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An *in vitro* investigation of the immunomodulatory effects of a traditional polyherbal traditional medicine product

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Declaration

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Abstract

In South Africa, human immunodeficiency virus (HIV) remains one of the major public health concerns. HIV is a highly infectious lentivirus which causes a progressive degeneration of the immune system. When not treated, it leads to the development of acquired immunodeficiency syndrome (AIDS). AIDS is an immunosuppressive disease that results in opportunistic infections that can lead to death. Antiretroviral therapy (ART) is the most common and widely used treatment. Despite the availability of ART, HIV prevalence is on the rise. This has led to a surge in traditional herbal preparations that are purported as immune boosters for treatment in HIV patients. It is known that many patients switch from using ART to traditional herbal medicine due to side effects associated with ART. In South Africa and on the African continent, traditional medicine is not used only as an alternative and supplementary medicine, but also as a primary source of health care due to its ease of accessibility, affordability and also as it forms part of traditional and cultural practices. Safety and efficacy of many traditional medicines is not yet established and the pharmacological properties of the latter are based on anecdotal evidence with no scientific evidence. Prijap Health is a herbal traditional medicine preparation that is sold as an immune booster for immunocompromised patients. Prijap Health traditional herbal medicine is comprised of the following plants: *Acorus calamus*, *Aloe arborescens*, *Artemisia afra*, *Drimia robusta, Elephantorrhiza elephantina, Erythrina lysistemon, Persea americana, Senecio serratuloides* and *Xysmalobium undulatum.* It is claimed to have anti-viral, antioxidant, anti-inflammatory, blood cleansing and appetising properties. It is currently traded in select stores across South Africa. The aim of this study was to investigate the immune modulating properties of Prijap Health herbal concoction and the individual plant species that comprise the concoction.

Water was used as a solvent to prepare plant extracts mimicking the traditional healer's preparation technique. Phytochemical analysis was carried out using thin layer chromatography (TLC) and ultra-high-performance liquid chromatography coupled with mass spectrometry (UPLC-MS). Cytotoxicity of the extracts was evaluated by the sulphorhodamine B (SRB) protein staining assay using differentiated human acute leukaemia monocytic (THP-1) and human histiocytic lymphoma (U937) cell lines after incubation for 72 h. The free-radical scavenging activity of the extracts

was evaluated against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radicals to determine the antioxidant potential. Evaluation of prostaglandin E_2 concentration in phorbol-12-myristate 13-acetate (PMA)-differentiated and lipopolysaccharide (LPS)-activated THP-1 and U937 was conducted using enzyme-linked immunosorbent assay (ELISA). Determination of Th1/Th2/Th17 (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A) and human inflammatory (IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70) cytokines concentrations was done using BD cytometric bead array (CBA) kits.

The TLC revealed the presence of flavonoids in the extracts of *A. afra*, *E. elephantina*, *P. americana* and *S. serratuloides*. Sterols were detected in the extracts of *A. arborescens*, *E. elephantina*, *P. americana*, *X. undulatum*. Saponins were present in the extracts of *A. afra*, *A. calamus*, *D. robusta*, *E. elephantina* and *P. americana*. Alkaloids were not detected in any of the extracts. Phytochemical markers were identified in the plant extracts using UPLC-MS. These include uzarin (*X. undulatum*), β-asarone (*A. calamus*), catechin (*P. americana*), (-)-epicatechin (*E. elephantina*), rutin (*A. afra*), D-saccharic acid (*D. robusta*), 3-caffeoylquinic acid (*S. serratuloides*) and aloesin (*A. arborescens*).

From the SRB assay, it was evident that the hot water extracts of *A. afra, A. arborescens, A. calamus, E. elephantina, E. lysistemon, P. americana and S. serratuloides* had no cytotoxic effects in both cell lines at the highest concentration tested (IC_{50}>100 µg/mL). The THP-1 cells were found to be more sensitive and indicated lower cell viability than the U937 cells. The *D. robusta, X. undulatum* extracts and Prijap Health traditional herbal medicine displayed a potential for toxicity in the THP-1 cell line with IC_{50} = 39.29±1.76, 76.05±1.21 and 74.52±1.31 µg/mL, respectively.

The DPPH and ABTS radical scavenging activity was most potent for the *E. elephantina* extract (DPPH scavenging EC_{50}= 6.98±1.04 µg/mL; ABTS scavenging EC_{50}= 2.45±1.05 µg/mL) and was found to be comparable to that of Trolox, the positive control (DPPH EC_{50} = 7.39±1.04 µg/mL; ABTS EC_{50} = 1.79±1.04 µg/mL). Prijap Health traditional herbal medicine showed greater antioxidant activity than all the individual plant extracts with the exception of *E. elephantina*.

Curcumin (positive control), Prijap, *D. robusta* and *X. undulatum* extracts decreased the extracellular and intracellular PGE_2 concentration in THP-1 cells, but not in U937 cells. Cytokine concentration determination by flow cytometry revealed that curcumin (positive control), *A. calamus*, Prijap Health traditional herbal medicine, and *E. elephantina* significantly ($p<0.05$) reduced IL-1β concentration. Curcumin, *A. calamus* and Prijap significantly ($p<0.05$) reduced TNF-α concentration. *E. elephantina, A. arborescens* and *P. americana* increased IL-10 concentration whereas curcumin, *A. afra* and Prijap significantly ($p<0.05$) reduced IFN-ɣ concentration in both THP-1 and U937 cells. These findings suggest that the above-mentioned extracts and Prijap Health traditional herbal medicine have the potential to be used as anti-inflammatory agents.

In conclusion, the results of the present study provide evidence of the antioxidant, anti-inflammatory, immune modulatory potential as well as safety of use of Prijap Health traditional herbal medicine. With this said, *E. elephantina* as a single plant showed to contain better activity than Prijap Health traditional herbal medicine concoction. Further studies in drug-herb interaction and antiviral properties should be conducted to investigate all the pharmacological properties of Prijap Health traditional herbal medicine.

Table of contents

Declaration i

Acknowledgements ii

Abstract iii

Table of contents vi

List of Figures viii

List of Tables xi

List of abbreviations xiii

Chapter 1: Literature review 1

1.1. General introduction 1

1.2. The immune system 2

1.2.1. Innate immunity 2

1.2.2. Adaptive immunity 4

1.2.3. Cytokines and immunity 5

1.3. An overview of HIV immunopathology 6

1.3.1. Progression from HIV infection to AIDS 6

1.3.2. Role of cytokines in HIV pathogenesis 7

1.4. Current treatment of HIV/AIDS 8

1.4.1. Conventional drugs 8

1.4.2. Traditional medicine 10

1.4.3. Polyherbal medicine investigated 14

1.5. Aim and objectives 21

Chapter 2: Materials and methods 23

2.1. Plant material 23

2.2. Preparation of extracts 24

2.3. Phytochemical analysis 25

2.3.1. Thin layer chromatography 25

2.3.2. Ultra-performance liquid chromatography-mass spectrometry 26

2.4. Antioxidant activity 27
2.4.1. 2, 2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid radical scavenging assay 27
2.4.2. 2, 2-Diphenyl-1-picrylhydrazyl radical scavenging assay 28
2.5. Cytotoxicity determination 29
2.5.1. Cell culture and maintenance 29
2.5.2. Sulphorhodamine B assay 29
2.6. Anti-inflammatory activity 31
2.6.1. Cytokine evaluation 31
2.6.2. Prostaglandin E_2 production 32
2.7. Statistical analysis 34
Chapter 3: Results and discussion 35
3.1. Phytochemical analysis 35
3.1.1. Thin layer chromatography 35
3.1.2. Ultra-performance liquid chromatography-mass spectrometry 39
3.2. Cytotoxicity 58
3.3. Antioxidant activity 63
3.4. Anti-inflammatory activity 70
3.4.1. Prostaglandin E2 determination 71
3.4.2. Cytokine analysis 73
Chapter 4: Conclusion 88
Chapter 5: Recommendations and limitations 90
References 91
Appendix I: Ethical clearance certificate 110
Appendix II: List of reagents and preparation 111
Appendix III: Gradient profiles used in UPLC-PDA analysis 114

List of Figures

Figure 1: Cellular components which constitute the innate and adaptive immune systems. 2

Figure 2: Schematic representation of the different pathogen associated molecular patterns and pattern recognition receptors, detection by TLRs and subsequent innate immune response by an effector cell. 4

Figure 3: Progression from onset of HIV infection AIDS with reference to viral load (orange graph) and CD4+ T cell count (black graph). 7

Figure 4: A) Prijap, the traditional medicine product under study, B) label indicating the uses of Prijap, C) the plant ingredients and directions of use. 15

Figure 5*:* Plant species used in the preparation of Prijap Health traditional herbal medicine. A) *Acorus calamus;* B) *Aloe arborescens;* C) *Artemisia afra;* D) *Drimia robusta;* E) *Elephantorrhiza elephantina;* F) *Erythrina lysistemon;* G) *Persea americana;* H) *Senecio serratuloides;* I) *Xysmalobium undulatum*. 20

Figure 6: Waters gradient curve profiles. The gradient curve profiles (G) are represented by the numbers 1-11. 26

Figure 7: UPLC-ESI-MS chromatogram of the hot water extract of the roots of *A. calamus*.. 41

Figure 8: The UPLC-ESI-MS chromatogram of the hot water extract of the leaves *A. arborescens*.. 43

Figure 9: The UPLC-ESI-MS chromatogram of the hot water extract of the leaves of *A. afra* following electrospray ionization in negative mode.. 45

Figure 10: UPLC-ESI-MS chromatogram of the hot water extract of the bulb of *D. robusta* Electrospray ionization in negative mode.. 47

Figure 11: UPLC-ESI-MS chromatogram of the hot water extract of the roots of *E. elephantina*..... 49

Figure 12: UPLC-ESI-MS chromatogram of the hot water extract of *E. lysistemon*. 51

Figure 13: UPLC-ESI-MS chromatogram of the hot water extract prepared from the leaves of *P. americana*..... 53

Figure 14: UPLC-ESI-MS negative ionisation mode chromatogram of the hot water extract prepared from the leaves of *P. americana*..... 53

Figure 15: UPLC-ESI-MS chromatogram of the hot water extract of *S. serratuloides*. 55

Figure 16: UPLC-ESI-MS Electrospray ionization in positive mode chromatogram of the hot water extract of *X. undulatum*..... 57

Figure 17: Effect of plant extract on viability of THP-1 (■) and U937 (▲) cells..... 62

Figure 18: The percentage DPPH radical scavenging activity of the plant species investigated (n=9)..... 66

Figure 19: The percentage ABTS radical scavenging activity of plants investigated. 67

Figure 20: Determination of PGE_2 concentration in the presence of plant extracts..73

Figure 21: Concentration of IL-1β in differentiated THP-1 and U937 cells after stimulation with LPS in the presence of plant extracts. 76

Figure 22: Concentration of IL-2 in differentiated THP-1 and U937 cells after stimulation with LPS in the presence of plant extracts. 77

Figure 23: Concentration of IL-4 in differentiated THP-1 and U937 cells after stimulation with LPS in the presence of plant extracts. 78

Figure 24: Concentration of IL-6 in differentiated THP-1 and U937 cells after stimulation with LPS in the presence of plant extracts. 79

Figure 25: Concentration of IL-8 in differentiated THP-1 and U937 cells after stimulation with LPS in the presence of plant extracts.. 80

Figure 26: Concentration of IL-10 in differentiated THP-1 and U937 cells after stimulation with LPS in the presence of plant extracts. 81

Figure 27: Concentration of IL-12p70 in differentiated THP-1 and U937 cells after stimulation with LPS in the presence of plant extracts. 82

Figure 28: Concentration of IL-17A in differentiated THP-1 and U937 cells after stimulation with LPS in the presence of plant extracts. 83

Figure 29: Concentration of IFN-γ in differentiated THP-1 and U937 cells after stimulation with LPS in the presence of plant extracts. 84

Figure 30: Concentration of TNF-α in differentiated THP-1 and U937 cells after stimulation with LPS in the presence of plant extracts. 85

List of Tables

Table 1: Current drugs used in the treatment of patients who are human immunodeficiency syndrome virus positive as approved by the Food and Drug Administration.[49] 10

Table 2: Detection reagents employed to assess the phytochemical classes in the respective plant species. 25

Table 3: The highest non-toxic dose used for anti-inflammatory analysis. The doses were informed by the cytotoxicity results. 31

Table 4: Extract yields of the plant species under investigation. 35

Table 5: Phytochemical classes detected in hot water extracts from the plant species investigated using TLC. 37

Table 6: List of the phytochemicals identified from the hot water extract of the roots of *A. calamus* using UPLC-ESI-MS. 42

Table 7: Phytochemicals identified from the hot water extract of the leaves of *A. arborescens* by UPLC-ESI-MS. 44

Table 8: List of phytochemicals identified from the hot water extract of *A. afra* by UPLC-ESI-MS. 46

Table 9: The compound identified from the hot water extract of *D. robusta*. 47

Table 10: Compounds identified from the hot water extract of *E. elephantina* as determined by UPLC-ESI -MS analysis. 50

Table 11: A list of all the phytochemicals identified from the hot water extract of the leaves of *P. americana* by UPLC-ESI-MS. 54

Table 12: Compounds identified from the hot water extract of *S. serratuloides* as determined by UPLC-TOF-ESI-MS. 56

Table 13: A list of all the compounds identified from the hot water extract of the roots of *X. undulatum* as determined by UPLC-ESI-MS. 57

Table 14: The IC_{50} cytotoxicity concentrations (µg/mL) for THP-1 and U937 cells treated with hot water extracts of the individual plants and Prijap Health herbal traditional mixture. 63

Table 15: The EC_{50}±SEM values for plant extracts as determined by the DPPH and ABTS radical scavenging assays. 68

Table 16: Significant changes in cytokine secretion alterations. 86

List of abbreviations

A

ABTS	2, 2′-Azinobis 3-ethylbenzthiazoline-6-sulphonic acid
AIDS	Acquired immunodeficiency syndrome
ANOVA	One-way analysis of variance
ART	Antiretroviral therapy
ARVs	Antiretroviral drugs
ATCC	American type culture collection

C

C	Carbon
CBA	Cytometric bead array
CCR5	C-C chemokine receptor type 5
CD4+	Cluster of differentiation 4
cm	Centimetres
CSIR	Council for Scientific and Industrial Research
CTC_{50}	Concentration of an analyte where there's a 50% cell survival

D

DNA	Deoxy-ribonucleic acid
dH_2O	Distilled water
DPPH	2, 2-Diphenyl-1-picrylhydrazyl

E

EC_{50}	Half maximum effective concentration
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionosation

F

FBS	Foetal bovine serum
FRAP	Ferric reducing antioxidant power

G

g	Gravitational force
g	Gram/s

H

h	Hour/s
HAART	Highly active antiretroviral therapy
HILIC	Hydrophilic interaction chromatography
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus Type-1
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
HSCCC	High speed counter current chromatography

I

IC_{50}	Half maximal inhibitory concentration
IFN	Interferons
IIs	Integrase inhibitors
IKK	Ikβ kinase complex
IL	Interleukins

K

$K_2S_2O_8$	Potassium persulfate
kg	Kilogram

L

LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide

M

mDa	milliDalton
MHC	Major-histocompatibility-complex
Min	Minute/s
MTA	Material transfer agreement

mg	Milligram
mL	Milliliter
N	
NF-kβ	Nuclear factor kappa beta
NO	Nitric oxide
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside-analogue reverse transcriptase inhibitors
O	
OD	Optical density
OIs	Opportunistic infections
ORAC	oxygen radical absorption capacity
P	
PAMPs	Pathogen-associated patterns
PDA	Photodiode array
PGE2	Pro-inflammatory prostaglandins
PIs	Protease inhibitors
R	
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
S	
SANBI	South African Biodiversity Institute
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SRB	Sulphorhodamine B
T	
TCA	Trichloroacetic acid
TEAC	Trolox equivalence antioxidant capacity
Th1	T-helper 1
Th2	T-helper 2

THP	Traditional Health Practitioner
THP-1	Human monocyte cell line
TLC	Thin layer chromatography
TLRs	Toll-like receptors
TM	Traditional medicines
TNF	Tumour necrosis factor
TOF	Time-of-Flight

U

U937	Human myeloid cells
UNAIDS	Joint United Nations Programme on HIV/AIDS
UPLC	Ultra-high-performance liquid chromatography
UPLC-ESI-MS	Ultra-high-performance liquid chromatography-electrospray ionisation mass spectrometry
UPLC-PDA	Ultra-high-performance liquid chromatography-photodiode array (PDA)
µL	Microliter

V

v/v	Volume/volume percentage

W

w/v	Weight/volume percentage
WHO	World Health Organization

Chapter 1: Literature review

1.1. General introduction

Human immunodeficiency virus (HIV) belongs to the lentivirus subfamily and is one of the highly infectious pathogens which cause a progressive degeneration of the immune system.[1,2] This pathogen is responsible for the development of acquired immunodeficiency syndrome (AIDS). The AIDS can be ascribed as an immunosuppressive disease that results in variety of life-threatening opportunistic infections which can result in death.[2]

In a report published by the Joint United Nations Programme on HIV and AIDS (UNAIDS) (2000), about 36.1 million individuals worldwide were living with HIV and 3 million died from HIV/AIDS.[3] In Sub-Saharan Africa, the AIDS-related deaths were estimated at 790,000, while 1.4 million new cases of HIV infections were reported in 2014.[4] There was a decrease in the mortality from AIDS complications during 2000–2015 which is ascribed to the roll-out of antiretroviral drugs at both public and private health care hospitals to manage the progression of HIV in patients.[5] In 2016, 36.7 million individuals were living with HIV, with an estimated total of 1,8 million new HIV infections reported.[6] UNAIDS reported a total of 1,0 million AIDS-related deaths in 2016.[6]

AIDS is one of the greatest challenges facing South Africa today amongst other diseases such as tuberculosis, cancer and diabetes.[7] The National Department of Statistics reported that about 6.2 million people were living with HIV in South Africa in 2012, which is an increase from 4.02 million people reported in 2002.[7] In 2013, HIV/AIDS was ranked as the 3rd leading cause of death in South Africa, moving up from 7th place in 2011 in South Africa.[8] In 2015, approximately 531 965 deaths were recorded on the national registry and of these, 30.5% were due to HIV/AIDS. In 2017, Statistics South Africa reported that 7,06 million individuals are living with HIV, up from 6,93 million reported in 2016.[9]

Based on epidemiological studies it is evident that the incidence and prevalence of HIV infection and mortality due to AIDS complications, particularly within the South African context, is still a major health problem.

1.2. The immune system

The immune system can be described as a collection of cellular responses in an organism with the main function of combatting and eliminating foreign particles or infection. There are two types of immune responses against invading pathogens namely; the innate (non-specific) immune response and the adaptive (acquired or specific) immune response.[10]

1.2.1. Innate immunity

The innate immune system is the host's first line of defence during an infection and therefore plays a vital role in early recognition and subsequent removal of the infectious agent after triggering of an inflammatory response.[11] The main effector cells of the innate immune response are macrophages, natural killer cells, dendritic cells, mast cells and granulocytes (basophils, eosinophils and neutrophils) (Figure 1).[13]

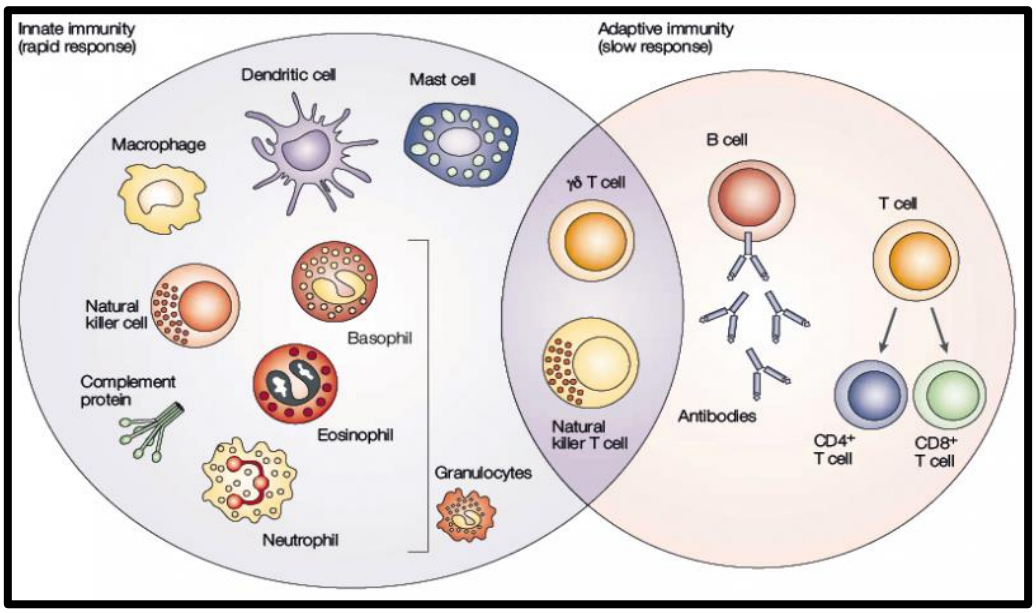


Figure 1: Cellular components which constitute the innate and adaptive immune systems.[13]

After an infection with a pathogen (such as viruses, fungi and bacteria), immune cells such as macrophages, neutrophils and dendritic cells are activated to protect the body. Pathogens are recognised by the pattern recognition receptors (PRRs) on the surface of the immune cells. The most widely studied and distinguished PRR are Toll-like receptors (TLRs) on the surface of the immune cells (Figure 2). The latter are considered the primary detectors of pathogens. The activated macrophages, through their TLRs, bind to the pathogen via its pathogen-associated patterns (PAMPs).[14]

The PAMPs can be described as molecular motifs which are evolutionary conserved within a particular class of microorganisms, and are typically crucial for the organism's survival. These can be either the flagellin, peptidoglycan, lipoteichoic acid (Gram-positive bacteria), lipopolysaccharide (LPS) (Gram-negative bacteria), unmethylated DNA, lipophosphoglycan (protozoa), β-glucans (fungi), single/double stranded nucleic acids and glycoproteins (viruses).[12] Binding of the macrophages to the PAMPs via the TLRs activates the Ikβ kinase complex (IKK complex) in the cytoplasm that in turn activates the nuclear factor kβ (NF-kβ) transcriptional pathway. The transcribed NF-kβ is then translocated from the cytoplasm into the nucleus where it initiates the production and secretion of cytokines, chemokines, nitric oxide (NO) and reactive oxygen species (ROS)[14] (Figure 2).

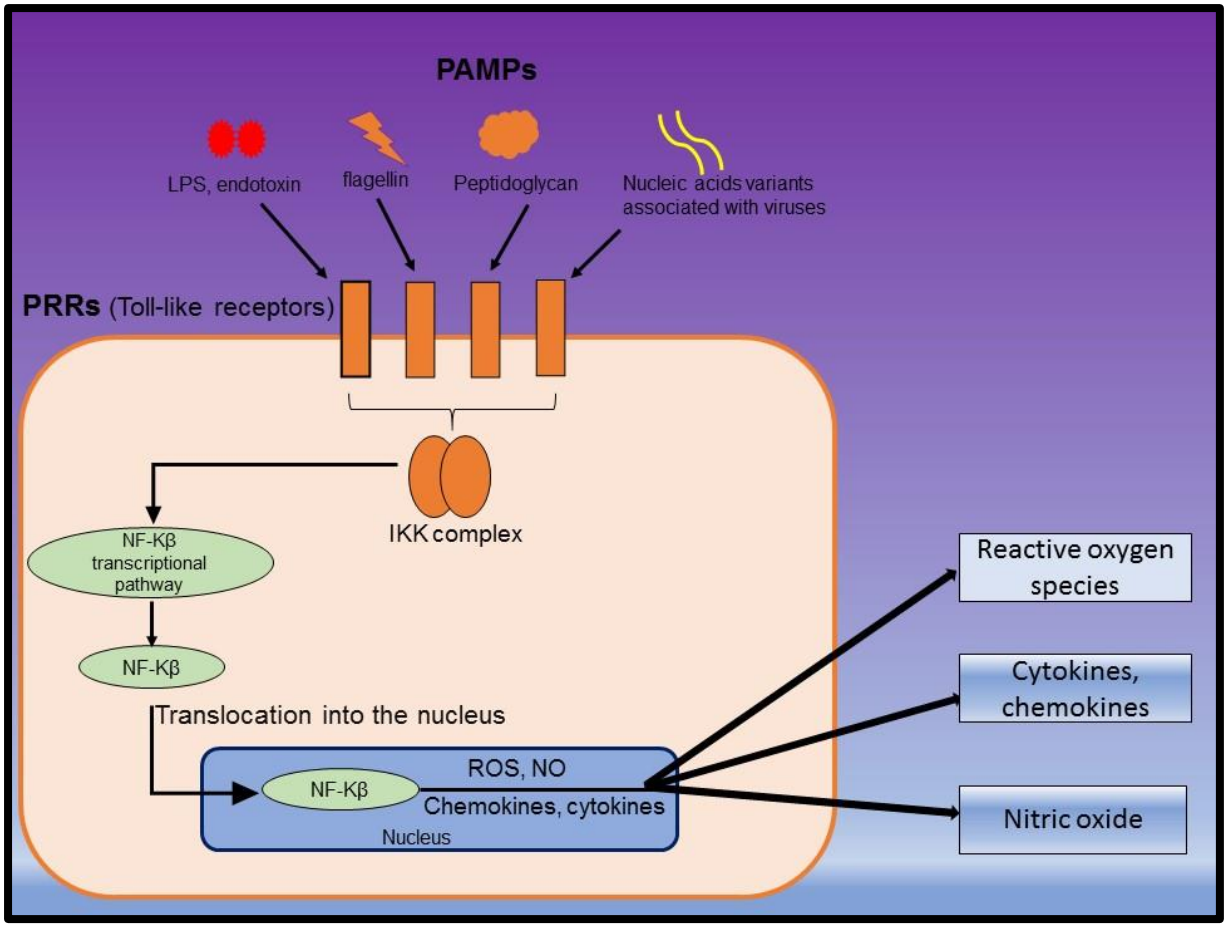


Figure 2: Schematic representation of the different pathogen associated molecular patterns and pattern recognition receptors, detection by TLRs and subsequent innate immune response by an effector cell.

Upon binding to the pathogen and subsequent release of pro-inflammatory cytokines, the effector cells of the innate immunity engulf the pathogen (antigen) by phagocytosis. Phagocytosis is carried out by three types of PRR-expressing cells: macrophages, neutrophils and dendritic cells, commonly referred to as phagocytes. The engulfed antigen by macrophages and dendritic cells is “presented” and recognized by helper T cells of the adaptive immune response. The helper T cells signal B cells to produce antibodies against that specific antigen to initiate an antigen-mediated removal of the antibody.[15]

1.2.2. Adaptive immunity

The immune system is comprised of various cells that communicate to rid the body of harmful stimuli. Adaptive immunity consists of antigen-specific reactions through T and B lymphocytes (Figure 1). Cells of the adaptive immune response include T and B

cells (Figure 1). The T cells contain specialized receptors that recognize fragments of antigens on the surfaces of infected or cancerous cells.[13] Macrophages present pathogens as antigens to T lymphocytes.[16] T lymphocytes are then activated and signal B lymphocytes to produce antibodies specific to the pathogen presented.[17] The antibodies then eliminate the pathogen (antigen) from the body.[16,17] Initially, the adaptive response is slow to take effect (can take several days or weeks). The adaptive response retains memory of the pathogen/stimuli in question, so that later exposure to the same pathogen leads to a stronger and more rapid response, although this is not immediate.[10] The T cells contribute to immune defence by directing and regulating immune responses whereas others (such as phagocytes and macrophages) directly attack cells infected with the pathogen.[10]

Furthermore, the T cells are differentiated into different subsets which include helper T cells (Th), cytotoxic T cells, memory T cells, suppressor T cells and natural killer T cells.[18] Helper T cells are involved in coordinating the immune responses by communicating and stimulating nearby B cells to produce antibodies against foreign particles, while other T cells recruit phagocytes.[19] Natural killer T cells are a lethal kind of white blood cells, or lymphocyte. These cells are armed with granules filled with potent chemicals. Natural killer cells recognize cells lacking self-major-histocompatibility-complex (MHC) molecules. They bind to, lyse and destroy foreign particles. Natural killer T cells have the potential to attack many types of foreign cells.[17] The B cells act by secreting antibodies into the body's fluids upon stimulation by helper T cells. Antibodies are responsible for attacking and binding to antigens circulating in the bloodstream.[16]

1.2.3. Cytokines and immunity

Cytokines are small proteins with a molecular weight of about 40 kDa. They are produced by a variety of cells in human body including B cells, helper T cells, macrophages, mast cells, neutrophils, basophils and eosinophils.[20] Cytokines play an important role in the activation and differentiation of the immune response by mediating communication between the different immune cells against the pathogen.[21]

There are five families of cytokines: the interleukins (IL), which are communicators between white blood cells;[22] interferons (IFN), which protect cells against viral infections;[21,23] tumour necrosis factor (TNF), which initiate inflammatory response of autoimmune diseases;[24–26] chemokines, which induce the migration of leukocytes into the area of infection or tissue injury;[21] and hematopoietic cytokines, which control the pathway of stem cell differentiation into red and white blood cells.[21]

Approximately 18 cytokines known to belong to the IL family. The cytokines which promote inflammation are referred to as pro-inflammatory cytokines whereas cytokines that suppress the activity of pro-inflammatory cytokines are known as anti-inflammatory cytokines. Pro-inflammatory cytokines include IL-1β, IL-2, IL-6, IL-8 and IL-12p70 and anti-inflammatory cytokines, IL-4 and IL-10.[20] Cytokines play an important role in controlling the homeostasis of the immune system.

1.3. An overview of HIV immunopathology

1.3.1. Progression from HIV infection to AIDS

When HIV type 1 enters the host organism, it infects some of the immune system cells, such as monocytes, macrophages, CD4+ T lymphocytes, dendritic cells, and macrophages.[12,22,27] Primary infection with HIV is also known as the asymptomatic phase. This is because the majority of infected people maintain normal health and/or are unaware that they have contracted the infection and the symptoms are not yet pronounced as the body's immune system tries to control the infection.[28,29] The peripheral blood CD4+ T-lymphocyte cell is above 800 cells/mm^3 at this point. This phase occurs in the first 0-3 weeks following infection (Figure 3). The second stage is acute HIV infection which is marked by increased viral load, declining CD4+ T lymphocyte cell count (less than 800 cells/mm^3) and when early symptoms start to manifest (fever, unexplained weight loss, recurrent diarrhoea, fatigue and headache).[30] This phase occurs 3-9 weeks following infection. The third stage is known as clinical latency which occurs a year following infection with HIV.[23] This stage is characterized by a significant decrease in CD4+ T cell count (less than 400 cell/mm^3), significant decrease in viral load (above 500 HIV copies/mL) and is

associated with a high risk of contracting AIDS related opportunistic infections.[22,31] After 7 years of infection, the HIV-related symptoms start to occur and manifest, this is coupled by a further decline in CD4+ cells (below 200 cell/mm^3) and an exponential increase in viral load. This is followed by a further decrease in CD4+ cells and an increase in HIV viral load and eventually death after 11 years.

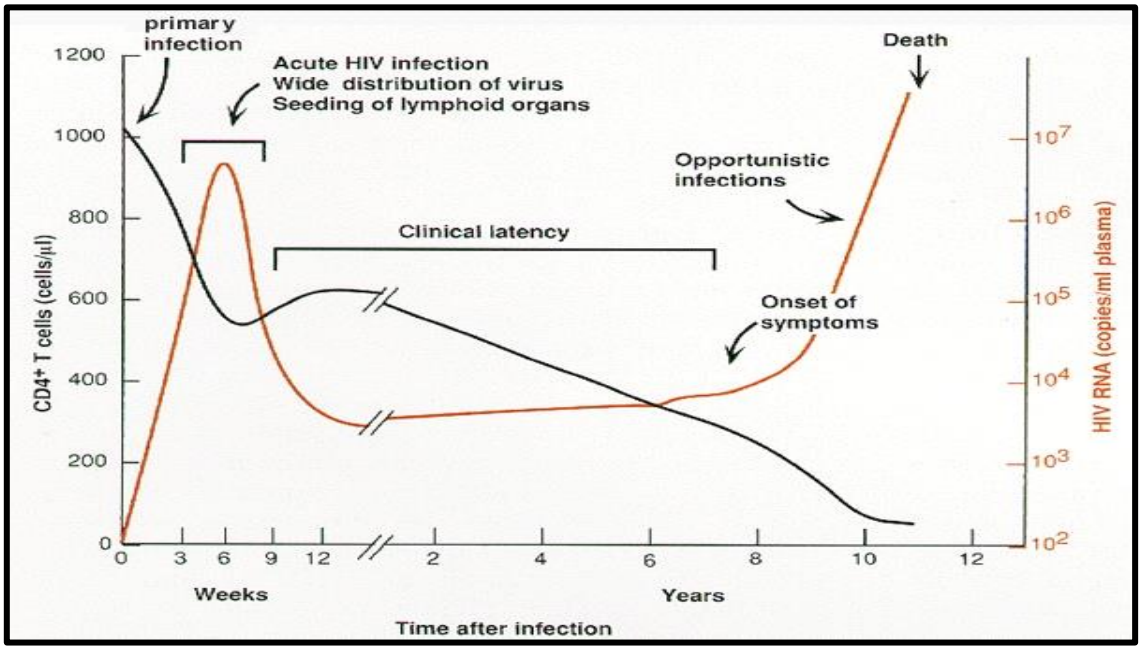


Figure 3: Progression from onset of HIV infection AIDS with reference to viral load (orange graph) and CD4+ T cell count (black graph)[1]

1.3.2. Role of cytokines in HIV pathogenesis

Infection of cells in the immune system with HIV has been shown to result in the dysregulation of the cytokine profile. Cytokine dysregulation is a major immune-pathogenic factor in HIV infection.[32] During HIV infection, there is a decrease in the secretion of T-helper 1 (Th1) cytokines, such as interleukin (IL)-2 and interferon (IFN)-γ, which are important in controlling intracellular infection, whilst the production of T-helper 2 (Th2) cytokines, which are essential for controlling extracellular infection is increased.[33]

As there is a decreased Th1 response and an increased Th2 response, cytokine imbalance develops. The Th2 cytokines inhibit the macrophages responsible for mediating killing of microbes. Consequently, this leads to failure of macrophage activation and killing of HIV.[33]

The pro-inflammatory cytokines, which are produced by monocytes and macrophages, play an important role in the activation process of neutrophils, monocytes and macrophages.[34,35] Therefore, production of appropriate amounts of TNF-α, IL-1 and IL-6 is important in response to infection.[36–38] The increase in the production of these cytokines, in particular TNF-α, has been indicated in acute and inflammatory conditions, such as in trauma, sepsis, infection and rheumatoid arthritis.[39] The increase in the pro-inflammatory cytokines following cell infection causes a dysfunction in the immune system's response to pathogens.[33,39,40]

TNF-α plays an important role in the pathogenesis of HIV infection and the associated complications, particularly in enhancing the viral replication and mediating apoptosis of CD4+ T cells.[41,42] Furthermore, TNF-α induces HIV transcription in both macrophages and T lymphocytes through the NF-Kβ pathway.[43,44] IL-1 increases HIV replication in pro-monocytic cell lines by enhancing TNF-α-mediated induction of NF-Kβ.[24,45]

1.4. Current treatment of HIV/AIDS

There is currently no treatment that cures HIV and AIDS but rather treatments that slow the progression of HIV into AIDS.

1.4.1. Conventional drugs

The most noteworthy advancement in HIV treatment or management has been the use of antiretroviral drugs (ARVs).[1] These drugs are classified into five distinct classes based on of their mode of action:

i. Protease inhibitors (PIs): This class of drugs inhibit the protease enzyme in the final stage of HIV replication which results in the formation of non-infective viral particles.[46–48]
ii. Integrase inhibitors (IIs): Prevent the transfer or integration of viral DNA strands into the chromosomal DNA of the host organism.[46–48]

iii. Nucleoside-analogue reverse transcriptase inhibitors (NRTIs): This class of drugs prevent transcription of viral RNA to a cDNA.[46–48]
iv. Non-nucleoside reverse transcriptase inhibitors (NNRTIs): Work by altering the conformation of the catalytic site of reverse transcriptase and directly inhibits the catalytic action of the reverse transcriptase enzyme. Thus, inhibiting formation of cDNA from the RNA strand.
v. Co-receptor antagonist/Fusion inhibitors: Bind to the viral glycoprotein 41 or120 or the host cell's CD4+ receptor or chemokine (CCR5) co-receptors and inhibit binding of the HIV to the host cell. This ultimately inhibits entry of HIV into the host cells.[46–48]

In recent years, the treatment and management of HIV/AIDS has been based on highly active antiretroviral therapy (HAART), which can be accessed by HIV and AIDS patients on prescription from the clinics. The HAART consists of at least three FDA-approved ARV drugs with different mechanisms of action.[49,50] HAART has been shown to significantly reduce viral replication and remarkably increase CD4+ T lymphocyte cell count.[51]

The most commonly used antiretroviral agents in HAART are NRTIs, PIs and fusion inhibitors in South Africa.[50] Despite the beneficial action of HAART, the drugs used in the therapy have been reported to be associated with detrimental side effects. These side effects include but are not limited to fatigue or tiredness, rashes, headaches, rectal bleeding, painful joints, chest pains, lack of appetite and muscle aches.[48,51–54]

To date, 24 drugs have been approved by the Food and Drug Administration for the treatment of HIV (Table 1).[49]

Table 1: Current drugs used in the treatment of patients who are human immunodeficiency syndrome virus positive as approved by the Food and Drug Administration[49]

Classes of ARV drugs	
Protease inhibitors (PIs)	**Approval date**
Atazanavir	June 2003
Ritonavir	March 1996
Tipranavir	June 2005
Saquinavir	December 1995
Nelfinavir	March 1997
Darunavir	June 2006
Indinavir	March 1996
Fosamprenavir	October 2003
Integrase Inhibitors	
Elvitegravir	September 2014
Dolutegravir	August 2013
Raltegravir	October 2007
Fusion Inhibitors	
Enfuvirtide	March 2003
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	
Tenofovir disoproxil fumarate	October 2001
Zidovudine	March 1987
Lamivudine	November 1995
Stavudine	June 1994
Emtricitabine	July 2003
Didanosine	October 1991
Abacavir	December 1998
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	
Efavirenz	September 1998
Nevirapine	June 1996
Delavirdine	April 1997
Rilpivirine	May 2011
Etravirine	January 2008

1.4.2. Traditional medicine

The use of plants for the treatment of diseases is as old as mankind.[55] Medicinal plants form part of the primary healthcare system in South Africa and other developing countries. About 80% of people from developing countries rely on traditional medicine

(TM) for treatment of diseases including HIV and AIDS.[56] It is estimated that between 60% and 80% of the population consult a Traditional Health Practitioner (THP) for treatment.[57,58] Reasons for use of TMs include easy accessibility, affordability and cultural beliefs.[57]

A third of drugs on the market originated from plants, such as taxol (paclitaxel), vincristine, quinine, atropine, cocaine, morphine, to name a few.[59] Medicinal plant research has gained an increased interest worldwide due to the potential for discovery of novel drugs.

Phytochemicals are biologically active, naturally occurring chemical compounds present in plants.[60–62] They are divided into primary and secondary metabolites. Primary metabolites are the compounds that are involved in the fundamental metabolic pathways by the plant itself for growth and development. This includes components such as sugars, proteins, amino acids, purines and pyrimidines of nucleic acids and chlorophyll.[63,64] Secondary metabolites are compounds that perform non-essential functions in plants.[65] They provide protection and defence against pathogens, ultraviolet (UV) exposure, predators, pollution and also help the plants preserve their aroma, colour and flavour.[64]

Phytochemicals have therapeutic effects in humans and animals. They may stimulate the immune system, have anti-carcinogenic properties, protect from oxidative stress by scavenging free radicals, prevent deoxyribonucleic acid (DNA) damage, have antimicrobial effect and have anti-cancer properties, to name a few.[60] Phytochemicals are chemotaxonomically classified into classes: alkaloids, terpenes, flavonoids, tannins, steroids, saponins, phenolics, coumarins, anthraquinones and glucosides.[66]

Flavonoids are polyphenolic compounds characterized by a heterocyclic benzo-γ-pyrone structure.[67] They consist of two aromatic C6 rings connected to the heterocyclic ring that contains one oxygen atom. Flavonoids are synthesized by the phenylpropanoid pathway with phenylalanine (amino acid) as the initial component of the pathway.[67,68] Flavonoids are chemically and structurally diverse depending on the degree of hydroxylation, other substitutions and conjugations, and degree of polymerization, which has led to further subcategorization of these compounds. These

are flavones, flavanones, flavonols, isoflavones, catechins, chalcones and anthocyanidins.[69] Flavonoids have been reported to possess antioxidant and anti-inflammatory activity. Antioxidants protect cells against the damaging effects of reactive oxygen species (ROS) and oxidative stress. An imbalance between antioxidants and ROS leads to oxidative stress.[70]

Saponins are high molecular weight glycosides which comprise of a nonpolar polycyclic aglycone ring connected to one or more polar sugars.[71,72] The aglycone, also referred to as sapogenin, is present as either a steroid or a triterpene depending on the number of carbon atoms in the ring. It is considered a steroid if it has 27 carbon atoms (C27) and a triterpenoid if it is a 37 carbon (C37) aglycone ring.[73,74] The aglycone part is hydrophobic (fat-soluble) whereas the sugar part is hydrophilic (water-soluble).[75] They have been shown to possess anti-cancer, anti-inflammatory, immune-stimulating, antioxidant and hypocholesterolaemic properties.[76–78] Industrial applications of saponins include their use in fire extinguishers, detergents, cosmetics, as food additives and as ingredients in photographic emulsions.[78] In pharmaceutical industries, saponins are sought after for use as starting material in the process of the production of steroid hormones.[79]

Terpenoids are secondary metabolites with molecular structures which contain carbon backbones made up of isoprene (2-methylbuta-1, 3-diene) units.[80] Isoprene contains five carbon atoms and as a result, the number of carbon atoms in any terpenoid is in multiples of five. The terpenoids consists of two isoprene units, i.e. ten carbon atoms.[81] The classification of terpenoids is based on the number of carbons and isoprene units. These are monoterpenes [10 carbons (C10) and 2 isoprene rings], sesquiterpenes (C15), diterpenoids (C20), triterpenoids (C30), tetraterpenoids (C40) and polymeric terpenoids (C>40).[81] Terpenoids have shown significant pharmacological activities, such as anti-viral, anti-bacterial, anti-malarial, anti-inflammatory, inhibition of cholesterol synthesis and anti-cancer (such as taxol) activities.[82–84]

Phytosterols are present in plants as free glycosides and alcohols.[85] Sterols contain between 27-30 carbons in their long chains with a side chain at a carbon positioned at 17 (C-17). Sterols differ from cholesterol in that they have an alkyl group attached at C-24.[86,87] The most common phytosterols include sitosterol, camphesterol and

stigmasterol. In plants, sterols play a defensive role against various types of pathogens.[85] They also serve as precursors for hormones involved in signal transduction.[88,89] Sterols have been shown to have a wide variety of beneficial health effects in humans such as cholesterol lowering ability,[90,91] and anti-inflammatory effects.[92,93]

Phenolic acids are plant secondary metabolites with an aromatic phenol ring bearing one or more hydroxyl groups. Phenolic acids are divided into two subcategories: the hydroxycinnamic acids and hydroxybenzoic acids. Hydroxycinnamic acids are phenolic acids with a 3-carbon sidechain [(C-6-C-3)] whereas hydroxybenzoic acids have 1-carbon sidechain.[94] The most common phenolic acids are gallic, caffeic, vanillic and coumaric acids. Various activities have been ascribed to phenolic acids, including; antioxidant, anti-inflammatory, anti-diabetic, anti-allergenic, cardioprotective and vasodilating effects.[95–98]

Alkaloids are chemical compounds which consist of a nitrogen (N) base and a heterocyclic ring. They are synthesized from amino acids such as phenylalanine, tyrosine and tryptophan as building blocks. Various radicals replace one or more hydrogen atoms in the peptide ring.[99] Alkaloids have a low molecular weight and make up about 20% of plant based secondary metabolites. The presence of one or more nitrogen molecules contributes to the basicity of the alkaloids.[100] The extent of the basicity differs greatly amongst alkaloids depending on the structure of the molecule, as well as the presence (number of N atoms) and location of the functional groups. Some alkaloids exist in solid state whereas some are liquids. Alkaloids have and still play an important role in human health and lifestyle as narcotics, poisons, analgesics, antibiotics, and stimulants. Alkaloids of therapeutic importance of plant origin include addictive stimulants such as cocaine, nicotine and morphine,[101,102] anticancer agents (vinblastine), antibiotics (sanguinafine) and sedatives (scopolamine).[101,102]

Plants commonly used in the formulation of a traditional medicine for oral application are assumed not toxic. This is based on their long usage in the treatment of ailments according to knowledge gathered over centuries. In addition, the non-toxic effect of traditional medicine is based on the fact that none of the patients or users of the medicines report any side effects after treatment (Semenya and Potgieter 2014;

Mabona and van Vuuren 2013). However, recent scientific research investigations have shown that some plants used in traditional medicines can be potentially toxic, mutagenic and carcinogenic (Semenya & Potgieter 2014; Nair 2010; Verschaeve & Van Staden 2008).

1.4.3. Polyherbal medicine investigated

Prijap Health traditional herbal medicine is a TM product used in South Africa to treat HIV (Figure 4A). According to the THP, the product is claimed to have anti-inflammatory, anti-viral, detoxifying and appetite, immune and energy boosting properties (Figure 4B). However, these claims are not supported by scientific evidence. The THP has obtained a patent in South Africa based on the method of preparing Prijap Health traditional herbal medicine product.

Prijap Health traditional herbal medicine is a mixture of leaves, roots and bulbs of the nine plant species, namely *Acorus calamus* L., *Aloe arborescens* Mill., *Artemisia. afra* Jacq. ex Willd. var., *Elephantorrhiza elephantina* (Burch.) Skeels, *Erythrina lysistemon* Hutch., *Drimia robusta* Baker, *Persea americana* Mill., *Senecio serratuloides* DC and *Xysmalobium undulatum* (L.) Aiton f. var. *ensifolium* Burch. ex Scott-Elliot (Figure 4). The product is being traded commercially by the THP in retail shops in South Africa.

Figure 4: A) Prijap Health traditional herbal medicine under study, B) label indicating the uses of Prijap Health traditional herbal medicine, C) the plant ingredients and directions of use

The plant species that Prijap Health traditional herbal medicine product consists of are described individually:

i) *Acorus calamus* L.

Acorus calamus is commonly known as 'Sweet flag' and belongs to the family Araceae.[103] It is found in some parts of South Africa such as North West and KwaZulu-Natal provinces but can also be found in Northern America and the South East Asia.[103,104] It is a reed-like, semi-aquatic plant with a stout aromatic rhizome (Figure 5A).[103] The flowers, which are not clearly visible, are compactly arranged on a long, fleshy axis, surrounded by a large leaf-like spathe. All parts of the plant have a strong, but not unattractive smell.[103]

The rhizomes are used to treat ailments such as stomach aches, dysentery, toothache, gastrointestinal complains and respiratory ailments.[103] It is also used as a carminative, stimulant and aphrodisiac.[103,105]

ii) *Aloe arborescens* Mill.

Aloe arborescens (Asphodelaceae) is a grey, green-leaf, rose-shaped succulent shrub with pale thorns arranged along the margin of the leaves.[106] During the winter season, a yellow to red flower spike emerges from the centre of the plant (Figure 5B). The plant species is largely distributed along the Cape peninsula, Eastern Coastal region through to Kwa-Zulu Natal, Mpumalanga and Limpopo provinces.[106] *A. arborescens*, which is indigenous to South Africa, is commonly known as the kranz aloe. Its leaf decoction is given to women to ease childbirth. Furthermore, *A. arborescens* is commercially cultivated in Japan as a remedy to treat burns and wounds.[106]

iii) *Artemisia afra* Jacq. ex Willd. var. *afra*

Artemisia afra is a small to medium-sized multi-stemmed and highly aromatic perennial plant that grows up to two metres in height (Figure 5C).[103] The plant species belongs to the family Asteraceae and has various common names; African wormwood (English), Lengana or Mhlanyane (Southern Sotho), Alsem (Afrikaans) and Umhlonyane (isiXhosa).[103,107,108]

The leaves of the plant are finely divided and feathery.[103] The alternately arranged greyish/silver to green leaves grow up to 80 mm long and 40 mm wide and are oval in shape.[103,107] The flowers are pale yellow and borne along branch ends (Figure 5C).[103] The bisexual flower heads are pale yellow and occur in an elongated panicle. *A. afra* is indigenous to South Africa, with distribution extending from the Cederberg mountains in the Western Cape to Limpopo province. It is also found in tropical east Africa as far up into North Africa as Ethiopia.[103,108]

It is known for its ability to remove worms, hence its common name wormwood.[107] The leaves are boiled to make a tea which is used to treat coughs, colds, convulsion, diabetes, heart problems, rheumatism, gout, malaria, intestinal worms and bladder and kidney problems.[103] For inflammation and fever, the leaves are made into a poultice with brandy or vinegar and wrapped around the affected area. The roots are also used to treat colds and fever The leaves can be inserted into the nostrils to clear a blocked nose.[103]

iv) *Drimia robusta* Baker

Drimia robusta (Hyacinthaceae) has a large green purplish brown bulb that sprouts in spring time with succulent strap shaped leaves and an erect or spreading stalk that has purple, green or white raceme crowded flowers (Figure 5D). The plant species is largely distributed in the North Eastern regions of South Africa, which includes Limpopo, Mpumalanga, KwaZulu-Natal and Eastern Cape provinces.[103]

The plant species is commonly known as brandui in Afrikaans and indonga-zibomvana in isiZulu.[109] It is widely used to treat broken bones, as an enema, for blood purification, to cleanse the bladder uterus, as an anti-inflammatory, anti-hypertensive and for treating dropsy.[109–112]

v) *Elephantorrhiza elephantina* (Burch.) Skeels

Elephantorrhiza elephantina is commonly known as Elephant-root in English or Intolwane in isiZulu or isiXhosa. It is an underground tree, arising from a very large underground tuberous root.[103] The visible portion of the plant has a stem of up to 60 cm long. The leaves occur in increasing number of pinnae high on the stem (Figure 5E). A creamy yellow catkin-like cluster of flowers emanate from the ground which turn brown as they mature.[103] This plant belongs to the family Fabaceae.

The plant species is found in most parts of the southern region of Africa such as Angola, Botswana, Namibia, Mozambique, South Africa and Zimbabwe.[103,113] In South Africa, the plant species grows in all provinces with the exception of the Western Cape province.[103]

E. elephantina is used to treat intestinal disorders, dysentery and diarrhoea and to stop bleeding. It is also reported to be used as treatment for heart problems and skin diseases.[103,114]

vi) *Erythrina lysistemon* Hutch.

Erythrina lysistemon is a widely used plant species in various areas of South Africa and belongs to the family Fabaceae. It is commonly known as the coral tree (English), umsinsi (isiZulu), mokhungwane (Sesotho), gewone koraalboom (Afrikaans), nsisimbane (isiTsonga), muvhale (Venda) and umsintsi (isiXhosa). The plant species is a deciduous, stocky, and small to medium-sized tree that grows up to ten meters in height.[103] The branches are thick and thorny.

The thorns are small, hooked and sprout from the branches and leaf stalks. The dark, smooth bark is a grey to grey-brown in colour. The flowers are bright red and grouped in elongated clusters on long thick stalks which carry trifoliate leaves (Figure 5F).[103] In South Africa, *E. lysistemon* grows in the North West, Limpopo, Gauteng, Mpumalanga, KwaZulu-Natal and Eastern Cape provinces.[103]

A leaf infusion of the plant species is used in the form of drops to treat earache.[103] The bark and leaves when applied topically are used in the treatment of wounds, sores, abscesses, arthritis and pain (toothache).[103,115]

vii) *Persea americana* Mill.

Persea americana, commonly known as the avocado tree, is an exotic plant cultivated in South Africa. It is native to Mexico and Central America.[116] The tree yields shiny evergreen elliptic leaves with a leathery texture (Figure 5G).[116] The fruit berry that is produced varies in colour from green, black, purple to reddish when mature. This plant belongs to the family Lauraceae.[116]

In South Africa this plant is sought after for its fruit which is believed to have aphrodisiac and emmenagogue properties. In West Africa, the leaves are used as a diuretic, to treat diabetes, hypertension and as an antitussive in West Africa.[116]

viii) *Senecio serratuloides* DC

Senecio serratuloides, also known as the two-day plant (English), insukumbili, umaphozisa, ichazampukane, umkutelo (Zulu),[103,117] is indigenous plant to South Africa[118,119] and belongs to the family Asteraceae. It is an herbaceous perennial plant which has upright stems sprouting from the rootstock and grows up to 60 cm in height.[118] The flowers are small, yellow and occur in sparse clusters near branch-ends. This plant usually grows in damp or marshy areas (Figure 5H).[103] *Senecio serratuloides* is found in the Northern and Eastern parts of South Africa, occurring in Limpopo, Gauteng, Mpumalanga, Eastern Cape and some parts of the North West province.[103]

The powdered leaves are used to treat burns, cuts, sores, swollen gums and chest pains.[120] It is sometimes used in combination with *Lippia javanica*, *Eucalyotus grandis* or *Psidium gujava* to treat the diseases mentioned.[119] A tea made from the leaves is taken to treat infection or leaves are applied directly to sores to discharge pus.[117] It is also used to treat skin disorders[118,121] or in combination with *Senecio deltoids* or *Ranunculus multifidus* to treat infertility, to ease labour pains, pelvic pains and to cleanse blood in pregnant women.[122]

ix) *Xysmalobium undulatum* (L.) Aiton f. var. *ensifolium* Burch. ex Scott-Elliot

Xysmalobium undulatum, commonly known as uzara (English), is an annual geophyte (plant with an underground storage organ) that sprouts in spring from the rootstocks.[103] It is characterized by thick and hairy erect branches with roughly heart shaped leaves (Figure 5I). During October to December, creamy green to yellowish brown flowers appear. The tips of the flowers are recurved and having short stout hairs. The roots of the plant are brown and fleshy and when damaged produce a white latex.[103] Uzara is found in the Eastern parts of South Africa, Angola, Malawi, Tanzania and Zambia. In South Africa, it is mainly concentrated in the grassland biome.[103] This plant belongs to the family Apocynaceae.

In South Africa, the Zulu people use it as an emetic in cases of poisoning, the Xhosas use it for treating hysteria, while the Nama people chew on the root to calm the

stomach and treat diarrhoea.[125] A powder formulation of this plant also has been used to treat dysentery, colic, intestinal problems[137,138] and topically to treat abscesses.[125]

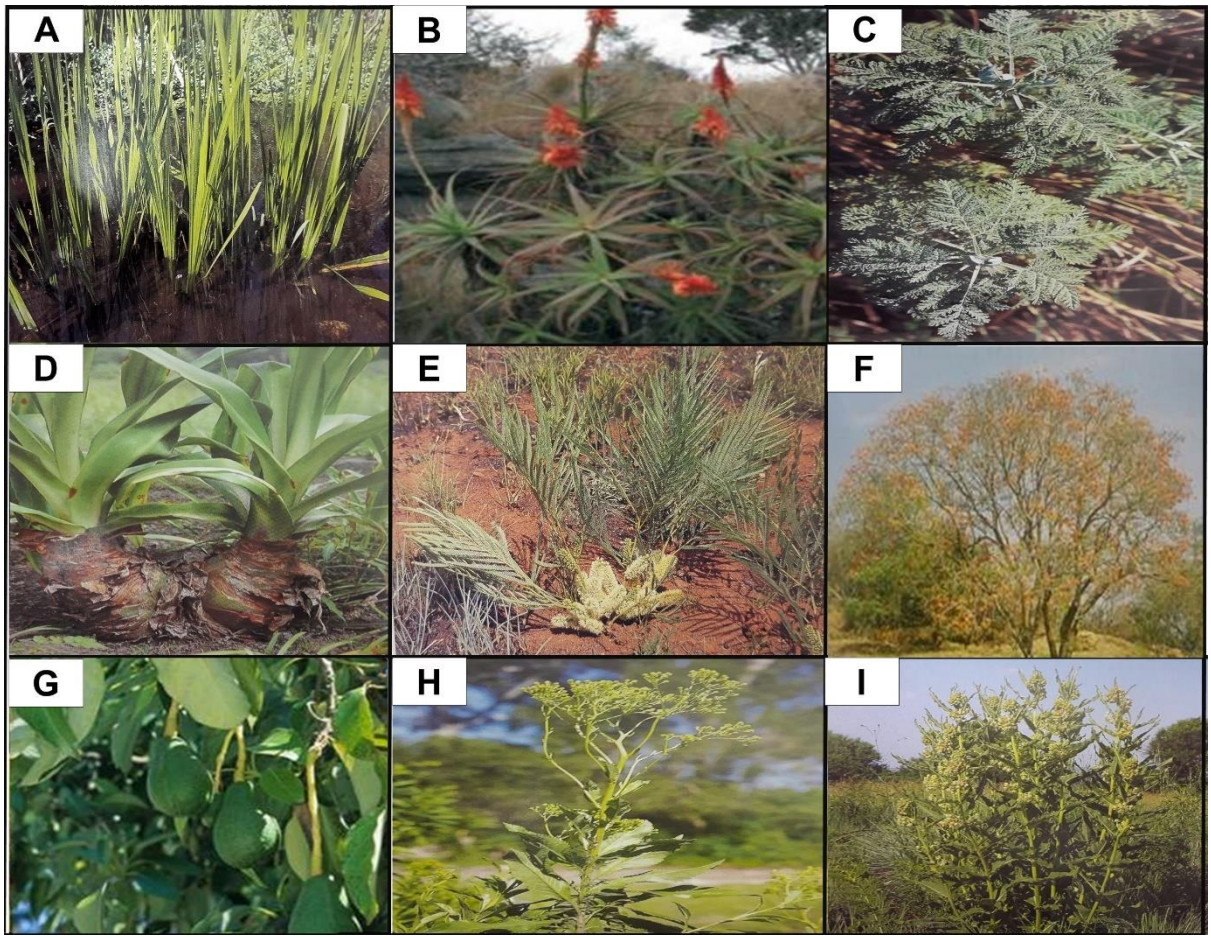


Figure 5*:* Plant species used in the preparation of Prijap Health traditional herbal medicine. A) *Acorus calamus;* B) *Aloe arborescens;* C) *Artemisia afra;* D) *Drimia robusta;* E) *Elephantorrhiza elephantina;* F) *Erythrina lysistemon;* G) *Persea americana;* H) *Senecio serratuloides;* I) *Xysmalobium undulatum*

1.5. Aim and objectives

The aim of this study was to determine the immunomodulatory effects of a polyherbal TM, Prijap Health traditional herbal medicine and its individual plant species ingredients.

The objectives of this study were as follows:

- Studies have demonstrated that medicinal plants contain phytochemicals that produce definite physiological actions in the body. Phytochemicals have been reported to have great potency as anti-inflammatory, antioxidant, antiviral, antidiabetic and anticancer agents. In order to identify the phytochemicals present in each plant species that comprises the mixture, TLC and UPLC/MS were carried out to achieve this objective.
- As stated earlier in this thesis (please refer to Chapter 1), oxidative stress contributes towards the destruction of the immune cells and the depletion of antioxidant status in the AIDS stage of HIV infection as a result of the increased level of reactive oxygen species (ROS). This study therefore assessed the potential of the extracts to act as antioxidants. The antioxidant capacity of the crude hot water extracts of the individual plants and of the polyherbal mixture were determined by the 2,2′-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays.
- With regards to the mechanism of action of immunomodulation by the extracts, the THP-1 and U937 macrophage cell lines were used to assess:
    - Cytokine secretion (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF-α and IFN-γ) in differentiated macrophages using flow Cytometric Bead Array (CBA) kits.
    - The pro-inflammatory prostaglandin E_2 (PGE_2) production using enzyme-linked immunosorbent assay (ELISA) kit.
- For many of traditional herbal remedies in use, very little is known about their active and/or toxic constituents. Herbal medicines are not subjected to the same regulatory standards as pharmaceutical drugs in terms of efficacy and safety. This raises concern on their safety and implications for their use as medicines. Toxicity testing can reveal some of the risks that may be associated with the use of herbal remedies, therefore avoiding potential harmful effects when used

as medicine. This study therefore tested for cytotoxicity of the individual plant extracts and polyherbal mixture for cytotoxicity in THP-1 and U937 cell lines. The SRB assay was used to determine the toxicity of the extracts.

Chapter 2: Materials and methods

Ethics approval to carry out this study was obtained from the Ethics committee of the Faculty of Health Sciences, University of Pretoria (Appendix I).

A list of all the reagents and their preparation is provided in Appendix II.

Access to traditional knowledge

The study was informed by the traditional knowledge and the associated polyherbal traditional medicine product which the Traditional Health Practitioner (THP) uses to treat patients with HIV and AIDS. Access to traditional knowledge of the Traditional Health Practitioner was obtained under the legal framework which comprised the Non-Disclosure Agreement (NDA) and Material Transfer Agreement (MTA), to protect the confidential information of the Traditional Health Practitioner. The legal aspects are aligned with the South African National Environmental Management, Biodiversity Act, 2004 and Bioprospecting, Access and Benefit Sharing Regulations, 2008.

South African National Environmental Management, Biodiversity Act, 2004 and Bioprospecting, Access and Benefit Sharing Regulations, 2008 requires that any research institution that conducts research on South African indigenous plant species and associated traditional knowledge, must issue a notification to the Department of the Environmental Affairs informing the Minister about the project. A notification in terms of the Bioprospecting, Access and Benefit Sharing Regulations, 2008 was subsequently processed to the National Department of Environmental to notify the Minister. The acknowledgement letter was subsequently received.

2.1. Plant material

The plant species investigated in this study were harvested by the THP from the wild and supplied to the Council for Scientific and Industrial Research (CSIR) under the MTA. This agreement, which is part of the framework of the Bioprospecting, Access and Benefit Sharing Regulations of 2008, stipulates the conditions and terms for the utilisation of the plant species throughout the research process. The plants were

submitted to the South African Biodiversity Institute (SANBI) in Pretoria to confirm the botanical identity and voucher specimens are deposited at SANBI.

2.2. Preparation of extracts

Fresh plant materials were spread evenly on a tray and oven-dried at 50ºC for 3-7 days. Thereafter, the plant materials were ground to a fine powder and stored in airtight containers to avoid moisture absorption and microbial contamination. To prepare aqueous extracts, 60 g of dried plant material was placed in 600 mL of boiling water for 3 h. The hot water extracts were filtered through a clean cloth and the filtrates were stored in the freezer at -80°C until freeze drying. The samples were freeze-dried (LABCONCO, Freezone 6) and stored at -80°C until use.

For the plant combination, a freshly prepared concoction of Prijap Health was supplied to the CSIR by Mr Prince Msomi, the THP who formulated and patented the polyherbal remedy. The polyherbal mixture was partitioned into smaller sample bottles and also stored at -20ºC until further processing.
The percentage yield was determined gravimetrically:

$$\%\text{Yield} = \frac{\text{weight of extract recovered (g)}}{\text{weight of initial plant material (g)}} \text{X } 100\%$$

To prepare stock solutions of 1 mg/mL of plant extracts, 1 mg of each plant extract was weighed and dissolved in 1 mL of water (for phytochemistry), DMSO (for antioxidant analysis) or cell culture media (for cytotoxicity & anti-inflammatory analysis) and sonicated for 15 min. The stock solutions were stored in the freezer until further use.

2.3. Phytochemical analysis

2.3.1. Thin layer chromatography

Thin layer chromatography (TLC) was carried out to identify major phytochemical classes within the crude aqueous extracts according to the method described by Stahl.[125] In brief, about 10 µL of extract was spotted onto the 5 cm × 10 cm TLC glass plates coated with silica gel 60 F_{254} (Merck, Darmstadt, Germany). To ensure that the extracts had dried, plates were placed in an oven (50°C) for 5 min. Plates were developed using various mobile phases: acetonitrile:water (3:1), acetonitrile:water (2:1) or acetonitrile:methanol:water (8:1:1). Plates were allowed to dry after development and visualized under ultraviolet (UV) light (254 nm and 366 nm).

To determine the presence of specific phytochemical classes, specific reagents were used (Table 2).

Table 2: Detection reagents employed to assess the phytochemical classes in the respective plant species.

Phytochemical class	Detection solution	Colour change indication
Flavonoids	3% Sodium nitrate:1% Aluminium chloride	Dark spot/yellow
Saponins	1% Vanillin: Sulphuric acid	Pink
Terpenoids	1% Vanillin: Sulphuric acid	Blue / violet / purple
Sterols	85% Phosphoric acid: Water	Blue / yellow spot
Phenolic acids	Ferric chloride	Red
Alkaloids	Dragendorff reagent	Yellow

2.3.2. Ultra-performance liquid chromatography-mass spectrometry

2.3.2.1. UPLC analysis

A Waters UPLC coupled in tandem to a Waters photodiode array (PDA) detector and a SYNAPT G1 HDMS mass spectrometry was used to generate accurate mass data. Optimisation of the chromatographic separation for each plant was done utilizing a Waters HSS T3 C18 UPLC column (150 mm x 2.1 mm, 1.8µm) and the column temperature was controlled at 60°C. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (pH 2.3) and acetonitrile (Eluent B) containing 10 mM formic acid. The individual analytical method for each plant extract is as shown in appendix III. Each plant extract was analysed using an automated system gradient profile (Figure 6) to promote optimum separation of the compounds present within the extract.

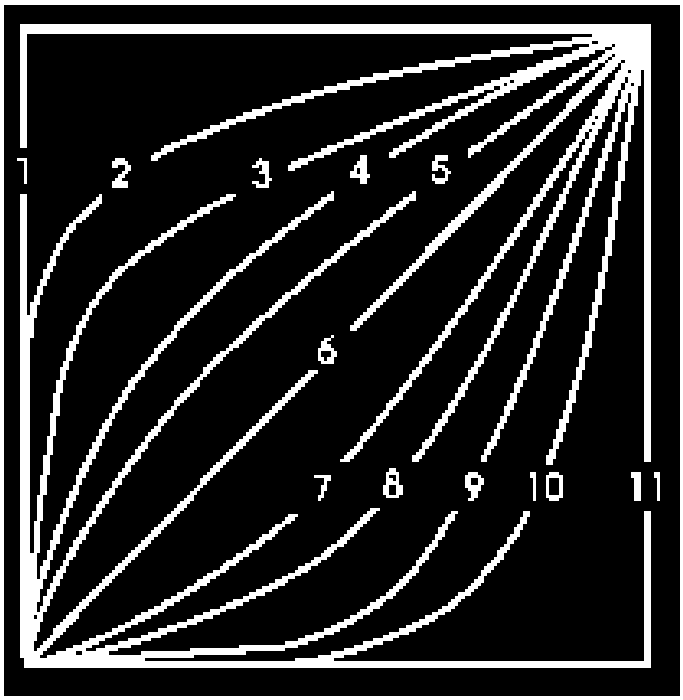


Figure 6: Waters gradient curve profiles. The gradient curve profiles (G) are represented by the numbers 1-11. G1: Immediately goes to specified conditions; G2-5: Convex; G6: Linear; G7-10: Concave; G11: Maintains start condition until next step. Source: MassLynx

The gradient profiles for *A. calamus, A. arborescens, A. afra, D. robusta, E. elephantina, E. lysistemon, P. americana, S. serratuloides and X. undulatum* are provided in Appendix III.

2.3.2.2. Time-of-Flight Mass spectrometric analysis

The SYNAPT G1 mass spectrometry was used in V-optics and operated in electrospray ionization (ESI) mode to enable detection of phenolic and other ESI-compatible compounds. Leucine encephalin (50 pg/mL) was used as a reference calibrant to obtain typical mass accuracies between 1 and 5 mDa. The mass spectrometer was operated in ESI positive and negative modes with a capillary voltage of 2.5 kV, the sampling cone at 30 V and the extraction cone at 4.5 V. The scan time was 0.1 s covering the 100–1000 Dalton mass range. The source temperature was set at 120°C and the desolvation temperature at 450°C. Nitrogen gas was used as the nebulisation gas at a flow rate of 550 L/h and cone gas was added at 50 L/h. The software used to control the hyphenated system and do all data manipulation was MassLynx 4.1 (SCN 872).

2.3.2.3. Identification of compounds

Compounds from the individual plants were identified by comparison of their ion mass [M+H] +/[M+H]-, double-bond equivalence and predicted molecular formula to those of the known compounds that were isolated from the respective plants. The Dictionary of Natural Products, ChemSpider and PubChem databases were used to identify the compounds based on the above-mentioned criterion. The identities of the products were considered for compounds with a normalized theoretical isotope (iFIT) of less than 0.5. The compound structures were acquired with the use of ChemDraw software.

2.4. Antioxidant activity

2.4.1. 2, 2’-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid radical scavenging assay

The antioxidant capacity of the extracts was determined using the ABTS$^{\bullet}$ radical scavenging activity assay as described by Re *et al*.[126] In brief, 20 µL of plant extracts

(0.781-100 µg/mL), Trolox (0.781-100 µg/mL), or DMSO (10% v/v) was added to 180 µL of ABTS• radical in a 96-well microtiter plate. A blank well containing only ABTS• radical was included. The plates were shaken and incubated in the dark for 30 min. Thereafter, the absorbance was read at a wavelength of 734 nm (Synergy 2, Bio-Tek Instruments, Inc). The EC_{50} value of each sample was determined by non-linear regression from the ABTS• radical inhibition curve.

The reference standard compound was Trolox. The ABTS radical scavenging activity of the test samples and Trolox was calculated using the following equation:

$$\text{ABTS radical scavenging activity } (\%) = \left[\frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}}\right] \times 100$$

Where:

Acontrol is the absorbance of the blank and

Asample is the absorbance of the test sample

2.4.2. 2, 2-Diphenyl-1-picrylhydrazyl radical scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability of the extracts was determined using the method as described by Gymfi *et al*.[127] Briefly, 180 µL of 0.135 mM solution of DPPH was added to 20 µL of plant extract (0.781-100 µg/mL), DMSO (10% v/v) or Trolox (0.781-100 µg/mL) in a 96-well plate. The plates were shaken and incubated for 30 min in the dark at room temperature. Thereafter absorbance was measured spectrophotometrically (Synergy 2, Bio-Tek Instruments, Inc) at 515 nm. The EC_{50} value of each sample was determined by non-linear regression from the DPPH radical inhibition curve.

The reference standard compound used was Trolox. The DPPH radical scavenging activity of the test samples and Trolox was calculated using the following equation:

$$\text{DPPH radical scavenging activity } (\%) = \left[\frac{\text{Acontrol} - \text{Asample}}{\text{Acontrol}}\right] \times 100$$

Where:

Acontrol is the absorbance of the blank and

Asample is the absorbance of the test sample

2.5. Cytotoxicity determination

2.5.1. Cell culture and maintenance

The THP-1 and U937 cell lines were purchased from American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 cell culture medium supplemented with 10% heat-inactivated foetal calf serum and antibiotics (1% penicillin-streptomycin) at 37 ˚C in a humidified atmosphere of 5% carbon dioxide (CO_2). Cells were maintained in 25 cm^2 culture flasks and RPMI-1640 medium was replaced every 3 days. Before the experiment, cells were washed in serum-free RPMI-1640 medium and re-suspended in fresh medium.

2.5.2. Sulphorhodamine B assay

The cytotoxicity of crude extracts was determined using the sulphorhodamine B (SRB) assay as described by Vichai *et al.*[128] with minor modifications. In brief, cells were harvested and centrifuged at 200 *g* for 5 min. Cells were resuspended in RPMI-1640 media and counted using the trypan blue exclusion method and a haemocytometer, and resuspended in a 96-well plate at 2 x 10^5 cells/well for 24 h prior to treatment.

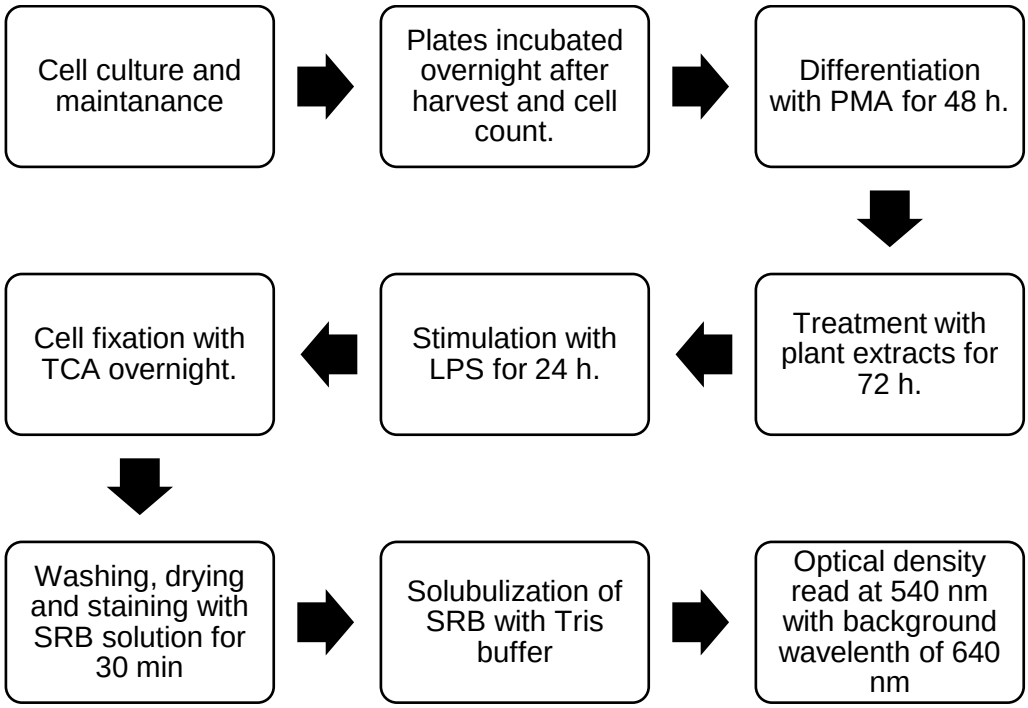
The THP-1 and U937 cells were differentiated by treatment with 0.1 µg/mL phorbol-12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, USA) for 48 h. Thereafter, the cells were treated with either crude plant extracts (0.781–100 µg/mL), curcumin (0.391-50 µg/mL) or 1% (w/v) saponin (positive control) and incubated for 72 h. Following incubation, the cells were stimulated with 1 µg/mL lipopolysaccharide (LPS) for 24 h. Thereafter, the cells were fixed by adding 50 µL of ice-cold trichloro acetic acid (TCA) overnight at 4^{o}C. The plates were then washed with sterile distilled water and dried.

One hundred microliters of SRB solution (0.057% w/v in 1% v/v acetic acid) was added to each well and the plates incubated at room temperature for 30 min in the dark. Any unbound or excess SRB solution was removed by washing the plates 3 times with 1% v/v acetic acid where after the plates were air-dried. The bound SRB solution was solubilized by adding 100 µL of 100 mM Tris buffer (pH 10.5) to each well and plates

shaken for 45 min. The 96-well plates were then read in a 96-well plate reader (Synergy 2, Bio-Tek Instruments, Inc) at a wavelength of 540 nm and a background wavelength of 630 nm. The optical density (OD) or absorbance of SRB in each well is directly proportional to the cell number. The percentage of surviving cells was calculated as follows:

$$\%\ Cell\ survival = \frac{OD(experimental)}{OD(untreated\ control)} X\ 100\%$$

The flow diagram below summarizes the cytotoxicity assay of the plant extracts using the SRB assay.



2.6. Anti-inflammatory activity

Table 3: The highest non-toxic dose used for anti-inflammatory analysis. The doses were informed by the cytotoxicity results

Extract	Concentration (µg/mL)	
	THP-1	U937
A. calamus	100	100
A. arborescens	100	100
A. afra	100	100
E. elephantina	100	100
E. lysistemon	100	100
D. robusta	20	20
P. americana	100	100
S. serratuloides	100	100
X. undulatum	50	50
Prijap Health	50	50
Curcumin	5	5

2.6.1. Cytokine evaluation

2.6.1.1. Cell preparation

Cells were harvested and differentiated as described in section 2.6.2. Thereafter, cells were treated with crude extracts (Table 3) or 5 µg/mL curcumin (positive control) and incubated for 72 h. Following incubation, the cells were treated with 1 µg/mL lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, USA) from *Escherichia coli* for 24 h to promote cytokine production and secretion.

2.6.1.2. Collection of supernatants

After incubation, plates were centrifuged (Allegra™ X-12R, Beckman Coulter, USA) at 2000 *g* for 10 min. Cell membrane integrity was confirmed via trypan blue exclusion after centrifugation at 2000 *g* to ensure that no cell rupture occurred.

Supernatants were aspirated from each well and stored at -80°C for determination of extracellular cytokine concentrations. Cells were lysed with 0.01% w/v sodium dodecyl sulphate (SDS) for 30 min. The cell lysates were centrifuged at 2000 *g* for 10 min then the supernatants were aspirated from each well and stored at -80 °C for determination of intracellular cytokines concentration.

2.6.1.3. Cytokine determination

Determination of intracellular and extracellular cytokine levels was carried out using two BD CBA kits by using an Accuri™ C6 Plus Beckton Dickson (BD) flow cytometer. The Human Inflammatory Kit contains beads loaded with antibodies directed against IL-6, IL-8, IL-10, TNF-α and IL-12p70. The Human Th1/Th2 Cytokine Kit contains antibodies directed against IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ.

The assays were performed according to the manufacturer's instructions. Briefly, a series of calibration standards (0-5000 pg/mL) were prepared. Mixed capture beads were added to each assay tube. Thereafter, 50 µL of appropriate cytokine standard or cell supernatant sample was added and incubated for 3 h to allow capture of the soluble cytokines onto the capture beads. The beads were then washed to remove any unbound protein, centrifuged (200 *g*, 5 min) and the supernatants discarded. Fifty microliters of the detection reagent were added and the suspension incubated for a further 30 min. After a second brief centrifugation, the supernatant was again discarded and the bead pellet re-suspended in wash buffer and 50 µL of each standard or test sample transferred to a well of a 96-well plate for analysis on the flow cytometer.

2.6.2. Prostaglandin E_2 production

2.6.2.1. Sample preparation

The THP-1 and U937 macrophages were cultured, harvested, differentiated and treated with test compounds (aqueous extracts and curcumin) as described in section 2.6.2. Supernatants were collected as described in section 2.7.1.2.

2.6.2.2. Prostaglandin E_2 determination

The amount of prostaglandin E_2 (PGE_2) secreted by LPS-activated macrophages was determined using commercially available ELISA kits purchased from Biocom Africa (Pty) Ltd. The assay was performed as per the manufacturer's instructions. Briefly, the PGE_2 standards were prepared by serially diluting the supplied standard with tissue culture medium (RPMI-1640) to obtain a concentration range of 39-2500 pg/mL. One hundred microliters of PGE_2 standards, plant extracts and curcumin (positive control) were pipetted into the wells of the provided 96-well ELISA plate. This was followed by addition of 50 µL of the assay buffer.

Thereafter, 50 µL of the PGE_2-AP conjugate and Anti-PGE_2 antibody were added into appropriate wells and the plate incubated for 2 h at room temperature while shaking. After washing thrice with 1X wash buffer, 5 µL of PGE_2-AP conjugate was added to the appropriate wells. Two hundred microliters of the pNpp substrate solution was then added to every well and the plates incubated at 37 ºC for 1 h after which 50 µL of the stop solution was added to each well. The plates were then read immediately at an optical density (OD) of 405 nm using a reference wavelength of 570 nm (Synergy 2, Bio-Tek Instruments, Inc).

To calculate the average net optical density bound for each standard and test sample, the average optical density from the non-specific binding (NSB) wells was subtracted from the average OD bound using the formula:

Average net OD = Average bound OD-Average NSB OD

The binding of each duplicate standard was calculated as a percentage of the maximum binding wells (Bo) as follows:

$$\%\text{Bound} = \frac{Average\ net\ OD}{Net\ Bo\ OD} X\ 100$$

2.7. Statistical analysis

All experiments were carried out in triplicates and repeated on three separate occasions. Data is presented as mean ± standard error of mean (SEM). The EC_{50} and IC_{50} values were calculated from the non-linear curve for each test sample. Results were analysed using a Graph Pad Prism (version 6.0).

The data for cytokine analysis is presented as mean ± SEM of duplicate experiments. Standard curves were generated for all cytokine standards through non-linear regression using BD FCAP Array Analysis software.

Data was considered statistically significant when $p<0.05$.

Chapter 3: Results and discussion

Extract yields

The amount of extract recovered from 60 g of starting plant material of the respective plants is provided in Table 4. The most common and widely used solvent for extraction of phytochemicals by THPs is water. The amount of raw starting material, solvent volume (amount of raw material and solvent volume determines the solid-to-liquid ratio), extraction temperature and time directly affect the extract yield during phytochemical extraction.[129]

Additionally, the type of extraction solvent (in terms of polarity) used plays a crucial role in terms of the type of phytochemicals that will be extracted. Water has been shown to be an effective extractant for polar compounds, better than ethanol, ethyl acetate and hexane.[130]

Table 4: Extract yields of the plant species under investigation

Plant species	Plant part used	Extract recovered (g)	Yield (%)
A. afra	Leaves and twigs	6.43	10.5
A. arborescens	Leaves	11.63	19.38
A. calamus	Roots	7.71	12.85
D. robusta	Bulbous roots	12.97	21.62
E. elephantina	Roots	15.78	26.30
E. lysistemon	Stem	3.50	5.83
P. americana	Leaves and twigs	5.14	8.57
S. serratuloides	Leaves and twigs	7.27	12.12
X. undulatum	Roots	12.11	20.18

3.1. Phytochemical analysis

3.1.1. Thin layer chromatography

Thin-layer chromatography (TLC) is a relatively old and well-established technique for the separation and isolation of compounds present in a complex mixture. Thin layer

chromatography is a solid-liquid separation technique. The two phases are the stationary phase (solid) and a mobile phase (liquid).[131] The achievement of separation in a complex mixture by TLC is greatly depended on the choice of the solid stationary phase. The solids used in TLC are silica gel, polymeric ion exchange, alumina, chemical-bonded silica gel, impregnated silica gel, cellulose, polyamide and chiral phase.[132] The most commonly used solid phase is the silica gel and is the most suitable solid for analysis.[132] TLC is relatively easy to carry out, it is fast, simple, inexpensive and allows for the simultaneous analysis of a large number of samples and does not require complicated instrumentation.[133]

The phytochemical classes detected in nine plant species using TLC is summarised in Table 5. The plant species studied were analysed for the presence of phenolic acids, flavonoids, alkaloids, sterols, saponins and terpenoids. Phenolic acids and alkaloids were not detected in any of the plant extracts. Initially, several different solvent systems were used to optimize TLC analysis for migration and separation of polar compounds, non-polar compounds and compounds with intermediate polarity. However, there was no success in separating the non-polar compounds and compounds with intermediate polarity from the baseline. This appeared to indicate the absence of non-polar compounds and compounds with intermediate polarity due to the use of water as an extractant.

Table 5: Phytochemical classes detected in hot water extracts from the plant species investigated using TLC

Plant extract	Phytochemical class					
	Phenolic acids	Flavonoids	Alkaloids	Sterols	Saponins	Terpenoids
A. calamus	-	-	-	-	+	+
A. arborescens	-	-	-	+	-	-
A. afra	-	+	-	-	+	+
D. robusta	-	-	-	-	+	-
E. elephantina	-	+	-	+	+	-
E. lysistemon	-	-	-	-	-	-
P. americana	-	+	-	+	+	-
S. serratuloides	-	+	-	-	-	-
X. undulatum	-	-	-	+	-	+
Prijap Health	+	+	+	-	+	+

+: indicates presence

-: indicates absence

In the present study, flavonoids were detected in the extracts of *A. afra, E. elephantina, P. americana and S. serratuloides*. No alkaloids were detected in all the extracts under investigation in this study (Table 5). However, the UPLC-TOF-MS analysis data (section 3.2.2) indicated the presence of nitrogenous compounds in the extract of only *E. lysistemon* of which none were successfully identified. Species of the *Erythrina* genus have been reported to be rich in alkaloids.[134]

Sterols were detected by TLC in extracts of *A. arborescens*, *E. elephantina, P. americana* and *X. undulatum*. Saponins were detected in the extracts of *A. calamus*, *A. afra*, *D. robusta*, *E. elephantina* and *P. americana*. Saponins have been shown to possess anticancer activity *in vitro* and *in vivo*.[135] Terpenoids were detected in the

extracts of *A. calamus, A. afra*, and *X. undulatum*. Terpenoids have been shown to exhibit immunosuppressive activity.[83]

The methanol extract of *A. afra* has been found to contain saponins, phenols, flavonoids, steroids, terpenoids and alkaloids.[136] Phenols, flavonoids and proanthocyanidins have been reported from the water extract of *A. afra*.[137] The presence of phenolic acids, anthrones, isoflavones, flavonoids and chromones have been reported in the leaf water extract of *A. arborescens*.[138]

Phenolic acids, flavonoids, saponins, alkaloids, sterols and terpenoids have been reported to be present in the methanol extract of *A. calamus*.[139,140] Flavonoids, saponins, tannins and sterols were reported as constituents of the ethanol extract of *P. americana*[141] whereas the presence of terpenoids, alkaloids, cardiac glycosides and flavonoids were previously reported in the ethanol extract of *P. americana*.[142]

The water, ethanol, methanol and acetone extracts of *X. undulatum* have been previously reported to contain tannins, flavonoids and alkaloids as secondary metabolites.[113] The methanol, hexane and ethyl acetate extracts of *E. elephantina* have been reported to contain saponins, phenolic acids, flavonoids, alkaloids, sterols and terpenoids.[142]

The water, ethanol and ethyl acetate extracts of *D. robusta* have been found to contain cardiac glycosides, saponins, bufadienolides, sterols and flavonoids.[109] In the methanol extract of *E. lysistemon*, alkaloids, flavones, isoflavones, pterocarpans as well as phenolic acids were identified as constituents.[143] Hepatotoxic alkaloids were identified in the methanol extract of *S. serratuloides*.[144]

There is a variation in terms of the phytochemicals identified from the plant species under investigation and what has been previously reported. Previous studies have suggested that environmental factors such as climate, geographical location, soil type, soil pH, light intensity, exposure to soil microorganisms and nutrients play a role in the phytochemical variability of plants of the same species.[145–149] Different agro-climatic conditions (such as temperature, precipitation and wind patterns) have been shown to affect the phytochemical composition and antioxidant activity of plants.[150] The plant

part, solvent (polarity), extraction time and temperature are also responsible for the variation in phytochemical profile observed within the same plant species.[129,130,151,152] The latter could be the reason for the observed variation in phytochemical profile between the findings in the current study and previous reports.

The presence of saponins, terpenoids, alkaloids, flavonoids and phenolic acids was confirmed in Prijap Health traditional herbal medicine. Due to the complexity of the mixture, it was not analysed by UPLC-MS. The presence of alkaloids in Prijap Health could be contributed by the *E. lysistemon* plant species which has been shown by UPLC-ESI-MS to contain alkaloids, although none were identified. Alkaloids were not detected using TLC in *E. lysistemon* but with UPLC-ESI-MS, this could be due to the sensitivity and detection limits of the two assays.[153,154] Also, the starting plant raw material directly affects the extract (and phytochemical) yield.[129]

During extract preparation, significantly larger amounts of *E. lysistemon* were used to prepare Prijap Health traditional medicine whereas small amounts were used to prepare the extracts of the individual plant species. The resulting difference in phytochemical yield could explain the detection of alkaloids in Prijap Health traditional medicine but not in *E. lysistemon* extract.

3.1.2. Ultra-performance liquid chromatography-mass spectrometry

UPLC (derived from HPLC) is a chromatographic technique used for the separation of the individual compounds in a complex mixture. It is based on the use of a stationary phase consisting of particle less than 2 μm and is governed by the Van Deemter equation which describes the relationship between flow rate (velocity) and column efficiency with respect to particle size.[153] The UPLC underlying principle is that as the particle size decreases, efficiency and resolution increases. The advantages of UPLC are that it allows for faster analysis, decreases run time, increases sensitivity, reduces solvent consumption, separation is performed under high pressures and gives increased peak resolution. Its main disadvantages are a reduced lifespan of columns due to increased pressure, higher prices of instruments and non-regenerable stationary phase (less than 2 μm).[153,155] Chromatography techniques are usually combined with mass spectrometry for downstream analysis of samples. Mass

spectrometry (MS) is a technique used to detect and determine the amount (nominal mass), elemental composition and aspects of the molecular structure of an analyte.[156] The concept of MS is to form ions from an analyte, separate the ions based on their mass-to-charge (*m/z*) ratio and measure the abundance of the ions.[157]

Acorus calamus

Ultra-high-performance liquid chromatography-mass spectrometry (UPLC-ESI-MS) chromatogram of the hot water extract of *A. calamus* analysed in both ESI-positive and ESI-negative modes indicated the presence of six compounds (Figure 7). The identified compounds are calamusin D (**1**), 1-hydroxyacoramone (**2**), acoramone (**3**), isoacoramone (**4**), asaronaldehyde (**5**) and β-asarone (**6**). The molecular weights, UPLC-ESI-MS retention times, chemical formulae, phytochemical class and compound structures are listed in Table 6. β-asarone was isolated and identified from the ethanol extract and essential oil of the rhizomes[105,158] and was determined as the phytochemical marker for the chemotaxonomic identification of *A. calamus*.[159] The sesquiterpenes calamusin D, neo-acorane A and acoric acid were also reported in the ethanol extract of the rhizomes.[160] Moreover, the chemical compounds: Linalool, β-asarone, α-asarone, asaronaldehyde, elemicin, 2,4,5-trimethoxybenzoic acid and 28 other phenylpropanoids have been reported from the essential oils of the leaves and rhizomes of *A. calamus*.[161]

In this study, calamusin D (**1**), asaronaldehyde (**5**) and β-asarone (**6**) are tepernoids identified through UPLC-ESI-MS and thus corroborates with terpenoids identified using TLC (Section 3.2.1). On the other hand, compound (**2**), (**3**) and (**4**) are phenylpropanoids and have not been detected/tested for using TLC. All the compounds identified in this study have all been previously reported in literature.

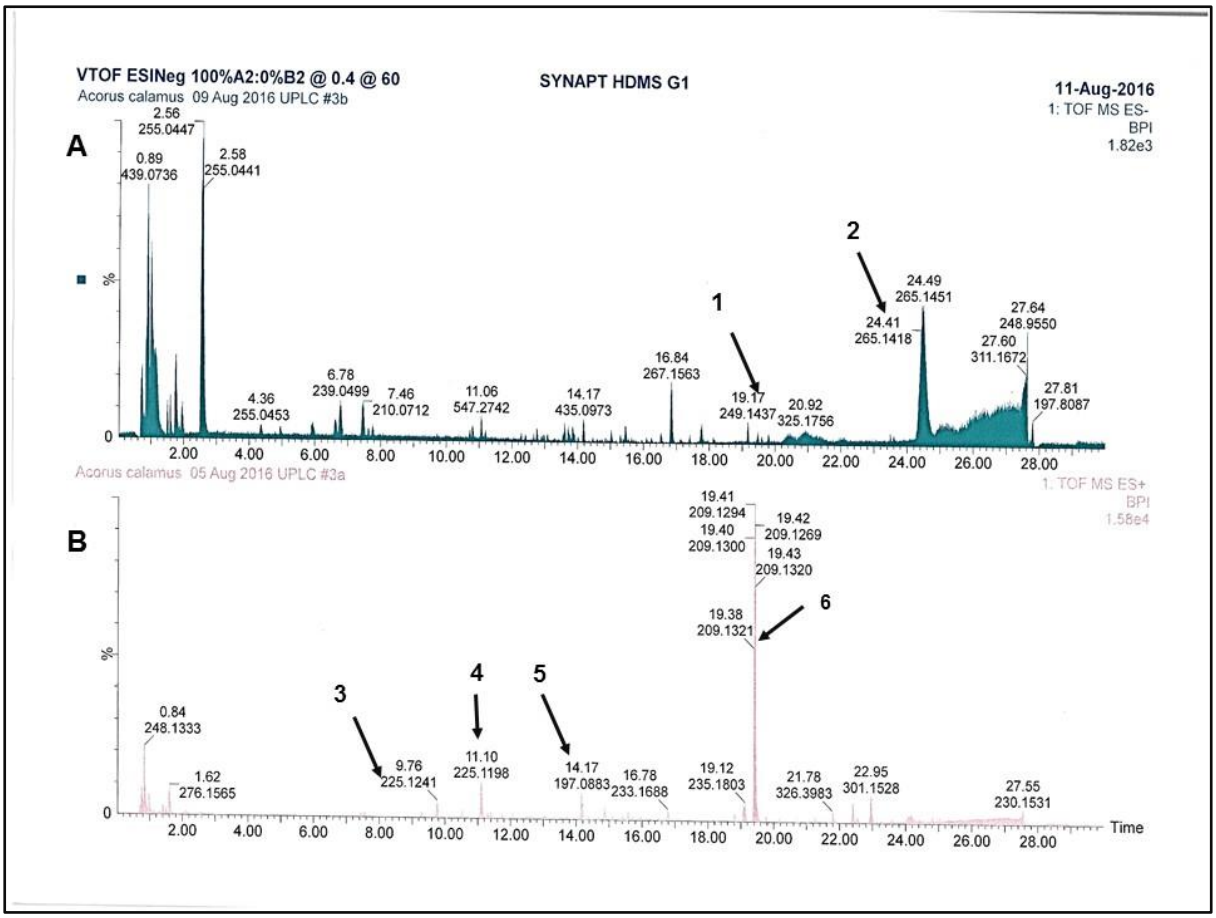
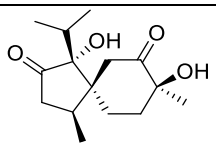
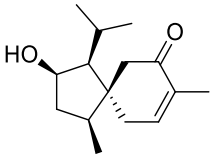
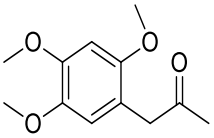
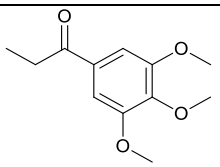
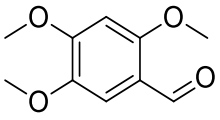
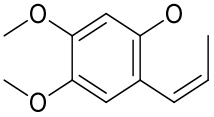


Figure 7: UPLC-ESI-MS chromatogram of the hot water extract of the roots of *A. calamus*. A) Electrospray ionization in positive mode. B) Electrospray ionization in negative mode. Calamusin D (**1**), 1-hydroxyacoramone (**2**), acoramone (**3**), I\isoacoramone (**4**), asaronaldehyde (**5**), β-asarone (**6**)

Table 6: List of the phytochemicals identified from the hot water extract of the roots of *A. calamus* using UPLC-ESI-MS.

Compound number	Compound name	UPLC retention time (min)	Molecular weight ($g.mol^{-1}$)	Chemical formula	Chemical structure	Phytochemical class
1	Calamusin D (Rel-Calamusin D)	16.84	268.1596	$C_{15}H_{23}O_4$		Sesqueterpenoid
2	1-Hydroxyacorenone	19.17	250.1559	$C_{15}H_{21}O_3$		Phenylpropanoid
3	Acoramone	9.76	224.2561	$C_{12}H_{17}O_4$		Phenylpropanoid
4	Isoacoramone	11.10	224.2561	$C_{12}H_{17}O_4$		Phenylpropanoid
5	Asaronaldehyde	14.17	196.0814	$C_{10}H_{12}O_4$		Sespuiterpenoid
6	β-asarone	19.38	208.1178	$C_{12}H_{17}O_3$		Sespuiterpenoid

Aloe arborescens

The ultra-high-performance liquid chromatography-mass spectrometry (UPLC-ESI-MS) chromatogram of the hot water extract of the leaves of *A. arborescens* analysed in ESI-negative mode is provided in Figure 8. The Time-of-flight (TOF) mass spectrometric analysis led to the ionization and identification of Aloesin (**7**) and Aloenin (**8**). The molecular weights, UPLC-ESI-MS retention times, chemical formulae, phytochemical class and compound structures are listed in Table 7.

Aloesin, aloesone, 8-C-glucosyl-noreugenin, aloeresin E, aloenin, Aloin, aloe-barbendol, aloesaponarin II and other flavones and hydroxycinnamics were previously identified from the leaf ethanol extract[162] while the chemical compounds barbaloin, aloeresin and aloenin were identified from the leaf exudate of *A. arborescens*.[163] From the methanol extract of the leaves of *A. arborescens*, chemical compounds aloesin, aloenin, aloeresin D and aloin A & B have been reported.[164]

Aloesin (**7**) is a chromone whereas aloenin (**8**) is a phenylpyrone, both phytochemical classes were not tested for/detected through TLC.

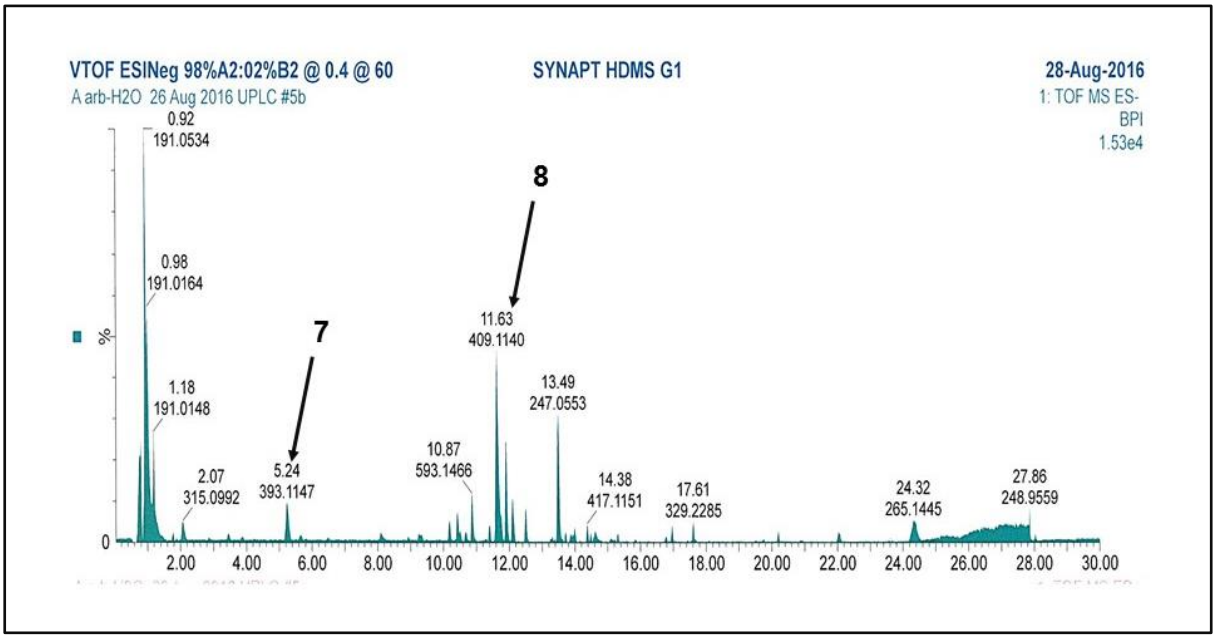
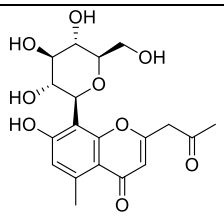
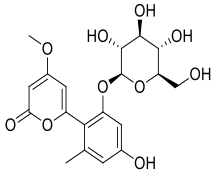


Figure 8: The UPLC-ESI-MS chromatogram of the hot water extract of the leaves *A. arborescens*. Aloesin (**7**), aloenin (**8**)

Table 7: Phytochemicals identified from the hot water extract of the leaves of *A. arborescens* by UPLC-ESI-MS.

Compound number	Name	UPLC retention time (min)	Molecular weight ($g.mol^{-1}$)	Chemical formula	Chemical structure	Phytochemical class
7	Aloesin	5.24	394.376	$C_{19}H_{21}O_9$		Chromone
8	Aloenin	11.63	410.1	$C_{19}H_{21}O_{10}$		Phenylpyrone

Artemisia afra

An ultra-high-performance liquid chromatography-mass spectrometry (UPLC-ESI-MS) chromatogram of the hot water extract of *A. afra* analysed in ESI-negative mode is provided in Figure 9. The Time-of-flight (TOF) mass spectrometric analysis led to the ionization and identification of five compounds, namely scopoletin (**9**), 3-caffeoylquinic acid (**10**), scopolin (**11**), rutin hydrate (**12**) and 3,4-dicaffeoylquinic acid (**13**). The molecular weights, UPLC-ESI-MS retention times, chemical formulae, phytochemical class and compound structures are listed in Table 8.

The sesquiterpene, isoalantolactone, has been reported from the ethanol extract of the leaves of *A. afra*.[165] scopoletin, *α*-amyrin, acacetin**,** 12*α*,4*α*-dihydroxybishopsolicepolide, phytol and betulinic acid compounds were present in the ethanol extract prepared from the leaves of the plant species.[166] Luteolin was reported from the aqueous extract of the leaves.[167] The essential oil of the leaves of *A. afra* was analysed by capillary gas chromatography and gas chromatography-mass spectrometry and was found to contain artemisyl acetate, yogomi alcohol, artemesia ketone,[168] β-thujone (major constituent), α-thujone, 1,8-cineole, chrysanthenyl acetate,[169] camphene, β-pinene, artemisia ketone and santolina alcohol.[170]

All the compounds identified in the current study for the extract of *A. afra* have been previously reported in literature. Rutin hydrate (**12**) is a representative of flavonoid compounds detected in the extract of *A. afra* by TLC. However, no steroidal compounds were identified in the extract of *A. afra.* The UPLC-ESI-MS results partly corroborates the TLC results.

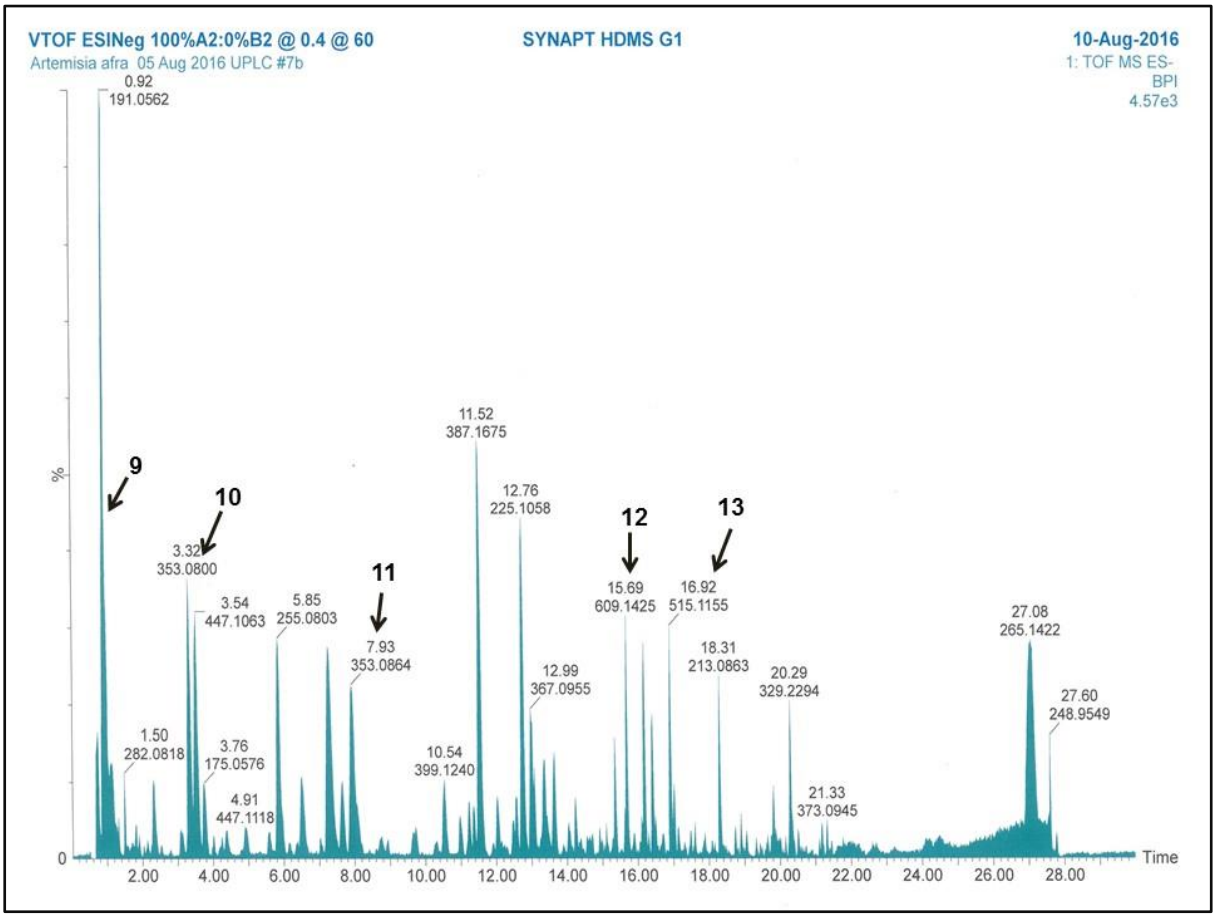
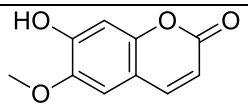
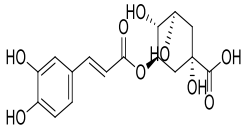
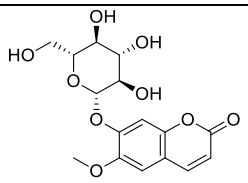
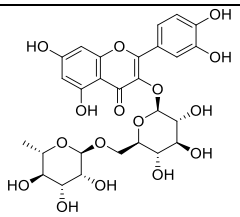
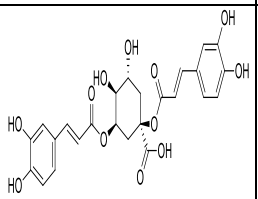


Figure 9: The UPLC-ESI-MS chromatogram of the hot water extract of the leaves of *A. afra* electrospray ionization in negative mode. Scopoletin (**9**), 3-caffeoylquinic acid (**10**), scopolin (**11**), rutin hydrate (**12**) and 3,4-dicaffeoylquinic acid (**13**) were identified

Table 8: List of phytochemicals identified from the hot water extract of *A. afra* by UPLC-ESI-MS

Compound number	Name	UPLC retention time (min)	Molecular weight ($g.mol^{-1}$)	Chemical formula	Chemical structure	Phytochemical class
9	Scopoletin	0.92	192.168	$C_{10}H_7O_4$		Coumarin
10	3-Caffeoylquinic acid	3.32	354.309	$C_{16}H_{17}O_9$		Phenylpropanoid
11	Scopolin	7.93	354.309	$C_{16}H_{17}O_9$		Glycoside
12	Rutin hydrate	15.69	610.518	$C_{27}H_{29}O_{16}$		Flavonoid
13	3,4-Dicaffeoylquinic acid	16.92	516.450	$C_{25}H_{23}O_{12}$		Phenylpropanoid

Drimia robusta

The ultra-high-performance liquid chromatography-mass spectrometry (UPLC-ESI-MS) chromatograms of the hot water extract of the bulb of *D. robusta* analysed in ESI-negative mode is provided in Figure 10. The Time-of-flight (TOF) mass spectrometric analysis led to the ionization and identification of a single compound, D-saccharic acid (Table 9). Previous studies, on the other hand, reported the presence of bufadienilide compounds such as scilliroside, 12β-hydroxyscillirosidin, 12β-hydroxyscilliroside, hellebrigenin-3-0-β-glucoside, 16β-hydroxyhellebrigenin, 16β-hydroxyhellebrigenin-3-O-β-glucoside and 5β,16β-dihydroxybufalin-3-O-β-glucoside from the chloroform extract of the bulbs of *D. robusta*.[171]

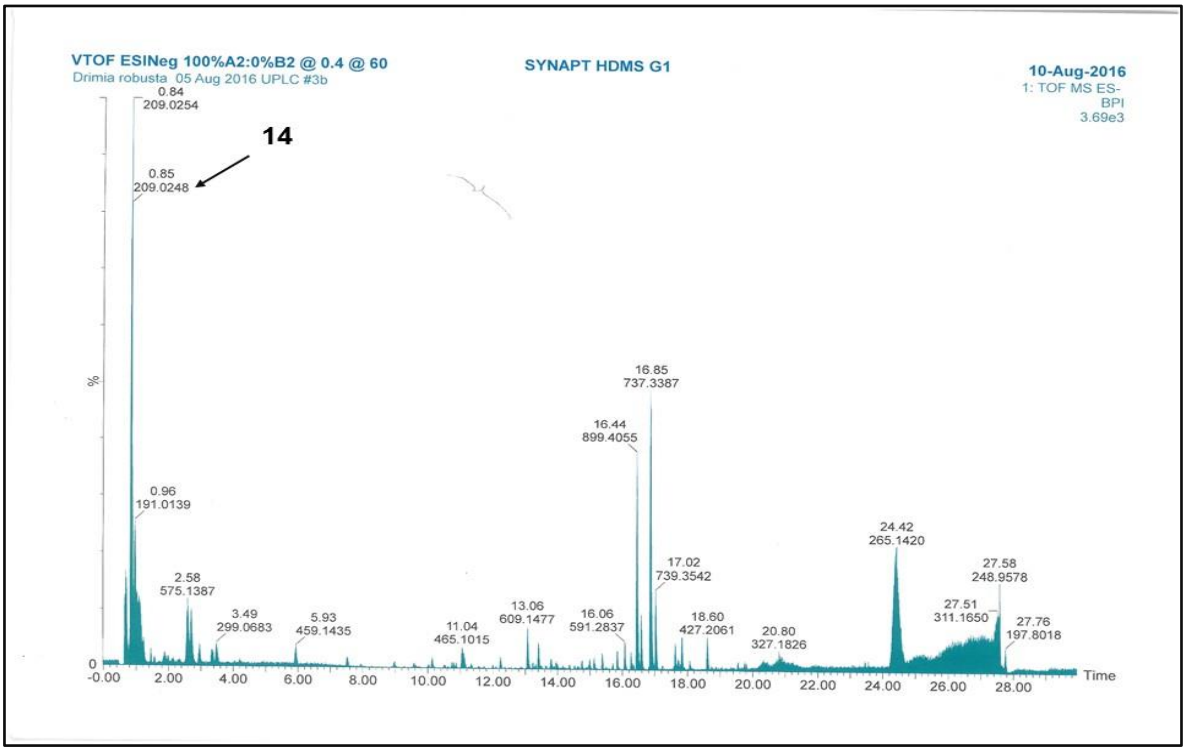
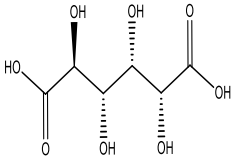


Figure 10: UPLC-ESI-MS chromatogram of the hot water extract of the bulb of *D. robusta* Electrospray ionization in negative mode. D-saccharic acid (**14**) was identified

Table 9: The compound identified from the hot water extract of *D. robusta*.

Compound number	Name	UPLC retention time (min)	Molecular weight ($g.mol^{-1}$)	Chemical formula	Chemical structure	Phytochemical class
14	D-Saccharic acid	0.84	210.139	$C_6H_9O_8$		Carboxylic acid

Elephantorrhiza elephantina

The ultra-high-performance liquid chromatography-mass spectrometry (UPLC-ESI-MS) chromatogram of the hot water extract of the roots of *E. elephantina* analysed in both ESI-negative and ESI-positive modes. The Time-of-flight (TOF) mass spectrometric analysis led to the ionization and identification of eight compounds, namely gallic acid (**15**); catechin (**16**); eriodictyl-7-O-glucoside (**17**); (-)-epicatechin

(**18**); taxifolin-3-O-glucoside (**19**); quercetin 3-O-glucoside (**20**) and taxifolin (**21**) (Figure 11).

The molecular weights, UPLC-ESI-MS retention times, chemical formulae, phytochemical class and compound structures are listed in Table 10. Previous phytochemical studies on *E. elephantina* reported the presence of catechin, (-)-epicatechin, gallic acid, quercetin 3-O-β-D-glucoside, β-sitosterol, taxifolin 3'-O-β-O-glucoside, methyl gallate, and 3-O-galloyl-3,3',5',5,7-pentahydroxyflavone from the isobutanol and hexane fractions of the aqueous extract as the phytochemical constituents of the rhizomes.[172] From the rhizome extract, epigallocatechin gallate, taxifolin, (-)-epicatechin and epicatechin gallate have been reported.[173]

Only phenolic compounds (flavonoids (**16**-**21**) and phenolic acid (**15**)) were identified by UPLC-ESI-MS and correspond to the flavonoids detected through TLC. No compounds of the sterol and saponin classes, which were detected through TLC, were identified by UPLC-ESI-MS.

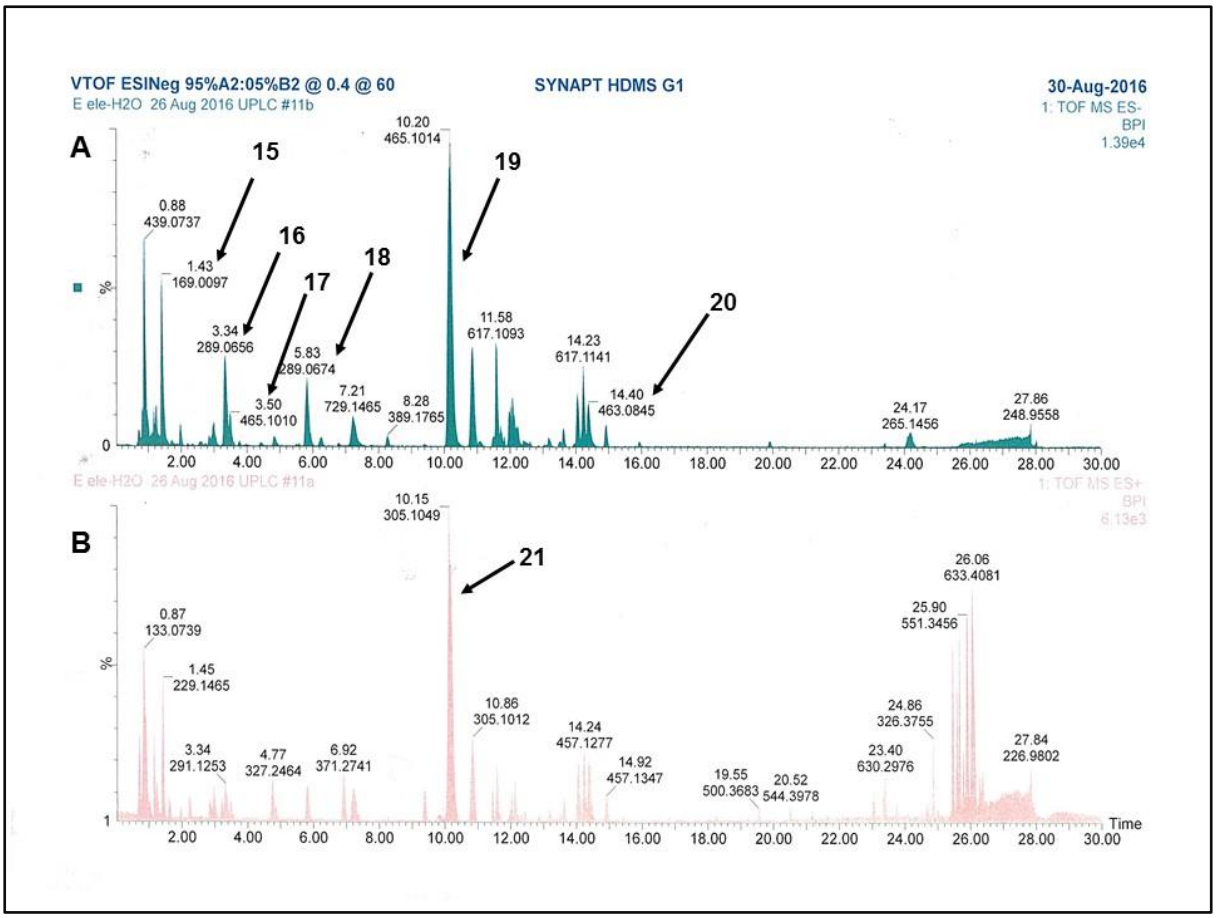
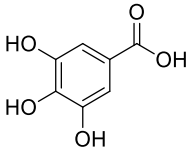
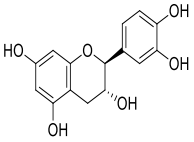
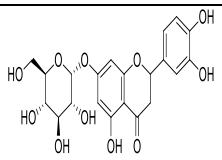
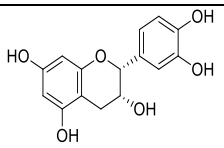
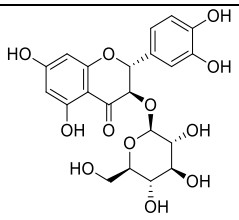
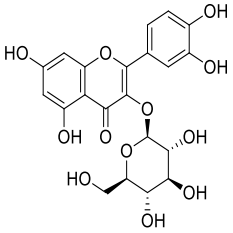
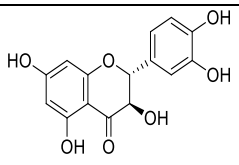


Figure 11: UPLC-ESI-MS chromatogram of the hot water extract of the roots of *E. elephantina*. A) Electrospray ionization in negative mode. B) Electrospray ionization in positive mode. Gallic acid (**15**); catechin (**16**); eriodictyl-7-O-glucoside (**17**); (-)-epicatechin (**18**); taxifolin-3-O-glucoside (**19**); quercetin 3-O-glucoside (**20**) and taxifolin (**21**) were identified

Table 10: Compounds identified from the hot water extract of *E. elephantina* as determined by UPLC-ESI -MS analysis.

Compound number	Name	UPLC retention time (min)	Molecular weight ($g.mol^{-1}$)	Chemical formula	Chemical structure	Chemical group
15	Gallic acid	1.43	170.1	$C_7H_5O_5$		Phenolic acid
16	Catechin	5.83	290.1	$C_{15}H_{13}O_6$		Flavonoid
17	Eriodictyl-7-O-glucoside	4.82	450.1	$C_{21}H_{21}O_{11}$		Flavonoid
18	(-)-Epicatechin	3.34	290.1	$C_{15}H_{13}O_6$		Flavonol
19	Taxifolin-3-O-glucoside	10.20	466.395	$C_{21}H_{21}O_{12}$		Flavonoid
20	Quercetin 3-O-glucoside	14.40	464.376	$C_{21}H_{19}O_{12}$		Flavonoid
21	Taxifolin	10.15	304.10	$C_{15}H_{12}O_7$		Flavonoid

Erythrina lysistemon

The ultra-high-performance liquid chromatography-mass spectrometry (UPLC-ESI-MS) chromatograms of the hot water extract of *E. lysistemon* analysed in both ESI-

negative and ESI-positive modes are as shown in Figure 12. The Time-of-flight (TOF) mass spectrometric analysis led to the ionization of compounds, but no possible identifications of the compounds were made in the hot water extract of *E. lysistemon*. However, the majority of the detected ions were nitrogenous compounds, which greatly suggested presence of alkaloids. However, previous studies have isolated and identified pterocarpans (erylysins A, B and C), flavonoids (isoerysenegalensein E, lysisteisoflavanone, isosenegalensin, abyssinone V-4'-methylether, alpinumisoflavone, wighteone, burttinone) and alkaloids ((+)-erysotrine, (+)-erythrabine, 11β-hydroxyerysotramidine and 14 other alkaloids) through their NMR spectra from the stem bark, flowers and pods of *E. lysistemon*.[134,174–178] Abyssinone V-4'-methylether and alpinumisoflavone were generally found to be major compounds of *E. lysistemon*.[175]

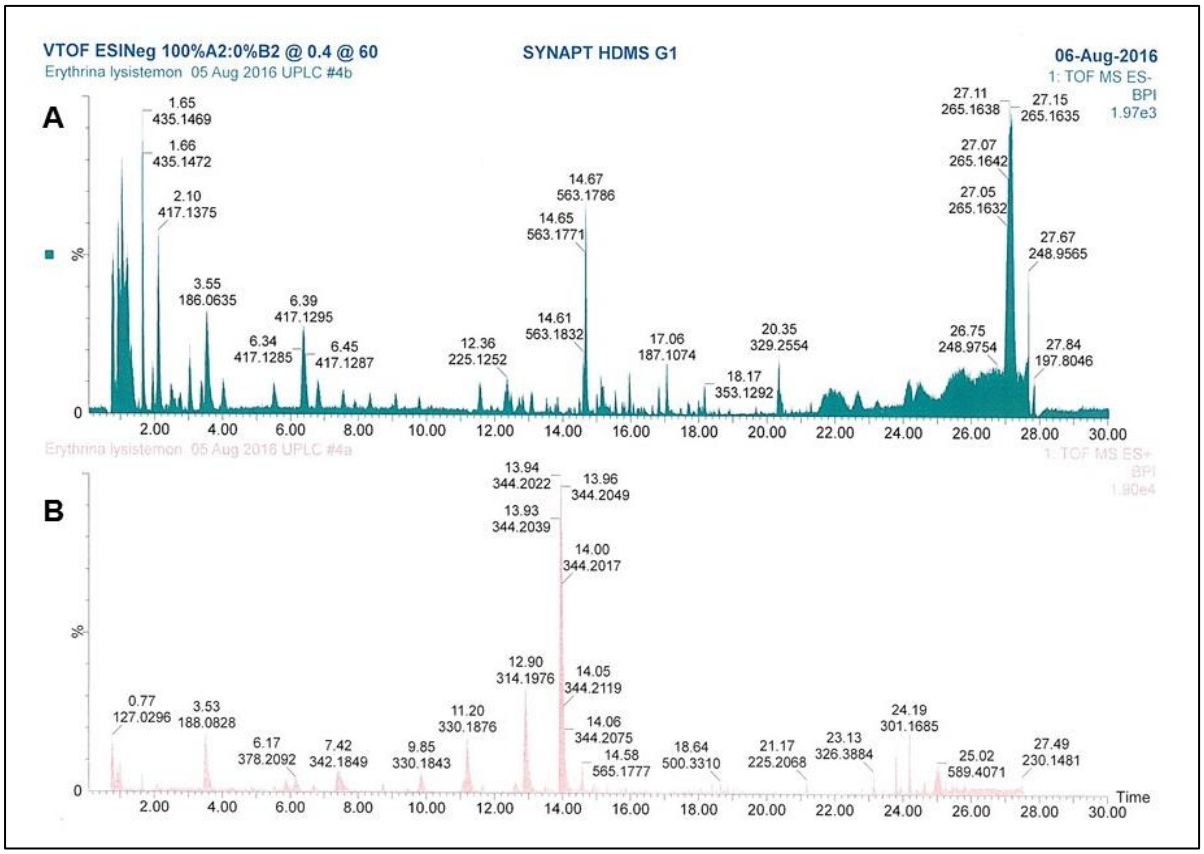


Figure 12: UPLC-ESI-MS chromatogram of the hot water extract of *E. lysistemon*. A) Electrospray ionization in positive mode. B) Electrospray ionization in negative mode

Persea americana

The ultra-high-performance liquid chromatography-mass spectrometry (UPLC-ESI-MS) chromatograms of the hot water extract of the leaves and twigs of *P. americana* analysed in ESI-negative modes is as shown in Figures 13 and 14. The Time-of-flight (TOF) mass spectrometric analysis led to the ionization and identification of seven compounds, procyanidin B1 (**22**); quercetin 3,7 diglucoside (**23**); rutin (**24**); nicotiflorin (**25**); catechin (**26**); (-)-epicatechin (**27**) and quercetrin (**28**). Compound names, UPLC-ESI-MS retention times, molecular weights and chemical formulas of the identified compounds are indicated in Table 11 together with their chemical structures.

Other studies reported flavonoids such as quercetin, rutin, apigenin, lutienol and isorhamnetin have been previously identified from the ethanol extract of the leaves of *P. americana*.[179,180] The essential oil of the leaves of *P. americana* was found to contain cis-9-hexadecanoic acid, 12-methyl-E,E-2,13-octadecadien-1-ol, eicosane, tetratetracontane, pentatriacontane, hentriacontane, phytol, diisooctyl phthalate, squalene, 2-methyloctacosane, vitamin E, oxirane and hexatriacontane as phytochemical constituents.[179] Squalene was also reported as the major chemical constituent of the essential oil.[179]

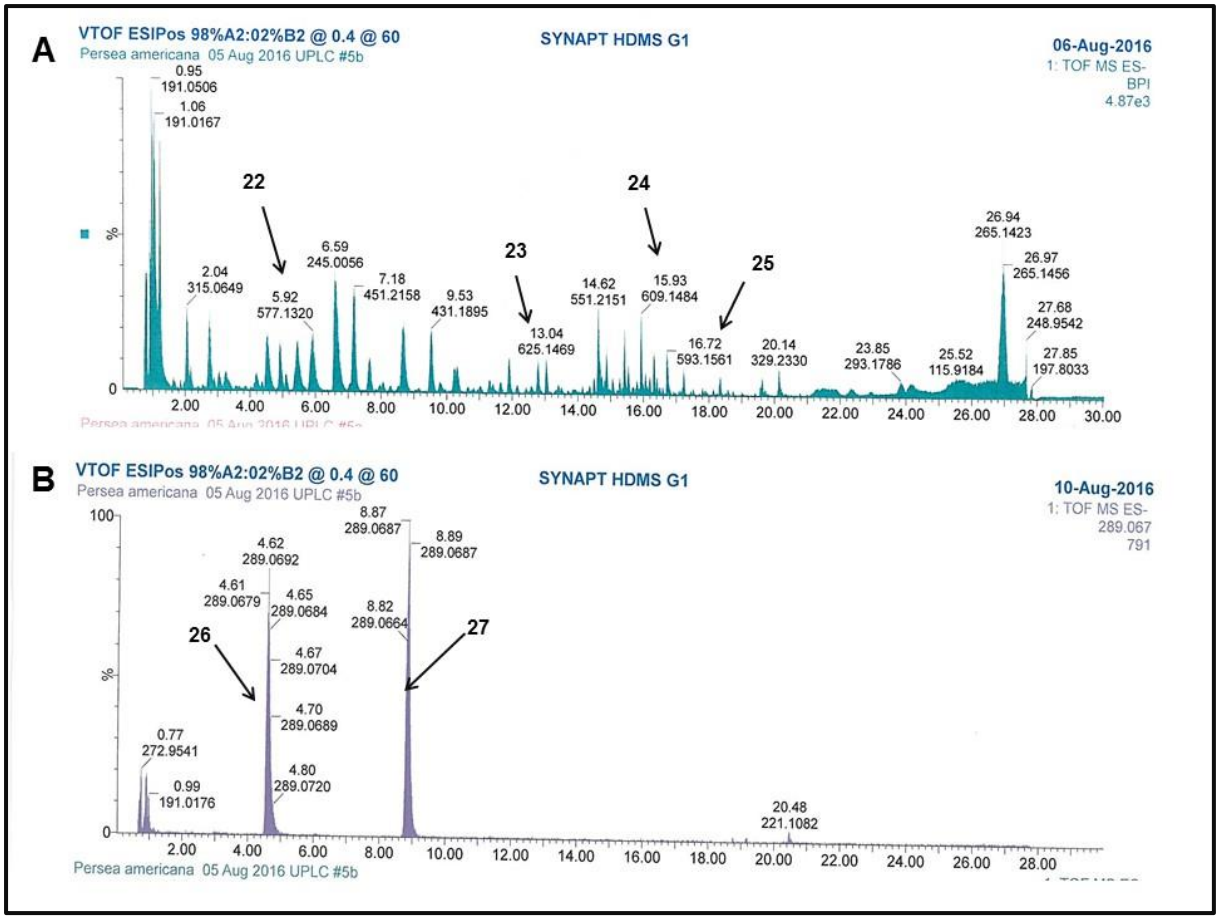


Figure 13: UPLC-ESI-MS chromatogram of the hot water extract prepared from the leaves of *P. americana* ran in electrospray ionization in negative mode. Procyanidin B1 (**22**); quercetin 3,7 diglucoside (**23**); rutin (**24**); nicotiflorin (**25**); catechin (**26**) and (-)-epicatechin (**27**) were identified

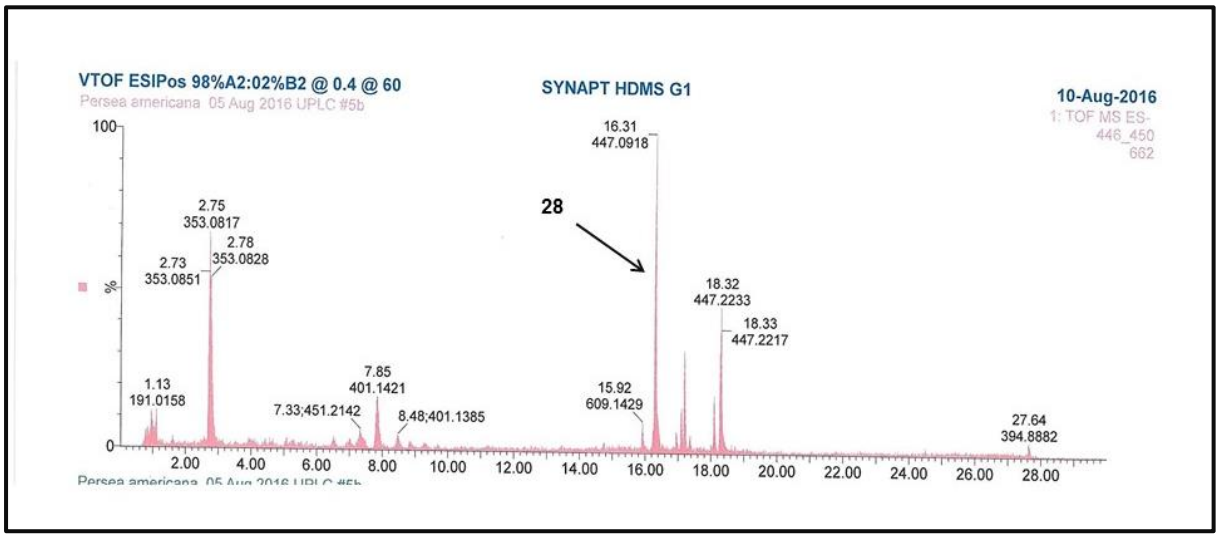
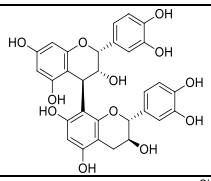
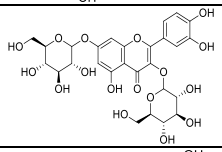
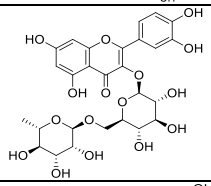
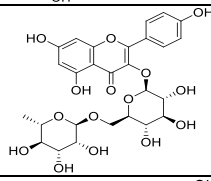
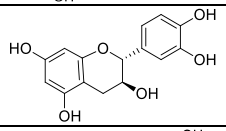
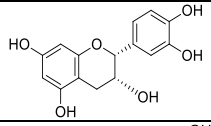
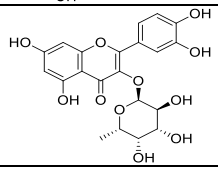


Figure 14: UPLC-ESI-MS negative ionisation mode chromatogram of the hot water extract prepared from the leaves of *P. americana*. Quercetrin (**28**) was identified

Table 11: A list of all the phytochemicals identified from the hot water extract of the leaves of *P. americana* by UPLC-ESI-MS

Compound number	Name	UPLC retention time (min)	Molecular weight ($g.mol^{-1}$)	Chemical formula	Chemical structure	Phytochemical class
22	Procyanidin B1	05.92	578.10	$C_{30}H_{25}O_{12}$		Flavonoid
23	Quercetin 3,7 diglucoside	13.04	626.52	$C_{27}H_{29}O_{17}$		Flavonoid
24	Rutin	15.93	610.52	$C_{27}H_{30}O_{16}$		Flavonoid
25	Nicotiflorin	16.72	594.52	$C_{27}H_{30}O_{15}$		Flavonoid
26	Catechin	04.62	290.26	$C_{15}H_{13}O_6$		Flavonoid
27	(-)-Epicatechin	08.89	290.27	$C_{15}H_{13}O_6$		Flavonoid
28	Quercetrin	16.31	448.38	$C_{21}H_{19}O_{11}$		Glycoside

Senecio serratuloides

The ultra-high-performance liquid chromatography-mass spectrometry (UPLC-ESI-MS) chromatograms of the hot water extract of *S. serratuloides* analysed in both ESI-negative and ESI-positive modes are provided in Figure 15. The Time-of-flight (TOF) mass spectrometric analysis led to the ionization and identification of two compounds namely 3-caffeoylquinic (**29**) acid and 3,4-dicaffeoylquinic acid (**30**) (Table 12). Compounds 3-caffeoylquinic and 3,4-dicaffeoylquinic acid were identified through tandem mass spectrometric analysis of non-significant peaks (peaks whose retention

times and *m/z* mass weren't captured on the chromatogram). These compounds are phenylpropanoids. TLC results revealed the presence of flavonoids in the extract of *S. serratuloides*, none of which were successfully identified through UPLC-ESI-MS.

There is limited scientific studies based on the phytochemical analysis of the *S. serratuloides* plant species. However, plant species belonging to the genus *Senecio* have been reported to contain pyrrolizidine alkaloids.[181]

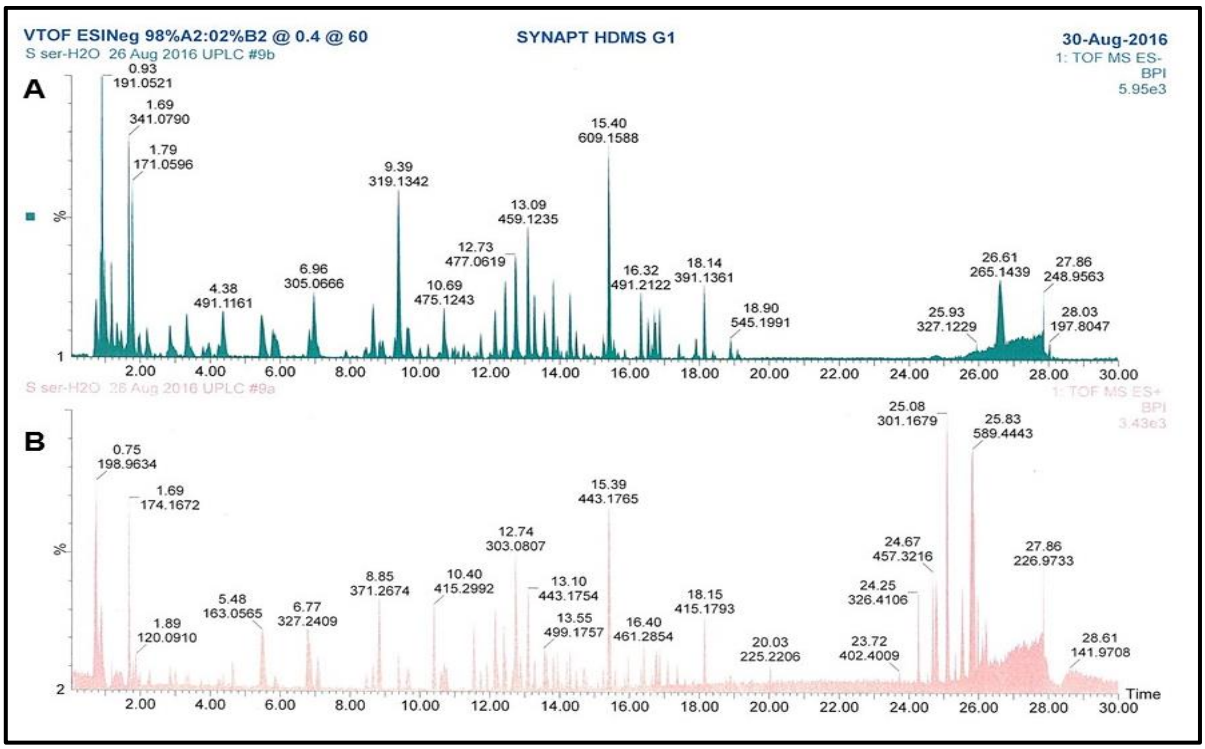
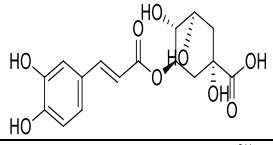
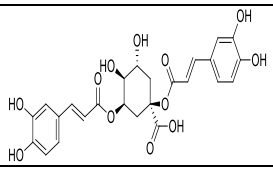


Figure 15: UPLC-ESI-MS chromatogram of the hot water extract of *S. serratuloides*. A) Electrospray ionization in positive mode. B) Electrospray ionization in negative mode

Table 12: Compounds identified from the hot water extract of *S. serratuloides* as determined by UPLC-TOF-ESI-MS.

Compound number	Name	Molecular weight	Chemical formula	Chemical structure	Phytochemical class
29	3-caffeoylquinic acid	354.311	$C_{16}H_{17}O_9$		Phenylpropanoid
30	3,4-Dicaffeoylquinic acid	516.455	$C_{16}H_{17}O_9$		Phenylpropanoid

Xysmalobium undulatum

The ultra-high-performance liquid chromatography-mass spectrometry (UPLC-ESI-MS) chromatogram of the hot water extract of *X. undulatum* analysed in both ESI-negative and ESI-positive modes are shown in Figure 16. The Time-of-flight (TOF) mass spectrometric analysis led to the ionization and identification of four compounds, corogluacigenin (**31**), uzarigenin (**32**), uzarin (**33**) and xysmalorin (**34**) (Table 13). The UPLC-ESI-MS retention times, molecular weights and chemical formulas of the identified compounds are indicated in Table 11 together with their chemical structures.

The previous studies on the phytochemical analysis based on the ethanol extract prepared from the roots of *X. undulatum* found the presence of uzarin, xysmalorin, allouzarin, alloxysmalorin, uzarigenin and xysmalogenin, with uzara as the major chemical constituent.[182,183] The findings of the current study are corroborated by previous phytochemical studies on the phytochemical composition of *X. undulatum*.

All the *X. undulatum* compounds identified in this study (**31**-**34**) are cardenolide glycosides, which have not been tested for by TLC in this study. TLC revealed the presence of sterols and terpenoids, of which none were identified by UPLC-ESI-MS in the current study. Isolation of compounds would have to be carried out in order to identify the compounds within the *X. undulatum* extract.

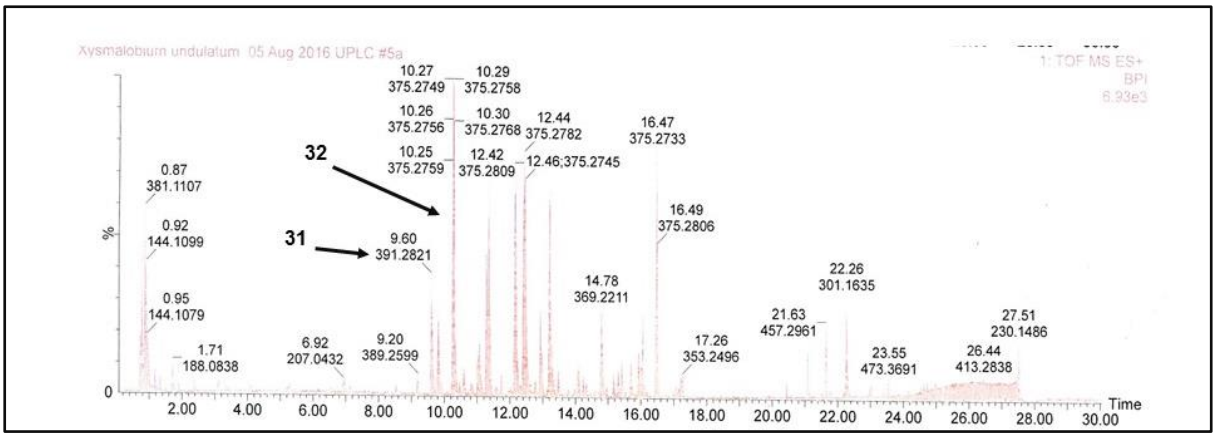
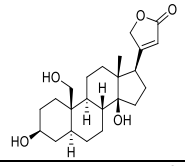
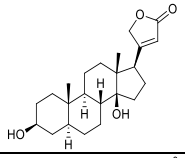
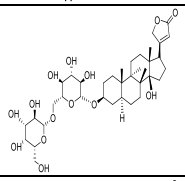
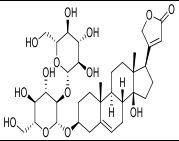


Figure 16: UPLC-ESI-MS Electrospray ionization in positive mode chromatogram of the hot water extract of *X. undulatum*. Corogluacigenin (**31**) and uzarigenin (**32**)

Table 13: A list of all the compounds identified from the hot water extract of the roots of *X. undulatum* as determined by UPLC-ESI-MS.

Compound number	Name	UPLC retention time (min)	Molecular weight ($g.mol^{-1}$)	Chemical formula	Chemical structure	Phytochemical class
31	Corogluacigenin	14.31	390.22	$C_{23}H_{33}O_5$		Cardenolide aglycone
32	Uzarigenin	10.27	374.25	$C_{23}H_{35}O_4$		Cardenolide aglycone
33	Uzarin	-	698.34	$C_{35}H_{53}O_{14}$		Cardenolide glycoside
34	Xysmalorin	-	696.33	$C_{35}H_{51}O_{14}$		Cardenolide glycoside

3.2. Cytotoxicity

There are a variety of cytotoxicity assays used in *in vitro* toxicological studies. The most commonly used assays for determination of cell viability include methyl tetrazolium (MTT) assay and the lactate dehydrogenase (LDH) leakage assay. A study conducted by Cordier and Steenkamp (2015)[184] compared four assays (neutral red uptake, sulphorhodamine B staining, MTT and resazurin conversion assays) for toxicity assessment on plant extracts. The sulphorhodamine B staining (SRB) assay was determined to be the most reproducible assay compared to the neutral red uptake and MTT assays.[184] In this study, SRB was used for cytotoxicity evaluation.

The sulphorhodamine B (SRB) assay relies on the uptake of the negatively charged pink aminoxanthine dye, sulphorhodamine B, by basic amino acids in the cells. The greater the number of cells, the greater amount of dye is taken up. After fixing, when the cells are lysed, the dye is released and gives a more intense colour and greater absorbance at 510 nm.[128] The SRB assay is sensitive, simple, reproducible[184] and more rapid and gives better linearity, a good signal-to-noise ratio and has a stable end-point that does not require a time-sensitive measurement than the formazan-based assays.[185]

The hot water extracts of *A. calamus*, *A. arborescens*, *A. afra*, *E. elephantina*, *E. lysistemon*, *P. americana* and *S. serratuloides* were not found to be cytotoxic to the THP-1 and U937 cell lines at concentration $\leq$ 100 µg/mL (Figure 17). All the above-mentioned extracts were associated with an increased cell density at lower concentrations (≤25 µg/mL) in both cell lines, though greater cell density was displayed with the U937 cell line. The difference in cell densities between the two cell lines could be due to difference in cellular response by the cells against the same treatment.

Similar findings with respect to the cytotoxicity of these individual plant species were found in some literature studies. For instance, the cytotoxicity of the ethanol and aqueous extracts prepared from the leaves of *A. afra* was evaluated on U937 and HeLa cancer cell lines using CellTiter-Blue® and MTT cell viability assays. It was found that both extracts were not toxic (IC_{50} = 18.21 µg/mL against U937 and 31.88 µg/mL against HeLa cells).[186] The ethanol leaf extract of *A. arborescens* was found to be non-

toxic to mouse melanocytes cells (B16-F10), where cell viability was more than 80% at the highest concentration of 500 µg/mL.[187]

The XTT and *Allium cepa* root tip assays were used to assess the cytotoxicity of the methanol and aqueous extracts of the rhizomes of *A. calamus*. The aqueous extract was found to be non-toxic with determined IC_{50} values of 63.65±8.30 µg/mL 85.22±11.40 µg/mL against human breast carcinoma (MDA-MB-435s) and human hepatoma (Hep3B) cells, respectively. On the other hand, the methanol extract was found to be cytotoxic with IC_{50} values of 13.71±6.66 µg/mL (MDA-MB-435s) and 32.73±4.55 µg/mL (Hep3B).[104] Moderate cytotoxicity was reported for the crude methanol extract and fractions (chloroform, ethanol, ethyl acetate and water) of the rhizomes of *A. calamus* on MCF-7 cell line with CTC_{50} of between 110-360 µg/mL.[188]

The aqueous extract prepared from the bulb of *E. elephantina* was investigated *in vitro* for cytotoxicity against raw macrophages, U937, MeWo and Vero cells using MTT cell viability assay. The *E. elephantina* was found to be non-cytotoxic with an inhibition of no greater than 20% even at the highest concentration of 125 µg/mL (U937) and 100 µg/mL (MeWo) and 50 µg/mL (macrophages).[189] The crude chloroform/methanol extract of the flowers and pods *E. lysistemon* was determined to be toxic with a LC_{50} of 23 ppm using the brine-shrimp lethality assay.[134] This is in contradiction with the results obtained in the current study, where the hot water extract of the stem of *E. lysistemon* was found to be not toxic.

On the other hand, the ethanol extract of the seed of *P. americana* was found to be non-toxic (IC_{50} = 99.74 µg/mL against MCF-7 and 100 µg/mL against Vero cells) while the n-hexane fraction was found to be cytotoxic with IC_{50} of 12.5 µg/mL against Vero cells and 80.05 µg/mL against MCF-7 cells.[190] Furthermore, the aqueous and ethanol extracts of the seeds of *P. americana* were found to be non-toxic against breast cancer cells (T47D) with IC_{50} values of 560.2 µg/mL and 107.15 µg/mL, respectively.[191]

S. serratuloides was investigated for wound healing activity and toxicity *in vivo* on pig models when applied for 16 days.[144] It was observed that the functions of the enzymes of the liver were no different to that of the controls and also no signs of liver

toxicity were observed on histological studies when *S. serratuloides* was applied topically on wounds generated in pigs.[144]

In the present study, the aqueous extract of *D. robusta* was found to be toxic at concentrations of ≥25 µg/mL in both cell lines with a 40-60% growth inhibition (Figure 17). However, the lowest dose of 0.781 µg/mL displayed a growth stimulatory effect on the cell lines. The IC_{50} of *D. robusta* against THP-1 and U937 cells was determined as 39.29±1.76 and 42.34±1.62 µg/mL, respectively. *D. robusta* was reported to be toxic to animals and humans in previous studies.[109] Patients taking traditional medication containing *D. robusta* have shown signs of toxicity, including vomiting, nausea and seizure.[109] Chemical analysis determined the presence of cardiac glycosides, bufadienolides and saponins as constituents. The toxicity of the plant is reported to be due to the presence of cardiac glycoside and caustic sap in the plant.[109] However, cardiac glycoside and bufadienolides were not detected/tested for in the current study, the toxicity displayed by *D. robusta* could be by the latter or other phytochemicals we are not aware of in the extract.

The aqueous extract of *X. undulatum* showed a concentration-dependant growth inhibitory effect on the THP-1 cells. The % cell survival plateaus at 50% from the 25 µg/mL concentration up to the highest concentration of 100 µg/mL. No toxicity was observed within the U937 cells by the same extract (Figure 17). The difference in cytotoxicity profiles between the two cell lines could be due to the fact that different cells respond differently to the same treatment. The U937 cells were more resilient to the *X. undulatum* extract as opposed to the THP-1 cells. A preliminary pilot randomized crossover study comprising of 60 female participants evaluated the efficacy for pain relief and safety of *X. undulatum*.[192] Treatment with 80 mg/8 hours for two doses, then 40 mg/8 hours for 5 days effectively relieved pain with no side effects or toxicities.[192]

The cytotoxicity results of the current study are in agreement with what was previously reported for *E. elephantina,*[189] *P. americana,*[190] *S. serratuloides,*[144] and *X. undulatum*[192] extracts which have been found to be non-toxic against the respective cell lines in each study. The extract of *D. robusta* was found to be toxic, similar to previous reports. However, the extracts of *A. calamus* and *E. lysistemon* have been

previously reported to be toxic, contradictory to the results in the current study, which were found to be non-toxic. The geographical location, season of harvesting, plant part and solvent of extraction are the reasons for the differences in toxicities observed within the same plant.[129,149,152,161] This is due to the fact that the latter results in the difference in the phytochemical profile of the same plant species which ultimately affect the pharmacological activity, explaining the difference in toxicity profiles.

Prijap Health traditional herbal mixture displayed moderate toxicity with a greater than 50% inhibition across a concentration gradient of 25-100 µg/mL in the THP-1 cells (Figure 17). No toxicity was observed within the U937 cells after treatment with Prijap Health traditional medicine. However, the lowest dose (0.781 µg/mL) showed a growth stimulatory effect in both cell lines. The IC_{50} of Prijap Health herbal concoction was determined as 74.52±1.31 µg/mL (Table 14) by non-linear aggression. Similarly, curcumin displayed toxicity at high doses with a greater than 50% growth inhibition in both the THP-1 and U937 cell lines, although lower doses had no effect on cell viability. The IC_{50} values are provided in Table 14.

Curcumin was the most cytotoxic test sample with the IC_{50} of 32.32±1.06 µg/mL and 38.14±1.15 µg/mL against THP-1 and U937 cells respectively. Curcumin, a polyphenol curcumoid, is a constituent of the rhizomes of *Curcuma longa* Linn plant species commonly referred to as turmeric. It is one of the active ingredients in turmeric and has been reported to target signalling molecules and to have multiple health benefits. It has been shown to have great potency as an antioxidant to relieve oxidative stress.[193,194] The anti-inflammatory effects of curcumin have also been investigated and reported.[195–197] Clinical studies have also demonstrated the anti-depressant efficacy and safety of curcumin in human subjects.[198,199]

With reference to the cytotoxicity results and the amount of each plant species used in the preparation of Prijap Health traditional herbal medicine, it is recommended that the amount of *D. robusta* used in the formulation be reduced since it displayed a high potential for toxicity in this study. *D. robusta* is the primary suspect for the moderate toxicity displayed by Prijap herbal concoction. However, further studies to investigate the interaction of the plants needs to be done to ascertain this.

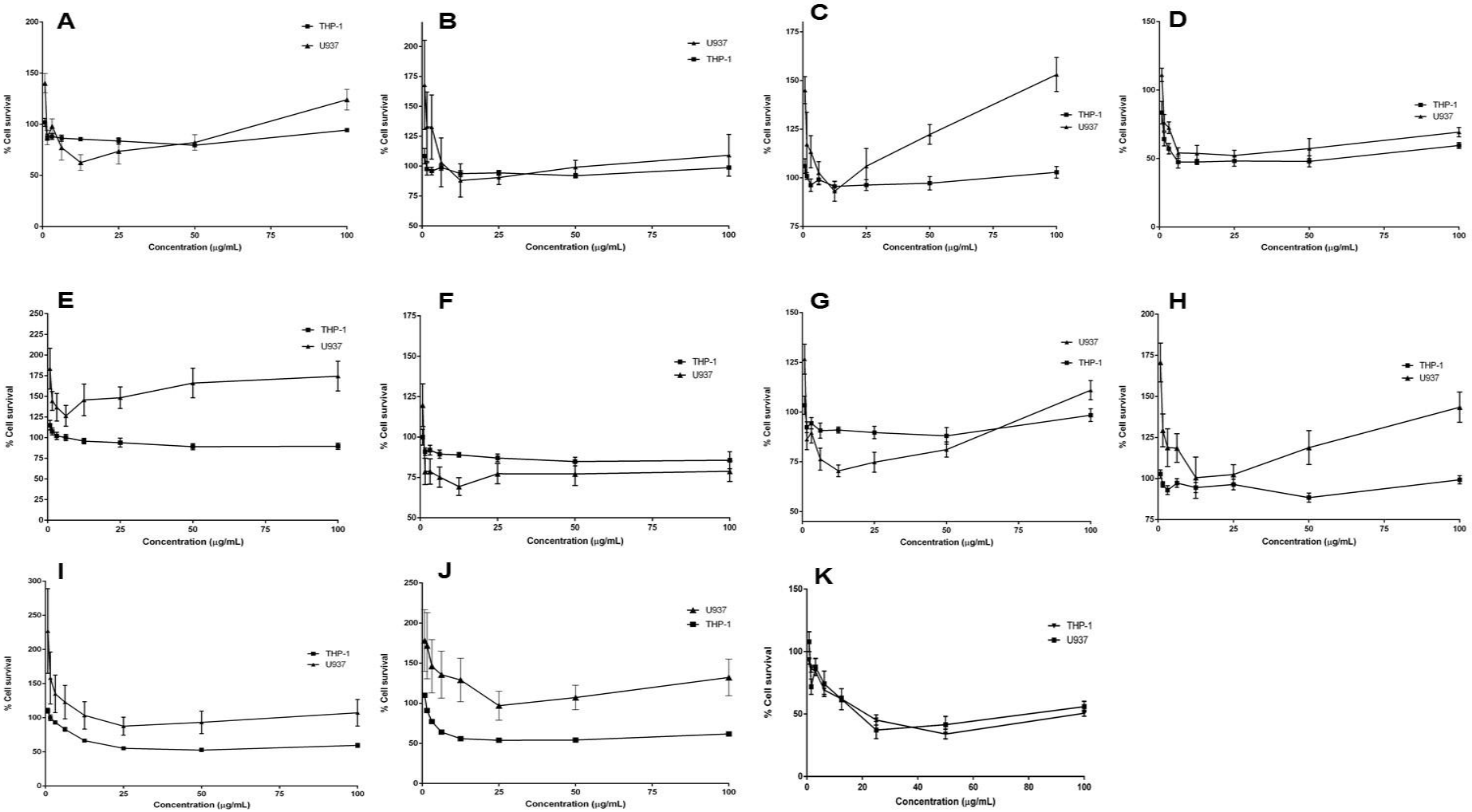


Figure 17: Effect of plant extract on viability of THP-1 (■) and U937 (▲) cells. A) *A. calamus*; B) *A. arborescens*; C) *A. afra*; D) *D. robusta*; E) *E. elephantina*; F) *E. lysistemon*; G) *P. americana*; H) *S. serratuloides*; I) *X. undulatum*; J) Prijap Health traditional herbal medicine; K) Curcumin

Table 14: The IC_{50} cytotoxicity concentrations (µg/mL) for THP-1 and U937 cells treated with hot water extracts of the individual plants and Prijap Health herbal traditional mixture

Plant	IC_{50} (µg/mL)		Toxicity
	THP-1	U937	
A. calamus	>100	>100	Non-toxic
A. arborescens	>100	>100	Non-toxic
A. afra	>100	>100	Non-toxic
E. elephantina	>100	>100	Non-toxic
E. lysistemon	>100	>100	Non-toxic
D. robusta	39.29±1.76	42.34±1.62	Toxic
P. americana	>100	>100	Non-toxic
S. serratuloides	>100	>100	Non-toxic
X. undulatum	76.05±1.21	-	Non-toxic
Prijap Health	74.52±1.31	-	Non-toxic
Curcumin	32.33±1.06	38.14±1.15	Toxic

-: IC_{50} not determined

3.3. Antioxidant activity

There are numerous techniques which have been developed to assess the antioxidant potential of food products and medicinal plant extracts.[200] Some of the most commonly used techniques in laboratories to investigate the antioxidant properties of medicinal plants include the DPPH free radical scavenging potential, the Trolox antioxidant potential which uses ABTS (TEAC), the oxygen radical absorption capacity (ORAC) and the ferric reducing antioxidant power (FRAP).[201,202] These assays measure the antioxidant potential by either single electron transfer or by hydrogen atom transfer mechanism.[200]

The DPPH radical scavenging assay is based on the reduction of an unstable $DPPH^+$ radical to a more stable radical by the antioxidant. The $DPPH^+$ radical has a purple colour and absorbs maximally at 517 nm.[203,204] When an antioxidant reacts with $DPPH^+$, the $DPPH^+$ is reduced to DPPHH by pairing off with an electron transferred by the antioxidant. As a result, decolorization from purple to a yellow colour occurs with

respect to the number of electrons captured.[204] The intensity of the decolorization (to yellow) is directly proportional to the $DPPH^+$ reducing ability of the antioxidant.[203,205]

The ABTS assay measures the ability of an analyte the ABTS in comparison to Trolox [Trolox antioxidant equivalence capacity (TAEC)]. The ABTS is generated in aqueous phase by reaction of a strong oxidizing agent (e.g. potassium persulfate) with ABTS salt to form a blue-green coloured ABTS radical.[206] ABTS in its radical form has a characteristic absorbance at 734 nm. When reacted with an antioxidant, the blue-green decolourization to a colourless ABTS occurs as a result of hydrogen donation by the antioxidant.[206]

Antioxidants are substances which can inhibit the oxidation of other molecules and are capable of removing potentially cell-damaging reactive oxygen species (ROS) in living organisms.[207] Medicinal plants have been shown to contain phytochemicals with antioxidant activity which prevent oxidative damage to cells.[208]

In this study, the majority of the plant species investigated were found to scavenge the DPPH radical, with the exception of *X. undulatum, E. lysistemon* and *A. calamus.* The hot water extracts of *A. arborescens, E. lysistemon, X. undulatum* and *A. calamus* did not elicit a 50% radical inhibition even at the highest concentration tested (100 µg/mL) as depicted in Figure 18. The negative values obtained for *X. undulatum* and *A. calamus* are possibly indicative of a pro-oxidative effect.

All the extracts with the exception of *X. undulatum* (at the highest concentration, 100 µg/mL) were found to have antioxidant activity when employing the ABTS radical scavenging assay (Figures 19). The activity was dose-dependent. The EC_{50} values are listed in Table 15. The radical inhibition activity of the extracts in this study is generally higher for the ABTS radical as compared to that of DPPH radical. This is probably due to the selectivity of the radicals on the basis of the stereo arrangement of the atoms.

Of all the extracts tested in this study, only *E. elephantina* extract displayed a high antioxidant activity [EC_{50} = 6.98±1.04 µg/mL (DPPH) and 2.45±1.05 µg/mL (ABTS)] with up to 95% (DPPH) and 90% (ABTS) radical inhibition. The antioxidant activity of

E. elephantina compares favourably with that of Trolox (positive control) with EC_{50} of 7.39±1.04 µg/mL against DPPH and 1.79±1.04 µg/mL against ABTS (Table 15). This is indication of good antioxidant capacity of the hot water extract of *E. elephantina*. Prijap Health herbal traditional medicine mixture had greater antioxidant activity than all the extracts of the individual plant species, with the exception of *E. elephantina*.

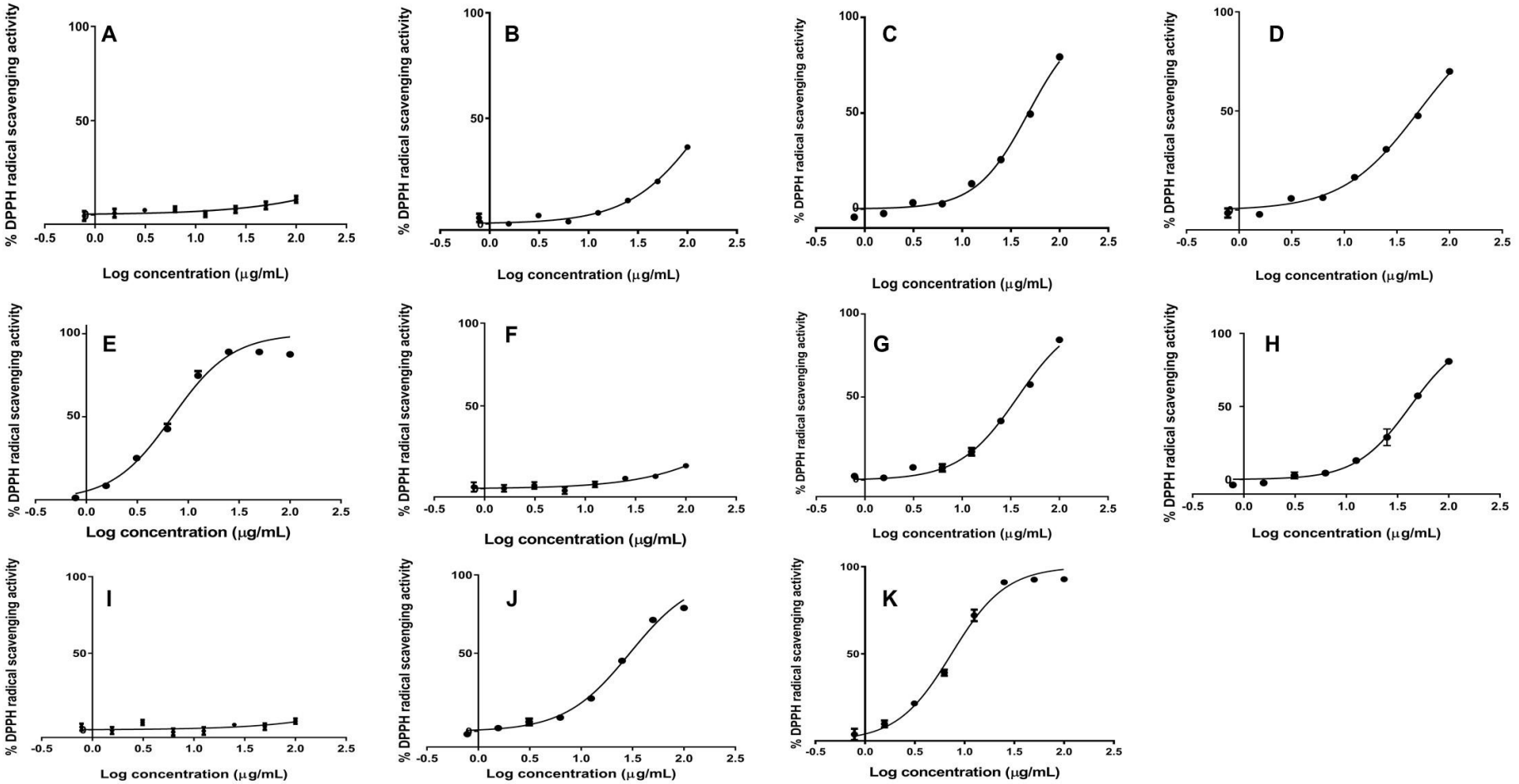


Figure 18: The percentage DPPH radical scavenging activity of the plant species investigated (n=9). A) *A. calamus;* B) *A. arborescens;* C) *A afra;* D) *D. robusta*; E) *E. elephantina;* F) *E. lysistemon;* G) *P. americana;* H) *S. serratuloides*; I) *X. undulatum;* J) Prijap Health herbal traditional medicine; K) Trolox (positive control)

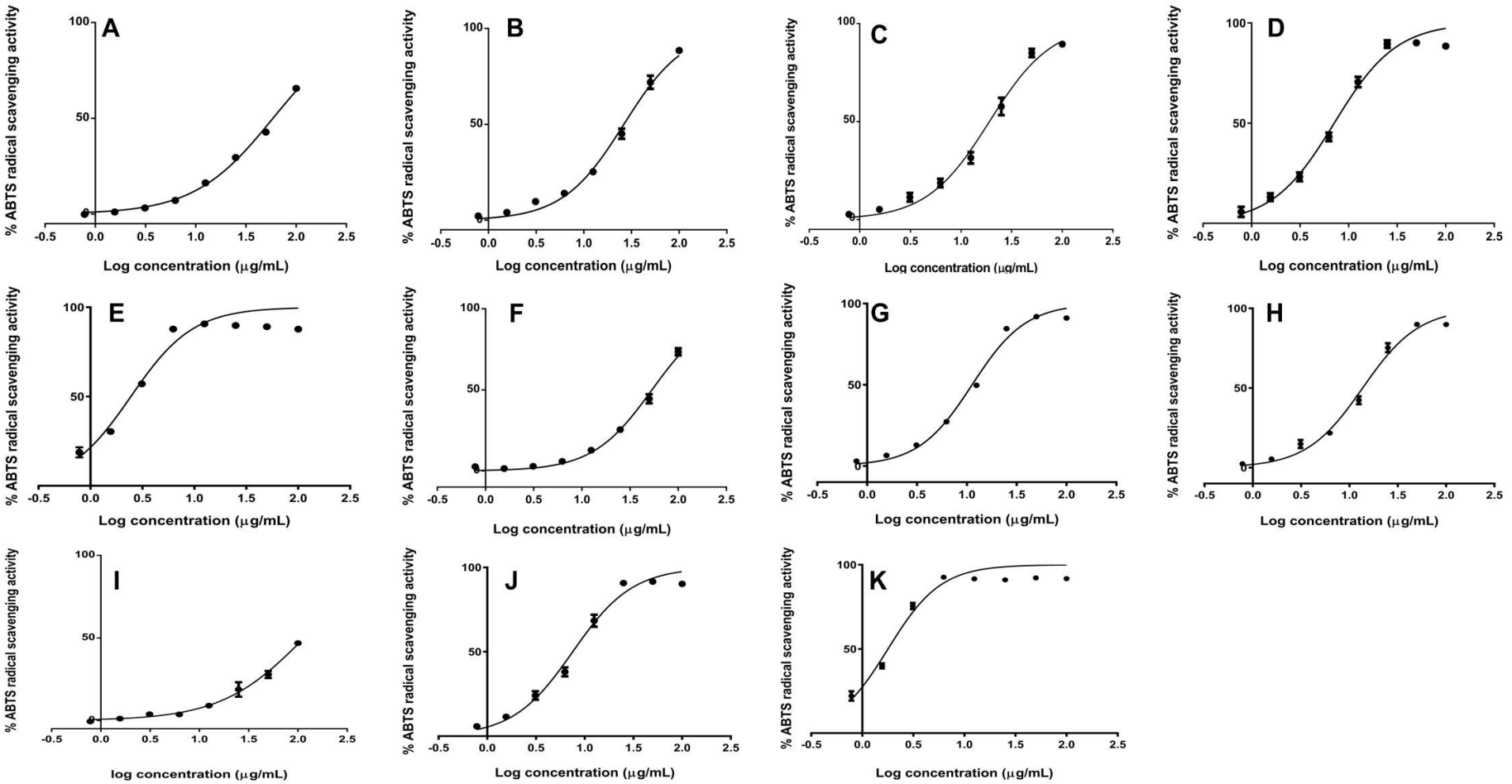


Figure 19: The percentage ABTS radical scavenging activity of plants investigated. A) *A. calamus;* B) *A. arborescens;* C) *A. afra;* D) *D. robusta*; E) *E. elephantina*; F) *E. lysistemon;* G) *P. americana;* H) *S. serratuloides;* I) *X. undulatum;* J) Prijap Health herbal traditional medicine; K) Trolox (positive control)

Table 15: The EC_{50}±SEM values for plant extracts as determined by the DPPH and ABTS radical scavenging assays

Extract	Antioxidant activity EC_{50} (µg/mL)	
	DPPH	ABTS
A. calamus	>100	58.72±1.02
A. arborescens	>100	26.31±1.03
A. afra	47.62±1.02	19.07±1.04
D. robusta	51.16±1.03	7.11±1.03
*E. elephantina**	6.98±1.04	2.45±1.05
E. lysistemon	>100	53.46±1.03
P. americana	37.33±1.03	11.30±1.02
S. serratuloides	42.03±1.04	13.70±1.03
X. undulatum	>100	>100
Prijap Health	29.75±1.03	7.54±1.04
Trolox^{+}	7.39±1.04	1.79±1.04

+: Positive control

*: Noteworthy DPPH radical scavenging activity compared to the positive control.

The aqueous extract of the leaves of *A. afra* has been reported to significantly (p<0.05) reduce the DPPH, ABTS and ferric radicals with more than 80% inhibition in a concentration dependent manner.[137] The antioxidant capacity of *A. afra* aqueous extract was investigated in streptozotocin-enduced diabetic rats. Its ability to reduce oxidative stress by reducing lipid peroxidase, glutathione peroxidase, superoxide dismutase and glutathione levels were reported.[137] The results of the current study are corroborated by previous studies.

Aqueous extracts of *A. calamus* were found to contain antioxidant activity against DPPH and ferric radicals.[209] The present results are corroborated for *E. elephantina* where the water extracts were found to have DPPH scavenging activity.[113]

With the use of DPPH and ferric reducing power antioxidant assays, aqueous and methanol extracts of *P. americana* were found to have antioxidant activity.[210] The ethanol extract of *P. americana* has been found to have a radical inhibition of 58.28%

against DPPH while the essential oil extracts have been shown to possess a high radical scavenging activity with an IC_{50} of 4.68±0.02 mg/mL.[142]

Juma *et al*[134] investigated and reported the antioxidant activity of crude water-methanol extract of *E. lysistemon* using the DPPH radical scavenging assay. In the study, *E. lysistemon* was reported to have a moderate (IC_{50} = 56 µg/mL) DPPH radical inhibition activity. Further isolation of individual compounds was done and were also evaluated for their antioxidant activity against the DPPH free radical.

Lucini *et al*[162] evaluated and reported the antioxidant activity of *A. arborescens* using the DPPH and ORAC assays and reported the IC_{50} as 71±07 µM and 26±08 µM Trolox equivalences respectively. The antioxidant activity of the boiled leaf extract of *A. arborescens* was investigated by Sonoda *et al*[211] in alloxan and streptozotocin-enduced induced hydroxyl (-OH) radicals in pancreatic islet B cells. The authors reported *A. arborescens* to protect the B cells destruction by alloxan, streptozotocin or hypoxanthine-xanthine oxidase.

Fawole *et al.*[120] determined the antioxidant activity of *S. serratuloides* using the DPPH radical scavenging assay. The aqueous-methanol extract of the *S. serratuloides* was reported to have antioxidant activity with a determined IC_{50} of 10.40±0.42 µg/mL. Adewusi and Steenkamp[212] reported a radical scavenging activity of less than 50% at the highest dose administered (0.125 mg/mL) when evaluating *X. undulatum* using the DPPH assay. Research on the antioxidant activity of *D. robusta* is scarcely reported in literature. There is limited scientific research done pertaining to the pharmacology and phytochemistry of the *D. robusta* plant species.

The *X. undulatum* extract had the least radical scavenging activity in this study, with the EC_{50} of >100 µg/mL in both DPPH and ABTS assays. This is in agreement with what was previously reported in a study by Adewusi and Steenkamp[212] where *X. undulatum* was found to not elicit a 50% DPPH inhibition even at the highest dose concentration tested (0.125 mg/mL).

The phytochemical analysis results (Section 3.2) revealed the presence of flavonoids, phenolic acids, terpenoids and saponins in the hot water extracts. These secondary plant metabolites have been demonstrated extensively to have great potency as

antioxidants, anti-inflammatory, anti-bacterial and anti-cancer agents.[70,213–215] Gallic acid (phenolic acid) and flavonoids such as catechin, (-)-epicatechin, taxifolin, rutin, and quercetin (including their glucosides) are compounds with potent antioxidant activity[216–220] and have been identified in the extracts of *E. elephantina* and *P. americana* in the current study. Rutin was identified in the extract of *A. afra*. The antioxidant activity displayed by these extracts against DPPH and ABTS radicals is therefore attributed to the above-mentioned compounds. Phenolic acids, flavonoids and saponins were not detected in the extracts of *X. undulatum*, *E. lysistemon* and *D. robusta*. This could explain the poor antioxidant activity displayed by these extracts.

In the present study, it is worth noting that the EC_{50} values for antioxidant activity of the extracts are way lower than the IC_{50} (IC_{50}>100 µg/mL) values for cytotoxicity as determined by the SRB assay. This implies that the extracts and Prijap Health have potency as antioxidants at concentrations that are non-toxic.

3.4. Anti-inflammatory activity

Inflammation is a process that occurs when infectious microorganisms such as viruses, bacteria or fungi invades the body and also during tissue injury and cell death.[26,221] The innate immune response is the first line of defence against invading pathogens or tissue injury. Inflammatory mediators are synthesised and secreted by various macrophages during inflammation. These can be either pro- or anti-inflammatory mediators and include ILs, IFNs, TNFs and PGs.[29,221,222]

IL-1β, TNF-α and INF-ɣ are potent pro-inflammatory mediators which are secreted from various macrophages. The TNF-α has been linked with immune and inflammatory diseases such as cancer and psychiatric disorders. Interleukins such as IL-10 and IL-4 on the other hand, are potent anti-inflammatory mediators and their activity exceeds that of pro-inflammatory mediators.[30,31] Other inflammatory mediators such as IL-6, cytokines of the IL-12 family and IL-1α have both pro- and anti-inflammatory properties.[24] Prostaglandin E_2 is a cell signalling molecule produced in the arachidonic acid pathway mediated by cyclooxygenases (COX-1 & COX-2) and prostaglandin synthases. The PGE_2 molecules are produced and secreted in response to stimulation by an invading pathogen or tissue injury. PGE_2 plays an important role in regulating

the immune system by initiating the inflammatory response, cytokine expression and T cell activation. Excessive amounts of PGE_2 have been shown to shift the immune response from Th1 to Th2 which leads to a reduced protective ability against pathogens.[221]

3.4.1. Prostaglandin E2 determination

Enzyme-linked immunosorbent assay (ELISA) is an immunoassay plate technique that is used to detect and quantify antibodies, proteins, hormones etc. It is a modification of the radioimmunoassay whereby an enzyme is used as opposed to a radioactive label.[223] The ELISA assay relies on antibodies (capture antibodies – usually coated to a 96-well microtiter plate) to detect antigens by using antibodies conjugated to an enzyme substrate and a fluorophore.[224] Reaction of the enzyme results in colour change, which is indication of a positive reaction (presence of analyte of interest) and the absorbance can be measured spectrophotometrically.[223,224] The ELISA assay has a high sensitivity and specificity, provides a higher accuracy, requires less sophisticated equipment, is relatively easy to perform and the results are reproducible. The disadvantages of ELISA are that are the temporary readouts, that is, readouts need to be read in a short span of time because detection is based on enzyme-substrate reaction and the colour change diminishes quicker.[225,226]

In the current study, LPS significantly (p<0,05) stimulated PGE_2 secretion in both THP-1 and U937 cells (Figure 20). Both the extracellular and intracellular PGE_2 levels were significantly (p<0,05) decreased by curcumin (positive control) in THP-1 cells. Prijap Health displayed a significant (p<0,05) decrease in intracellular and extracellular PGE_2 levels in THP-1 cells upon LPS-stimulated PGE_2 production and secretion (Figure 20).

With regards to the individual plant extracts investigated, the *D. robusta* and *X. undulatum* extracts significantly reduced both extracellular and intracellular PGE_2 levels (Figure 20). The *E. elephantina* and *A. afra* extracts displayed a pro-inflammatory response by significantly increasing PGE_2 levels in U937 cells. Although not statistically significant, the extract of *E. elephantina* exhibited an increase in both extracellular and intracellular PGE_2 levels in THP-1 cells (Figure 20). The remainder

of the extracts had no significant effect in neither increasing nor decreasing PGE_2 levels in both cell lines.

A significant decrease in the PGE_2 levels secreted into the extracellular environment is indicative of anti-inflammatory activity. It has been established that HIV+ patients have increased levels of PGE_2 which contribute to the severity of CD4+ cells destruction by altering Th1/Th2 response and exacerbation of the inflammatory response.[227] Prijap Health herbal traditional medicine mixture can potentially help overcome this effect by effectively inhibiting PGE_2 production and secretion and thus curbing the excessive inflammatory response.

The overall result of reduction in PGE_2 levels by Prijap Health herbal traditional medicine can be attributed to the *D. robusta* and *X. undulatum* extracts. However, this does not rule out the fact that this overall effect is a result of the interaction between all the extracts acting either synergistically, additively or antagonistically to display the observed result.

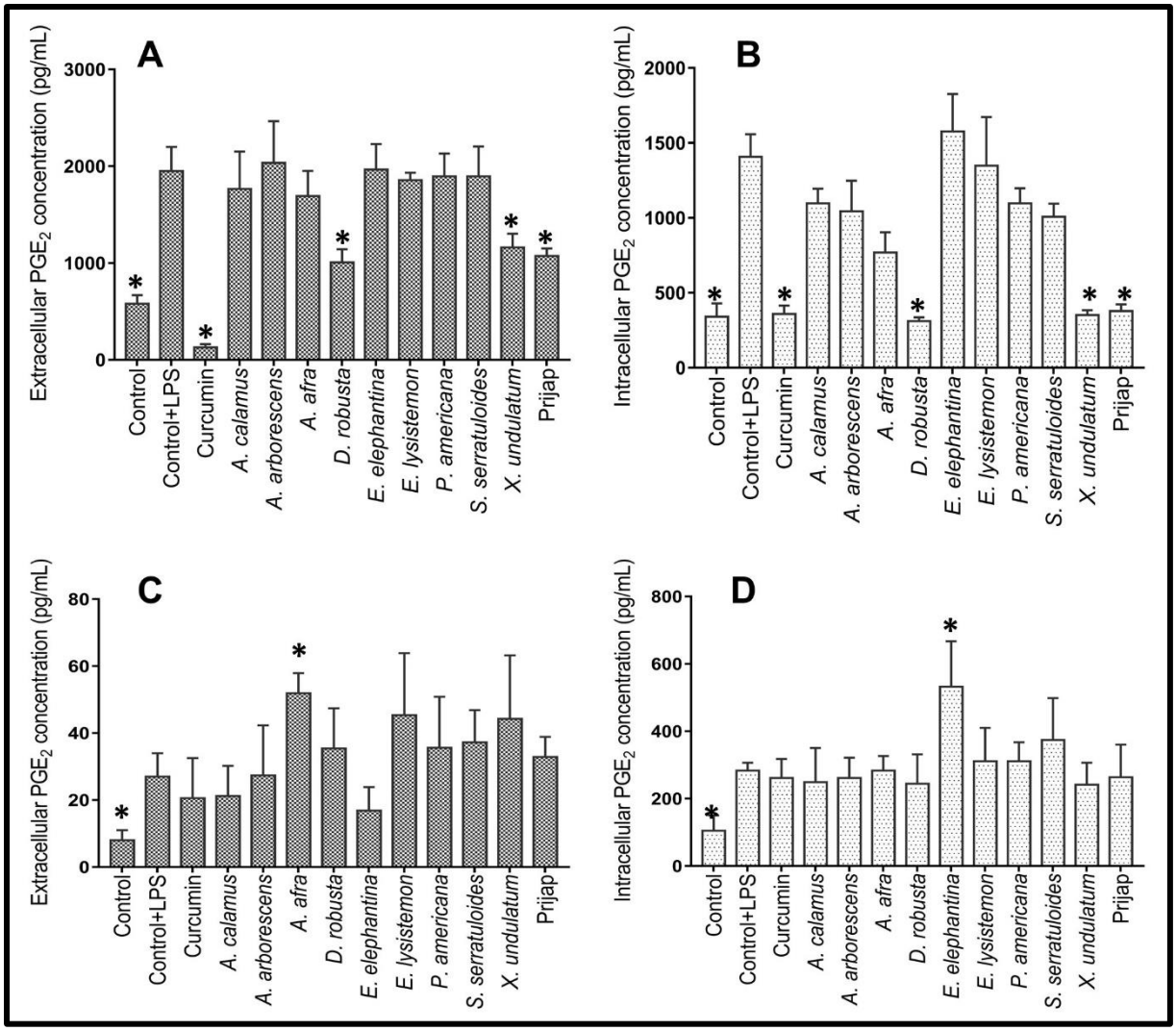


Figure 20: Determination of PGE_2 concentration in the presence of plant extracts. A) Extracellular PGE_2 concentration in THP-1 cells; B) Intracellular PGE_2 concentration in THP-1 cells; C) Extracellular PGE_2 concentration in U937 cells; D) Intracellular PGE_2 concentration in U937 cells. *: statistically significant difference compared to the control+LPS $p<0,05$

3.4.2. Cytokine analysis

The intracellular and extracellular IL-1β secretion, when treated with extracts of *A. calamus*, *E. elephantina*, Prijap Health, significantly ($p<0,05$) decreased in both cell lines as compared to the control (Figure 21). Curcumin (positive control) also significantly ($p<0,05$) decreased IL-1β concentration in both cell lines (Figure 21).

The extracellular and intracellular concentrations of IL-2, IL-4, IL-6, IL-8, IL-12p70 and IL-17A in differentiated THP-1 and U937 macrophages are provided in Figures 22 to

28. No significant changes were observed with regards to the concentrations of the cytokines when compared to the control and when treated with the plant extracts or curcumin (positive control) in either of the cell lines.

The extracts of *E. elephantina* and *A. arborescens* extracts showed a significant increase in both extracellular and intracellular IL-10 concentration in both THP-1 and U937 cells (Figure 26). The extract of *P. americana* significantly ($p<0.005$) increased extracellular IL-10 secretion only in THP-1 cells. Curcumin induced an increase in IL-10 concentration in both cell lines, however a significant increase in intracellular IL-10 was observed in THP-1 cells only (Figure 26). The remainder of the extracts, including Prijap Health, showed no noteworthy changes in IL-10 concentration as compared to the control in both cell lines.

The secretion of the IL-12p70 (Figure 27) and IL-17A (Figure 28) did not change significantly when treated with plant extracts and positive control (curcumin). With regards to IFN-γ, curcumin (positive control), *A. afra* extract and Prijap Health significantly ($p<0.05$) decreased the extracellular and intracellular IFN-γ concentration in both U937 and THP-1 cells (Figure 29). The TNF-α secretion was significantly ($p<0.05$) decreased by *A. calamus* extract, Prijap Health and the positive control (curcumin) (Figure 30).

A decrease in IL-1β, TNF-α, IFN-γ, PGE_2 and an increase in IL-10 secretions is indicative of an anti-inflammatory activity. A summary of the most noteworthy alterations in cytokine secretion is provided in Table 16. Prijap Health traditional herbal medicine has great potency as an anti-inflammatory agent as it displayed a significant decrease in pro-inflammatory mediators in IL-1β, TNF-α, IFN-γ and PGE_2. The role of these pro-inflammatory cytokines and PGE_2 towards the severity of CD4+ destruction during HIV infection has been described earlier. Prijap Health can act as an effective immunosuppressant by suppressing the immune activation that is caused by HIV through inhibition of pro-inflammatory cytokine secretion and PGE_2. The hot water extracts of *A. afra*, *A. calamus*, *E. elephantina* and *P. americana* have been found to significantly reduce pro-inflammatory cytokines as well, although fewer cytokines were affected by individual extracts as opposed to Prijap Health, which affected secretion

of multiple cytokines. On the other hand, the hot water extract of *A. arborescens* increased the secretion of a potent anti-inflammatory IL-10 cytokine.

Water, ethanol and ethyl acetate extracts of *D. robusta* have been previously reported to inhibit cyclooxygenase enzyme activity.[109,111] This is contrary to the results obtained for the hot water extract of *D. robusta* in the current study, which showed no significant alterations in cytokine and PGE_2 secretions in both THP-1 and U937 cells. The aqueous extracts of *P. americana* were found to reduce carrageen-induced rat paw oedema in a dose-dependent manner, which is indicative of the anti-inflammatory activity of *P. americana*.[210]

The aqueous extracts of *E. elephantina* have been found to reduce carrageen-histamine induced inflammation and pain in Wilstar rats being superior to indomethacin.[114] In contrary, it has been implied that the *E. elephantina* may possess pro-inflammatory effects as it induced nitric oxide (NO) secretion in resting macrophages.[189] The anti-inflammatory results of the current study for *E. elephantina* water extract are in agreement with the reports of Maphosa *et al*[114], which found *E. elephantina* extract to have anti-inflammatory activity.

The anti-inflammatory ability of the methanol extract of the leaves of *A. arborescens* was demonstrated by inhibition of cyclooxygenase enzymes, which also infers the inhibition of PGE_2 production by the extract.[164] The ethanol extract of *A. arborescens* was found to act as an immune-stimulant by upregulating the transcription of IL-1*β*, COX-2, TGF-*β* and TNF-α immune related genes in SAF-1 cells.[228] The ability of *A. afra* ethanol extracts to reduce the IL-10, IFN-ɣ and IL-6 cytokine concentrations *in vitro* has been shown.[229]

Moreover, Shi *et al.*[230] evaluated the anti-inflammatory activity of *A. calamus* in RAW 264.7 cells following stimulation with LPS. The authors reported the aqueous extract of *A. calamus* to effectively inhibit mRNA expression of TNF-α, IL-6, IL-1β and iNOS - all of which are pro-inflammatory mediators. The immunomodulatory activity of the volatile oil, alcohol and petroleum ether extracts of *A. calamus* was further demonstrated by phagocytosis stimulation test and nitro-blue tetrazolium assays in human neutrophil cells.[231]

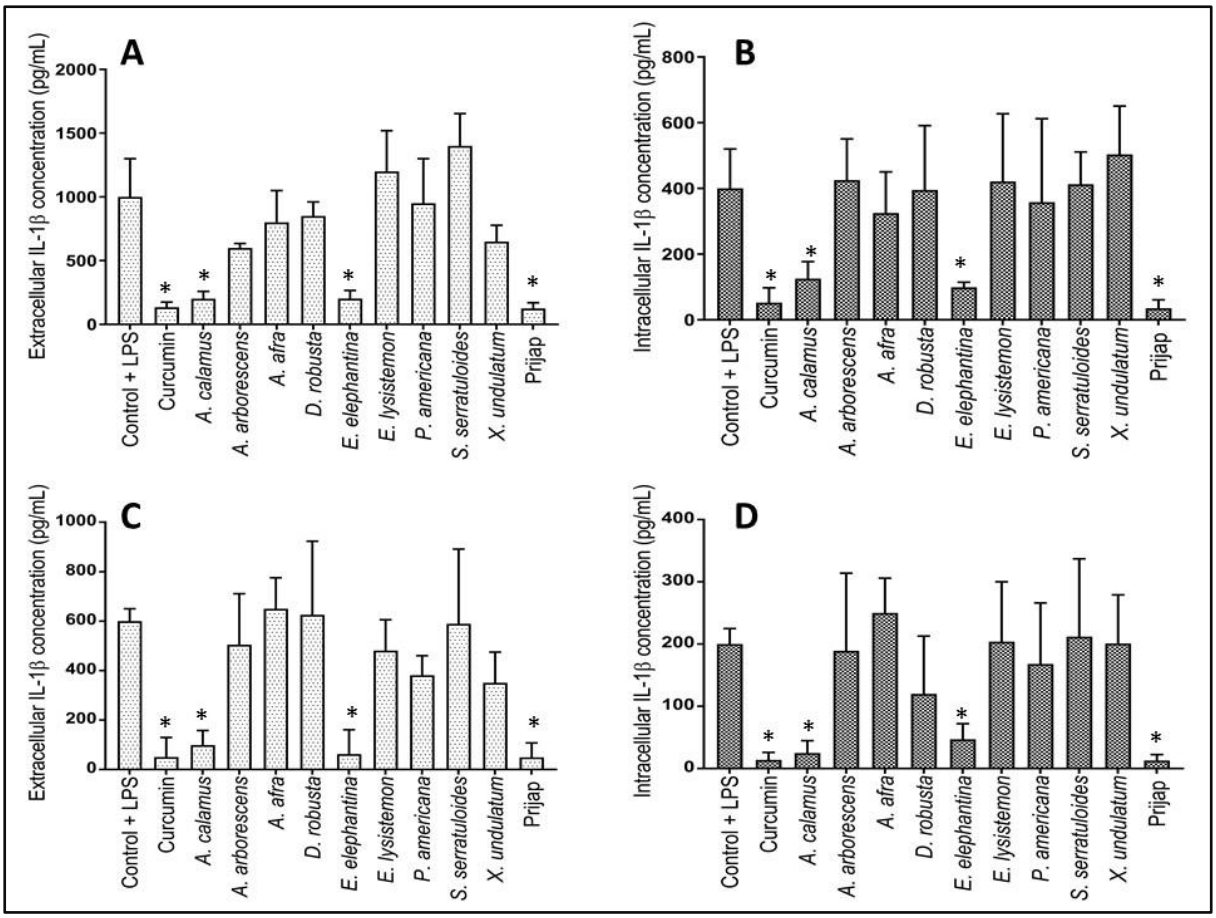


Figure 21: Concentration of IL-1β in differentiated THP-1 and U937 cells. A) Extracellular IL-1β in THP-1 cells, B) intracellular IL-1β in THP-1 cells, C) extracellular IL-1β in U937 cell, C) intracellular IL-1β in U937
*****: Indicates statistically significant difference relative to the control (p<0,05).

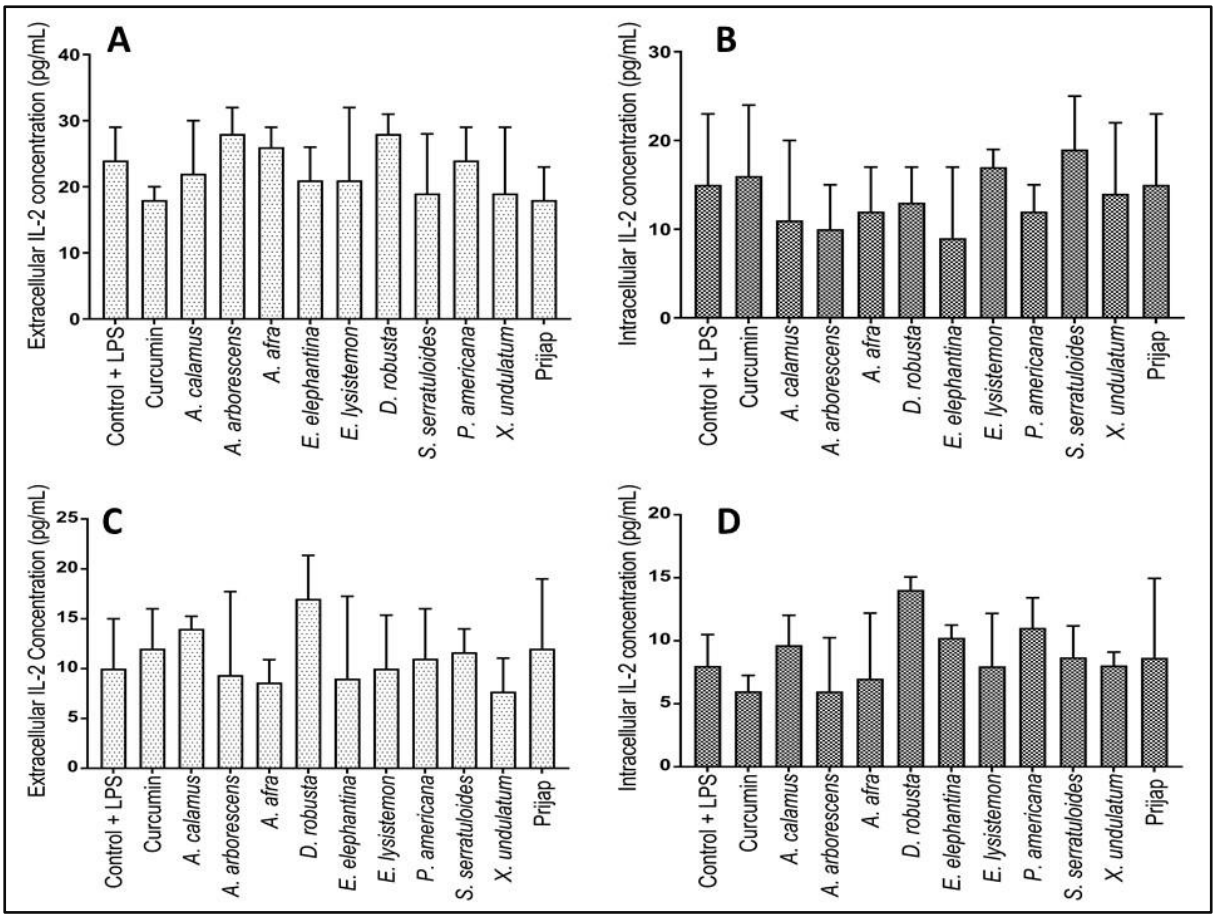


Figure 22: Concentration of IL-2 in differentiated THP-1 and U937 cells. A) Extracellular IL-2 concentration in THP-1 cells, B) intracellular IL-2 in THP-1 cells, C) extracellular IL-2 in U937 cell, D) intracellular IL-2 in U937

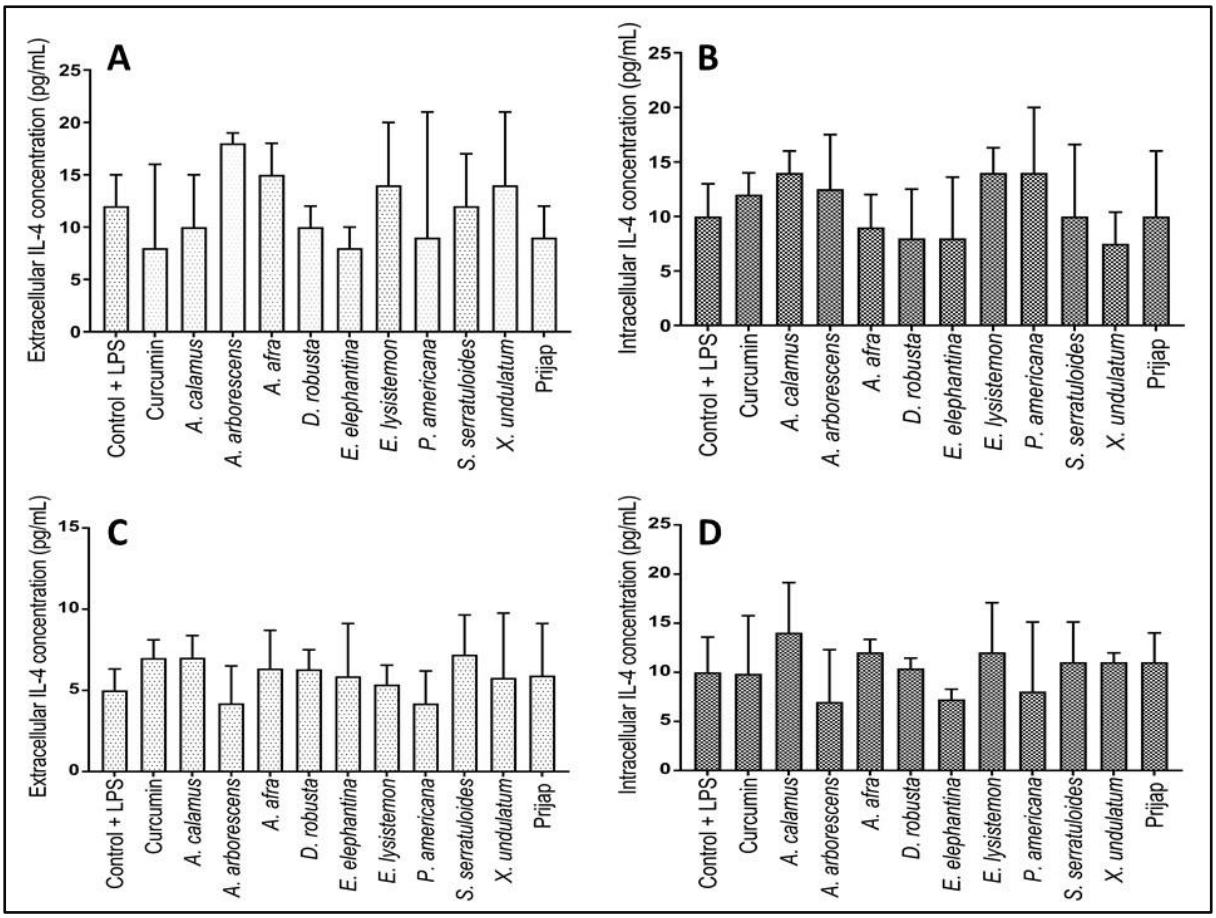


Figure 23: Concentration of IL-4 in differentiated THP-1 and U937 cells. A) Extracellular IL-4 in THP-1 cells, B) intracellular IL-4 in THP-1 cells, C) extracellular IL-4 in U937 cell, D) intracellular IL-4 in U937.

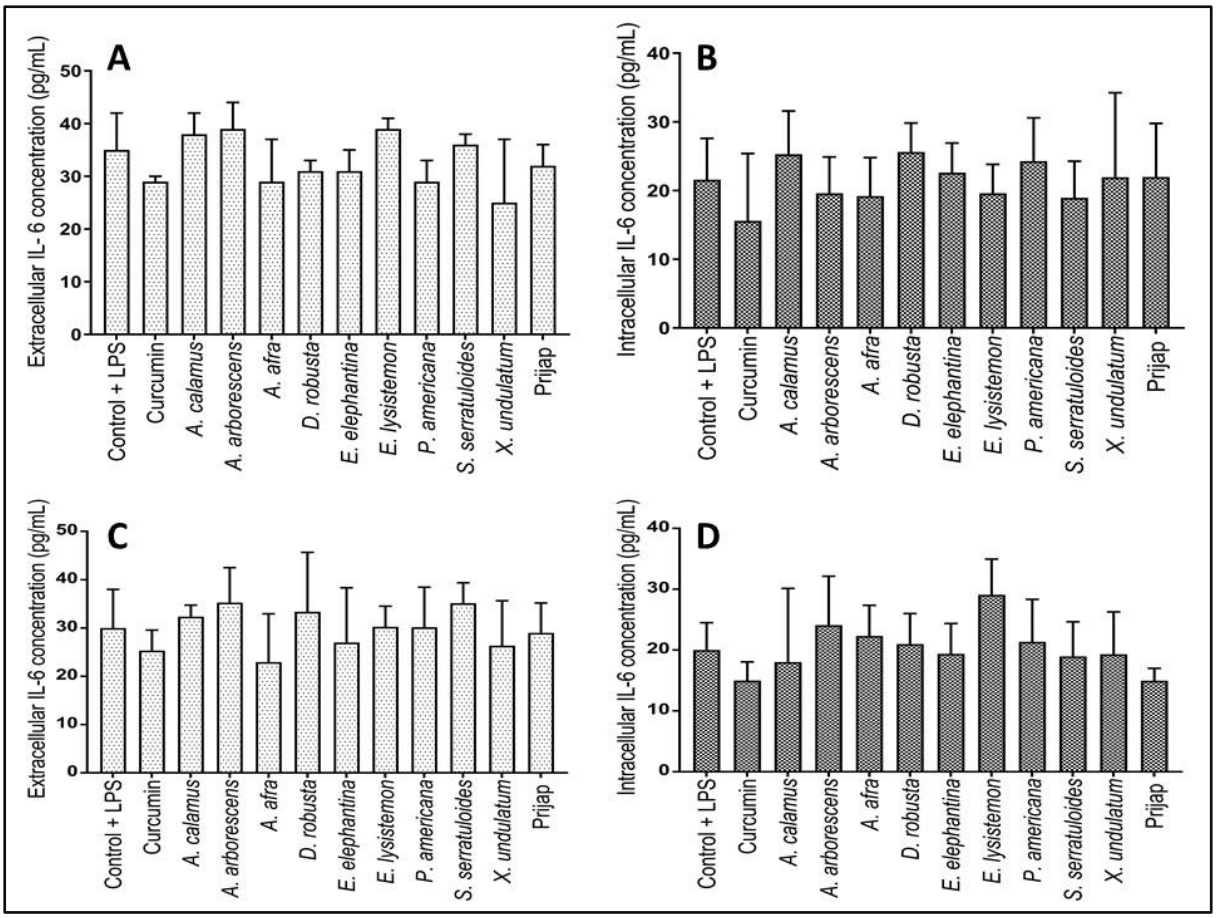


Figure 24: Concentration of IL-6 in differentiated THP-1 and U937 cells. A) Extracellular IL-6 in THP-1 cells, B) intracellular IL-6 in THP-1 cells, C) extracellular IL-6 in U937 cells, D) intracellular IL-6 in U937 cells.

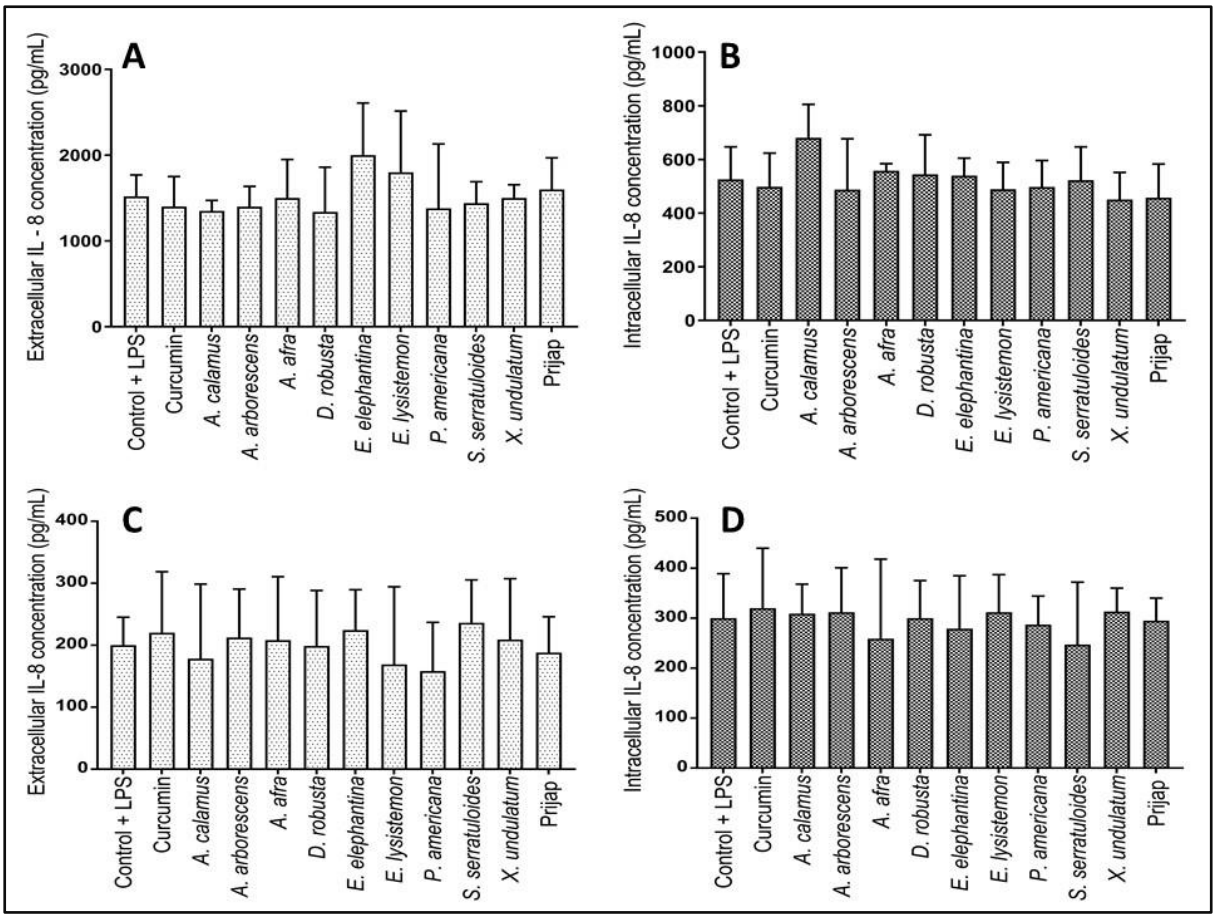


Figure 25: Concentration of IL-8 in differentiated THP-1 and U937 cells. A) Extracellular IL-8 in THP-1 cells, B) intracellular IL-8 in THP-1 cells, C) extracellular IL-8 in U937 cells, D) intracellular IL-10 in U937

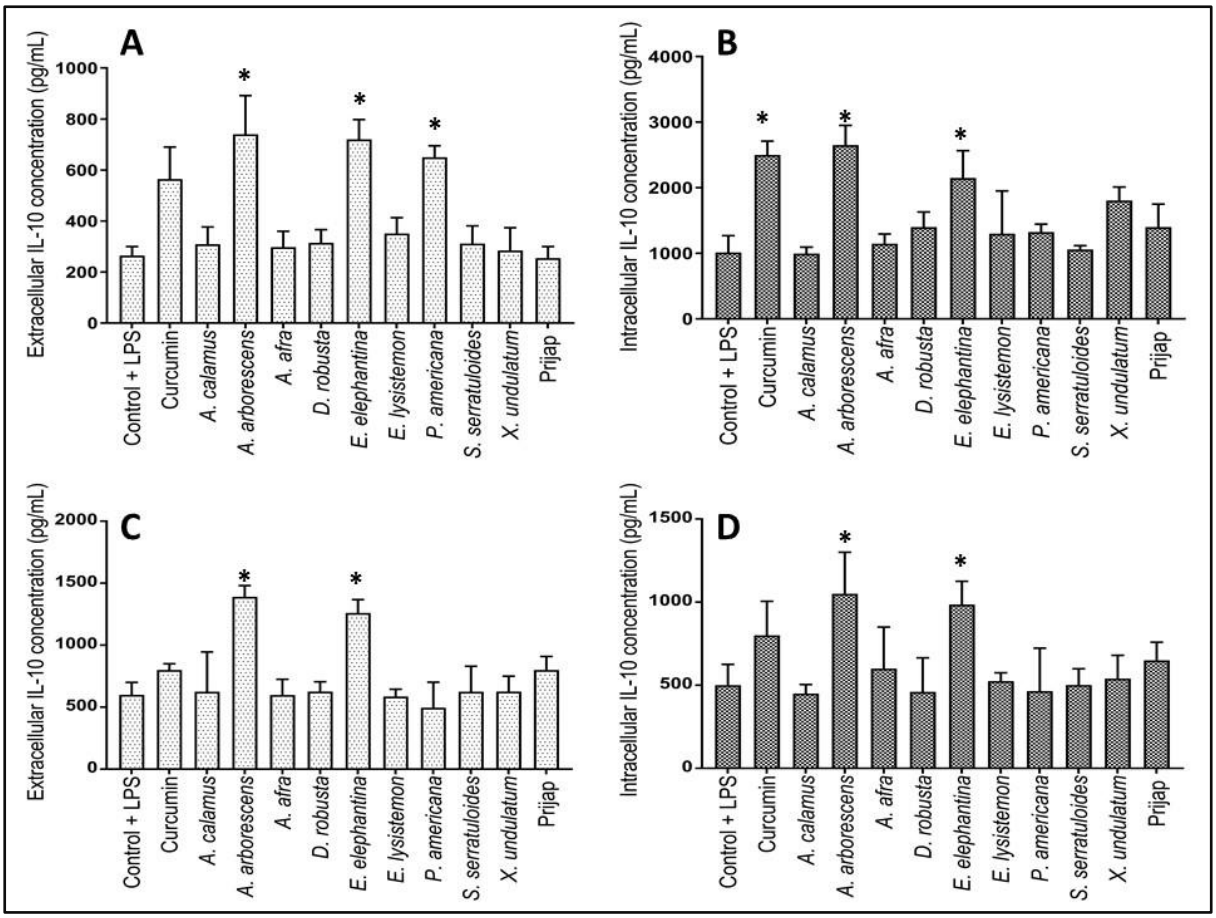


Figure 26: Concentration of IL-10 in differentiated THP-1 and U937 cells. A) Extracellular IL-10 in THP-1 cells, B) intracellular IL-10 in THP-1 cells, C) extracellular IL-10 in U937 cells, D) intracellular IL-10 in U937
***:** Indicates statistically significant difference relative to the control (p<0,05)

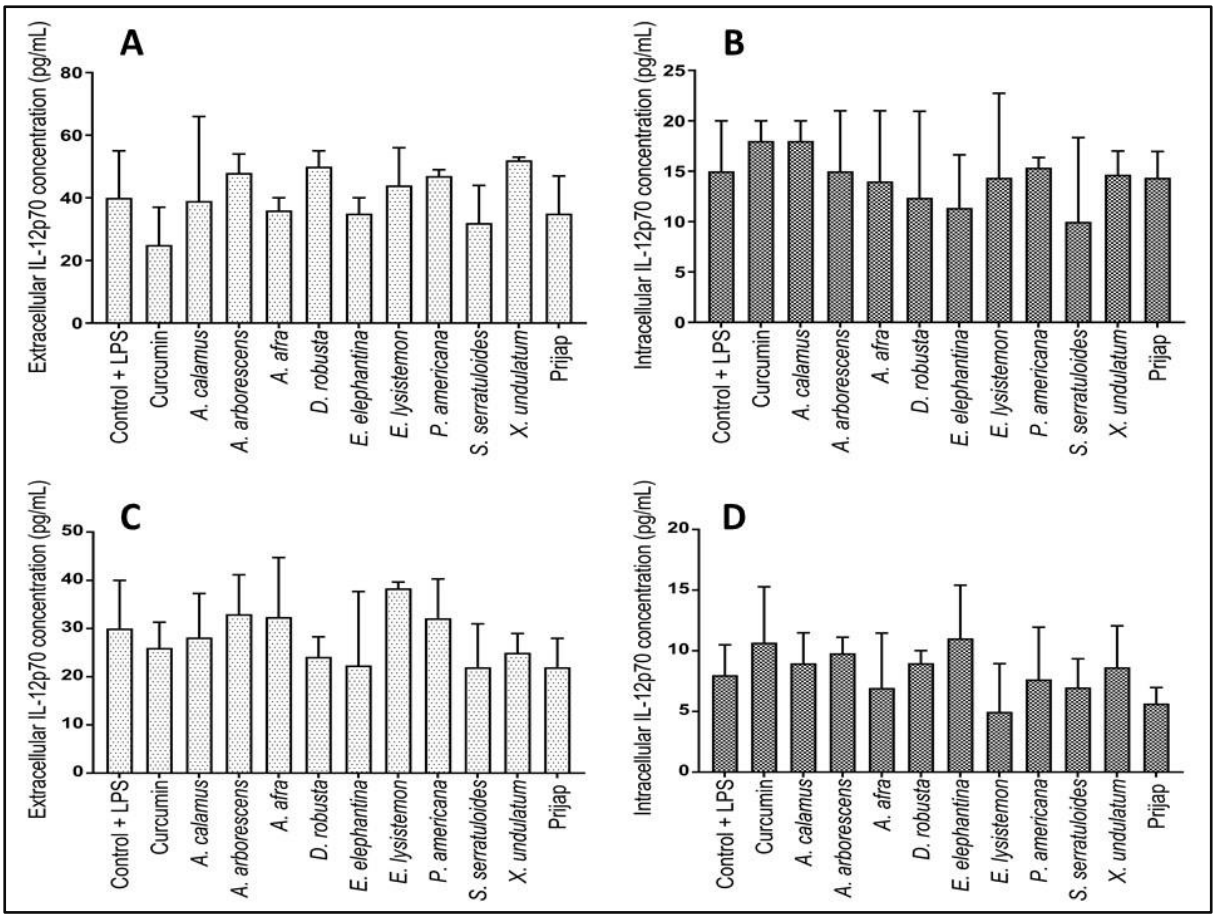


Figure 27: Concentration of IL-12p70 in differentiated THP-1 and U937 cells. A) Extracellular IL-12p70 in THP-1 cells, B) intracellular IL-12p70 in THP-1 cells, C) extracellular IL-12p70 in U937 cell, D) intracellular IL-12p70 in U937 cells.

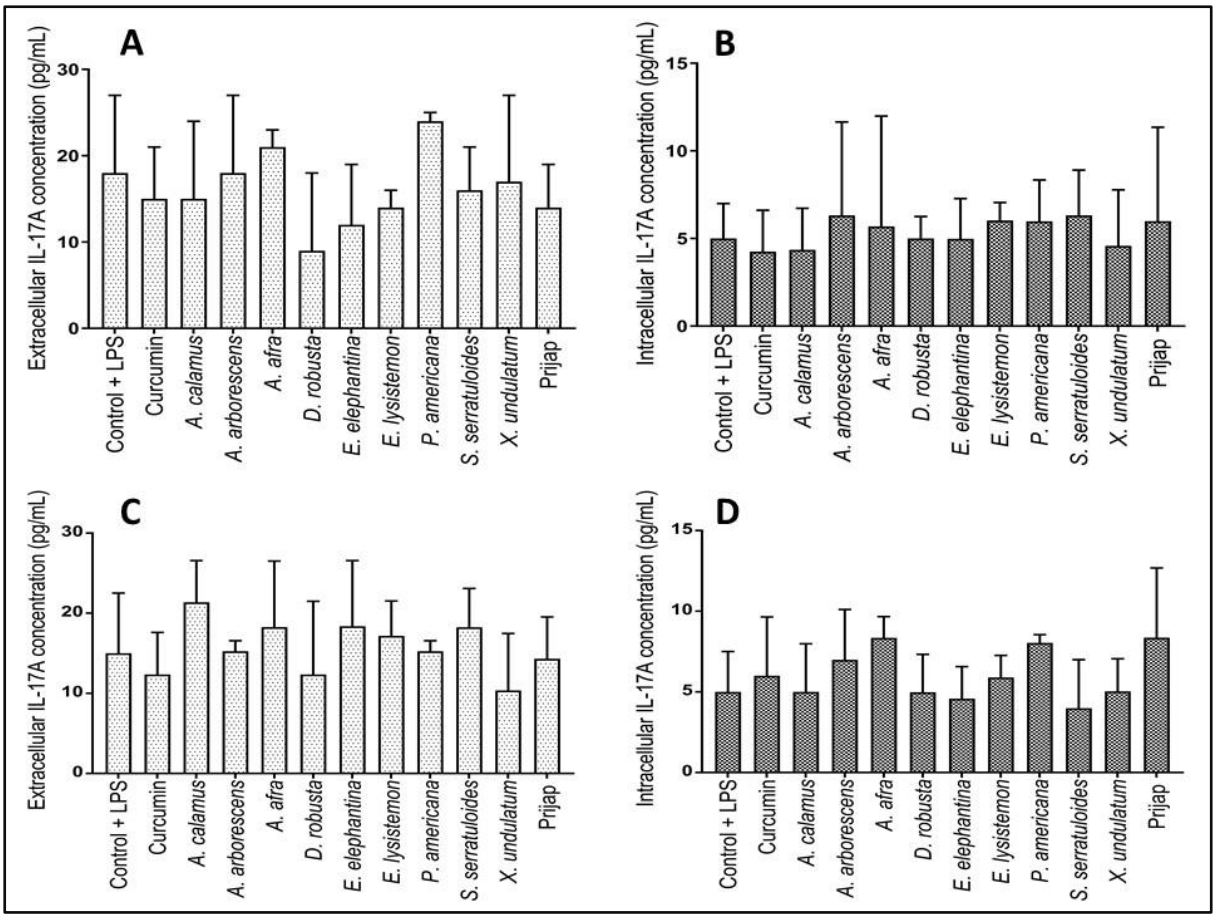


Figure 28: Concentration of IL-17A in differentiated THP-1 and U937 cells. A) Extracellular IL-17A in THP-1 cells, B) intracellular IL-17A in THP-1 cells, C) extracellular IL-17A in U937 cells, D) intracellular IL-17A in U937 cells

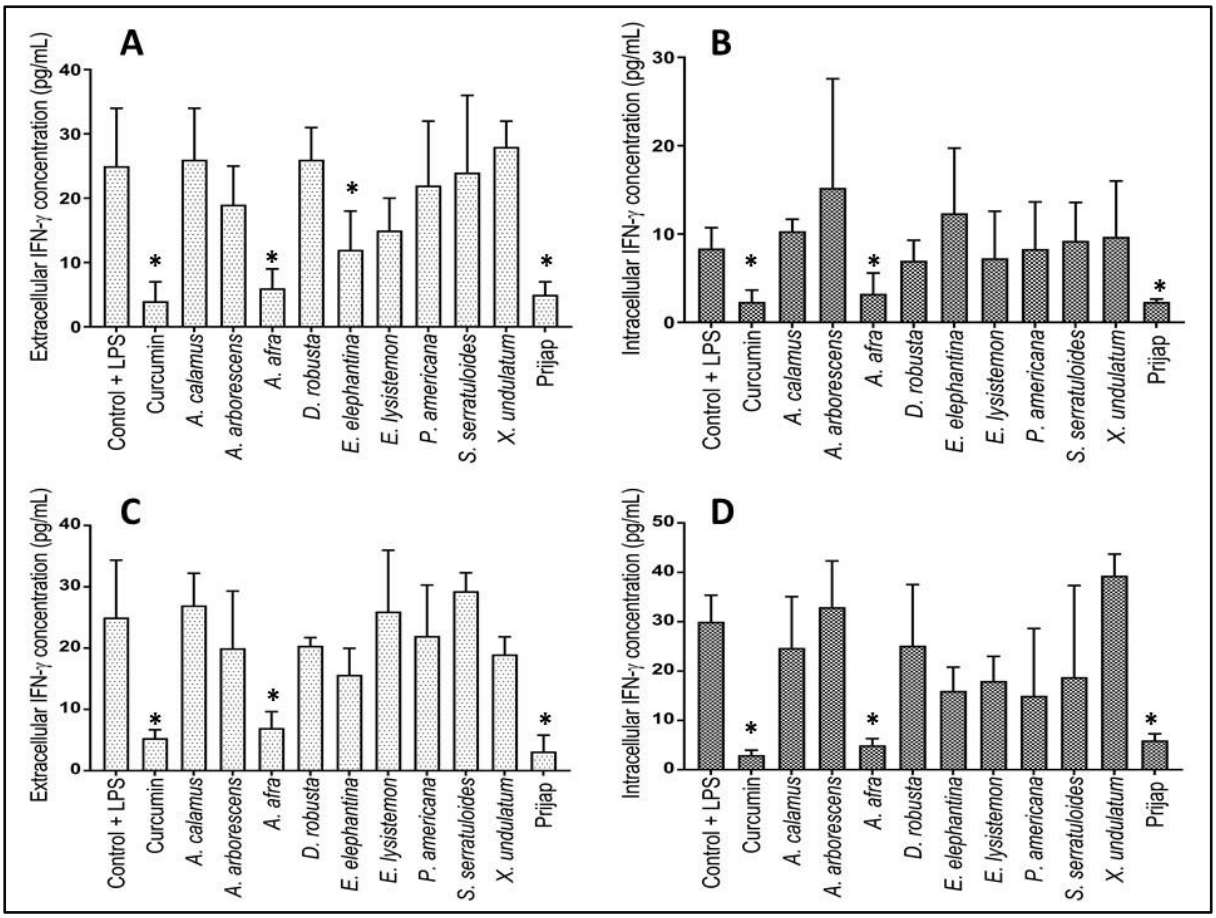


Figure 29: Concentration of IFN-γ in differentiated THP-1 and U937 cells. A) Extracellular IFN-γ in THP-1 cells, B) intracellular IFN-γ in THP-1 cells, C) extracellular IFN-γ in U937 cells, D) intracellular IFN-γ in U937 cells
***:** Indicates statistically significant difference relative to the control (p<0,05)

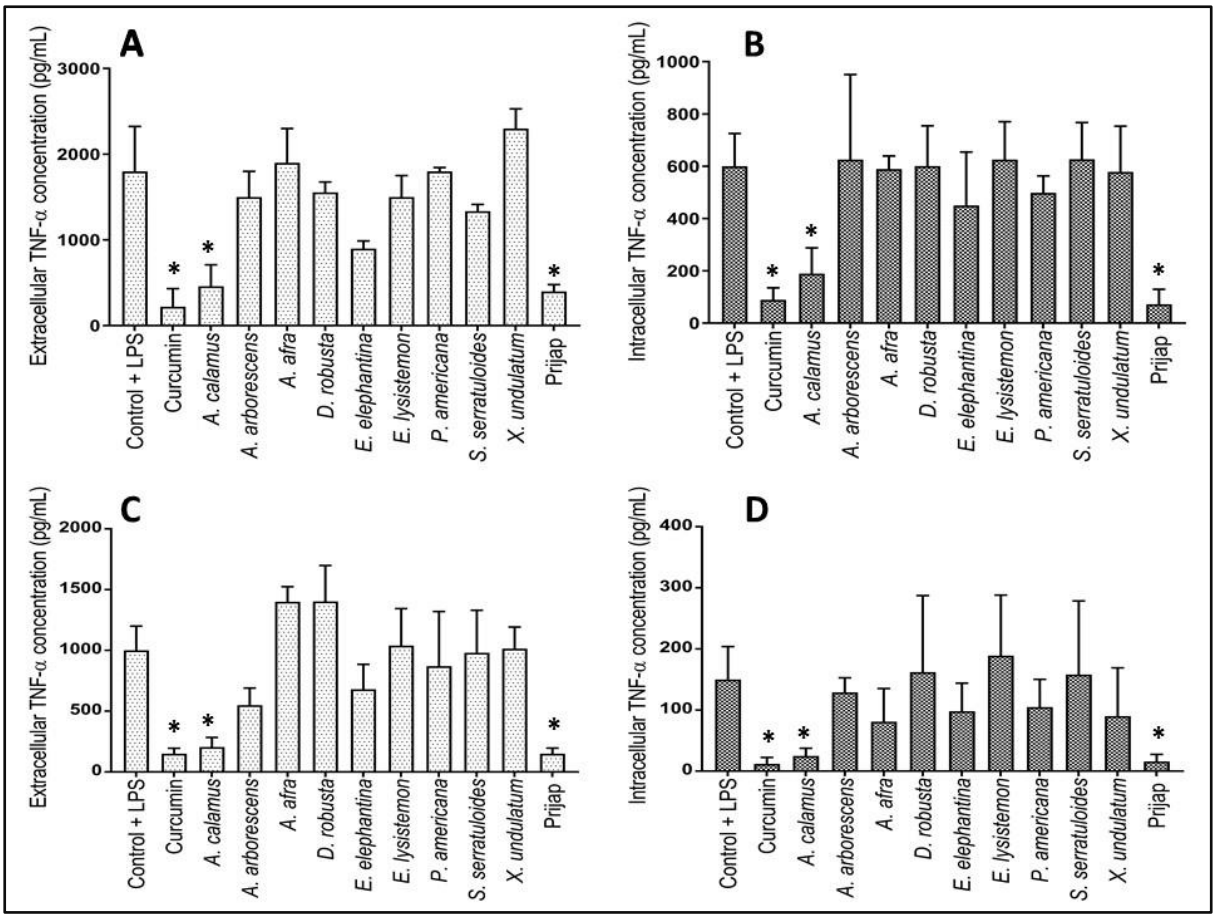


Figure 30: Concentration of TNF-α in differentiated THP-1 and U937 cells. A) Extracellular TNF-α in THP-1 cells, B) intracellular TNF-α in THP-1 cells, C) extracellular TNF-α in U937 cells, D) intracellular TNF-α in U937 cells. ***:** Indicates statistically significant difference relative to the control $p<0,05$.

Table 16: Significant changes in cytokine and PGE_2 secretion alterations.

Extract	Cytokine					Effect
	TNF-α	IL-1β	IL-10	IFN-γ	PGE_2	
A. afra	-	-	-	↓	↑	Anti-inflammatory
A. arborescens	-	-	↑	-	-	Anti-inflammatory
A. calamus	↓	↓	-	-	-	Anti-inflammatory
Curcumin	↓	↓	-	↓	↓	Anti-inflammatory
D. robusta	-	-	-	-	↓	Anti-inflammatory
E. elephantina	-	↓	↑	-	↑	Anti-inflammatory
P. americana	-	-	↑	-	-	Anti-inflammatory
X. undulatum	-	-	-	-	↓	Anti-inflammatory
Prijap Health traditional herbal medicine	↓	↓	-	↓	↓	Anti-inflammatory

↑: Significant increase (p<0,05)
↓: Significant decrease (p<0,05)
–: No difference relative to the control

Flow cytometry is a laser-based platform that is most often used to detect specific markers on or in cells. The principle of flow cytometry is based on the flow or passaging of cells through a tube in single files. The cells or cell components are labelled with a fluorescent dye and pass in front of a laser where they are excited to emit light. The fluorescence intensity is directly proportional to the amount of the analyte of interest.[232,233] In recent years, bead-based immunoassays which combine the traditional immunoassays with flow cytometry have been added. These methods use microspheres (beads) conjugated with antibodies. The beads are mixed with a biological sample containing antigens/antibodies of interest.[234,235] The assays are developed by addition of fluorescent detection antibodies. The amount of the analyte is directly proportional to the fluorescence intensity generated from the bead-analyte fluorescent detection antibody.[234]

Multiplex bead-based flow cytometry assays were developed by using beads of either differing or same sizes conjugated with multiple antibodies with differing levels of fluorescence. Similarly, the beads pass through a laser where they are excited and emit multiple fluorescence (due to multiple antibodies conjugated to the same bead with differing florescence intensities) and the amount of analyte is directly proportional

to fluorescence intensity.[235] The analyte amount is determined from a standard calibration curve. Multiplex bead-based assays allow for measurement of multiple analytes at once from a single sample, this reduces sample volume required for analysis. It also offers good precision, is highly sensitive and gives a way better or higher resolution data as opposed to cell-based flow cytometric analysis.[234,235]

Chapter 4: Conclusion

Prijap Health traditional herbal concoction consists of a combination of nine plant species. The medicine is used traditionally as an immune booster, appetizer, detoxifier, energy booster, antiviral and anti-inflammatory agent. In this study, Prijap Health traditional herbal medicine and its plant species constituents were assessed for their phytochemical profile, antioxidant, cytotoxicity and immunomodulatory activities. Prijap Health traditional herbal medicine and its individual plant species were found to contain the following phytochemicals: flavonoids, phenolic acids, terpenoids, alkaloids and saponins. The majority of compounds identified from most of the extracts were flavonoids.

The *in vitro* cytotoxicity test conducted in both cell lines showed that the majority (seven out of a total of nine) of the individual plant species are non-toxic and have shown some growth stimulation even at the highest dose tested but the combined Prijap Health product displayed moderate toxicity at high doses, which is concerning.

The DPPH and ABTS assays both showed that the polyherbal remedy as well as its individual plant species possess some antioxidant activity, with *E. elephantina* showing the highest antioxidant activity comparable to that of trolox in both assays. This study provides scientific evidence with regards to the antioxidant potential of Prijap Health traditional herbal medicine and the contribution of the activity by the individual medicinal plant species. The antioxidant activity is ascribed to the presence of flavonoids and phenolic acids in both the polyherbal concoction and the individual plants. The anti-inflammatory analysis results demonstrated that Prijap Health traditional herbal medicine can significantly reduce pro-inflammatory mediators such as PGE_2, TNF-α, IFN-ɣ and IL-1β. This is indicative of the potent anti-inflammatory activity of the product. Contribution of anti-inflammatory activity by extracts of *E. elephantina*, *A. afra*, *A. calamus*, *A. arborescens* and *P. americana* has been demonstrated. The Prijap Health traditional herbal medicine product appears to modulate the innate immune system response by altering secretion of cytokines and chemokines in a manner that doesn't favour the inflammatory response.

Prijap Health herbal traditional medicine has shown great potency as an antioxidant and immunomodulatory agent. With the exception of *E. elephantina*, some individual plant species showed good antioxidant activity and a poor immunomodulatory potential and vice versa. *E. elephantina* displayed antioxidant activity exceeding that of Prijap Health traditional herbal medicine. However, Prijap Health traditional herbal medicine decreased ($p<0.05$) the secretion of two more pro-inflammatory markers (PGE_2 and IFN-ɣ) than *E. elephantina* did. *E. elephantina* showed pro-inflammatory potential by increasing ($p>0.05$) PGE_2 secretion in THP-1 cells.

Prijap Health traditional herbal medicine is more useful as a whole and no single plant proved to be as effective with regards to anti-inflammatory activity. The extracts of *D. robusta* and *E. lysistemon* displayed poor antioxidant activity, concerning cytotoxicity (*D. robusta*) and no immunomodulatory activity. It is suggested that these two plants be excluded from the herbal mixture and activity be re-assessed to determine if the polyherbal medicine mixture would contain more superior activity than the current mixture on the market.

Chapter 5: Recommendations and limitations

Only water extracts are used as it is the way the medicine is prepared by the THP associated to the Prijap Health traditional herbal medicine. Other solvent extracts could be more active and developed as pharmaceutical drugs. The current study conforms to the use of experimentally acceptable concentrations of both plant extracts and Prijap Health herbal concoction. The daily recommended dose of the herbal product is not taken into consideration and thus this makes it difficult to draw the conclusion as to whether the experimental data directly address the medication and its uses. It is therefore recommended that the daily recommended dose of Prijap Health traditional herbal medicine be used in further studies.

The results of the PGE_2 analysis with the use of ELISA were obtained from two experiments done and these data are not viable to do statistical analysis. More ELISA experiments could be done to make the data more viable and thus reliable.

Dicaffeoylquinic acids (3,5 Dicaffeoylquinic acid),[236] gallotannins,[237] gallic acid,[238] catechins[239] and tannins[240] have been previously shown to have anti-HIV properties by inhibition of HIV-1 associated enzymes. In the present study, 3,5 Dicaffeoylquinic acid, gallic acid and catechin have been identified through UPLC-ESI-MS as constituents of *A. afra*, *E. elephantina* and *P. americana*, respectively. The activity of the individual compounds should be tested to determine whether they are responsible for the activity noted. Furthermore, it is recommended that, individual plant species and Prijap Health herbal traditional medicine be tested for anti-HIV properties or inhibitory activity against HIV-related enzymes. Also, drug-herb interaction studies should be conducted in order to investigate how Prijap Health traditional herbal medicine interacts with ARVs as these are used concurrently. Furthermore,

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Appendix I: Ethical clearance certificate

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 28 August 2018.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

24/11/2016

Approval Certificate
New Application

Ethics Reference No.: 465/2016

Title: A in vitro investigation of the immunomodulatory effects of a polyherbal traditional medicine product.

Dear Tebatso Martin Moloto

The **New Application** as supported by documents specified in your cover letter dated 17/10/2016 for your research received on the 17/10/2016, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 23/11/2016.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years
- Please remember to use your protocol number (**465/2016**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Additional Conditions:
- Researcher to take note: P16 - fusion inhibitors are not frequently used in South Africa. The most common classes are NRTIs and NNRTIs, followed by PIs and then Integrase Inhibitors (please note that these are not intergrase inhibitors, as stated on P17).

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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Appendix II: List of reagents and preparation

ABTS

2, 2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and potassium persulfate (K_2S_2O8) was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. The ABTS radical was generated by dissolving 38.40 mg ABTS and 6.62 mg K_2S_2O8 in 10 mL of distilled water and incubating at 4^{o}C for 16 h to make a 7.46 mM ABTS and 2.5 mM K_2S_2O8 solution.

Acetic acid

Acetic acid was obtained from Sigma-Aldrich (Pty) Ltd (Kempton Park, South Africa). A 10% acetic acid solution was prepared by dissolving 10 mL of acetic acid in 990 mL of dH_2O and stored at room temperature in a clear glass container until use.

DMSO

DMSO was obtained from Sigma-Aldrich (St. Louis, USA) and used undiluted.

DPPH

DPPH was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A stock solution of 600 µM was prepared by dissolving 11.8 mg DPPH in 50 mL methanol and sonicated for 20 min. A working solution of 120 µM was prepared by diluting 20 mL of stock solution with 80 mL methanol and sonicated for 20 min.

Dragendorff's reagent

Bismuth nitrate and potassium iodide was obtained from Merck Chemicals (Darmstadt, Germany) in powdered form. Two solutions will be prepared: A) 1.7% bismuth nitrate in 20% aqueous acetic acid and B) 13% potassium iodide solution in 30% aqueous acetic acid. Solution A and B was mixed in 4:1 ratio prior to use.

FCS

FCS was obtained from The Scientific group (Gauteng, South Africa) and heat-inactivated through heating at 56^{o}C for 45 min. The solution was stored at -20^{o}C until use.

Folin-Ciocalteau reagent

Folin-Ciocalteau reagent (2 N) was obtained from Sigma-Aldrich (St. Louis, USA) in liquid form and used undiluted.

PBS

BBL™ FTA hemagglutination buffer was obtained from BD Scientific (France) in powder form. A 0.9% solution was prepared in distilled water and stored at 4°C until use.

Penicillin/Streptomycin

A penicillin (10 00 U) and streptomycin (10 000 µg/mL) solution was obtained from Bio Whittaker (Walkersville, USA). The solution was added to the culture medium to a final concentration of 1%.

Phosphoric acid (85%) in dH_2O

Phosphoric acid was obtained from Merck Chemicals (Darmstadt, Germany) as a liquid. An 85% phosphoric acid solution was prepared by adding 0.85 mL of phosphoric acid to 1.5 mL of dH_2O.

RPMI-1640

RPMI-1640 medium was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 1.04% solution was prepared in autoclaved, ultra-pure, pyrogen-free deionized water and adjusted to pH 7.4 using sodium hydroxide carbonate obtained from Merck Chemicals (Darmstadt, Germany) in powder form. The solution was filtered three times using *in vacuo* filtration (Sartorius, 0.22 µm) and supplemented with 1% penicillin/streptomycin and stored at 4°C until use.

Sodium nitrate (3%), Aluminium chloride (1%)

Sodium nitrate powder was obtained from Merck Chemicals (Darmstadt, Germany) and aluminium chloride powder from Sigma-Aldrich (St. Louis, USA). The solution was prepared by dissolving 0.45 g of sodium nitrate and 0.15 g of aluminium chloride in 15 mL of dH_2O.

Solvents

Acetone (≥99.8%, ACS, ISO, Reag. Ph Eur), ammonium hydroxide (28-30%, ACS, Reag. Ph Eur), chloroform (≥98%, ACS), ethanol (≥99.9%, Reag. Ph Eur, ACS, ISO), ethyl acetate (≥99.5%, Reag. Ph Eur, ISO, ACS), hexane (≥96%, Reag. Ph Eur, ACS), methanol (≥99.9%, ACS, ISO, Reag. Ph Eur)), sulphuric acid (95%, ACS), formic acid (≥98%, ACS), acetonitrile (≥99.9%, Reag. Ph Eur, ISO, ACS) and toluene (≥99.9%, Reag. Ph Eur, ACS, ISO) were obtained from Merck (Pty) Ltd (Modderfontein, South Africa) as liquids and stored at room temperature.

SRB

SRB was obtained from Sigma-Aldrich (Kempton Park, South Africa) in powdered form. SRB powder (285 mg) was dissolved in 500 mL 1% acetic acid and stored in a foil-covered plastic container until use.

Tris-base buffer solution

Tris-base powder was purchased from Research Organics Inc. (Cleveland, USA). Tris-base was prepared by dissolving 600 mg of powder in 500 mL dH_2O. The solution was adjusted to pH 10.5 and stored in a 500 mL plastic container at room temperature till use.

Trypan blue

Trypan blue was obtained from Sigma-Aldrich (St. Louis, USA) in powder form. A 0.1% solution was prepared by dissolving 0.1 mg of powder in 100 mL of dH_2O and stored in a foil-covered plastic tube at room temperature.

Vanillin (1%) Sulphuric acid (5%)

Vanillin powder was obtained from Sigma-Aldrich (St. Louis, USA). Prior to use, 0.1 g of vanillin powder was dissolved in 0.5 mL of sulphuric acid. This solution will further be dissolved in 9.5 mL of dH_2O.

Appendix III: Gradient profiles used in UPLC-PDA analysis

Acorus calamus

Step	Time (min)	Flow rate (mL/min)	% A	% B	Curve (G)	Segment duration (min)
initial	0	0.4	100	0	-	0.0
2	1	0.4	100	0	**11**	1.0
3	2	0.4	5	95	**7**	25.0
4	25	0.4	5	95	**1**	2.0
5	30	0.4	100	0	**1**	2.0

Aloe arborescens

Step	Time (min)	Flow rate (mL/min)	% A	% B	Curve (G)	Segment duration (min)
initial	0	0.4	100	0	-	0.0
2	1	0.4	100	0	**11**	1.0
3	2	0.4	5	95	**8**	25.0
4	25	0.4	5	95	**1**	2.0
5	30	0.4	100	0	**1**	2.0

Artemisia. afra

Step	Time (min)	Flow rate (mL/min)	% A	% B	Curve (G)	Segment duration (min)
initial	0	0.4	95	5	-	-
2	1	0.4	95	5	**11**	1.0
3	2	0.4	5	95	**8**	25.0
4	25	0.4	5	95	**1**	2.0
5	30	0.4	95	5	**1**	2.0

Drimia robusta

Step	Time (min)	Flow rate (mL/min)	% A	% B	Curve (G)	Segment duration (min)
initial	0	0.4	100	0	-	0.0
2	1	0.4	100	0	**11**	1.0
3	2	0.4	5	95	**8**	25.0
4	25	0.4	5	95	**1**	2.0
5	30	0.4	100	0	**1**	2.0

Elephantorrhiza elephantina

Step	Time (min)	Flow rate (mL/min)	% A	% B	Curve (G)	Segment duration (min)
initial	0	0.4	98	2	-	0.0
2	1	0.4	98	2	**11**	1.0
3	2	0.4	5	95	**8**	25.0
4	25	0.4	5	95	**1**	2.0
5	30	0.4	95	5	**1**	2.0

Erythrina lysistemon

Step	Time (min)	Flow rate (mL/min)	% A	% B	Curve (G)	Segment duration (min)
initial	0	0.4	100	0	-	0.0
2	1	0.4	100	0	**11**	1.0
3	2	0.4	5	95	**8**	25.0
4	25	0.4	5	95	**1**	2.0
5	30	0.4	100	0	**1**	2.0

Persea americana

Step	Time (min)	Flow rate (mL/min)	% A	% B	Curve (G)	Segment duration (min)
initial	0	0.4	98	2	-	0.0
2	1	0.4	98	2	**11**	1.0
3	2	0.4	5	95	**8**	25.0
4	25	0.4	5	95	**1**	2.0
5	30	0.4	98	2	**1**	2.0

Senecio serratuloides

Step	Time (min)	Flow rate (mL/min)	% A	% B	Curve (G)	Segment duration (min)
initial	0	0.4	98	2	-	0.0
2	1	0.4	98	2	**11**	1.0
3	2	0.4	5	95	**8**	25.0
4	25	0.4	5	95	**1**	2.0
5	30	0.4	95	5	**1**	2.0

Xysmalobium undulatum

Step	Time (min)	Flow rate (mL/min)	% A	% B	Curve (G)	Segment duration (min)
Initial	0	0.4	90	10	**-**	0.0
2	1	0.4	90	10	**11**	1.0
3	2	0.4	5	95	**7**	25.0
4	25	0.4	5	95	**1**	2.0
5	30	0.4	90	10	**1**	2.0