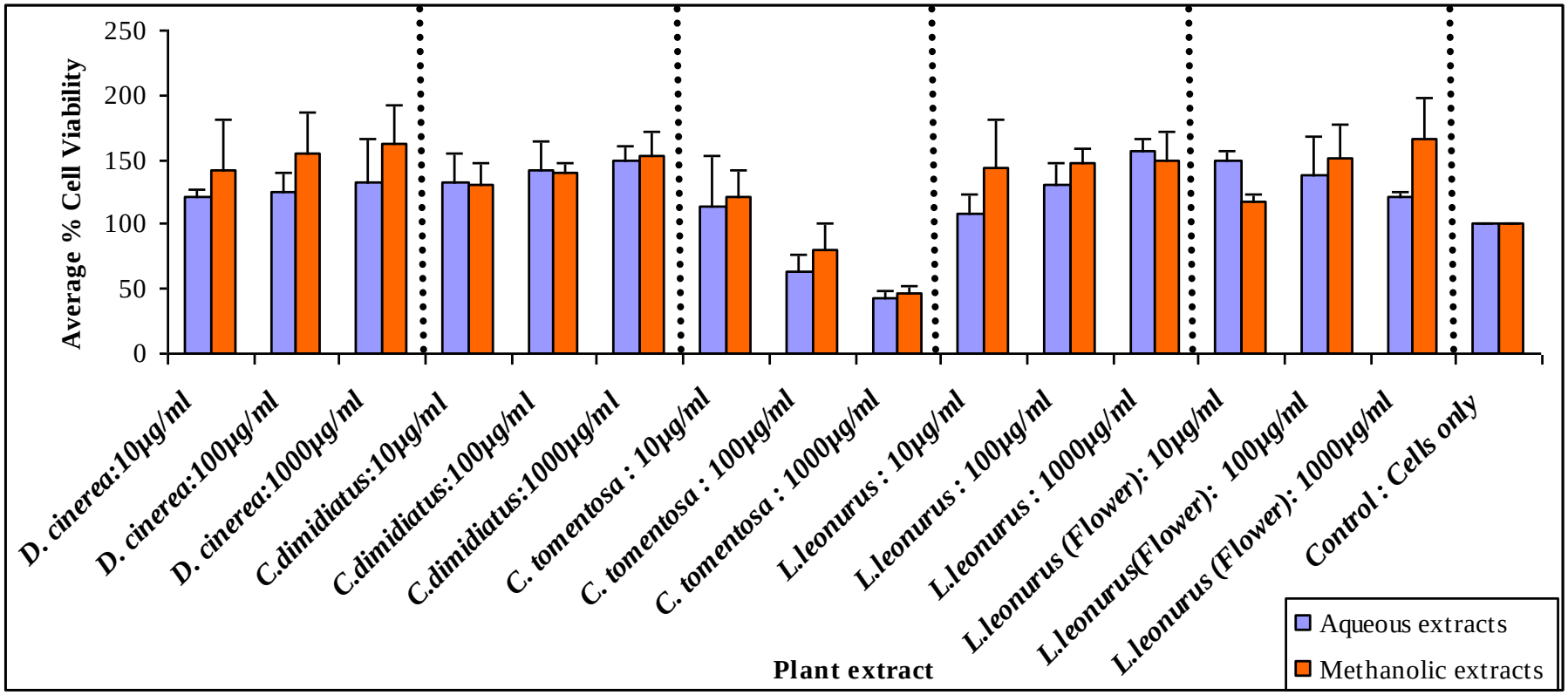


Fig. 21. Cytotoxic effect of K 562 cells exposed to the methanolic and aqueous extracts of *D. cinerea*, *C. dimidiatus*, C. *tomentosa* and *L. leonurus.* Activity indicated by percentage cell viability

Values are mean±SD (n = 3)

4.3 Immmunomodulatory activities of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus*

4.3.1 Optimum concentration of the controls Con A, LPS, PHA, CSA and DMSO

The optimum concentrations of Con A (20 µg/ml, 10 µg/ml, 5 µg/ml), LPS (20 µg/ml, 10 µg/ml, 5 µg/ml), PHA: positive controls (250 µg/ml, 125 µg/ml), CSA: negative control (20 µg/ml, 10 µg/ml, 5 µg/ml) and DMSO: diluent (10 µl, 5 µl) on PBMC using the MTT Assay is shown in Fig. 22.

The maximum proliferation of PBMC was at concentrations of 20 µg/ml for Con A, and 20 µg/m LPS, 250 µg/ml for PHA and 5 µg/ml for CSA.

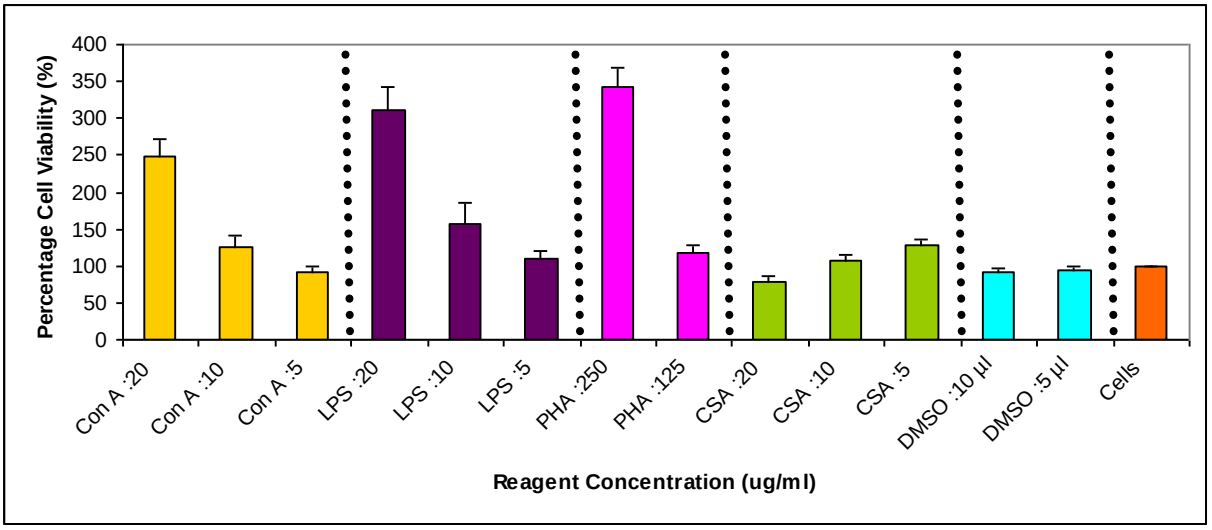


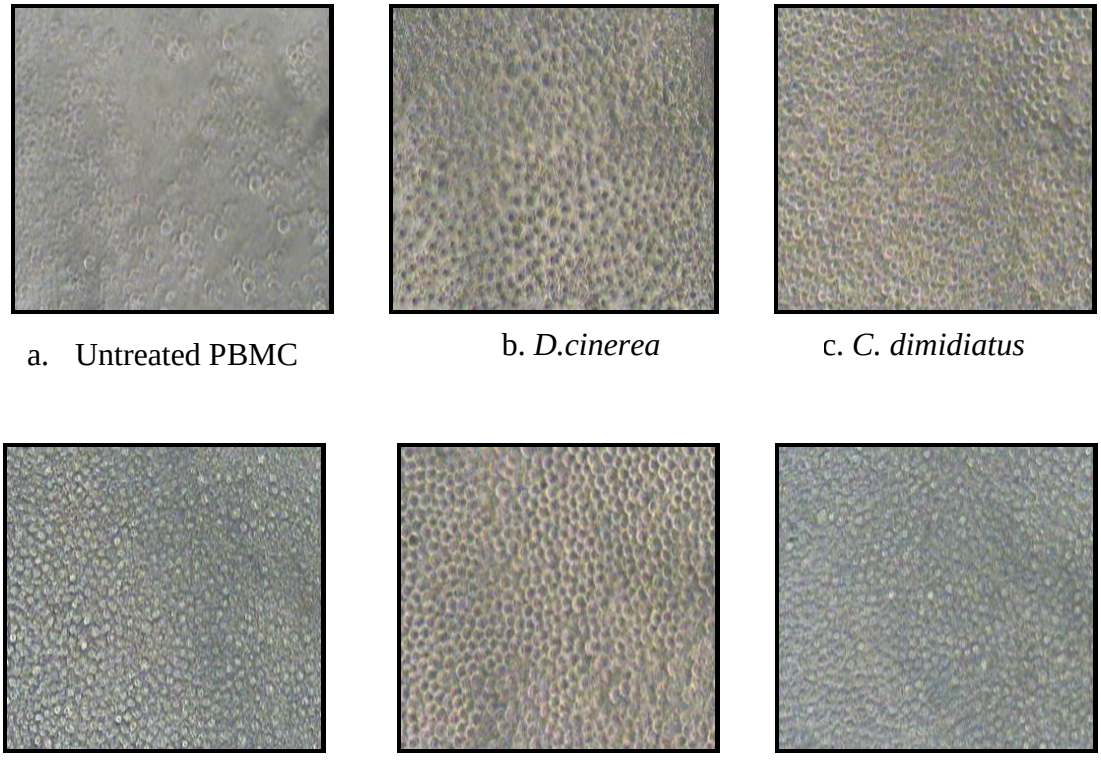
Fig. 22: PBMC exposed to different concentrations of Con A(20 µg/ml, 10 µg/ml, 5 µg/ml), LPS(20 µg/ml, 10 µg/ml, 5 µg/ml), PHA (250 µg/ml, 125 µg/ml), CSA(20 µg/ml, 10 µg/ml, 5 µg/ml), DMSO (10 µl, 5 µl).

Values are mean±SD (n = 3)

4.3.2 Morphological effect of the methanolic and aqueous extracts of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* (250 µg/ml) on lymphocytes.

Lymphocytes exposed to *C. dimidiatus, C. tomentosa, D. cinerea* and *L. leonurus* aqueous and methanolic extracts appeared healthy with distinct mononucleus, a thin cytoplasmic region and intact cell membrane (Fig.23 and 24) showed signs of proliferation in comparison to the untreated cells. Lymphocytes exposed to the solvent control DMSO (10 µl) for 24 h showed slight signs of membrane disruption was cytoplasmic expulsion was observed.

4.3.2.1 PBMC treated with methanolic extracts of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* (250 µg/ml)

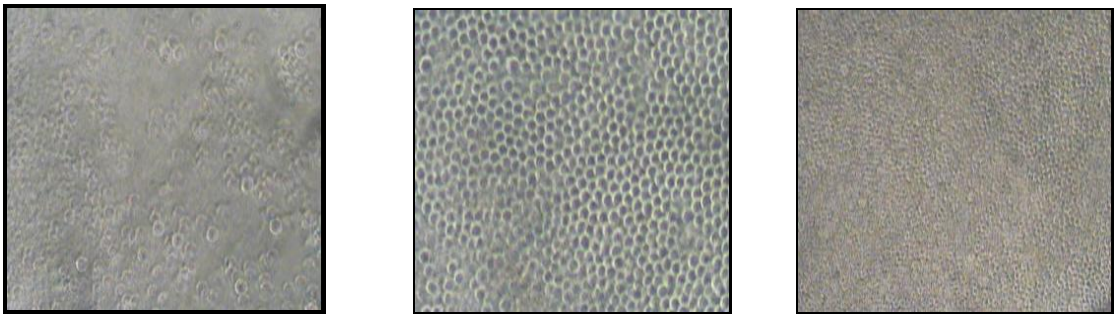


a. Untreated PBMC b. *D.cinerea* c. *C. dimidiatus*

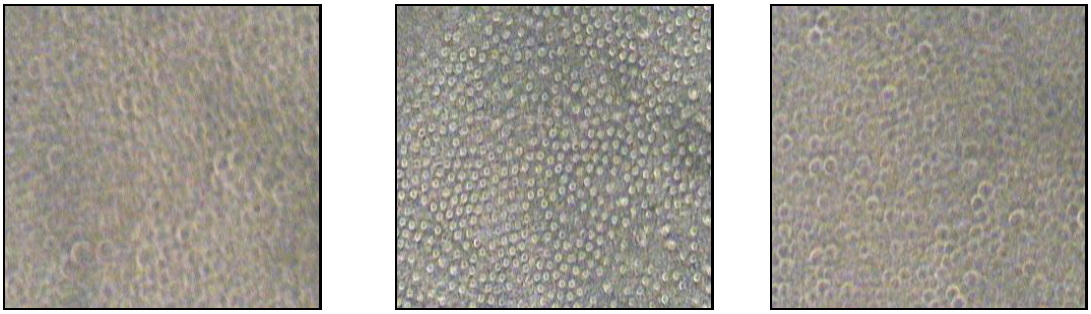
d. *C. tomentosa* e. *L. leonurus* f. *L. leonurus* flowers

Fig. 23. Morphological effect of the methanolic extracts of *C. dimidiatus, C. tomentosa, D. cinerea* and *L. leonurus* (250 µg/ml) on lymphocytes using a light microscope X 250

4.3.2.2 PBMC treated with aqueous extracts of *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus* (250 µg/ml)



a. Untreated PBMC b. *D.cinerea* c. *C dimidiatus*



d. *C. tomentosa* e. L. *leonurus* f. L. *leonurus* flowers

Fig. 24. Morphological effect of the aqueous extracts of *C. dimidiatus, C. tomentosa, D. cinerea* and *L. leonurus* (250 µg/ml) on lymphocytes using a light microscope X 250

4.3.3 Effect of *D. cinerea, C. dimidiatus, C. tomentosa,* and *L. leonurus* on PBMC

4.3.3.1 Cell viability activity of *D. cinerea*

PBMC exposed to methanolic and aqueous extracts of *D. cinerea* showed a two fold increase in comparison to untreated cells and this was also comparable to Con A which also demonstrated a two fold increase. This stimulation was dose dependent and at demonstrated a maximum proliferation at 250 µg/ml. This proliferation was observed with Con A, LPS and PHA as they are known to stimulate PBMC. The effect over 48 h period showed signs of dying after 24 h period

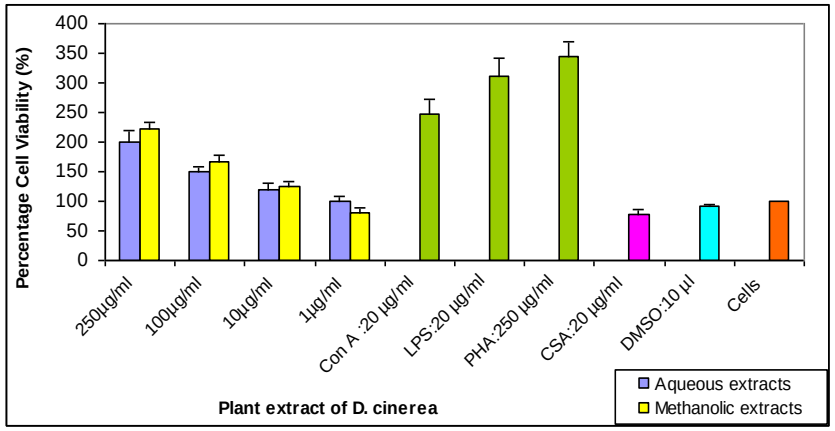


Fig. 25. Effect of *D. cinerea* extract (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 24h period

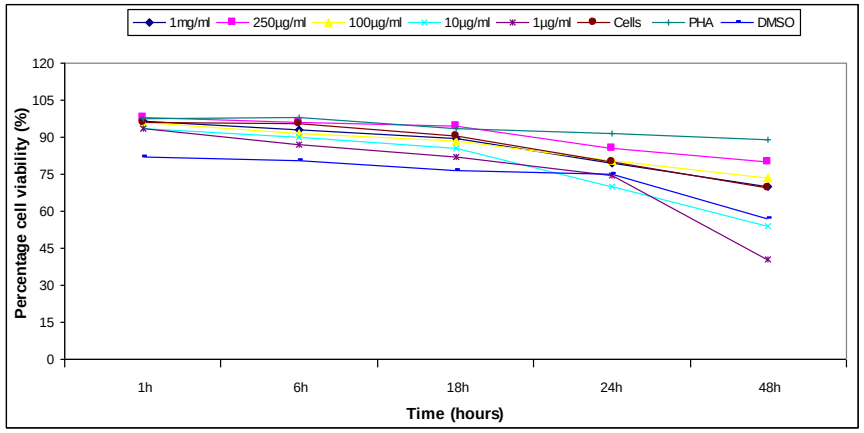


Fig. 26. Effect of *D. cinerea* extract *(*1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 48h period

4.3.3.2 Cell viability activity of *C. dimidiatus*

PBMC exposed to methanolic and aqueous extracts of *C. dimidiatus* and the positive control Con A, showed a two and half fold increase in comparison to unexposed cells (Fig. 27). In comparison with the untreated cells, PHA displayed a three half fold increase, whereas LPS displayed a three fold increase. The methanolic and aqueous extracts of *C. dimidiatus* demonstrate that stimulation was dose dependent and at a concentration of 250 µg/ml (24 h) the methanolic and aqueous extracts demonstrated a higher proliferation than that of the untreated cells and also stimulated the lymphocytes equivalent to Con A. The cells showed signs of death after 24 h (Fig. 28).

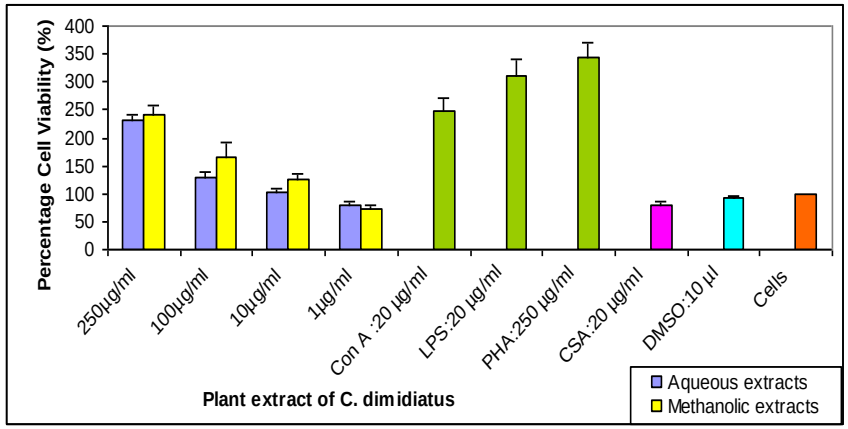


Fig. 27. Effect of *C. dimidiatus* extract (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 24h period

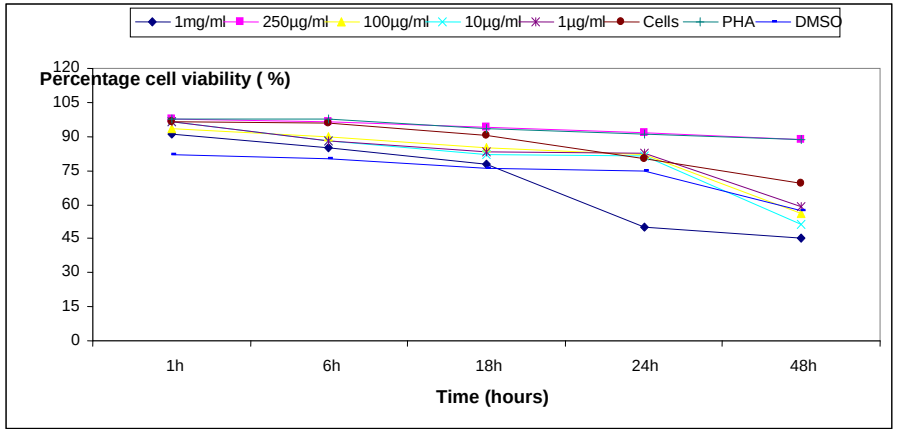


Fig. 28. Effect of *C. dimidiatus* extract (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 48h period

4.3.3.3 Cell viability activity of *C. tomentosa*

The aqueous extracts of *C. tomentosa* did not show proliferation of lymphocytes, however there was two fold increase in the number of lymphocytes by the methanolic extracts at 250 µg/ml and 100 µg/ml which was equal to the activity of Con A when treated for 24 h . This increase in cell number was dose dependent (Fig. 29) and was maximum until 24 h, but the cell started dying at 48 h (Fig. 30).

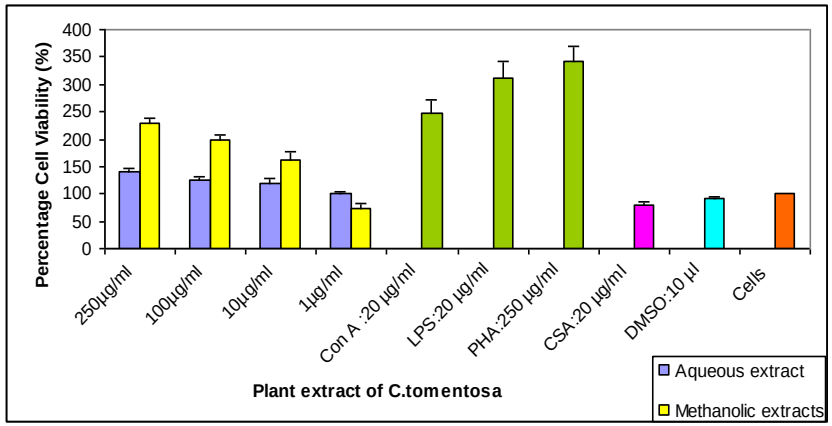


Fig. 29. Effect of *C. tomentosa* extract (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 24h period

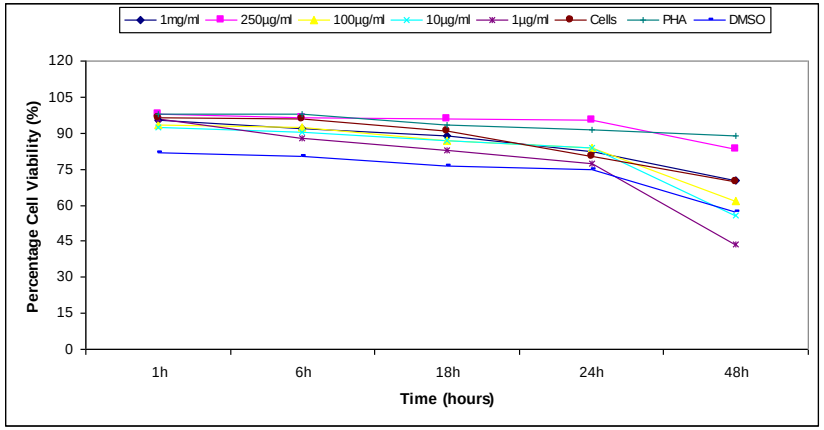


Fig. 30. Effect of *C. tomentosa* extract (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 48h period

4.3.3.4 Cell viability activity of *L. leonurus* leaf extract

The methanolic and aqeuouos extract of *L. leonurus* stimulated the growth of the PBMC at a dose dependent manner. The aqueous extract demonstrated a very low level of proliferation even at the highest concentration of 250 µg/ml in comparison to PHA and the methanolic extract. At concentration of 250 µg/ml the methanolic extract demonstrated a proliferation equal to Con A and a three fold increase to the untreated cells (Fig.31). All the cells displayed signs of dying after 24 h exposure period including unexposed cells (Fig.32).

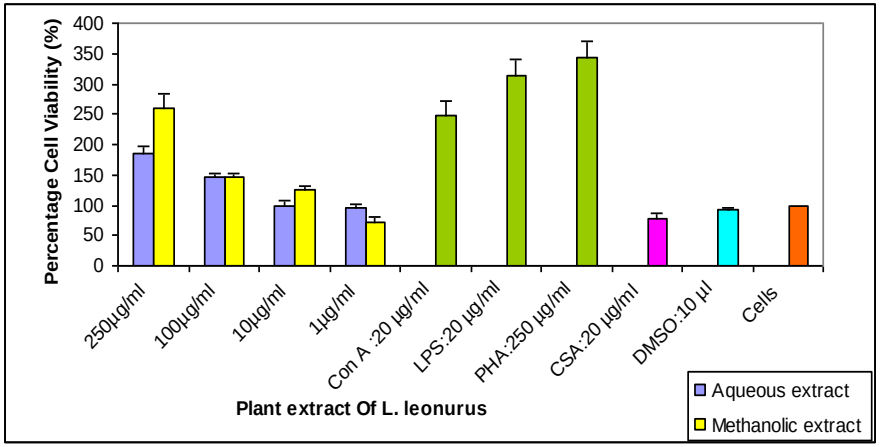


Fig. 31. Effect of *L. leonurus* extract (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 24h period

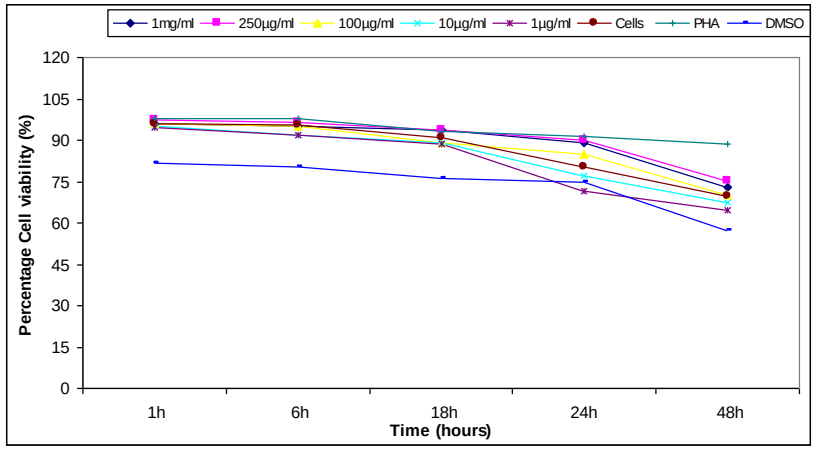


Fig. 32. Effect of *L. leonurus* extract (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 48h period

4.3.3.5 Cell viability activity of the *L. leonurus* flower

The methanolic and aqueous extract of *L. leonurus* flowers stimulated the growth of the PBMC in a dose dependent manner. The aqueous extract demonstrated a two fold increase in comparison to the untreated cells at the highest concentration of 250 µg/ml which is also demonstrated by Con A. The methanolic extract at concentration of 250 µg/ml demonstrated a proliferation equal to Con A and a three fold increase to the untreated cells (Fig. 33). All the cells displayed signs of dying after 24 h exposure period including unexposed cells (Fig. 34).

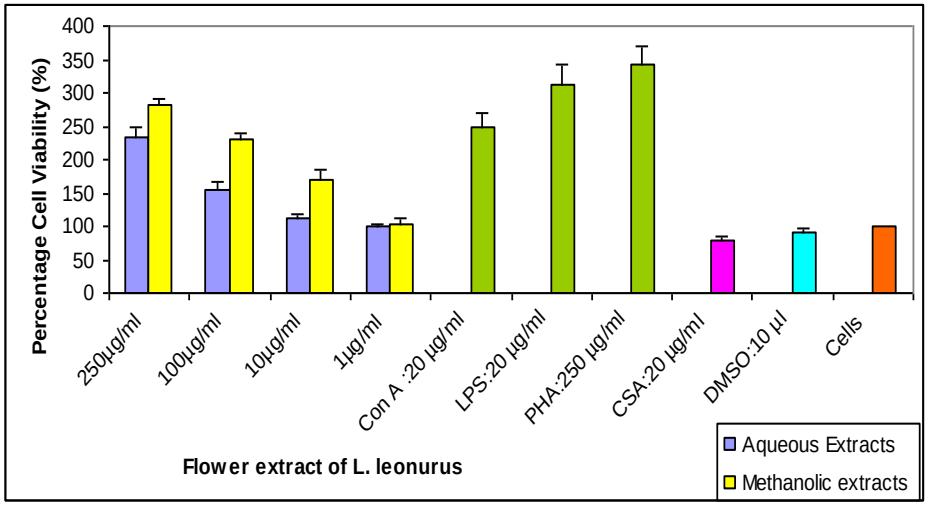


Fig. 33. Effect of flower extract of *L. leonurus* (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 24h period

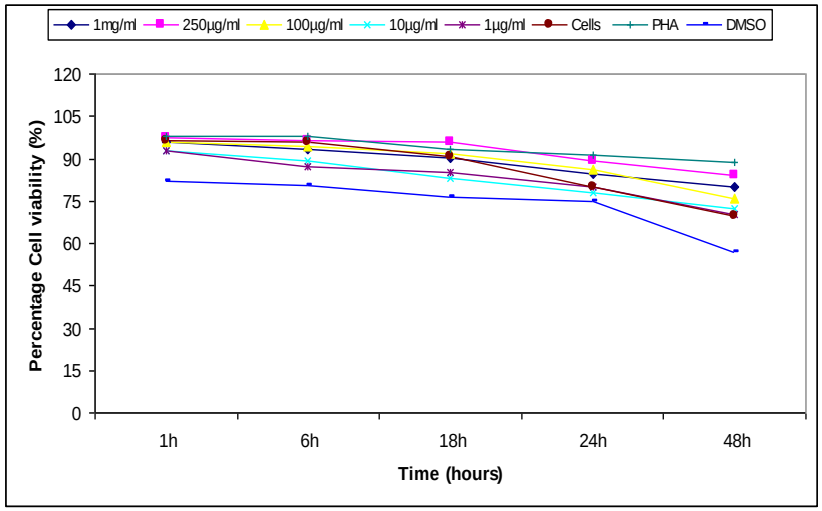


Fig. 34. Effect of flower extract of *L. leonurus* (1 µg/ml, 10 µg/ml, 100 µg/ml, 250 µg/ml, 1 mg/ml) on PBMC over a 48h period

4.3.4 Immunomodulatory activity evaluated by the lymphocyte secretion of IFNγ and IL-10.

When exploring the immunostimulating phytochemicals, results showed that at a concentration of 250 μg/ml, the four plants significantly stimulated the proliferation of resting PBMC, whereas the plant extract stimulated the secretion of IFN-γ and IL-10 in comparsion to untreated cells shown in Table 16. Actual results obtained are in Appendix 2.5.

IFN-γ secretion has been well-known to be associated with lymphocyte activation after stimulation by antigens or mitogens, and PHA and Con A showed this typical response. However, there are 3 types of immunostimulation after treatment with a phytochemical according to Cherng *et al*.(2008).

- Type 1 stimulated resting lymphocyte proliferation and inhibited IFN-γ secretion.
- Type 2 immunostimulation, which stimulated the proliferation of resting PBMC at high doses and augmented the secretion of IFN-γ.
- Type 3 immunostimulation which enhanced the proliferation of resting PBMC but did not stimulate IFN-γ secretion (Table 16 and Table 17).

Aqueous and methanolic extracts from *L. leonurus* (plant and flower), stimulated the secretion of IFN-γ and IL-10 and at concentration of 100 μg/ml and 250 μg/ml. Whereas *C.dimidiatus, C.tomentosa, D. cinerea* aqueous and methanolic extracts stimulated the secretion of IFN-γ and IL-10 at high concentration (250 μg/ml), and at lower concentration it did not stimulate IFN-γ and IL-10 secretion.

Table 16: Cytokine activity (IFN-γ and IL- 10) by *D. cinerea, C. dimidiatus, C. tomentosa* and *L. leonurus*

Plants	Secretion of IFN-γ (pg/ml)				Secretion of IL- 10 (pg/ml)			
	Aqueous extracts		Methanolic extracts		Aqueous extracts		Methanolic extracts	
	(250 µg/ml)	(100 µg/ml)	(250 µg/ml)	(100 µg/ml)	(250 µg/ml)	(100 µg/ml)	(250 µg/ml)	(100 µg/ml)
D. cinerea	70	35	90	45	205	115	465	145
C. dimidiatus	45	30	77	45	480	200	540	290
C. tomentosa	25	35	50	40	200	120	210	145
L. leonurus (leaves)	88	47	100	56	290	125	460	150
L. leonurus (Flower)	110	47	120	77	345	155	480	165
Cells	40	40	40	40	190	205	210	205
DMSO (10 µl)	50	50	50	50	25	30	25	30
Con A (20 µg/ml)	175	175	175	175	545	495	545	495
PHA (250 µg/ml)	230	230	230	230	545	490	545	490

4.3.5 Effect of plant extracts on T-cell sub-population

To determine which types of lymphocytes direct the patterns of immunostimulation, we used flow cytometric analysis. The results from the control groups showed that medium control decreased the fraction of total B cells while solvent control increased the fraction of active T cells is shown in Table 17 . Actual results obtained are in Appendix 3. The immunostimulatory effects by PHA exhibited significant elevation of $CD8^+$ T and activated PBMC, whereas the $CD4^+$ T and total B cells were slightly decreased. Whereas *L. leonurus, C. dimidiatus, C. tomentosa* and *D. cinerea* exhibited significant elevation of $CD4^+$, activated PBMC and total B cells whereas the $CD8^+$ T and were slightly decreased.

Table 17: Immunomodulatory activity evaluated by flow cytometry of PBMC treated with *D. cinerea*, *C. dimidiatus*, *C. tomentosa* and *L. leonurus*

	Stimulation Index							
Lymphocyte	***D. cinerea***	***C. dimidiatus***	***C. tomentosa***	***L. leonurus***	***L. leonurus* Flower**	**Untreated Cells**	**Control: DMSO**	**Control: PHA**
Activated PBMC	**0.134** ±0.0064	**0.145** ±0.0113	**0.157** ±0.0028	**0.136** ±0.0064	**0.131** ±0.0099	**0.128** ±0.0099	**0.102** ±0.0058	**0.168** ±0.0021
T cells	**0.560** ±0.0361	**0.651** ±0.0007	**0.653** ±0.0226	**0.684** ±0.0050	**0.672** ±0.0028	**0.618** ±0.0050	**0.625** ±0.0099	**0.711** ±0.0085
Activated T cells	**0.117** ±0.0064	**0.164** ±0.0042	**0.189** ±0.0078	**0.151** ±0.0071	**0.160** ±0.0028	**0.128** ±0.0099	**0.089** ±0.0021	**0.262** ±0.0057
CD 4 +	**0.580** ±0.0014	**0.578** ±0.0156	**0.644** ±0.0262	**0.588** ±0.0078	**0.592** ±0.0021	**0.558** ±0.0587	**0.477** ±0.0311	**0.264** ±0.0021
CD 8 +	**0.292** ±0.0552	**0.276** ±0.0057	**0.229** ±0.0255	**0.279** ±0.0021	**0.260** ±0.0057	**0.217** ±0.0014	**0.170** ±0.0354	**0.730** ±0.0014
Total B cells	**0.435** ±0.0318	**0.347** ±0.0014	**0.345** ±0.0219	**0.309** ±0.0021	**0.326** ±0.0028	**0.216** ±0.0678	**0.237** ±0.0021	**0.287** ± 0.01
NK cells	**0.070** ±0.0226	**0.096** ±0.0028	**0.126** ±0.0007	**0.124** ±0.0021	**0.091** ±0.0205	**0.198** ± 0.016	**0.019** ±0.0007	**0.346** ±0.021

T-cell sub-population : Total T (CD3+), Total B ($CD3^-$, HLA-DR^+), Active T ($CD3^+$, HLA-DR^+), $CD4^+$ T ($CD3^+$, $CD4^+$), $CD8^+$ T ($CD3^+$, $CD8^+$), NK cell ($CD3^-$, $CD16^+$, $CD56^+$), Activated PBMC ($CD25^+$) according to Cherng *et al.*,(2008)

Actual results in Appendix 3

Chapter Five: Discussion

5.1 D. cinerea

D. cinerea is investigated in other countries for the chemical properties and biological activities such as Sri Lanka where it is commonly used as an aphrodisiac and for the treatment of eye diseases (Wijesundara, 2003). In Zimbabwe it is used as a treatment for sexually transmitted diseases (Kambizi and Afolayan, 2001), and in Sudan it is used for the treatment of wounds (Eisa *et al.,* 1999). The phytochemicals present in the leaves are saponins, tannins, flavonoids, sterols and triterpenes (Eisa *et al.*, 1999).

***Antimicrobial*:** The extracts of *D. cinerea* at a concentration of 1000 µg/ml displayed antibacterial activity against *E. coli, P. aeruginosa, K. oxytoca, S. typhimurium, S. marcescens* and *B. Cereus* (Table 6). These results are similar to those of Eisa *et al.* (1999) where the leaf extract of *D. cinerea* inhibited the growth of *Bacillus* sp., *S. aureus, E. coli* and *P. aeruginosa.* The antibacterial activity of *D. cinerea* was found mainly against Gram negative bacteria whilst the Gram positive bacteria appear to be more resistant to the extracts. (Kambizi and Afolayan, 2001; Paz *et al.,* 1995).

In this study, the extract from *D. cinerea,* did not inhibit *Penicillium* sp., *Rhizopus* sp., *Geotrium* sp., *Fusarium* sp., *A. niger, Cladosporium* sp., *S. cerevisiae* and *C. albicans* and only showed activity against *Tricoderm sp* (Table 8). *Trichoderma* spp. possesses innate resistance to most agricultural chemicals, including fungicides, although individual strains differ in their resistance. Due to lack of information on the antifungal activity of *D. cinerea* in the literature, it is difficult to make recommendations regarding the specific antimicrobial

activity of *D. cinerea.* However, this can be attributed to the phytochemicals present in the plant (Hostettman *et al.*, 1995; Eisa *et al.*, 1999). Therfore a further investigation of the chemicals present need to assessed for the antifungal activity against *Tricoderm sp.*

Antioxidant activity: The methanolic and aqueous extracts of *D. cinerea* demonstrated excellent scavenging capability in comparison to the positive control rutin (Table 9). This high scavenging capacity is attributed to the phytochemicals presence in the plant such as polyphenols which are known antioxidants (Balasundram *et al*., 2005). Currently researchers have been discovering the importance of polyphenolic compounds present in plants, which until recently was considered unnecessary for the human diet. There is now proof that polyphenols are efficient antioxidants, and are stronger than antioxidant vitamins (Bravo, 1998; Cieslik, 2004).

During the last decade natural antioxidants, particularly polyphenols have been under very close scrutiny as potential therapeutic agents against a wide range of aliments including, neurodegenerative diseases, cancer, diabetes, cardiovascular dysfunctions, inflammatory diseases and also ageing (Soobrattee *et al.*, 2005). Many medicinal actions of polyphenols are mostly attributed to their antioxidant capacity, free radical scavenging, chelation of redox active metal ions, modulation of gene expression and interaction with the cell signalling pathways (Cao *et al.*, 1997). *D. cinerea* belongs to the family Fabaceae which is characterized by impressive phytochemical diversity. Polyphenols, especially flavonoids and tannins, are common (Raven *et al.*, 1999).

***Anti-inflammatory*:** The methanolic extract of *D. cinerea* was screened for anti-inflammatory properties, and this which showed poor activity in comparison to NDGA (Fig. 17). The

methanolic extracts where only analysed as most anti-inflammatory compounds are found in the polar solvents. The control selected was NDGA and is the bioactive component of the Mexican desert plant, *Larrea divaricata*, *w*hich are an antioxidant and lipooxygenase inhibitor (anti-inflammatory) as well as an inhibitor of peptido-leukotriene biosynthesis.

Safety and toxicity**:** *D. cinerea* displays no toxicity effects to the brine shrimp. All shrimps survived after an incubation of 24 h. The concentration used in the assement of the plant extract was assessed for toxicity. According to Meyer *et al.* (1982) an LC_{50} value higher than 1000 µg /ml is considered bioactive in toxicity evaluation of the plant extract by the brine shrimp lethality assay. Krishnaraju *et al.* (2005) reported that plants from the family Fabaceae recorded LC_{50} ranging as low as 60 µg /ml to some species as high as 5000 µg /ml in the brine shrimp lethality assay. Ajaiyeoba *et al.* (2006) reported that the family Fabaceae had an LC_{50} from 808.8 – 795.8 µg /ml in the brines shrimp lethality assay.

The Ames test result revealed that *D. cinerea* had no mutagenic potential towards both of the tester strains, TA 100 and TA 98 (Table 14; 15). In the cytotoxcity assay (MTT assay), using the cell line K 562, the extract had a stimulatory effect at concentration of 1000 µg/ml (Fig. 20).

Anti-mosquito**:** The results from the *A. arabiensis* larvacidal assay with the methanolic and aqueous *D. cinerea* extracts, at concentration of 1000 µg/ml are shown in Table 10. Plant extracts tested displayed no effect on the immature third in-star stage of *A. arabiensis* mosquitoes over the 7 day period. Compared to the positive control which is an organophosphate (Mostop), all larvae were dead on the first day. The plant extract displayed no irregularities (inhibition or mutation) of growth pattern throughout the seven day trial. All

larvae developed to pupae stages normally. *D. cinerea* aqueous and methanolic plant extracts showed repellence but no knockdowns or mortality (Table 11). The insecticidal assay of *D. cinerea* aqueous and methanolic plant extracts has exhibited knockdown in the first half hour and again after one hour and mortality after 24 hour period (Table 12).

D. cinerea demonstrated good repellence and average mortality. This could be attributed to the chemicals such as polyphenols (especially flavonoids and tannins) present mostly in the family Fabaceae (Raven *et al.*, 1999). No skin irritation or rashes were observed during the experiment or in the following 24 h period, after which time observation ceased. *D. cinerea* offers a potential source for bioactive compounds against *A. arabiensis*, particularly in its mosquito repellency ability. Further studies of the active compounds involved and their modes of action, formulate preparation for enhancing potency and stability, toxicity and effects on non target organisms and the environment.

Immunological studies: The MTT assay test was used to measure the viability of lymphocytes when exposed to different concentrations of the methanolic and aqueous extract of *D. cinerea* (Fig. 25). The methanolic and aqueous extract showed that, in a dose dependent manner extracts significantly stimulated the proliferation of human PBMC in comparison to the controls (CSA, DMSO and untreated cells). The methanolic extract demonstrated a higher rate of proliferation than the aqueous extract. The optimum concentration was found to be 250 µg/ml at this concentration, the cells proliferated. The time period after which the methanolic extracts were exposed to proliferated lymphocytes was 48 h (Fig. 26). The optimum time period was between 18 – 24 h periods, in which time the lymphocyte proliferation stabilized. Therefore the optimum concentration and time is 250 µg/ml for 24 h for the most effective

treatment of the PBMC with the plant extracts. The optimized concentration in comparison to the untreated cells, for the positive control is at doses 20 µg/ml for Con A, LPS and 250 µg/ml for PHA, for the negative control is at 20 µg/ml for CSA and 10 µl for diluent DMS0.

Microscopic analysis of cell proliferation**,** of lymphocytes exposed to methanolic and aqueous extracts of *D. cinerea* (250 µg/ml) was evaluated using an invert phase contrast light microscope (Fig. 23; 24). Treatment with most of the extracts slightly stimulated cell proliferation but did not induce changes in cell morphology at concentration from 1 µg/ml to 1 000 µg/ml. In the same concentration range, extracts of slightly reduced cell proliferation, and at the higher concentration of 250 µg/ml, so did most of the extracts.

The immunomodulatory activity was evaluated by flow cytometry analysis (Table 17) and secretion of IFN- γ (Table 16). Lymphocyte transformation or activation as an *in vitro* technique commonly is used to assess cellular immunity in human and other animals and this response refers to an *in vitro* correlate of an *in vivo* immune response (Horsmanheimo, 1974). IFN- γ is produced by T lymphocytes, natural killer cells, macrophages and neutrophils and has receptors on virtually all cell types of the body. It is a hallmark of Th-1 type response and exerts a multitude of cellular biological effects. Thus high-level production of IFN -γ is typically associated with effective host defense against intracellular pathogens and cancer (Cherng *et al* , 2008).

The extracts significantly stimulated the proliferation of the human lymphocytes and the secretion of IFN- γ in comparison to untreated cells. The immunostimulating activity may probably be due partly to the phenolic compounds and flavonoids, which have been shown to possess this activity (Cherng *et al.*, 2008). IFN- γ was first identified in mitogen–activated

lymphocyte supernatant as a distinctive antiviral activity and this pleotropic cytokine plays an important role in modulating nearly all phases of immune and inflammatory responses (Cherng *et al.*, 2007).

Hence *D. cinerea* activated lymphocytes and secretion of IFN- γ is in accordance to Type 1 immunodulation similar to the controls phytomitogen (PHA and ConA). To determine which types of lymphocytes directed the patterns of immunomodulation flow cytometric analysis was used. The result showed that phytomitogen PHA increased CD 8^+ T cells, whereas *D. cinerea*, elevated CD 4^+ T cells. Thus enhanced secretion of IFN- γ might be due to the CD 8^+ T cells because they were significantly elevated in the PHA but not in the *D. cinerea*, extracts. This is in accordance with the research of Cherng *et al* (2008) which noted that the CD 8^+ T cells were the main cell type to secrete IFN- γ. However, slight elevation of IFN-γ and IL-10 secretion after treatment with *D. cinerea* might be correlated to the activated $CD4^+$ T cells.

To elucidate which type of CD 4^+ T cells regulated the patterns of immunomodulation , the results indicated that immunostimulation of *D. cinerea* might be due to Th1 cells owing to the fact that they slightly elevated IFN- γ but significantly inhibited IL-10 secretion.

D. cinerea extract acted to affect lymphocytes as immunomodulating agents that is directly enhancing of lymphocytes activation and slight elevation in the secretion of multi-potent cytokine IFN- γ.

5.2 C. dimidiatus

C. dimidiatus is traditionaly used to treat dysentery laryngitis, acts as soothing cure for blue bottle sting, ringworm, eczema, dermatitis, herpes, nappy rash, thrush, cold sores, earache and eye infections (Van der Watt and Pretorius, 2001; Van Wyk *et al.*, 2002; Watt and Breyer-Brandwijk., 1962). *Carpobrotus* species displays the phytochemical profile of flavonoids, tannins, alkaloids, phytosterols and aromatic acids. The flavonoids present in *Carpobrotus* species are rutin, neohesperidin, hyperoside, cathecin and ferulic acid which are shown to have biological properties such as antimicrobial, antioxidant, anti-inflammatory (Van der Watt and Pretorius, 2001; Springfield *et al.*, 2003).

Antimicrobial: *C. dimidiatus* extracts showed activity against *E. coli, P. aeruginosa, K. oxytoca, S. typhimurium, B. cereus* and *Tricoderm sp* (Table 6 ; 8). In studies by Van der Watt and Pretorius, (2001) they found that *C. edulis* from the family Mesembryanthemaceae showed antimicrobial activity against *B. subtilis, S. epidermidis, S. aureus, S. pneumoniae, S. pyrogenes, M. catarrhalis, H. influenzae, E. coli,* and *P. aeruginosa*. Studies by Springfield *et al*. (2003) showed that *C. muirii* and *C. quadrifidus* show antimicrobial activity against *S. aureus* and *Mycobacterium smegmatis*. Martins *et al*. (2004) reported that extracts of *C. edulis* inhibits the growth of multidrug resistant *Mycobacterium tuberculosis* and methicillin resistant *S. aureus.*

The antibacterial effect of *C. dimidiatus* can be attributed to the flavonoids and tannins and not a single active compound as they act synergistically to provide an antimicrobial agent (Van der Watt and Pretorius, 2001). He further reported that the active flavoniods present are rutin, neohesperidin, hyperoside, cathecin and ferulic acid. No particular compound was

antibacterial but together displayed excellent antibacterial activity against both Gram negative and Gram positive bacteria.

***Antioxidant activity*:** The extracts of *C. dimidiatus* demonstrated insignificant antioxidant activity relative to rutin which had a IC_{50} value of 6 µg/ml. The methanolic extract had an IC_{50} value of 240 µg/ml and the aqueous extract had an IC_{50} value of 1125 µg/ml shown in Table 9.

***Anti-inflammatory activity*:** In this study we did not find any inflammatory activity (Fig. 17). However, many other *Carpobrotus* species are known to possess a hyperoside. Hyperoside is quite common in a wide range of plants, which contributes to the anti-inflammatory properties (Bruneton, 1995).

Safety and toxicity: *C. dimidiatus* displays no toxic effects on the brine shrimp. The Ames test revealed that *C. dimidiatus* had no mutagenic potential towards both of the tester strains, TA 100 and TA 98 shown in Table 14; 15. In the cytotoxity assay (MTT assay), using the cell line K 562 the extract had a stimulatory effect in a concentration dose dependent manner (Fig. 20).

Anti-mosquito: The extracts tested showed that *C. dimidiatus* does not affect the larvae of *A. arabiensis* (Table 10). The aqueous extract did show 97% repellency (Table 11) and the methanolic extracts 86.5 ± 9.19% but none of the extracts showed any mortality. The insecticidal assay (Table 12) using *C. dimidiatus* aqueous plant extract has exhibited 11.67 ± 2.35 % knockdown in the first half hour and 43.34 ± 9.43 % knockdown in the first hour and a mortality of 41.67 ± 11.79 % after the 24 hour period. *C. dimidiatus* methanolic plant

extract has exhibited 15 ± 2.35 % knockdown in the first half hour and still in the first hour and a mortality rate of 33.34 ± 4.72 % after the 24 hour period. Thus, *C. dimidiatus* had displayed great potential as a repellent, and insecticide.

The repellency against *A. arabiensis* could be attributed to the fact that it belongs to the family Mesembryanthemaceae which has a high concentration of chemicals such tannins, malic acid and citric acid (Van Wyk *et al.,* 2002) which have been reported to demonstrate repellency activity (Choochote *et al.,* 2004). The use of plant extracts in insect control as an alternative pest control method for minimizing the noxious effects of some pesticidal compounds on the environment (Fatope *et al.,* 1993). In this way, the results obtained suggest that the leaf extract of *C. dimidiatus* is promising as a repellent against *A. arabiensis*. These results could be useful in the search for newer, more selective, and biodegradable natural compounds. Further investigation is required to isolate the active compound.

Immunological studies: *C. dimidiatus* demonstrated similar results as *D. cinerea* and refer to this section under Immunological studies section of *D.cinerea* (Page 102).The results obtained demonstrated that *C. dimidiatus* extract acted to affect lymphocytes as immunomodulating agents that is directly enhancing of lymphocytes activation and slight elevation in the secretion of multi-potent cytokine IFN- γ.

5.3 *C. tomentosa*

This plant is used traditionally as an anti-inflammatory and anti-convulsive agent, and for the treatment of rheumatism, insanity, jaundice, malaria, headache, coughs and pneumonia (Van Wyk *et al.,* 2002).

***Antibacterial*:** The methanolic and aqueous leaf extract of *C. tomentosa* inhibited *P. aeruginosa*, *S. typhimurium* (Table 6) and *Tricoderm sp* (Table 8). Studies by Steenkamp *et al*. (2004) showed that the aqueous extract of *C. tomentosa* showed activity against *S. aureus* and *S. pyogenes*, and in studies by Sama *et al.* (2006) the hexane and ethanolic extracts displayed activity against *S. aureus*, *B. cereus*, *C. albicans* and *A. flavus*. The results indicate that the traditional use of *C. tomentosa* extract to treat diseases such as coughs and pneumonia is justified and the need to identify the presence of active compounds need to be identified. The antimicrobial activity of *C. tomentosa* could be attributed to the phytochemicals present in the plant: alkaloids, anthranoids, flavonoids, glycosides, polyphenols, saponins and steroids (Amusan *et al.*, 2007). Flavonoids are known to possess antimicrobial properties (Hostettman *et al.* , 1995). Other plants of the Family Capparidaceae have been investigated for their antimicrobial activity. The methanol extract of *Cappris thonningii* displayed antibacterial activity in accordance with the concentration by inhibiting the growth of *S. aureus* and mild activity against *E. coli*, *C. albicans* and A. *flavus* (Sama and Ajaiyeoba, 2006) and they reported that the leaves, stem, bark and roots of *Ritchiea capparoides* were found to possess antifungal properties.

***Antioxidant*:** The extracts of *C. tomentosa* demonstrated excellent scavenging capability when compared to rutin (Table 9). This result indicates that the extract can be used for prevention of degenerative diseases such as Parkinson's, Alzheimer's, cancer, diabetes, heart disease, arthritis, osteoporosis etc (Percival, 1998). They also provide important health benefits in the form of antioxidant activity by preventing aging (Lindsey *et al*., 2002).

This high scavenging capacity is attributed to the phytochemicals present in the plant: alkaloids, anthranoids, flavonoids, glycoside, polphenols, saponins and steroids (Amusan *et al.*, 2007). The medicinal actions of polyphenols are mostly attributed to their antioxidant capacity, free radical scavenging, chelation of redox active metal ions, modulation of gene expression and interaction with the cell signalling pathways (Cao *et al.*, 1997).

***Anti-inflammatory*:** In our study we did not find significant anti-inflammatory activity when compared with NDGA which is a potent anti-inflammatory compound (Fig. 17). Van Wyk *et al.* (1997) reported that *C. tomentosa* is used to treat rheumatism because it contains L-stachydrine and 3-hydroxy-4-methoxy-3-methyl-oxindole, a known anti-inflammatory compound. Contrary to results obtained in our study. Stachydrine is commonly found in a wide range of plants, and contributes to the anti-inflammatory properties of these plants (Bruneton, 1995).

***Safety and toxicity*:** *C. tomentosa* displays no toxic effects on the brine shrimp. The Ames test revealed that *C. tomentosa* had no mutagenic potential towards both of the tester strains, TA 100 (Table 15) and TA 98 (Table 14). In the cytotoxity assay (MTT assay), using the cell line K 562 the extract had a stimulatory effect at concentration 1000 µg/ml (Fig. 20). These results

are contradictory to reports made by Ahmed *et al.* (1993) where *C. tomentosa* had a toxic effect on goats fed with the leaves. However, Mlambo *et al.* (2004) reported that the leaves and fruit do not need to be detoxified to be fed to goats. But Getahun *et al.* (1967) reported that both goat and camels die instantly if consumed.

***Anti-mosquito*:** *C. tomentosa* showed good repellence against the malarial vector, *A. arabiensis*. This could be attributed to the fact that it belongs to the family Capparaceae which has a high concentration of chemicals such as stachydrine and 3-hydroxy-4-methoxy-3-methly-oxindole (Raven *et al.,* 1999). The methanolic extract displayed greater activity because polar solvents extract more volatiles. Volatiles have been reported to have a strong repellency and mortality activity (Choochote *et al.,* 2004).

Due to the repellency and insecticidal potential, *C. tomentosa* can therefore be recommended as a probable source of some biologically active compounds useful in the development of potential alternatives for vector control, in areas where mosquitoes are resistant to conventional insecticides.

Immunological studies: *C. tomentosa* demonstrated similar results as *D. cinerea* and refer to this section under immunological studies section of *D. cinerea* (Page 102).The results obtained demonstrated that *C. tomentosa* extract acted to affect lymphocytes as immunomodulating agents that is directly enhancing of lymphocytes activation and slight elevation in the secretion of multi-potent cytokine IFN- γ.

5.4 L. leonurus

L. leonurus belongs to the family Lamiaceae. Plants from the family Lamiaceae contain various phenolic compounds such as flavonoids, phenolic diterpenes, monoterpenoids, glycosides (iridoids), rosmarinic acid and other derivatives of caffeic acid (Erdemoglu *et al.*, 2006; Raven et al., 1999). Essential oils from the leaf and flower of *L. leonurus* illustrate diverse phytochemicals such as limonene, (Z)-β-ocimene, terpinene, caryophyllene, humulene and germacrene. These phenolic compounds allow the plants from the Lamiaceae family to have a diverse range of biological activity (Steenkamp *et al*., 2004).

Antimicrobial: Extracts of *L. leonurus (*1000 µg/ml) showed inhibitory activity against *P. aeruginosa, K. oxytoca, S. typhimurium* and *B. cereus* (Table 6). However the other bacteria display poor or no activity. Studies by Kamatou *et al.* (2006) showed that *E. coli, S. aureus, K. pneumoniae* and *Bacillus sp.* were inhibited at MIC values ranging from 2000 – 3000 µg/ml. As these are twice and thrice the concentration used in this experiment, it could explain the difference in the results. Similarly Steenkamp *et al.* (2004) report antibacterial activity against *S. pyogenes, E. coli* and *P. aeruginosa*, with a MIC values of 4000 µg/ml. Other studies (Hutchings *et al.*, 1996; Kelmanson *et al.*, 2000) show no antibacterial activity which could be due to low concentration of *L. leonurus* plant extracts.

L. leonurus antibacterial activity could be attributed to caffeic acid and terpenoids, which are known antimicrobial agents (Van Wyk *et al.*, 2002)

Antioxidant activity: The extracts (leaves and flowers) of *L. leonurus* showed moderate anti-oxidative activity with IC_{50} value ranging from 20 µg/ml to 125 µg/ml (Table 9). Frum *et al.* (2006) noted that the methanolic extract of *L. leonurus* was far more effective than the

aqueous extract due to the factor that flavonoids and tannins are readily more soluble in methanol than in water. Methanolic flower extracts displayed strong radical scavenging capacity due to the intense flower colour, which has a greater concentration of flavonoids and phenolic compounds. Flavonoids and phenolic compound are known to demonstrate free radical scavenging capacity (Bravo, 1998). The antioxidant activity of plants from the family Lamiaceae was reported to be attributed mainly to the presence of phenolic compounds such as rosmaric acid, flavonoids and diterpenes, because of their ability to scavenge free radicals and active oxygen species such as singlet oxygen, superoxide anion radical and hydroxyl radicals (Nurgun *et al.,* 2006). Methanolic extracts were generally more active than aqueous extracts as antioxidant agents.

Plants in the family Lamiaceae are strong free radical scavengers and are therefore considered as good source of natural antioxidants for medicinal uses in ageing and other diseases related to radical mechanisms (Erdemoglu *et al.,* 2006). The family Lamiaceae contains phenolic compounds and flavonoids, which inhibit the oxidation of lipids by donating hydrogen atoms to scavenge free radicals (Zhang, 1998), flavonoids are known natural antioxidants (Erdemoglu *et al.*, 2006). Determination of the natural antioxidant compounds of plant extracts will help to develop new drug candidate for antioxidant therapy (Zhang, 1998).

Anti-inflammatory: Poor anti-inflammatory activity was found in the extracts shown in Fig. 17. An interesting observation was made by Stafford *et al*. (2004), with regards to the Cyclooxygenase (COX-1) inhibition anti-inflammatory assay, where methanolic extract of *L. leonurus* demonstrated a 100 % inhibition and also noted that the aqueous and methanolic

extracts showed an increase in activity after storage of the plant material however the methanolic extract displayed a better anti-inflammatory response.

Safety and toxicity: *L. leonurus* displays no toxicity to brine shrimp or human cell line (Fig. 20) and did not display any mutagenicity (Table 14; 15) . McGaw *et al*. (2000) reported that *L. leonurus* demonstrates no activity in the brine shrimp lethality assay. There have been no mutagenic reports on *L. leonurus* in the literature.

Anti-mosquito: *A. arabiensis* repellence assay using the leaves and flowers of *L. leonurus* showed strong repellency in comparison to the two negative controls acetone and distilled water. All *L. leonurus* extracts showed excellent repellence against the mosquitoes, it also showed 'knockdown' which is the rapidly and normally reversible paralysis of the mosquitoes, and mortality. At the end of the two minutes exposure period about 50 % of the mosquitoes became inactive (knockdown). The *L. leonurus* leaf and flower aqueous plant extract showed repellence but no knockdowns and mortality. The *L. leonurus* methanolic flower extracts displayed repellence and knockdowns after two minute exposure time and mortality after 24 hours. The methanolic extract of *L. leonurus* showed a greater repellency in comparison to the aqueous plant extract. Plants such as *Ocimum basilicum* (basil), *Mentha citrata* (bergamot), *Nepeta cataria* (catnip), *Salvia sclarea* (clary sage), *Mentha arvensis* (cornmint), *Lavandula angustifolia* (lavender) and common herbs from the family Lamiaceae such as thyme, marjoram, sweet marjoram, oregano, rosemary, sage and spearmint all display anti- mosquito activity (Pavela, 2005). The chemistry of these plants is well documented and includes monoterpenes, sesquiterpenes and phenols (Pavela, 2005).

The *L. leonurus* flower extracts showed a stronger repellence and mortality than *L. leonurus* leaf extracts. *L. leonurus* had displayed great potential as a repellent, insecticide and has also been shown to demonstrate a high mortality rate against *A. arabiensis.* This could be attributed to the fact that it belongs to the family Lamiaceae which has a high concentration of chemicals such as rosmarinic acid and other derivatives of caffeic acid (Raven *et al.,* 1999) and diterpenoids (Labdane type lactones) (Van Wyk *et al.*, 2002). Pavela, (2005) reported that plants from the family Lamiaceae demonstrate a strong anti-mosquito activity.

Immunological studies: *L. leonurus* demonstrated similar results as *D. cinerea* and refer to this section under immunological studies section of *D.cinerea* (Page 102).The results obtained demonstrated that *L. leonurus* extract acted to affect lymphocytes as immunomodulating agents that is directly enhancing of lymphocytes activation and slight elevation in the secretion of multi-potent cytokine IFN- γ.

Chapter Six: Conclusion

Safe and effective pharmaceuticals are based on bioactivities of plant compounds. Knowledge of these active compounds provides a basis for understanding herbal medicinal efficacy, for nutritional and therapeutic value. In this study, antimicrobial activity was determined by evaluating the bactericidal and fungicidal activity using the agar disc diffusion assay. Anti-oxidative property was tested using the DPPH photometric assay and anti-inflammatory properties with 5-lipooxygenase assay. The larvacidal, repellency and insecticidal assay against the malaria transmission vector *A. arabiensis* were also investigated. The safe use of these plants was investigated with the brine shrimp assay, an *in vitro* cell culture toxicity assay and a mutagenicity assay. The immune modulatory activity on PBMC was evaluated by lymphocyte transformation, and by determination of B cells, T cells, activated T cells, CD 4^+, CD 8^+, NK cells with flow cytometry. The type of immune response was then determined by correlating the secretion of interferon-gamma and interleukin 10.

The extracts showed anti-microbial activity against *E. coli, P. aeruginosa, K. oxytoca, S. typhimurium, S. marcescens, B. cereus* and against *Tricoderm* sp. The highest activity was shown by methanolic and aqueous extracts of *L. leonurus* leaves followed by methanolic and aqueous extracts of *D. cinerea*.

D. cinerea and *C. tomentosa* demonstrated a higher degree of free radical scavenging capacity indicating the extracts have strong antioxidant properties. None of the plants demonstrated any anti-inflammatory activity. In the anti-mosquito assays, the extracts showed strong repellency and insecticidal activity. *L. leonurus* extracts demonstrated the highest insecticidal

and repellency activity against the mosquito, and was also found to cause 'knockdown' and mortality of the malarial vector. The extracts display no toxicity, cytoxicity or mutagenicity. The results of the immune-stimulation showed that the methanolic extracts of these plants induced a Th_1- predominant immune response because they significantly suppressed the secretion of IL-10 and augmented the IFN-γ production.

Information from traditional sources indicates that these plants are used in the treatment of a multitude of diseases and scientific knowledge shows that these plants have a vast number of secondary metabolites. From the chemicals that are described in these plants *viz.* polyphenols (especially the flavonoids alkaloids and tannins) in these species, and the antibacterial activities found, many of the traditional medicinal uses can be justified. *D. cinerea, C. dimidiatus, C. tomentosa and L. leonurus* showed antibacterial activity against many of the Gram negative organisms tested; good potential as anti-oxidative agents, and also activity against the insect *A. arabiensis*, a malarial vector. The plant extracts also have immune stimulatory properties and are nontoxic.

This study also provides new information, with respect to the potential use of these plants in producing a mosquito repellent. The most significant results are probably those related to the immune function which indicates its use as an immune stimulant, and the fact that all the *in vitro* toxicity results indicated that there are non toxic at the concentration assessed (10-1000 ug/ml).

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APPENDIX 1

PREPARATION OF REAGENTS

SECTION 1

Antioxidant Activity

Preparation of Flavonoid

The flavonoid chosen was Rutin which is a potent antioxidant, is found in the buckwheat plant *Fagopyrum esculentum*. Rutin hydrate (HPLC grade) was purchased from Sigma. A stock solution of 1000 μg/ml was prepared which was diluted to 500 μg/ml, 250 μg/ml, 100 μg/ml, 10 μg/ml, and 1μg/ml with ethanol.

SECTION 2

Solution and media for Ames test

1. **O.5 Mm histidine / 0.5 Mm biotin solution for top agar**

Ingredients	**per 250 ml**
• D-Biotin	30.5 mg
• L-Histidine	26.2 mg
• Double distilled water (ddH_2O)	250 ml

Dissolve biotin and histindine in warm ddH_2O and filter sterilize

2. **Top Agar**

Ingredients	**per 1 000 ml**
• Bacteriological Agar	6 g
• NaCl	5 g
• Double distilled water (ddH_2O)	1000 ml

Microwave to dissolve the agar. Mix thoroughly and make 100 ml aliquots in sterile 250 ml glass bottles with scew caps. Autoclave at 120 ° C for 20 minutes. Cool the agar and tighten caps. Add 1 / 10 volume (10 ml) of the 0.5 mM histidine/ 0.5 biotin solution to molten top agar. Mix thoroughly by swirling.

3. **Vogel- Bonner medium E stock for minimal agar base**

Ingredients	**per 1 000 ml**
• Warm ddH_2O (45 °C)	670 ml
• $MgSO_4$. 7 H_2O	10 g
• Citric acid monohydrate	100 g
• K_2HPO_4	500 g
• $NaHNH_4PO_4$. 4 H_2O	175 g

Add salts in order indicated to warm water in a 2 L flask placed on a magnetic stirring hot plate. Allow each salt to dissolve completely before adding the next. Adjust the volume to one liter distribute into two 1 L glass bottles. Autoclave at 120 ° C for 20 minutes.

4. 40 % glucose solution

Ingredients	**per 1 000 ml**
• Glucose	400 g
• ddH_2O	1000 ml

Dissolve glucose in ddH_2O at and autoclave 120 ° C for 20 minutes

5. Minimal glucose agar plates

Ingredients	**per 1 000 ml**
• Bacteriological Agar	15 g
• ddH_2O	930 ml
• Vogel- Bonner medium E stock	20 ml
• 40 % glucose solution	50 ml

Add ddH_2O to agar in a 2L flask. Place a large magnetic stirring bar inside for later mixing and autoclave 120 ° C for 20 minutes. When the gar has cooled add the Vogel-Bonner medium E stock and glucose solution. Stir the mix thoroughly. Pour 30 ml into each Petri dish.

SECTION 3

Media and reagents for continuous cell line culture

1. Dulbecco's Modified Eagle's Medium

With 4.5g/lglucose, 0.110/L Sodium pyruvate with L-glutamine (Highveld Biological, South Africa).

2. Foetal Calf Serum (FCS)

Was filter sterilized and gamma irradiated at 25 to 28 kGy under conditions, which preserve the biological integrity of serum. The serum was aliquoted into 10ml sterile flasks and kept frozen (-20º) until use. (Highveld Biological, South Africa).

3. **Non- Essential Amino acids (NEAA)**

Was aliquoted into 10ml sterile flasks and kept frozen (-20º) until use. (Highveld Biological, South Africa).

4. Penicillin/ Streptomycin (Pen/Strep)

Mixture contained 100mg/ml penicillin G sodium and 100mg/ml streptomycin sulphate in double reverse osmosis water. One ml aliquots were frozen (-20ºC) until use (Highveld Biological, South Africa).

5. Phosphate Buffered Saline (PBS)

Ingredients	**per 1 000 ml**
• Sodium Chloride	8g
• Potassium Chloride	0.2g
• Potassium dihyhydrogen phosphate	0.12g
• Sodium hydrogen phosphate	0.91g
• Double distilled water (ddH_2O)	1000 ml

Mix Sodium Chloride, Potassium Chloride, Potassium dihyhydrogen phosphate, Sodium hydrogen phosphate in one litre of double distilled water. And was equilibrated to pH 7.2

filter sterilized and autoclaved aliquoted into 100ml sterile McCartney's Bottles and kept (4ºC) until use. (Merck NT, South Africa)

6. **Dimethyl sulphoxide (DMSO)**

Was pharmaceutical grade (tested in tissue culture) was filter sterilized and aliquoted into 100ml sterile Dark Bottles and kept away from direct sunlight and excessive temperature until use. (Highveld Biological, South Africa).

7. **70% ethanol**

Contained 70ml Analar grade ethanol (100%) and 30ml distilled water was combined to provide general tissue culture disinfectant (Merck NT, South Africa).

8. **Complete Culture Media (CCM)**

Contained 20 ml FCS, 10ml NEAA, 2ml Pen/Step mixture in 68ml DMEM and stored in sterile bottle at 4ºC.

9. ***Reagents for Microscopy***

0.2 % Trypan Blue Solution (w/v) was made in PBS pH 7.4. Biowhittaker, Walkersville, USA.

SECTION 4

Reagents for Cytotoxicity assays

1. 3-{4,5-dimethylthylthiazol-2-yl}-2,5 diphenyl tetratrazolium bromide (MTT) reagent was prepared by dissolving 5 mg MTT in 1 ml PBS (pH 7.5). The solution was filter sterilized and stored in the fridge. (Sigma –Aldrich, South Africa)

SECTION 5

Immuno-modulation

1. Concanavalin A (ConA)

ConA (purchased from Sigma) is lectin protein originally extracted from the jackbean, *Canavalia ensiformis*. Lectin has a number of effects on cell metabolism: it induces mitosis and affects the cell membrane in regard to transport and permeability to proteins and thus serves as a positive control. 1mg of lyophilized ConA powder was added to 1 ml of phosphate buffered saline to make the stock of 1mg/ml. This was further diluted to in RPMI-1640 (with antibiotics) to make the working stocks of 20µg/ml, 10µg/ml, 5µg/ml.

2. Lipopolysaccharide (LPS)

LPS (purchased from Sigma) are a major constituent of the cell wall of gram negative bacteria, *Escherichia coli*. LPS directly activate B cells, regardless of their antigenic specificity. Plasma B cells are terminally differentiated and therefore cannot undergo mitosis. Memory B cells can proliferate to produce more memory cells or plasma B cells, hence LPS induces mitosis in B cells. 1mg of lyophilized LPS powder was added to 1 ml of phosphate

buffered saline to make the stock of 1mg/ml. This was further diluted to in RPMI-1640 (with antibiotics) to make the working stocks of 20 µg/ml, 10 µg/ml, 5 µg/ml.

3. Cyclosporin A (CSA)

CSA (purchased from Sigma) is a non-polar cyclic oligopeptide produced by the fungus, *Tolypocladium inflatum*. It is a potent immunosuppressive agent affecting primarily T-lymphocytes. Hence it is used as a negative control.

CSA is an extremely hydrophobic substance. 1 mg CSA was added to 100 µl of absolute ethanol and 20 µl of Tween 80 respectively. This was made up to a final volume of 1 ml with RPMI-1640 to give a stock solution of 1 mg/ml. This was further diluted to in RPMI-1640 (with antibiotics) to make the working stocks of 20 µg/ml, 10 µg/ml, 5 µg/ml.

4. Phytohaemagglutinin (PHA)

PHA (purchased from Sigma) is an extract of *Paseolus vugaris* seed. It stimulates progressive non-specific lymphocyte mitosis in cell culture and thus serves as a positive control. It is stored lyophilized at 2-8 ºC, however if reconstituted PHA can be stored at – 80 ºC for 6- 12 months or stored at 4 ºC for 4 weeks. 2mg of lyophilized PHA powder was added to 2 ml of phosphate buffered saline (pH) to make the stock of 1mg/ml. This was further diluted to a 1: 4 dilution of 2 ml of stock solution in 6 ml of RPMI-1640(with antibiotics) to make the working stock of 250µg/ml.

Appendix 2

Actual Results

2.1. Antioxidant assay (methanol)

Table 1: Free radical scavenging activity of D. *cinerea,* C.dimidiatus*; C.tomentosa* and *L. leonurus.*

Plant extract	Abs 1	Abs2	Abs3	Avg	%S.C	RXN
C. dimidiatus (aqueous)						
1. 1mg/ml	0.105	0.102	0.108	0.105	83	+
2. 500µg/ml	0.237	0.196	0.246	0.226	64	+
3. 250µg/ml	0.242	0.266	0.251	0.253	60	+
4. 100µg/ml	0.410	0.573	0.516	0.499	20	-
5. 10µg/ml	0.592	0.599	0.599	0.597	5	-
6. 1µg/ml	0.663	0.627	0.661	0.650	0	-
C .dimdiatus (methanolic)						
1. 1mg/ml	0.336	0.346	0.348	0.343	45	Slight change
2. 500µg/ml	0.524	0.467	0.522	0.504	20	-
3. 250µg/ml	0.478	0.545	0.573	0.532	15	-
4. 100µg/ml	0.595	0.644	0.621	0.620	1	-
5. 10µg/ml	0.604	0.694	0.599	0632	0	-
6. 1µg/ml	0.622	0.673	0.792	0.715	0	-
C. tomentosa (aqueous)						
1. 1mg/ml	0.019	0.017	0.019	0.018	97	+
2. 500µg/ml	0.013	0.022	0.021	0.019	97	+
3. 250µg/ml	0.027	0.031	0.028	0.029	95	+
4. 100µg/ml	0.058	0.071	0.045	0.058	90	+
5. 10µg/ml	0.308	0.301	0.431	0.347	45	+
6. 1µg/ml	0.499	0.438	0.384	0.440	30	+
C .tomentosa (methanolic)						
1. 1mg/ml	0.054	0.052	0.058	0.055	91	+
2. 500µg/ml	0.056	0.052	0.062	0.057	90	+
3. 250µg/ml	0.067	0.073	0.058	0.066	89	+
4. 100µg/ml	0.123	0.074	0.089	0.095	85	+
5. 10µg/ml	0.128	0.133	0.130	0.130	79	+
6. 1µg/ml	0.455	0.511	0.582	0.516	18	+
L. leorunus (aqueous)						
1. 1mg/ml	0.049	0.043	0.039	0.044	93	+
2. 500µg/ml	0.057	0.051	0.061	0.056	91	+
3. 250µg/ml	0.056	0.061	0.068	0.062	90	+

4. 100µg/ml	0.736	0.700	0.646	0.694	49	+
5. 10µg/ml	0.736	0.700	0.646	0.694	0	-
6. 1µg/ml	0.730	0.733	0.745	0.736	0	-
L. leorunus (methanolic)						
1. 1mg/ml	0.048	0.042	0.047	0.046	92	+
2. 500µg/ml	0.057	0.070	0.053	0.060	90	+
3. 250µg/ml	0.070	0.065	0.062	0.066	89	+
4. 100µg/ml	0.086	0.089	0.071	0.082	87	+
5. 10µg/ml	0.446	0.609	0.643	0.566	10	-
6. 1µg/ml	0.611	0.593	0.582	0.595	6	-
L. leorunus Flower (aqueous)						
1. 1mg/ml	0.030	0.034	0.031	0.032	95	+
2. 500µg/ml	0.044	0.041	0.041	0.042	93	+
3. 250µg/ml	0.192	0.194	0.188	0.191	70	+
4. 100µg/ml	0.371	0.343	0.355	0.356	43	+
5. 10µg/ml	0.588	0.594	0.618	0.600	5	-
6. 1µg/ml	0.553	0.588	0.797	0.646	0	-
L. leorunus Flower (methanolic)						
1. 1mg/ml	0.031	0.031	0.032	0.031	95	+
2. 500µg/ml	0.028	0.030	0.032	0.030	95	+
3. 250µg/ml	0.036	0.038	0.037	0.037	94	+
4. 100µg/ml	0.039	0.035	0.041	0.038	94	+
5. 10µg/ml	0.576	0.454	0.482	0.504	20	-
6. 1µg/ml	0.565	0.456	0.599	0.540	15	-
D. cinerea (aqueous)						
1. 1mg/ml	0.016	0.016	0.018	0.017	97	+
2. 500µg/ml	0.022	0.029	0.034	0.028	96	+
3. 250µg/ml	0.027	0.028	0.032	0.029	95	+
4. 100µg/ml	0.037	0.038	0.032	0.036	94	+
5. 10µg/ml	0.455	0.511	0.582	0.516	18	-
6. 1µg/ml	0.575	0.582	0.570	0.576	8	-
D. cinerea (methanolic)						
1. 1mg/ml	0.022	0.031	0.029	0.027	96	+
2. 500µg/ml	0.034	0.035	0.035	0.035	94	+
3. 250µg/ml	0.045	0.049	0.069	0.054	91	+
4. 100µg/ml	0.060	0.055	0.054	0.056	91	+
5. 10µg/ml	0.101	0.108	0.118	0.109	83	+
6. 1µg/ml	0.547	0.600	0.524	0.557	11	-
Positive control: Rutin	**0.048**	**0.053**	**0.066**	**0.056**	**91**	**+**
Negative control : Methanol	**0.602**	**0.642**	**0.644**	**0.629**	**-**	**-**

2. 2 Anti-inflammatory

Table 2: Anti-inflammatory (5-lipoxygenase) activity of *D. cinerea, C.dimidiatus; C.tomentosa* and *L. leonurus.*

Concentration of sample dissolved in DMSO (ug/ml)	Dichrostachys cinera (ug/ml)	Leonotis leonurus (ug/ml)	Leonotis leonurus (flowers) (ug/ml)	Capparis tomentosa	Carpobrotus dimidiatus	NDGA
100	27.0	37.1	18.3	21.4	16.6	
200	42.7	42.9	24.0	28.7	25.1	
300	51.4	58.2	35.1	33.5	29.4	
400	59.6	65.7	44.8	51.9	35.8	
500	59.6	65.2	49.3	56.1	41.6	
600	-	-	52.1	56.6	47.2	
700	-	-	52.0	56.6	51.0	
800	-	-	-	-	51.0	
1						19.4
2						43.7
3						59.6
4						77.9
5						91.0
6						91.0
$IC_{50\ (ug/ml)}$	270	220	560	450	450	2.5

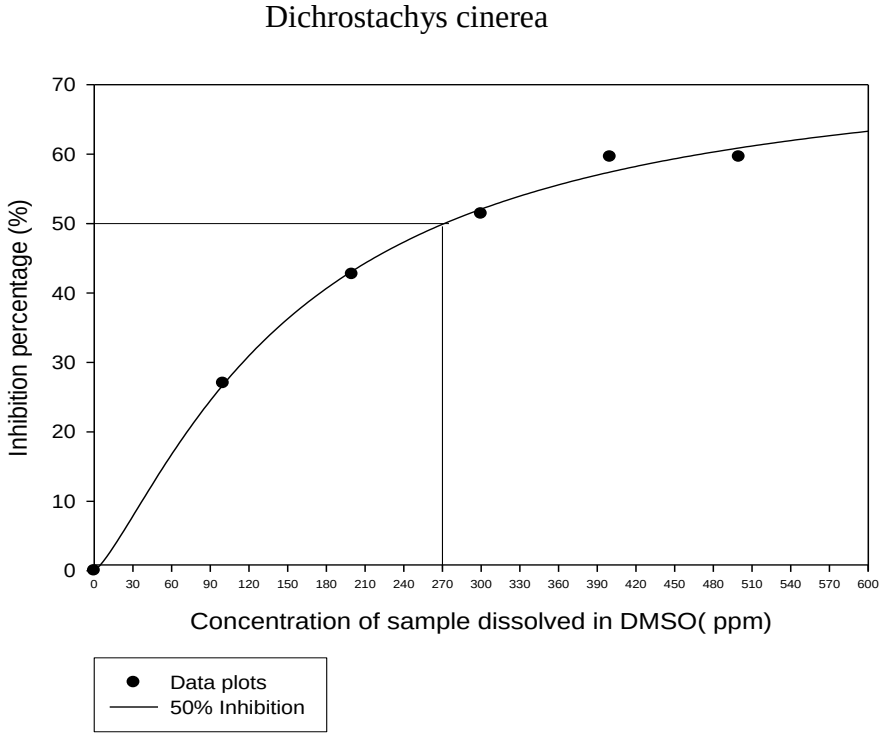


Figure 1: Anti-inflammatory (5-lipoxygenase) activity of *D. cinerea,*

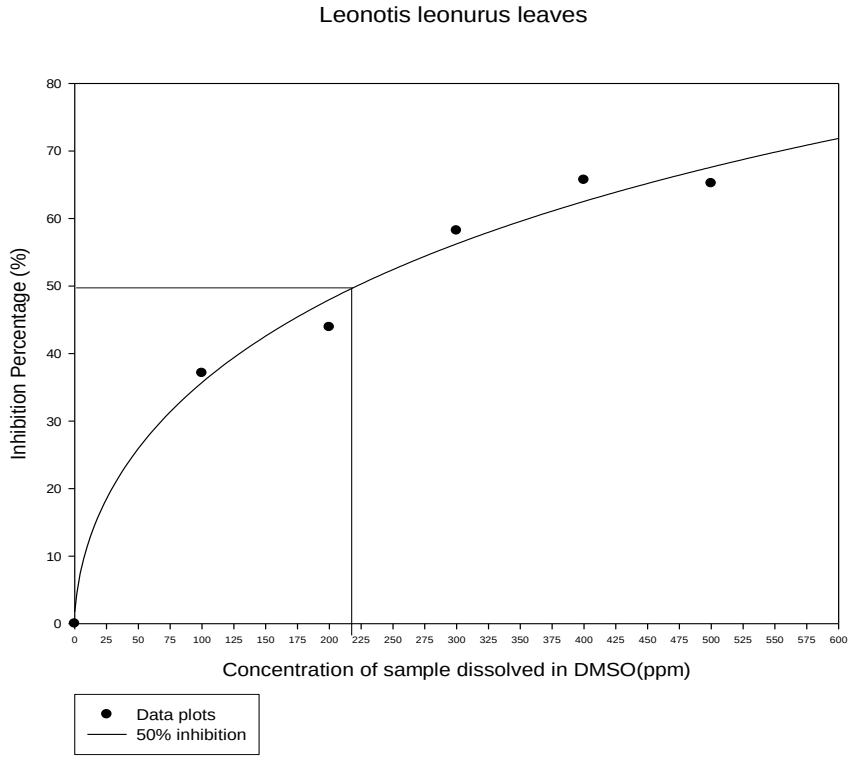


Figure 2: Anti-inflammatory (5-lipoxygenase) activity of L. *leonurus.*

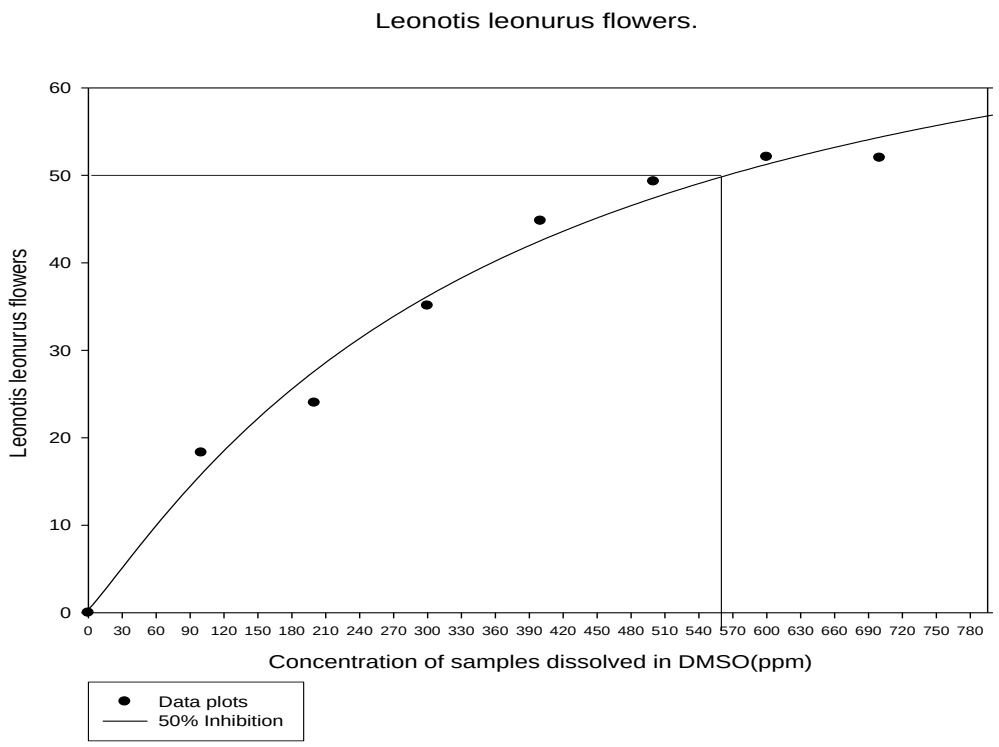


Figure 3. Anti-inflammatory (5-lipoxygenase) activity of *L. leonurus* flowers.

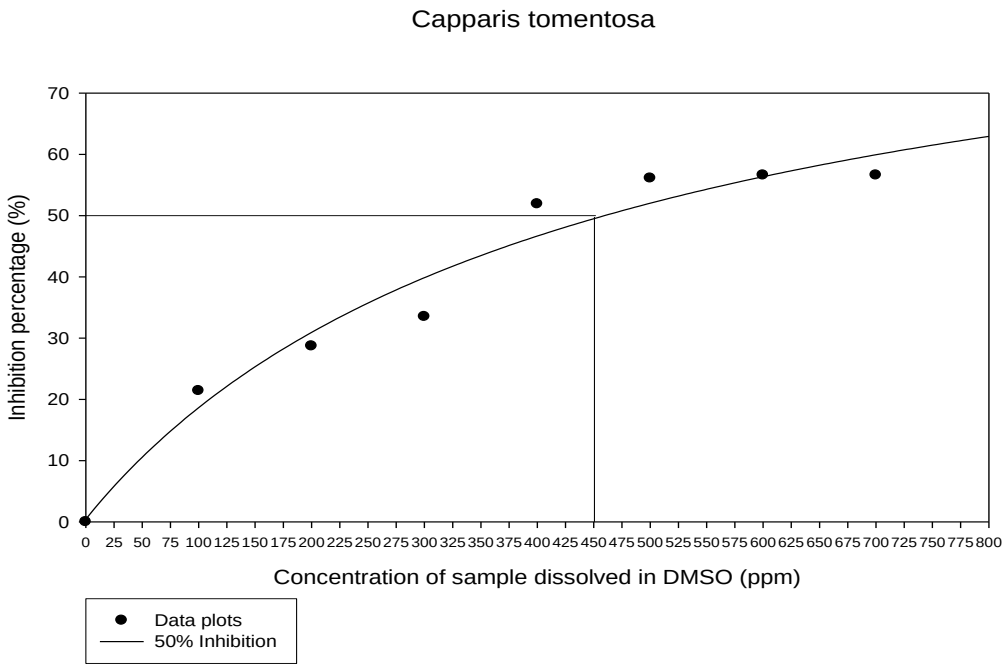


Figure 4: Anti-inflammatory (5-lipoxygenase) activity of *C.tomentosa.*

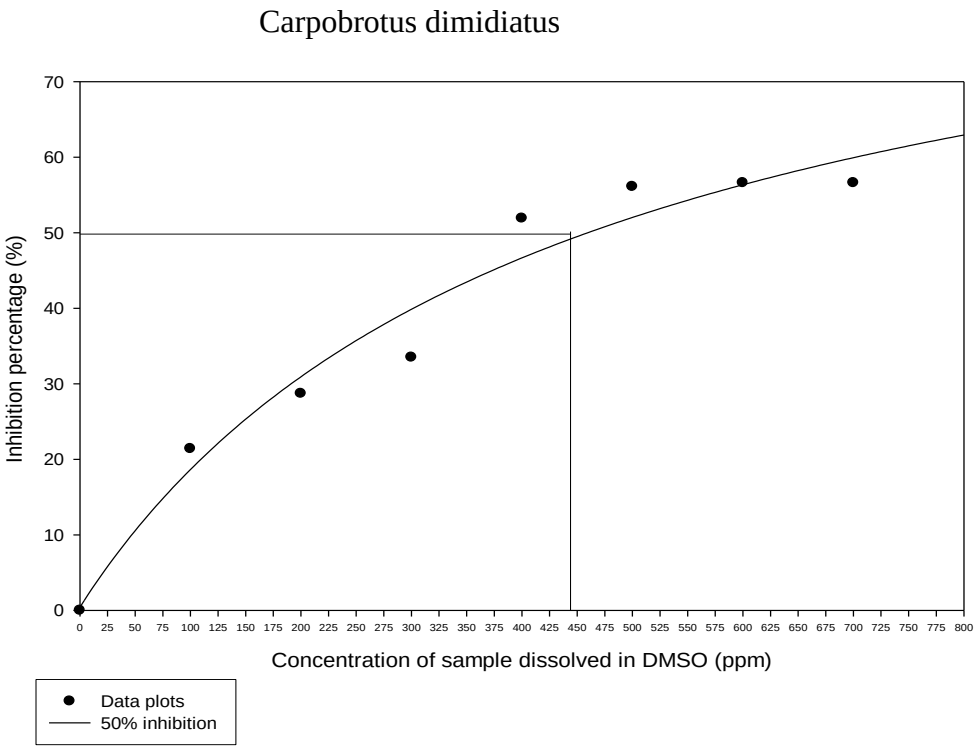


Figure 5: Anti-inflammatory (5-lipoxygenase) activity of *C.dimidiatus*

2.3. Cytotoxicity

Table 3: Percentage Cell viability after treatment with *D. cinerea, C.dimidiatus; C.tomentosa* and *L. leonurus.*

PLANT			ABS1	ABS2	ABS3	AVG	%CELL VIABILITY
C.tomentosa	Methanolic	1000 µg/ml	0.054	0.048	0.043	0.048	47
		100 µg/ml	0.061	0.099	0.090	0.083	81
		10 µg/ml	0.124	0.103	0.146	0.124	121
C.tomentosa	Aqueous	1000 µg/ml	0.044	0.049	0.037	0.043	42
		100 µg/ml	0.060	0.080	0.047	0.062	61
		10 µg/ml	0.105	0.083	0.162	0.116	112
D. cinerea	Methanolic	1000 µg/ml	0.096	0.164	0.126	0.129	125
		100 µg/ml	0.122	0.139	0.152	0.138	137
		10 µg/ml	0.122	0.130	0.126	0.126	122
D. cinerea	Aqueous	1000 µg/ml	0.156	0.199	0.115	0.157	152
		100 µg/ml	0.192	0.161	0.128	0.160	156
		10 µg/ml	0.193	0.125	0.122	0.147	142
C.dimidiatus	Methanolic	1000 µg/ml	0.150	0.166	0.146	0.154	150
		100 µg/ml	0.118	0.157	0.161	0.145	141
		10 µg/ml	0.112	0.155	0.145	0.137	133
C.dimidiatus	Aqueous	1000 µg/ml	0.111	0.143	0.130	0.127	124
		100 µg/ml	0.149	0.146	0.135	0.143	139
		10 µg/ml	0.114	0.144	0.143	0.134	130
L. leonurus Flower	Methanolic	1000 µg/ml	0.100	0.128	0.126	0.118	115
		100 µg/ml	0.178	0.131	0.117	0.142	138
		10 µg/ml	0.162	0.153	0.146	0.154	150
L. leonurus Flower	Aqueous	1000 µg/ml	0.179	0.117	0.169	0.155	150
		100 µg/ml	0.194	0.178	0.139	0.170	165
		10 µg/ml	0.117	0.127	0.119	0.12	117
L. leonurus	Methanolic	1000 µg/ml	0.198	0.118	0.138	0.151	147
		100 µg/ml	0.144	0.162	0.140	0.149	144
		10 µg/ml	0.175	0.149	0.130	0.151	147
L. leonurus	Aqueous	1000 µg/ml	0.150	0.169	0.163	0.160	156
		100 µg/ml	0.116	0.141	0.147	0.135	131
		10 µg/ml	0.104	0.102	0.130	0.112	108
CONTROL			0.107	0.114	0.089	0.103	
DMSO			0.102	0.108	0.083	0.097	

2.4. Cytokine graphs

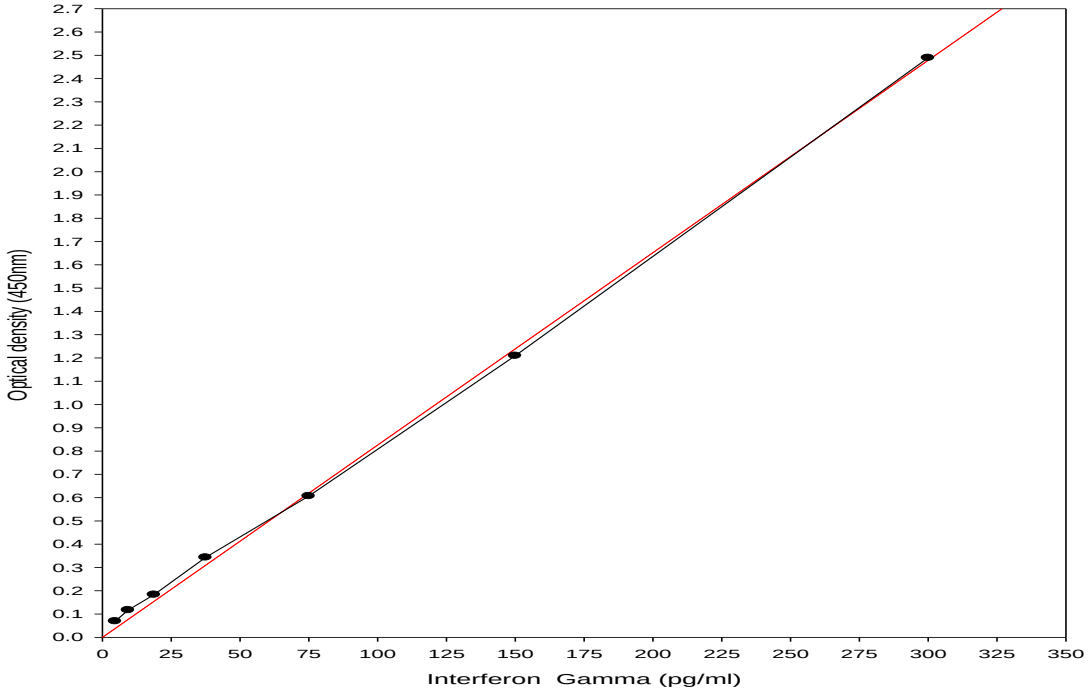


Figure 6. Standard graph of Interferon gamma (pg/ml).

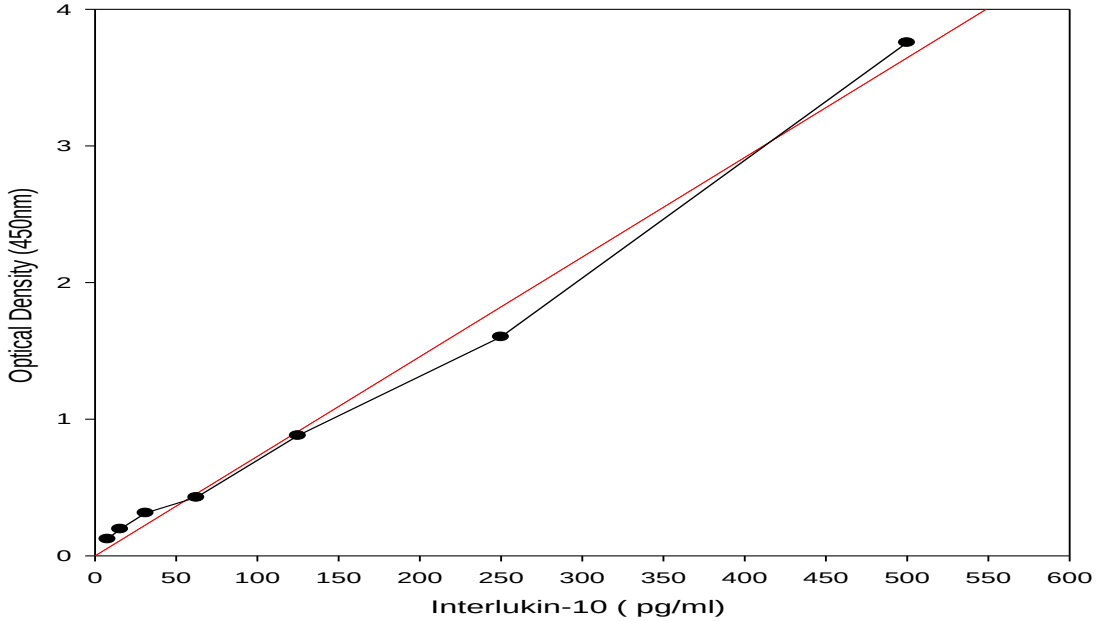


Figure 7. Standard graph of Interlukin – 10 (pg/ml).

2.5. Flow cytometry results:

Table 7. The flow cytometry results with PBMC treated with *C. dimidiatus*

***Carpobrotus dimidiatus* flow results**				
	Stimulation Index			
Lymphocyte	**1**	**2**	**Mean**	**Std dev**
Activated PBMC	0.137	0.153	**0.145**	0.011314
T cells	0.65	0.651	**0.6505**	0.000707
Activated T cells	0.161	0.167	**0.164**	0.004243
CD 4 +	0.589	0.567	**0.578**	0.015556
CD 8 +	0.272	0.28	**0.276**	0.005657
Total B cells	0.348	0.346	**0.347**	0.001414
NK cells	0.094	0.098	**0.096**	0.002828

Table 8. The flow cytometry results with PBMC treated with *C. tomentosa*

***Capparis tomentosa* flow results**				
	Stimulation Index			
Lymphocyte	**1**	**2**	**Mean**	**Std dev**
Activated PBMC	0.155	0.159	**0.157**	0.002828
T cells	0.637	0.669	**0.653**	0.022627
Activated T cells	0.194	0.183	**0.1885**	0.007778
CD 4 +	0.662	0.625	**0.6435**	0.026163
CD 8 +	0.211	0.247	**0.229**	0.025456
Total B cells	0.36	0.329	**0.3445**	0.02192
NK cells	0.126	0.125	**0.1255**	0.000707

Table 9. The flow cytometry results with PBMC treated with *D.cinerea*

***Dichrostachys cinerea* flow results**				
	Stimulation Index			
Lymphocyte	**1**	**2**	**Mean**	**Std dev**
Activated PBMC	0.138	0.129	**0.1335**	0.006364
T cells	0.585	0.534	**0.5595**	0.036062
Activated T cells	0.112	0.121	**0.1165**	0.006364
CD 4 +	0.579	0.581	**0.58**	0.001414
CD 8 +	0.253	0.331	**0.292**	0.055154
Total B cells	0.457	0.412	**0.4345**	0.03182
NK cells	0.054	0.086	**0.07**	0.022627

Table 10. The flow cytometry results with PBMC treated with *L. leonurus* leaf extracts

***Leonotis leonurus* leaf flow results**				
	Stimulation Index			
Lymphocyte	**1**	**2**	**Mean**	**Std dev**
Activated PBMC	0.131	0.14	**0.1355**	0.006364
T cells	0.68	0.687	**0.6835**	0.00495
Activated T cells	0.156	0.146	**0.151**	0.007071
CD 4 +	0.582	0.593	**0.5875**	0.007778
CD 8 +	0.28	0.277	**0.2785**	0.002121
Total B cells	0.307	0.31	**0.3085**	0.002121
NK cells	0.122	0.125	**0.1235**	0.002121

Table 11. The flow cytometry results with PBMC treated with *L.leonurus* flower extracts

***Leonotis leonurus* flower flow results**				
	Stimulation Index			
Lymphocyte	**1**	**2**	**Mean**	**Std dev**
Activated PBMC	0.138	0.124	**0.131**	0.009899
T cells	0.67	0.674	**0.672**	0.002828
Activated T cells	0.158	0.162	**0.16**	0.002828
CD 4 +	0.593	0.59	**0.5915**	0.002121
CD 8 +	0.264	0.256	**0.26**	0.005657
Total B cells	0.328	0.324	**0.326**	0.002828
NK cells	0.076	0.105	**0.0905**	0.020506

Table 12. The flow cytometry results with untreated PBMC

Cells only flow results				
	Stimulation Index			
Lymphocyte	**1**	**2**	**Mean**	**Std dev**
Activated PBMC	0.121	0.135	**0.128**	0.009899
T cells	0.621	0.614	**0.6175**	0.00495
Activated T cells	0.121	0.135	**0.128**	0.009899
CD 4 +	0.599	0.516	**0.5575**	0.05869
CD 8 +	0.216	0.218	**0.217**	0.001414
Total B cells	0.218	0.214	**0.216**	0.002828
NK cells	0.186	0.209	**0.1975**	0.016263

Table 13. The flow cytometry results with PBMC treated with PHA

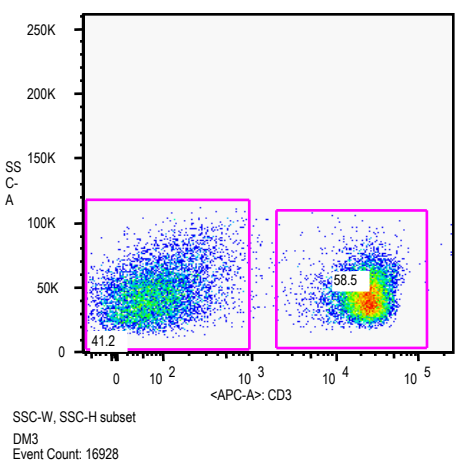
PHA flow results				
	Stimulation Index			
Lymphocyte	**1**	**2**	**Mean**	**Std dev**
Activated PBMC	0.169	0.166	**0.1675**	0.002121
T cells	0.717	0.705	**0.711**	0.008485
Activated T cells	0.266	0.258	**0.262**	0.005657
CD 4 +	0.729	0.731	**0.73**	0.001414
CD 8 +	0.262	0.265	**0.2635**	0.002121
Total B cells	0.28	0.294	**0.287**	0.009899
NK cells	0.365	0.327	**0.346**	0.02687

Table 14. The flow cytometry results with PBMC treated with DMSO

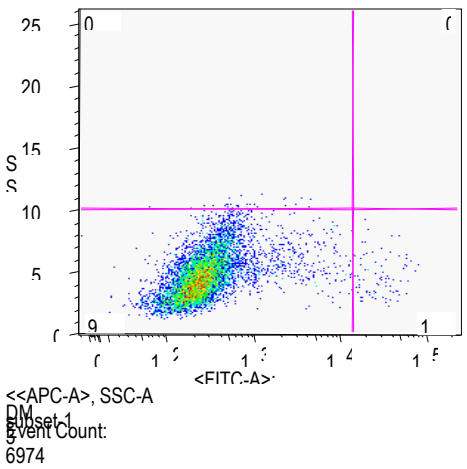
DMSO flow results				
	Stimulation Index			
Lymphocyte	**1**	**2**	**Mean**	**Std dev**
Activated PBMC	0.0978	0.106	**0.1019**	0.005798
T cells	0.632	0.618	**0.625**	0.009899
Activated T cells	0.09	0.087	**0.0885**	0.002121
CD 4 +	0.455	0.499	**0.477**	0.031113
CD 8 +	0.195	0.145	**0.17**	0.035355
Total B cells	0.238	0.235	**0.2365**	0.002121
NK cells	0.018	0.019	**0.0185**	0.000707

Appendix 3

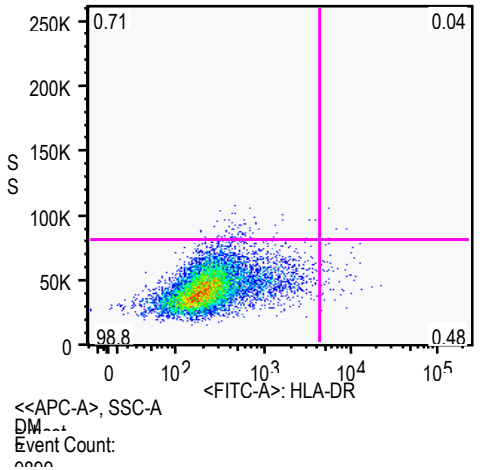
FLOW CYTOMETRY GRAPHS



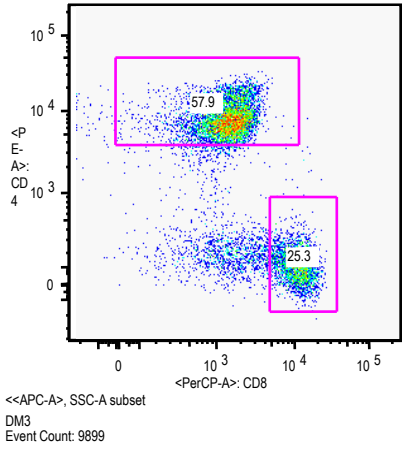
CD 3+ and HLA-DR+



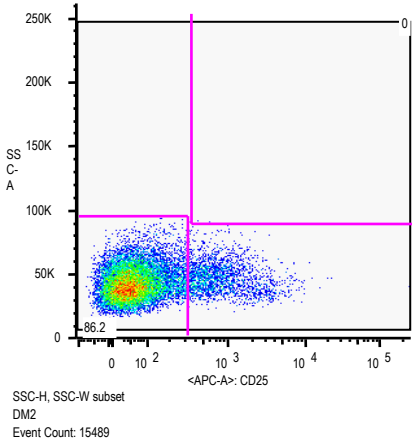
CD3- and CD3



+ CD3-, HLA-DR+

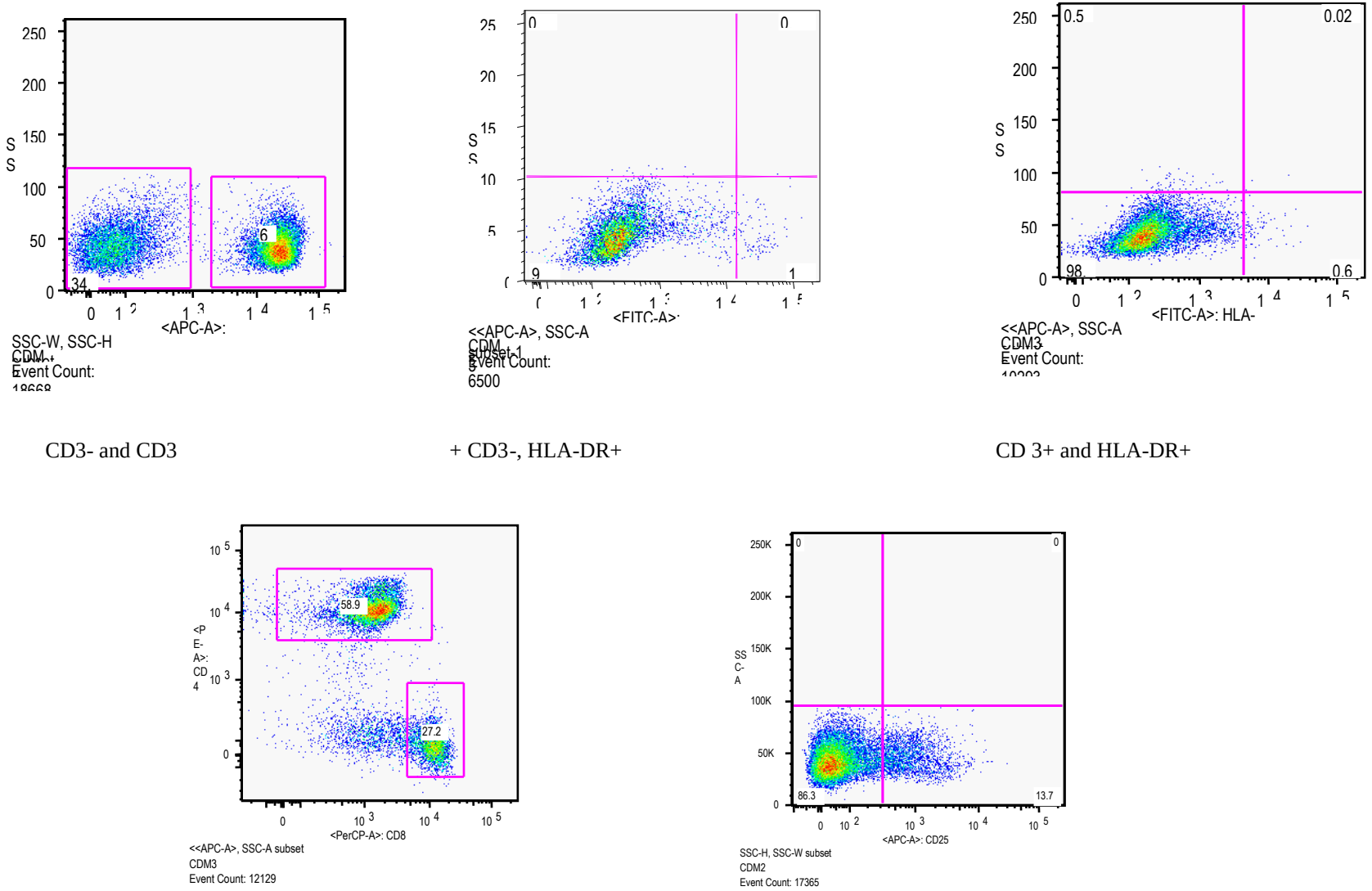


CD 25+



CD 4 +

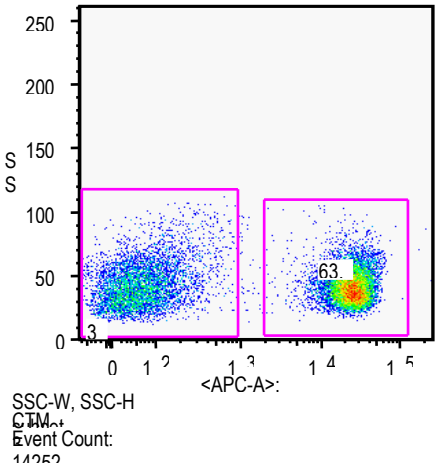
Fig 8. The flow cytometry results with PBMC treated with *D.cinerea*



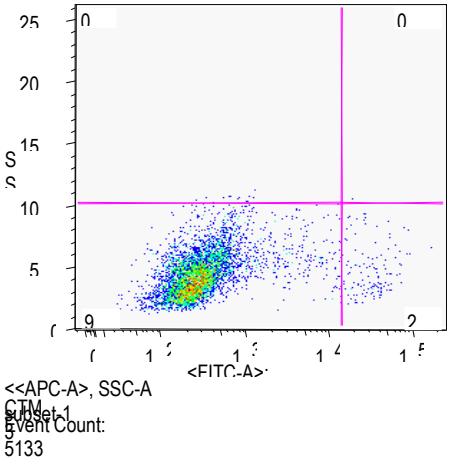
CD3- and CD3 + CD3-, HLA-DR+ CD 3+ and HLA-DR+

CD 25+ CD 4 +

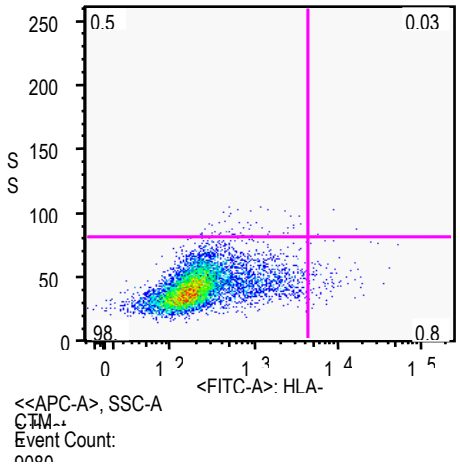
Fig 9. The flow cytometry results with PBMC treated with *C. dimidiatus*



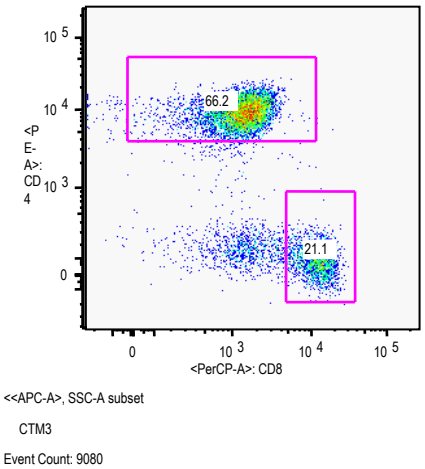
CD3- and CD3



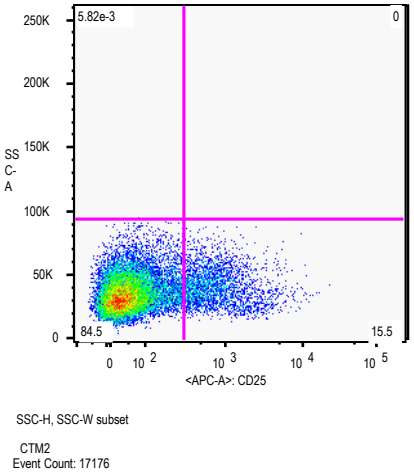
+ CD3-, HLA-DR+



CD 3+ and HLA-DR+

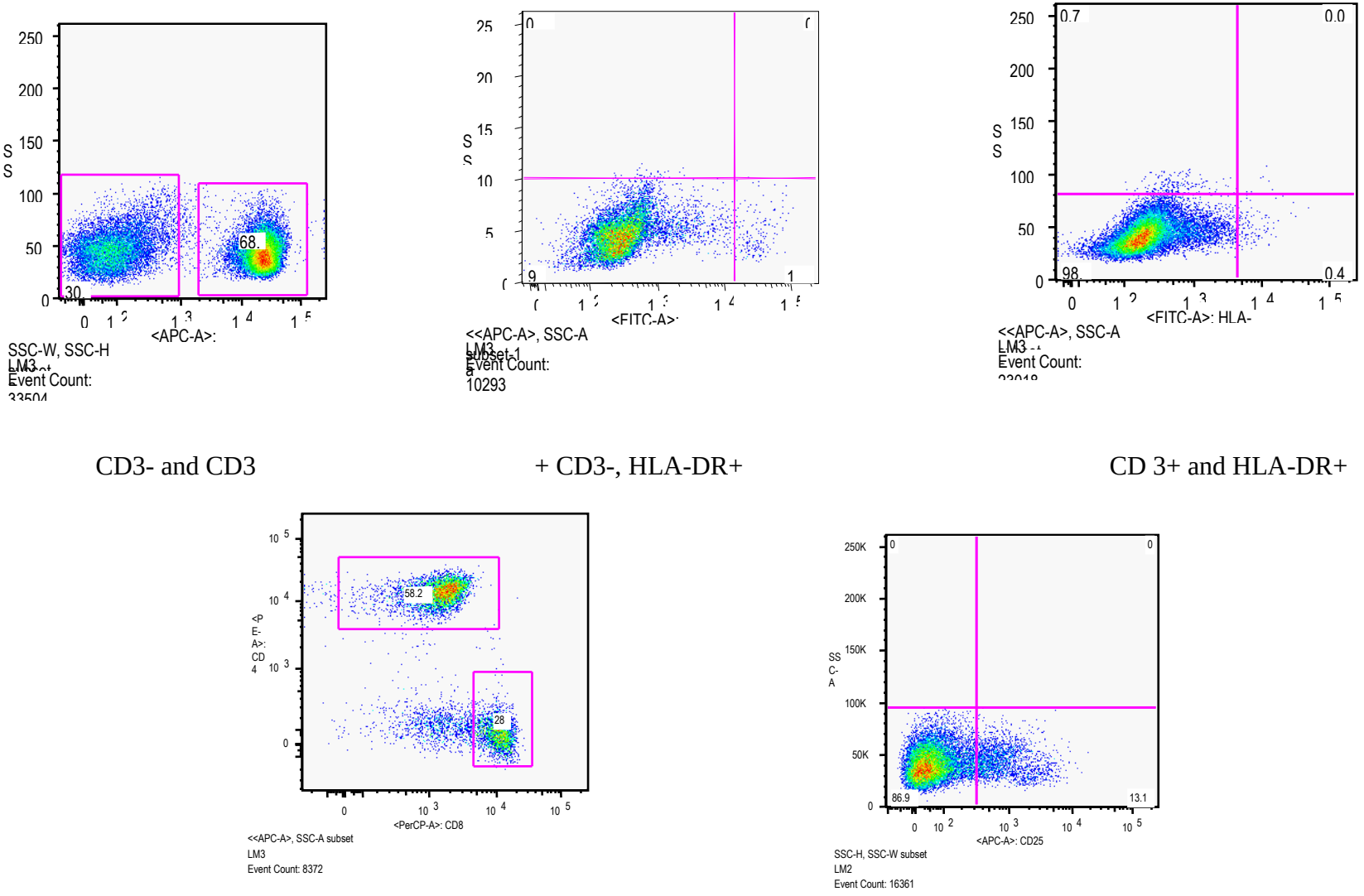


CD 4 +



CD 25+

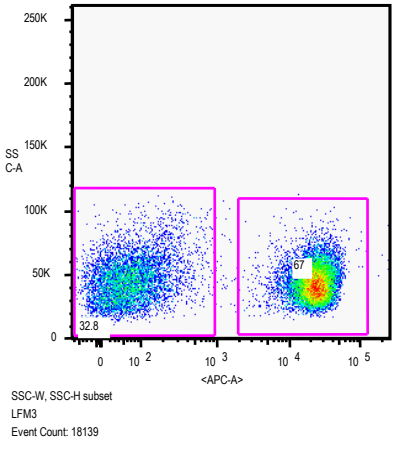
Fig 10. The flow cytometry results with PBMC treated with *C. tomentosa*



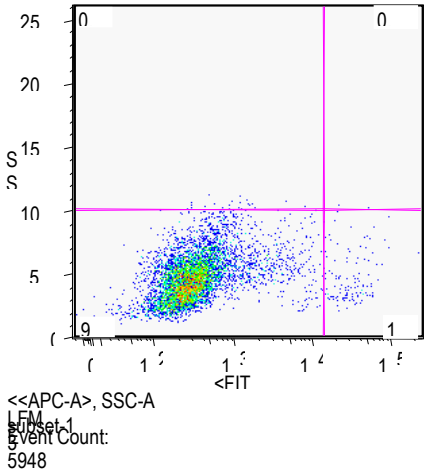
CD3- and CD3 + CD3-, HLA-DR+ CD 3+ and HLA-DR+

CD 4 + CD 25+

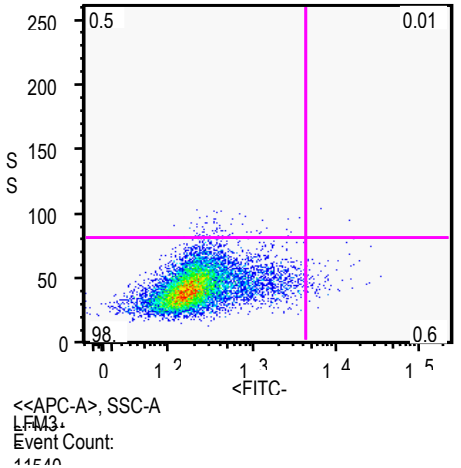
Fig 11. The flow cytometry results with PBMC treated with *L. leonurus*



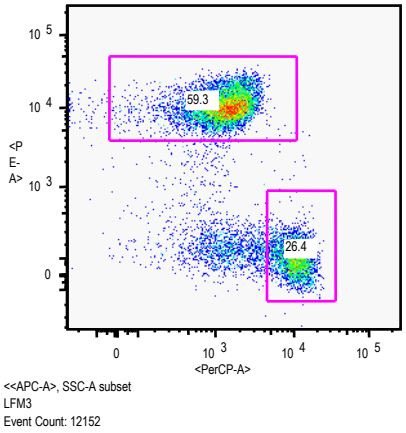
CD3- and CD3



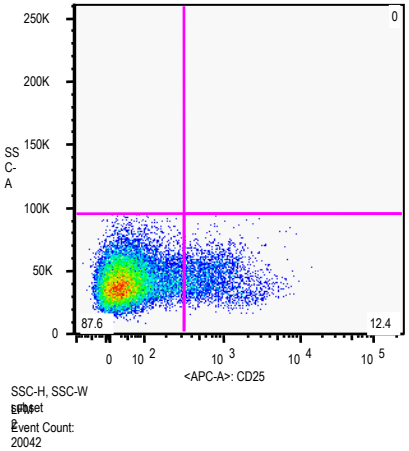
+ CD3-, HLA-DR+



CD 3+ and HLA-DR+

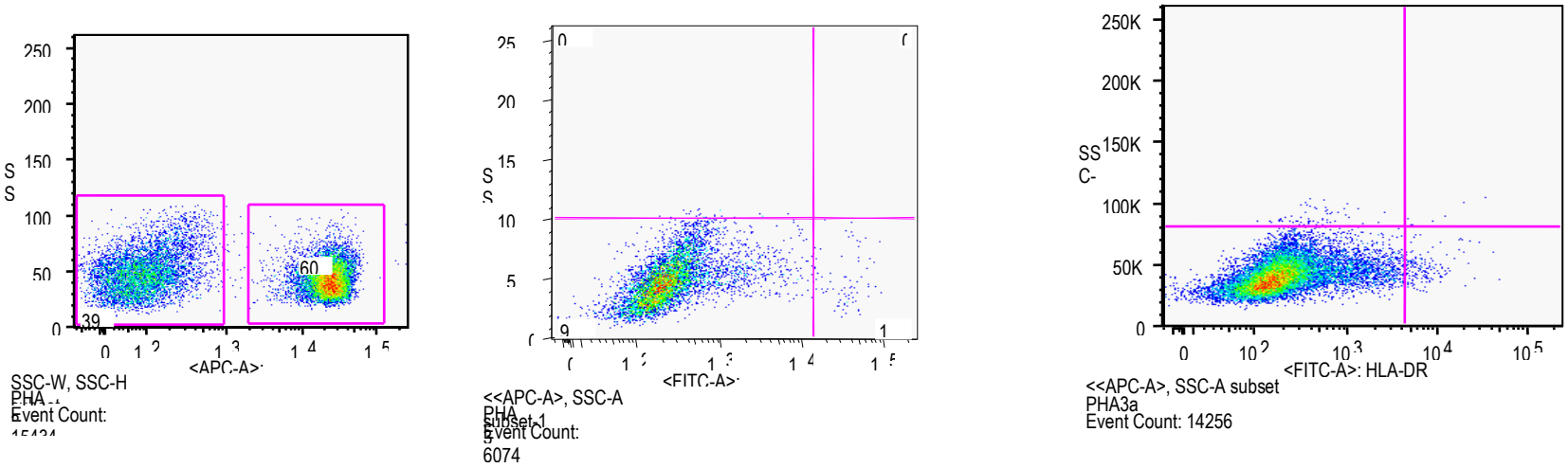


CD 4 +

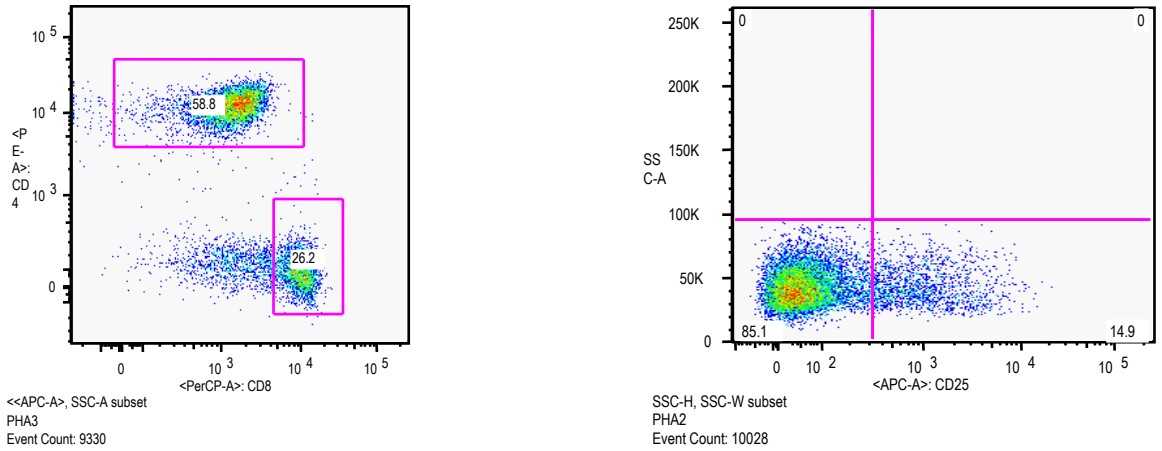


CD 25+

Fig 12. The flow cytometry results with PBMC treated with *L. leonurus*

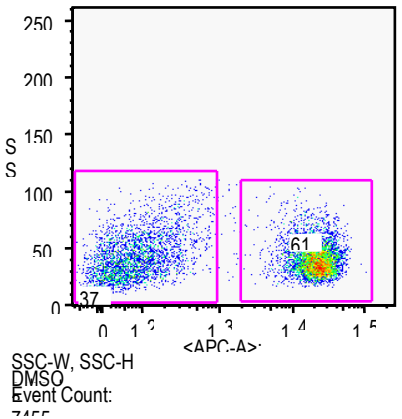


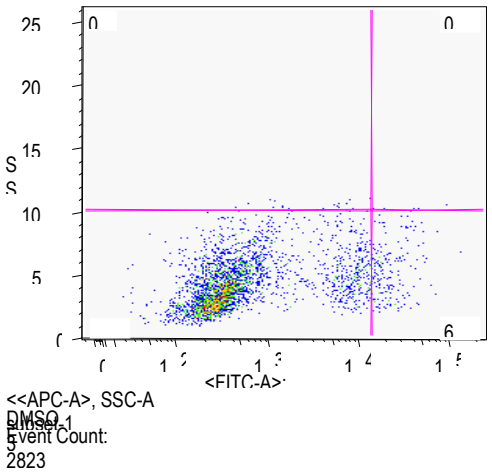
CD3- and CD3 + CD3-, HLA-DR+ CD 3+ and HLA-DR+

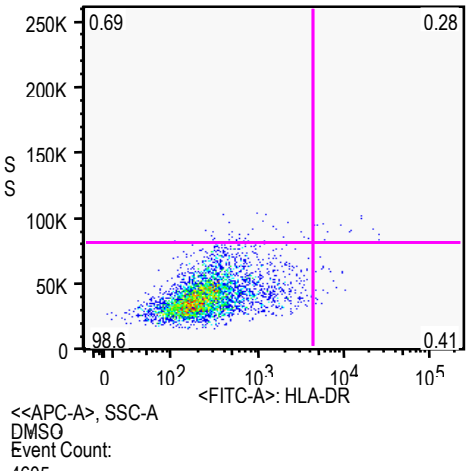


CD 4 + CD 25+

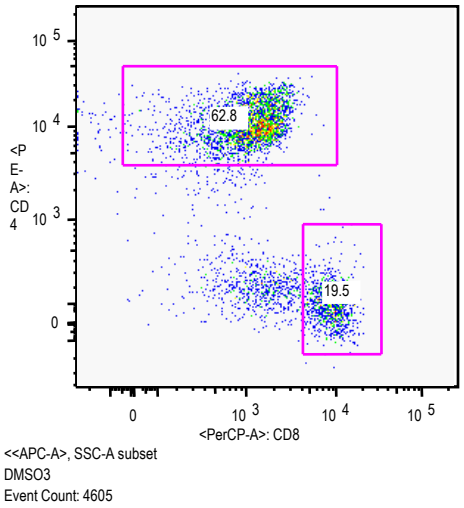
Fig 13.The flow cytometry results with PBMC treated with PHA

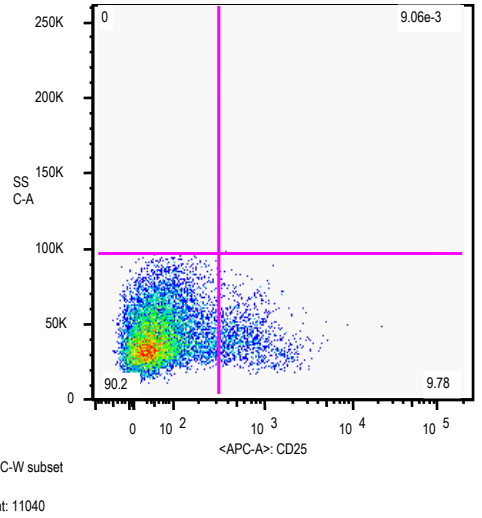






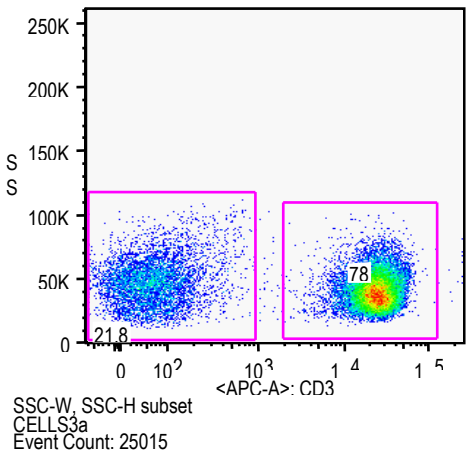
CD3- and CD3 + CD3-, HLA-DR+ CD 3+ and HLA-DR+



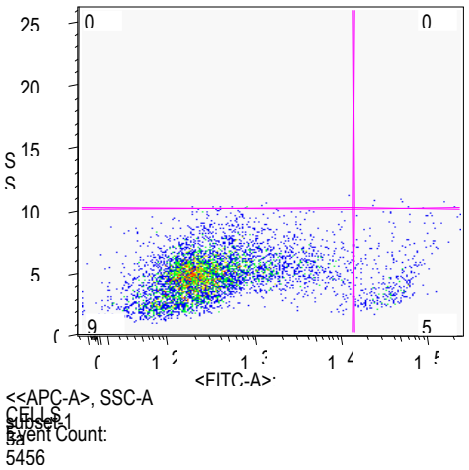


CD 4 + CD 25+

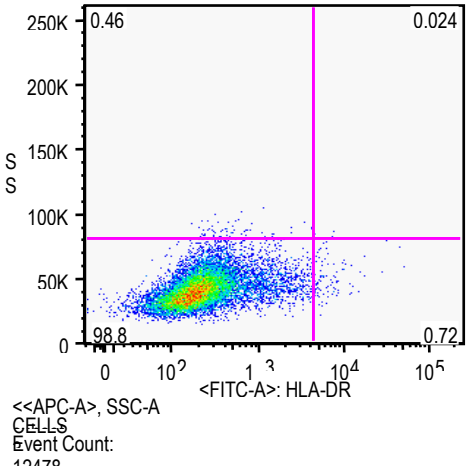
Fig 14. The flow cytometry results with PBMC treated with DMSO



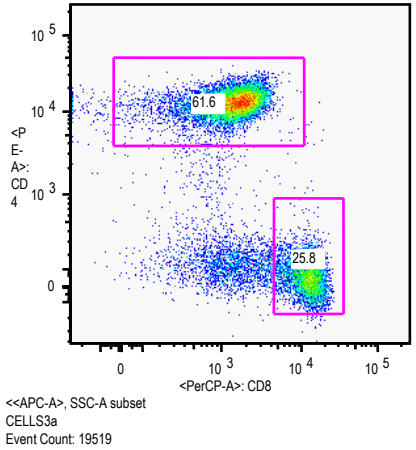
CD3- and CD3



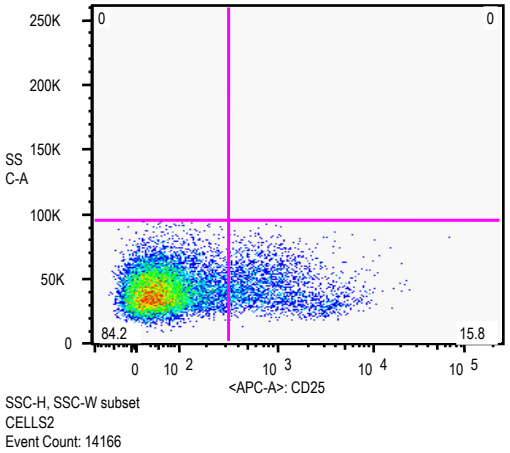
+ CD3-, HLA-DR+



CD 3+ and HLA-DR+



CD 4 +



CD 25+

Fig 15. The flow cytometry results with untreated PBMC