



**UNIVERSITY OF
KWAZULU-NATAL**

**INYUVESI
YAKWAZULU-NATALI**

**AN *IN VITRO* INVESTIGATION INTO THE ANTI-PROLIFERATIVE AND
ANTI-INFLAMMATORY PROPERTIES OF *CENTELLA ASIATICA* (LINNAEUS)
URBAN (LEAF) AND *WITHANIA SOMNIFERA* (LINNAEUS) DUNAL (ROOT)
EXTRACTS**

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Doctor of Philosophy (Medical Biochemistry)

in the Discipline of Medical Biochemistry and Chemical Pathology

School of Laboratory Medicine and Medical Science

College of Health Sciences

University of KwaZulu-Natal

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DECLARATION

I, Dhaneshree Bestinee Naidoo declare that:

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DEDICATION

To my extraordinary parents, Mr. D. C. Naidoo and Mrs. B. Naidoo, this would not have been possible without your endless sacrifices and unconditional love.

To the late Dr. M. Govender, you were a very special person, an exceptional medical doctor and a loving father figure. You will forever be remembered and missed.

To all cancer patients who have fought and/ or still fighting to defeat this disease. Never give up.

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3. Article title: *Centella asiatica* decreases nuclear factor kappa-beta (p50, p65) protein expression, decreases pro-inflammatory cytokine levels and modulates cell death in leukaemic (THP-1) cells
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PRESENTATIONS

1. Title: *Centella asiatica*: Its potential in cancer cachexia
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antioxidant pathway and enhances apoptotic cell death in human embryonic kidney (HEK293) cells.

LIST OF ABBREVIATIONS

<i>W. somnifera</i>	<i>Withania somnifera</i>
<i>C. asiatica</i>	<i>Centella asiatica</i>
THP-1	Leukaemic cell line
PBMC's	Peripheral blood mononuclear cells
W_{RE}	<i>W. somnifera</i> aqueous root extract
C_{LE}	<i>C. asiatica</i> ethanolic leaf extract
C	<i>C. asiatica</i> ethanolic leaf extract
C3	<i>C. asiatica</i> ethanolic leaf extract purified fraction-3
A549	Cancerous lung cells
HEK293	Embryonic kidney cells
H	Hours
IC₅₀	Half maximal inhibitory concentration
GSH	Glutathione
GSSG	Oxidized glutathione
ATP	Adenosine triphosphate
TNF-α	Tumour necrosis factor alpha
IL	Interleukin
IROS	Intracellular reactive oxygen species
MDA	Malondialdehyde
LDH	Lactate dehydrogenase

NF-κB	Nuclear factor kappa beta
Nrf-2	Nuclear factor erythroid 2-related factor 2
GPx	Glutathione peroxidase
Bcl-2	B cell lymphoma-2
Bax	Bcl-2 associated X
HSP	Heat shock protein
c-PARP	Cleaved poly(ADP-ribose) polymerase-1
SOD	Superoxide dismutase
CAT	Catalase
OGG-1	8-oxoguanine glycosylase
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
WSF	<i>Withania somnifera</i> formulation
HL-60	Human promyelocytic leukaemia
Con A	Concanavalin A
IFN-γ	Interferon-gamma
NO	Nitric oxide
LPS	Lipopolysaccharide
iNOS	Inducible nitric oxide synthase
COX-2	Cyclooxygenase-2
PGE2	Prostaglandin E2

O₂⁻	Superoxide radical
DPPH	2, 2-diphenyl-1 picrylhydrazyl
MK-1	Human gastric adenocarcinoma
B16F10	Murine melanoma
SK-MEL-2	Human melanoma cells
RB	Retinoblastoma-associated
LMF	Lipid mobilizing factor
PIF	Proteolysis inducing factor
NPY	Neuropeptide Y
CRF	Corticotrophin-releasing factor
EE	Energy expenditure
AA	Asiatic acid
WS	<i>Withania somnifera</i>
CC	<i>Cinnamomum camphora</i>
THC	Δ^9 tetrahydrocannabinol
HP	<i>Harpagophytum procumbens</i>
CRP	C reactive protein
FA's	Fatty acids
TAG	Triacylglycerols
LPL	Lipoprotein lipase
PKA	Protein kinase A

HSL	Hormone sensitive lipase
E1	Ubiquitin-activating enzyme
E2	Carrier protein
MAC16	Colon carcinoma
RHD	Rel homology domain
IκB	Inhibitory kappa B
IKK	IκB kinase
CA	<i>Centella asiatica</i>
SF	<i>Sutherlandia frutescens</i>
H	Harpagoside
MPA	Medroxyprogesterone
EPA	Eicosapentaenoic acid
H₂O₂	Hydrogen peroxide
TICs	Tumour-initiating cells
KEAP1	Kelch-like ECH-associated protein 1
GCL	Glutamate–cysteine ligase
PKR	RNA-dependent protein kinase
eIF2α	Eukaryotic initiation factor 2
PARP-1	Poly(ADP-ribose)polymerase-1
TNFR1	TNF receptor
CD95	Fas receptor

DISC	Death-inducing signalling complex
Apaf-1	Apoptotic protease-activating factor-1
AIF	Apoptosis inducing factor
IAPs	Inhibitors of apoptosis proteins
MCF7	Breast cancer cells
MDA MB 231	Breast cancer cells
HeLa	Human cervical/ uterine carcinoma
B16F1	Mouse melanoma
BHK-21	Normal hamster kidney
MK-1	Human gastric adenocarcinoma
B16F10	Murine melanoma
HepG2	Human hepatoma
HT-29	Human colon adenocarcinoma
DNA	Deoxyribonucleic acid
SA	South Africa
ELISA	Enzyme-linked immunosorbant assay
FCS	Foetal calf serum
PSF	Pen/ Strep Amphotericin B
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate
BHT	Butylated hydroxytoluene

RT	Room temperature
min	Minute
PBS	Phosphate buffered saline
RLU	Relative light units
DMSO	Dimethyl sulphoxide
ANOVA	One way analysis of variance
DCM	Dichloromethane
HEX	Hexane
EA	Ethyl acetate
TLC	Thin layer chromatography
GC-MS	Gas chromatography mass spectrometry
PS	Penstrepfungizone
L-GLUT	L-glutamine
TTBS	Tween-20 wash buffer
RBD	Relative band density
JC-1	5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolcarbocyanine iodide
H2DCF-DA	2', 7'-dichlorodihydrofluorescein diacetate
C_{ct}	Control for <i>Centella asiatica</i> crude leaf extract
C_{ct3}	Control for <i>Centella asiatica</i> purified fraction-3
DCF	20, 70-dichlorofluorescein

TBARS	Thiobarbituric acid assay
$\Delta\Psi_m$	Mitochondrial depolarisation
SCGE	Single cell gel electrophoresis
CTL's	Comet tail lengths
AML	Acute myeloid leukaemia
ETC	Electron transport chain
8-oxoG	8-Oxo-7,8-dihydroguanine
ARE	Anti-oxidant response element
EMEM	Eagle's minimum essential medium
OD	Optical density
AKI	Acute kidney failure
DMEM	Dulbucco's minimum essential

ABSTRACT

Background: The cachectic syndrome is primarily associated with malignancy. Cachexia leads to abnormal weight loss through adipose tissue and skeletal muscle depletion. Previously, antioxidant mechanisms and inflammatory cytokines have been associated with cancer cachexia. In cancer cells, increased antioxidant levels assist cancer cells to evade reactive oxygen species (ROS) induced cell death whereas increased pro-inflammatory cytokine levels initiate metabolic events such as increased cancer cell proliferation, lipolysis, and proteolysis. Thus decreasing pro-inflammatory cytokine levels and antioxidant mechanisms within cancer cells may be an effective treatment to decrease the progression of cancer and cachexia. The scientific exploration into the usage of traditional medicinal plants in the prevention and treatment of cancer cachexia is increasing. *Withania somnifera* (Linnaeus) Dunal (*W. somnifera*) and *Centella asiatica* (Linnaeus) Urban (*C. asiatica*) are commonly used in traditional medicine due to their many therapeutic properties (antioxidant, anti-inflammatory and anti-tumour potential).

Aims: We investigated the modulation of cytokines, antioxidants and apoptosis in leukaemic (THP-1) cells and healthy peripheral blood mononuclear cells (PBMC's) by *W. somnifera* aqueous root extract (W_{RE}) and *C. asiatica* ethanolic leaf extract (C_{LE}). We also investigated the anti-inflammatory, antioxidant and anti-proliferative effects of *C. asiatica* ethanolic leaf extract (C) and purified fraction-3 (C3) in THP-1 cells. Additionally, we investigated the antioxidant and anti-proliferative/ cytotoxic effects of C3 in cancerous lung (A549) cells and embryonic kidney (HEK293) cells.

Methods: Cytotoxicity of plant extracts/ fractions were determined (cell viability assay 24 and 72 hours). C3 was obtained by silica column fractionation and identified using thin layer chromatography. Gas chromatography mass spectrometry determined the bioactive compounds present in C and C3. Oxidant scavenging activity was evaluated using the 2, 2-diphenyl-1-picrylhydrazyl assay and cytokine levels were evaluated using the enzyme-linked immunosorbant assay. Reduced glutathione, oxidized glutathione, adenosine triphosphate levels and caspase

activities were measured by luminometry. The level of nitrites (griess assay), intracellular ROS, mitochondrial membrane potential (flow cytometry), malondialdehyde, lactate dehydrogenase (spectrophotometry), and DNA damage (comet assay) were assessed. Protein expression and gene expression were quantified by western blotting and quantitative polymerase chain reaction (qPCR), respectively.

Results: In PBMC's and THP-1 cells, W_{RE} and C_{LE} proved to effectively modulate antioxidants, inflammatory cytokines and cell death. Notably, in THP-1 cells, W_{RE} and C_{LE} decreased pro-inflammatory cytokine levels which may alleviate cancer cachexia and excessive leukaemic cell growth. In THP-1 cells, C and C3 have been shown to decrease nuclear factor kappa beta (NF- κ B: p50 p65) protein expression, decrease pro-inflammatory cytokine (tumour necrosis factor alpha (TNF- α), interleukin (IL)-6 and IL-1 β) levels and modulate THP-1 apoptosis. Notably, C3 more effectively decreased pro-inflammatory cytokines levels, suggesting that C3 may be effective in combating cancer cachexia. In THP-1 cells, the Nrf-2 antioxidant response was increased by C whilst decreased by C3. Both C and C3 exerted anti-proliferative effects in THP-1 cells by increasing apoptosis. Notably, C3 more effectively induced THP-1 apoptosis which may be associated with the decreased antioxidant responses. In A549 and HEK293 cells, C3 diminished the antioxidant gene expression and induced anti-proliferative/ cytotoxic effects.

Conclusion: W_{RE} and C_{LE} demonstrated antioxidant, anti-inflammatory and anti-proliferative activities. Interestingly, C3 elicited higher anti-inflammatory and anti-proliferative activities than C.

INTRODUCTION

Worldwide, cancer is a leading cause of morbidity and mortality. It is a hyper-proliferative disorder involving cellular transformation, mutations, evasion of apoptosis, invasion, angiogenesis and metastasis [1]. Annually there are approximately ten million newly diagnosed cancer cases and greater than six million cancer related deaths [2]. Therefore, research into the discovery/development of new and more effective anti-cancer therapies is imperative to combat the rapid progression of cancer and the increasing related mortality.

Homeostatic inflammatory responses may be beneficial due to its anti-tumour activity [3]. However, chronic inflammation has been implicated in carcinogenesis [1,4,5,6], with increased levels of reactive oxygen species (ROS), inflammatory cytokines, and nuclear factor-kappa beta (NF- κ B) expression contributing to inflammation-induced carcinogenesis [7].

The cachectic syndrome is primarily associated with malignant conditions and is known as ‘a multi-factorial syndrome defined by an ongoing loss of skeletal muscle mass (with/ without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment’ [8]. ‘The pathophysiology is characterized by a negative protein and energy balance driven by a variable combination of reduced food intake and abnormal metabolism’ [8]. The occurrence of cachexia varies with tumour types with the highest frequency present in pancreatic or gastric cancer patients (83–87%), followed by the unfavourable non-Hodgkin lymphoma, colon, prostate and lung cancer patients (48–61%), the lowest frequency is in patients with favourable subtypes of non-Hodgkin lymphoma, breast cancer, acute non-lymphocytic leukaemia and sarcomas (31–40%) [9]. Weight loss (>10%), systemic inflammation (C-reactive protein >10 mg/l) and reduced food intake (<1,500 kilo calories per day) are key features of cachexia [10]. The syndrome can be divided into three stages, namely precachexia, cachexia, and refractory cachexia [11]. Precachexia patients present with anorexia, impaired glucose tolerance and involuntary weight loss (\leq 5%) [11]. A number of factors determine the progression of cachexia including cancer type and stage, systemic inflammation, decreased food intake and poor responsiveness to anti-cancer therapy [11]. Patients with cachexia experience greater than 5% weight loss over 6 months and the body mass index is less than 20 kg/m² [11]. In refractory cachexia there is active catabolism and the management of weight loss becomes improbable due to

advanced/ rapidly progressive cancers which are unresponsive to anti-cancer therapy [11]. The inability to ingest/ use nutrients [12] and the negative energy balance present in cachectic patients leads to catalysis of muscle and fat stores for energy production [13]. Cachectic cancer patients lose up to 85% of adipose tissue and 75% of skeletal muscle [14]. Neuropeptides, hormones, pro-inflammatory cytokines and tumour-related factors contribute to the development and progression of cancer cachexia. Increased pro-inflammatory cytokine levels associated with cancer progression initiate metabolic events such as decreased lipogenesis, increased lipolysis and proteolysis which lead to tissue wasting [14]. Additionally, NF- κ B plays an important role in the inflammatory process by regulating the expression of many inflammatory molecules [15,16] as well as in protein degradation by inducing the ubiquitin–proteasome pathway [17]. Cancer cachexia decreases the quality of life of patients, the responsiveness to chemotherapy [18] and is responsible for 20–25% of cancer deaths [19,20] (or approximately 2 million deaths annually) [21] with the degree of cachexia being inversely proportional to survival time [19]. Therefore, it is essential to discover an effective cancer cachectic treatment to alleviate the development and progression of cachexia, ultimately prolonging the survival rate of cancer patients.

Depending on the level of ROS, ROS can be tumourigenic by increasing DNA damage, cell proliferation, survival, and migration [22] as well as anti-tumourigenic by inducing cellular damage and cell death [22]. Oxidative stress is a result of ROS accumulation due to an imbalance between ROS generation and antioxidant defence mechanisms [23]. Antioxidants such as glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) protect cells against oxidative damage and cell death [24]. In mammalian cells, GSH is the most abundant thiol that functions as a major antioxidant by detoxifying ROS and maintaining the redox status [25]. GSH also aids in tumour cell survival [23]. In numerous cell types, GSH depletion is an early hallmark of cell death progression [26,27]. Within the cell, the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf-2) is an important regulator of antioxidant gene expression [23]. Previous studies have indicated that Nrf-2 protects tumours and cancerous cell lines from chemotherapeutic drugs [28,29,30]. Additionally, Nrf-2 has been directly associated with resistance to apoptosis [31]. In normal cells an effective antioxidant defence system is essential for the detoxification of elevated ROS levels [32]. However, cancer cells exploit antioxidant mechanisms to infer survival properties, increased malignancy and resistance to anti-cancer drugs [33]. Therefore targeting antioxidant mechanisms in cancer cells may be a promising therapeutic strategy [23].

For centuries, medicinal plants have been employed for chemotherapy and chemoprevention due to their vast number of naturally occurring chemicals [34]. In Asian and African countries, the population is dependent on complementary and traditional medicine for the prevention and treatment of diseases [34]. Additionally, many phytochemicals found in medicinal plants have been shown to possess anti-cancer and chemo-preventive properties [34].

The medicinal plant, *Withania somnifera* (Linnaeus) Dunal (*W. somnifera*) has been shown to have pharmacological value as an anti-inflammatory, anti-tumour and antioxidant agent [35]. *W. somnifera* contains many active compounds such as alkaloids, steroidal lactones, saponins, withaferins and withanolides [35]. Previously, withaferin A and 3- β -hydroxy-2, 3 dihydro withanolide F were shown to possess anti-inflammatory properties [36]. *W. somnifera* root powder possesses immune modulating properties [36] and inhibits immune functions [36]. *In vitro*, a *W. somnifera* formulation (WSF) significantly increased the Th-1 cytokine response in concanavalin A (Con A) sensitized splenocytes and induced a significant production of interferon-gamma (IFN- γ) whilst having no effect on interleukin (IL)-4 levels [37]. Additionally, *W. somnifera* proved effective in rheumatologic conditions that may be related to its anti-inflammatory properties [35]. In Ayurvedic and Unani systems, *W. somnifera* leaves have been used in the treatment of tumours, with withanolide steroidal lactones exhibiting anti-tumour properties [35]. The leaves and roots of *W. somnifera* contain antioxidant components [38]. *W. somnifera* leaves contain withaferin-A, a compound that delays tumour progression, induces apoptosis, inhibits NF- κ B activation, tumour cell angiogenesis, and macrophage cytokine production (IL-6 and tumour necrosis factor-alpha (TNF- α)), increases GSH, glutathione *S* transferase, SOD, and CAT [39]. In Swiss albino mice exposed to a skin cancer-causing agent, *W. somnifera* root extract decreased the incidence and average number of skin lesions [40]. The antioxidant activity of *W. somnifera* root extract may be responsible for its chemopreventive activity [40]. Additionally, withanolides were shown to inhibit proliferation of human cancer (breast, lung, and colon) cell lines comparable to doxorubicin (a known cancer drug), *in vitro* [41]. Interestingly, withaferin A inhibited breast and colon cancer cell growth more effectively than doxorubicin [41]. Withaferin A and withanolide (D and E) also demonstrated anti-tumour activity *in vitro* (cells derived from human epidermoid carcinoma of nasopharynx) and *in vivo* (Ehrlich ascites carcinoma, Sarcoma (180 and Black) and E 0771 mammary adenocarcinoma in mice) [42].

Centella asiatica (Linnaeus) Urban (*C. asiatica*) is a medicinal plant commonly consumed as a vegetable [43]. Previous studies have indicated the safe consumption, minimal toxicity and effectiveness of *C. asiatica* [44]. The compounds isolated from *C. asiatica* include triterpenes saponins (asiatic acid, asiaticoside, madecassic acid, madecassoside) as well as flavonoids (quercetin and kaempferol) [45]. The chemical constituents of *C. asiatica* possess medicinal properties [46] and have been used in the treatment of inflammation, immune system deficiencies [47] and certain cancers [48]. Previously, *C. asiatica* water extracts and asiaticoside showed anti-inflammatory properties by inhibiting nitric oxide (NO) synthesis [49]. In lipopolysaccharide (LPS) stimulated RAW 264.7 murine macrophage cells, asiatic acid and madecassic acid inhibited the expression of enzymes (inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2)) and inflammatory cytokines (IL-6, IL-1 β , TNF- α) by down-regulating NF- κ B [50,51]. Asiaticoside also inhibited LPS stimulated TNF- α , IL-6, COX-2 protein expression and prostaglandin E2 (PGE2) production [52]. *C. asiatica* elicited significantly high antioxidant potential with the leaves showing the highest antioxidant activity [53]. Additionally, *C. asiatica* ethanol extract demonstrated higher antioxidant activity than the water extract [54]. *C. asiatica* leaves were found to have very high superoxide (O₂⁻) free radical scavenging activity (86.4%), 2, 2-diphenyl-1 picrylhydrazyl (DPPH) radical scavenging activity (92.7%) and inhibition of linoleic acid peroxidation (98.2%) [55,56]. In rats, *C. asiatica* extract and powder decreased oxidative stress [57]. Cell proliferation of human gastric adenocarcinoma (MK-1), human uterine carcinoma (HeLa), and murine melanoma (B16F10) were inhibited by *C. asiatica* constituents [58]. Asiatic acid prevents skin tumours, inhibits tumour development, induces apoptosis of cancer cells [59] and has anti-inflammatory effects [50]. In breast cancer (MCF-7) cells, asiaticoside stimulates apoptosis, disrupts mitochondrial function and increases caspase-3 activity [60].

There is growing interest in the use of traditional medicinal plants in the treatment of cancer and cachexia owing to their antioxidant, anti-inflammatory and anti-tumour potential. The discovery of a medicinal plant that down-regulates pro-inflammatory cytokine concentrations, decreases antioxidant mechanisms and promotes cell death in cancer cells will be beneficial in the treatment of cancer and cachexia.

RATIONALE, AIMS AND OBJECTIVES

The cancer cachectic syndrome is responsible for 20–25% of cancer deaths due to cardiac and respiratory conditions. Previously cancer and cachexia were associated with increased pro-inflammatory cytokine levels. Anti-cancer and anti-cachectic treatments are very expensive requiring specialized drugs, facilities and health personnel. Most South Africans are unable to afford these treatments; hence there is a need for the development of an alternate and affordable treatment for cancer and cachexia. This study screened two South African medicinal plants for anti-cachectic activity by investigating the modulation of inflammatory cytokines associated with cachexia. A decrease in pro-inflammatory cytokines levels may decrease development and progression of cancer cachexia. Additionally, the anti-cancer activity of plant extracts/ fractions were assessed for antioxidant potential and induction of apoptosis.

Aims

1. This study investigated the anti-inflammatory, antioxidant and apoptosis inducing potential of *W. somnifera* aqueous root extract and *C. asiatica* ethanolic leaf extract in various cell lines.

Objectives

1. To determine the oxidant scavenging potential, modulation of inflammatory cytokines and cell death induction by *W. somnifera* aqueous root extract in healthy peripheral blood mononuclear cells (PBMC's) and leukaemic (THP-1) cells.
2. To determine the oxidant scavenging potential, modulation of inflammatory cytokines and cell death induction by *C. asiatica* ethanolic leaf extract in healthy PBMC's and THP-1 cells.
3. To determine the oxidant scavenging potential, modulation of inflammatory cytokines and apoptosis induction by *C. asiatica* (crude extract (C) and purified fraction-3 (C3)) in leukaemic (THP-1) cells.

4. To determine the antioxidant and anti-proliferative effects of C and C3 in leukaemic (THP-1) cells.
5. To determine the antioxidant and anti-proliferative/ cytotoxic effects of C3 in lung cancer (A549) cells.
6. To determine the antioxidant and anti-proliferative/ cytotoxic effects of C3 in embryonic kidney (HEK293) cells.

CHAPTER ONE

Literature Review

1.1 Cancer

Human tumourigenesis is a multi-step process in which normal cells progressively become neoplastic cells [61]. There are six hallmarks of cancer namely ‘sustaining proliferative signal, evading growth suppressors, resisting cell death, inducing angiogenesis, activating invasion as well as metastasis and enabling replicative immortality’ [61]. These distinctive and complementary hallmark capabilities enable developing cancer cells to become tumourigenic, metastatic and malignant [61].

1.1.1 The Hallmarks of cancer

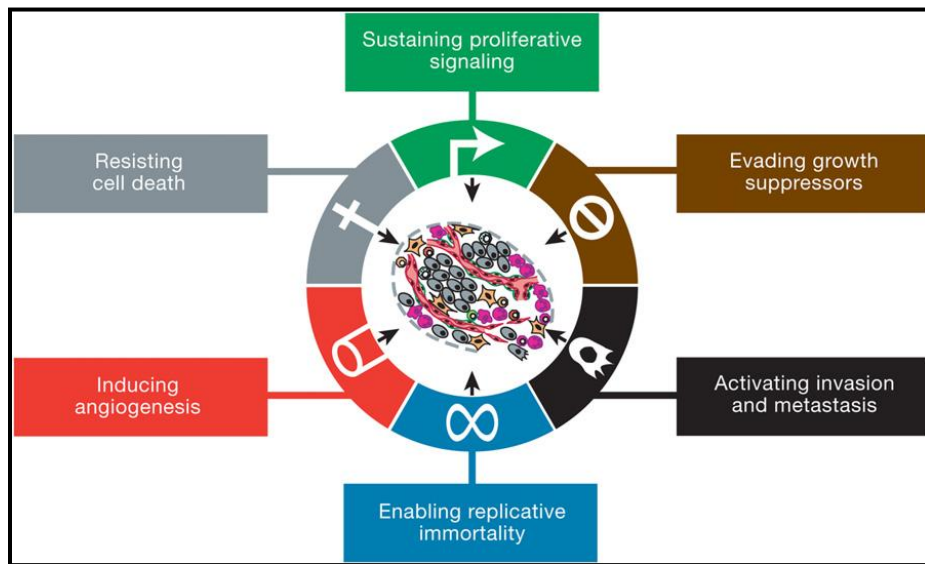


Figure 1.1: The hallmarks of cancer [61].

Notably, sustained chronic proliferation is a fundamental trait of cancerous cells [61,62]. In normal tissues, the production and release of growth-promoting signals are tightly regulated thereby

maintaining homeostatic cell number and function [61]. However in cancer cells, the growth-promoting signals are deregulated and favours sustained proliferation (Figure 1.1) [61,62]. The potential of cancer cells to sustain proliferative signalling is acquired [61] by cancerous cells producing growth factor ligands and stimulating normal cells to produce growth factors [63]. Additionally, deregulation of receptor signalling may render cancerous cells hyper-responsive and facilitates ligand-independent firing [61].

In order to sustain proliferative signalling, cancer cells need to evade signals which suppress cell proliferation (Figure 1.1) [61,62]. Tumour suppressor genes function in the negative regulation of cell proliferation [61]. Two tumour suppressors encode for the retinoblastoma-associated (RB) and TP53 proteins [61]. These proteins play important roles in determining whether cells undergo proliferation or apoptotic cell death [61]. However, cancer cells have defects in RB pathway which allows for persistent cell proliferation [61]. In the event of extensive genome damage and/ or low growth-promoting signals, TP53 can stop cell-cycle progression or induce apoptosis [61]. In cancer cells, the TP53 function is lost which eliminates TP53 apoptosis-inducing function [61]. Additionally, cancer cells corrupt the TGF- β pathway and abolish its anti-proliferative effects [61].

Cell death processes are essential barriers to cancer pathogenesis [61,62]. Cancer cells attenuate apoptosis by increasing anti-apoptotic protein expression and survival signals while decreasing pro-apoptotic protein expression (Figure 1.1) [61,62]. By successfully resisting apoptosis, cancer cells may achieve malignancy and resistance to anti-cancer therapy (Figure 1.1) [62,64]. Necrosis may be beneficial in counteracting cancer cell hyper-proliferation; however necrosis also has tumour promoting potential [61]. Necrosis releases pro-inflammatory signals and recruits inflammatory cells which are capable of promoting angiogenesis, cell proliferation and invasiveness [61].

Proliferation has two barriers namely senescence (a non-proliferative but viable state) and crisis (cell death) [61]. In normal cells, these barriers limit the number of consecutive cell growth and division cycles [61]. However, cancer cells need the ability to replicate uncontrollably (Figure 1.1) [61,62]. Previous studies indicate that telomeres play a role in unlimited proliferation [65]. Telomeric DNA length determines the number of consecutive cell growth and division cycles a cell may undergo before cell death is signalled [61]. The DNA polymerase, telomerase increases telomeric DNA length by adding telomere repeat segments [61]. Telomerase is almost absent in normal cells but highly expressed in cancer cells and allows for unlimited cell growth and division

cycles [61]. Telomerase activity has also been correlated with a resistance to induce senescence and apoptosis [61].

Angiogenesis is the formation of new blood vessels which allows for the delivery of nutrients and oxygen to cells as well as to evacuate metabolic waste from cells [61]. In order to proliferate and metastasize, cancerous cells require a wide vascular network (Figure 1.1) [66]. Thus, cancer cells activate an “angiogenic switch” that facilitates the continuous development of new blood vessels which aids in tumorigenesis and tumour progression (Figure 1.1) [67]. Notably, an inadequate vascular network may lead to tumour cell necrosis and/ or apoptosis [66,68,69].

In order to increase cancer progression, primary malignant cells need to invade and metastasize (Figure 1.1) [61,62]. E-cadherin (a cell-to cell adhesion molecule) functions in forming adheren junctions between cells subsequently assembling cell sheets and maintaining the order of the cells [61]. In carcinoma cells, the E-cadherin functions are lost [61].

1.1.2 Emerging Hallmarks and Enabling characteristics

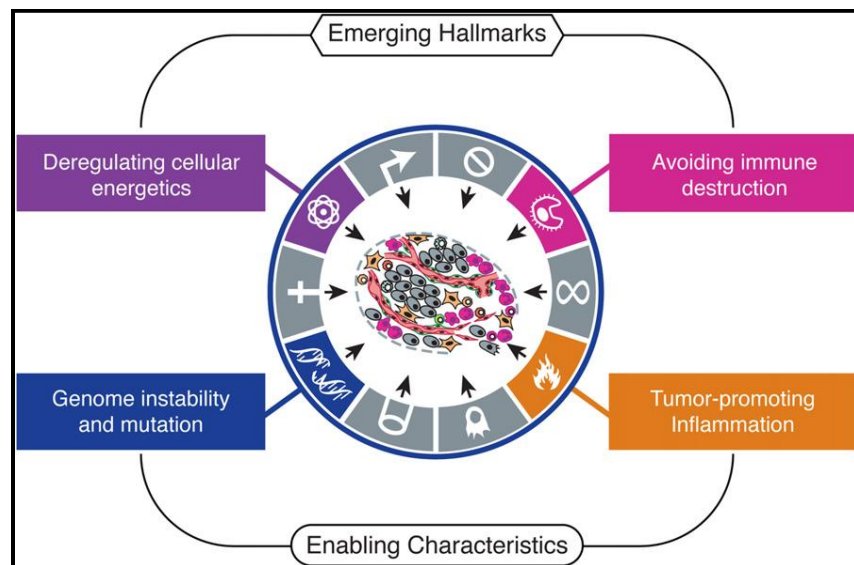


Figure 1.2: The emerging hallmarks and the enabling characteristics [61].

The emerging hallmarks facilitate human cancer development and progression [61]. The first emerging hallmark is the deregulation of cellular energetics (Figure 1.2) which involves

reprogramming of cellular energy metabolism, ultimately encouraging the continuous growth and proliferation of tumour cells [61]. The second emerging hallmark is avoiding immune destruction (Figure 1.2), which entails the ability of cancer cells to evade attack and elimination by immune cells [61].

The acquisition of the hallmarks of cancer is facilitated by two enabling characteristics [61]. The first and most prominent enabling characteristic is genome instability and mutation (Figure 1.2) [61]. Within cancer cells, an increase in genomic instability will induce mutations and genetic changes which may orchestrate the hallmark capabilities, ultimately enhancing tumour progression [61]. The second enabling characteristic is tumour-promoting inflammation (Figure 1.2), which takes into account the inflammatory state of malignant lesions [61]. Within the tumour microenvironment, inflammation supplies several bioactive molecules (growth, survival and pro-angiogenic factors) which allows tumour cells to sustain proliferation, evade cell death and facilitate angiogenesis, invasion, and metastasis [70,71,72,73]. In addition, inflammatory cells release ROS, which are mutagenic and increases genetic evolution of cancer cells, ultimately enhancing malignancy [70].

1.2 Inflammation

The role of inflammation in carcinogenesis has been extensively researched [74] and confirmed by anti-inflammatory therapies which demonstrated its effectiveness in cancer prevention and treatment [22]. There are two stages of inflammation namely the acute and chronic. Acute inflammation occurs for short time periods and is usually beneficial (in tissue repair, pathogen elimination, tumour suppression and anti-tumour activity) to the host [3,22,70,74]. Inflammation that persists for extended time periods is referred to as chronic inflammation which may predispose the host to oncogenic transformation by inducing genomic instability, increasing angiogenesis and cell proliferation [1,7]. Chronic inflammation has been associated with a higher risk of cancer as well as implicated in many steps involved in carcinogenesis [1,4,5,6]. Previous studies suggest that 25% of all cancers are due to chronic inflammation [75] which may be seen as the seventh hallmark of cancer [76]. In addition, increased ROS, inflammatory cytokines, and NF- κ B expression contribute to inflammation-induced carcinogenesis [7]. Taken together, modulation of inflammatory responses may be beneficial in reducing inflammation-induced carcinogenesis [7].

1.3 The Cachectic syndrome

Cachexia is a condition involving the abnormal loss of body weight (greater than 5% of total body mass per month) [14] and is mainly found in malignancy [20]. It is a complex metabolic status resulting in adipose tissue and skeletal muscle depletion [20]. The cachectic syndrome is associated with hormones, neuropeptides, increased pro-inflammatory cytokine production; increased energy expenditure and the release of the tumour factors, lipid mobilizing factor (LMF) and proteolysis inducing factor (PIF) [12].

1.3.1 Hormones and neuropeptides

The loss of body weight is related to the regulation of hormones and neuropeptides. Neuropeptide Y (NPY) is a potent feeding stimulant and is down-regulated by the hypothalamic orexigenic network [77]. Disruption in NPY signalling inhibits food and energy intake [77] thus leading to anorexia. Corticotrophin-releasing factor (CRF) is a part of the anorexigenic neuropeptide network and functions to inhibit NPY [77]. Cytokines including IL-1, IL-6, TNF- α and IFN- γ stimulate CRF which in turn inhibits NPY leading to decreased food intake and increased energy expenditure (EE) [77] (Figure 1.3).

Leptin is secreted from adipocytes [20] and plays an important role in weight regulation [12]. It acts on the central nervous system as a suppresser of food intake and stimulator of energy consumption [14]. Decreased leptin levels in the brain increases hypothalamic orexigenic signals which lead to stimulation of feeding and decreased EE [78,79]. Previously, leptin levels were shown to be inversely correlated with cytokine levels [80,81,82]. Pro-inflammatory cytokines have been shown to inhibit feeding by mimicking the hypothalamic negative-feedback signalling effect of leptin [80] (Figure 1.3). In cachexia, the leptin feedback loop is disrupted, increased levels of leptin block the release of NPY [20] and stimulate CRF, thus decreasing food intake and increasing EE [77].

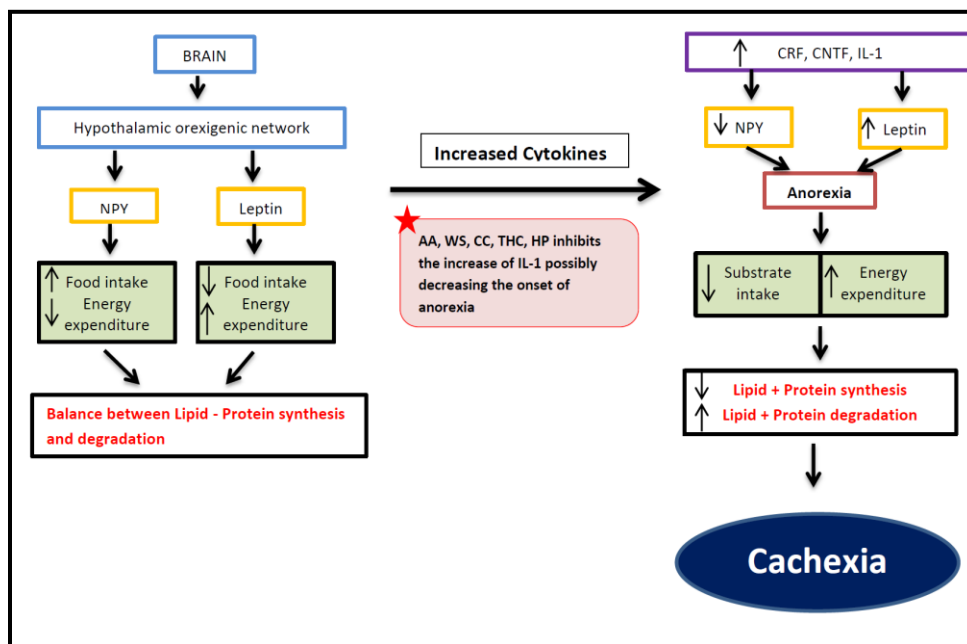


Figure 1.3: Role of leptin and NPY in the cachectic syndrome with possible therapeutic effects of medicinal plants through cytokine modulation (Prepared by author).

Key: AA – Asiatic acid, WS – *Withania somnifera*, CC – *Cinnamomum camphora*, THC – Δ^9 tetrahydrocannabinol, HP – *Harpagophytum procumbens*

Ghrelin is produced by stomach endocrine cells [83] and has been associated with a number of cachectic states such as anorexia nervosa [84]. It plays a role in weight gain by increasing food intake, decreasing food utilisation and inhibiting leptin-induced feeding reduction [85]. Serum levels of adipocyte derived adiponectin are inversely correlated with body weight [86,87] and elevated adiponectin levels have been observed during weight loss and anorexia nervosa [88,89]. Kemik *et al* (2010) have shown C reactive protein (CRP) and leptin concentrations were significantly higher while adiponectin and ghrelin serum concentrations were significantly lower in cachectic colon cancer patients compared to the controls [90]. Insulin is a hormone produced in the pancreas and plays a pivotal role in weight regulation. It is responsible for allowing glucose to enter skeletal muscle and adipose tissue for substrate anabolism [14]. In mononuclear cells, glucose intake initiates acute pro-inflammatory changes and increased NF- κ B binding, suggesting that elevated plasma glucose levels may lead to muscle atrophy in the presence of insulin resistance/ low insulin levels due to the induction of the ubiquitin–proteasome pathway by NF- κ B [17].

1.3.2 Adipose tissue and lipolysis

In cancer cachexia, fat loss is more rapid than muscle loss [91]. Adipose tissue depletion may result from decreased food intake, tumour factors and inflammatory cytokines which inhibit lipogenesis or promote lipolysis [91]. Fatty acids (FA's) are stored in adipose tissue in the form of triacylglycerols (TAG) which accounts for 90% of energy stores [14]. Lipoprotein lipase (LPL) hydrolyses FA's from plasma lipoproteins and transports FA's into adipose tissue for TAG production (Figure 1.4) [14]. Previously, cachexia has been associated with increased serum triglycerides [92] and decreased serum LPL activity [93]. Lipolysis is induced by catabolic hormones stimulating cAMP, which binds and activates protein kinase A (PKA), which in turn phosphorylates and activates hormone sensitive lipase (HSL) [91]. HSL functions in the breakdown of TAG's into FA's and glycerol (Figure 1.4) [14]. In adipocytes from cachectic patients, the expression of HSL mRNA and protein were increased by 50% and 100% respectively [14]. Thus, decreased LPL and increased HSL activity contributes to cachexia by decreasing lipogenesis and increasing lipolysis.

1.3.3 Skeletal muscle and proteolysis

The ATP-ubiquitin-dependent proteolytic pathway has shown to be responsible for the accelerated proteolysis seen in a variety of wasting conditions including cancer cachexia [94]. Ubiquitin is activated by ubiquitin-activating enzyme (E1) and proteins are marked for degradation by the attachment of activated ubiquitin [94]. Thereafter ubiquitin is transferred to a carrier protein (E2) which leads to the ligation of ubiquitin to the target protein directly or by ubiquitin protein ligase activity [94]. Proteolysis occurs within a multi-subunit complex known as the proteasome [94,95] (Figure 1.4). The proteasome consists of a central catalytic chamber (20S proteasome) and two terminal regulatory subunits (19S complex/ PA700 and 11S regulator/or PA28) [94,95]. The 26S active proteasome is formed by the attachment of the regulatory subunits at both ends of the central chamber in opposite orientations [94]. The process requires adenosine triphosphate (ATP) with at least six ATPases associated with the 26S proteasome to provide energy for proteolysis [95]. An increased expression of ubiquitin, E2, and proteasome subunits were found in cachexia rat models with Yoshida ascites hepatoma transplants [96], Yoshida sarcoma transplants [97] and in mice with colon carcinoma (MAC16) transplants [98]. In the muscle of gastric cancer patients, proteasome activity and muscle ubiquitin mRNA expression were increased, indicative of ATP-ubiquitin-dependent proteolysis [21]. This provides evidence that the ATP-ubiquitin-dependent pathway

plays a role in muscle wasting in cancer [94]. The pathway is independent of protein consumption thus nutritional supplementation is unable to prevent or reverse muscle catabolism [94]. Inhibiting the ubiquitin pathway may contribute to decreasing muscle wasting.

1.3.4 The Nuclear Factor kappa beta pathway

NF- κ B is a transcription factor which regulates immune responses, inflammation, cell survival, cell proliferation, invasion, angiogenesis and apoptosis by the induction of numerous genes [74,99,100,101,102,103]. There are five homologous subunits namely RelA/p65, c-Rel, RelB, NF- κ B1/p50, and NF- κ B2/p52. Processing of precursors NF- κ B1/p105 and NF- κ B2/p100 leads to the production of p50 and p52 respectively [104]. The Rel homology domain (RHD) which is present in all NF- κ B members is essential for DNA binding, dimerization (homo and hetero), nuclear localization and inhibitory kappa B (I κ B) binding [104]. The I κ B family regulates NF- κ B signalling by binding to NF- κ B dimers and blocking their nuclear localization [105,106]. In non-stimulated cells, NF- κ B is located in the cytoplasm by interaction with I κ B proteins [107]. NF- κ B is activated by various stimuli such as cytokines (TNF- α , IL-1 β , IFN- γ , IL-6) [108,109], PIF [110] and oxidative stress [111]. In response to these various stimuli, I κ B's are rapidly phosphorylated and degraded via I κ B kinase (IKK) signalosome complex which results in the release of NF- κ B dimers [112]. The dimers translocate to the nucleus and function in the transcription of target genes (inflammatory genes, cell cycle genes, anti-apoptotic genes) [7,105,106,112] (Figure 1.4). The classical or canonical pathway (p50: RelA/p65 and p50: c-Rel dimers) is essential for innate immunity and the inhibition of apoptosis [102,113].

NF- κ B activation has been observed in many human cancers (eg. colon, pancreatic, ovarian, hepatocellular, breast, lymphomas, leukaemia's etc) [114,115]. The association between elevated NF- κ B activation and malignancy development is due to the potential of NF- κ B to induce many cancer hallmarks [7,116,117]. NF- κ B contributes to tumour development by increasing the expression of anti-apoptotic genes, growth factor genes, proto-oncogene *c-Myc*, and cell cycle regulator cyclin D1 which ultimately suppresses apoptosis and stimulates cell proliferation [76,114,118,119]. Previously, NF- κ B expression has been shown to promote cell proliferation while NF- κ B inhibition blocked cell proliferation [120]. In lymphoma and myelogenous leukaemia cells, TNF and IL-1 suppression has been shown to decrease NF- κ B expression and inhibit proliferation

[121]. In certain cancers, increased basal NF- κ B activity has been associated with tumour resistance to chemotherapy and radiation [122]. In breast cancer cells, the over expression of NF- κ B led to increased cell survival and resistance to ionizing radiation [123]. Interestingly, NF- κ B inhibition blocks adaptive radio-resistance [124] and shows promise as a therapeutic approach that stimulates apoptosis and inhibits DNA repair [22].

In immune cells, NF- κ B activation increases the expression of cytokines, COX-2 [125], iNOS [126] and growth factors [18] that leads to the induction of the ubiquitin–proteasome pathway [17]. In mice, muscle specific transgenic expression of activated I κ B kinase β leads to NF- κ B activation which induces extensive muscle wasting through increased gene expression of the 20S proteasome and 19S regulatory subunits of the proteasome [17]. Activated NF- κ B further contributes to proteolysis by suppressing MyoD expression which plays an important role in replenishing wasted muscle [108]. However, cachexia seems to be reversed by NF- κ B antisense therapy without altering tumour growth [127]. Suppressing NF- κ B activation may decrease tissue wasting and preserve muscle mass.

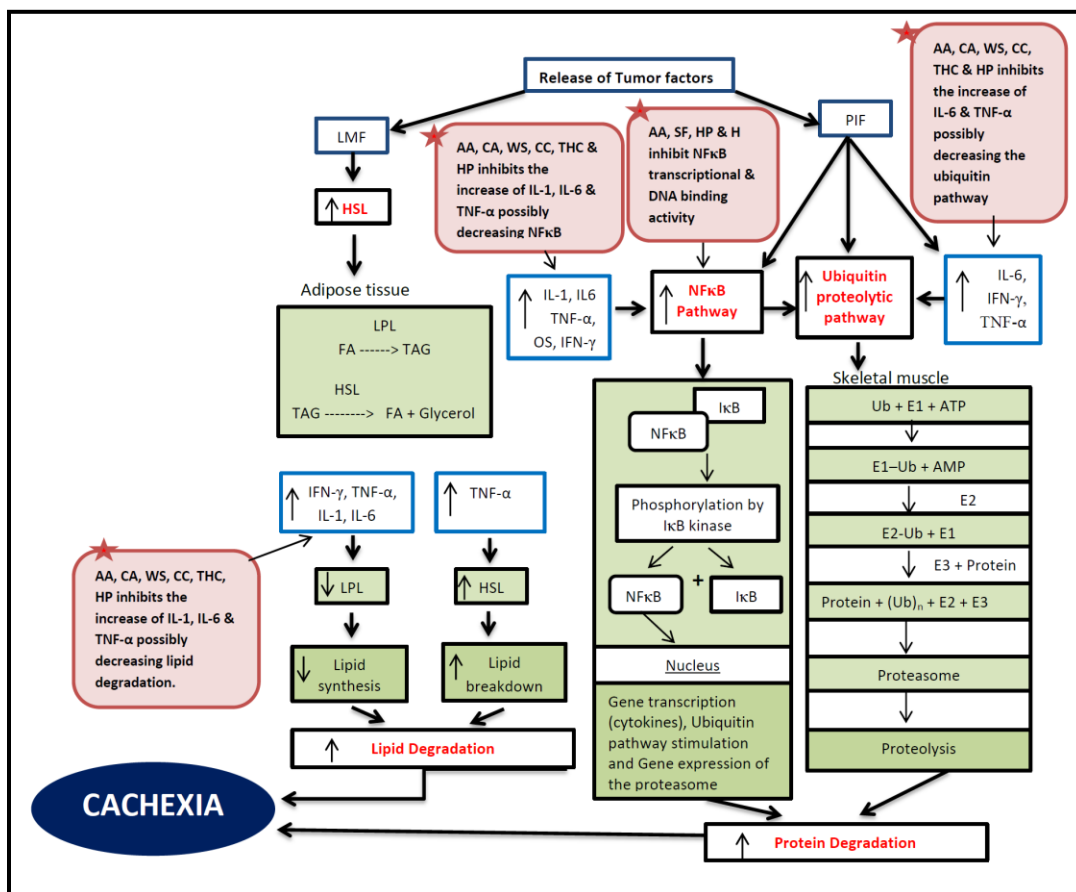


Figure 1.4: The effect of increased levels of tumour factors and cytokines in the depletion of adipose tissue and skeletal muscle via the HSL, NF-κB and Ubiquitin pathways, as well as the therapeutic effects of medicinal plants (Prepared by author).

Key: AA – Asiatic acid, WS – *Withania somnifera*, CC – *Cinnamomum camphora*, THC – Δ^9 tetrahydrocannabinol, HP – *Harpagophytum procumbens*, CA – *Centella asiatica*, SF – *Sutherlandia frutescens*, H – Harpagoside

1.3.5 Tumour effects

1.3.5.1 Energy inefficiency

Tumours produce increased lactate levels resulting in gluconeogenesis via the Cori Cycle (conversion of lactate to glucose) [14] which is energy inefficient (Figure 1.5). Tumours use up to 40 times more glucose than healthy cells due to energy inefficient processes [14] with a significant increase in hepatic glucose production and recycling seen in patients experiencing weight loss [14]. Mitochondrial DNA mutation results in a dysfunctional mitochondrion which prevents the use of

the tricarboxylic acid cycle, preventing complete combustion of pyruvic acid [14] thus leading to increased dependence on glycolysis [128]. Previous studies have found a correlation between increased Cori Cycle activity and weight loss [129]. The tumour is energy inefficient, therefore increases EE [130] and decreases food intake hence contributing to the progression of cancer cachexia [131] (Figure 1.5).

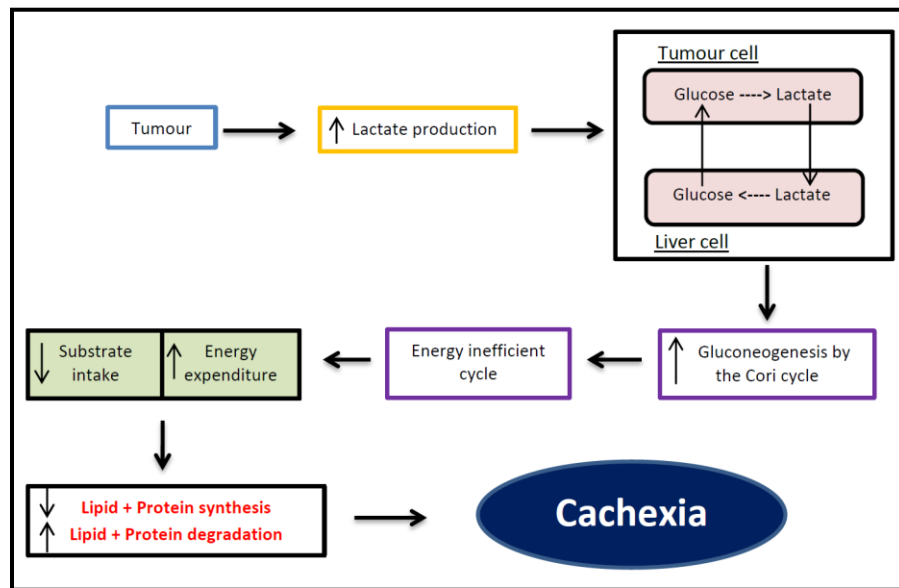


Figure 1.5: Effect of tumour lactate production in the development and progression of cancer cachexia (Prepared by author).

1.3.5.2 Tumour factors: proteolysis inducing factor and lipid mobilizing factor

Tumours release substances such as PIF and LMF which affect pathways leading to cachexia. The proteoglycan, PIF inhibits proteogenesis and induces proteolysis by increasing NF- κ B expression and the ubiquitin-proteasome pathway [132] (Figure 1.4). A study involving gastrocnemius muscles of weight-losing mice revealed that PIF induces the accumulation of ubiquitin-protein conjugates [94] and in murine studies (*in vitro* and *in vivo*) PIF induced skeletal muscle breakdown through NF- κ B dependent activation of the ubiquitin-proteasome pathway [133]. Indicating the important role played by the ubiquitin proteolytic pathway in PIF-induced protein catabolism [98]. Additionally, PIF triggers the release of cytokines (TNF- α , IL-6 and IL-8) from isolated Kupffer

cells, subsequently activating NF- κ B leading to additional pro-inflammatory cytokine production by hepatocytes [134]. LMF is highly abundant in cachexigenic tumours and has been associated with induction of lipolysis [135]. Serum levels of LMF are proportional to the extent of weight loss [136] and LMF has been shown to induce lipolysis in murine adipocytes [137]. This factor may induce lipolysis via HSL activation [14] (Figure 2). LMF possibly increases lipid mobilization and substrate utilization by increasing the mitochondrial oxidative pathway in brown adipose tissue [14] and increasing oxidation of released FA's possibly via the induction of uncoupling protein expression [138].

1.4 Pro-inflammatory cytokines in cancer and cachexia

Inflammatory cytokines enhance carcinogenesis in various ways [139]. Cytokines activate pathways which lead to cell proliferation, angiogenesis, and apoptosis [7]. Continuous exposure to elevated pro-inflammatory cytokine levels is considered to be pro-tumourigenic [7]. Pro-inflammatory cytokines are also important in the induction, promotion and development of experimental cancer cachexia [18]. Concepts of cachexia have proposed that the ongoing stimulus for lipolysis and proteolysis is due to deregulation of TNF- α , IL-1, and IL-6 [140]. Previously, increased pro-inflammatory cytokine levels (tumour-derived, or tumour induced but host-derived) have been correlated with cancer and the prevalence of cachexia [91]. The inflammatory mediators associated with cachexia include IL-1, IL-6, TNF- α , INF- γ [14], IL-8, IL-10, IL-15, leukaemia inhibitory factor and oncostatin-M ciliary neurotrophic factor [141]. In human subjects, pro-inflammatory cytokine (TNF- α , IL-1, IL-6) plasma concentrations have been investigated in relation to the development of cancer cachexia [10]. In cachectic colon cancer patients, significantly higher concentrations of IL-1 α , IL-1 β , IL-6, IL-8, IL-10, and TNF- α were observed compared to the controls [90]. Cytokines injected into animals leads to decreased skeletal muscle protein mass, increased rates of proteolysis and decreased proteogenesis [142]. In addition, pro-inflammatory cytokines have been associated with decreased appetite and defective host energy metabolism contributing to tissue wasting [143]. In mice, studies have shown an improvement in anorexia, cachexia and decreased tumour growth by monospecific neutralizing antibodies targeted to certain cytokines implicated in the cachectic syndrome [144] thus decreasing these cytokine levels are beneficial in treating cachexia.

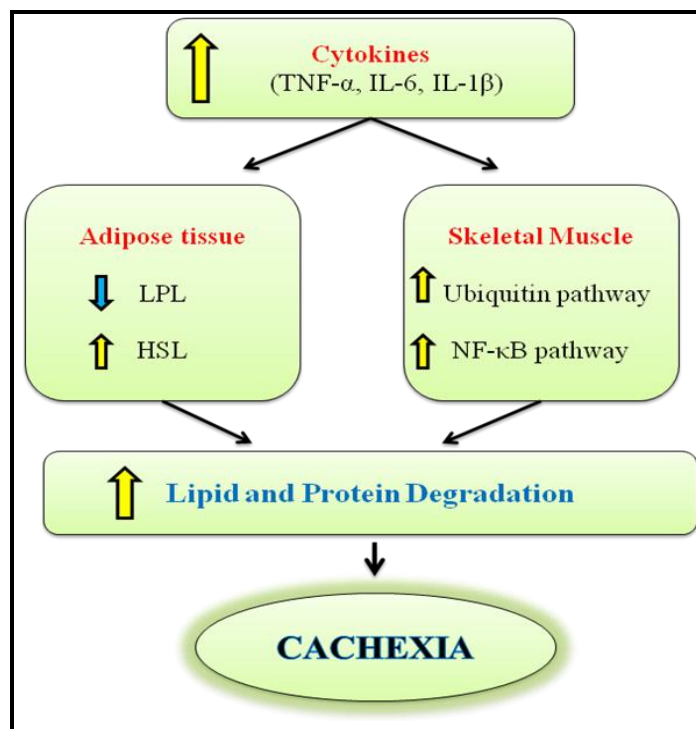


Figure 1.6: Pro-inflammatory cytokines in the development and progression of cancer cachexia (Prepared by author).

1.4.1 Tumour necrosis factor alpha

TNF functions in stimulating cellular change, initiating an inflammatory cascade, tissue remodelling, tissue damage as well as tissue damage recovery [145]. It has both anti-cancer and pro-cancer actions [145]. In tumours, administration of a high TNF dose may have necrotic effects however; TNF also induces NF-κB activation, which has anti-apoptotic activity [145]. Additionally, in tumours TNF can induce DNA damage, inhibit DNA repair [146], act as a growth factor [147], promote angiogenesis, promote tumour growth [145,148,149], and enhance invasion and metastatic potential [7,150]. Notably, TNF-α inhibition reduces tumour burden and metastasis [7].

TNF-α is considered a cachectin due to its catabolic action [151] and has the capability to induce IL-1 production which in turn can induce IL-6 production [152] ultimately resulting in anorexia and muscle wasting [153]. In experimental animals, episodic TNF administration was unsuccessful at inducing cachexia while other studies showed that increased TNF doses are needed to maintain cachectic effects [154]. TNF-α inhibits LPL production and reduces the rate of LPL gene

transcription [155,156,157] thus preventing the formation of new lipid stores while stimulating HSL and increasing lipolysis [158]. *In vivo*, TNF- α administration lead to decreased LPL activity in adipose tissue of rat, mouse, and guinea pig; however TNF- α was unable to decrease LPL in human primary cultures of isolated adipocytes [130]. In human adipose tissue maintained in organ culture, TNF- α dose-dependently suppressed LPL activity and in 3T3-L1 (pre-adipocyte cell line) cells, TNF- α decreased LPL activity as well as mRNA expression [130]. Decreased LPL activity results in decreased uptake of exogenous lipids by adipose tissue and increased circulating TAG's in rats [130]. In addition, TNF- α has been shown to increase skeletal muscle breakdown associated with increased gene expression of free and conjugated ubiquitin and may directly induce ubiquitin-dependant proteolysis [159]. In limb muscles of intact rats, acute intravenous injection of TNF- α results in time-dependent increases in free and conjugated ubiquitin [160] and ubiquitin mRNA [161]. In isolated rat soleus muscle, TNF- α also increased ubiquitin gene expression however no change in proteasome subunit expression was observed [162]. Although an up-regulation of the ubiquitin-dependent proteolytic pathway is suggested, the up-regulation may not be interpreted as increased muscle proteolysis [94]. Increased ubiquitin mRNA may be related to increased signalling for cell death through apoptosis [94].

The early mediators of TNF- α action are ROS and NF- κ B which are sensitive to IL-1 and IL-6 [151]. In skeletal muscle cells, TNF- α stimulates the rapid, dose-dependent NF- κ B activation, nuclear translocation [111,163] and binding activity [127]. Activated NF- κ B may increase the activity of the ubiquitin-proteasome pathway, accelerating proteolysis and promoting muscle weakness [151]. Additionally, in differentiated C2C12 myotubes and in mice gastrocnemius muscle *in vivo*, TNF- α down-regulated MyoD expression thus decreasing muscle replenishment [21].

1.4.2 Interleukin-1

In cancer, IL-1 β increases immune suppression, tumour adhesiveness, invasion and angiogenesis [164]. IL-1 also was shown to enhance metastasis through the induction of adhesion molecules [165]. IL-1 plays a role in anorexia and cachexia that develops during inflammation and cancer [166]. The initiation of anorexia by IL-1 is associated with blocking NPY activities [167]. Studies have indicated that IL-1 α was able to induce profound cachexia [168] whereas IL-1 β regulates immune responses by inducing cytokine expression (IL-6 and IL-12) [169]. Previous studies suggest that IL-1 directly modulates lipid metabolism by suppressing LPL activity [170]. IL-1 also

enhances protein turnover and leads to muscle proteolysis [171]. In animals with IL-1 α -over-expressing cell-derived tumours, cachexia correlated with increased serum levels of leptin and reduced triglyceride levels [168]. In breast cancer cell lines, increased IL-1 α expression correlated with NF- κ B DNA binding, IL-6 expression, and anti-apoptotic gene expression [168] suggesting protein degradation. In mice bearing the colon 26 adenocarcinoma, intra-tumoural injections of IL-1 receptor antagonist significantly reduced cachexia; however rats bearing the Yoshida ascites hepatoma, the injections did not prevent protein catabolism, suggesting the IL-1 receptor antagonist activity may be carcinoma specific [94].

1.4.3 Interleukin-6

The pro-tumourigenic activity of IL-6 has been indicated in many cancers due to its growth-promoting and anti-apoptotic properties [172,173]. Increased IL-6 levels are associated with elevated tumour size, weight loss and poor prognosis [174]. IL-6 produced by macrophage-like Kupffer cells was shown to promote liver injury, inflammation, cell proliferation, and carcinogenesis [175]. Additionally, in breast cancer, elevated IL-6 is regarded as a predisposing genetic factor that contributes to poor prognosis [176]. Increased IL-6 in murine colon 26 adenocarcinoma correlated with the cachexia development [14]. Previously, a pancreatic cancer study showed that IL-6 levels and mRNA expression were significantly elevated in tumours of cancer patients with cachexia compared with tumours of cancer patients without cachexia [140]. In addition, IL-6 protein concentration was increased by 18-fold in clinically localized prostate cancer compared with normal prostatic tissue [177] and IL-6 concentrations were significantly up-regulated in patients with cachexia compared to patients without cachexia [178]. Interestingly, it was demonstrated that the tumour was the main source of IL-6 and it triggers IL-6 expression in PBMC's hence further up-regulating IL-6 levels [178]. In pancreatic cancer, the tumour is a source of cytokines associated with cachexia and PBMC's of cachectic pancreatic cancer patients are sensitized and stimulated by pancreatic cancer cells (T3M4-IL-6-positive) to produce large quantities of IL-6 [140]. *In vivo*, IL-6 injected into mice decreases LPL activity in adipose tissue, suggesting that IL-6 may have an important role in adipose tissue depletion [179]. IL-6 may also play a role in muscle depletion possibly through cathepsin and proteasome pathways [94]. An *in vitro* study using C2C12 myotubes found IL-6 increased the activity of the 26S proteasome and cathepsins B and L suggesting the activation of both the non-lysosomal (proteasomes) and lysosomal (cathepsin) proteolytic pathways [94]. Studies have shown that administration of an IL-6

antibody reverses cachexia parameters [180] and despite high plasma IL-6 concentrations, increased IL-10 levels in colon 26 adenocarcinoma-bearing mice reversed the cachectic syndrome [181].

1.4.4 Interleukin-10

Anti-inflammatory cytokine IL-10 plays an anti-tumourigenic role by suppressing NF- κ B activity, subsequently decreasing pro-inflammatory TNF- α , and IL6 levels [182]. IL-10 also has an inhibitory effect on inflammation-associated cancer [7].

1.5 Treatment targets in cachexia

In the search for cachexia treatments, studies have investigated whether nutritional supplementation may increase body weight and reverse cachexia. However, nutritional supplementation was found to be unsuccessful in reversing cachexia [14]. A possible treatment for cancer cachexia is medroxyprogesterone (MPA), a synthetic derivative of progesterone. Progestagens stimulate appetite by increasing NPY release and may down-regulate cytokine synthesis and release [183]. MPA has the ability to increase appetite by NPY secretion [184] and in patients with advanced malignant disease, MPA improved appetite but not body weight [94]. MPA also decreased PBMC cytokine production in advanced stage cancer patients [183]. In cachectic cancer patients, cyproheptadine, a serotonin antagonist, also increased appetite but not body weight [14]. Hydrazine sulphate was initially proposed to improve appetite and reduce weight loss, but subsequent trials showed no improvements in quality of life and survival of patients [21]. Prednisolone/dexamethsone increases appetite and well-being however weight and muscle wasting were not improved [10]. This suggests that in cachectic cancer patients, anorexia is not a major contributor to loss of lean body mass [94]. Thalidomide has been used for treatment of cachexia possibly due to its anti-inflammatory potential and in advanced cachectic pancreatic cancer patients; thalidomide was shown to attenuate weight loss [183]. In an experimental cancer cachexia model, low doses of indomethacin decreased TNF- α and IL-6 levels, NF- κ B activities, delayed body weight loss and muscle atrophy [18]. In cachectic individuals, NF- κ B suppression by indomethacin could be used to improve the catabolic status [18]. Decreased body weight and muscle wasting can also be prevented by treatment with antioxidants (D- α -tocopherol/ BW755c) or a nitric oxide synthase inhibitor (nitro-L-arginine) [94]. Additionally, omega-3 polyunsaturated FA's such as eicosapentaenoic acid (EPA) are able to modulate the levels of pro-inflammatory cytokines and tumour-derived factors, potentially reversing the effects of cachexia [184]. The anti-tumour and anti-cachectic potential of

EPA have been confirmed by laboratory and clinical studies [185]. Tumour-induced lipolysis in mice with the MAC16 tumour and increased proteolysis in skeletal muscle of cachectic animals were inhibited by EPA [186]. In experimental conditions, EPA has been shown to down-regulate increased ubiquitination [94], interfere with proteasome activity [21] and prevent NF- κ B accumulation in the nucleus which may decrease proteolysis [185].

1.6 Oxidative stress and antioxidant defence mechanisms

1.6.1 Oxidative stress

ROS include free radicals such as O_2^- , hydroxyl, peroxy, and alkoxy radicals, as well as non-radical species such as hydrogen peroxide (H_2O_2) [187]. Superoxide is a relatively unreactive free radical, produced by the mitochondrial electron transport chain [188]. H_2O_2 may be produced spontaneously from molecular oxygen in peroxisomes and from O_2^- by SOD activity [189]. It is less reactive; however it induces many cellular injuries thus contributing to carcinogenesis [189]. In mammalian cells, the hydroxyl radical is responsible for the main injurious effects of ROS [190]. Additionally, both enzymatic and non-enzymatic reactions produce ROS [23]. The concentration of ROS determines its pro-tumourigenic or anti-tumourigenic potential [191]. Acute low ROS levels are involved in cell proliferation, differentiation, and activation of survival pathways [23,192]. Therefore regulating redox homeostasis is essential to maintain normal cellular functions [23]. Elevated ROS levels causes increased genomic instability, lipid peroxidation, angiogenesis and metastatic potential of tumours [7,191]. However, acute high ROS levels can also inhibit tumour formation [7] by causing damage to cellular components (DNA, proteins and lipids) and inducing cell death [23,192,193]. ROS may also function as signal transduction molecules which induce pro-inflammatory cytokine production and the activation of the NF- κ B pathway [194,195].

An imbalance between ROS production and detoxification leads to oxidative stress [193]. Oxidative stress interacts with all three stages (initiation, promotion, and progression) of carcinogenesis [22]. During cancer initiation, ROS may cause gene mutations [22]. In cancer promotion, ROS can increase cell proliferation and/ or decrease apoptosis [22]. In cancer progression, ROS may cause further DNA alterations [22]. In addition, ROS plays a role in normal stem cell renewal and differentiation [196]. Cancer stem cells (tumour-initiating cells (TICs)) have similar phenotypes to normal stem cells, however there is minimal knowledge regarding the redox status of TICs [23]. If TICs growth is essential during the initial stages of tumourigenesis, then the maintenance of low

ROS levels in TICs may be vital for pre-neoplastic foci survival [23]. Chemo and radio therapy treatments induce ROS production which is useful in eliminating majority of cancer cells; however these treatments may fail to cure the patient due to the TICs ability to survive in high ROS conditions by increasing antioxidant levels [23]. The oxidative stress causes by chemo and radio therapy treatments may also increase DNA damage and mutations which may result in the development of drug-resistant tumour cells [23]. High ROS levels induce cell death thus cancer cells elicit a high antioxidant capacity to combat/ regulate high ROS levels which allows for tumour cell proliferation [23]. Targeting antioxidant mechanisms in cancerous cells may be a promising therapeutic strategy to specifically kill cancer cells (as well as TICs) without damaging normal cells [23].

1.6.2 Antioxidants

Antioxidants such as GSH, GPx, SOD and CAT protect against oxidative cellular damage [24,32]. The tripeptide, GSH (L- γ -glutamyl-L-cysteinyl-L-glycine) is formed enzymatically by glycine, cysteine, and glutamate [25]. GSH is a highly soluble, abundant antioxidant found in all cell compartments and plays a central role in maintaining cellular homeostasis [187,197].

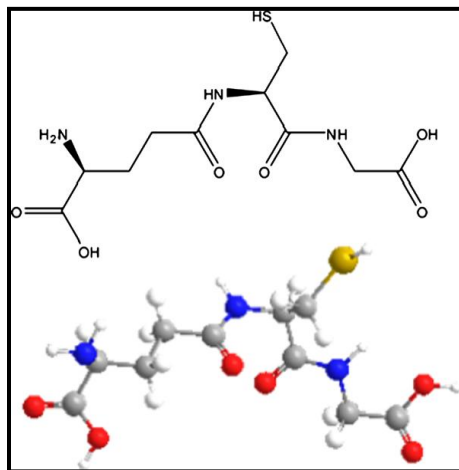


Figure 1.7: GSH stereochemical and ball and stick figure [198].

SOD detoxifies O_2^- into H_2O_2 and molecular oxygen, thereafter H_2O_2 is further detoxified by GPx and CAT into oxygen and water [24] (Figure 1.8). During GSH detoxification of H_2O_2 and lipid peroxides, GSH is oxidized to GSSG, a reaction is catalyzed by GPx [197].

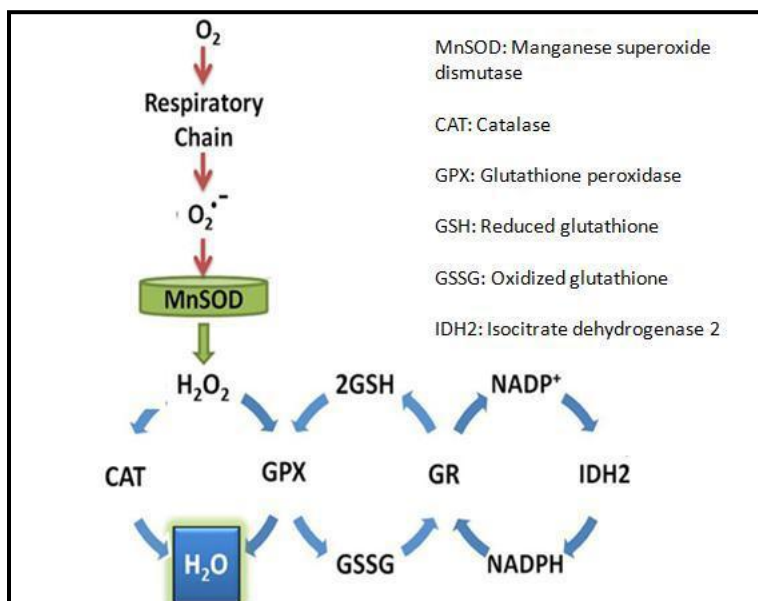


Figure 1.8: Anti-oxidant detoxification of ROS [199].

The GSH/ GSSG ratio is an important, simple and convenient indicator of cellular oxidative stress [197]. Usually, there is a high cellular reduced GSH/ GSSG ratio which is decreased during oxidative stress and apoptosis [187,200]. Due to the potentially toxic nature of GSSG, GSSG is actively reduced to GSH by glutathione reductase [198].

GSH regulates apoptosis by preventing ROS accumulation [25]. Previous studies have demonstrated that elevated GSH levels have been associated with resistance to apoptosis [201,202]. GSH supplementation prevents apoptosis [25,203] while GSH replenishment protects against apoptosis [202]. Decreased GSH levels are associated with an imbalanced cellular redox status and ROS-mediated apoptosis [187,204]. The correlation between GSH depletion and the progression of apoptosis has also been demonstrated [25]. GSH depletion can predispose cells to apoptosis or directly trigger cell death by modulating the permeability transition pore formation triggering cytochrome c release, activating Bax and executioner caspases [205,206,207]. *In vitro* studies have demonstrated that decreased GSH levels are required for apoptosome formation [208]. On the other

hand, Bcl-2 over-expression increases GSH levels and inhibits intrinsic cell death [209]. During apoptosis, an increase in intracellular GSSG has been shown to occur before cytochrome c release and caspase-3 activation [25]. Under physiological conditions, GSSG is maintained at low levels, however during stressful conditions GSSG levels are increased [25]. Previous studies have shown that after oxidant exposure, an initial increase in GSSG production preceded the activation of intrinsic apoptotic cell death hours later [210,211,212,213]. Notably, thiol antioxidant administration and cellular GSH/ GSSG redox status stabilisation after oxidant-induced GSH oxidation did not prevent apoptosis [211].

1.6.3 Nuclear factor erythroid 2-related factor 2 pathway

Nrf-2 is a functionally active transcription factor and is expressed to control gene expression [214]. During resting conditions, Nrf-2 is bound and constitutively degraded by Kelch-like ECH-associated protein 1 (KEAP1) in the cytosol [215] (Figure 1.9). Under conditions of increased oxidative stress, KEAP1 is oxidised and modified thereby allowing for Nrf-2 release and translocation to the nucleus where it increases gene transcription [215] (Figure 1.9). Nrf-2 stimulates the transcriptional activation of various cytoprotective and detoxification genes (SOD, CAT and GPx) rapidly changing the sensitivity of cells to ROS [216]. In addition, Nrf-2 is the master regulator of enzymes responsible for GSH production and utilization through the expression of glutathione *S*-transferases and GPx [23,217]. Glutathione reductase is also Nrf-2 dependent [218]. Nrf-2 increases the expression of the subunits [the modifier subunit and the catalytic subunit] that forms glutamate–cysteine ligase (GCL). GCL catalyses the rate limiting step in GSH synthesis [23]. Also, Nrf-2 controls the abundance of cysteine which is the rate limiting substrate of GSH synthesis [23].

Nrf-2 may protect against tumourigenesis by decreasing ROS, DNA damage and inhibiting cell migration [218,219,220]. However, Nrf-2 may promote tumourigenesis by increasing antioxidant gene expression which combats increased ROS, ultimately maintaining the redox balance and decreasing ROS induced cell death [218]. The sensitivity of cells to ROS induced cell death is partly determined by the Nrf-2 pathway [221]. Therefore cancer cells take advantage of the Nrf-2 pathway to increase their malignant growth [33]. Elevated Nrf-2 levels also increase cancer cell resistance to radio/ chemo therapy due to Nrf-2 activation and regulation of expression of

detoxification enzymes, antioxidant genes/ proteins and xenobiotic transporters [222]. Thus Nrf-2 is a crucial role player in tumour cell survival and the development of radio/ chemo resistance [221].

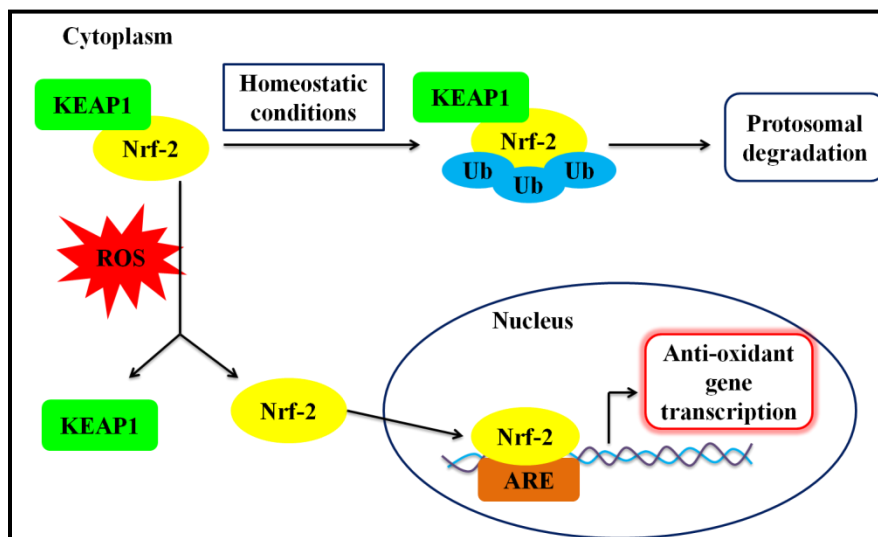


Figure 1.9: Nrf-2 degradation under homeostatic conditions and Nrf-2 activation by ROS (Prepared by author).

Antioxidants aid in cancer cell survival and progression by providing growth signals and combating increased ROS levels, thus antioxidant inhibitors are regarded as promising anti-cancer agents [23]. Antioxidant inhibitors in combination with radio/ chemo therapeutic drugs may be beneficial in killing cancer cells by ROS induced cell death [23].

1.6.4 Oxidative stress and cachexia

Oxidative stress and apoptosis play an important role in the imbalance of catabolic/ anabolic processes, initiation of tissue wasting and cachexia development [223]. In skeletal muscle, an efficient antioxidant system is essential to combat the elevated oxidative capacity and maintain redox homeostasis [224]. Studies indicate that in cancer patients there are increased serum ROS levels and decreased antioxidant levels resulting in a pro-oxidative shift [224]. In cachectic skeletal muscle there are increased O_2^- levels and a defective antioxidant system to dismutate O_2^- indicating that O_2^- is implicated in cancer cachexia [224]. In wasted muscles of mice with hepatoma, lipid peroxidation, and protein oxidation levels were increased [225]. Increased levels of aldehyde products of lipid peroxidation and increased iNOS were associated with muscle wasting

and decreased body weight [94]. However, muscle wasting was prevented by treatment with antioxidants and a nitric oxide synthase inhibitor [94]. Oxidative stress causes gene expression of NF- κ B and caspases leading to inflammation and apoptosis [226]. An important initial step in muscle proteolysis is caspase-3 activation resulting in cleavage of certain proteins, which are eventually degraded by the ubiquitin proteasome system [17]. A study has indicated that caspase (-3 and -8) inhibitors reduced the auto-phosphorylation of RNA-dependent protein kinase (PKR). This indicates that activation of caspases (-3 and -8), activates PKR leading to ROS production, which is essential for proteolysis [17]. Additionally, PKR is cleaved by caspases (-3, -7, -8), releasing the kinase domain and leading to eukaryotic initiation factor 2 (eIF2 α) phosphorylation [17]. This suggests that caspases increases proteolysis owing to PKR-induced increase in the expression of the ubiquitin–proteasome pathway [227] and decreases proteogenesis through eIF2 phosphorylation [17]. The increased proteolytic rate in cachexia has also been shown to be associated with apoptosis and DNA fragmentation [228]. In skeletal muscle of tumour-bearing animals and rats with Yoshida AH-130 ascites hepatoma there was increased DNA laddering [229]. The laddering of DNA increased with tumour burden whereas non-tumour-bearing controls showed no DNA laddering [229]. In addition, weight losing upper gastro intestinal tract cancer patients showed a 3-fold increase in DNA fragmentation, associated with increased poly(ADP-ribose)polymerase-1 (PARP-1) cleavage and decreased MyoD protein [14] suggesting increased apoptosis and decreased muscle replenishment.

1.7 Cell Death Pathways

1.7.1 An overview

There are different types of cell death namely necrosis, autophagy and apoptosis. Intracellular ATP levels play an important role in determining the mode of cell death [230]. High ATP levels facilitate apoptosis while low ATP levels encourage necrosis [230]. Thus, ATP depletion leads to a change from apoptotic to necrotic cell death [231]. Apoptosis is a tightly regulated process involving a number of check points before an irreversible point is reached [232]. Apoptosis affects individual or clusters of cells whereas necrosis is generally an uncontrolled process usually affecting large fields of cells [233]. Morphological features of apoptosis include cell shrinkage, intact cell membrane and no inflammation whereas necrosis leads to cell swelling, disruption of the cell membrane and inflammation [233]. During apoptosis, activated caspases cleave cellular proteins and activate

DNAase which degrades nuclear DNA [234]. The intrinsic (or mitochondrial) and extrinsic (or death receptor) are the two main apoptotic pathways [234] (Figure 1.10).

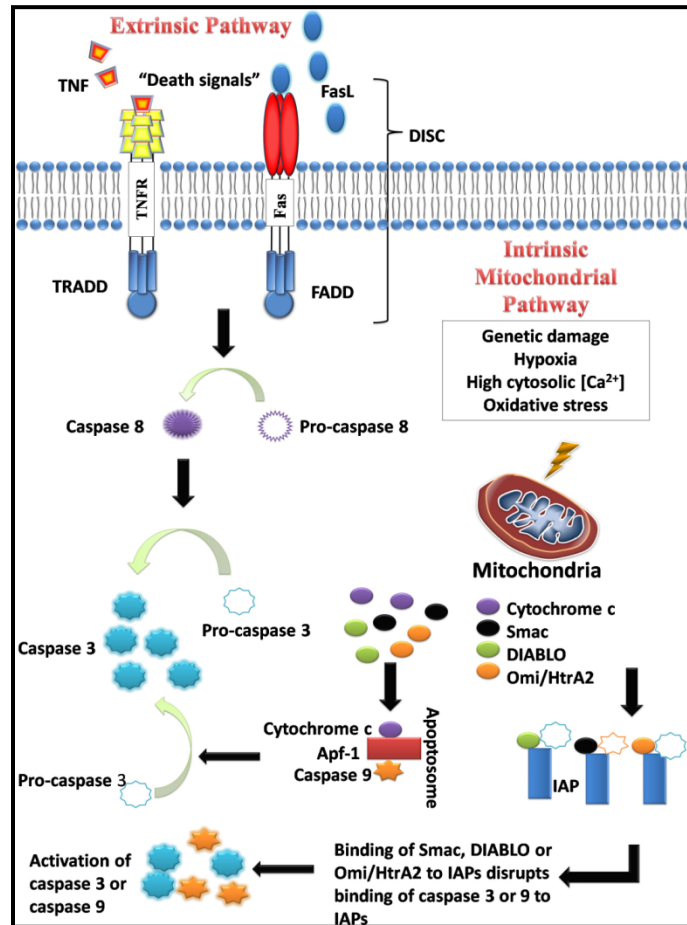


Figure 1.10: Extrinsic and intrinsic apoptotic cell death pathways [234].

1.7.2 The extrinsic apoptotic pathway

During extrinsic apoptosis, the death ligands (TNF and Fas) binds to corresponding death receptors (TNF receptor (TNFR1) and Fas (CD95)) leading to the binding of adapter proteins (FADD and TRADD) [235,236]. Thereafter FADD associates with procaspase-8 which forms a death-inducing signalling complex (DISC) resulting in the auto-catalytic activation of procaspase-8 [237].

1.7.3 The intrinsic apoptotic pathway

The intrinsic apoptotic pathway is activated by various stimuli such as ROS and mitochondrial DNA damage which promote outer membrane permeabilization and the release of apoptotic mediators from the mitochondrial intermembrane space [187,238]. Thereafter caspase-dependent or caspase-independent apoptosis occurs [239]. During caspase-dependent apoptosis, the apoptosome is formed by cytochrome c forming a complex with apoptotic protease-activating factor-1 (Apaf-1) and recruiting procaspase-9 [187]. Caspase-independent apoptosis is mediated by apoptosis inducing factor (AIF) and the induction of nuclear chromatin condensation and DNA fragmentation [240].

1.7.4 Bcl-2 family of proteins

Mitochondrial integrity is regulated by the Bcl-2 family of proteins, including anti-apoptotic members (Bcl-2) and pro-apoptotic members (Bax) [241]. During stressful conditions such as increased ROS, Bax is activated, undergoes conformational changes, translocates to mitochondria and integrates into the outer mitochondrial membrane resulting in membrane permeabilization and subsequent release of pro-apoptotic proteins [242]. Anti-apoptotic protein, Bcl-2 is localized in the mitochondria, where it is responsible for transmembrane potential stabilization, reduces membrane permeability and inhibits pro-apoptotic protein release [241]. The relative ratio of Bcl-2: Bax is important in determining the cell sensitivity or resistance to apoptosis [238].

1.7.5 p53

In human cancer, the *p53* gene is commonly mutated [243]. In about one half of all human cancers, the loss of *p53* function has been demonstrated [244]. The *p53* network can initiate pathways that lead to cell cycle arrest (blockage of G-1 or G-2), cellular senescence or apoptosis [245]. In cells, *p53* protein activation results from the mutational inactivation of some tumor suppressor genes (retinoblastoma (Rb), and adenomatous polyposis coli (APC)) or the mutational activation of some oncogenes (*ras* and *myc*) [245]. In response to stress, the *p53* protein is activated, mediated and regulated by protein kinases, histone acetyl-transferases, methylases, ubiquitin and sumo ligases [245]. The *p53* protein can also be inactivated by phosphatases, histone deacetylases, ubiquitinases or even inhibitors of ubiquitin ligases [245]. In cells, the Rb protein forms a complex with MDM-2

and *p53* which results in increased *p53* activity and apoptotic activity [246]. The *p53* response is changed from G-1 arrest to apoptosis in the presence of elevated levels of active E2F-1 (not bound to Rb) [245]. Cyclin ECDK2 phosphorylates and inhibits Rb and MDM-2 [245]. Activated *p53* stimulates p21 protein synthesis which inhibits cyclin ECDK2 activity, ultimately promoting *p53* activity and apoptosis [245]. Additionally, *p53* regulates genes that initiate the intrinsic (bax, noxa, puma) and the extrinsic (Fas ligand) apoptotic pathway [245]. Under stressful conditions, the *p53* protein is stabilized by phosphorylation [247] and stabilized *p53* accumulates in the nucleus. In the nucleus, *p53* binds to DNA sequences and transactivates a number of pro-apoptotic genes (Bax, and Apaf-1) [248,249,250,251]. Cytoplasmic *p53* can also induce cell death by activating cytosolic Bax [252].

There are three subfamilies in the MAP kinase signaling pathways namely SAPK/c-Jun N-terminal protein kinase (JNK), the p38 mitogen-activated protein kinase and the extracellular signal related kinase (ERK) [253,254]. Activation of the MAP kinases can phosphorylate *p53* which will affect the cell growth and apoptosis pathways [255]. p38 is activated by stressful conditions and apoptosis [243]. Studies have demonstrated the important role of p38 in *p53* activation [243]. Stress induced p38 activation phosphorylates *p53* at various serine residuals which activate *p53* ultimately resulting in apoptosis [243]. Nitric oxide induced p38 activation phosphorylates *p53* at serine 15 resulting in *p53* protein accumulation and apoptosis induction [256]. Upon osmotic shock, activated p38 phosphorylates *p53* at serine 33 and causes G1 arrest [257]. Upon UVB radiation, JNK phosphorylates *p53* at serine 20 ultimately increasing *p53* transcriptional activity [258]. During oxidative stress, JNK also phosphorylates *p53* at serine 15 which leads to protein accumulation and apoptosis [259]. In response to DNA damage, JNK phosphorylates *p53* at threonine 81 and the substitution of this site attenuates JNK mediated *p53* stabilization and impairs *p53* mediated G1 arrest [260]. ERK is generally activated by growth factors [243]. Previously in ovarian cells, ERK was needed for *p53* phosphorylation at serine 15 and cisplatin induced apoptosis [261]. Also, resveratrol induced *p53* activation and apoptosis is mediated by ERK phosphorylating *p53* at serine 15 [262].

Cell cycle progression is tightly regulated by a variety of proteins [263]. Cyclin-dependent kinases (CDKs) ensure cell proliferation control [264]. CDK activation is dependent on phosphorylation and cyclin association [264]. CDKs are negatively controlled by cyclin-dependent kinase inhibitors

(CDKIs) [244,265]. There are two families of CDKIs namely INK4 and CIP/KIP [266,267,268]. The CIP/KIP family (p21Waf1, p27Kip1, and p57Kip2) are potent inhibitors of cyclin–CDK complexes implicated in the G1 and S phase [268,269]. CDKs and cyclins form heterodimeric protein complexes [270] and facilitate the orderly progression of the cell cycle [271]. The transcription factor *p53* regulates downstream genes involved in cell-cycle arrest, DNA repair, and programmed cell death [272]. Loss of *p53* function results in genomic instability, impaired apoptosis, and diminished cell-cycle restraint [264].

The p21 protein is a product of the WAF1/CIP1 gene [264]. p21 plays an vital role in regulating the G1–S transition of the cell cycle [268]. In response to DNA damage, wild-type *p53* binds to the promoter region of the p21WAF1/CIP1 gene which induces p21WAF1/CIP1 expression [264]. The expression of p21WAF1/CIP1 inhibits cyclin/CDK complex activity ultimately blocking cell-cycle progression [264]. In cells arrested at G0 and G1 phases, p27 is highly expressed [264]. At the G1/S phase checkpoint, p27 blocks the cell cycle [264].

1.7.6 Heat shock proteins

In cancer, heat shock protein (HSP)-70 is over expressed and correlates with elevated tumour grade, drug resistance, poor prognosis and survival [273]. HSPs function as inhibitors of apoptosis [242]. Previously, HSP-70 was shown to inhibit cytochrome c release, interacts with Apaf-1 and prevents apoptosome formation and caspase activation [242]. HSP-70 also inhibits apoptosis by decreasing Bax mitochondrial translocation and consequently decreasing mitochondrial pro-apoptotic protein release [242]. In BCR-ABL-expressing cells, HSP-70 binds to the death receptors, inhibiting DISC assembly thus inhibiting the extrinsic apoptotic pathway [273]. In addition, HSPs have been reported to contribute to chemo resistance [242].

1.7.7 Caspases

Caspases belong to a family of cysteine proteases that cleave aspartate residues and execute apoptosis [274]. Under normal conditions, caspases are expressed as inactive zymogen monomers in the cytosol [275]. During stressful conditions, apoptotic signals activate caspases [275]. There are initiator and executioner caspases [274]. Initiator caspases activate apoptosis at a death receptor (caspase-8) or in the cytosol (caspase-9) [274]. Initiator caspases cleave executioner caspases (-3/7) which results in a conformational change and caspase activation [276]. Notably, one activated

executioner caspase can cleave and activate other executioner caspases resulting in positive feedback loop of caspase activation [276]. Apoptotic cell death is executed by caspase-3/7 [274]. Caspase activation is prevented by inhibitors of apoptosis proteins (IAPs) which bind and suppress caspase activity [277]. During apoptosis, Smac/Diablo and Omi/HtrA2 proteins inhibit IAPs which allows for caspase dependant apoptosis [278,279].

1.7.8 DNA fragmentation

Caspase-3/7 activation leads to the activation of cytoplasmic endonucleases which degrade nuclear material [233]. Endonucleases G, ICAD and AIF are release from the mitochondria [233]. Cleavage of ICAD by caspase-3 releases CAD [280] and CAD is responsible for oligonucleosomal DNA degradation and advanced chromatin condensation [281]. Endonucleases G cleave nuclear chromatin to produce oligonuclease DNA fragments [282] whereas AIF leads to DNA fragmentation into 50-300kb pieces and condenses peripheral nuclear chromatin [283]. The DNA fragmentation caused by endonucleases G and AIF are caspase independent [233].

1.7.9 DNA repair mechanisms

1.7.9.1 Poly (ADP-ribose) polymerase-1

The nuclear enzyme, PARP-1 functions in DNA repair, DNA stability, and transcriptional regulation [284]. Depending on the amount of DNA strand breaks, PARP-1 may activate repair and cell cycle control machineries (limited DNA damage) or activate cell death process (extensive DNA damage) [284]. PARP-1 cleavage was shown to be a universal phenomenon occurring during apoptosis [284]. ATP is essential for apoptotic cell death therefore PARP-1 is cleaved and inactivated to preserve cellular ATP for apoptosis [284]. Increased PARP-1 activation leads to energy depletion and necrotic cell death [284]. Caspases cleave PARP-1 at the DNA binding domain leading to the formation of the 24 and 89 kDa polypeptides which abolishes PARP-1 catalytic activity [284]. The PARP-1 89 and 24 kD fragments play a role in apoptotic cell death [284]. The 89 kD fragment binds to intact PARP-1 and prevents the homodimerization essential for PARP-1 activity [284]. The 24 kD fragment binds to DNA strand breaks and RNA transcripts however cannot be poly(ADPriboseyl)ated and released [284]. As a result, DNA repair, transcription and poly(ADP-ribose) synthesis is inhibited [284].

1.7.9.2 8-oxo-7,8-dihydroguanine and OGG-1

Oxidative DNA damage causes single/ double-strand breaks, base modifications, deoxyribose modification and DNA cross-linking which may lead to DNA mutations [285]. DNA mutations, replication errors, cell death and genomic instability occur due to the absence of DNA repair before DNA replication. In mammalian cells, 8-oxo-7,8-dihydroguanine (8-oxoGua) is most abundant oxidative DNA lesion produced and is highly mutagenic [286]. In addition, previous studies have shown increased 8-oxoGua levels in various human cancers [287,288,289]. Thus, 8-oxoGua is used as a biomarker of oxidative DNA damage as well as a measure of oxidative stress [290]. In various cancer types (e.g., leukaemia, renal, lung etc), 8-oxoGua levels were shown to be increased in tumour tissue as compared to healthy tissue [287,291,292,293,294,295]. In cells, these DNA lesions are not lethal however they are highly mutagenic [296]. The base excision repair pathway is one of the main DNA lesion repair pathways [296]. A glycosylase, OGG-1 is an important DNA repair enzyme which recognises and removes 8-oxoG lesions [297].

1.8 Medicinal plants in the modulation of inflammatory cytokines and apoptotic cell death

In developing countries, the population depend on traditional medicines for the treatment of various diseases [298]. On the other hand, developed countries are searching for new ecofriendly treatments [298].

1.8.1 *Withania somnifera* (Linnaeus) Dunal

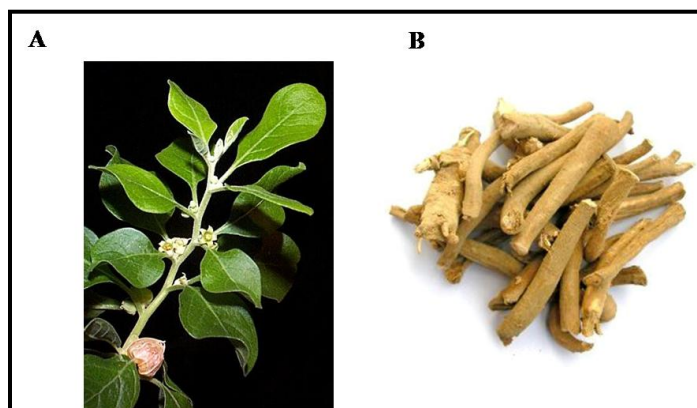


Figure 1.11: *W. somnifera* leaves (A) and roots (B) [299].

Withania somnifera (Linnaeus) Dunal belongs to the Solanaceae family and is commonly referred to as Ashwagandha [35]. It is cultivated in India, East Asia and Africa [35]. *W. somnifera* has shown various properties such as an anti-stress, immunomodulator, antioxidant activities and anabolic effects [300] with potent rejuvenative and life prolonging properties [41]. *W. somnifera* is traditionally used to increase energy, endurance, strength, vital fluids, muscle, and fat [41].

For centuries, Ayurvedic practitioners have used *W. somnifera* roots to increase vitality, longevity and treat health conditions [35]. *W. somnifera* roots were found to contain greater than 35 chemical constituents [35]. Biologically active chemical constituents include alkaloids (withanine, isopellertierine, anferine), steroidal lactones (withanolides, withaferins), saponins (sitoindoside VII and VIII), and withanoloides (sitonidoside XI and X) [35]. *W. somnifera* roots mainly contain compounds known as withanolides [35]. Two main withanolides (withaferin A and withanolide D) are mostly responsible for the pharmacological activity of *W. somnifera* [35].

1.8.1.1 Antioxidant activity

In experimental animals and clinical studies, the antioxidant properties of *W. somnifera* root may be responsible for the anti-stress, anti-inflammatory and anti-aging effects [301]. *W. somnifera* contains various antioxidants (caffeic acid, chlorogenic acid, ellagic acid, ferulic acid, gallic acid, catechin, tannic acid, kaempferol, quercetin and rutin) [35,40]. The leaves and roots of *W. somnifera* is a good source of nonenzymatic (ascorbic acid, tocopherol and GSH) and enzymatic (SOD, ascorbate peroxidase, CAT, peroxidase) antioxidant components [38]. In the skin and liver, *W. somnifera* significantly increased antioxidant enzymes (GSH, GPx and CAT) as well as inhibited lipid peroxide levels [302]. Additionally, *W. somnifera* active constituents (sitoindosides VII-X and Withaferin A) were shown to increase antioxidant (SOD, CAT and GPx) activity in rat brain frontal cortex and striatum [42].

1.8.1.2 Immunomodulatory and anti-inflammatory activity

W. somnifera was found to be an immune-stimulant in immune-suppressed animal models [303] whereas an immune regulator in immune inflammation animal models [304]. The immunosuppressive action may be due to the presence of withanolides, steroidal lactones and

flavanoids [305]. Previous studies investigated the capacity of *W. somnifera* to relieve inflammatory conditions and have proven the effectiveness of *W. somnifera* as an anti-inflammatory agent [35]. In rats, *W. somnifera* root powder and withaferin A demonstrated anti-inflammatory activity comparable to that of hydrocortisone (a commonly prescribed anti-inflammatory drug) [42,306]. However, hydrocortisone-treated animals lost weight whereas withaferin A treated animals gained weight [42]. In cancerous cell lines, *W. somnifera* decreased NF- κ B levels, suppressed TNF, potentiated apoptotic signalling [307] and reduced tumour size [308]. Withaferin A also inhibits NF- κ B and AP-1 levels [309]. *W. somnifera* extract suppressed LPS stimulated cytokine (TNF- α and IL-1 β) production in rheumatoid arthritis patients and decreased LPS stimulated NO production in RAW 264.7 cells [310]. The extract also inhibited IkappaB-alpha phosphorylation as well as NF- κ B and AP-1 nuclear translocation in normal individuals and rheumatoid arthritis patients [310].

1.8.1.3 Cytotoxic and anti-proliferative activity

W. somnifera and its constituents are effective in cancer (eg. lung, blood, breast, renal etc) prevention and treatment [300]. Clinical studies have suggested the use of *W. somnifera* as an anti-tumour (eg. sarcoma, brain cancer, uterine tumour) and immunomodulatory agent [300]. *W. somnifera* ethanolic extracts demonstrated cytotoxic activity against breast (MCF7, MDA MB 231) and cervical (HeLa) cancer cells with no induction of apoptosis in non-cancerous HaCaT cells [299]. On the other hand, *W. somnifera* aqueous extracts did not show any cytotoxic effect [299]. Interestingly, *W. somnifera* ethanolic extracts induced apoptosis, but not necrosis in breast cancer cells [299]. Previous studies have shown that *W. somnifera* root extracts prevented DMBA-induced squamous cell carcinoma in Swiss albino mice, [40], inhibited tumour growth and increased survival rate in Ehrlich ascites carcinoma mouse model [311], inhibited skin carcinogenesis in mice [312], and elicited anti-angiogenic effects against human laryngeal carcinoma [313]. WSF has been shown to induce the loss of mitochondrial membrane potential, translocation of Bax, release of cytochrome c and Smac/DIABLO suggesting the activation of the intrinsic apoptotic pathway [37]. Cells treated with WSF also showed an over expression of TNF-R1 and DR-4 with associated activation of caspase-8 indicating the involvement of the extrinsic apoptotic pathway [37].

In human promyelocytic leukaemia (HL-60) cells amongst other cancer cells, withaferin A induces ROS generation and mitochondrial dysfunction which trigger events leading to apoptosis [314]. Withaferin A also demonstrated anti-metastatic activity in breast cancer as well as inhibiting the

growth of human breast cancer cells (MCF-7 and MDA-MB-231, *in vitro*) and xenografts (MDA-MB-231, *in vivo*) by inducing apoptosis [315,316]. In experimental tumours (*in vivo*), both *W. somnifera* alcoholic root extract and withaferin A demonstrated anti-tumour and radio-sensitizing effects, without inducing any visible systemic toxicity [317]. *In vitro*, withanolides were shown to inhibit the growth of human cancer (breast, lung, and colon) cell lines comparable to doxorubicin, a cancer chemotherapy drug [41]. Interestingly, withaferin A inhibited cancer (breast and colon) cell line growth to a greater extent than doxorubicin [41]. Taken together, *W. somnifera* extracts may prevent/ inhibit cancerous cell growth and induce apoptosis, suggesting its potential as a chemotherapeutic agent [40].

Agents capable of selectively inducing apoptosis in cancer cells, while minimally effecting normal cells, are of interest in developing cancer chemotherapeutic drugs [318]. *W. somnifera* alone can be used as alternative cancer medicine or as a complimentary cancer medicine in combination with chemo/ radio therapy [300]. During radio/ chemo therapy normal cells are also killed and body immunity is weakened. *W. somnifera* may aid in preventing the adverse effects of conventional cancer therapies and improve patient life styles [300].

1.8.2 *Centella asiatica* (Linnaeus) Urban



Figure 1.12: *C. asiatica* leaves (Photographed by Dr. Kathleen Immelman and colleagues, Department of Botany at the Walter Sisulu University, SA).

Centella asiatica (Linnaeus) Urban belongs to the Apiaceae family (previously known as Umbelliferae) [46]. It is indigenous to South- East Asia, India, Sri Lanka, parts of China, the

Western South Sea Islands, Madagascar, South Africa, South East USA, Mexico, Venezuela, Columbia and Eastern South America [319]. In various countries, *C. asiatica* is referred to as pegaga (Malaysia), Indian pennywort, gotu Kola (Europe and America), mandookaparni (India), pegagan/ kaki kuda (Indonesia) and Luei Gong Gen/ Tung Chain (China) [320]. Over the years *C. asiatica* has become a plant of interest due to its potential as an alternative natural antioxidant [46]. In the international market of medicinal plant trade, *C. asiatica* has been reported to be an important medicinal plant [46]. However, *C. asiatica* wild stocks are markedly depleted due to its large scale usage and limited cultivation [46]. Thus, *C. asiatica* is listed as threatened and endangered plant species by the International Union for Conservation of Nature and Natural Resources [46,321]. *C. asiatica* is commonly used in food and beverages due to its vast range of health benefits including antioxidant [54,322], anti-inflammatory, wound healing and memory enhancing properties [46,323]. Additionally, *C. asiatica* consumption within the recommended dosage elicits no known side effects [323].

The main biologically active components found in *C. asiatica* are triterpenes saponins [46] such as asiatic acid, madecassic acid, asiaticoside, madecassoside [324]. *C. asiatica* also contains a vast range of flavonoids (quercetin, kaempferol, catechin, rutin, apigenin and naringin), volatile oils (caryophyllene, farnesol and elemene) [325,326], vitamins and minerals (A, C, B1, B2, niacin, carotene, chloride, sulphate, phosphate, iron, calcium, magnesium, sodium and potassium) [46]. Previously, *C. asiatica* leaves were reported to contain the highest phytochemicals concentration compared to the petioles and the roots [327].

1.8.2.1 Antioxidant activity

Compared to other plant parts *C. asiatica* leaves showed the highest antioxidant activity and contained the highest phenolic compounds suggesting that *C. asiatica* phenolic compounds are mainly responsible for the antioxidant potential [53]. Among 20 traditional leafy vegetables cultivated in South Africa and 11 edible Indian leafy vegetables, *C. asiatica* showed highest antioxidant activity [56,328]. Additionally, *C. asiatica* had the highest total phenol and flavonoid content compared to other leafy vegetables [56]. The plant has been shown to decrease lipid peroxidation by decreasing malondialdehyde (MDA) levels and increasing CAT activity ultimately preventing H₂O₂ accumulation [329]. The antioxidant activity of *C. asiatica* was higher in the ethanolic leaf extract than the aqueous leaf extract [330]. *C. asiatica* phenolic content was also the

highest in the ethanolic extract [331]. Previously, the antioxidant activity of *C. asiatica* (84%) has been shown to be comparable to the activity of rosemary, sage, vitamin C (88%) and grape seed extract (83%) [332,333]. In Sprague Dawley rats, *C. asiatica* (extract and powder) was shown to decrease H₂O₂-induced oxidative stress by adjusting antioxidant defence mechanisms and decreasing lipid peroxidation [334]. In lymphoma-bearing mice, oral treatment of *C. asiatica* methanol extract significantly increased antioxidant enzymes such as SOD, CAT and GPx [335]. Additionally, in rats, exogenous SOD from *C. asiatica* is capable of increasing endogenous SOD activities, which decreases formaldehyde induced oxidative damage [193].

The antioxidant potential of *C. asiatica* offers an effective and safe way of increasing the body defence against ROS [323], therefore *C. asiatica* should be explored to as a source of natural antioxidants [46].

1.8.2.2 Immunomodulatory and anti-inflammatory activity

C. asiatica has been used in the treatment of inflammation and immune system deficiencies [47]. In a mouse macrophage cell line, *C. asiatica* aqueous extract increased NO and TNF- α production and lead to TNF- α expression (*C. asiatica* alone and in conjunction with LPS) [336]. In contrast, *C. asiatica* ethanolic extract suppressed iNOS and TNF- α expression which correlated with decreased NO and TNF- α protein levels [336]. In PBMC's, cell proliferation and TNF- α production were increased by *C. asiatica* water extract however inhibited by the ethanol extract [337]. Asiatic acid dose-dependently inhibits LPS induced NO and PGE2 production [50] while asiaticoside mildly inhibited NO and PGE2 production [50]. In a study by Yun *et al* (2008), pre-treatment of RAW264.7 cells with asiatic acid significantly reduced IL-6 production and mRNA expression, whereas TNF- α and IL-1 β production and mRNA expressions were only slightly inhibited [50]. Additionally, in RAW 264.7 cells transfected with an NF- κ B-dependent luciferase reporter plasmid, LPS induced a 4-fold increase in NF- κ B transcription activity which decreased dose-dependently with asiatic acid treatment [50]. This indicates that asiatic acid inhibits the LPS-induced DNA binding activity of NF- κ B and the nuclear translocations of p65 and p50 proteins [50].

1.8.2.3 Cytotoxic and anti-proliferative activity

In vitro, *C. asiatica* methanolic extract showed anti-proliferative activity in a number of cancer cells including mouse fibrosarcoma, hepatocarcinoma, gastric adenocarcinoma, murine melanoma, and keratinocytes [338]. In male F344 rats, *C. asiatica* aqueous extract exerted chemo-preventive effects on colon tumourigenesis [339]. In MCF-7 breast cancer cells, *C. asiatica* dose dependently inhibited cell proliferation and induced apoptosis by nuclear condensation, externalization of phosphatidyl serine, loss of mitochondrial membrane potential and DNA damage [338]. In primary human respiratory epithelial cells, *C. asiatica* aqueous extract dose dependently decrease cell proliferation and at high concentrations may cause cytotoxicity [340]. *C. asiatica* juice inhibited HepG2 human hepatoma proliferation through apoptosis and DNA damage [341]. In addition, *C. asiatica* aqueous extract showed cytotoxic activity against human breast cancer (MDA-MB 231) and mouse melanoma (B16F1) whereas *C. asiatica* was not cytotoxic in human lung carcinoma (A549) and normal hamster kidney (BHK-21) cell lines [322].

Human gastric adenocarcinoma (MK-1), human uterine carcinoma (HeLa), and murine melanoma (B16F10) cell proliferation was inhibited by *C. asiatica* constituents [58]. A partially purified fraction of *C. asiatica* methanol extract inhibited tumour cell growth whereas induced no toxic effects on lymphocytes [46]. In HepG2, HT-29 human colon adenocarcinoma, and MCF7 cells, asiatic acid has been shown to inhibit cell growth and induce apoptosis [342,343,344]. In A549 cells, asiatic acid induced growth inhibition and cell death [345]. Asiatic acid has also shown to decrease cell viability, induce apoptosis, and increase basal ROS levels and caspase-3 activity [59]. However, the antioxidant Trolox has been shown to efficiently suppress the induction of cell death and caspase-3 activation by asiatic acid [59]. In HepG2 cells, asiatic acid induces apoptosis through increased intracellular Ca^{2+} and consequently increased *p53* expression [342]. However, in SK-MEL-2 cells, it is suggested that *p53* has no role in asiatic acid induced apoptosis, since no change in the nuclear accumulation of mutant *p53* was noted [59].

1.9 Conclusion

Cachexia is the most debilitating and life threatening aspect of cancer [18] resulting in 20–25% of cancer deaths. It is essential to establish an effective cancer and cachexia treatment to prolong the survival rate of cancer patients and improve patient quality of life. Understanding and targeting the factors and processes that contribute to cancer and cachexia progression will aid in the invention of

new treatments. Cancer as well as the cachectic syndrome is associated with increased pro-inflammatory cytokine production [14], oxidative stress and cell death. Therefore, the discovery of a medicinal plant/ active fraction capable of down regulating pro-inflammatory cytokine levels, decreasing cancer cell antioxidant mechanisms, and increasing cancerous cell death may lead to the development of a natural inexpensive treatment for cancer cachexia.

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CHAPTER TWO

***Withania somnifera* modulates cancer cachexia associated inflammatory cytokines and cell death in leukaemic THP-1 cells and peripheral blood mononuclear cells (PBMC's)**

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Abstract

Background: Cancer and inflammation are associated with cachexia. *Withania somnifera* (*W. somnifera*) possesses antioxidant and anti-inflammatory potential. We investigated the potential of an aqueous root extract of *W. somnifera* (W_{RE}) to modulate cytokines, antioxidants and apoptosis in leukaemic THP-1 cells and peripheral blood mononuclear cells (PBMC's).

Methods: Cytotoxicity of W_{RE} was determined at 24 and 72 hours (h). Oxidant scavenging activity of W_{RE} was evaluated (2, 2-diphenyl-1-picrylhydrazyl assay). Glutathione (GSH) levels, caspase (-8, -9, -3/7) activities and adenosine triphosphate (ATP) levels (Luminometry) were then assayed. Tumour necrosis factor- α (TNF- α), interleukin (IL)-6, IL-1 β and IL-10 levels were also assessed using enzyme-linked immunosorbant assay.

Results: At 24 h, W_{RE} (0.2–0.4 mg/ml) decreased PBMC viability between 20–25%, whereas it increased THP-1 viability between 15–23% ($p < 0.001$). At 72 h, W_{RE} increased PBMC viability by 27–39% (0.05, 0.4 mg/ml W_{RE}) whereas decreased THP-1 viability between 9–16% (0.05–0.4 mg/ml W_{RE}) ($p < 0.001$). Oxidant scavenging activity was increased by W_{RE} (0.05–0.4 mg/ml, $p < 0.0001$). PBMC TNF- α and IL-10 levels were decreased by 0.2–0.4 mg/ml W_{RE} , whereas IL-1 β levels were increased by 0.05–0.4 mg/ml W_{RE} ($p < 0.0001$). In THP-1 cells, W_{RE} (0.05–0.4 mg/ml) decreased TNF- α , IL-1 β and IL-6 levels ($p < 0.0001$). At 24 h, GSH levels were decreased in PBMC's, whilst increased in THP-1 cells by 0.2–0.4 mg/ml W_{RE} ($p < 0.0001$). At 72 h, W_{RE} (0.1–0.4 mg/ml) decreased GSH levels in both cell lines ($p < 0.0001$). At 24 h, W_{RE} (0.2–0.4 mg/ml) increased PBMC caspase (-8, -3/7) activities whereas W_{RE} (0.05, 0.1, 0.4 mg/ml) increased THP-1 caspase (-9, -3/7) activities ($p < 0.0001$). At 72 h, PBMC caspase (-8, -9, 3/7) activities were increased at 0.05–0.1 mg/ml W_{RE} ($p < 0.0001$). In THP-1 cells, caspase (-8, -9, -3/7) activities and ATP levels were increased by 0.1–0.2 mg/ml W_{RE} , whereas decreased by 0.05 and 0.4 mg/ml W_{RE} (72 h, $p < 0.0001$).

Conclusion: In PBMC's and THP-1 cells, W_{RE} proved to effectively modulate antioxidant activity, inflammatory cytokines and cell death. In THP-1 cells, W_{RE} decreased pro-inflammatory cytokine levels, which may alleviate cancer cachexia and excessive leukaemic cell growth.

Key words: Cancer, Cachexia, Cytokines, Apoptosis, *Withania somnifera*

2.1 Introduction

Chronic inflammation plays an essential role in malignancies [1] through the initiation, promotion and progression of tumours [2]. Usually, the host-mediated anti-tumour activity overcomes the tumour-mediated immunosuppressive activity leading to the elimination of cancerous cells [2]. However, in the presence of an inadequate host anti-tumour defence, the pro-inflammatory tumour microenvironment is enhanced and promotes tumour development, invasion, angiogenesis and metastasis [2].

The cachectic syndrome is prominent in malignancies occurring in up to 50% of all cancer patients [3]. It is a progressive, debilitating condition leading to abnormal weight loss, as a result of adipose tissue (85%) and skeletal muscle (75%) depletion [3-5]. Modulation of lipogenesis and lipolysis is essential in maintaining adipose tissue mass. Lipoprotein lipase (LPL) hydrolyses fatty acids (FA's) from plasma lipoproteins, thereafter FA's are transported to adipose tissue for triacylglycerol (TAG) production, whereas hormone sensitive lipase (HSL) hydrolyses TAG's into FA's and glycerol [3]. Literature shows that decreased serum LPL levels/activity and increased HSL levels/activity are associated with cachexia [6-8]. Additionally, increased proteolysis [9] and decreased proteogenesis have been established in cachectic patients [10]. The ATP-ubiquitin-dependent proteolytic pathway has been shown to be responsible for the accelerated proteolysis seen in a variety of wasting conditions, including cancer cachexia [11].

Inflammatory cytokines, oxidative stress and apoptosis have been implicated in the initiation and progression of cancer, imbalance of catabolic/anabolic processes [12] and development of cachexia [13]. Production of inflammatory cytokines [tumour necrosis factor- α (TNF- α), interleukin (IL) – 6, and IL-1 β] is activated by lipopolysaccharide (LPS) that potently stimulates macrophages [14, 15]. The LPS signal is transduced by LPS binding to LPS binding protein, delivered to CD14 and transferred to Toll like receptor-4 [16]. Nuclear factor kappa B (NF- κ B) is subsequently activated and regulates the transcription of genes associated with inflammation, proliferation, invasion, angiogenesis and apoptosis [1, 17-19]. Pro-inflammatory cytokines (TNF- α , IL-6 and IL-1) have been shown to decrease LPL activity [20-23], which reduces the uptake of exogenous lipids by adipose tissue [21], ultimately decreasing lipogenesis. Additionally, previous studies have indicated that TNF- α increased ubiquitin (concentrations and mRNA), while IL-6 increased the 26S proteasome and cathepsins activities, which may activate proteolytic pathways [4, 24-26],

ultimately increasing proteolysis. In combination, excessive levels of pro-inflammatory cytokines increase tumour suppressive activity [2] and tissue wasting [3].

Reactive oxygen species (ROS) have been associated with tumour initiation, inflammation [2, 27] and muscle wasting [28]. However, antioxidants have been shown to decrease muscle wasting by neutralizing ROS [1, 28]. Elevated ROS levels activate apoptotic pathways, ultimately activating caspase-3 [29]. Caspase-3 activation plays an essential role in the execution of apoptosis, as well as muscle proteolysis [30]. In addition, in weight losing upper gastro-intestinal tract cancer patients, deoxyribonucleic acid (DNA) fragmentation and poly (ADP-ribose) polymerase (PARP) cleavage were increased, whereas MyoD protein was decreased, indicating increased apoptosis and decreased muscle replenishment [3].

Cancer patients suffer from a wide range of side-effects caused by current cancer chemotherapeutic and radiotherapeutic agents. Patients are constantly seeking alternative traditional remedies to alleviate their discomfort. *Withania somnifera* (L.) Dunal (*W. somnifera*) is a well known medicinal plant cultivated in India, parts of East Asia and Africa [31]. It is commonly referred to as Ashwagandha and belongs to the Solanaceae family [31]. Compounds isolated from *W. somnifera* include withaferin A and 3- β -hydroxy-2, 3 dihydro withanolide F [32]. The major constituent of *W. somnifera* root extract is withanolide-A [33]. *W. somnifera* is frequently used in Ayurvedic medicine due to its various medicinal properties [31]. These properties include anti-inflammatory [34], antioxidant and immune-modulatory activity [35]. *W. somnifera* was found to be an immune regulator in inflammation animal models [36]. The immunosuppressive action of *W. somnifera* may be due to the presence of withanolides, steroidal lactones and a few flavanoids [37]. In addition, *W. somnifera* formulation (WSF) has shown anti-proliferative potential in human promyelocytic leukemia (HL-60) cells, by activating the intrinsic and extrinsic apoptotic pathways [38]. When used together, *W. somnifera* formulations aid the host to effectively fight cancer and reduce the harmful effects of chemotherapy and radiotherapy [39].

There is a need for the discovery of an inexpensive cancer cachectic treatment to improve the prognosis of cancer patients and to establish a mechanism of regulation of the immune system, inflammasome and apoptosis in order to prevent/decelerate the rapid depletion of skeletal muscle and adipose tissue. We investigated the effect of an aqueous root extract of *W. somnifera* (W_{RE}) on

antioxidant capacity, inflammatory cytokine levels and cell death induction in leukaemic THP-1 cells and peripheral blood mononuclear cells (PBMC's).

2.2 Materials and Methods

2.2.1 Materials

W. somnifera roots were collected on the 11th of March 2011 (collectors number: Immelman 427) from the Eastern Cape [the Ntubeni Location near Dwesa Reserve], South Africa (SA) and identified by Dr. Kathleen Immelman from the Department of Botany at the Walter Sisulu University, SA. Voucher specimens were deposited at the KEI herbarium (13995). THP-1 cells were obtained from American Type Culture Collection (ATCC, University Boulevard Manassas, Virginia, USA). RPMI-1640 and BD OptEIA enzyme-linked immunosorbant assay (ELISA) cytokine kits were purchased from The Scientific Group (Johannesburg, SA). Foetal calf serum (FCS) and Pen/Strep Amphotericin B (PSF) were acquired from Whitehead Scientific (Cape Town, SA). Dimethyl sulphoxide (DMSO) was purchased from Merck (Johannesburg, SA). Histopaque-1077, LPS and 2, 2-diphenyl-1 picrylhydrazyl (DPPH) were purchased from Sigma (Aston Manor, SA). The 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) cell proliferation reagent was purchased from Roche (Johannesburg, SA). Promega (Madison, USA) supplied the caspase (-3/7, -8, -9), adenosine triphosphate (ATP) and glutathione (GSH) kits.

2.2.2 Plant Description and Extraction

The plants official name is *Withania somnifera* (L.) Dunal and has been confirmed by using the plant list [40]. The common name is Ashwagandha.

W. somnifera roots were dried and milled. Dried plant material was sequentially extracted in ethanol and distilled water. For the ethanol extraction, ethanol (200–350 ml) was added to the milled plant (10–30 g) and extracted overnight by shaking (4 × g, 37 °C). Ethanol extracts were filtered, rotor evaporated, dried (37 °C) and stored (4 °C). For the sequential water extraction, the remaining plant material from the ethanol extraction was used, distilled water was added (200–350 ml) and extracted for 6 hours (h) by shaking (4 × g, 75 °C). Water extracts were filtered, dried and stored (4 °C).

2.2.3 The 2, 2-diphenyl-1 picrylhydrazyl assay

W_{RE} (0.05–0.4 mg/ml) and butylated hydroxytoluene (BHT) (60–300 μ M) dilutions were prepared in methanol (99.5% and grade AR). A 50 μ M DPPH solution was prepared from a stock solution of 0.135 mM DPPH in methanol. W_{RE} , BHT dilutions and methanol (1 ml) were aliquoted into 15 ml polypropylene tubes, followed by the 50 μ M DPPH solution (1 ml). Reaction mixtures were vortexed and incubated [room temperature (RT), 30 minutes (min)] in the dark. Absorbance of samples was read at 517 nm using a Varine Cary 50 UV-visible spectrophotometer (McKinley Scientific, New Jersey, US).

2.2.4 Isolation of Peripheral blood mononuclear cells

Buffy coats containing PBMC's were obtained from the South African National Blood Service (2011/09). PBMC's were extracted by differential centrifugation. Buffy coats (5 ml) were layered onto equivolume histopaque-1077 (5 ml) in 15ml polypropylene tubes and centrifuged (400 \times g, 21 $^{\circ}$ C for 30 min). After centrifugation, the PBMC's were transferred to sterile 15 ml polypropylene tubes, phosphate buffered saline (PBS) was added (0.1 M, 10 ml) and tubes were centrifuged (400 \times g, 21 $^{\circ}$ C, 15 min). Cell density of isolated PBMC's was adjusted (1 \times 10⁶ cells/ml) using the trypan blue exclusion test and cryo-preserved (10 % FCS, 10 % DMSO) using a NELGENE cryo freezing container and stored at -80 $^{\circ}$ C.

2.2.5 Tissue Culture

THP-1 cells were grown in the appropriate tissue culture conditions in a 75 cm³ tissue culture flask (37 $^{\circ}$ C, 5% CO₂). The growth media comprised of RPMI-1640, FCS (10%) and PSF (2%). Cells were thawed, seeded into a 75 cm³ tissue culture flask at a concentration of 3 \times 10⁵ cells/ml and incubated (37 $^{\circ}$ C, 5% CO₂). THP-1 cells were allowed to grow for 2 – 3 days before the cells were centrifuged (162 \times g, 10 min) and re-suspended in fresh growth media. The number of cells should not exceed 8 \times 10⁵ cells/ml, therefore the cells/ml was quantified daily by trypan blue staining. Once the cell count reached 8 \times 10⁵ cells/ml the THP-1 cells were split/ diluted to 3 \times 10⁵ cells/ml

with media and incubated. Subsequent experiments were conducted once the cell numbers were sufficient.

2.2.6 Cell Viability Assay

Cytotoxicity of W_{RE} in PBMC's and THP-1 cells was measured using the WST-1 assay (Roche, Johannesburg, SA). PBMC and THP-1 cells (10,000 cells/well, 96-well plate, in triplicate wells) were stimulated with LPS (20 μ g/ml, 37 °C, 5% CO_2 , 4 h) before exposure to W_{RE} (0.05–0.4 mg/ml) for 24 and 72 h (37 °C, 5 % CO_2). Similarly, controls received media. Thereafter, plates were centrifuged (162 \times g, 10 min), supernatant removed, cell pellets re-suspended in growth media (100 μ l/well), WST-1 reagent (10 μ l/well) added and plates incubated (37 °C, 5 %, CO_2 , 3h). Optical density was measured at 450 nm (620 nm reference wavelength) with a BIO-TEK μ Quant spectrophotometer (Analytical and Diagnostic Products, SA). This experiment was conducted independently on three occasions.

2.2.7 Stimulation and treatment of cells

PBMC's and THP-1 cells (1×10^5 cells/ml) were transferred into 24-well plates and stimulated with LPS (20 μ g/ml, 37 °C, 5% CO_2 , 4 h) before exposure to W_{RE} (0.05–0.4 mg/ml) for 24 h (TNF- α) and 72 h (IL-1 β , IL-6, IL-10) (37 °C, 5% CO_2). After incubation, plates were centrifuged (162 \times g, 10 min) and supernatant was collected and stored (-80 °C) for cytokine analysis. Cell pellets were used to conduct the caspase (-8, -9, -3/7) activity, as well as ATP and GSH assays. The experiments were conducted independently (twice) for all subsequent assays.

2.2.8 Quantification of Cytokines

Cytokine levels were estimated using the BD OptEIA ELISA kits (The Scientific Group, SA) and the procedure was followed as per the instruction manual. ELISA plates were coated with capture antibody overnight (100 μ l/well, 4 °C). Thereafter, plates were washed (3x) with wash buffer and blocked with assay diluent (200 μ l/well, 1 h, RT). Standard solutions were prepared by diluting a stock solution [TNF- α , IL-10 (500 pg/ml), IL-6 (300 pg/ml), IL-1 β (250 pg/ml)] serially [TNF- α ,

IL-10 (500–7.8 pg/ml), IL-6 (300–4.7 pg/ml), IL-1 β (250–3.9 pg/ml)]. Plates were washed (3x), standards and samples (100 μ l/well) were aliquoted into appropriate wells and plates were incubated (2 h, RT). Plates were washed (5x), working detector (100 μ l/well) added and plates incubated (1 h, RT). The plates were washed (7x), substrate solution (100 μ l/well) added and plates were incubated (30 min, RT) in the dark. Finally, stop solution (50 μ l/well) was added and the absorbance was read at 450 nm (570 nm reference wavelength) with a Multiskan FC micro-plate reader (Thermo Scientific). Cytokine concentrations were calculated by extrapolation from a standard curve.

2.2.9 The Glutathione assay

The GSH-GloTM assay (Promega, Madison, USA) was performed to measure GSH levels. Standard GSH solutions were prepared by diluting a 5 mM stock solution serially (1.56–50 μ M) and PBS (0.1 M) was the standard blank. Cells (50 μ l/well, 2×10^5 cells/ml) and standards were added into an opaque 96-well plate, followed by GSH-GloTM reagent (25 μ l/well) and allowed to incubate (30 min, RT) in the dark. Luciferin detection reagent (50 μ l/well) was subsequently added and plates incubated (15 min, RT) in the dark. The absorbance was read on a ModulusTM microplate luminometer (Turner Biosystems, Sunnyvale, USA) and GSH concentrations were calculated by extrapolation from a standard curve.

2.2.10 Caspase and ATP assays

Caspase activity and ATP levels were determined using the Caspase-Glo[®]-3/7, -8, -9 and ATP assay kits (Promega, Madison, USA). Caspase-Glo[®]-3/7, -8, -9 and ATP Reagents were reconstituted according to the manufacturer's instructions. Cells (100 μ l, 2×10^5 cells/ml) were added into duplicate wells of a microtitre plate for each assay, thereafter caspase -3/7, -8, -9 and ATP reagents (100 μ l/well) were added into appropriate wells. The plate was incubated (30 min, RT) in the dark. Luminescence was measured on a ModulusTM microplate luminometer (Turner BioSystems) and expressed as relative light units (RLU).

2.2.11 Statistical Analysis

Statistical analysis was performed using the STATA and GraphPad Prism statistical analysis software. The one-way analysis of variance (ANOVA) was used to compare between groups, followed by the Tukey multiple comparisons test, with $p < 0.05$ defining statistical significance.

2.3 Results

2.3.1 The oxidant scavenging potential of W_{RE}

The oxidant scavenging activity of W_{RE} using the DPPH assay is shown in Figure 2.1. W_{RE} (0.05–0.4 mg/ml) significantly increased DPPH scavenging activity by 13.33–46.38% (Figure 2.1, $p < 0.0001$).

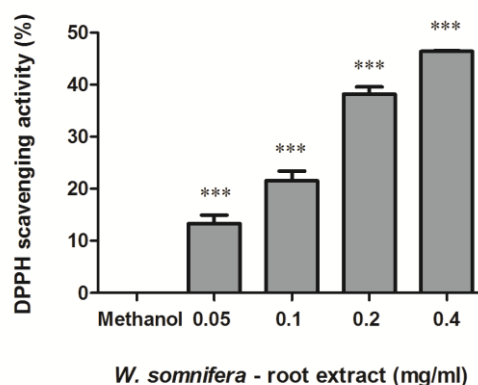


Figure 2.1: Percentage DPPH scavenging activity of W_{RE} (Values expressed as mean \pm SD, * $p < 0.05$, *** $p < 0.0001$, compared to control).

2.3.2 The *in vitro* cytotoxicity of W_{RE}

The WST-1 assay was used to determine cell viability of THP-1 cells and PBMC's after treatment with W_{RE} (Figure 2.2). At 24 h, W_{RE} (0.05–0.4 mg/ml) decreased PBMC viability by 20.69–25.15% while W_{RE} (0.2–0.4 mg/ml) increased THP-1 viability by 15.99–22.54% as compared to the controls (Figure 2.2A and 2.2C, $p < 0.001$). This result suggests that PBMC's are more sensitive to W_{RE} treatment than THP-1 cells.

At 72 h, PBMC viability was increased (27.16–38.58%) by W_{RE} (0.05, 0.4 mg/ml), as compared to the control (Figure 2.2B, $p < 0.0001$). In the same time period, W_{RE} (0.05–0.4 mg/ml) decreased THP-1 viability by 9.07–16.09% relative to the control (Figure 2.2D, $p=0.0002$).

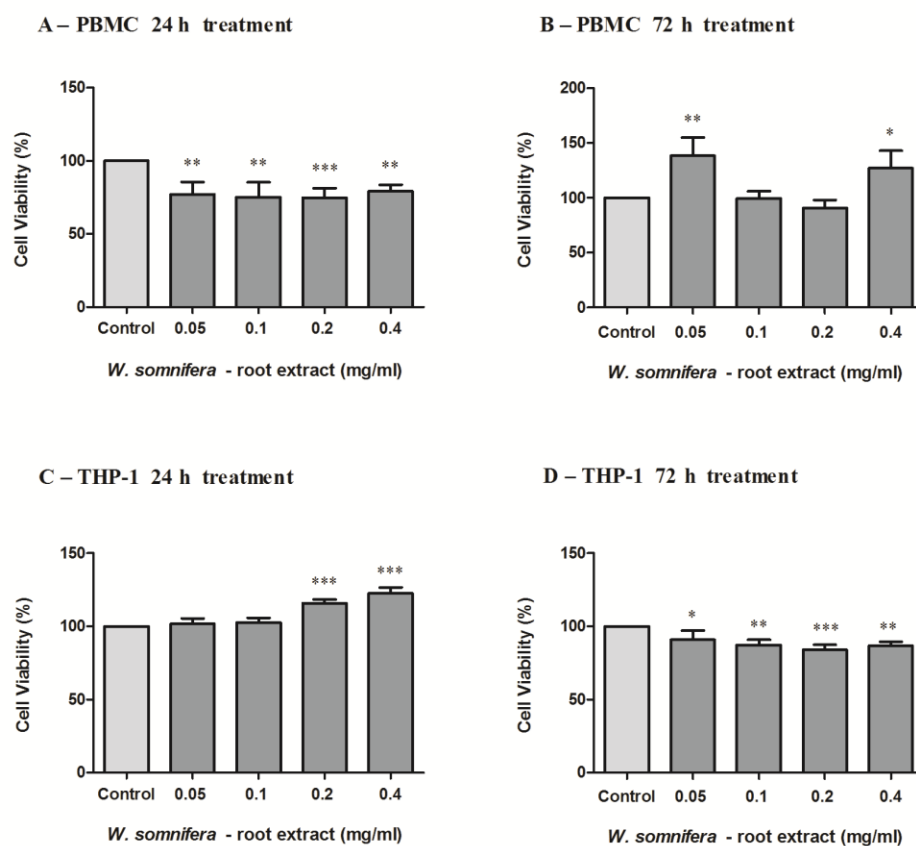


Figure 2.2: Cell viability of PBMC's (A – 24 h, B – 72 h) and THP-1 (C – 24 h, D – 72 h) cells treated with W_{RE} for 24 and 72 h (Values expressed as mean \pm SD, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$ compared to the control).

2.3.3 The immune suppressive properties of W_{RE}

W_{RE} altered cytokine levels in PBMC's and THP-1 cells (Figure 2.3 and 2.4). The levels of TNF- α , IL-1 β , IL-6 and IL-10 produced by LPS stimulated PBMC's was 336.218, 168.100, 657.878 and

46.990 pg/ml respectively. W_{RE} (0.2–0.4 mg/ml) decreased PBMC TNF- α and IL-10 levels as compared to the control (Figure 2.3A and 2.3D, $p < 0.0001$). In PBMC's, IL-6 levels were decreased by 0.4 mg/ml W_{RE} , whereas IL-1 β levels were increased by 0.05–0.4 mg/ml W_{RE} relative to the control (Figure 2.3B and 2.3C, $p < 0.0001$).

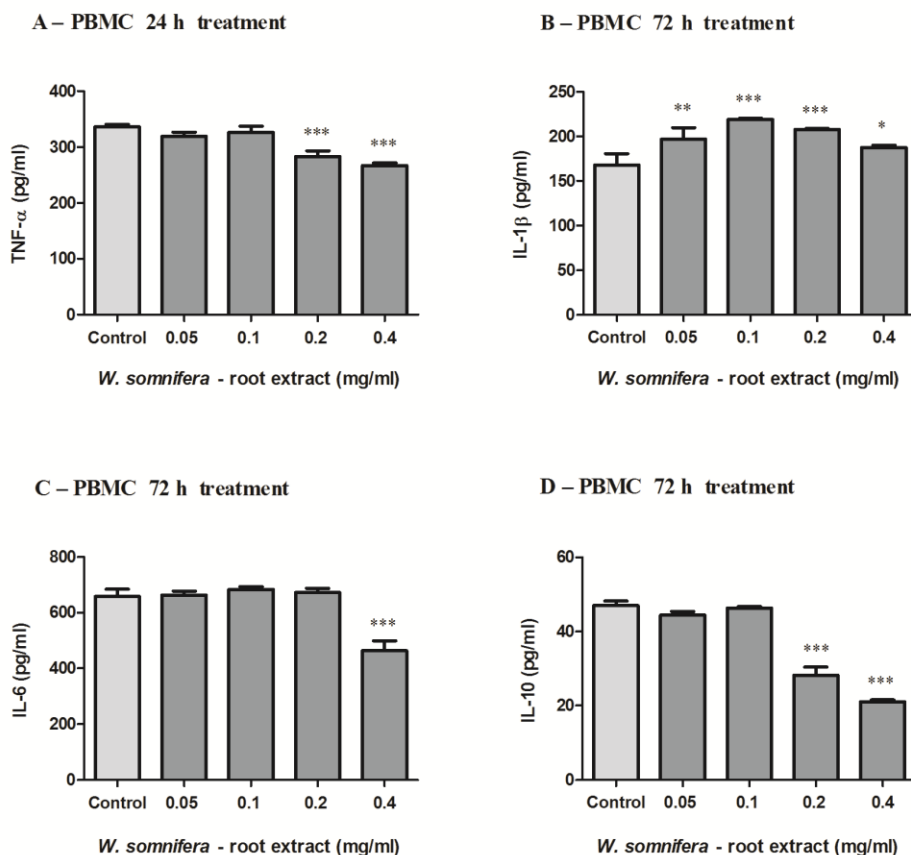


Figure 2.3: Concentration of TNF- α (A), IL-1 β (B), IL-6 (C) and IL-10 (D) in LPS stimulated and W_{RE} treated PBMC's (Values expressed as mean \pm SD, * $p < 0.05$, *** $p < 0.0001$, compared to the control).

The levels of TNF- α , IL-1 β , IL-6 and IL-10 produced by LPS stimulated THP-1 cells were 13.285, 21.947, 78.622 and 2.705 pg/ml respectively. In THP-1 cells, TNF- α , IL-1 β and IL-6 levels were

decreased by 0.05–0.4 mg/ml W_{RE} , whilst IL-10 levels were decreased by 0.4 mg/ml W_{RE} as compared to the control (Figure 2.4, $p < 0.003$).

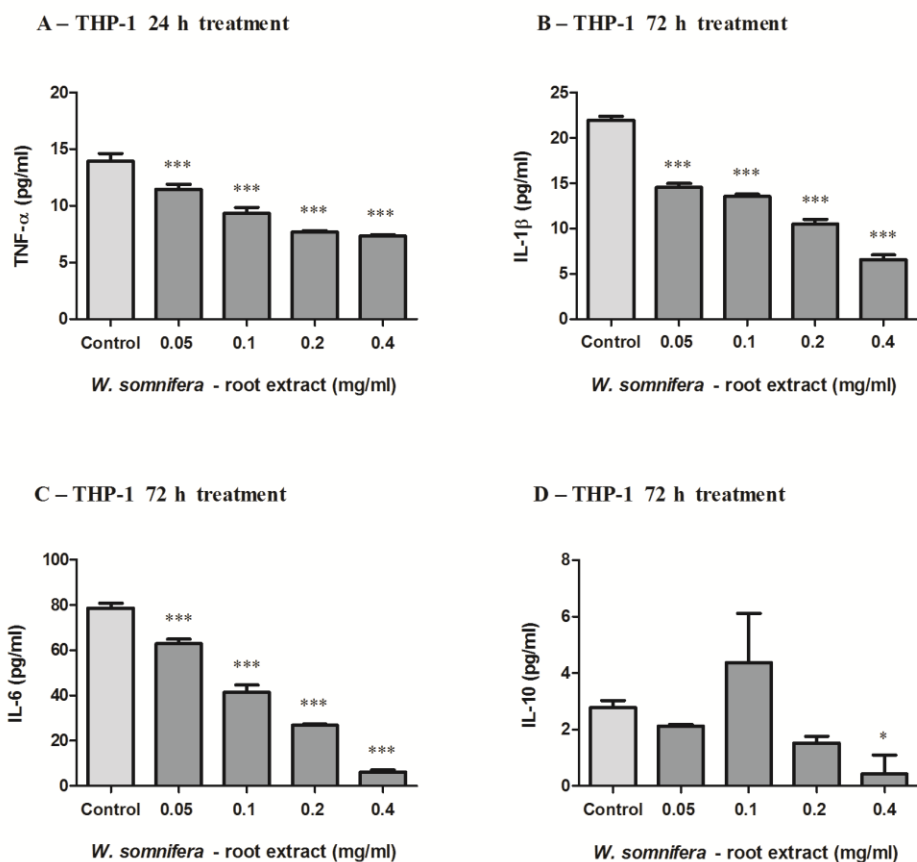


Figure 2.4: Concentration of TNF- α (A), IL-1 β (B), IL-6 (C) and IL-10 (D) in LPS stimulated and W_{RE} treated THP-1 cells (Values expressed as mean \pm SD, * $p < 0.05$, *** $p < 0.0001$ compared to the control).

2.3.4 The antioxidant potential of W_{RE}

The endogenous antioxidant activity of W_{RE} was determined by measuring GSH levels in both cell lines (Table 2.1). At 24 h, GSH levels in PBMC's were decreased by W_{RE} (0.05, 0.2, 0.4 mg/ml) relative to the control (Table 2.1, $p < 0.0001$). In THP-1 cells, GSH levels were decreased at 0.05

mg/ml W_{RE} whereas increased (0.41–1.62 μ M) at 0.1–0.4 mg/ml W_{RE} compared to the control (Table 2.1, 24 h, $p < 0.0001$).

At 72h, PBMC GSH levels were increased at 0.05 mg/ml whereas decreased 0.1–0.4 mg/ml W_{RE} compared to the control (Table 2.1, $p < 0.0001$). W_{RE} (0.05–0.4 mg/ml) decreased GSH levels in THP-1 cells relative to the control (Table 2.1, 72 h $p < 0.0001$).

Table 2.1: Glutathione levels in LPS stimulated and W_{RE} treated PBMC's and THP-1 cells.

Glutathione (μ M)				
W_{RE} (mg/ml)	24 h Treatment		72 h Treatment	
	PBMC	THP-1	PBMC	THP-1
Control	1.613 \pm 0.017	1.632 \pm 0.004	4.799 \pm 0.008	1.608 \pm 0.004
0.05	1.442 \pm 0.024 ***	1.267 \pm 0.004 ***	5.232 \pm 0.011 ***	1.548 \pm 0.002 ***
0.1	1.617 \pm 0.002	2.045 \pm 0.002 ***	4.015 \pm 0.001 ***	1.589 \pm 0.004 ***
0.2	1.390 \pm 0.001 ***	3.253 \pm 0.017 ***	2.323 \pm 0.005 ***	1.401 \pm 0.006 ***
0.4	1.321 \pm 0.006 ***	2.785 \pm 0.005 ***	4.697 \pm 0.003 ***	1.411 \pm 0.005 ***

(Values expressed as mean \pm SD, *** $p < 0.0001$, compared to the control).

2.3.5 W_{RE} modulates caspase (-8, -9, -3/7) activities and ATP levels

Luminometry assays were used to determine caspase activity and ATP levels in THP-1 cells and PBMC's after treatment with W_{RE} . The pro-apoptotic effect of W_{RE} in PBMC's treated for 24 h is shown in Table 2.2. At 24 h, PBMC caspase-8 activity was decreased by 0.05 mg/ml W_{RE} whereas increased by 0.2–0.4 mg/ml W_{RE} compared to the control (Table 2.2, $p < 0.0001$). PBMC caspase-9 activity was increased by 0.05 and 0.2 mg/ml W_{RE} but decreased by 0.1 and 0.4 mg/ml W_{RE} relative to the control (Table 2.2, $p < 0.0001$). In PBMC's, the increased caspase activity may be related to the decreased GSH levels at 24 h. A decrease in GSH levels may allow for an increase in ROS levels which can activate apoptotic pathways. Caspase-3/7 activity was increased in PBMC's by

0.05–0.4 mg/ml W_{RE} compared to the control (Table 2.2, $p < 0.0001$), suggesting an increased execution of apoptotic cell death. In azoxymethane-induced colon cancer in mice, *W. somnifera* has been shown to modulate TCA cycle enzymes and the electron transport chain [41]. The PBMC ATP levels were increased by 0.1, 0.4 mg/ml W_{RE} but decreased by 0.05, 0.2 mg/ml W_{RE} compared to the control (Table 2.2, $p < 0.0001$), which may be related to the modulation of the electron transport chain by *W. somnifera*.

Table 2.2: Modulation of caspase (-8, -9, -3/7) activities and ATP levels in LPS stimulated and 24 h W_{RE} treated PBMC's.

W_{RE} (mg/ml)	Caspase-8 (RLU x 10^5)	Caspase-9 (RLU x 10^5)	Caspase-3/7 (RLU x 10^5)	ATP (RLU x 10^5)
Control	0.185 ± 0.006	0.366 ± 0.0003	7.756 ± 0.006	4.714 ± 0.004
0.05	0.155 ± 0.0002 ***	0.376 ± 0.001 ***	8.109 ± 0.094 **	2.783 ± 0.017 ***
0.1	0.192 ± 0.0002	0.253 ± 0.0002 ***	11.504 ± 0.253 ***	5.208 ± 0.005 ***
0.2	0.246 ± 0.0003 ***	0.397 ± 0.0005 ***	8.961 ± 0.015 ***	3.741 ± 0.033 ***
0.4	4.814 ± 0.006 ***	0.351 ± 0.001 ***	17.095 ± 0.089 ***	6.965 ± 0.039 ***

(Values expressed as mean ± SD, ** $p < 0.005$, *** $p < 0.0001$ compared to the control).

W_{RE} pro-apoptotic effects in THP-1 cells treated for 24 h are shown in Table 2.3. At 24 h, THP-1 caspase-9 activity was decreased by 0.2 mg/ml W_{RE} but increased by 0.05, 0.1, 0.4 mg/ml W_{RE} compared to the control (Table 2.3, $p < 0.0001$). At 0.2 mg/ml W_{RE} , the decreased caspase-9 activity may be related to the increased GSH levels. An increase in GSH levels may decrease ROS levels thus minimising mitochondrial depolarisation and the activation of the intrinsic apoptotic pathway. In THP-1 cells, W_{RE} (0.05–0.4 mg/ml) decreased caspase-8 activity, whereas increased caspase-3/7 activity and ATP levels relative to the control (Table 2.3, $p < 0.0001$). Elevated caspase (-9, -3/7) activities suggests the initiation of the mitochondrial apoptotic pathway.

Table 2.3: Modulation of caspase (-8, -9, -3/7) activities and ATP levels in LPS stimulated and 24 h W_{RE} treated THP-1 cells.

W_{RE} (mg/ml)	Caspase-8 (RLU x 10^5)	Caspase-9 (RLU x 10^5)	Caspase-3/7 (RLU x 10^5)	ATP (RLU x 10^5)
Control	10.207 ± 0.0114	1.040 ± 0.007	1.251 ± 0.016	2.636 ± 0.011
0.05	8.440 ± 0.039 ***	2.365 ± 0.005 ***	1.315 ± 0.005 ***	3.726 ± 0.005 ***
0.1	2.413 ± 0.005 ***	2.459 ± 0.002 ***	2.294 ± 0.006 ***	5.132 ± 0.014 ***
0.2	7.149 ± 0.027 ***	0.775 ± 0.002 ***	3.406 ± 0.006 ***	29.838 ± 0.186 ***
0.4	2.456 ± 0.033 ***	3.197 ± 0.0001 ***	9.428 ± 0.004 ***	10.282 ± 0.195 ***

(Values expressed as mean ± SD, *** $p < 0.0001$ compared to the control).

The pro-apoptotic effect of W_{RE} in PBMC's treated for 72 h is shown in Table 2.4. At 72 h, PBMC caspase-8 activity was increased by 0.05–0.2 mg/ml W_{RE} but decreased by 0.4 mg/ml W_{RE} compared to the control (Table 2.4, $p < 0.0001$). PBMC caspase-9 activity was increased by 0.05–0.1 mg/ml W_{RE} but decreased by 0.2–0.4 mg/ml W_{RE} relative to the control (Table 2.4, $p < 0.0001$). In PBMC's, caspase-3/7 activity was increased by 0.05, 0.1, 0.4 mg/ml W_{RE} whereas it decreased by 0.2 mg/ml W_{RE} compared to the control (Table 2.4, $p < 0.0001$). At 0.05–0.1 mg/ml W_{RE} , the increased caspase-3/7 activity is consistent with the significantly increased caspase -8 and -9 activity. At 0.2 mg/ml W_{RE} , caspase-8 activity was minimally increased and caspase-9 activity significantly decreased which lead to the decreased caspase-3/7 activity. At 0.4 mg/ml W_{RE} , although both caspase -8 and -9 activities were decreased, caspase-3/7 activity was increased. A previous study has demonstrated that one activated executioner caspase can cleave and activate other executioner caspases resulting in positive feedback loop of caspase activation [42] which may account for the increased caspase-3/7 activity at 0.4 mg/ml W_{RE} . W_{RE} (0.05–0.4 mg/ml) decreased PBMC ATP levels relative to the control (Table 2.4, $p < 0.0001$).

Table 2.4: Modulation of caspase (-8, -9, -3/7) activities and ATP levels in LPS stimulated and 72 h W_{RE} treated PBMC's.

W_{RE} (mg/ml)	Caspase-8 (RLU x 10^5)	Caspase-9 (RLU x 10^5)	Caspase-3/7 (RLU x 10^5)	ATP (RLU x 10^5)
Control	42.651 ± 0.039	115.041 ± 3.848	155.556 ± 0.387	20.574 ± 0.316
0.05	53.840 ± 0.026 ***	143.861 ± 3.929 ***	196.471 ± 0.338 ***	10.223 ± 0.046 ***
0.1	52.109 ± 0.009 ***	129.033 ± 0.289 ***	192.695 ± 0.233 ***	12.506 ± 0.373 ***
0.2	42.751 ± 0.039 ***	105.494 ± 4.247 ***	154.203 ± 0.224 ***	13.210 ± 0.043 ***
0.4	29.656 ± 0.007 ***	92.718 ± 0.021 ***	165.139 ± 0.096 ***	13.361 ± 0.279 ***

(Values expressed as mean ± SD, ** $p < 0.005$, *** $p < 0.0001$ compared to the control).

W_{RE} pro-apoptotic effects in THP-1 cells treated for 72 h are shown in Table 2.5. At 72 h, THP-1 caspase (-8, -9, -3/7) activity and ATP levels were increased by 0.1–0.2 mg/ml W_{RE} as compared to the control (Table 2.5, $p < 0.0001$), suggesting an increase in THP-1 apoptotic cell death. THP-1 caspase (-8, -9, -3/7) activity and ATP levels were decreased by 0.05, 0.4 mg/ml W_{RE} relative to the control (Table 2.5, 72 h, $p < 0.0001$), suggesting a decrease in THP-1 apoptosis.

Table 2.5: Modulation of caspase (-8, -9, -3/7) activities and ATP levels in LPS stimulated and 72 h W_{RE} treated THP-1 cells.

W_{RE} (mg/ml)	Caspase-8 (RLU x 10^5)	Caspase-9 (RLU x 10^5)	Caspase-3/7 (RLU x 10^5)	ATP (RLU x 10^5)
Control	0.991 ± 0.001	5.738 ± 0.002	7.463 ± 0.012	4.332 ± 0.002
0.05	0.978 ± 0.0001 ***	5.562 ± 0.009 ***	6.919 ± 0.003 ***	4.133 ± 0.005 ***
0.1	1.216 ± 0.001 ***	7.045 ± 0.005 ***	8.211 ± 0.002 ***	4.889 ± 0.005 ***
0.2	1.095 ± 0.001 ***	6.091 ± 0.001 ***	7.532 ± 0.006 **	4.576 ± 0.004 ***
0.4	0.952 ± 0.0003 ***	5.639 ± 0.003 ***	6.626 ± 0.007 ***	4.039 ± 0.0003 ***

(Values expressed as mean ± SD, *** $p < 0.0001$ compared to the control).

2.4 Discussion

Cachexia patients experience excessive weight loss due to increased lipolysis and proteolysis which have been linked to elevated levels of pro-inflammatory cytokines, oxidative stress and apoptosis [3, 5, 30]. Previously, *W. somnifera* root powder has shown immune modulatory properties [43] and WSF has been shown to increase caspase-3 activity, subsequently inducing apoptosis [38]. The objective of this study was thus to investigate the modulation of cytokines, antioxidants and cell death by W_{RE} in PBMC's and THP-1 cells.

Dhanani *et al* (2017) showed that 50 percent inhibition of DPPH was seen at 0.4 mg/ml *W. somnifera* root extract [44]. Our results indicated that W_{RE} has oxidant scavenging potential ranging between 13–46% at 0.05–0.4 mg/ml. ROS plays an essential role in tumour initiation, inflammation, protein degradation and apoptosis. The antioxidant potential of W_{RE} may decrease inflammatory cytokine levels as well as ROS induced apoptosis.

At 24 h, the WST-1 results indicated that W_{RE} decreased PBMC viability whereas it increased THP-1 viability. However at 72 h, W_{RE} increased PBMC viability whereas it decreased THP-1 viability.

In contrast, the growth of various cell lines (HT-29, HCT-15, SW620, 502713, Colo-205, A549, HOP-62 and Hep-G2) were dose dependently inhibited by WSF and 50% cell growth inhibition was seen at 30 µg/ml WSF [38].

The pivotal role of inflammatory cytokines in malignancies and cachexia has been extensively documented [3]. Dhuley (1997) previously reported that *W. somnifera* inhibits macrophage production of inflammatory cytokines (IL-1, TNF- α) [45]. Our results showed that W_{RE} decreased PBMC TNF- α , IL-10 and IL-6 levels, although it increased IL-1 β levels. In THP-1 cells, pro-inflammatory cytokine (TNF- α , IL-1 β , IL-6) levels were significantly decreased by W_{RE} .

Pro-inflammatory cytokines, over a longstanding time period, stimulate the production of genotoxic molecules [nitric oxide (NO), ROS] and tumour progression by promoting angiogenesis and metastasis [1, 2]. In addition, pro-inflammatory cytokines activate NF- κ B which regulates the expression of genes involved in the suppression of tumour apoptosis, stimulation of tumour cell cycle progression and enhancement of inflammatory mediators [1, 2]. NF- κ B promotes tumour progression, invasion, angiogenesis and metastasis [1, 2].

Previous literature has shown that IL-1 stimulates growth and invasion of malignant cells [2]. Additionally, IL-6 has been shown to target cell cycle progression and anti-apoptotic genes leading to tumour proliferation and anti-apoptotic potential [2]. The ability of W_{RE} to increase pro-inflammatory cytokines such as IL-1 β in PBMC's may aid in cancerous cell elimination through increased host anti-tumour activity. Conversely, in THP-1 cells, the decrease in TNF- α , IL-6 and IL-1 β levels by W_{RE} may prevent excessive activation of NF- κ B, diminish cytokine induced tumour immunosuppressive activity and cancer progression.

With regard to cancer cachexia, IL-6 decreased LPL activity in adipose tissue of mice [22] and IL-1 directly modulates lipid metabolism by suppressing LPL activity [23]. TNF- α decreased LPL activity in adipose tissue of human (maintained in organ culture), rat, mouse, and guinea pigs [21]. Additionally, TNF- α inhibits the production of LPL and reduces the rate of LPL gene transcription in mouse 3T3-L1 adipocytes, hence preventing the formation of new lipid stores while stimulating HSL and increasing lipolysis [3, 20, 46]. The potential of W_{RE} to decrease pro-inflammatory cytokine levels in PBMC's and THP-1 cells suggests a decrease in LPL inhibition and HSL

stimulation, thus maintaining lipogenesis and minimizing lipolysis. IL-6 and TNF- α further contribute to cachexia by stimulating muscle catabolism via the activation of proteasome pathways [24, 25, 47]. In cachexia, NF- κ B activation induces ubiquitin–proteasome pathway activity and suppresses MyoD expression [48], thereby increasing proteolysis and reducing muscle replenishment [49]. By decreasing TNF- α and IL-6 levels in PBMC's and THP-1 cells, W_{RE} may prevent excessive activation of NF- κ B and proteasome pathways, ultimately decreasing proteolysis associated with the cachectic syndrome. Taken together, W_{RE} may be able to decrease tissue wasting through the down regulation of pro-inflammatory cytokine levels.

The immunosuppressive and anti-inflammatory cytokine, IL-10, inhibits tumour development, tumour progression, modulates apoptosis and suppresses angiogenesis during tumour regression [1, 2]. Additionally, IL-10 inhibits NF- κ B activation and subsequently inhibits pro-inflammatory cytokine production (TNF- α , and IL-6) [2]. In PBMC's and THP-1 cells, the decreased IL-10 levels may be due to IL-10 combating increased pro-inflammatory cytokines levels (TNF- α , IL-6, IL-1 β).

Antioxidants protect cells from increased oxidative stress [50]. GSH is a potent antioxidant that effectively scavenges ROS both directly and indirectly [50]. *W. somnifera* has previously been shown to possess chemo-preventive activity which may be a consequence of its antioxidant capacity [39]. The 24 h results showed that W_{RE} decreased GSH levels in PBMC's, whereas it increased GSH levels in THP-1 cells. However, at 72 h, W_{RE} decreased GSH levels in both cell lines. Notably, GSH levels (72 h) were higher in control PBMC's (4.79 μ M) compared to control THP-1 cells (1.61 μ M), suggesting a higher oxidant defence in PBMC's.

The extrinsic (death receptors) and intrinsic (mitochondria) pathways are the two main apoptotic pathways [29]. Activation of initiator caspases (-8, -9) leads to the activation of executioner caspases (-3/7) resulting in activation of cytoplasmic endonucleases [29]. In HL-60 cells, WSF treatment led to a loss of mitochondrial membrane potential, translocation of Bax to mitochondria, release of cytochrome c, Smac/DIABLO and apoptosis inducing factor, suggesting the activation of the intrinsic apoptotic pathway [38]. Additionally, WSF treated HL-60 cells showed an over-expression of TNF receptor-1 and death receptor-4 with associated caspase-8 activation, suggesting the activation of the extrinsic apoptotic pathway [38]. Our 24 h results showed that W_{RE} increased PBMC caspase -8, -9 and -3/7 activities at different concentrations, suggesting the activation of

extrinsic and intrinsic apoptotic pathways. In the same time period, W_{RE} increased THP-1 caspase -9 and -3/7 activities, suggesting initiation of apoptosis through the intrinsic pathway. At 72h, W_{RE} (0.05–0.1 mg/ml) increased caspase (-8, -9, -3/7) activities, suggesting an increased initiation of PBMC apoptotic cell death. However, 0.4 mg/ml W_{RE} decreased initiator caspase (-8, -9) activities, suggesting a decreased initiation of PBMC apoptotic cell death. In THP-1 cells, 0.1–0.2 mg/ml W_{RE} (72 h) increased caspase (-8, -9, -3/7) activities, suggesting initiation of apoptosis through the intrinsic and extrinsic pathways. However, at 0.05 and 0.4 mg/ml W_{RE} (72 h), THP-1 caspase (-8, -9, -3/7) activities were decreased, suggesting a decrease in THP-1 apoptosis. Previous studies have indicated that *W. somnifera* may activate the extrinsic and intrinsic apoptotic pathways [38], therefore our results prove to be consistent with other studies.

Increased caspase-3 activity, proteasome activity and E3 ubiquitin-conjugating enzyme expression is associated with increased proteolysis [51]. Therefore the ability of W_{RE} (0.4 mg/ml, 72 h) to down regulate caspase activity in PBMC's and THP-1 cells may decrease proteolysis and the progression of cancer cachexia.

A successful anti-cancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells [39]. The potential of W_{RE} to regulate PBMC apoptosis while increasing cancerous THP-1 cell apoptosis may be beneficial to cancer patients by preventing excessive cancerous cell growth while minimally effecting healthy PBMC's.

2.5 Conclusion

The cachectic syndrome decreases the quality of life of patients, the responsiveness to chemotherapy and leads to 20-25% of cancer deaths [3]. Our results show that W_{RE} increased oxidant scavenging activity, modulated GSH and pro-inflammatory cytokine levels and regulated caspase activity in normal PBMC's and THP-1 cells. The discovery of a medicinal plant capable of decreasing the levels of pro-inflammatory cytokines may decrease tissue wasting. In this study, *W. somnifera* root extract has shown promising results in modulating the production of cytokines associated with cancer cachexia. The ability of W_{RE} to decrease pro-inflammatory cytokine levels

and increase cancerous cell death may decrease the development and progression of cancer and cachexia. W_{RE} may therefore be effective in cancer cachexia.

2.6 Abbreviations

LPL: lipoprotein lipase; HSL: hormone sensitive lipase; FA's: fatty acids; TAG: triacylglycerol; TNF- α : tumour necrosis factor- α ; IL: interleukin; LPS: lipopolysaccharide; NF- κ B: Nuclear factor kappa B; DNA: Deoxyribonucleic acid; PARP: poly (ADP-ribose) polymerase; *W. somnifera*: *Withania somnifera*; WSF: *W. somnifera* formulation; HL-60: Human promyelocytic leukemia; W_{RE} : *W. somnifera* aqueous root extract; PBMC's: peripheral blood mononuclear cells; THP-1: leukemic cell line; SA: South Africa; ELISA: enzyme-linked immunosorbant assay; FCS: Foetal calf serum; PSF: Pen/Strep Amphotericin B; DPPH: 2, 2-diphenyl-1 picrylhydrazyl; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; ATP: Adenosine triphosphate; GSH: Gluthatione; h: Hours; BHT: butylated hydroxytoluene; RT: room temperature; min: Minute; PBS: phosphate buffered saline; RLU: Relative light units; NO: nitric oxide; ROS: reactive oxygen species.

2.7 Declarations

Ethics approval and consent to participate

Collection of PBMC's was ethically approved by the South African Medical Research Council Ethics Committee (EC09-018).

2.8 Consent for publication

Not applicable

2.9 Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

2.10 Competing interests

The author(s) declare that they have no competing interests.

2.11 Role of Funding Source

Sources of funding included the National Research Foundation, the South African Medical Research Council and Department of Science and Technology. The funding sources were not involved in study design, collection of samples, analysis of data, interpretation of data, writing of the report and decision to publish. Scientific out-put is a requirement of the National Research Foundation.

2.12 Authors' contributions

DBN contributed to experimental design and carried out all experimentation except the luminometry (Caspase, ATP, GSH) assays. DBN analysed and interpreted data, performed statistical analysis, drafted and revised the manuscript. AC and AP carried out luminometry assays and revised manuscript critically for important intellectual input. KPG, KS and VS gave substantial contributions to conception, design and revised manuscript critically for important intellectual input. All authors read and approved the final manuscript.

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Bridging paragraph:

In the search to identify a medicinal plant capable of decreasing the development and progression of cancer cachexia. An initial screening experiment was conducted to determine the potential of South African medicinal plants to modulate inflammatory cytokines associated with cancer cachexia. This *in-vitro* study investigated the oxidant scavenging activity and the modulation of inflammatory cytokine levels (TNF- α , IL-6, IL-1 β , and IL-10) by *Aloe ferox* (*A. ferox*), *C. asiatica*, *Elytropappus rhinocerotis* (*E. rhinocerotis*), *Tulbaghia violacea* (*T. violacea*) (leaf and root) and *W. somnifera* (leaf and root) extracts (ethanolic and aqueous) in LPS stimulated THP-1 cells (Appendix 2). The results indicated that *W. somnifera* root (aqueous) and *C. asiatica* leaf (ethanolic) extracts possessed significant antioxidant and anti-inflammatory potential. Therefore, further experimentation was conducted on a range of concentrations of *W. somnifera* root (aqueous) and *C. asiatica* leaf (ethanolic) extracts.

The following manuscript is entitled *Centella asiatica* modulation of cancer cachexia associated inflammatory cytokines and cell death in THP-1 cells and PBMC's (Supplementary information: Appendix 3). There is no direct link to the previous manuscript except that both medicinal plants were shown to decrease pro-inflammatory cytokines in THP-1 cells. However, the experimental techniques conducted are the same as the previous manuscript.

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Please note: The manuscript was formatted for BMC Complementary and Alternative Medicine, however, the margins, font, line spacing, numbering of sections and figures were adjusted for thesis consistency.

CHAPTER THREE

***Centella asiatica* modulates cancer cachexia associated inflammatory cytokines and cell death in leukaemic THP-1 cells and peripheral blood mononuclear cells (PBMC's)**

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Abstract

Background: Cancer cachexia is associated with increased pro-inflammatory cytokine levels. *Centella asiatica* (*C. asiatica*) possesses antioxidant, anti-inflammatory and anti-tumour potential. We investigated the modulation of antioxidants, cytokines and cell death by *C. asiatica* ethanolic leaf extract (C_{LE}) in leukaemic THP-1 cells and normal peripheral blood mononuclear cells (PBMC's).

Methods: Cytotoxicity of C_{LE} was determined at 24 and 72 hours (h). Oxidant scavenging activity of C_{LE} was evaluated using the 2, 2-diphenyl-1 picrylhydrazyl (DPPH) assay. Glutathione (GSH) levels, caspase (-8, -9, -3/7) activities and adenosine triphosphate (ATP) levels (Luminometry) were then assayed. The levels of tumour necrosis factor- α (TNF- α), interleukin (IL)-6, IL-1 β and IL-10 were also assessed using enzyme-linked immunosorbant assay.

Results: C_{LE} decreased PBMC viability between 33.25–74.55 % (24 h: 0.2–0.8 mg/ml C_{LE} and 72 h: 0.4–0.8 mg/ml C_{LE}) and THP-1 viability by 28.404 % (72 h: 0.8 mg/ml C_{LE}) ($p < 0.0001$). Oxidant scavenging activity was increased by C_{LE} (0.05–0.8 mg/ml) ($p < 0.0001$). PBMC TNF- α and IL-10 levels were decreased by C_{LE} (0.05–0.8 mg/ml) ($p < 0.0001$). However, PBMC IL-6 and IL-1 β concentrations were increased at 0.05–0.2 mg/ml C_{LE} but decreased at 0.4 mg/ml C_{LE} ($p < 0.0001$). In THP-1 cells, C_{LE} (0.2–0.8 mg/ml) decreased IL-1 β and IL-6 whereas increased IL-10 levels ($p < 0.0001$). In both cell lines, C_{LE} (0.05–0.2 mg/ml, 24 and 72 h) increased GSH concentrations ($p < 0.0001$). At 24 h, caspase (-9, -3/7) activities was increased by C_{LE} (0.05–0.8 mg/ml) in PBMC's whereas decreased by C_{LE} (0.2–0.4 mg/ml) in THP-1 cells ($p < 0.0001$). At 72 h, C_{LE} (0.05–0.8 mg/ml) decreased caspase (-9, -3/7) activities and ATP levels in both cell lines ($p < 0.0001$).

Conclusion: In PBMC's and THP-1 cells, C_{LE} proved to effectively modulate antioxidant activity, inflammatory cytokines and cell death. In THP-1 cells, C_{LE} decreased pro-inflammatory cytokine levels whereas it increased anti-inflammatory cytokine levels which may alleviate cancer cachexia.

Key words: Cancer, Cachexia, Cytokines, Apoptosis, *Centella asiatica*

3.1 Introduction

The role of inflammation in carcinogenesis has been extensively documented [1]. Although inflammatory responses have shown beneficial effects in tissue repair and pathogen elimination [1, 2], chronic inflammation has been implicated in tumour initiation, promotion and progression [3]. During ideal conditions, the host-mediated anti-tumour activity combats the tumour-mediated immunosuppressive activity and cancerous cells are sentenced to cell death [3]. In the event that the host anti-tumour activity is weakened/ inadequate, the persistent and enhanced pro-inflammatory tumour microenvironment will facilitate tumour development, invasion, angiogenesis and metastasis [3].

Many malignancies are associated with the cachectic syndrome [4], a disorder characterised by abnormal weight loss [5] due to adipose tissue (85%) and skeletal muscle (75%) depletion [6]. The enzyme lipoprotein lipase (LPL) hydrolyses fatty acids (FA's) and transports FA's into adipose tissue for triacylglycerol (TAG) production, whereas hormone sensitive lipase (HSL) breaks down TAG's into FA's and glycerol [6]. Studies have revealed that decreased serum LPL levels/activity [7, 8] and increased HSL levels/activity are associated with cachexia [9]. Additionally, increased proteolysis and decreased proteogenesis have been reported in cachectic patients [10]. The ATP-ubiquitin-dependent proteolytic pathway has been shown to be responsible for the excessive proteolysis seen in cancer cachexia [11].

Oxidative stress, inflammatory cytokines and apoptosis play a pivotal role in the initiation and development of cancer cachexia [12]. Inflammatory cytokine production is increased by lipopolysaccharide (LPS) potently stimulating macrophages [13]. The LPS signal is transduced by LPS binding to LPS binding protein, delivered to CD14 and transferred to Toll like receptor-4 [14]. This subsequently activates nuclear factor kappa B (NF- κ B), which regulates the transcription of genes associated with inflammation, proliferation, invasion, angiogenesis and apoptosis [1, 15-17]. Previously, IL-1 [18], IL-6 (mice) [19] and TNF- α (rat, mouse and guinea pigs) [20] were shown to decrease LPL activity in adipose tissue. Decreased LPL activity reduces the uptake of exogenous lipids by adipose tissue [20], which decreases lipogenesis. Additionally, previous literature showed that TNF- α increased ubiquitin (concentrations and mRNA), while IL-6 increased the 26S proteasome and cathepsin activities, suggesting the activation of proteolytic pathways [21-24]. The activation of proteolytic pathways causes extensive muscle wasting through proteolysis. Taken

together, an excessive increase in pro-inflammatory cytokine levels may increase tumour immunosuppressive activity [3], as well as tissue wasting [6]

Oxidative stress has been associated with tumour initiation, inflammation [2, 3] and muscle wasting [25]. However, antioxidants have been shown to decrease muscle wasting by neutralizing reactive oxygen species (ROS) [1, 25]. Elevated ROS levels activate apoptotic pathways, ultimately activating caspase-3 [26]. The activation of caspase-3 plays an important role in the execution of apoptosis as well as muscle proteolysis [27]. Additionally, in weight-losing upper gastrointestinal tract cancer patients, deoxyribonucleic acid (DNA) fragmentation and poly (ADP-ribose) polymerase (PARP) cleavage were increased, whereas MyoD protein was decreased [6], suggesting increased apoptosis and decreased muscle replenishment.

There is a constant need for alternative traditional medicines to improve the prognosis of cancer patients and prevent chemotherapy and radiotherapy induced discomfort. The tropical medicinal plant *Centella asiatica* (*C. asiatica*) is native to India, China, and South Africa [28]. It belongs to the Apiaceae family and is commonly referred to as Gotu kola, Asiatic pennywort and Tiger herb [28]. *C. asiatica* is widely used in Ayurvedic and Chinese traditional medicines due to its various medicinal properties. These properties include its hepato-protective, cardio-protective, anti-diabetic, antioxidant, anti-inflammatory and anti-tumour potential [28]. The major active compounds in *C. asiatica* are triterpene saponosides such as asiatic acid, madecassic acid and asiaticoside [28]. *C. asiatica* also contains flavonoid derivatives, vitamins, minerals, polysaccharides, sterols and phenolic acids [28]. *C. asiatica* has previously been used in treatment of inflammation due to its promising anti-inflammatory effects [29, 30]. Additionally, *C. asiatica* extracts have demonstrated high antioxidant [31, 32] and anti-proliferative activity in many cancerous cell lines [33].

There is a need for the discovery of an inexpensive cancer cachectic treatment. The ability of a plant extract to regulate inflammatory cytokines and cell death may elevate cancerous cell death and diminish tissue wasting. We investigated the potential of a *C. asiatica* ethanolic leaf extract (C_{LE}) to modulate inflammatory cytokines, antioxidants and cell death in leukaemic THP-1 cells and normal peripheral blood mononuclear cells (PBMC's).

3.2 Materials and Methods

3.2.1 Materials

C. asiatica leaves were collected on the 7th of March 2011 (collectors number: Immelman 411) from the Eastern Cape [Langeni forest, roadside (S31°28.135', E28°32.681')], South Africa (SA) and identified by Dr. Kathleen Immelman from the Department of Botany at the Walter Sisulu University, SA. Voucher specimens were deposited at the KEI herbarium (13979). The THP-1 cells were obtained from American Type Culture Collection (ATCC, University Boulevard Manassas, Virginia, USA). RPMI-1640 and BD OptEIA enzyme-linked immunosorbant assay (ELISA) cytokine kits were purchased from The Scientific Group (Johannesburg, SA). Foetal calf serum (FCS) and Pen/Strep Amphotericin B (PSF) were acquired from Whitehead Scientific (Cape Town, SA). Dimethyl sulphoxide (DMSO) was purchased from Merck (Johannesburg, SA). Histopaque-1077, LPS and 2, 2-diphenyl-1 picrylhydrazyl (DPPH) were purchased from Sigma (Aston Manor, SA). The 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) cell proliferation reagent was purchased from Roche (Johannesburg, SA). Promega (Madison, USA) supplied the caspase (-3/7, -8, -9), adenosine triphosphate (ATP) and glutathione (GSH) kits.

3.2.2 Plant Description and Extraction

The plants official name is *Centella asiatica* (L.) Urb and has been confirmed by using the plant list [34]. The English name is Tiger herb. *C. asiatica* leaves were dried and milled. Ethanol (200–350 ml) was added to milled plant material (10–30 g) and extracted overnight by shaking (4 × g, 37 °C). Ethanol extracts were filtered, rotor evaporated, dried (37 °C) and stored (4 °C).

3.2.3 The 2, 2-diphenyl-1 picrylhydrazyl assay

C_{LE} (0.05–0.8 mg/ml) and butylated hydroxytoluene (BHT) (60–300 µM) dilutions were prepared in methanol (99.5 % and grade AR). A 50 µM DPPH solution was prepared from a stock solution of 0.135 mM DPPH in methanol. C_{LE}, BHT dilutions and methanol (1 ml, triplicate tubes) were aliquoted into 15ml polypropylene tubes, followed by the 50 µM DPPH solution (1 ml). Reaction mixtures were vortexed and incubated (room temperature (RT) for 30 minutes (min)) in the dark.

Absorbance of samples was read at 517 nm using a Varine Cary 50 UV-visible spectrophotometer (McKinley Scientific, New Jersey, US).

3.2.4 Isolation of Peripheral blood mononuclear cells

Buffy coats containing PBMC's were obtained from the South African National Blood Service (2011/09). PBMC's were extracted by differential centrifugation. Buffy coats (5 ml) were layered onto equivolume histopaque-1077 (5 ml) in 15ml polypropylene tubes and centrifuged ($400 \times g$, 21 °C for 30 min). After centrifugation, the PBMC's were transferred to sterile 15 ml polypropylene tubes, phosphate buffered saline (PBS) was added (0.1 M, 10 ml) and tubes were centrifuged ($400 \times g$, 21 °C, 15 min). Cell density of isolated PBMC's was adjusted (1×10^6 cells/ml) using the trypan blue exclusion test and cryo-preserved (10 % FCS, 10 % DMSO) using a NELGENE cryo freezing container and stored at -80°C.

3.2.5 Tissue Culture

THP-1 cells were grown in the appropriate tissue culture conditions in a 75 cm³ tissue culture flask (37 °C, 5% CO₂). The growth media comprised of RPMI-1640, FCS (10 %) and PS (2 %). Cells were thawed, seeded into a 75 cm³ tissue culture flask at a concentration of 3×10^5 cells/ml and incubated (37 °C, 5% CO₂). THP-1 cells were allowed to grow for 2 – 3 days before the cells were centrifuged ($162 \times g$, 10 min) and re-suspended in fresh growth media. The number of cells should not exceed 8×10^5 cells/ml, therefore the cells/ml was quantified daily by trypan blue staining. Once the cell count reached 8×10^5 cells/ml the THP-1 cells were split/ diluted to 3×10^5 cells/ml with media and incubated. Subsequent experiments were conducted once the cell numbers were sufficient.

3.2.6 Cell Viability Assay

Cytotoxicity of C_{LE} in PBMC's and THP-1 cells was measured using the WST-1 assay (Roche, Johannesburg, SA). PBMC and THP-1 cells (10,000 cells/well, 96-well plate, in triplicate wells) were stimulated with LPS (20 µg/ml, 37 °C, 5 % CO₂, 4 hours (h)) before exposure to C_{LE} (0.05–0.8

mg/ml) for 24 and 72 h (37 °C, 5 % CO₂). Similarly, controls received media containing DMSO (0.2 %). Thereafter, plates were centrifuged (162 × g, 10 min), supernatant removed, cell pellets re-suspended in growth media (100 µl/well), WST-1 reagent (10 µl/well) added and plates incubated (37 °C, 5 %, CO₂, 3 h). Optical density was measured at 450 nm (620 nm reference wavelength) with a BIO-TEK µQuant spectrophotometer (Analytical and Diagnostic Products, SA). This experiment was conducted independently on three occasions.

3.2.7 Stimulation and treatment of cells

PBMC's and THP-1 cells (1 × 10⁵ cells/ml) were transferred into 24-well plates, stimulated with LPS (20 µg/ml, 37 °C, 5 % CO₂, 4 h) before exposure to C_{LE} (0.05–0.8 mg/ml) for 24 h (TNF-α) and 72 h (IL-1β, IL-6, IL-10) (37 °C, 5 % CO₂). After incubation, plates were centrifuged (162 × g, 10 min) and supernatant was collected and stored (-80°C) for cytokine analysis. Cell pellets were used to conduct the caspase (-8, -9, -3/7) activity, ATP and GSH assays. The experiments were conducted independently (twice for all subsequent assays).

3.2.8 Quantification of Cytokines

Cytokine levels were estimated using the BD OptEIA ELISA kits (The Scientific Group, SA) and the procedure was followed as per the instruction manual. ELISA plates were coated with capture antibody overnight (100 µl/well, 4 °C). Thereafter, plates were washed (3x) with wash buffer and blocked with assay diluent (200 µl/well, 1 h, RT). Standard solutions were prepared by diluting a stock solution [TNF-α, IL-10 (500 pg/ml), IL-6 (300 pg/ml), IL-1β (250 pg/ml)] serially [TNF-α, IL-10 (500–7.8 pg/ml), IL-6 (300–4.7 pg/ml), IL-1β (250–3.9 pg/ml)]. Plates were washed (3x), standards and samples (100 µl/well, triplicate wells) were aliquoted into appropriate wells and plates were incubated (2 h, RT). Plates were washed (5x), working detector (100 µl/well) added and plates incubated (1 h, RT). The plates were washed (7x), substrate solution (100 µl/well) added and plates were incubated (30 min, RT) in the dark. Finally, stop solution (50 µl/well) was added and the absorbance was read at 450 nm (570 nm reference wavelength) with a Multiskan FC micro-plate reader (Thermo Scientific). Cytokine concentrations were calculated by extrapolation from a standard curve.

3.2.9 Glutathione assay

The GSH-Glo™ assay (Promega, Madison, WI, USA) was used to measure GSH levels. Standard GSH solutions were prepared by diluting a 5 mM stock solution serially (1.56–50 μM) and PBS (0.1 M) was the standard blank. Cells (50 μl/well, 2×10^5 cells/ml) and standards were added into an opaque 96-well plate (duplicate wells), followed by GSH-Glo™ reagent (25 μl/well) and allowed to incubate (30 min, RT) in the dark. Subsequently, luciferin detection reagent (50 μl/well) was added and plates incubated (15 min, RT) in the dark. The absorbance was read on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA) and GSH concentrations were calculated by extrapolation from a standard curve.

3.2.10 Caspase and ATP assays

Caspase activity and ATP levels were determined using the Caspase-Glo®-3/7, -8, -9 and ATP assay kits (Promega, Madison, WI, USA). Caspase-Glo®-3/7, -8, -9 and ATP Reagents were reconstituted according to the manufacturer's instructions. Cells (100 μl, 2×10^5 cells/ml) were added into duplicate wells of a microtitre plate for each assay, thereafter caspase -3/7, -8, -9 and ATP reagents (100 μl/well) were added into appropriate wells. The plate was incubated (30 min, RT) in the dark. Luminescence was measured on a Modulus™ microplate luminometer (Turner BioSystems) and expressed as relative light units (RLU).

3.2.11 Statistical Analysis

Statistical analysis was performed using the STATA and GraphPad Prism (v5) statistical analysis software. The one-way analysis of variance (ANOVA) was used to make comparisons between groups, followed by the Tukey multiple comparisons test, with $p < 0.05$ indicating significant results.

3.3 Results

3.3.1 The oxidant scavenging potential of C_{LE}

The oxidant scavenging activity of C_{LE} using the DPPH assay is shown in Figure 3.1. C_{LE} (0.05–0.8 mg/ml) significantly increased DPPH scavenging activity by approximately 45–84 % (Figure 3.1, $p < 0.0001$).

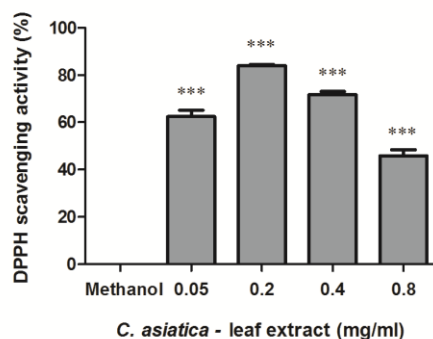


Figure 3.1: Percentage DPPH scavenging activity of C_{LE} (Values expressed as mean \pm SD, *** $p < 0.0001$ compared to control).

3.3.2 The *in vitro* cytotoxicity of C_{LE}

The WST-1 assay was used to determine cell viability of THP-1 cells and PBMC's after treatment with C_{LE} (Figure 3.2). At 24 h, C_{LE} (0.2–0.8 mg/ml) dose dependently decreased PBMC viability by 33.25–61.85 % (Figure 3.2A, $p < 0.0001$), whereas THP-1 viability was not significantly altered as compared to the control (Figure 3.2C, $p = 0.0003$). At 72 h, C_{LE} decreased both PBMC (Figure 3.2B, 34.268–74.547 %) and THP-1 (Figure 3.2D, 28.404 %) viability at 0.4–0.8 mg/ml and 0.8 mg/ml respectively as compared to the control ($p < 0.0001$), suggesting that PBMC's are more sensitive to C_{LE} treatment than THP-1 cells.

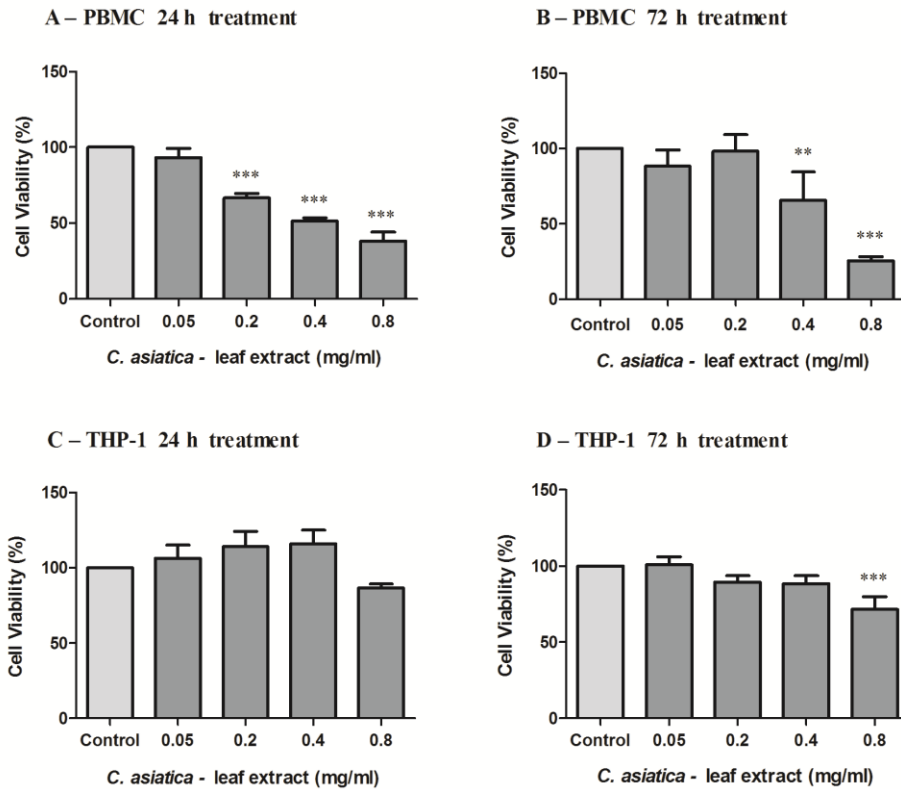


Figure 3.2: Cell viability of PBMC (A – 24 h, B – 72 h) and THP-1 (C – 24 h, D – 72 h) cells treated with C_{LE} for 24 and 72 h (Values expressed as mean \pm SD, ** $p < 0.005$, *** $p < 0.0001$ compared to the control).

3.3.3 The immune suppressive properties of C_{LE}

C_{LE} altered cytokine levels in PBMC's and THP-1 cells which are shown in Figure 3.3 and Figure 3.4 respectively. The levels of TNF- α , IL-1 β , IL-6 and IL-10 produced in LPS stimulated PBMC's was 309.60, 152.83, 626.33 and 23.55 pg/ml respectively. C_{LE} (0.05–0.2 mg/ml) increased PBMC IL-1 β and IL-6 concentrations relative to the control (Figure 3.3B and 3.3C, $p < 0.0001$). In PBMC's, TNF- α , IL-1 β and IL-6 concentrations were decreased at 0.05–0.8 mg/ml C_{LE} , 0.4–0.8 mg/ml C_{LE} and 0.4 mg/ml C_{LE} respectively as compared to the control (Figure 3.3A, 3.3B and 3.3C, $p < 0.0001$).

The levels of TNF- α , IL-1 β , IL-6 and IL-10 produced in LPS stimulated THP-1 cells was 5.96, 25.92, 98.63, and 2.46 pg/ml respectively. TNF- α concentration in THP-1 cells was increased by C_{LE} (0.05, 0.8 mg/ml, Figure 3.4A, $p < 0.0001$) relative to the control. In THP-1 cells, IL-1 β and IL-6 concentrations were increased by 0.05 mg/ml C_{LE} whereas decreased by 0.2–0.8 mg/ml C_{LE} as compared to the control (Figure 3.4B and 3.4C, $p < 0.0001$). Concentration of the anti-inflammatory cytokine, IL-10 was decreased in PBMC's while increased in THP-1 cells by C_{LE} (0.05–0.8 mg/ml) relative to the control (Figure 3.3D and Fig. 3.4D, $p < 0.0001$).

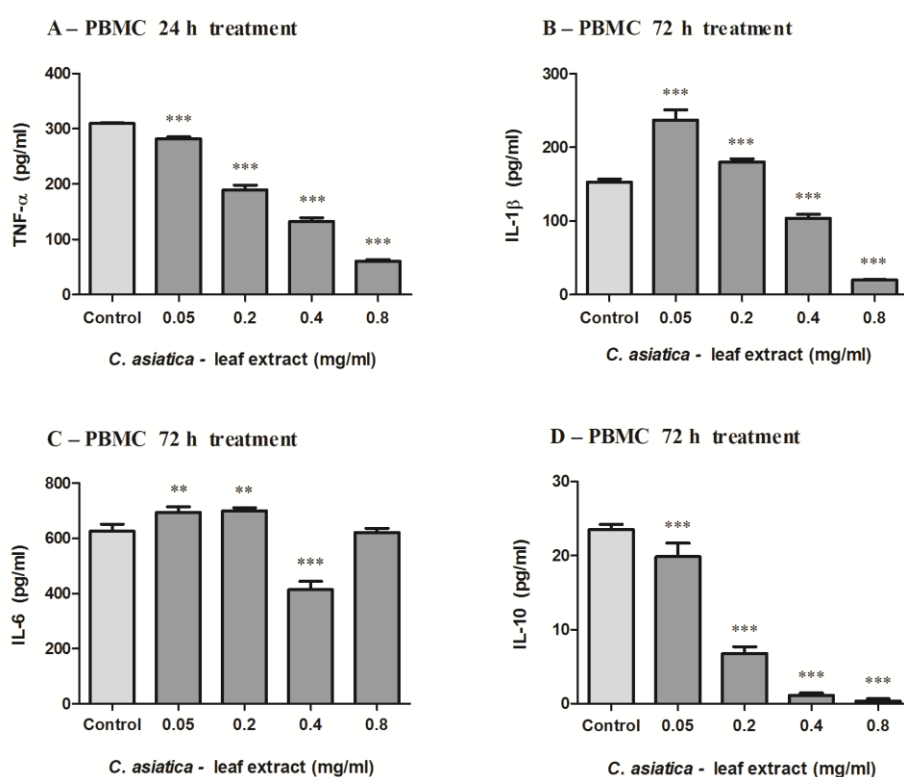


Figure 3.3: Concentration of TNF- α (A), IL-1 β (B), IL-6 (C) and IL-10 (D) in C_{LE} treated PBMC's (Values expressed as mean \pm SD, * $p < 0.05$, *** $p < 0.0001$, compared to the control).

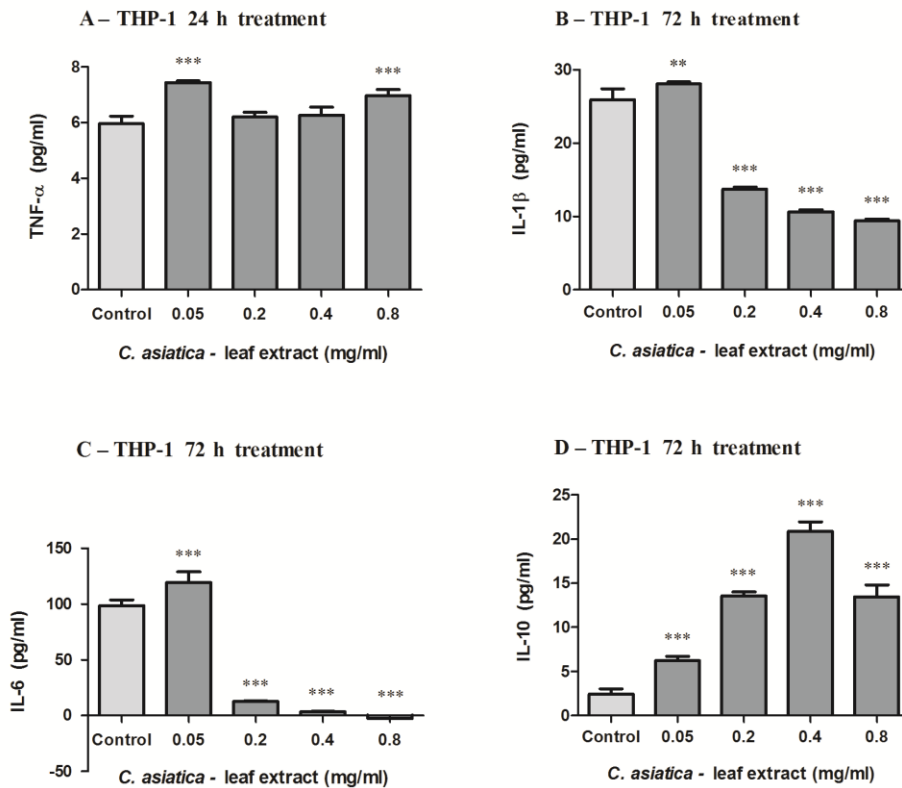


Figure 3.4: Concentration of TNF- α (A), IL-1 β (B), IL-6 (C) and IL-10 (D) in C_{LE} treated THP-1 cells (Values expressed as mean \pm SD, ** $p < 0.005$, *** $p < 0.0001$ compared to the control).

3.3.4 The antioxidant potential of C_{LE}

The endogenous antioxidant activity of C_{LE} was determined by measuring GSH levels in both cell lines (Table 3.1). At 24 h, GSH levels in PBMC's were increased by 0.05–0.2 mg/ml C_{LE} but decreased by 0.4–0.8 mg/ml C_{LE} relative to the control (Table 3.1, $p < 0.0001$). In THP-1 cells, C_{LE} (0.05–0.8 mg/ml) increased GSH levels as compared to the control (Table 3.1, 24 h, $p < 0.0001$). At 24 h, GSH concentrations were increased to a greater extent in THP-1 cells (0.068–3.890 μ M) than PBMC's (0.191–1.746 μ M). At 72 h, C_{LE} (0.05–0.8 mg/ml) increased GSH concentrations in PBMC's and THP-1 cells by 1.13–5.91 μ M and 0.12–0.19 μ M respectively as compared to the control (Table 3.1, $p < 0.0001$). Notably, C_{LE} increased GSH levels to a greater extent in PBMC's as compared to THP-1 cells at 72 h.

Table 3.1: Glutathione levels in C_{LE} treated PBMC's and THP-1 cells.

C _{LE} (mg/ml)	Glutathione (μM)			
	24 h treatment		72 h treatment	
	PBMC	THP-1	PBMC	THP-1
Control	1.238 ± 0.007	1.713 ± 0.002	3.842 ± 0.009	1.449 ± 0.002
0.05	1.429 ± 0.007 ***	4.125 ± 0.004 ***	9.138 ± 0.082 ***	1.576 ± 0.007 ***
0.2	2.984 ± 0.004 ***	5.603 ± 0.004 ***	4.972 ± 0.003 ***	1.568 ± 0.007 ***
0.4	0.959 ± 0.002 ***	1.781 ± 0.002 ***	5.534 ± 0.011 ***	1.610 ± 0.009 ***
0.8	1.073 ± 0.015 ***	2.495 ± 0.005 ***	9.749 ± 0.015 ***	1.634 ± 0.004 ***

(Values expressed as mean ± SD, *** $p < 0.0001$, compared to the control).

3.3.5 C_{LE} modulates caspase (-8, -9, -3/7) activities and ATP levels

Luminometry assays were used to determine caspase activity and ATP levels in THP-1 cells and PBMC's after treatment with C_{LE}. The pro-apoptotic effect of C_{LE} in PBMC's treated for 24 h is shown in Table 3.2. At 24 h, PBMC caspase-8 activity was increased by 0.05–0.2 mg/ml C_{LE}, whereas decreased by 0.4–0.8 mg/ml C_{LE} as compared to the control (Table 3.2, $p < 0.0001$). C_{LE} (0.05–0.8 mg/ml, 24 h) increased PBMC caspase -9 and -3/7 activities relative to the control (Table 3.2, $p < 0.0001$). Increased caspase activity led to the initiation and execution of PBMC apoptosis at 24 h. The PBMC ATP levels were increased by 0.4mg/ml C_{LE}, whereas decreased by 0.05, 0.2 and 0.8 mg/ml C_{LE} (Table 3.2, $p < 0.0001$).

Table 3.2: Modulation of caspase (-8, -9, -3/7) activities and ATP levels in 24 h C_{LE} treated PBMC's.

C _{LE} (mg/ml)	Caspase-8 (RLUx10 ⁵)	Caspase-9 (RLU x10 ⁵)	Caspase-3/7 (RLU x10 ⁵)	ATP (RLU x10 ⁵)
Control	0.146 ± 0.001	0.265 ± 0.002	5.861 ± 0.028	3.486 ± 0.011
0.05	0.176 ± 0.001 ***	0.293 ± 0.001 ***	6.066 ± 0.032	3.168 ± 0.006 ***
0.2	0.256 ± 0.003 ***	0.364 ± 0.002 ***	6.264 ± 0.031 **	3.074 ± 0.002 ***
0.4	0.135 ± 0.001 ***	0.397 ± 0.0003 ***	16.407 ± 0.263 ***	4.180 ± 0.013 ***
0.8	0.101 ± 0.001 ***	0.307 ± 0.0004 ***	6.331 ± 0.007 ***	0.796 ± 0.002 ***

(Values expressed as mean ± SD, ** $p < 0.005$, *** $p < 0.0001$ compared to the control).

C_{LE} pro-apoptotic effects in THP-1 cells treated for 24 h is shown in Table 3.3. At 24 h, C_{LE} (0.05–0.8 mg/ml) increased THP-1 caspase-8 activity as compared to the control (Table 3.3, $p < 0.0001$). In THP-1 cells, caspase-9 activity and ATP levels were decreased by 0.05–0.4 mg/ml C_{LE}, whereas increased by 0.8 mg/ml C_{LE} relative to the control (Table 3.3, 24 h, $p < 0.0001$). The THP-1 caspase-3/7 activity was decreased by 0.2–0.4 mg/ml C_{LE}, whereas increased by 0.05 and 0.8 mg/ml C_{LE} as compared to the control (Table 3.3, 24 h, $p < 0.0001$). THP-1 caspase (-8, -9, -3/7) activities was increased by 0.8 mg/ml C_{LE}, suggesting an increased initiation and execution of THP-1 apoptosis.

Table 3.3: Modulation of caspase (-8, -9, -3/7) activities and ATP levels in 24 h C_{LE} treated THP-1 cells.

C _{LE} (mg/ml)	Caspase-8 (RLUx10 ⁵)	Caspase-9 (RLUx10 ⁵)	Caspase-3/7 (RLUx10 ⁵)	ATP (RLUx10 ⁵)
Control	8.517 ± 0.001	1.933 ± 0.012	9.980 ± 0.008	17.551 ± 0.088
0.05	11.494 ± 0.006 ***	0.415 ± 0.002 ***	10.348 ± 0.218 **	12.507 ± 0.398 ***
0.2	18.909 ± 0.085 ***	0.675 ± 0.001 ***	3.974 ± 0.001 ***	15.586 ± 0.215 ***
0.4	12.276 ± 0.028 ***	1.119 ± 0.003 ***	4.046 ± 0.033 ***	3.948 ± 0.042 ***
0.8	16.191 ± 0.013 ***	2.261 ± 0.002 ***	18.189 ± 0.104 ***	19.496 ± 0.267 ***

(Values expressed as mean ± SD, ** $p < 0.005$, *** $p < 0.0001$ compared to the control).

The pro-apoptotic effect of C_{LE} in PBMC's treated for 72 h is shown in Table 3.4. At 72 h, PBMC caspase-8 activity was increased by 0.4 mg/ml C_{LE}, whereas decreased by 0.05, 0.2, 0.8 mg/ml C_{LE} relative to the control (Table 3.4, $p < 0.0001$). C_{LE} (0.05–0.8 mg/ml) decreased PBMC caspase (-9, -3/7) activities and ATP levels as compared to the control (Table 3.4, 72 h, $p < 0.0001$). Decreased PBMC caspase activity suggests a decrease in PBMC apoptotic cell death.

Table 3.4: Modulation of caspase (-8, -9, -3/7) activities and ATP levels in 72 h C_{LE} treated PBMC's.

C _{LE} (mg/ml)	Caspase-8 (RLUx10 ⁵)	Caspase-9 (RLUx10 ⁵)	Caspase-3/7 (RLUx10 ⁵)	ATP (RLUx10 ⁵)
Control	30.688 ± 0.006	83.054 ± 0.009	132.624 ± 0.118	14.567 ± 0.184
0.05	21.726 ± 0.015 ***	56.070 ± 0.003 ***	128.471 ± 0.253 ***	4.061 ± 0.014 ***
0.2	10.436 ± 0.021 ***	25.014 ± 0.007 ***	57.946 ± 0.024 ***	2.343 ± 0.029 ***
0.4	42.625 ± 0.003 ***	11.887 ± 0.005 ***	35.842 ± 0.036 ***	0.855 ± 0.002 ***
0.8	14.157 ± 0.045 ***	32.499 ± 0.288 ***	43.376 ± 0.028 ***	3.117 ± 0.007 ***

(Values expressed as mean ± SD, *** $p < 0.0001$ compared to the control).

C_{LE} pro-apoptotic effects in THP-1 cells treated for 72 h is shown in Table 3.5. At 72 h, THP-1 caspase-8 activity was increased by 0.4 mg/ml C_{LE} whereas decreased by 0.05, 0.2, 0.8 mg/ml C_{LE} relative to the control (Table 3.5, $p < 0.0001$). C_{LE} (0.05–0.8 mg/ml) decreased THP-1 caspase (-9, -3/7) activities and ATP levels as compared to the control (Table 3.5, 72 h, $p < 0.0001$). Decreased THP-1 caspase activity suggests a decrease in THP-1 apoptotic cell death.

Table 3.5: Modulation of caspase (-8, -9, -3/7) activities and ATP levels in 72 h C_{LE} treated THP-1 cells.

C _{LE} (mg/ml)	Caspase-8 (RLUx10 ⁵)	Caspase-9 (RLUx10 ⁵)	Caspase-3/7 (RLUx10 ⁵)	ATP (RLUx10 ⁵)
Control	1.068 ± 0.002	6.694 ± 0.002	8.218 ± 0.002	4.552 ± 0.029
0.05	1.021 ± 0.001 **	6.343 ± 0.009 ***	6.293 ± 0.001 ***	4.252 ± 0.039 ***
0.2	0.972 ± 0.0003 ***	5.442 ± 0.034 ***	4.954 ± 0.002 ***	3.852 ± 0.039 ***
0.4	11.246 ± 0.034 ***	4.271 ± 0.001 ***	3.596 ± 0.005 ***	3.013 ± 0.005 ***
0.8	0.286 ± 0.0001 ***	1.720 ± 0.001 ***	0.497 ± 0.001 ***	1.65 0.011 ***

(Values expressed as mean ± SD, ** $p < 0.005$, *** $p < 0.0001$ compared to the control).

3.4 Discussion

Cancer and cachexia have been associated with increased levels of oxidative stress, pro-inflammatory cytokines and apoptosis [6, 27]. The medicinal plant, *C. asiatica* possesses anti-inflammatory [29] and anti-tumor activity [35], which can be beneficial in the treatment of cancer cachexia.

Previously, Zainol *et al* (2003) reported that *C. asiatica* possessed antioxidant potential, possibly associated with phenolic compounds [36]. The DPPH assay revealed that C_{LE} has oxidant scavenging potential ranging between 45–84 % at 0.05–0.8 mg/ml C_{LE}. ROS plays a pivotal role in tumour initiation, inflammation, protein degradation and apoptosis. The significant oxidant scavenging potential of C_{LE} may decrease inflammatory cytokine levels and ROS induced apoptosis.

At 24 h, C_{LE} dose dependently decreased PBMC viability, whereas THP-1 viability remained unchanged. However, at 72 h, C_{LE} significantly decreased both PBMC and THP-1 viability. *C. asiatica* derived compounds, asiatic acid and asiticoside, were shown to reduce RAW 264.7 cell

viability (120 μ M, 24 h) by 82 % and 71 % respectively [37]. Additionally, *C. asiatica* extracts inhibited breast (MCF-7) and liver (HepG2) cancer cell proliferation [33, 38], indicating our data on C_{LE} cytotoxicity is in agreement with previous studies.

Inflammatory cytokines play an essential role in tumourgenesis and the cachectic syndrome [6]. Previously, Punturee *et al* (2004) reported that *C. asiatica* ethanolic extract modulated/ suppressed TNF- α production in mouse macrophages [39]. Our results also show that C_{LE} decreased TNF- α concentration in PBMC's. Yun *et al* (2008) reported that the pre-treatment of RAW264.7 cells with asiatic acid significantly reduced IL-6 production with minimal effects on TNF- α and IL-1 β levels [37]. Our findings, however, suggest that C_{LE} modulates pro-inflammatory cytokine levels. In both PBMC's and THP-1 cells, IL-1 β and IL-6 levels were increased by the lower 0.05 mg/ml C_{LE} concentration but decreased at the higher 0.4 mg/ml C_{LE} concentration. Pro-inflammatory cytokines, over a chronic time period, stimulate the production of genotoxic molecules [nitric oxide (NO), ROS] and tumour progression by promoting angiogenesis and metastasis [1, 3]. Previous literature has shown that IL-1 stimulates malignant cell growth and invasiveness [3]. In addition, IL-6 exerts its tumour proliferative and anti-apoptotic potential by targeting genes involved in cell cycle progression and the suppression of apoptosis [3]. The ability of C_{LE} to increase pro-inflammatory cytokines such as IL-1 β in PBMC's may aid in cancerous cell elimination through increased host anti-tumour activity. Conversely, in THP-1 cells, the decrease in IL-6 and IL-1 β concentrations by C_{LE} may diminish cytokine induced tumour immunosuppressive activity and cancer progression.

With regard to the cachectic syndrome, TNF- α inhibits the production of LPL and reduces the rate of LPL gene transcription [40-42], thereby preventing the formation of new lipid stores while stimulating HSL and increasing lipolysis [43]. In adipose tissue (*in vivo*), IL-6 decreased LPL activity leading to tissue wasting in cachectic individuals [19]. The potential of C_{LE} (0.4 mg/ml) to decrease IL-6 and IL-1 β concentrations in PBMC's and THP-1 cells suggests a decrease in LPL inhibition and HSL stimulation, thus contributing to lipogenesis maintenance and minimal lipolysis. IL-6 and TNF- α further contribute to cachexia by stimulating muscle catabolism through the activation of the ubiquitin-proteasome pathway [21, 22, 44]. Furthermore, pro-inflammatory cytokines activate NF- κ B which regulates the expression of genes involved in the suppression of tumour apoptosis, stimulation of tumour cell cycle progression and enhancement of inflammatory mediators [1, 3]. Taken together, NF- κ B promotes tumour progression, invasion, angiogenesis and metastasis [1, 3]. In cachexia, NF- κ B activation induces ubiquitin-proteasome pathway activity and

suppresses MyoD expression [45], thereby increasing proteolysis and decreasing muscle replenishment [46]. By decreasing IL-6 and IL-1 β concentrations in PBMC's and THP-1 cells, C_{LE} (0.4 mg/ml) may prevent excessive activation of NF- κ B and proteasome pathways, ultimately decreasing proteolysis. Thus, C_{LE} may be able to decrease tissue wasting through the down regulation of pro-inflammatory cytokine levels.

The immunosuppressive and anti-inflammatory cytokine IL-10, inhibits tumour development, tumour progression, modulates apoptosis and suppresses angiogenesis during tumour regression [1, 3]. Additionally, IL-10 inhibits NF- κ B activation and subsequently inhibits pro-inflammatory cytokine production (TNF- α , and IL-6) [3]. With regard to tissue wasting, increased IL-10 levels in colon 26- bearing mice was reported to reverse the cachectic syndrome [47]. The decreased PBMC IL-10 concentration may be due to IL-10 combating increased pro-inflammatory cytokine levels (IL-6 and IL-1 β). In THP-1 cells, the potential of C_{LE} to increase IL-10 levels will facilitate a decrease in pro-inflammatory cytokine levels, a decrease in malignant cell progression and possibly alleviate the cancer cachectic syndrome.

GSH, a potent antioxidant [48], effectively scavenges ROS both directly and indirectly [49]. In PBMC's and THP-1 cells, C_{LE} increased GSH concentrations. At 72 h, C_{LE} (0.4 mg/ml) increased GSH levels more significantly in PBMC's (1.45-fold) than THP-1 cells (1.11-fold). This suggests that C_{LE} induces a higher antioxidant defense in normal PBMC's than cancerous THP-1 cells at 72 h.

Apoptosis is a tightly regulated process involving a number of check points before an irreversible point is reached [50]. The extrinsic (death receptors) and intrinsic (mitochondria) pathways are the two main apoptotic pathways [26]. Activation of initiator caspases (-8, -9) leads to the activation of execution caspases (-3/7) resulting in activation of cytoplasmic endonucleases [26].

Previous studies reported that asiatic acid decreased cell viability, induced apoptosis and DNA fragmentation [51, 52]. In PBMC's, C_{LE} (0.4–0.8 mg/ml, 24 h) decreased caspase-8 activity. An increase in TNF- α levels initiates the extrinsic apoptotic pathway subsequently activating caspase-8. However, C_{LE} decreased PBMC TNF- α levels which may have contributed to the decreased caspase-8 activity. At 24 h, C_{LE} increased PBMC caspase (-8 (0.05–0.2 mg/ml), -9, -3/7 (0.05–0.8 mg/ml)) activities, suggesting the activation of the extrinsic and intrinsic apoptotic pathways. GSH

regulates apoptosis by preventing ROS accumulation [53]. Previous studies have demonstrated that elevated GSH levels have been associated with resistance to apoptosis [54, 55]. In PBMC's, the decrease in GSH levels and the increase in caspase (-9, -3/7) activities by C_{LE} (0.4–0.8 mg/ml, 24 h) may have increased apoptosis ultimately decreasing PBMC cell viability. In THP-1 cells, C_{LE} (0.05–0.4 mg/ml) increased caspase-8 activity and decreased caspase-9 activity, suggesting initiation of apoptosis through the extrinsic pathway (24 h). In C_{LE} treated THP-1 cells, the decreased caspase-9 activity may have been a consequence of the increased GSH levels. Although extrinsic apoptosis was activated in THP-1 cells, C_{LE} (0.2–0.4 mg/ml) decreased caspase-3/7 activity, indicating that apoptosis was not fully executed (24 h). Interestingly, C_{LE} increased THP-1 caspase (-8, -9, -3/7) activities at 0.8 mg/ml (24 h), suggesting an increased initiation and execution of THP-1 apoptosis.

At 72 h, caspase activities were decreased in both cell lines, suggesting a decreased activation of apoptosis. In PBMC's and THP-1 cells, the increase in GSH levels and the decrease in caspase (-9, -3/7) activities by C_{LE} (0.05–0.8 mg/ml, 72h) may have decreased apoptotic cell death. However, PBMC and THP-1 cell viability was decreased at 0.4–0.8 mg/ml C_{LE} and 0.8 mg/ml C_{LE} respectively, suggesting an alternative form of cell death occurred.

Increased caspase-3 and proteasome activity, as well as E3 ubiquitin-conjugating enzyme expression are associated with increased proteolysis [56]. Thus the ability of C_{LE} to down regulate caspase activities in PBMC's and THP-1 cells may decrease proteolysis and the progression of cancer cachexia.

The cachectic syndrome is characterized by a negative energy balance due to reduced food intake and abnormal metabolism [57]. The inability to ingest/ use nutrients [5] and the negative energy balance present in cachectic patients leads to catalysis of muscle and fat stores for energy production [58]. In PBMC's, C_{LE} decreased ATP levels, a possible consequence of the decreased cell viability. Cancer cells require high levels of ATP for cellular proliferation [59]. In THP-1 cells, C_{LE} decreased ATP levels which may decrease THP-1 cell proliferation. However in cachexia, a decrease in ATP levels may contribute to tissue wasting.

The potent feeding stimulant neuropeptide Y (NPY) promotes food and energy intake [60]. Increased cytokine (IL-1, IL-6, TNF- α) levels may inhibit NPY signalling leading to decreased food intake and increased energy expenditure [60]. Leptin functions as a suppresser of food intake and stimulator of energy consumption [6]. Pro-inflammatory cytokines may inhibit feeding by mimicking the hypothalamic negative-feedback signalling effect of leptin [61]. Thus, the ability of C_{LE} to decrease pro-inflammatory cytokine levels may increase food intake, decrease energy expenditure and possibly combat the negative energy balance associated with cancer cachexia.

3.5 Conclusion

Our results show that C_{LE} increased oxidant scavenging activity and GSH levels, modulated pro-inflammatory cytokine levels and regulated apoptosis and caspase activity in normal PBMC's and THP-1 cells. C_{LE} may thus be effective in cancer cachexia.

3.6 Abbreviations

LPL: lipoprotein lipase; FA's: fatty acids; TAG, triacylglycerol; HSL: hormone sensitive lipase; LPS: lipopolysaccharide; NF- κ B: Nuclear factor kappa B; IL: interleukin; TNF- α : tumour necrosis factor- α ; ROS: reactive oxygen species; DNA: Deoxyribonucleic acid; PARP: poly (ADP-ribose) polymerase; *C. asiatica* *Centella asiatica*; C_{LE}, *C. asiatica* ethanolic leaf extract; PBMC's: peripheral blood mononuclear cells; THP-1: a leukaemic cell line; SA: South Africa; ELISA: enzyme-linked immunosorbant assay; FCS: Foetal calf serum; PSF: Pen/Strep Amphotericin B; DMSO: Dimethyl sulphoxide; DPPH: 2, 2-diphenyl-1 picrylhydrazyl; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; ATP: Adenosine triphosphate; GSH: Glutathione; BHT: butylated hydroxytoluene; RT: room temperature; Min: Minute; PBS: phosphate buffered saline; h: Hours; RLU: Relative light units; ANOVA: one way analysis of variance; NO: nitric oxide; NPY: neuropeptide Y.

3.7 Declarations

Ethics approval and consent to participate

Collection of PBMC's was ethically approved by the South African Medical Research Council Ethics Committee (EC09-018).

3.8 Consent for publication

Not applicable

3.9 Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

3.10 Competing interests

The author(s) declare that they have no competing interests.

3.11 Role of Funding Source

Sources of funding included the National Research Foundation, the South African Medical Research Council and Department of Science and Technology. The funding sources were not involved in study design, collection of samples, analysis of data, interpretation of data, writing of the report and decision to publish. Scientific out-put is a requirement of the National Research Foundation.

3.12 Authors' contributions

DBN contributed to experimental design and carried out all experimentation except the luminometry (Caspase, ATP, GSH) assays. DBN analysed and interpreted data, performed

statistical analysis, drafted and revised the manuscript. AC and AP carried out luminometry assays and revised manuscript critically for important intellectual input. KPG, KS and VS gave substantial contributions to conception, design and revised manuscript critically for important intellectual input. All authors read and approved the final manuscript.

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3.14 References

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Bridging paragraph:

There was limited *W. somnifera* aqueous root extract available therefore experimentation ceased. The previous manuscript demonstrated that *C. asiatica* ethanolic leaf extract (C) decreased IL-6 and IL-1 β whereas increased IL-10 concentration in THP-1 cells. Additionally, C modulated cell death in PBMC's and THP-1 cells.

To identify active fractions/ compounds responsible for the modulation of inflammatory cytokines and cell death by C. Silica column fractionation was conducted to separate *C. asiatica* compounds based on their polarity. Thereafter, thin layer chromatography analysis revealed fractions with similar spot patterns which were combined and pooled into one fraction (Appendix 4). There were a total of 13 pooled fractions. Preliminary experimentation revealed that fraction-3 (C3) significantly increased oxidant scavenging activity, decreased pro-inflammatory cytokine levels and increased an anti-inflammatory cytokine level (Appendix 5). Thereafter, gas chromatography mass spectrometry determined the compounds present in C and C3 (Appendix 6).

The following manuscript is entitled *Centella asiatica* decreases nuclear factor kappa-beta (p50, p65) protein expression; decreases pro-inflammatory cytokine levels and modulates cell death in leukaemic (THP-1) cells (Supplementary information: Appendix 7). This manuscript compared the potential of C and C3 [0.1 mg/ml] to modulate nitrite levels, inflammatory cytokine levels, NF- κ B (p50, p65) protein expression and cell death in THP-1 cells.

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Please note: The manuscript was formatted for Herbal medicine, however, the margins, font, line spacing, numbering of sections and figures were adjusted for thesis consistency.

CHAPTER FOUR

***Centella asiatica* decreases nuclear factor kappa-beta (p50, p65) protein expression, decreases pro-inflammatory cytokine levels and modulates cell death in leukaemic (THP-1) cells**

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Abstract

Centella asiatica (*C. asiatica*) possesses antioxidant, anti-inflammatory and anti-tumour potential. We investigated the anti-inflammatory and anti-proliferative effects of *C. asiatica* ethanolic leaf extract (C) and purified fraction-3 (C3) in leukaemic (THP-1) cells.

C3 was obtained by silica column fractionation and identified using thin layer chromatography. Gas chromatography mass spectrometry determined the bioactive compounds present in C and C3. Cytotoxicity of C and C3 in healthy PBMC's and THP-1 cells were evaluated (cell viability assay; 24 and 72 hours (h); [0.1 mg/ml]). Oxidant scavenging activity (spectrophotometry), cytokine (tumour necrosis factor alpha (TNF- α), interleukin (IL)-6, IL-1 β and IL-10) concentrations (enzyme-linked immunosorbant assay), nitrite levels (griess assay), caspase (-8, -9, -3/7) activities (luminometry) and nuclear factor kappa beta (NF- κ B: p50, p65) protein expressions (western blotting) were assessed.

THP-1 viability was decreased at 24 h whereas increased at 72 h by C and C3 ($p < 0.003$). At 72 h, C3 decreased PBMC viability ($p < 0.0001$). Both C and C3 increased oxidant scavenging activity ($p < 0.0001$). In THP-1 cells, C and C3 decreased nitrite, TNF- α , IL-1 β , IL-6 levels and NF- κ B (p50, p65) protein expressions whereas increased IL-10 levels ($p < 0.0001$). At 24 h, THP-1 caspase (-8, -9, -3/7) activities were increased by C and C3 ($p < 0.0001$). At 72 h, C increased THP-1 caspase -8 and -9 whereas decreased caspase-3/7 activity ($p < 0.009$). C3 (72 h) decreased THP-1 caspase-8 whereas did not alter caspase -9 and -3/7 activities ($p < 0.009$).

Thus *C. asiatica* elicited anti-inflammatory and anti-proliferative effects on THP-1 cells.

Key words: Cancer cachexia, cytokines, apoptosis, *Centella asiatica*

Abbreviations:

LPL, lipoprotein lipase; FA's, fatty acids; TAG, triacylglycerol; HSL, hormone sensitive lipase; NF- κ B, Nuclear factor kappa-beta; TNF- α , tumour necrosis factor-alpha; IL, interleukin; I κ B, NF- κ B inhibitor; *Centella asiatica*, *C. asiatica*; C, *C. asiatica* crude extract; C3, *C. asiatica* pooled fraction-3; PBMC's, peripheral blood mononuclear cells; THP-1, a leukaemic cell line; SA, South Africa; DCM, dichloromethane; HEX, hexane; EA, ethyl acetate; DMSO, dimethyl sulphoxide; TLC, thin layer chromatograph; GC-MS, gas chromatography mass spectrometry; ELISA, enzyme-linked immunosorbant assay; FCS, foetal calf serum; PS, penstrepfungizone; L-GLUT, L-glutamine; LPS, lipopolysaccharide; DPPH, 2, 2-diphenyl-1 picrylhydrazyl; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; BHT, butylated hydroxytoluene; Min, minute; PBS, phosphate buffered saline; h, hours; RT, room temperature; RLU, relative light units; ROS, reactive oxygen species; TTBS, tween-20 wash buffer; RBD, relative band density; iNOS, inducible nitric oxide synthase.

4.1 Introduction

Cancer cachexia is a multi-factorial syndrome that decreases patient quality of life and responsiveness to chemotherapy (Zhou et al., 2003). It is responsible for 20–25% of cancer deaths with the degree of cachexia being inversely proportional to survival time (Lelbach et al., 2007; Martignoni et al., 2003). Key features of cachexia include abnormal loss of body weight (up to 85% adipose tissue and 75% skeletal muscle) (Tisdale, 2009), systemic inflammation and reduced food intake (Fearon, 2008). Lipogenesis occurs through lipoprotein lipase (LPL) hydrolysing fatty acids (FA's) from plasma lipoproteins and transporting FA's into adipose tissue for triacylglycerols (TAG) production whereas lipolysis is due to hormone sensitive lipase (HSL) breaking down TAG's into FA's and glycerol (Tisdale, 2009). Previously, decreased serum LPL levels/ activity (Lanza-Jacoby et al., 1984; Vlassara et al., 1986) and increased HSL levels/ activity have been associated with the cachectic syndrome (Thompson et al., 1993). Additionally, the ubiquitin-dependent proteolytic pathway and the nuclear factor kappa-beta (NF- κ B) pathway have been shown to be responsible for the excessive proteolysis seen in cancer cachexia (Tisdale, 2009). Cancer and the prevalence of cachexia are commonly correlation with elevated pro-inflammatory cytokine levels (Fearon et al., 2012). Increased pro-inflammatory cytokine levels have been shown

to initiate metabolic events such as decreasing appetite and lipogenesis while increasing lipolysis and proteolysis which contributes to tissue wasting (Fearon et al., 1999; Tisdale, 2009).

The transcription factor, NF- κ B regulates the transcription of genes associated with inflammation, proliferation, invasion, angiogenesis and apoptosis (Asehnoune et al., 2004; Janssen-Heininger et al., 2000; Landskron et al., 2014; Park et al., 2004). Under basal conditions, NF- κ B inhibitor (I κ B) proteins regulate NF- κ B signalling by binding to NF- κ B dimers and blocking their nuclear localization (Baldwin, 1996; Ghosh et al., 1998). Proteasomal degradation of I κ B proteins allows for the release and nuclear translocation of NF- κ B dimers (Baldwin, 1996; Ghosh et al., 1998). Activation of the classical/ canonical NF- κ B pathway (p50:p65 dimers) is essential for innate immunity and the inhibition of apoptosis (Bonizzi and Karin, 2004; Ghosh and Karin, 2002). In many human cancers, NF- κ B is frequently activated (Karin et al., 2002; Staudt, 2010). NF- κ B activation is associated with malignancy development due to its role in tumour promotion and anti-apoptotic gene expression (Garg and Aggarwal, 2002). In cancer cachexia, NF- κ B activation leads to the induction of the ubiquitin–proteasome pathway which induces extensive muscle wasting in mice (Russell et al., 2009). In addition, elevated NF- κ B activity leads to increased pro-inflammatory cytokine production [tumour necrosis factor- α (TNF- α), interleukin (IL) – 6, and IL-1 β] further contributing to lipid and protein degradation (Martignoni et al., 2005). Thus, NF- κ B inhibition is regarded a potential therapeutic target for cancer and the cachectic syndrome.

In the search for cancer therapies, medicinal plants are of great interest due to their antioxidant and anti-inflammatory potential. The tropical medicinal plant *Centella asiatica* (*C. asiatica*, Apiaceae family) is native to India, China, and South Africa (Orhan, 2012). It is commonly referred to as Gotu kola, Asiatic/ Indian pennywort and Tiger herb (Orhan, 2012). Major active compounds in *C. asiatica* are triterpene saponosides (asiatic acid, madecassic acid, asiaticoside and madecassoside) (Orhan, 2012). Additionally, *C. asiatica* contains potent antioxidants (flavonoids, quercetin, catechin and rutin), vitamins, minerals, polysaccharides, sterols, and phenolic acids (Hussin et al., 2007; Orhan, 2012). *C. asiatica* possesses antioxidant, anti-inflammatory and anti-tumour potential (Orhan, 2012). Previously, in J774.2 mouse macrophages, *C. asiatica* ethanolic extract suppressed TNF- α expression and decreased TNF- α protein levels (Punturee et al., 2004). In addition, *C. asiatica* methanolic extract has demonstrated anti-proliferative activity in many cancer cell lines (Babykutty et al., 2008).

The cancer cachectic syndrome is a progressive and debilitating condition responsible for approximately two million deaths annually (Muscaritoli et al., 2006). The discovery of a medicinal plant/ active compound capable of decreasing pro-inflammatory cytokines and increasing cancerous cell death may alleviate cancer cachexia and pro-long the life span of patients. Thus, this study investigated the potential of an ethanolic leaf extract of *C. asiatica* (C) and *C. asiatica* pooled fraction-3 (C3) to modulate inflammatory responses and cell death in leukaemic (THP-1) cells.

4.2 Materials and methods

4.2.1 Materials

C. asiatica leaves were collected in March 2011 (collectors number: Immelman 411) from the Eastern Cape [Langeni forest, roadside (S31°28.135', E28°32.681')], South Africa (SA) and identified by Dr. Kathleen Immelman (Department of Botany, Walter Sisulu University, SA). Voucher specimens were deposited at the KEI herbarium (13979). THP-1 cells were obtained from ATCC (University Boulevard Manassas, USA) and whole blood (EC09-018) was obtained from a healthy donor (Durban, SA). Solvents [dichloromethane (DCM), hexane (HEX), ethyl acetate (EA), methanol, dimethyl sulphoxide (DMSO)], sulphuric acid, silica gel 60, thin layer chromatography (TLC) silica gel 60 sheets and 4-methoxybenzaldehyde were purchased from Merck (Johannesburg, SA). The RPMI-1640 and BD OptEIA enzyme-linked immunosorbant assay (ELISA) cytokine kits were purchased from The Scientific group (Johannesburg, SA). Foetal calf serum (FCS), penstrepfungizone (PS) and L-glutamine (L-Glut) were acquired from Whitehead scientific (Cape Town, SA). Histopaque-1077, lipopolysaccharide (LPS) and 2, 2-diphenyl-1 picrylhydrazyl (DPPH) were purchased from Sigma (Aston Manor, SA). The 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) cell proliferation reagent was purchased from Roche (Johannesburg, SA). Caspase (-8, -9, -3/7) activity kits were acquired from Promega (Madison, USA). Western blot reagents were purchased from Bio-Rad (Johannesburg, SA) and the NF- κ B family member antibody sampler kit was purchased from Cell Signalling Technology (Johannesburg, SA). ECL-LumiGlo® chemiluminescent substrate kit was purchased from Gaithersburg (USA).

4.2.2 Plant extraction and fractionation

The plant's official name is *Centella asiatica* (L.) Urb and has been confirmed by using the plant list (www.theplantlist.org). The local names are icudwane (Zulu) and varkoortjies (Afrikaans). The English names are tiger herb and pennywort. *C. asiatica* leaves were dried and milled. Ethanol (200–350 ml) was added to milled plant material (10–30 g) and extracted overnight by shaking (4 x g, 37 °C). Ethanol extracts were filtered, rotor evaporated, dried (37 °C) and stored (4 °C). Silica column fractionation was used to separate *C. asiatica* compounds based on their polarity. A column (height: 85 cm, diameter: 17 cm) was assembled by adding a tap (25 mm), a cotton wool stop, a thin layer of sand, carefully pouring silica mixed with DCM: HEX (50: 50) into a column and allowing the silica to set overnight. *C. asiatica* crude extract (17 g) was dissolved in DCM: HEX (70: 30) and a portion of the extract was further dissolved in methanol. Thereafter, *C. asiatica* crude extract was layered onto the silica and 50 ml fractions were collected using a series of solvent systems [DCM: HEX (70: 30), DCM: EA (70: 30), DCM: EA (50: 50), DCM: EA: methanol (50: 40: 10), DCM: EA: methanol (30: 50: 20), EA: methanol (70: 30), EA: methanol (40: 60), EA: methanol (20: 80), methanol (100)]. The 50 ml fractions were boiled (100 °C) to allow evaporation of excess solvent and TLC was used to determine spot patterns and solvent changes. A total of 766 fractions were collected and TLC analysis revealed fractions with similar spot patterns which were combined and pooled into one fraction. Fractionation of *C. asiatica* yielded 13 pooled fractions. For all subsequent experiments, *C. asiatica* crude and fraction-3 extracts were used.

4.2.3 Gas chromatography mass spectrometry (GC-MS)

C. asiatica (C and C3) chemical composition has been subjected to GC-MS (Hewlett-Packard, USA) and the GCD-HP1800A system was used. Ionization energy (70 eV) was used for the GC-MS detection and the detection was passed under high vacuum (10⁻⁴ to 10⁻⁸ torr). Helium gas was used for analysis at a constant flow rate (1 mL/min). The mass transfer line and injector temperature were fixed at 280 and 250 °C. The bioactive compounds which are present in *C. asiatica* (C and C3) were matched with the NIST computer library and reported.

4.2.4 The 2, 2-diphenyl-1 picrylhydrazyl (DPPH) assay

C (0.1 mg/ml), C3 (0.1 mg/ml) and butylated hydroxytoluene (BHT) (60–300 μ M) dilutions were prepared in methanol (99.5% and grade AR). A 50 μ M DPPH solution was prepared from a stock solution of 0.135 mM DPPH in methanol. C, C3, BHT dilutions and methanol (100 μ l/well, triplicate wells) were aliquoted into a 96-well plate, followed by the 50 μ M DPPH solution (100 μ l/well). Reaction mixtures were incubated (room temperature (RT), 30 minutes (min)) in the dark and the optical density (OD) was measured at 517 nm with a BIO-TEK μ Quant spectrophotometer (Analytical and Diagnostic Products, SA). This experiment was conducted independently two times.

4.2.5 Isolation of peripheral blood mononuclear cells

Buffy coats containing PBMC's (EC09-018) were extracted from whole blood by differential centrifugation. Whole blood (5 ml) from a healthy donor was layered onto equivolume histopaque-1077 (5 ml) in 15 ml polypropylene tubes and centrifuged (400 \times g, 21°C, 30 min). After centrifugation, buffy coats were transferred to sterile 15 ml polypropylene tubes, phosphate buffered saline (PBS) was added (0.1 M, 10 ml) and tubes were centrifuged (400 \times g, 21°C, 15 min). Cell density of isolated PBMC's was adjusted (1×10^6 cells/ml) using the trypan blue exclusion test, cryo-preserved (10% FCS, 10% DMSO) using a NALGENE cooler and stored at -80°C.

4.2.6 Tissue culture

THP-1 cells were grown in the appropriate tissue culture conditions in a 75 cm³ culture flask (37 °C, 5% CO₂). The growth media comprised of RPMI-1640, FCS (10%), L-Glut (1%), PS (1%) and 2-mercaptoethanol (0.05 mM). Cells were seeded at a concentration of 3×10^5 cells/ml, quantified daily by trypan blue staining and were used for subsequent experiments once the cell density reached 8×10^5 cells/ml.

4.2.7 Cell viability assay

Cytotoxicity of C and C3 to PBMC's and THP-1 cells was measured using the WST-1 assay (Roche, Johannesburg, SA). PBMC and THP-1 cells (10,000 cells/well, 96 well plate, triplicate wells) were stimulated with LPS (20 µg/ml, 37 °C, 5% CO₂, 4 hours (h)) before exposure to C (0.1 mg/ml) and C3 (0.1 mg/ml) for 24 and 72 h (37 °C, 5% CO₂). Similarly controls received media containing DMSO (0.025%). Thereafter plates were centrifuged (162 × g, 10 min), supernatant removed, cell pellets re-suspended in growth media (100 µl/well), WST-1 reagent (10 µl/well) added and plates incubated (37 °C, 5%, CO₂, 3 h). OD was measured at 450 nm (620 nm reference wavelength) with a BIO-TEK µQuant spectrophotometer (Analytical and Diagnostic Products, SA). This experiment was conducted independently two times.

4.2.8 Stimulation and treatment of cells

THP-1 cells (1x10⁵ cells/ml) were transferred into 24 well plates in triplicate, stimulated with LPS (20 µg/ml, 37 °C, 5% CO₂, 4 h) before exposure to *C. asiatica* (C and C3 [0.1mg/ml]) for 24 h (TNF-α) and 72 h (IL-1β, IL-6, IL-10) (37 °C, 5% CO₂). After incubation, plates were centrifuged (162 × g, 10 min), supernatant was collected and stored (-80°C) for nitrite and cytokine analysis. Cell pellets were used to conduct the caspase (-8, -9, -3/7) activity assays and protein isolation. The following experiments were conducted independently two times for all subsequent assays.

4.2.9 The Griess assay

The griess assay was used to measure nitric oxide (NO) production. The level of nitrites (end product of NO generation) was determined as per Miranda *et al.*, (2001) (Miranda et al., 2001). A positive control of nitrate solution (25 µM in 0.1 M PBS) and a blank (0.1 M PBS) was prepared. Samples (100 µl/well) were dispensed into triplicate wells of a 96-well microtitre plate. Thereafter vanadium (III) chloride (100 µl/well, 8 mg/ml) was added followed rapidly by sulphanilamide (50 µl/well, 2%) and *N*-1 (naphthyl)ethylenediamine (50 µl/well, 0.1% in 5% HCl). The plate was incubated (37 °C, 45 min) in the dark. OD was measured at 540 nm (690 nm reference wavelength) with a BIO-TEK µQuant spectrophotometer (Analytical and Diagnostic Products, SA). The net

absorbance was calculated by subtracting the absorbance of the blank from the treated samples. The data is represented as mean OD values.

4.2.10 Quantification of cytokines

Cytokine levels were estimated using the BD OptEIA ELISA kits (The Scientific group, SA) and the procedure was followed as per instruction manual. ELISA plates were coated with capture antibody overnight (100 µl/well, 4 °C). Thereafter, plates were washed (3x) with wash buffer and blocked with assay diluent (200 µl/well, 1 h, RT). Standard solutions were prepared by diluting a stock solution [TNF- α , IL-10 (500 pg/ml), IL-6 (300 pg/ml), IL-1 β (250 pg/ml)] serially [TNF- α , IL-10 (500–7.8 pg/ml), IL-6 (300–4.7 pg/ml), IL-1 β (250–3.9 pg/ml)]. Plates were washed (3x), standards and samples (100 µl/well) were aliquoted into appropriate wells and plates were incubated (2 h, RT). Plates were washed (5x), working detector (100 µl/well) added and plates incubated (1 h, RT). The plates were washed (7x), substrate solution (100 µl/well) added and plates were incubated (30 min, RT) in the dark. Finally, stop solution (50 µl/well) was added and the absorbance was read at 450 nm (570 nm reference wavelength) with a BIO-TEK μ Quant spectrophotometer (Analytical and Diagnostic Products, SA). Cytokine concentrations were calculated by extrapolation from a standard curve.

4.2.11 Caspase assays

Caspase (-8, -9, -3/7) activities were determined using the Caspase-Glo[®] -3/7, -8 and -9 assay kits (Promega, Madison, USA). Caspase-Glo[®] -3/7, -8 and -9 reagents were reconstituted according to the manufacturer's instructions. Cells (50 µl/well, 2x10⁵ cells/ml) were added into triplicate wells of a microtitre plate for each assay; thereafter caspase -3/7, -8 and -9 reagents (25 µl/well) were added into appropriate wells. Plates were incubated (30 min, RT) in the dark. Luminescence was measured on a Modulus[™] microplate luminometer (Turner BioSystems) and expressed as relative light units (RLU).

4.2.12 Protein Isolation and Western Blotting

Western Blots were performed to determine the relative expression of NF- κ B proteins (p50, p65). Protein was isolated using Cytobuster™ reagent (Novagen, Pretoria, SA), supplemented with protease and phosphatase inhibitors (Roche, 05892791001 and 04906837001, respectively). Cytobuster (200 μ l) was added to the cells (10 min, 4 °C) and centrifuged (180 x g, 4 °C, 10 min). Protein was quantified using the bicinchoninic acid assay (Sigma, Germany) and standardised to 0.2 mg/ml. Laemmli buffer (30 μ l) was added to protein samples (120 μ l) and boiled (100 °C, 5 min). Protein samples (30 μ l) were electrophoresed (150 V, 1 h) in 10% SDS gels (Bio-Rad compact power supply). Separated proteins were transferred to nitrocellulose membrane using the Trans-Blot® Turbo Transfer system (Bio-Rad) (20 V, 45 min). Membranes were blocked (1 h) using bovine serum albumin (5%) in Tris-buffered saline containing Tween20 (0.05%) [TTBS: NaCl, KCL, Tris, Tween 20, dH₂O, pH 7.4]. Thereafter, membranes were immune-probed with primary antibody [NF- κ B1 p105/p50 (3035), NF- κ B p65 (8242), 1: 1000 (Cell Signalling, Danvers, USA)] overnight (4 °C). Membranes were then washed 5 \times with TTBS (10 min each) and incubated (1 h, RT) with the secondary antibody [anti-rabbit IgG (7074), 1: 2000 (Cell Signalling, Danvers, USA)]. Membranes were washed 5 \times with TTBS (10 min each). Horse radish peroxidase chemiluminescence detector (Bio-Rad) and enhancer solution were used for the antigen-antibody complex and the signal was detected with the Alliance 2.7 image documentation system (UViTech). To correct for loading error and to normalise relative protein expression, β -actin was assessed (A3854; 1: 5000). Protein expressions were analyzed with UViBand Advanced Image Analysis software v12.14 (UViTech) and data was expressed as relative band density (RBD) and fold change.

4.2.13 Statistics

Statistical analysis was performed using GraphPad Prism (v5) statistical analysis software. The one way analysis of variance (ANOVA) was used to compare between groups, followed by the Tukey multiple comparisons test, with $p < 0.05$ chosen as the significant level.

4.3 Results

4.3.1 Chemical composition of *C. asiatica* (C and C3)

The GC-MS chromatograms of C and C3 were recorded and presented in Supplementary Figures 4 and 5. Chemical composition of C and C3 constituents were identified after comparison with those available in the NIST computer library. The organic compounds that were identified in *C. asiatica* leaf extract namely caranol, oxymetholone methanol adduct, vitamin E, tocopherol and terpenes (phytosterol and stigmasterol) (Supplementary information: Table 1 and 2).

4.3.2 Oxidant scavenging activity of *C. asiatica* (C and C3)

The DPPH assay was used to assess oxidant scavenging activity. DPPH oxidant scavenging activity was significantly increased by C (78.205%) as compared to C3 (13.051%) relative to the control (Figure 4.1, $p < 0.0001$).

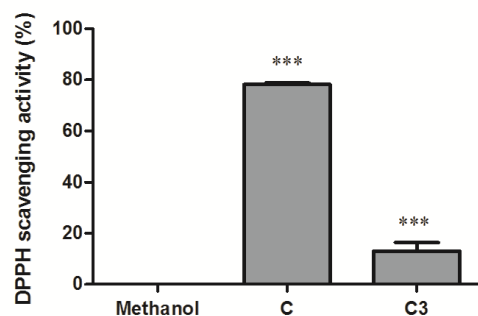


Figure 4.1: Percentage DPPH scavenging activity of *C. asiatica* (C and C3) (Values expressed as mean \pm SD, *** $p < 0.0001$)

4.3.3 Cytotoxicity of *C. asiatica* (C and C3) in PBMC's and THP-1 cells

At 24 h, *C. asiatica* (C and C3) did not significantly alter PBMC viability (Figure 4.2A, $p = 0.0215$) whereas significantly decreased THP-1 viability by 3.783–7.164% (Figure 4.2C, $p = 0.0025$) relative to their respective controls. At 72 h, C3 decreased PBMC viability by 16.703% (Figure 4.2B)

whereas C and C3 increased THP-1 viability by $\pm 11\%$ (Figure 4.2D) as compared to their respective controls ($p < 0.0001$).

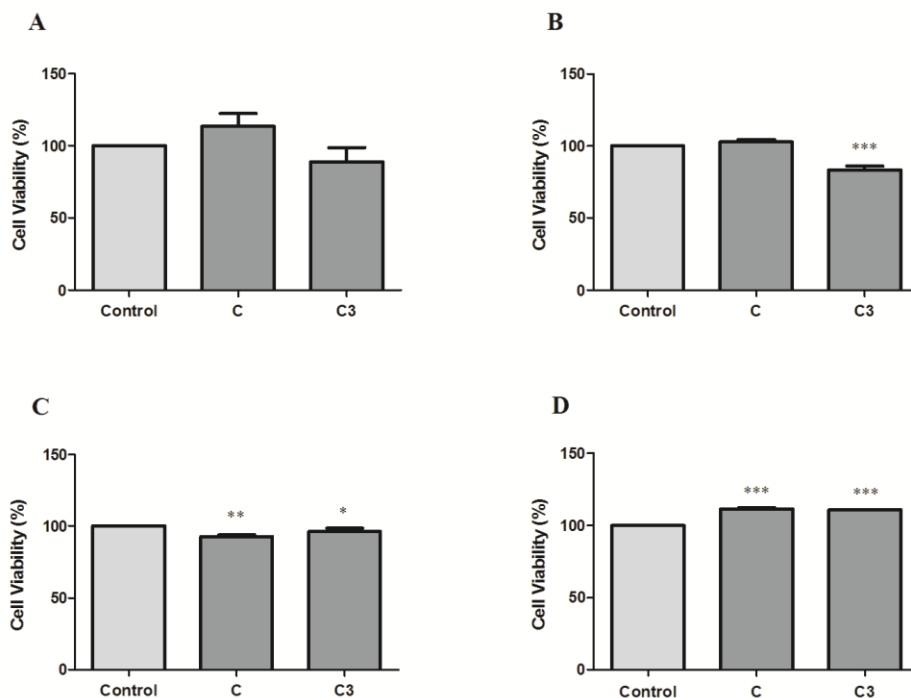


Figure 4.2: Cell viability of PBMC (A – 24 h, B – 72 h) and THP-1 (C – 24 h, D – 72 h) cells treated with *C. asiatica* (C and C3) for 24 and 72 h (Values expressed as mean \pm SD, * $p < 0.05$ ** $p < 0.005$ *** $p < 0.0001$ compared to the control).

4.3.4 Nitrite levels in *C. asiatica* (C and C3) treated THP-1 cells

In THP-1 cells, *C. asiatica* (C and C3) decreased nitrite levels at 24 (Figure 4.3A) and 72 h (Figure 4.3B) ($p < 0.0001$). Notably, C3 (1.464–1.767 fold) decreased nitrite levels to a greater extent than C (1.030–1.136 fold).

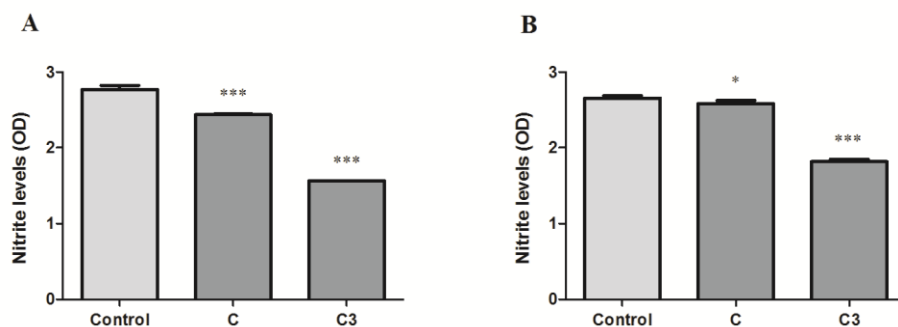


Figure 4.3: Nitrite levels in THP-1 (A – 24 h, B – 72 h) cells treated with *C. asiatica* (C and C3) for 24 and 72 h (Values expressed as mean \pm SD, * $p < 0.05$, *** $p < 0.0001$ compared to the control).

4.3.5 Modulation of inflammatory cytokine levels in *C. asiatica* (C and C3) treated THP-1 cells

Concentration of TNF- α , IL-1 β , IL-6 and IL-10 in LPS stimulated control THP-1 cells was 65.884, 117.059, 614.697 and 37.778 pg/ml respectively. THP-1 TNF- α concentration was decreased by C (4.603-fold) and C3 (> 65.883-fold) relative to the control (Figure 4.4A, $p < 0.0001$). In THP-1 cells, IL-1 β concentration was decreased by C (2.309-fold) and C3 (10.855-fold) as compared to the control (Figure 4.4B, $p < 0.0001$). THP-1 IL-6 concentration was decreased by both C (3.397-fold) and C3 (37.565-fold) relative to the control (Figure 4.4C, $p < 0.0001$). Notably, C3 decreased TNF- α , IL-1 β and IL-6 concentrations to greater extent than C. In THP-1 cells, the concentration of the anti-inflammatory cytokine IL-10 was increased by C (1.732-fold) and C3 (2.611-fold) compared to the control (Figure 4.4D, $p < 0.0001$). Interestingly, C3 increased IL-10 concentration to a greater extent than C.

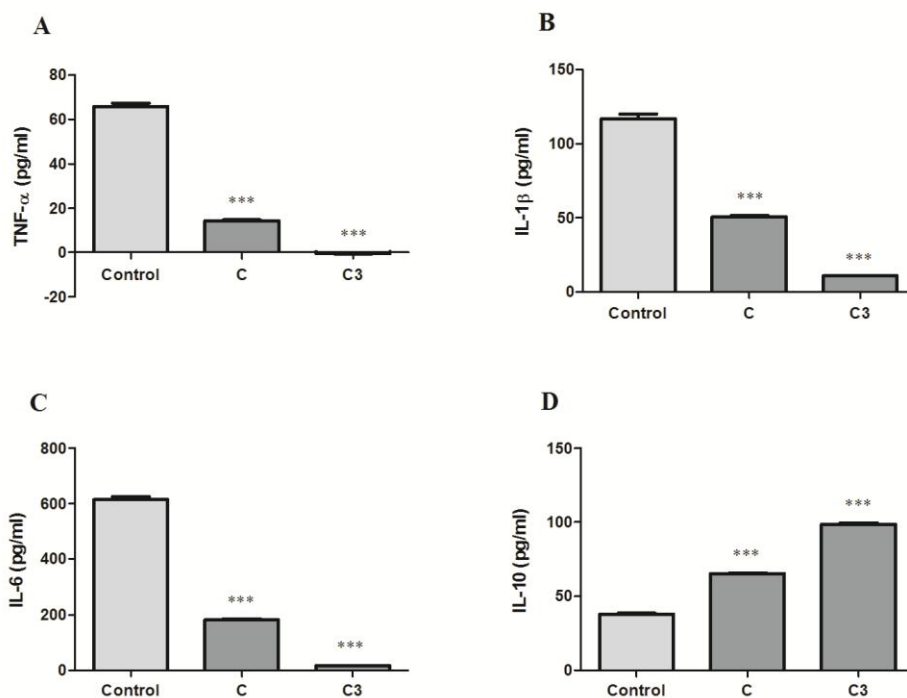


Figure 4.4: Concentration of TNF- α (A), IL-1 β (B), IL-6 (C) and IL-10 (D) in LPS stimulated and *C. asiatica* (C and C3) treated THP-1 cells (Values expressed as mean \pm SD, *** $p < 0.0001$ compared to the control).

4.3.6 Modulation of caspase (-8, -9, -3/7) activity in *C. asiatica* (C and C3) treated THP-1 cells

At 24 h, THP-1 caspase-8 (± 2.200 -fold), caspase-9 (± 2.300 -fold) and caspase-3/7 (± 2.200 -fold) activities were increased by C and C3 relative to the control (Table 4.1, $p < 0.0001$). The substantially increased executioner caspase-3/7 activity suggests an increase in apoptotic cell death.

Table 4.1: Modulation of caspase (-8, -9, -3/7) activities in LPS stimulated and 24 h *C. asiatica* (C and C3) treated THP-1 cells (Values expressed as mean \pm SD, *** $p < 0.0001$ compared to the control).

Extracts (mg/ml)	Caspase-8 (RLUx10 ⁵)	Caspase-9 (RLUx10 ⁵)	Caspase-3/7 (RLUx10 ⁵)
Control	0.242 \pm 0.019	2.989 \pm 0.053	3.338 \pm 0.124
C	0.485 \pm 0.018 ***	6.649 \pm 0.075 ***	7.966 \pm 0.073 ***
C3	0.578 \pm 0.104 ***	7.501 \pm 0.498 ***	6.825 \pm 0.048 ***

At 72 h, C increased THP-1 caspase-8 (1.528-fold, $p < 0.0001$) and caspase-9 (1.667-fold $p < 0.0001$) whereas decreased caspase-3/7 activity (1.260-fold, $p = 0.0089$) compare to the controls (Table 4.2). Although initiator caspase (-8 and -9) activity was increased, the decreased executioner caspase-3/7 activity suggests a decrease in apoptotic cell death. In C3 treated THP-1 cells, caspase-8 (1.259-fold, $p < 0.0001$) was decreased while caspase -9 and -3/7 activities remained unchanged relative to the controls (Table 4.2, $p < 0.009$).

Table 4.2: Modulation of caspase (-8, -9, -3/7) activities in LPS stimulated and 72 h *C. asiatica* (C and C3) treated THP-1 cells (Values expressed as mean \pm SD, ** $p < 0.005$, *** $p < 0.0001$ compared to the control).

Extracts (mg/ml)	Caspase-8 (RLUx10 ⁵)	Caspase-9 (RLUx10 ⁵)	Caspase-3/7 (RLUx10 ⁵)
Control	0.316 \pm 0.017	4.425 \pm 0.019	4.641 \pm 0.213
C	0.483 \pm 0.013 ***	7.378 \pm 0.914 ***	3.683 \pm 0.275 **
C3	0.251 \pm 0.010 ***	4.152 \pm 0.029	4.027 \pm 0.466

4.3.7 NF- κ B (p50, p65) protein expression in *C. asiatica* (C and C3) treated THP-1 cells

At 24 h, THP-1 p50 protein expression was decreased by C (1.715-fold) and C3 (1.688-fold) as compared to the control (Figure 4.5A, $p < 0.0001$). Also, THP-1 p65 protein expression was decreased by C (1.559-fold) and C3 (1.674-fold) relative to the control (Figure 4.5B, $p < 0.0001$).

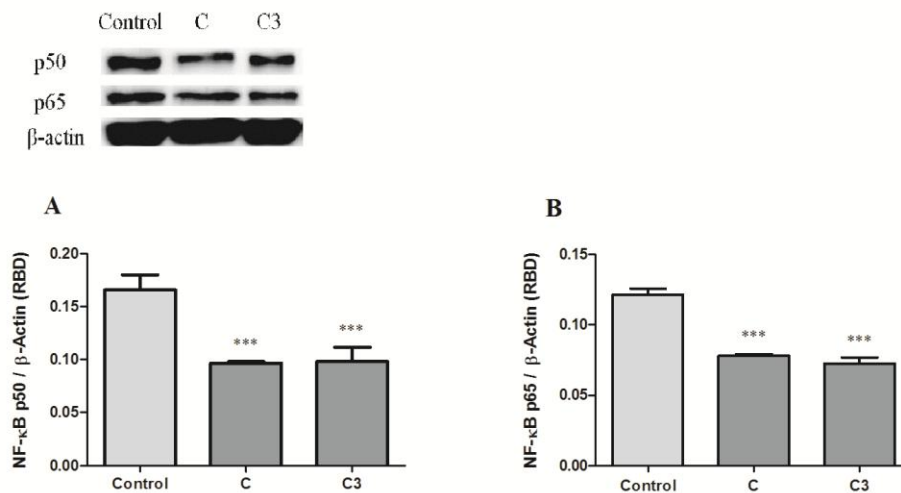


Figure 4.5: Protein expression of p50 (A) and p65 (B) in LPS stimulated and 24 h *C. asiatica* (C and C3) treated THP-1 cells (Values expressed as mean \pm SD, *** $p < 0.0001$ compared to the control).

Similarly at 72 h, THP-1 p50 protein expression was decreased by C (1.501-fold) and C3 (1.918-fold) compared to the control (Figure 4.6A, $p < 0.0001$). Also, THP-1 p65 protein expression was decreased by C (1.146-fold) and C3 (1.533-fold) relative to the control (Figure 4.6B, $p < 0.0001$). Notably, C3 decreased THP-1 p50 and p65 protein expressions to a greater extent than C.

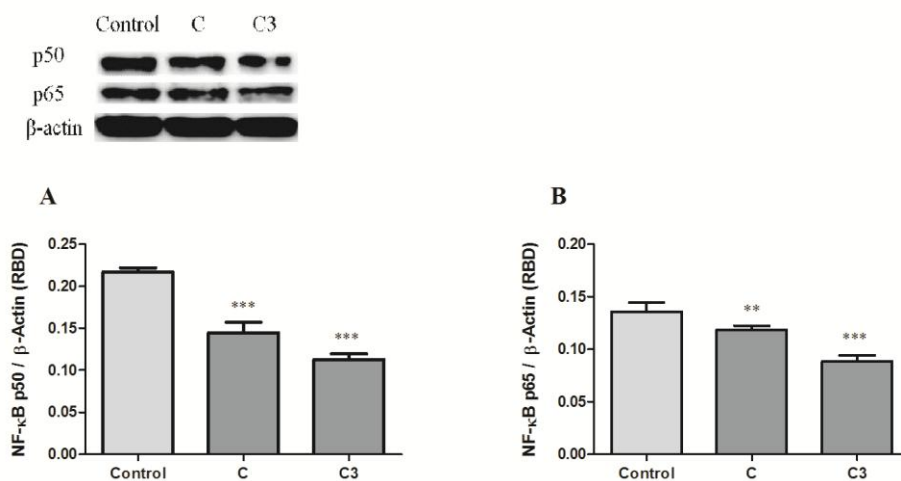


Figure 4.6: Protein expression of p50 (A) and p65 (B) in LPS stimulated and 72 h *C. asiatica* (C and C3) treated THP-1 cells (Values expressed as mean \pm SD, ** $p < 0.005$, *** $p < 0.0001$ compared to the control).

4.4 Discussion

The cancer cachectic syndrome leads to the abnormal loss of body weight as a result of adipose tissue and skeletal muscle degradation (Tisdale, 2009). Oxidative stress, pro-inflammatory cytokines and apoptosis play an important role in the initiation of tissue wasting, the imbalance of catabolic/ anabolic processes and the development of cancer cachexia (Sharma and Anker, 2002). Thus, this study investigated the anti-cancer and anti-cachectic potential of *C. asiatica* (C and C3) in THP-1 cells.

At 24 h, *C. asiatica* (C and C3) did not significantly alter PBMC viability whereas at 72 h C3 decreased PBMC viability. *C. asiatica* (C and C3) decreased THP-1 viability at 24 h whereas increased THP-1 viability at 72 h. Previous studies showed that *C. asiatica* extracts inhibited breast (MCF-7) and liver (HepG2) cancer cell proliferation (Babykutty et al., 2008; Hussin et al., 2014). Thus the results of the present study prove to be similar to previous studies.

C. asiatica has been reported to possess antioxidant potential possibly associated with phenolic compounds (Zainol et al., 2003). DPPH oxidant scavenging activity was significantly increased by C (78.20%) as compared to C3 (13.05%), indicating their antioxidant potential.

NO is produced by inducible nitric oxide synthase (iNOS) (Chung et al., 2001) and regulates many physiological processes such as immune responses and apoptosis (Muntané and De la Mata, 2010). During carcinogenesis, NO may cause genotoxic lesions, promote angiogenesis, tumour cell growth and invasion (Muntané and De la Mata, 2010). Our results indicated that *C. asiatica* (C and C3) decreased THP-1 nitrite levels which may decrease the carcinogenic process. Interestingly, C3 decreased nitrite levels to a greater extent than C.

Cancer-related inflammation has been considered the seventh hallmark of cancer (Colotta et al., 2009) due to the role of inflammation in tumour initiation, promotion, invasion, and metastasis (Grivennikov et al., 2009). During cancer and inflammation, IL-1 suppresses LPL activity (Beutler and Cerami, 1985), increases protein turnover, leads to muscle proteolysis and plays a role in the development of cachexia (Tocci and Schmidt, 1997; Tocco-Bradley et al., 1987). TNF- α has been shown to inhibit LPL production and reduce LPL gene transcription (Cornelius et al., 1988; Fried and Zechner, 1989; Zechner et al., 1988) thus preventing the formation of new lipid stores while stimulating HSL and increasing lipolysis (Elborn et al., 1993). An increase in IL-6 levels are associated with elevated tumour size, weight loss (Kuroda et al., 2007), decreased LPL activity (Greenberg et al., 1992) and the development of cachexia (Tisdale, 2009). Additionally, IL-6 and TNF- α stimulates muscle catabolism by activating proteasome pathways (Garcia-Martinez et al., 1993; Garcia-Martinez et al., 1995; Llovera et al., 1997; Tisdale, 2001).

In THP-1 cells, both C and C3 significantly decreased TNF- α , IL-6 and IL-1 β concentrations. The increased oxidant scavenging activity of C and C3 may have contributed to decreasing pro-inflammatory cytokine levels. Notably, TNF- α , IL-1 β and IL-6 concentrations were decreased to greater extent by C3 than C. The potential of C and C3 to down regulate pro-inflammatory cytokine production in THP-1 cells may decrease pro-inflammatory cytokine mediated LPL inhibition and proteasome pathway activation thus preventing excessive lipolysis and proteolysis.

In cancer patients, pro-inflammatory cytokines are elevated which increases the probability of successful cancer cell metastasis (Nguyen et al., 2009). Previous studies have shown that TNF- α and IL-6 can promote metastasis and survival of metastatic seeds (Nguyen et al., 2009). Additionally, IL-1 β contributes to increased tumour invasiveness and angiogenesis in chemical carcinogen-induced tumours (Krelin et al., 2007). Thus, the ability of C and C3 to decrease pro-inflammatory cytokine levels may decrease tumour cell growth and metastasis, ultimately preventing rapid tumour progression.

In adenocarcinoma colon 26-bearing mice, elevated concentrations of the anti-inflammatory cytokine, IL-10 has been reported to reverse the cachectic syndrome (Fujiki et al., 1997). Additionally, IL-10 inhibits NF- κ B activation and subsequently inhibits pro-inflammatory cytokine production (TNF- α and IL-6) (Lin and Karin, 2007). Both C and C3 increased IL-10 concentration in THP-1 cells. The ability of C and C3 to increase IL-10 concentration may decrease pro-inflammatory cytokine levels, malignant cell progression and alleviate cancer cachectic syndrome.

There are two main apoptotic pathways namely the extrinsic and intrinsic pathway. The intrinsic apoptotic pathway is mediated through the mitochondria, stimulus such as free radicals leads to the opening of mitochondrial permeability transition pores, loss of transmembrane potential and release of pro-apoptotic proteins (Saelens et al., 2004). The apoptosome is formed by cytochrome c binding Apaf-1 and pro-caspase-9 (Chinnaiyan, 1999; Hill et al., 2004). The extrinsic pathway is mediated through death receptors resulting in the auto-catalytic activation of procaspase-8 (Elmore, 2007). Activation of initiator caspases (-8, -9) leads to the activation of executioner caspases (-3/7) resulting in activation of cytoplasmic endonucleases which degrade nuclear material (Elmore, 2007). Previously in breast cancer cells, *C. asiatica* induced apoptosis through loss of mitochondrial membrane potential, increased annexin staining and DNA fragmentation (Babykutty et al., 2008). At 24 h, THP-1 caspase (-8, -9, -3/7) activity was increased by C and C3, indicating an increased execution of THP-1 apoptosis. The 24 h result demonstrates the anti-proliferative potential of *C. asiatica* (C and C3) in THP-1 cells. At 72 h, THP-1 caspase-8 and caspase-9 activity was increased however caspase-3/7 activity was decreased by C, suggesting a decreased in the execution of apoptosis. In C3 treated THP-1 cells (72 h), caspase-8 activity was decreased while caspase -9 and -3/7 activities were not altered, suggesting a decreased activation of apoptosis.

Depending on NO levels, NO may promote or inhibit apoptotic pathways (Chung et al., 2001; Muntané and De la Mata, 2010). Usually, low NO levels have protective effects while high NO levels led to apoptosis (Taylor et al., 2003). Caspases are essential in the initiation and execution of apoptosis (Chung et al., 2001). The cysteine (a thiol) at the caspase catalytic site is susceptible to redox modification (Chung et al., 2001). NO effectively modifies caspases by *S*-nitrosylation which decreases caspase activity, ultimately decreasing apoptosis (Chung et al., 2001). However, the level of other thiol containing compounds (such as glutathione) influences the capacity of NO to *S*-nitrosylate caspases (Chung et al., 2001; Taylor et al., 2003). In cells with normal thiol levels, NO induces apoptosis (Taylor et al., 2003). On the hand, in cells with low thiol levels, NO protects against apoptosis by *S*-nitrosylation of caspases (Taylor et al., 2003). Taken together, *C. asiatica* (C and C3) showed increased oxidant scavenging activity and decreased THP-1 nitrite levels which may have decreased NO capacity to *S*-nitrosylate caspases. This may have contributed to the increased caspase activity at 24 h. During the 72 h *C. asiatica* (C and C3) treatment, the level of thiol containing compounds may have decreased, allowing for NO to *S*-nitrosylate caspases, ultimately decreasing caspase activation.

Many stimuli activate NF- κ B pathways, such as TNF- α , IL-1 β , LPS and reactive oxygen species (ROS) (Fan et al., 2013). Production of cytokines and chemokines leads to a localized inflammatory response which also enhances premalignant cell survival through NF- κ B activation (Lin and Karin, 2007). In various cancer types, activation of the NF- κ B pathway is an essential survival mechanism (Wang et al., 2009). Elevated NF- κ B activity may increase certain tumourigenic adhesion proteins, chemokines and inhibitors of apoptosis which promote cell survival (Wang et al., 2009). Continuous NF- κ B activation up-regulates inflammatory cytokines (TNF α , IL-6, IL-1, IL-8) which are potent activators of NF- κ B thus creating a positive feedback loop (Fan et al., 2013). Additionally, in the tumour microenvironment, cytokines and NF- κ B induces iNOS expression which leads to NO production (Rajput and Wilber, 2010). At 24 and 72 h, THP-1 p50 and p65 protein expression were decreased by both C and C3. Notably, in C and C3 treated THP-1 cells, the decreased p50 and p65 protein expressions are consistent with the decreased nitrite levels and pro-inflammatory cytokine concentrations.

As mentioned earlier, elevated levels of pro-inflammatory cytokines increase catabolic pathways leading to tissue wasting (Martignoni et al., 2005). Additionally, NF- κ B directly stimulates the ubiquitin pathway which increases skeletal muscle breakdown (Russell et al., 2009). Thus, the

potential of C and C3 to diminish NF- κ B protein expressions and pro-inflammatory cytokine levels may decrease lipolysis, proteolysis and the progression of cancer cachexia.

Anti-apoptotic proteins work together to block or regulate apoptosis by targeting the various steps in the apoptotic cascade (Lin and Karin, 2003). Activation of the NF- κ B pathway inhibits apoptosis by inducing the expression of anti-apoptotic genes (Lin and Karin, 2003). Thus, inhibiting the NF- κ B pathway may decrease anti-apoptotic protein expression and allow for a pro-apoptotic shift. As seen earlier, the execution of THP-1 apoptosis was increased by C and C3 at 24 h, a possible consequence of the decreased THP-1 p50 and p65 protein expressions. However, at 72 h the decreased THP-1 p50 and p65 protein expressions did not influence THP-1 caspase activity.

In tumour cells, LPS induction of the metastatic growth response has been shown to be dependent on NF- κ B activation (Yoshimura, 2006). Previously, in tumour-associated inflammatory cells, NF- κ B activation enhanced pro-inflammatory cytokine synthesis which promotes tumour growth (Viatour et al., 2005). Additionally, NF- κ B inhibition has been shown to sensitize prostate cancer cells to apoptosis (Shukla and Gupta, 2004), decrease invasion, inhibit tumour growth, inhibit angiogenesis in metastatic cells (Huang et al., 2001) and promote tumour regression in carcinoma cells (Yoshimura, 2006). Thus, the ability of C and C3 to decrease the NF- κ B (p50, p65) protein expressions may greatly attenuate the carcinogenic process (Viatour et al., 2005).

4.5 Conclusion

Cachexia is a progressive and devastating syndrome that greatly diminishes the survival rate of cancer patients. In the present study, C and C3 have been shown to decreased NF- κ B (p50, p65) protein expressions, decrease pro-inflammatory cytokine (TNF- α , IL-6 and IL-1 β) levels and modulate THP-1 cell death. These results suggest that C and C3 may prove to be effective in combating the cancer cachectic syndrome, improving the quality of life and prolonging the survival time of cancer patients.

4.6 Conflict of interests

The author(s) declare that they have no competing interests.

4.7 Acknowledgements

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4.8 Role of funding source

The National Research Foundation and the South African Medical Research Council were not involved in study design, collection of samples, analysis of data, interpretation of data, writing of the report and decision to publish. Scientific out-put is a requirement of the National Research Foundation.

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Bridging paragraph:

In the previous manuscript, C3 proved to be more effective than C in decreasing THP-1 pro-inflammatory (TNF- α , IL-6, IL-1 β) cytokine levels while increasing the THP-1 anti-inflammatory (IL-10) cytokine levels, suggesting that C3 may be more effective in decreasing the development and progression of cancer cachexia. Additionally, C and C3 (24 h) were shown to increase caspase activity in THP-1 cells, suggesting an increased initiation and execution of apoptotic cell death. Therefore we investigated the antioxidant and anti-proliferative effects of C and C3 in THP-1 cells.

The following manuscript is entitled *Centella asiatica* modulates Nrf-2 antioxidant mechanisms and enhances ROS mediated apoptosis in leukaemic THP-1 cells (Supplementary information: Appendix 8). In this manuscript, an IC₅₀ for C and C3 (24 h) in THP-1 cells was determined then the antioxidant response signalling and apoptosis induction were evaluated.

Publication statuses: Submitted to Phytomedicine however the journal didn't provide a manuscript number in 4 weeks

The manuscript has been published in proceedings of the 2nd International Conference on Herbal and Traditional Medicine 2017 (HTM 2017), "Value-Added of Herbs and Phytotherapy: Challenges for the 21st Century". (T5 – O – ST – 014)

Please note: The manuscript was formatted for Phytomedicine, however, the margins, font, line spacing, numbering of sections and figures were adjusted for thesis consistency.

CHAPTER FIVE

***Centella asiatica* modulates Nrf-2 antioxidant mechanisms and enhances ROS mediated apoptosis in leukaemic THP-1 cells**

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Abstract

Background: *Centella asiatica* (*C. asiatica*) is commonly used in traditional medicine due to its many therapeutic properties. The effects of *C. asiatica* on leukaemic THP-1 cells have yet to be examined.

Hypothesis/ Purpose: We investigated the antioxidant and anti-proliferative effects of an ethanolic *C. asiatica* leaf extract (C) and purified fraction-3 (C3) in THP-1 cells.

Methods: C3 was obtained by silica column fractionation and identified using thin layer chromatography. Gas chromatography mass spectrometry determined the bioactive compounds present in C and C3. Cytotoxicity of C and C3 in THP-1 cells was evaluated (cell viability assay; 24 hours; [0.2–3 mg/ml]) to determine a half maximal inhibitory concentration (IC₅₀). Intracellular reactive oxygen species (IROS), mitochondrial membrane potential (flow cytometry), malondialdehyde (MDA) (spectrophotometry), reduced glutathione (GSH), oxidized GSH (GSSG), adenosine triphosphate (ATP) levels, caspase (-8, -9, -3/7) activity (luminometry) and DNA damage (comet assay) were evaluated. Protein expression [nuclear factor erythroid 2-related factor 2 (Nrf-2), glutathione peroxidase (GPx), B cell lymphoma-2 (Bcl-2) and cleaved poly(ADP-ribose) polymerase-1 (c-PARP)] and gene expression [*Nrf-2*, *GPx*, superoxide dismutase (*SOD*), catalase (*CAT*), *c-myc* and 8-oxoguanine glycosylase (*OGG-1*)] were quantified by western blotting and quantitative polymerase chain reaction, respectively.

Results: *C. asiatica* (C and C3) dose dependently decreased THP-1 cell viability and the IC₅₀ of C and C3 increased MDA, IROS and mitochondrial depolarisation ($p < 0.01$). Expression of anti-oxidant response genes ($p < 0.0001$), Nrf-2 protein expression and GSSG levels ($p < 0.01$) were increased by C but decreased by C3. Caspase activity and DNA damage were elevated by both C (-9, -3/7) and C3 (-8, -9, -3/7) ($p < 0.0001$). *C. asiatica* (C and C3) decreased GPx and Bcl-2 protein expressions ($p < 0.003$). Levels of c-PARP and *c-myc* were decreased by C whilst increased by C3 ($p < 0.002$). Only C3 reduced *OGG-1* gene expression ($p < 0.0003$)

Conclusion: Antioxidant responses were increased by C whilst decreased by C3. Both C and C3 exerted anti-proliferative effects in THP-1 cells by enhancing apoptosis. Notably, C3 more effectively induced apoptosis.

Key words: *Centella asiatica*, oxidative stress, antioxidants, apoptosis

¹ **Abbreviations:** (ROS), Reactive oxygen species; (Nrf-2), Nuclear factor erythroid 2-related factor 2; (KEAP1), Kelch-like ECH-associated protein 1; (GSH), Glutathione; (GSSG), oxidised glutathione; (GPx), Glutathione peroxidase; (SOD), Superoxide dismutase; (CAT), Catalase; (PARP-1), Poly(ADP-ribose)polymerase-1; (SA), South Africa; (DCM), Dichloromethane; (HEX), Hexane; (EA), Ethyl acetate; (DMSO), Dimethyl sulfoxide; (TLC), Thin layer chromatography; (FCS), Foetal calf serum; (PS), Penstrepfungizone; (L-GLUT), L-glutamine; (LPS), Lipopolysaccharide; (WST-1), 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; (JC-1), 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolcarbocyanine iodide; (H2DCF-DA), 2', 7'-dichlorodihydrofluorescein diacetate; (ATP), Adenosine triphosphate; (GC-MS), Gas chromatography mass spectrometry; (C), *Centella asiatica* crude leaf extract; (C3), *Centella asiatica* purified fraction-3; (h), Hours; (IC₅₀), Half maximal inhibitory concentration; (C_{ct}), Control for *Centella asiatica* crude leaf extract; (C_{ct3}), Control for *Centella asiatica* purified fraction-3; (DCF), 2, 7-dichlorofluorescein; (TBARS), Thiobarbituric acid assay; (MDA), Malondialdehyde; (IROS), Intracellular reactive oxygen species; (min), Minutes; (PBS), Phosphate buffer saline; (RT), Room temperature; ($\Delta\psi_m$), Mitochondrial depolarisation; (RLU), Relative light units; (SCGE), Single cell gel electrophoresis; (CTL's), Comet tail lengths, (RBD), Relative band density; (OGG-1), 8-oxoguanine DNA glycosylase; (AML), Acute myeloid leukaemia; (MCF-7), Breast cancer cells; (ETC), Electron transport chain, (8-oxoG), 8-Oxo-7,8-dihydroguanine.

5.1 Introduction

Cancer is a leading cause of morbidity and mortality worldwide. Annually there are about 10 million newly diagnosed cancer cases and greater than 6 million cancer deaths (Tariq et al., 2015). Characteristically, cancer is a hyper-proliferative disorder involving elevated anaerobic glycolysis, oxidative stress, mutations, evasion of apoptosis, invasion, angiogenesis and metastasis (Gorrini et al., 2013; Lin and Karin, 2007). Ongoing anti-cancer research is essential to combat the rapid progression of cancer and the increasing number of cancer deaths.

Reactive oxygen species (ROS) is involved in many biological processes and the modulation of ROS levels is important in maintaining cellular homeostasis (Gorrini et al., 2013). Acute low ROS levels is involved in cell proliferation, differentiation, and activation of survival pathways whereas acute high ROS levels cause damage to cellular components and induces apoptosis (Gorrini et al., 2013; Lau et al., 2008). In healthy cells, an efficient antioxidant defence system is essential for the detoxification of elevated ROS levels (Perricone et al., 2009). However, cancer cells exploit antioxidant mechanisms such as the nuclear factor erythroid 2-related factor 2 (Nrf-2) pathway to increase tumour cell survival, proliferation, and resistance to anti-cancer therapies (Bauer et al., 2013).

During normal conditions, Nrf-2 is bound to Kelch-like ECH-associated protein 1 (KEAP1) in the cytosol and marked for degradation (Kansanen et al., 2013). Elevated ROS levels lead to KEAP1 oxidation and modification which allows for Nrf-2 release and translocation to the nucleus where it increases antioxidant gene transcription (Kansanen et al., 2013). Nrf-2 stimulates the transcriptional activation of various cytoprotective and detoxification genes rapidly changing the sensitivity of the cells to ROS (Rushworth et al., 2012). Antioxidants such as glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) protects against oxidative cellular damage (Perricone et al., 2009; Weydert and Cullen, 2010). SOD detoxifies superoxide radicals into hydrogen peroxide and molecular oxygen, thereafter hydrogen peroxide is further detoxified by GPx and CAT into oxygen and water (Weydert and Cullen, 2010). The imbalance between ROS generation and elimination may lead to ROS accumulation which results in oxidative stress and apoptosis (Gorrini et al., 2013).

Apoptosis occurs through two main apoptotic pathways namely the intrinsic (or mitochondrial) and extrinsic (or death receptor) (Wong, 2011). The extrinsic pathway leads to caspase-8 activation (Wong, 2011) whilst the intrinsic pathway leads to apoptosome formation (cytochrome c, Apaf-1 and procaspase-9) and caspase-9 activation (Wong, 2011). Activation of initiator caspases (-8, -9) lead to activation of executioner caspases (-3/7) ultimately activating cytoplasmic endonucleases which degrade nuclear material (Wong, 2011). Nuclear enzyme, poly (ADP-ribose) polymerase-1 (PARP-1) functions in DNA repair, DNA stability, and transcriptional regulation (Bouchard et al., 2003). Depending on the amount of DNA damage, PARP-1 may activate DNA repair or apoptosis (Bouchard et al., 2003). During apoptosis, caspases cleave PARP-1 leading to the formation of the

89 and 24 kDa polypeptides which abolishes PARP-1 DNA repair activity and promotes apoptosis (Bouchard et al., 2003).

Anti-cancer research on medicinal plants is increasing. *Centella asiatica* is a tropical medicinal plant native to India, China, and South Africa (Orhan, 2012). It belongs to the Apiaceae family and is commonly referred to as Gotu kola, Asiatic/ Indian pennywort and Tiger herb (Orhan, 2012). *C. asiatica* is widely used in Ayurvedic and Chinese traditional medicines due to its vast health benefits such as antioxidant, anti-inflammatory and anti-tumour potential (Orhan, 2012). Major active compounds in *C. asiatica* are triterpene saponosides such as asiatic acid, madecassic acid, asiaticoside and madecassoside (Orhan, 2012). In addition, *C. asiatica* contains flavonoid derivatives, vitamins, minerals, polysaccharides, sterols, and phenolic acids (Orhan, 2012). *C. asiatica* possess high antioxidant activity, phenol and flavonoid content (Dasgupta and De, 2007; Odhav et al., 2007). Also a methanolic extract of *C. asiatica* showed anti-proliferative activity in many cancer cell lines (Babykutty et al., 2008).

There is limited scientific information on the effects of *C. asiatica* in leukaemic cells. We investigated the antioxidant response and ROS manipulation associated with leukaemic cells. This study investigated the effects of an ethanolic *C. asiatica* (crude (C) and fraction-3 (C3)) leaf extract in THP-1 cells and its associated antioxidant response signalling and apoptosis induction in these leukaemic cells.

5.2 Materials and Methods

5.2.1 Materials

C. asiatica leaves were collected in March 2011 (collectors number: Immelman 411) from the Eastern Cape [Langeni forest, roadside (S31°28.135', E28°32.681')], South Africa (SA) and identified by Dr. Kathleen Immelman (Department of Botany at the Walter Sisulu University, SA). Voucher specimens were deposited at the KEI herbarium (13979). THP-1 cells were obtained from ATCC (University Boulevard Manassas, USA). Solvents [Dichloromethane (DCM), Hexane (HEX), ethyl acetate (EA), methanol, Dimethyl sulphoxide (DMSO)] sulphuric acid, silica gel 60, thin layer chromatography (TLC) silica gel 60 sheets and 4-methoxybenzaldehyde were purchased from Merck (Johannesburg, SA). The RPMI-1640 was purchased from The Scientific group

(Johannesburg, SA). Foetal calf serum (FCS), penstrepfungizone (PS) and L-glutamine (L-Glut) were acquired from Whitehead scientific (Cape Town, SA). Lipopolysaccharide (LPS) was purchased from Sigma (Aston Manor, SA). The 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) cell proliferation reagent was purchased from Roche (Mannheim, Germany or Johannesburg, SA). The 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) kit and the 2', 7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) reagent were purchased from BD Biosciences (Johannesburg, SA). Caspase (-8, -9, -3/7), adenosine triphosphate (ATP) and GSH kits were acquired from Promega (Madison, USA). Western blot reagents were purchased from Bio-Rad (Johannesburg, SA) and qPCR primers were obtained from Inqaba Biotechnologies (Johannesburg, SA).

5.2.2 Plant Extraction and Fractionation

The plants official name is *Centella asiatica* (L.) Urb and has been confirmed by using the plant list (www.theplantlist.org). The local names are icudwane (Zulu) and varkoortjies (Afrikaans). The English names are tiger herb and pennywort. *C. asiatica* leaves were dried and milled. Ethanol (200–350 ml) was added to milled plant material (10–30 g) and extracted overnight by shaking (4 x g, 37 °C). Ethanol extracts were filtered, rotor evaporated, dried (37 °C) and stored (4 °C). Silica column fractionation was used to separate *C. asiatica* compounds based on their polarity. A column (height: 85 cm, diameter: 17 cm) was assembled by adding a tap (25 mm), a cotton wool stop, a thin layer of sand, carefully pouring silica mixed with DCM: HEX (50: 50) into a column and allowing the silica to set overnight. *C. asiatica* crude extract (17 g) was dissolved in DCM: HEX (70: 30) and a portion of the extract was further dissolved in methanol. Thereafter, *C. asiatica* crude extract was layered onto the silica and 50 ml fractions were collected using a series of solvent systems [DCM: HEX (70: 30), DCM: EA (70: 30), DCM: EA (50: 50), DCM: EA: methanol (50: 40: 10), DCM: EA: methanol (30: 50: 20), EA: methanol (70: 30), EA: methanol (40: 60), EA: methanol (20: 80), methanol (100)]. The 50 ml fractions were boiled (100 °C) to allow evaporation of excess solvent and TLC was used to determine spot patterns and solvent changes. A total of 766 fractions were collected and TLC analysis revealed fractions with similar spot patterns which were combined and pooled into one fraction. Fractionation of *C. asiatica* yielded 13 pooled fractions. For all subsequent experiments, *C. asiatica* (C and C3) was used.

5.2.3 Gas chromatography mass spectrometry (GC-MS)

C. asiatica (C and C3) chemical composition has been subjected to GC-MS (Hewlett-Packard, USA) and the GCD-HP1800A system was used. Ionization energy (70 eV) was used for the detection of GC-MS and the detection was passed under high vacuum (10⁻⁴ to 10⁻⁸ torr). Helium gas was used for the analysis at a constant flow rate (1 mL/min). The mass transfer line and injector temperature were fixed at 280 and 250 °C. The bioactive compounds present in *C. asiatica* (C and C3) were matched with the NIST computer library and reported.

5.2.4 Tissue Culture

THP-1 cells were grown in the appropriate tissue culture conditions in a 75 cm³ culture flask (37 °C, 5 % CO₂). The growth media comprised of RPMI-1640, FCS (10 %), L-Glut (1 %), PS (1 %) and 2-mercaptoethanol (0.05 mM). Cells were seeded at a concentration of 3 x 10⁵ cells/ml, quantified daily by trypan blue staining and were used for subsequent experiments once the cell density reached 8 x 10⁵ cells/ml.

5.2.5 Cell Viability Assay

Cytotoxicity of C and C3 to THP-1 cells was measured using the WST-1 assay (Roche, Johannesburg, SA). THP-1 cells (20,000 cells/well, 96 well plate, triplicate wells) were stimulated with LPS (20 µg/ml, 37 °C, 5 % CO₂, 4 hours (h)) before exposure to C and C3 (0.2–3 mg/ml) for 24 h (37 °C, 5 % CO₂). Controls received media containing DMSO (0.75 %). Thereafter plates were centrifuged (162 x g, 10 minutes (min)), supernatant removed, cell pellets re-suspended in growth media (100 µl/well), WST-1 reagent (10 µl/well) added and plates incubated (37 °C, 5 %, CO₂, 3 h). Optical density was measured at 450 nm (620 nm reference wavelength) with a BIO-TEK µQuant spectrophotometer (Analytical and Diagnostic Products, SA). This experiment was conducted on two separate occasions before using GraphPad Prism to calculate the half maximal inhibitory concentration (IC₅₀) [percentage cell viability vs. log concentration of *C. asiatica* (C and C3)]. Cells were treated with the IC₅₀ (C = 1.4 mg/ml and C3 = 0.618 mg/ml) in all subsequent experiments. Control (C_{ct} = control for C, C_{ct3} = control for C3) treatments were represented by an equal amount of DMSO (C_{ct} = 0.745 %, C_{ct3} = 0.309 %) in growth media without *C. asiatica* (C and C3).

5.2.6 Stimulation and treatment of cells

THP-1 cells (2×10^5 cells/ml) were transferred into 75 cm³ culture flasks, stimulated with LPS (20 µg/ml, 37 °C, 5 % CO₂, 4 h) before exposure to C (1.4 mg/ml) and C3 (0.618 mg/ml) for 24 h (37°C, 5% CO₂). Thereafter, media containing cells were transferred into 50 ml tubes and centrifuged (162 x g, 10 min). Cell pellets were used to conduct the JC-1, 20, 70-dichlorofluorescein (DCF), GSH, GSSG, caspase (-8, -9, -3/7) activity, ATP and comet assays; as well as protein and RNA isolation. The experiments were conducted independently two times for all subsequent assays.

5.2.7 Thiobarbituric acid assay

The thiobarbituric acid assay (TBARS) measures malondialdehyde (MDA), the end product of lipid peroxidation and was conducted as per the method previously described by Phulukdaree *et al* (2010) (Phulukdaree et al., 2010). Optical density was measured at 532 nm (600 nm reference wavelength) with a BIO-TEK µQuant spectrophotometer (Analytical and Diagnostic Products, SA). Malondialdehyde concentration was calculated by dividing the optical density for each sample by the absorption coefficient (156 mM⁻¹).

5.2.8 DCF assay

The DCF assay measures intracellular ROS (IROS) and was conducted as per the method previously described by Nagiah *et al* (2015) (Nagiah et al., 2015). Events were gated and the fluorescence measured on FL-1 channel (525 nm) of the AccuriTMC6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). A total of 50,000 events were acquired and analyzed using the CFlow Plus Software (BD Biosciences, San Jose, CA, USA).

5.2.9 GSH assay

The GSH-GloTM assay (Promega, Madison, WI, USA) was used to measure GSH and GSSG levels. The assay was conducted as per the method previously described by Nagiah *et al* (2015) (Nagiah et al., 2015). Luminescence was measured on a ModulusTM microplate luminometer (Turner BioSystems, Sunnyvale, CA, USA).

5.2.10 JC-1 assay

The JC-1 assay measures percentage mitochondrial depolarisation ($\Delta\psi_m$) and was conducted as per the method previously described by Nagiah *et al* (2015) (Nagiah et al., 2015). Scatter plots of 50,000 events per sample were acquired using the AccuriTMC6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using the CFlow Plus Software (BD Biosciences, San Jose, CA, USA).

5.2.11 Caspase and ATP assays

Caspase activities and ATP levels were determined using the Caspase-Glo[®]3/7, -8, -9 and ATP assay kits (Promega, Madison, WI, USA). Reagents were reconstituted according to the manufacturer's instructions. Cells (50 μ l/well, 2×10^5 cells/ml) were added into triplicate wells of a microtitre plate for each assay, thereafter caspase -3/7, -8, -9 and ATP reagents (25 μ l/well) were added into appropriate wells. Plates were incubated (30 min, RT) in the dark. Luminescence was measured on a ModulusTM microplate luminometer (Turner BioSystems, Sunnyvale, CA, USA) and expressed as relative light units (RLU).

5.2.12 Single cell gel electrophoresis (SCGE) or Comet assay

The SCGE assay was performed to assess DNA fragmentation and was conducted as per the method previously described by Singh *et al* (1988) (Singh et al., 1988). Cells were stained with GR red (1 μ l, 1000x) instead of ethidium bromide. Slides were viewed using an Olympus IX7I microscope with 510–560 nm excitation and 590 nm emission filters (Wirsam Scientific, Johannesburg, SA). Images from triplicate slides per treatment were captured. Comet tail lengths (CTL's) of 50 comets per treatment were measured using the Soft imaging system (Life Science - ©Olympus Soft Imaging Solutions v5) and average CTL's were calculated (μ m).

5.2.13 Western Blotting

Western Blotting was performed to determine relative protein expressions. Cytobuster (200 μ l, Novagen, San Diego, CA, USA) supplemented with protease and phosphatase inhibitors (Roche, Mannheim, Germany) was added to the cells (10 min, 4 °C) and centrifuged (13000 x g, 4 °C, 10 min). Protein was quantified using the bicinchoninic acid assay (Sigma, Germany) and standardised to 1 mg/ml. Western blotting was conducted as per the method previously described by Nagiah *et al*

(2015) (Nagiah et al., 2015). Membranes were immune-probed with Nrf-2 (ab89443), GPx (ab129911), Bcl-2 (CS2827), and c-PARP (CS9541) primary antibodies (1:1000). Anti-rabbit (sc-2054) or anti-mouse (ab97046) were the secondary antibodies used (1:5,000). Horse radish peroxidase chemiluminescence detector (Bio-Rad) and enhancer solution were used for the antigen-antibody complex and the signal was detected with the Alliance 2.7 image documentation system (UViTech). To correct for loading error and to normalise relative protein expression, β -actin was assessed (A3854; 1:5000, Sigma, St Louis, MO, USA). The expression of the proteins were analysed with UViBand Advanced Image Analysis software v12.14 (UViTech) and data was expressed as relative band density (RBD) and fold change.

5.2.14 Quantitative PCR

Total RNA was isolated using an in-house protocol (Chuturgoon et al., 2014). RNA was quantified (Nanodrop 2000, ThermoScientific, South Africa) and standardised (200 ng/ml). A reaction volume (10 μ l) containing RNA template (2 μ l), 5X iScript™ reaction mix (2 μ l), iScript reverse transcriptase (0.5 μ l) and nuclease free water (5.5 μ l) was used to synthesize cDNA (iScript™ cDNA Synthesis kit, BioRad, 107e8890). Thermocycler conditions were 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min and a final hold at 4 °C.

Gene expression of *SOD2* [Sense 5'-GAGATGTTACACGCCAGAG TAGC-3'; Antisense 5-AATCCCCAGCAGTGGGAATAAGG-3' (57 °C)], *CAT* [Sense 5'-TAAGACTGACCAGGGCATC-3'; Antisense 5'-CAACCTTGGTGAGATCGAA- 3' (58 °C)], *GPx* [Sense 5'-GACTACACCCAGATGAACGAGC- 3'; Antisense 5'-CCCACCAGGAA CTTCTCAAAG- 3' (58 °C)], *Nrf-2* (forward 5'-AGTGGATCTGCCAACTACTC-3'; reverse 5'-CATCTACAAACGGGAATGTCTG-3' (58 °C)), oxoguanine DNA glycosylase (*OGG-1*) (forward 5'-GCATCGTACTCTAGCCTCCAC-3'; reverse 5'-AGGACTTTGCTCCCTCCAC-3' (60 °C)) and *c-myc* (forward 5'-AGCGACTCTGAGGAGGAACAAG-3'; reverse 5'-GTGGCACCTCTTGAGGACCA-3' (56 °C)) were evaluated using the iQ™ SYBR® Green PCR kit (Bio-Rad; 170e880). The final reaction volume was 10.5 μ l [6.25 μ l 5 X iScript reaction mix, 0.5 μ l sense primer, 0.5 μ l anti-sense primer, 3.25 μ l nuclease-free water, and 2 μ l cDNA sample (triplicate wells per sample)] and all assays were carried out using CFX Touch™ Real Time PCR Detection System (Bio-Rad). The reaction included an initial denaturation (95 °C, 4 min), followed by 37 denaturation cycles (95 °C, 15 sec), annealing (primer specific temperature, 40 sec), extension (72 °C, 30 sec) and a plate read for 37 cycles. Under the same conditions, the *β -actin* [Sense 5'-

TGACGGGTCACCCACACTGTGCCCAT-3'; Antisense 5'-
CTAGAAGCATTGCGGTGGACGATGGAGGG-3'] and 18S: (Sense: 5'-
ACAGGGACAGGATTGACAGA-3'; Antisense: 5'-CAAATCGCTCCACCAACCTAA-3')
housekeeping genes were run. Using the method described by Livak and Schmittgen (2001), qPCR
results were analyzed, represented as fold change ($2^{-\Delta\Delta CT}$) relative to the house keeping genes (β -
actin and 18S) and control (Livak and Schmittgen, 2001).

5.2.15 Statistical Analysis

Statistical analysis was performed using the GraphPad Prism (v5) statistical analysis software. Statistical significance was set at 0.05 and comparisons were made using unpaired t tests with Welch correction.

5.3 Results

5.3.1 Chemical composition of *C. asiatica* (C and C3)

The GC-MS performance of C and C3 was recorded (Supplementary information: Figure 4 and 5). Chemical composition of C and C3 constituents were identified after comparison with those available in the NIST computer library. The supplementary information illustrates the organic compounds that were identified in *C. asiatica* leaf extract namely caranol, oxymetholone methanol adduct, vitamin E, tocopherol and terpenes (phytosterol and stigmaterol) (Supplementary information: Table 1 and 2).

5.3.2 Cytotoxicity of C and C3 in THP-1 cells

The WST-1 assay showed that both C and C3 decreased THP-1 viability with IC₅₀ values of 1.4 mg/ml and 0.618 mg/ml respectively (Figure 5.1). These IC₅₀ concentrations were used in all subsequent experiments.

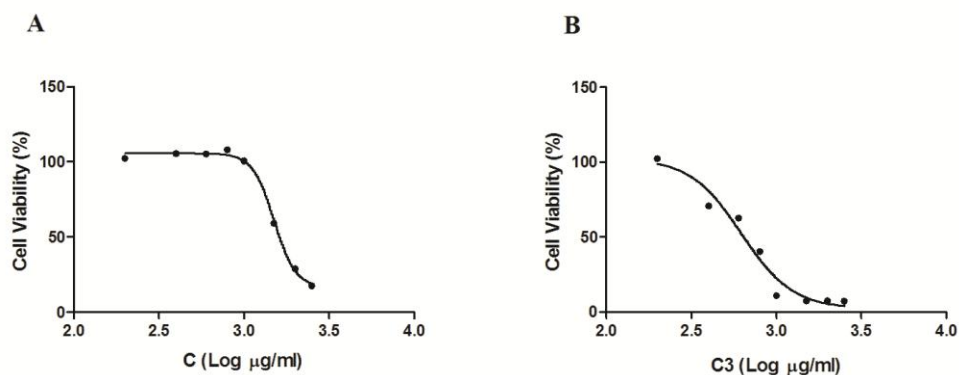


Figure 5.1: *C. asiatica* crude extract [0.2 – 3 mg/ml (A)] and C3 [0.2 – 3 mg/ml (B)] induced a dose dependent decrease in THP-1 cell viability (n = 6); IC₅₀ values of 1.4 mg/ml (C) and 0.618 mg/ml (C3).

5.3.3 The effect of C and C3 on oxidative stress and antioxidant mechanisms in THP-1 cells

The effect of C and C3 on lipid peroxidation and IROS production were assessed by the TBARS and DCF assays. MDA was significantly increased by C (2.621-fold, $p < 0.0001$) as compared to C3 (1.108-fold, $p = 0.0045$) relative to their respective controls (Figure 5.2A). Interestingly, IROS was increased to a greater extent by C3 (3.482-fold, $p = 0.0003$) than C (1.415-fold, $p = 0.0175$) compared to their controls (Figure 5.2B). Further the overall antioxidant effects of C and C3 were determined by measuring GSH and GSSG concentrations, Nrf-2 and GPx protein expressions as well as *Nrf-2*, *GPx*, *SOD* and *CAT* gene expressions. Intracellular GSH was slightly increased by C ($p = 0.0049$) and C3 (Figure 5.2C, $p < 0.0001$), however GSSG concentration was increased by C (1.561-fold $p = 0.0095$) whereas decreased by C3 (2.883-fold, $p = 0.001$) compared to their controls (Figure 5.2D).

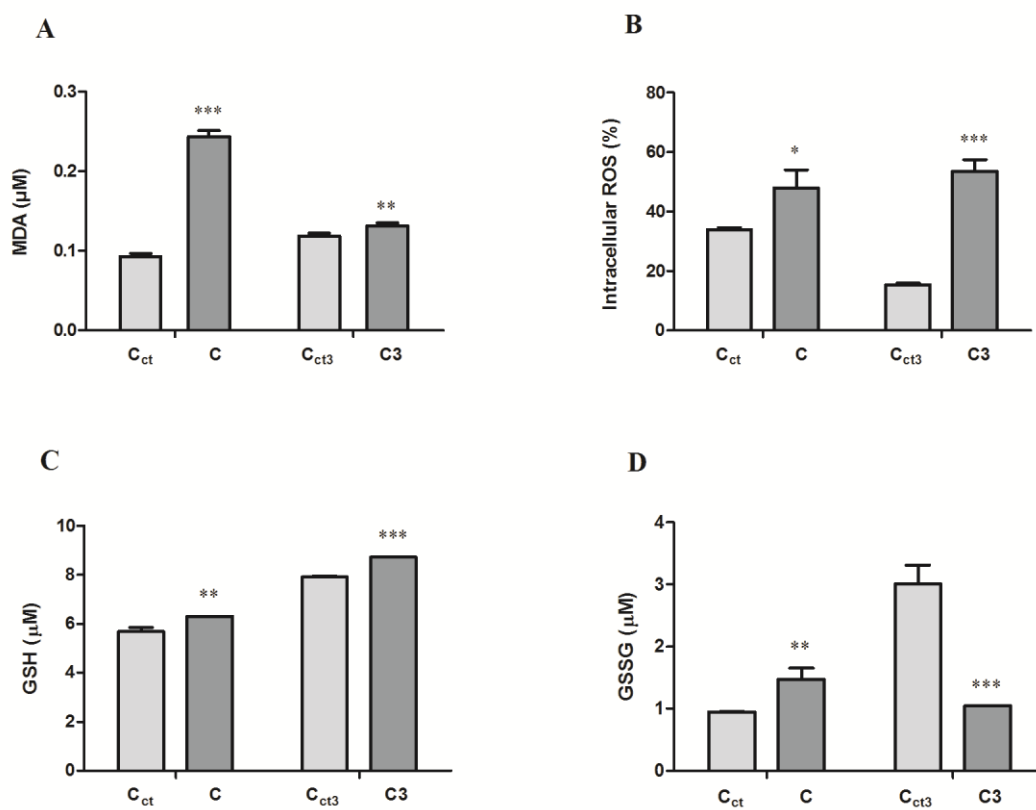


Figure 5.2: Levels of MDA (A), IROS (B), GSH (C) and GSSG (D) in LPS stimulated and *C. asiatica* (C and C3) treated THP-1 cells (n = 3), (Values expressed as mean ± SD, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$, compared to controls).

Interestingly, Nrf-2 protein expression was increased by C (1.503-fold, Figure 5.3A) whereas decreased by C3 (1.639-fold, Figure 5.3B) relative to the controls ($p < 0.0005$). Both C and C3 significantly decreased GPx protein expression. Notably, C3 (2.118-fold, Figure 5.3B, $p < 0.0001$) decreased GPx to a greater extent than C (1.281-fold, Figure 5.3A, $p = 0.0005$) compared to the controls.

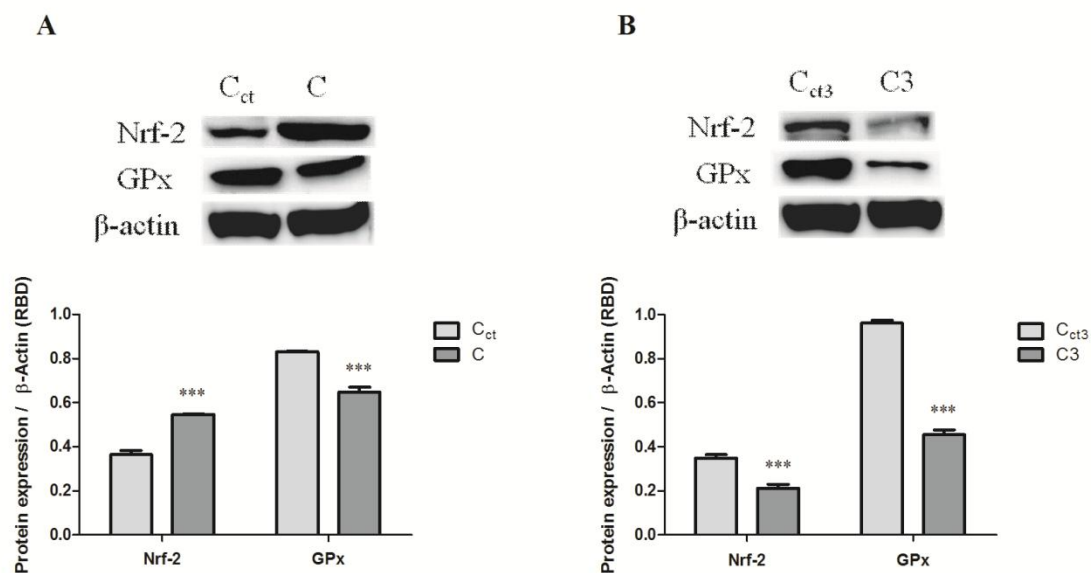


Figure 5.3: Protein expression of Nrf-2 and GPx in LPS stimulated C (A) and C3 (B) treated THP-1 cells (n = 3), (Values expressed as mean \pm SD, *** $p < 0.0001$, compared to controls).

The C extract significantly increased the antioxidant gene expression of *Nrf-2* (1.632-fold), *GPx* (6.249-fold) and *CAT* (4.853-fold) ($p < 0.0001$) while *SOD* (1.096-fold, $p = 0.1294$) remained unchanged relative to the control (Figure 5.4A). Interestingly, C3 on the other hand elicited the opposite effect by significantly suppressing *Nrf-2* (1.149-fold), *GPx* (21.98-fold), *SOD* (5.579-fold) and *CAT* (2.876-fold) gene expression compared to control (Figure 5.4B, $p < 0.0001$).

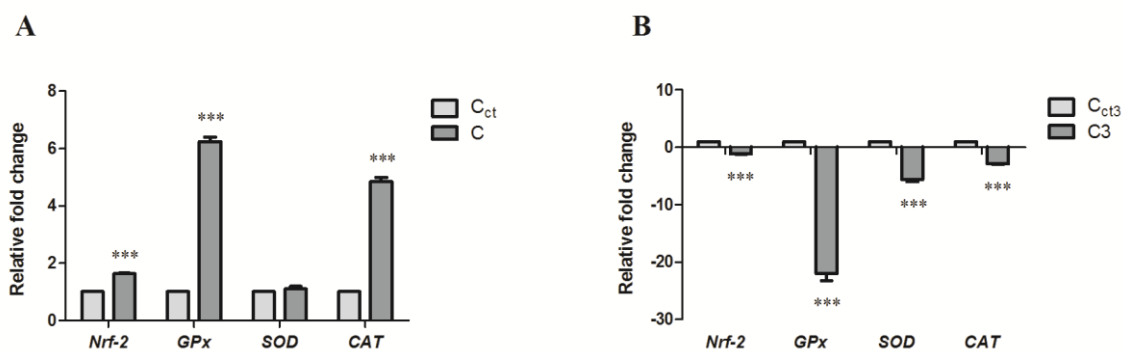


Figure 5.4: Gene expression of *Nrf-2*, *GPx*, *SOD* and *CAT* in LPS stimulated C (A) and C3 (B) treated THP-1 cells (n = 3), (Values expressed as mean \pm SD, *** $p < 0.0001$, compared to controls).

5.3.4 C and C3 induced apoptotic cell death in THP-1 cells

The JC-1 and ATP assays were used to determine $\Delta\psi_m$ and ATP levels respectively. Percentage $\Delta\psi_m$ was increased to a greater extent by C3 (2.896-fold) than C (1.891-fold) relative to the controls (Figure 5.5A, $p < 0.0001$). ATP levels were decreased by C and C3 compared to the control (Figure 5.5B, $p < 0.0001$).

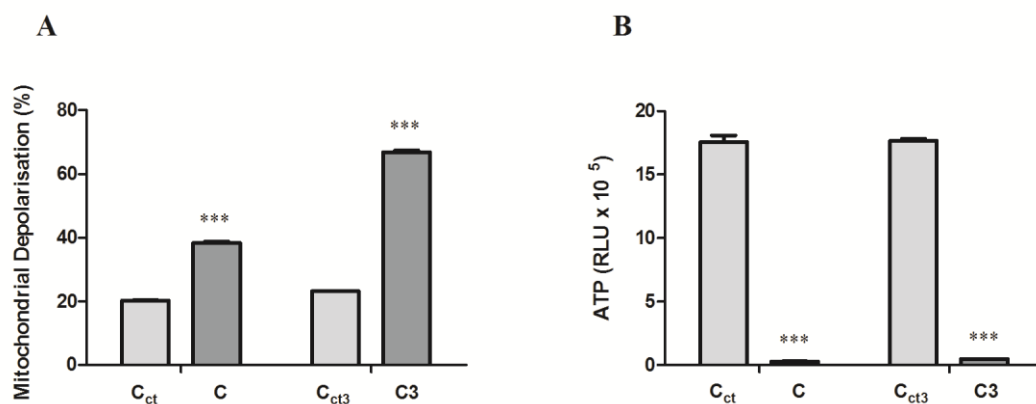


Figure 5.5: Mitochondrial depolarisation (A) and ATP levels (B) in LPS stimulated and *C. asiatica* (C and C3) treated THP-1 cells (n = 3), (Values expressed as mean \pm SD, *** $p < 0.0001$, compared to controls).

The initiation and execution of apoptosis was evaluated by measuring caspase activity. The C extract decreased caspase-8 (1.108-fold, $p = 0.0168$) whilst significantly increasing caspase -9 (1.311-fold) and -3/7 (1.816-fold) activity compared to the control (Table 5.1, $p < 0.0001$). Interestingly, C3 significantly elevated caspase activity [-8 (1.449-fold), -9 (2.142-fold) and -3/7 (2.763-fold)] to a greater extent than C, relative to the control ($p < 0.0001$, Table 5.1).

Table 5.1: Caspase (-8, -9, -3/7) activities in LPS stimulated C and C3 treated THP-1 cells (n = 3), (Values expressed as mean \pm SD, * $p < 0.05$, *** $p < 0.0001$, compared to controls).

	Caspase-8 (RLU x 10 ⁵)	Caspase-9 (RLU x 10 ⁵)	Caspase-3/7 (RLU x 10 ⁵)
C_{ct}	0.349 \pm 0.002	3.460 \pm 0.035	0.265 \pm 0.001
C	0.315 \pm 0.014 *	4.535 \pm 0.040 ***	0.481 \pm 0.001 ***
C_{ct3}	0.331 \pm 0.003	4.640 \pm 0.139	0.260 \pm 0.012
C3	0.479 \pm 0.007 ***	9.940 \pm 0.300 ***	0.717 \pm 0.006 ***

RLU – Relative light units

Cell death was further investigated by assessing Bcl-2 and c-PARP (24 kD) protein expression. Bcl-2 protein expression was decreased by both C (1.279-fold, $p = 0.0001$) and C3 (1.295-fold, $p = 0.0006$) relative to their respective controls (Figure 5.6A-B). Interestingly, C slightly decreased c-PARP (1.073-fold, $p = 0.0003$) whilst C3 significantly increased c-PARP (1.619-fold, $p < 0.0001$) compared to their respective controls (Figure 5.6A-B).

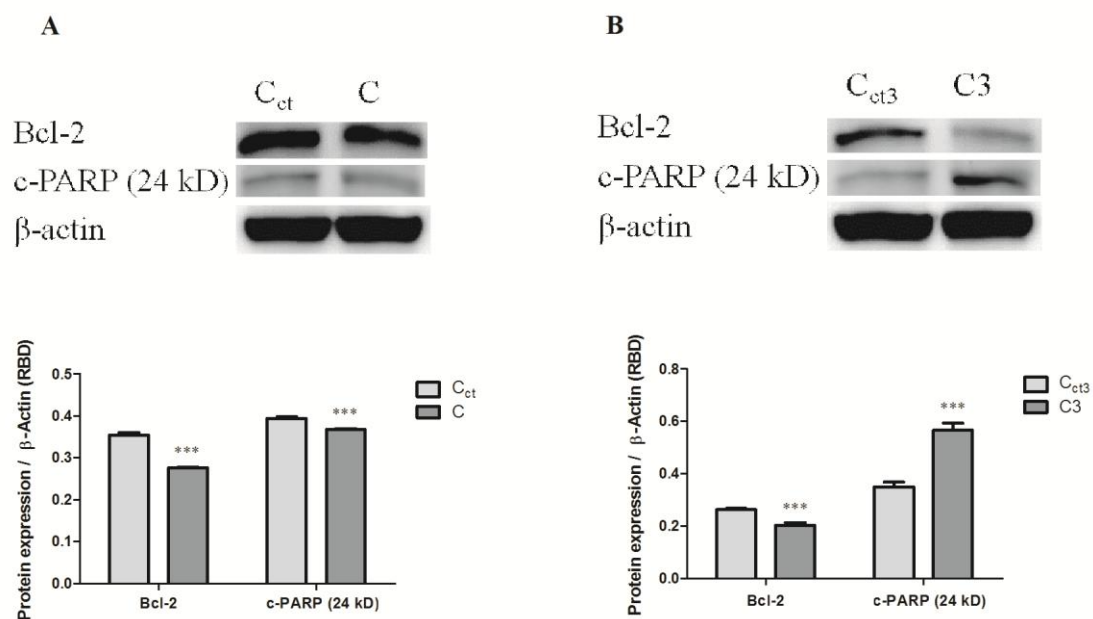


Figure 5.6: Protein expression of Bcl-2 and c-PARP (24 kD) in LPS stimulated C (A) and C3 (B) treated THP-1 cells (n = 3), (Values expressed as mean ± SD, *** $p < 0.0001$, compared to controls).

The effect of C and C3 on DNA damage was evaluated by the SCGE assay. CTL's were significantly increased by both C (1.596-fold) and C3 (1.730-fold) relative to their respective controls ($p < 0.0001$, Figure 5.7).

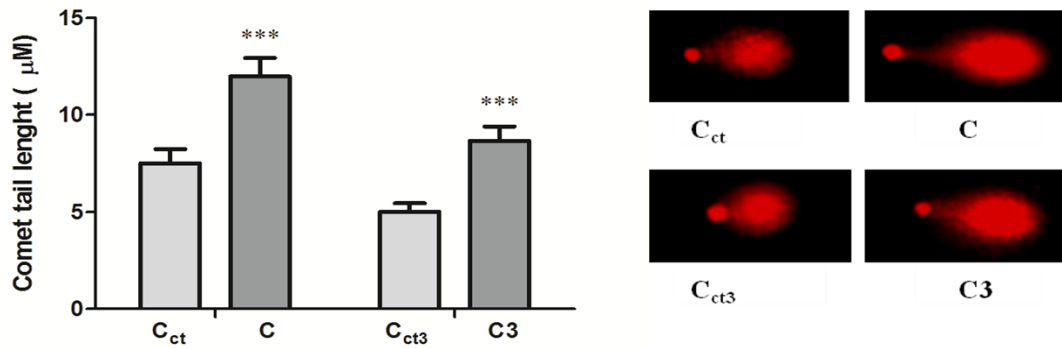


Figure 5.7: CTL's in LPS stimulated C and C3 treated THP-1 cells (n = 3), (Magnification: x 10, Values expressed as mean \pm SD, *** $p < 0.0001$, compared to controls).

Interestingly, *c-myc* gene expression was decreased by C (4.149-fold, $p < 0.0001$) whilst increased by C3 (1.579- fold, $p = 0.0019$) relative to the controls (Figure 5.8A-B). The *OGG-1* gene expression was not significantly altered by C ($p = 0.0508$) whereas significantly decreased by C3 (13.69-fold, $p = 0.0002$) compared to the controls (Figure 5.8A-B).

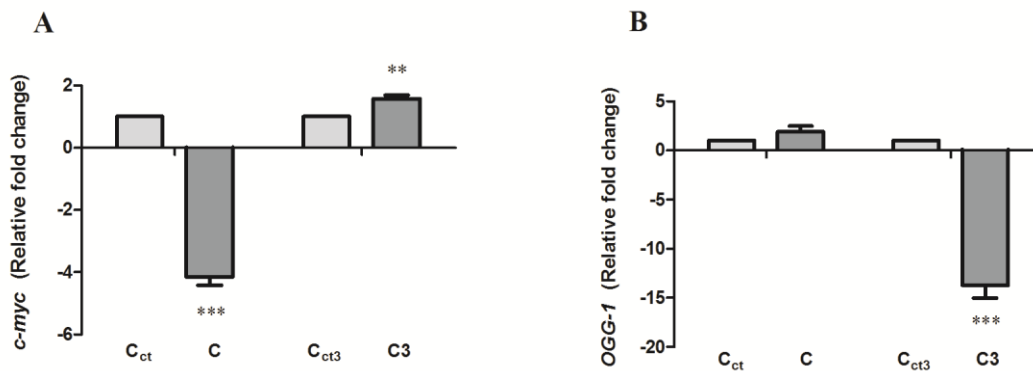


Figure 5.8: Gene expression of *c-myc* (A) and *OGG-1* (B) in LPS stimulated C and C3 treated THP-1 cells (n = 3), (Values expressed as mean \pm SD, ** $p < 0.005$, *** $p < 0.0001$, compared to controls).

5.4 Discussion

C. asiatica leaves possess high free radical scavenging activity [superoxide radical (86.4%), DPPH radical (92.7%)] and inhibits linoleic acid peroxidation (98.2%) (Vimala et al., 2003). Our findings, however, showed that *C. asiatica* (C and C3) increased lipid peroxidation and IROS in THP-1 cells. Interestingly, IROS was increased to a greater extent by C3 than C.

GSH protects against cellular damage by detoxifying ROS (Perricone et al., 2009). During ROS detoxification, GSH is oxidised to GSSG and the intracellular ratio of GSH/ GSSG is used as a predictor of the antioxidant capacity (Perricone et al., 2009). In THP-1 cells, both C and C3 minimally increased GSH levels, however C increased GSSG whilst C3 decreased it. The crude extract increased the antioxidant capacity as evidenced by the increased GSSG levels whilst C3 elicited the opposite effect by decreasing it.

Nrf-2 is the master regulator of enzymes responsible for GSH production and utilization through the expression of glutathione *S*-transferases and GPx (Gorrini et al., 2013; Harvey et al., 2009). During GSH detoxification of ROS, GPx catalyses the conversion of GSH to GSSG (Gorrini et al., 2013). In C treated THP-1 cells, protein expression of Nrf-2 was increased whilst GPx was decreased indicating the utilization of GSH to combat ROS. However in C3 treated THP-1 cells, protein expression of Nrf-2 and GPx were decreased. Diminished Nrf-2 expression decreases GSH utilization and GPx expression ultimately dampening the antioxidant response. In C3 treated THP-1 cells, the diminished antioxidant capacity corresponds with the substantially elevated IROS levels. A decrease antioxidant capacity combined with radiotherapy/ chemotherapeutic drugs may enhance tumour cell elimination by ROS induced cell death (Gorrini et al., 2013).

Under normal cellular conditions Nrf-2 is anti-cancerous due to its activation of cytoprotective and detoxification genes which protect cells against oxidative damage and cancer initiation (Rushworth et al., 2012). However, the Nrf-2 pathway plays an essential role in cancer cell survival by increasing antioxidant defences, decreasing apoptosis and enhancing cancer cell resistance to chemotherapeutic drugs (Rushworth et al., 2012). In THP-1 cells, C increased *Nrf-2*, *GPx* and *CAT* gene expression whereas C3 decreased *Nrf-2*, *GPx*, *SOD* and *CAT* gene expression, indicating that the antioxidant response was elevated by C and diminished by C3. The increased antioxidant gene expression by C is consistent with a previous study by Jayashree *et al* (2003) that showed *C.*

asiatica crude methanol extract substantially increased antioxidant enzymes (*SOD*, *CAT* and *GPx*) in lymphoma bearing mice (Jayashree et al., 2003).

Cancer cells possess a high antioxidant capacity to counteract excessive ROS and allow for tumour survival and proliferation (Gorrini et al., 2013). In most acute myeloid leukaemia (AML) patients, Nrf-2 stimulates the up-regulation of cytoprotective and detoxification genes which function in reducing apoptosis (Rushworth et al., 2012). The growth advantage and chemoresistance of AML cells may be a consequence of Nrf-2 activated genes (Rushworth et al., 2012). Targeting antioxidant mechanisms may be a promising therapeutic strategy to specifically kill cancer cells without damaging normal cells (Gorrini et al., 2013). We demonstrated that C3 did in fact diminish antioxidant responses in THP-1 cells.

Cancer cells require high levels of ATP for cellular proliferation (Gorrini et al., 2013). However, uncontrolled energy production leads to increased ROS which can then induce cellular damage and cell death (Gorrini et al., 2013). Previous studies showed that *C. asiatica* extracts inhibited breast (MCF-7) and liver (HepG2) cancer cell proliferation by inducing apoptosis (Babykutty et al., 2008; Hussin et al., 2014). Cell death may occur through various pathways including apoptosis, necrosis and autophagy. ROS leads to the loss of transmembrane potential and release of pro-apoptotic proteins (Wong, 2011) ultimately activating the mitochondrial apoptotic pathway. In THP-1 cells, increased ROS led to increased $\Delta\psi_m$ by both C and C3 ultimately inducing caspase activation. C3 as compared to C significantly increased $\Delta\psi_m$, a possible consequence of a decreased antioxidant response. The C extract activated the intrinsic apoptotic pathway with an increase in caspase -9 and -3/7 activities. C3 was more effective in inducing apoptosis as evidenced by increased caspase -8, -9 and -3/7 activities, strongly suggesting that both the intrinsic and extrinsic apoptotic pathways were activated. The mitochondrial protein gradient couples the electron transport chain (ETC) to produce ATP (Marchi et al., 2012). However, an increase in $\Delta\psi_m$ leads to the cessation of the ETC and uncoupling of ATP production (Marchi et al., 2012). Both C and C3 significantly decreased ATP levels consistent with the increased $\Delta\psi_m$. In addition, the activation of ATP dependent caspases may have contributed to the decreased ATP levels.

Mitochondrial integrity is regulated by the Bcl-2 family proteins, including anti-apoptotic members (Bcl-2, Bcl-xL, and Mcl-1) and pro-apoptotic members (Bax and Bak) (Jiang et al., 2011). Bax

activation results in membrane permeabilization and subsequent release of pro-apoptotic proteins (Yang et al., 2012). Transcription factor *p53* positively regulates Bax expression; however in THP-1 cells the *p53* gene is deleted therefore diminishing the pro-apoptotic activates of Bax. Taken together, both C and C3 activate THP-1 apoptosis independent of *p53* and Bax. Anti-apoptotic protein, Bcl-2 is responsible for transmembrane potential stabilization, reduces membrane permeability, and inhibits pro-apoptotic protein release (Jiang et al., 2011). Both C and C3 decreased the relative protein expressions of Bcl-2. The decrease in Bcl-2 protein expression diminishes Bcl-2 anti-apoptotic functions ultimately promoting apoptosis through increased $\Delta\psi_m$ and release of pro-apoptotic proteins.

Caspase-3/7 activates cytoplasmic endonucleases which are responsible for degrading nuclear material (Elmore, 2007). In THP-1 cells, both C and C3 increased DNA fragmentation. Cellular ATP is essential for apoptotic cell death therefore PARP-1 is cleaved and inactivated by caspases (Bouchard et al., 2003). Our results indicated that both C and C3 increased caspase-3/7 activity which led to PARP-1 cleavage. PARP-1 fragments (89 and 24 kD) play essential roles in apoptotic cell death (Bouchard et al., 2003). The 24 kD fragment binds to DNA strand breaks and RNA transcripts however cannot be poly(ADPriboseyl)ated and released (Bouchard et al., 2003). As a result, DNA repair, transcription and poly(ADP-ribose) synthesis is inhibited (Bouchard et al., 2003). C3 increased 24 kD PARP protein expression, suggesting that C3 decreases DNA repair ultimately promoting THP-1 apoptosis.

Increased ROS causes oxidative DNA damage leading to the formation of DNA base lesions such as 8-Oxo-7,8-dihydroguanine (8-oxoG) (Hooten et al., 2011). A glycosylase, *OGG-1* is an important DNA repair enzyme which recognises and removes 8-oxoG lesions (Hooten et al., 2011). Our findings demonstrated that C3 significantly decreased *OGG-1* gene expression in THP-1 cells thus decreasing DNA repair.

The proto-oncogene *c-myc* is a master regulator of cellular metabolism and proliferation (Miller et al., 2012). Majority of human cancers have shown elevated *c-myc* gene expression which increases cell growth and differentiation (Miller et al., 2012; Sagun et al., 2006). In tumours, *c-myc* inactivation has been shown to induce apoptotic cell death and tumour regression (Lu et al., 2010). In mice, suppression of *c-myc* has been shown to prevent leukaemia initiation by eliminating leukaemia initiation cell activity (Roderick et al., 2014). Additionally, inhibition of *c-myc* may

disrupt cell survival and extensive proliferation associated with leukaemia initiation cell activity (Roderick et al., 2014). We demonstrated that the C extract did in fact significantly decrease *c-myc* gene expression. These results are consistent with a study by Hussin *et al* (2014) indicating *C. asiatica* extract reduced *c-myc* gene expression in HepG2 cells (Hussin et al., 2014).

c-myc negatively regulates the Nrf-2 antioxidant response by interacting with Nrf-2, decreasing Nrf-2 stability and increasing Nrf-2 degradation (Levy and Forman, 2010). Additionally, *c-myc* replaces Nrf-2 at the electrophile response element complex and decreases antioxidant gene transcription (Levy and Forman, 2010). As a result, *c-myc* over expression leads to decreased intracellular thiol antioxidants, increased intracellular superoxide levels and causes oxidative DNA damage (Sagun et al., 2006). In C treated THP-1 cells, increased Nrf-2 protein expression and antioxidant gene expression may be a consequence of the decreased *c-myc* gene expression. C3 on the other hand elicited the opposite effect by increasing *c-myc* gene expression which may have contributed to the decreased Nrf-2 protein expression and antioxidant gene expression ultimately increasing ROS-induced cell death.

5.5 Conclusions

In THP-1 cells, the antioxidant response was elevated by C whilst diminished by C3. Both C and C3 increased IROS, $\Delta\psi_m$, caspase activity and DNA fragmentation indicating THP-1 apoptosis. Notably, C3 activated apoptosis more effectively than C, which may be related to the decreased antioxidant and DNA repair mechanisms.

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5.7 Disclosure statement

The author(s) declare that they have no competing interests.

5.8 References

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The National Research Foundation and the South African Medical Research Council were not involved in study design, collection of samples, analysis of data, interpretation of data, writing of the report and decision to publish. Scientific out-put is a requirement of the National Research Foundation.

Bridging paragraph:

In the previous manuscript, C increased antioxidant responses while C3 elicited the opposite effect by decreasing it. Additionally, both C and C3 elicited anti-proliferative effects in THP-1 cells. Notably, C3 more effectively induced THP-1 apoptosis than C, which may have been a consequence of the decreased antioxidant response.

Worldwide, lung cancer is the leading cause of cancer-related mortality. Although lung cancer has been extensively researched, survival rate of patients have not improved. Since C and C3 demonstrated anti-proliferative effects in cancerous THP-1 cells. We investigated the antioxidant and anti-proliferative/ cytotoxic effects of C and C3 in lung carcinoma (A549) cells.

The following manuscript is entitled *Centella asiatica* fraction-3 suppresses the Nrf-2 antioxidant pathway and enhances ROS mediated cell death in cancerous lung (A549) cells (Supplementary information: Appendix 9). In this manuscript, only an IC₅₀ for C3 in A549 cells was determined then the antioxidant response signalling and cell death induction were evaluated. The experimental techniques conducted are similar to the previous manuscript.

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Please note: The manuscript was formatted for Journal of Medicinal Food, however, the margins, font, line spacing, numbering of sections and figures were adjusted for thesis consistency.

CHAPTER SIX

***Centella asiatica* semi-purified fraction-3 suppresses the Nrf-2 antioxidant pathway and enhances ROS mediated cell death in cancerous lung A549 cells**

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Running title: *Centella asiatica* induced A549 cell death

Abstract

Centella asiatica (*C. asiatica*) is a tropical medicinal plant commonly used in traditional medicine. Medicinal properties of *C. asiatica* include antioxidant, anti-inflammatory and anti-cancer activity.

Aim: We investigated the antioxidant and anti-proliferative/ cytotoxic effects of a semi-purified fraction-3 of *C. asiatica* ethanolic leaf extract (C3) in cancerous lung A549 cells.

Methods: C3 was obtained by silica column fractionation and components were identified using thin layer chromatography and gas chromatography mass spectrometry. Cytotoxicity of C3 in A549 cells was evaluated (cell viability assay-WST-1; 24 hours; [0.2–3 mg/ml]) to determine an IC₅₀ concentration. Intracellular reactive oxygen species (IROS), mitochondrial membrane potential (flow cytometry), malondialdehyde (MDA), lactate dehydrogenase (LDH) (spectrophotometry), GSH, GSSG, ATP levels, caspase activity (luminometry) and DNA damage (comet assay) were evaluated. Protein expression (Nrf-2, p53, Bax, Bcl-2 and HSP-70) and gene expression (*Nrf-2*, *GPx*, *SOD*, *CAT*, *c-myc* and *OGG-1*) were quantified by western blotting and qPCR, respectively.

Results: C3 dose dependently decreased A549 cell viability. The IC₅₀ of C3 increased MDA, IROS, mitochondrial depolarisation, LDH, caspase (-8, -9, -3/7) activity, DNA damage, GSH levels, Nrf-2 protein expression, HSP-70 protein expression and *OGG-1* gene expression ($p < 0.05$). GSSG levels, antioxidant (*Nrf-2*, *GPx*, *SOD*) gene expression, p53, Bax and Bcl-2 protein expression were decreased by C3 ($p < 0.02$).

Conclusion: C3 diminished the antioxidant gene expression and induced anti-proliferative/ cytotoxic effects in A549 cells.

Key words: *Centella asiatica*, ROS, antioxidants, Nrf-2, caspases, cell death

6.1 Introduction

Worldwide, lung cancer is the leading cause of cancer-related mortality with a 5 year survival rate less than 15%.¹ Cancer is characterized by uncontrolled cell growth, dysregulation of apoptosis, invasion, angiogenesis and metastasis.² Cancerous cells require vast amounts of energy for rapid cell proliferation therefore mitochondrial function is accelerated leading to increased reactive oxygen species (ROS) production.³ Under homeostatic conditions, acute low ROS levels are involved in many biological processes such as cellular proliferation, differentiation and activation of survival pathways.^{4,5} On the other hand, stressful conditions led to acute high ROS levels that cause oxidative damage to DNA, lipids and proteins ultimately activating apoptosis.^{4,5} An efficient antioxidant defence system is required to combat ROS and maintain cellular homeostasis.⁴ However, tumour cells utilize antioxidant mechanisms such as the nuclear factor erythroid 2-related factor 2 (Nrf-2) pathway to enhance malignant cell growth, survival and resistance to chemotherapeutic agents.³

Transcription factor Nrf-2 regulates the expression of antioxidant and cytoprotective genes via the antioxidant response element (ARE).³ Under basal conditions, inhibitory protein, KEAP1 (Kelch-like ECH-associated protein 1) sequesters Nrf-2 in the cytosol and promotes its degradation.⁶ During stressful conditions such as elevated ROS levels, the oxidation and modification of KEAP1 allows Nrf-2 to escape degradation and translocate to the nucleus where it binds to ARE, subsequently increasing antioxidant gene transcription.⁶ Antioxidants prevent oxidative damage to cellular components by counteracting increased ROS levels.⁷ Nrf-2 activated antioxidants include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).⁷ SOD detoxifies superoxide radicals into hydrogen peroxide and molecular oxygen.⁸ Hydrogen peroxide is further detoxified into oxygen and water by GPx and CAT.⁸ Maintaining homeostatic ROS levels is essential to prevent ROS accumulation, subsequent oxidative stress and cell death.

Cell death may occur through various pathways such as apoptosis, necrosis and autophagy. Apoptosis affects individual or clusters of cells whereas necrosis usually affects large fields of cells.⁹ The intrinsic apoptotic pathway leads to pro-apoptotic protein release, apoptosome formation (cytochrome c binding Apaf-1 and pro-caspase-9) and caspase-9 activation whereas the extrinsic apoptotic pathway is initiated by the interaction between death ligands and death receptors resulting

in caspase-8 activation.¹⁰ Initiator caspases (-8, -9) activate executioner caspases (-3/7) subsequently activating cytoplasmic endonucleases which degrade nuclear material.⁹

For centuries, medicinal plants have been employed for chemo-prevention/ therapy due to their vast number of naturally occurring chemicals.¹¹ In Asian and African countries, about 80% of the population depend on complementary and traditional medicine for the treatment and prevention of diseases.¹¹ Medicinal plants contain many phytochemicals that possess anti-cancer and chemo-preventive properties.¹¹ The tropical medicinal plant *Centella asiatica* (*C. asiatica*) belongs to the Apiaceae family and is native to India, China, and South Africa.¹² The common names of *C. asiatica* include Gotu kola, Asiatic/ Indian pennywort and Tiger herb.¹² In Ayurvedic and Chinese traditional medicines, *C. asiatica* is frequently used due to its many medicinal properties including antioxidant, anti-inflammatory, anti-tumour and cytotoxic activity.¹² *C. asiatica* contains a vast range of compounds such as triterpene saponosides (eg. asiatic acid, madecassic acid, asiaticoside etc), flavonoid derivatives (quercetin, kaempferol, patuletin and rutin), vitamins, minerals, polysaccharides, sterols and phenolic acids.¹² The antioxidant activity of *C. asiatica* may be attributed to its high phenolic and flavonoid content.¹³⁻¹⁵ *C. asiatica* extracts (aqueous and methanolic) have also exhibited anti-proliferative/ cytotoxic activity against a number of cancerous cell lines.^{16, 17} However, *C. asiatica* aqueous extract was not cytotoxic in human lung carcinoma (A549).¹⁶

Even though lung cancer has been extensively researched, our understanding and therapies enhanced, the survival rate of lung cancer patients hasn't improved over the past twenty years.¹⁸ Therefore, there is an urgent requirement for combination therapies that inhibit cancer cell antioxidant mechanisms and enhance the toxicity of radio/ chemo therapy, ultimately increasing tumour cell elimination.¹⁸ We hypothesized that the inhibition of the Nrf-2 antioxidant pathway may enhance ROS induced cell death. This study aimed to investigate the effect of *C. asiatica* semi-purified fraction-3 (C3) on antioxidant response signalling and cell death induction in A549 cells.

6.2 Materials and Methods

6.2.1 Materials

C. asiatica leaves were collected in March 2011 (collectors number: Immelman 411) from the Eastern Cape [Langeni forest, roadside (S31°28.135', E28°32.681')], South Africa (SA) and identified by Dr. Kathleen Immelman (Department of Botany at the Walter Sisulu University, SA). Voucher specimens were deposited at the KEI herbarium (13979). A549 cells were obtained from Highveld Biologicals (Johannesburg, SA). Solvents [Dichloromethane (DCM), Hexane (HEX), ethyl acetate (EA), methanol, Dimethyl sulphoxide (DMSO)] sulphuric acid, silica gel 60, thin layer chromatography (TLC) silica gel 60 sheets and 4-methoxybenzaldehyde were purchased from Merck (Johannesburg, SA). Eagle's minimum essential medium (EMEM), foetal calf serum (FCS), penstrepfungizone (PS) and L-glutamine (L-GLUT) were acquired from Whitehead scientific (Cape Town, SA). The 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) cell proliferation reagent and lactate dehydrogenase (LDH) cytotoxicity detection reagent were purchased from Roche (Mannheim, Germany or Johannesburg, SA). The 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolcarbocyanine iodide (JC-1) kit and the 2', 7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) reagent were purchased from BD Biosciences (Johannesburg, SA). Caspase (-8, -9, -3/7), adenosine triphosphate (ATP) and glutathione (GSH) kits were acquired from Promega (Madison, USA). Western blot reagents were purchased from Bio-Rad (Johannesburg, SA) and qPCR primers were obtained from Inqaba Biotech.

6.2.2 Plant Extraction and Fractionation

The plants official name is *Centella asiatica* (L.) Urb and has been confirmed by using the plant list (www.theplantlist.org). The local names are icudwane (Zulu) and varkoortjies (Afrikaans). The English names are tiger herb and pennywort. *C. asiatica* leaves were dried and milled. Ethanol (200–350 ml) was added to milled plant material (10–30 g) and extracted overnight by shaking (4 x g, 37°C). Ethanol extracts (\pm 20 g) were filtered, rotor evaporated, dried (37°C) and stored (4°C). The yield of dried extract as a percentage weight of dried plant was approximately 65%. Silica column fractionation was used to separate *C. asiatica* compounds based on their polarity. A manual column (height: 85 cm, diameter: 17 cm) was assembled by inserting a tap (25 mm), a cotton wool stop, a thin layer of sand, carefully pouring silica mixed with DCM: HEX (50: 50) into a column and allowing the silica to set overnight. *C. asiatica* crude extract (17 g) was dissolved in DCM:

HEX (70: 30), however a portion of the crude extract did not dissolve in DCM: HEX (70: 30). This portion of the crude extract was further dissolved in methanol and added to the crude extract that dissolved in DCM: HEX (70: 30). Thereafter, the total *C. asiatica* crude extract was rotated evaporated, layered onto the silica and 50 ml fractions were collected using a series of solvent systems [DCM: HEX (70: 30), DCM: EA (70: 30), DCM: EA (50: 50), DCM: EA: methanol (50: 40: 10), DCM: EA: methanol (30: 50: 20), EA: methanol (70: 30), EA: methanol (40: 60), EA: methanol (20: 80), methanol (100)]. The 50 ml fractions were boiled to allow evaporation of excess solvent and TLC was used to determine spot patterns and solvent changes. A total of 766 fractions were collected and TLC analysis revealed fractions with similar spot patterns which were combined and pooled into one fraction. Fractionation of *C. asiatica* yielded 13 pooled fractions. For all subsequent experiments, *C. asiatica* crude leaf extract (C) and C3 were used.

6.2.3 Gas chromatography mass spectrometry (GC-MS)

C. asiatica (C and C3) chemical composition was analysed by GC-MS (Hewlett-Packard, USA) and the GCD-HP1800A system was used. Ionization energy (70 eV) was used for the detection of GC-MS and the detection was passed under high vacuum (10⁻⁴ to 10⁻⁸ torr). Helium gas was used for the analysis at a constant flow rate (1 mL/min). The mass transfer line and injector temperature were fixed at 280 and 250 °C. The bioactive compounds which are present in *C. asiatica* (C and C3) were matched with the NIST computer library and reported.

6.2.4 Tissue Culture

A549 cells were grown in the appropriate tissue culture conditions in a 25 or 75 cm³ tissue culture flasks (37°C, 5% CO₂). The A549 cells were cultured in EMEM supplemented with 10% FCS, 1% PS and 1% L-GLUT. Cells were seeded, allowed to attach overnight and for all subsequent assays the cells were treated at 80% confluency.

6.2.5 Cell Viability Assay

Cytotoxicity of C and C3 to A549 cells was measured using the WST-1 assay (Roche, Johannesburg, SA). A549 cells (20,000 cells/well, 96 well plate, in triplicate wells) were allowed to attach overnight (37°C, 5% CO₂). At 80% confluency, cells were washed twice with 0.1 M phosphate buffer saline (PBS) before exposure to C and C3 (0.2–3 mg/ml) for 24 hours (h) (37°C,

5% CO₂). Controls received media containing DMSO (0.750%). Following 24 h treatment, cells were washed twice with 0.1 M PBS, growth media (100 µl/well), WST-1 reagent (10 µl/well) was added and plates incubated (37°C, 5% CO₂, 3 h). The optical density was measured at 450 nm (620 nm reference wavelength) with a BIO-TEK µQuant spectrophotometer (Analytical and Diagnostic Products, SA). This experiment was conducted on two separate occasions before using GraphPad Prism to calculate the half maximal inhibitory concentration (IC₅₀) [percentage cell viability vs log concentration of *C. asiatica* (C and C3)]. *C. asiatica* crude extract did not influence A549 cell viability. However, an IC₅₀ of 1.437 mg/ml was obtained in C3 treated A549 cells and was used in all subsequent experiments. Control treatments were represented by an equal amount of DMSO (0.359%) in growth media without C3.

6.2.6 Treatment of cells

A549 cells (2 x 10⁵ cells/ml) were aliquoted into 75 cm³ tissue culture flask and allowed to attach overnight (37°C, 5% CO₂). At 80% confluency, cells were washed twice with 0.1 M PBS before exposure to C3 (1.437 mg/ml) for 24 h (37°C, 5% CO₂). Thereafter, cells were washed twice with 0.1 M PBS and were used to conduct the JC-1, 20, 70-dichlorofluorescein (DCF), GSH, GSSG, caspase (-8, -9, -3/7) activity, ATP, comet assays; as well as protein and RNA isolation. The experiments were conducted independently two times for all subsequent assays.

6.2.7 Thiobarbituric acid assay

The thiobarbituric acid assay (TBARS) measures malondialdehyde (MDA), the end product of lipid peroxidation and was conducted as per the method previously described by Phulukdaree *et al* (2010).¹⁹ Optical density was measured at 532 nm (600 nm reference wavelength) with a BIO-TEK µQuant spectrophotometer. Malondialdehyde concentration was calculated by dividing the optical density (OD) for each sample by the absorption coefficient (156 mM⁻¹).

6.2.8 The DCF assay

The DCF assay measures intracellular ROS (IROS) and was conducted as per the method previously described by Nagiah *et al* (2015).²⁰ Events were gated and the fluorescence measured on FL-1 channel (525 nm) of the AccuriTMC6 flow cytometer (BD Biosciences, Franklin Lakes, NJ,

USA). A total of 50,000 events were acquired and analyzed using the CFlow Plus Software (BD Biosciences, San Jose, CA, USA).

6.2.9 The GSH assay

The GSH-Glo™ GSH-Glo™ assay (Promega, Madison, WI, USA) was used to measure GSH and GSSG levels. The assay was conducted as per the method previously described by Nagiah *et al* (2015).²⁰ Luminescence was measured on a Modulus™ microplate luminometer (Turner BioSystems, Sunnyvale, CA, USA). GSH and GSSG concentrations (μM) were determined by extrapolation from a standard curve.

6.2.10 The JC-1 Mitoscreen assay

The JC-1 assay measures percentage mitochondrial depolarisation ($\Delta\psi_m$) and was conducted as per the method previously described by Nagiah *et al* (2015).²⁰ Scatter plots of 50,000 events per sample were acquired using the Accuri™C6 flow cytometer and analyzed using the CFlow Plus Software.

6.2.11 The LDH assay

The LDH cytotoxicity detection kit (Roche) was used to measure cell damage by C3. Stress compromises cell membrane integrity and leads to the leakage of LDH from cells. LDH activity in the supernatant (100 μl/ well; triplicate wells) was quantified by addition of LDH reagent (100 μl/ well) and incubation (25 min, RT). OD was measured at 500 nm with a BIO-TEK μQuant spectrophotometer. Results are represented as mean OD.

6.2.12 Caspase and ATP assays

Caspase activities and ATP levels were determined using the Caspase-Glo®3/7, -8, -9 and ATP assay kits (Promega, Madison, WI, USA). Reagents were reconstituted according to the manufacturer's instructions. Cells (50 μl/well, 2×10^5 cells/ml) were added into triplicate wells of a microtitre plate for each assay, thereafter caspase -3/7, -8, -9 and ATP reagents (25 μl/well) were

added into appropriate wells. Plates were incubated (30 min, RT) in the dark. Luminescence was measured on a ModulusTM microplate luminometer and expressed as relative light units (RLU).

6.2.13 Single cell gel electrophoresis (SCGE) or COMET assay

The SCGE assay was performed to assess DNA fragmentation and was conducted as per the method previously described by Singh *et al* (1988).²¹ Cells were stained with GR red (1µl, 1000x) instead of ethidium bromide. Slides were viewed using an Olympus IX7I microscope with 510–560 nm excitation and 590 nm emission filters (Wirsam Scientific, Johannesburg, SA). Images from triplicate slides per treatment were captured. Comet tail lengths (CTL's) of 50 comets per treatment were measured using the Soft imaging system (Life Science - ©Olympus Soft Imaging Solutions v5) and average CTL's were calculated (µm).

6.2.14 Western Blotting

Western Blotting was performed to determine relative protein expressions. Cytobuster (200 µl, Novagen, San Diego, CA, USA) supplemented with protease and phosphatase inhibitors (Roche, Mannheim, Germany) was added to the cells (10 min, 4°C) and centrifuged (13000 x g, 4°C, 10 min). Protein was quantified using the bicinchoninic acid assay (Sigma, Germany) and standardised to 1 mg/ml. Western blotting was conducted as per the method previously described by Nagiah *et al* (2015).²⁰ Membranes were immune-probed with Nrf-2 (ab89443), *p53* (CS9289), Bax (CS5023), Bcl-2 (CS2827), heat shock protein (HSP)-70 (CS4872) primary antibodies (1:1000, Cell Signalling, Danvers, USA). Anti-rabbit (sc-2054) or anti-mouse (ab97046) were the secondary antibodies used (1:5,000). Horse radish peroxidase chemiluminescence detector (Bio-Rad) and enhancer solution were used for the antigen-antibody complex and the signal was detected with the Alliance 2.7 image documentation system (UViTech). To correct for loading error and to normalise relative protein expression, β-actin was assessed (A3854; 1:5000, Sigma, St Louis, MO, USA). The expression of the proteins were analysed with UViBand Advanced Image Analysis software v12.14 (UViTech) and data was expressed as relative band density (RBD) and fold change.

6.2.15 Quantitative PCR

Total RNA was isolated using an in-house protocol. ²² RNA was quantified (Nanodrop2000, ThermoScientific, South Africa) and standardised (800 ng/ml). A reaction volume (10 µl) containing RNA template (2 µl), 5X iScript™ reaction mix (2 µl), iScript reverse transcriptase (0.5 µl) and nuclease free water (5.5 µl) was used to synthesize cDNA (iScript™ cDNA Synthesis kit, BioRad, 107e8890). Thermocycler conditions were 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and a final hold at 4°C.

Gene expression of *SOD2* [Sense 5'-GAGATGTTACACGCCAG TAGC-3'; Antisense 5'-AATCCCCAGCAGTGGAATAAGG-3'(57°C)], *CAT* [Sense 5'-TAAGACTGACCAGGGCATC-3'; Antisense 5'-CAACCTTGGTGAGATCGAA- 3'(58°C)], *GPx* [Sense 5'-GACTACACCCAGATGAACGAGC- 3'; Antisense 5'-CCCACCAGGAA CTTCTCAAAG-3'(58°C)], *Nrf-2* (forward 5'-AGTGGATCTGCCAACTACTC-3'; reverse 5'-CATCTACAAACGGGAATGTCTG-3' (58°C)), oxoguanine DNA glycosylase (*OGG-1*) (forward 5'-GCATCGTACTCTAGCCTCCAC-3'; reverse 5'-AGGACTTTGCTCCCTCCAC-3' (60°C)) and *c-myc* (forward 5'-AGCGACTCTGAGGAGGAACAAG-3'; reverse 5'-GTGGCACCTCTTGAGGACCA-3' (56°C)) were evaluated using the iQ™ SYBR® Green PCR kit (Bio-Rad; 170e880). The final reaction volume was 10.5 µl [6.25 µl 5X iScript reaction mix, 0.5 µl sense primer, 0.5 µl anti-sense primer, 3.25 µl nuclease-free water, and 2 µl cDNA sample (triplicate wells per sample)] and all assays were carried out using CFX Touch™ Real Time PCR Detection System (Bio-Rad). The reaction included an initial denaturation (95°C, 4 min), followed by 37 denaturation cycles (95°C, 15sec), annealing (primer specific temperature, 40sec), extension (72°C, 30sec) and a plate read for 37 cycles. Under the same conditions, the *β-actin* [Sense 5'-TGACGGGTCACCCACACTGTGCCCAT-3'; Antisense 5'-CTAGAAGCATTGCGGTGGACGATGGAGGG-3'] and *18S*: (Sense: 5'-ACAGGGACAGGATTGACAGA-3'; Antisense: 5'-CAAATCGCTCCACCAACCTAA-3') housekeeping genes were run. Using the method described by Livak and Schmittgen, qPCR results were analyzed, represented as fold change ($2^{-\Delta\Delta CT}$) relative to the house keeping genes (*β-actin* and *18S*) and control. ²³

6.2.16 Statistical Analysis

Statistical analysis was performed using the GraphPad Prism (v5) statistical analysis software. Statistical significance was set at 0.05 and comparisons were made using unpaired t tests with Welch correction.

6.3 Results

6.3.1 Chemical composition of *C. asiatica* (C and C3)

The GC-MS chromatograms of C and C3 were recorded and presented in Supplementary Figures 4 and 5. Chemical composition of C and C3 constituents were identified after comparison with those available in the NIST computer library. The organic compounds that were identified in *C. asiatica* leaf extract namely caranol, oxymetholone methanol adduct, vitamin E, tocopherol and terpenes (phytosterol and stigmasterol) (Supplementary information: Table 1 and 2).

6.3.2 Cytotoxicity of C3 against A549 cells

The WST-1 assay showed that A549 cell viability was not significantly altered by C however dose dependently decreased by C3 with an IC_{50} value of 1.437 mg/ml (Figure 6.1). The IC_{50} concentration was used in all subsequent experiments.

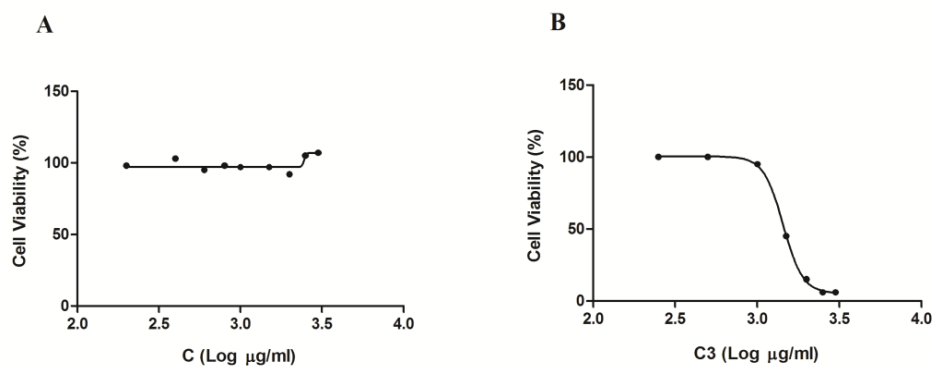


Figure 6.1: *C. asiatica* crude extract [0.2 – 3 mg/ml (A)] did not influence A549 cell viability whereas purified fraction-3 [0.2 – 3 mg/ml (B)] induced a dose dependent decrease in A549 cell viability; IC_{50} values of 1.437 mg/ml.

6.3.3 The effect of C3 on oxidative stress and antioxidant mechanisms in A549 cells

The effect of C3 on lipid peroxidation and IROS production were assessed by the TBARS and DCF assays. C3 significantly increased MDA (1.615-fold) and IROS (2.492-fold) levels ($p < 0.0001$, Figure 6.2A and 6.2B). Further the overall antioxidant effects of C3 were determined by measuring the concentration of GSH and GSSG, Nrf-2 protein expression as well as *Nrf-2*, *GPx*, *SOD* and *CAT* gene expression. The level of GSH was increased 1.420-fold ($p = 0.0009$, Figure 6.2C) whilst GSSG was decreased 1.759-fold ($p = 0.0102$, Figure 6.2D) by C3.

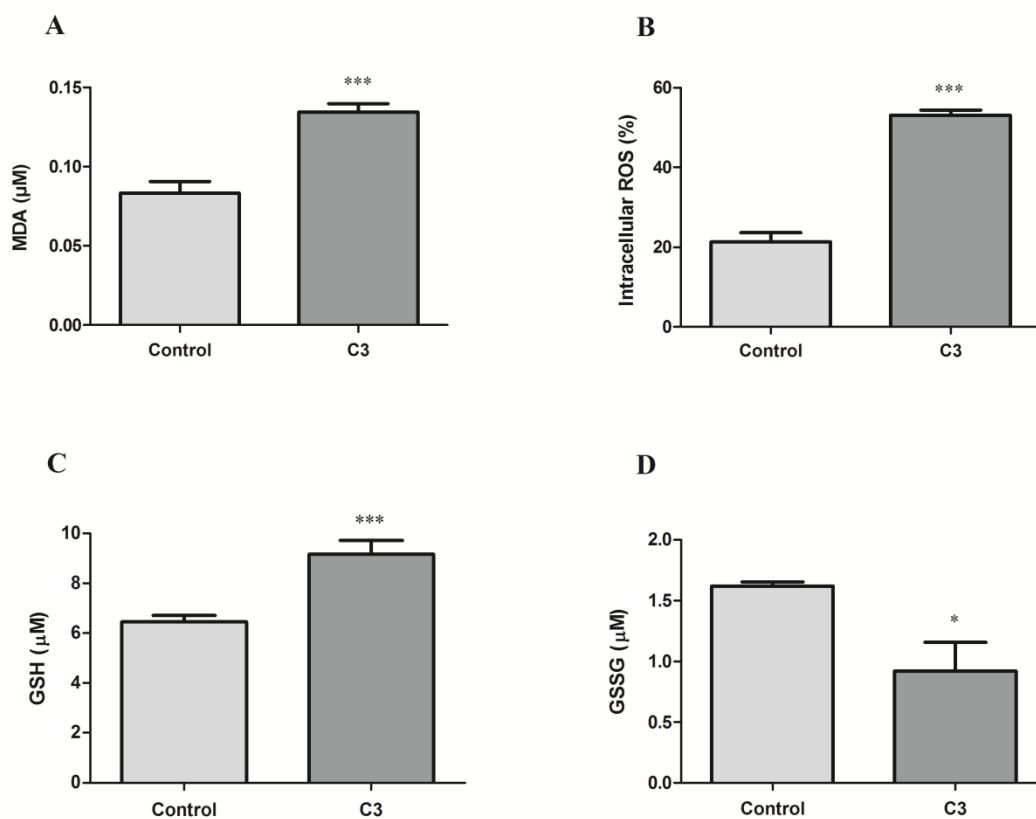


Figure 6.2: Levels of MDA (A), IROS (B), GSH (C) and GSSG (D) in C3 treated A549 cells (Values expressed as mean \pm SD, * $p < 0.05$, *** $p < 0.0001$, compared to controls).

C3 increased Nrf-2 protein expression 1.359-fold relative to the control ($p = 0.0002$, Figure 6.3).

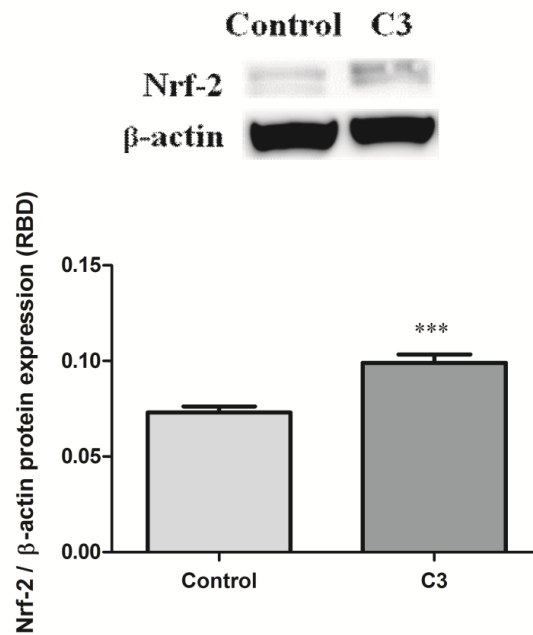


Figure 6.3: Protein expression of Nrf-2 in C3 treated A549 cells (Values expressed as mean \pm SD, *** $p < 0.0001$, compared to the control).

Interestingly, C3 significantly decreased *Nrf-2* (1.737-fold), *GPx* (3.431-fold) and *SOD* (1.959-fold) ($p < 0.0001$) whilst *CAT* (1.021-fold, $p = 0.0820$) gene expression was unchanged compared to the control (Figure 6.4).

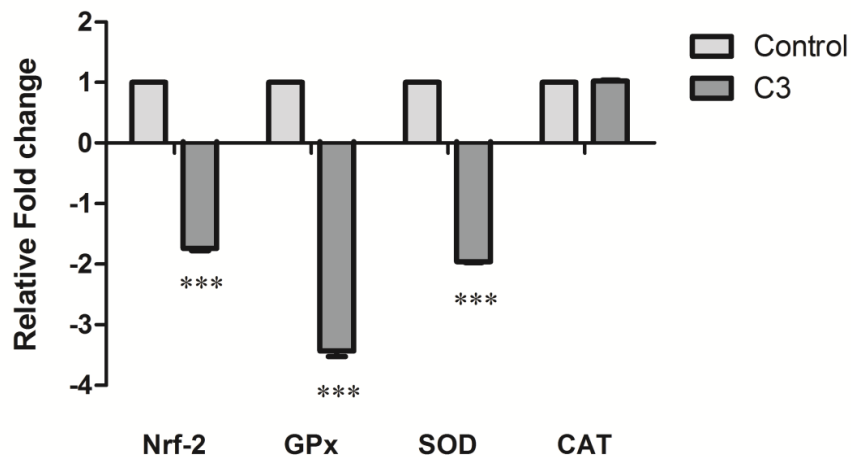


Figure 6.4: Gene expression of *Nrf-2*, *GPx*, *SOD* and *CAT* in C3 treated A549 cells (Values expressed as mean \pm SD, *** $p < 0.0001$, compared to the control).

6.3.4 C3 induced cell death pathways in A549 cells

The JC-1 and LDH assays were used to determine $\Delta\psi_m$ and cell membrane permeability respectively. C3 significantly increased $\Delta\psi_m$ 2.279-fold relative to the control ($p < 0.0001$, Figure 6.5A). LDH levels were also increased 1.711-fold by C3 compared to the control ($p < 0.0001$, Figure 6.5B).

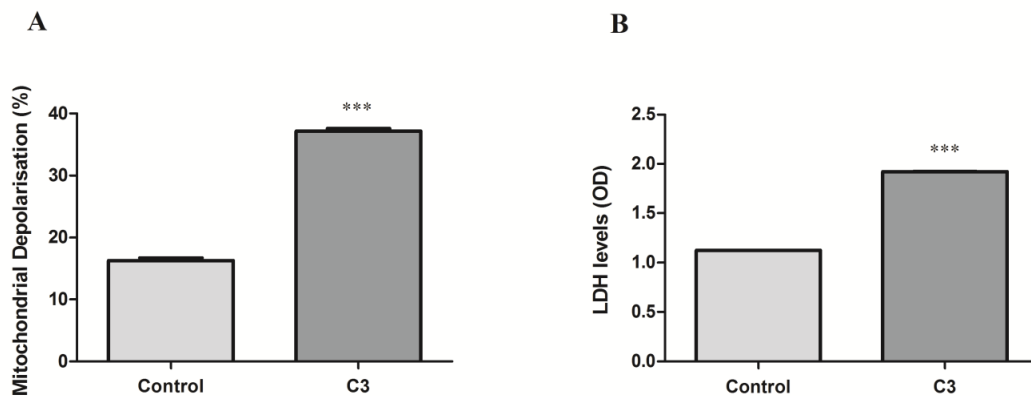


Figure 6.5: Mitochondrial depolarisation (A) and LDH (B) levels in C3 treated A549 cells (Values expressed as mean \pm SD, *** $p < 0.0001$, compared to the control).

Caspase activity was measured to evaluate the initiation and execution of apoptosis. Semi-purified fraction-3 increased caspase [-8, -9, -3/7, $p < 0.003$] activity approximately 1.2-fold and depleted ATP levels ($p < 0.0001$) compared to the control (Table 6.1).

Table 6.1: Caspase activity (-8, -9, -3/7) and ATP levels in C3 treated A549 cells (Values expressed as mean \pm SD, ** $p < 0.005$, *** $p < 0.0001$, compared to the control).

	Caspase-8 (RLU x 10 ⁵)	Caspase-9 (RLU x 10 ⁵)	Caspase-3/7 (RLU x 10 ⁵)	ATP (RLU x 10 ⁵)
Control	3.615 \pm 0.123	4.300 \pm 0.006	0.050 \pm 0.0005	14.300 \pm 0.468
C3	4.396 \pm 0.204 **	5.214 \pm 0.113 ***	0.063 \pm 0.001 ***	0.671 \pm 0.003 ***

Cell death was further investigated by assessing anti-apoptotic and pro-apoptotic protein expression. Protein expression of *p53* (2.958-fold), *Bax* (1.623-fold) and *Bcl-2* (1.206-fold) were significantly decreased by C3 relative to their respective controls ($p < 0.0005$, Figure 6.6). On the other hand, C3 increased HSP-70 protein expression relative to the control (1.157-fold, $p = 0.0007$, Figure 6.6).

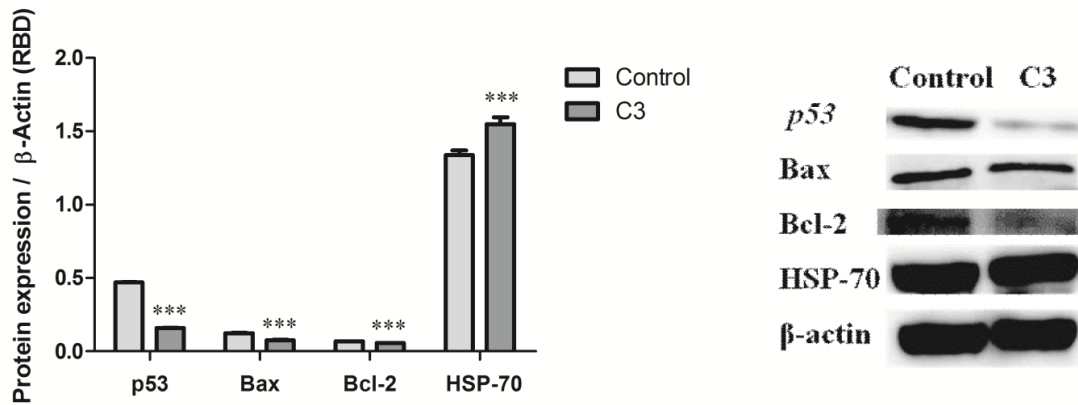


Figure 6.6: Protein expression of *p53*, Bax, Bcl-2 and HSP-70 in C3 treated A549 cells (Values expressed as mean \pm SD, ** $p < 0.005$, *** $p < 0.0001$, compared to the control).

The SCGE assay was used to determine the amount of DNA damage caused by C3. CTL's were increased 1.664-fold compared to the control ($p < 0.0001$, Figure 6.7).

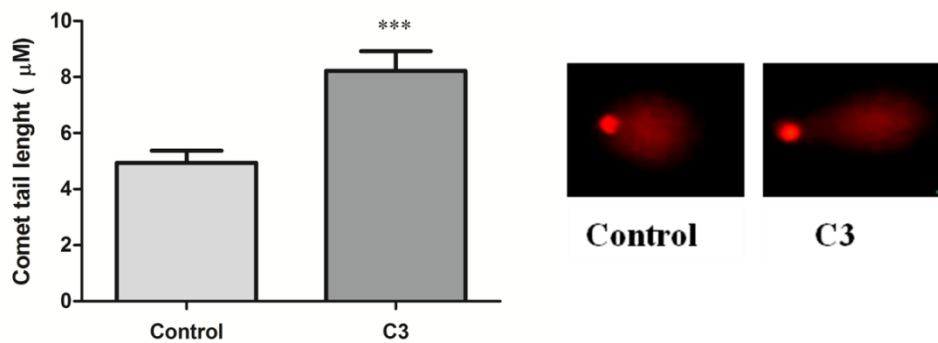


Figure 6.7: DNA fragmentation in C3 treated A549 cells (Values expressed as mean \pm SD, *** $p < 0.0001$, compared to the control).

C3 did not significantly influence *c-myc* gene expression (1.033-fold, $p = 0.276$, Figure 6.8A) but increased *OGG-1* gene expression (1.346-fold, $p = 0.0424$, Figure 6.8B) relative to the control (1.00 fold).

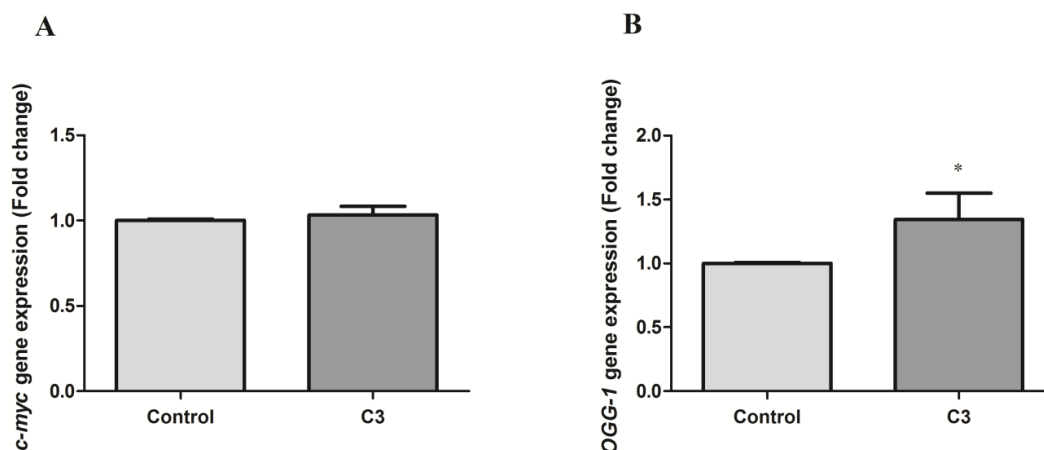


Figure 6.8: Gene expression of *c-myc* (A) and *OGG-1* (B) in C3 treated A549 cells (Values expressed as mean \pm SD, * $p < 0.05$, compared to the control).

6.4 Discussion

C. asiatica leaf extracts have shown excellent free radical scavenging capabilities [hydroxyl, superoxide (86.4%) and DPPH (92.7%)] and lipid peroxidation preventive properties.^{14, 24} Additionally, the antioxidant activity of *C. asiatica* (84%) is comparable to vitamin C (88%) and grape seed extract (83%).²⁵ Our results, however, showed that C3 increased lipid peroxidation and IROS compared to the controls.

Within the cell, GSH is the most abundant antioxidant that detoxify's hydrogen peroxide and free radicals, ultimately protecting cellular components from oxidative damage.²⁶ GSH production and utilization is controlled by Nrf-2 mediated expression of glutathione *S*-transferases and GPx.^{4, 27} During ROS detoxification, GPx oxidises GSH to GSSG and the intracellular GSH/ GSSG ratio is a predictor of the antioxidant capacity.²⁶ In C3 treated A549 cells, the increased Nrf-2 protein expression corresponds with the increased GSH levels; however GSSG levels were decreased compared to the control. Our findings indicate that C3 decreased the antioxidant capacity in A549

cells as evidenced by the decreased GSSG levels. Also, MDA and IROS levels were substantially elevated by C3, a possible consequence of the diminished antioxidant capacity. Cancerous cells utilize antioxidants to combat elevated ROS levels and encourage cell survival.⁴ Therefore, diminishing the antioxidant capacity is essential to enhance tumour cell elimination by ROS induced cell death.⁴

Through ROS generation, radio/ chemo therapeutic agents effectively induce apoptotic cell death. However, cancer cells prevent ROS mediated apoptosis by increasing antioxidant enzyme expression.¹⁸ Previously, elevated Nrf-2 expression has been shown to promote cancer cell growth and survival through increased antioxidant capacity, decreased apoptosis and increased drug resistance.⁷ In A549 cells, Nrf-2 expression has been shown to protect cells against ionizing radiation toxicity indicating that Nrf-2 may contribute to A549 cell survival and resistance to ionizing radiation.³ Our findings demonstrated that C3 decreased A549 antioxidant gene expression (*Nrf-2*, *GPx* and *SOD*). As a result, A549 cells are more susceptible to ROS mediated cell death. The *Nrf-2* gene expression was decreased; however the Nrf-2 protein expression was increased by C3. This may be a result of alterations in posttranslational modifications. Malignant cells possess a superior antioxidant defence system for survival and proliferation,¹⁸ therefore suppressing antioxidant mechanisms is a promising therapeutic strategy to facilitate cancer cell elimination.⁴ We demonstrated that the semi-purified fraction-3 (C3) of *C. asiatica* did in fact diminish antioxidant responses in A549 cells.

In chronic myeloid leukemia (CML), a chromosomal translocation t(9; 22) produces the Philadelphia chromosome which leads to the production of a fusion protein between Bcr and Abl.²⁸²⁹ BCR-ABL confers anti-apoptotic and proliferative properties.²⁸ In addition, BCR-ABL is associated with increased ROS, DNA damage and altered repair which may contribute to genomic instability and cancer progression.²⁸ Imatinib mesylate, (a tyrosine kinase inhibitor (TKI)) binds to and inhibits BCR-ABL tyrosine kinase.²⁹ In BCR-ABL positive CML patients, imatinib mesylate induces a positive clinical response.²⁸ In A549 cells, cell growth and proliferation was inhibited by imatinib mesylate.²⁹ Additionally, in A549 cells imatinib mesylate in combination with cisplatin induced cell death synergistically.²⁹ However, BCR-ABL mutations or BCR-ABL independent mechanisms lead to TKIs resistance and a poor clinical response.²⁸

In imatinib-resistant P210Mo7eIR cells, the down-regulation of Nrf-2 re-sensitizes cells to imatinib and significantly increases cell death.²⁸ On the other hand, in imatinib sensitive P210Mo7e cells, over-expression of Nrf-2 cDNA constructs results in imatinib resistance.²⁸ Thus, an up-regulation of the Nrf-2 pathway may be seen as another mechanism for TKI resistance.²⁸ Taken together, inhibition of Nrf-2 and its downstream targets (such as antioxidant genes) may be a good therapeutic strategy to minimize TKI resistance.²⁸ As stated earlier, C3 decreased Nrf-2, GPx and SOD gene expression in A549 cells, suggesting that C3 may decrease TKI resistance by down regulating the Nrf-2 antioxidant pathway. Thus, C3 in combination with conventional cancer therapies may further enhance cancer cell death by minimizing TKI resistance.

In vitro, *C. asiatica* methanolic extract showed anti-proliferative activity in a number of cancerous cell lines.¹⁷ In breast cancer (MCF-7) and liver cancer (HepG2) cells, *C. asiatica* extract inhibited cell proliferation through apoptosis and DNA damage.^{17,30} Decreased antioxidant mechanisms and increased ROS levels lead to oxidative damage and cell death.⁴ Semi-purified fraction-3 significantly increased $\Delta\psi_m$, a possible consequence of a decreased antioxidant response and increased IROS levels. The increased $\Delta\psi_m$ led to increased caspase [-8, -9, -3/7] activation, suggesting the initiation and execution of apoptosis. However, C3 also significantly elevated LDH levels, indicating that the cellular membrane integrity was compromised and necrosis may be occurring. The mitochondrial protein gradient couples the electron transport chain (ETC) to produce ATP.³¹ However, increased $\Delta\psi_m$ leads to the cessation of the ETC and uncoupling of ATP production.³¹ In A549 cells, C3 decreased ATP levels, consistent with the increased $\Delta\psi_m$. In addition, the activation of ATP dependent caspases (-8, -9, -3/7) may have contributed to the decreased ATP levels. Taken together, C3 induced anti-proliferative and cytotoxic effects in A549 cells.

The Bcl-2 family of proteins are responsible for maintaining mitochondrial membrane potential.³² An anti-apoptotic member such as Bcl-2 stabilizes transmembrane potential, reduces membrane permeability and inhibits pro-apoptotic protein release.³² Elevated ROS levels activate pro-apoptotic members such as Bax which translocates to the mitochondria, integrates into the outer mitochondrial membrane leading to membrane permeabilization and pro-apoptotic protein release.³³ In A549 cells, Bax and Bcl-2 protein expressions were decreased by C3. Decreased Bcl-2 protein expression diminishes Bcl-2 anti-apoptotic functions, ultimately promoting apoptosis.

Tumour suppressor *p53* has many biological functions including the maintenance of genetic integrity, cell cycle arrest, DNA repair and apoptosis.³⁴ With regard to cell death, *p53* activates Bax and promotes apoptosis.³⁴ On the other hand, HSP-70 over expression provides a survival advantage to cancer cells by inhibiting apoptosis.³⁵ HSP-70 inhibits apoptosis by preventing Bax mitochondrial translocation, Bax mediated membrane permeability and subsequent pro-apoptotic protein release.^{33, 35} Our results showed that C3 decreased *p53* and increased HSP-70 protein expression which may have contributed to the decreased Bax protein expression and limited caspase activation.

Cell death pathways lead to the activation of cytoplasmic endonucleases.⁹ Endonucleases G, ICAD and AIF are released from the mitochondria and are responsible for degrading nuclear material.⁹ Our results indicated that CTL's were increased by C3, a consequence of increased cell death.

Elevated ROS levels causes oxidative DNA damage leading to the formation of DNA adducts such as 8-oxo-7,8-dihydroxyguanine (8-oxoG).³⁶ Previously, 8-oxoG has been associated with several cancers due to its ability to induce mutations.³⁶ The base excision repair enzyme, *OGG-1* excises and repairs 8-oxoG adducts.³⁶ Previously, in A549 cells, mutant *OGG-1* over expression has been shown to block oxidant-induced mitochondrial dysfunction, caspase-9 activation, and DNA fragmentation by preserving mitochondrial aconitase.³⁶ Our results showed that C3 increased *OGG-1* gene expression in A549 cells thus increasing DNA repair. Also, the increased *OGG-1* gene expression may have suppressed oxidant induced caspase activation.

The *c-myc* transcription factor regulates the expression of genes involved in cell proliferation, growth and transformation.³⁷ In many human cancers, the *c-myc* gene is over expressed and cellular proliferation is not linked to growth-factor stimulation which results in uncontrolled cell growth.^{38, 39} In A549 cells, C3 did not influence *c-myc* gene expression, indicating that C3 does not increase *c-myc*-mediated cell proliferation.

6.5 Conclusion

The antioxidant capacity of cancerous A549 cells was decreased by C3 thus facilitating ROS induced cell death. Semi-purified fraction-3 exerts anti-proliferative and cytotoxic activity in A549 cells.

6.6 Acknowledgements

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6.7 Author Disclosure statement

The author(s) declare that they have no competing interests.

6.8 Abbreviations

(ROS), Reactive oxygen species; (Nrf-2), Nuclear factor erythroid 2-related factor 2; (ARE), Antioxidant response element; (KEAP1), Kelch-like ECH-associated protein 1; (SOD), Superoxide dismutase; (CAT), Catalase; (GPx), Glutathione peroxidase; (*C. asiatica*), *Centella asiatica*; (A549), Lung carcinoma; (SA), South Africa; (DCM), Dichloromethane; (HEX), Hexane; (EA), Ethyl acetate; (DMSO), Dimethyl sulfoxide; (EMEM), Eagle's minimum essential medium; (FCS), Foetal calf serum; (PS), Penstrepfungizone; (L-GLUT), L-glutamine; (WST-1), 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; (LDH), Lactate dehydrogenase; (JC-1), 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolcarbocyanine iodide; (H2DCF-DA), 2', 7'-dichlorodihydrofluorescein diacetate; (ATP), Adenosine triphosphate; (GSH), Glutathione; (TLC), Thin layer chromatography; (GC-MS), Gas chromatography mass spectrometry; (C), *C. asiatica* leaf extract; (C3), *C. asiatica* Purified fraction-3; (PBS), Phosphate buffer saline; (IC₅₀), Half maximal inhibitory concentration; (h), Hours; (DCF), 2, 7-dichlorofluorescein; (TBARS), Thiobarbituric acid assay; (MDA), Malondialdehyde; (iROS), Intracellular reactive oxygen species; (min), Minutes; (RT), Room temperature; ($\Delta\psi_m$), Mitochondrial depolarisation; (OD), Optical density; (RLU), Relative light units; (SCGE), Single cell gel electrophoresis; (CTL's), Comet tail lengths; (HSP), Heat shock protein; (RBD), Relative

band density; (*OGG-1*), oxoguanine DNA glycosylase; (MCF-7), Breast cancer cells; (HepG2), Liver cancer; (ETC), Electron transport chain; (8-oxoG), 8-Oxo-7,8-dihydroguanine.

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Bridging paragraph:

In the previous manuscript, C3 elicited anti-proliferative and cytotoxic effects in A549 cells which may have been a consequence of the decreased antioxidant response. Notably, in both cancerous cell lines (THP-1 and A459) C3 decreased the Nrf-2 antioxidant response and elicited anti-proliferative/ cytotoxic effects.

The kidney is a vital organ that functions in many biological processes including the excretion of waste products. Although, medicinal plants/ fractions possess many beneficial effects, excessive consumption of medicinal plants/ fractions may induce renal toxicity. Research into the toxic effects of medicinal plants/ fractions is essential to determine the appropriate dosage of medicinal plants/ fractions being administered. Therefore we investigated the antioxidant and anti-proliferative/ cytotoxic effects of C3 in a kidney cell line (HEK293).

The following manuscript is entitled *Centella asiatica* fraction-3 suppresses the Nrf-2 antioxidant pathway and enhances apoptotic cell death in human embryonic kidney (HEK293) cells (Supplementary information: Appendix 10). In this manuscript, only an IC₅₀ for C3 in HEK293 cells was determined then the antioxidant response signalling and cell death induction were evaluated. The experimental techniques conducted are similar to the previous manuscript.

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CHAPTER SEVEN

***Centella asiatica* purified fraction-3 suppresses the Nrf-2 antioxidant pathway and enhances apoptotic cell death in human embryonic kidney (HEK293) cells**

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Abstract

Centella asiatica (*C. asiatica*), a medicinal plant, is used in Chinese and Ayurvedic medicine due to its vast range of medicinal properties. We investigated the antioxidant and anti-proliferative effects of a chemically semi-purified fraction of *C. asiatica* ethanolic leaf extract (C3) in embryonic kidney (HEK293) cells.

C3 was obtained by silica column fractionation and identified using thin layer chromatography; gas and mass spectrometry determined the bioactive compounds present in crude leaf extract (C) and C3. Cytotoxicity of C3 in HEK293 cells was evaluated (cell viability assay; 24 hours; [0.2–3 mg/ml]) to determine an IC₅₀. Intracellular reactive oxygen species, mitochondrial membrane potential (flow cytometry), malondialdehyde, lactate dehydrogenase (spectrophotometry), reduced glutathione, oxidized GSH, ATP levels, caspases (-8, -9, -3/7) activity (luminometry) and DNA damage (comet assay) were evaluated. Protein expression of Nrf-2, p53, Bcl-2, Bax HSP-70 and gene expression of *Nrf-2*, *GPx*, *SOD*, *CAT*, *c-myc* and *OGG-1* were quantified by western blotting and qPCR, respectively.

C3 dose dependently decreased HEK293 cell viability. The IC₅₀ of C3 significantly increased MDA, mitochondrial depolarisation, LDH, caspase (-8, -9, -3/7) activity and DNA damage ($p < 0.0004$). Nrf-2 protein expression, GSH and GSSG levels were increased whereas antioxidant (*Nrf-2*, *GPx*, *SOD* and *CAT*) gene expression was significantly decreased by C3 ($p < 0.001$). C3 decreased both Bax and Bcl-2 protein expression ($p < 0.03$). Gene expression of *c-myc* was significantly increased whereas *OGG-1* was significantly decreased by C3 ($p < 0.05$).

C3 diminished the antioxidant gene expression and exerted anti-proliferative effects in HEK293.

Key words: *Centella asiatica*, Nrf-2, GSH, SOD, c-myc, caspases

Abbreviations: AKI, Acute kidney failure; ROS, Reactive oxygen species; Nrf-2, Nuclear factor erythroid 2-related factor 2; ARE, Antioxidant response element; KEAP1, Kelch-like ECH-associated protein 1; SOD, Superoxide dismutase; CAT, Catalase; GPx, Glutathione peroxidase; *C. asiatica*, *Centella asiatica*; HEK293, human embryonic kidney cells; C3, Purified fraction-3; SA, South Africa; DCM, Dichloromethane; HEX, Hexane; EA, Ethyl acetate; DMSO, Dimethyl sulfoxide; DMEM, Dulbecco's minimum essential; FCS, Foetal calf serum; PS, Penstrepfungizone; L-GLUT, L-glutamine; WST-1, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; LDH, Lactate dehydrogenase; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide; H2DCF-DA, 2', 7'-dichlorodihydrofluorescein diacetate; ATP, Adenosine triphosphate; GSH, glutathione; TLC, Thin layer chromatography; C, *C. asiatica* crude leaf extract; PBS, Phosphate buffer saline; h, Hours; IC₅₀, Half maximal inhibitory concentration; DCF, 2', 7'-dichlorofluorescein; TBARS, Thiobarbituric acid assay; MDA, Malondialdehyde; IROS, Intracellular reactive oxygen species; Min, Minutes; RT, Room temperature; TCEP, Tris (2-carboxyethyl) phosphine; $\Delta\Psi_m$, Mitochondrial depolarisation; OD, Optical density; RLU, Relative light units; SCGE, Single cell gel electrophoresis; CTL, Comet tail lengths; HSP, Heat shock protein; RBD, Relative band density; OGG-1, oxoguanine DNA glycosylase; ETC, Electron transport chain.

7.1 Introduction

The kidney is a vital organ that functions to excrete waste products and toxins, preserve bodily fluids and electrolytes as well as regulate blood pressure and hormone secretions [1]. Excretion of harmful substances may cause acute kidney injury (AKI) through the interaction of toxins with the glomeruli and tubules of kidneys [1]. Clinically, AKI has been associated with increased morbidity and mortality [1]. In the event of AKI, kidney function is compromised; as a result the maintenance of fluid, electrolyte and acid-base homeostasis are impaired [1]. Additionally, AKI has been related to oxidative stress, a consequence of increased reactive oxygen species (ROS) levels and decreased antioxidant capacity [1]. The modulation of ROS levels is essential in maintaining cellular homeostasis [2,3]. Acute low ROS levels is involved in many biological processes (cellular proliferation and activation of survival pathways) whilst acute high ROS levels cause oxidative damage to cellular components (DNA, lipids, proteins) and induces apoptosis [2,3]. Therefore, an efficient antioxidant defense system is required to combat excessive ROS levels and maintain cellular homeostasis, ultimately reducing tissue injury [2,4].

Nuclear factor erythroid 2-related factor 2 (Nrf-2) is an essential transcription factor in the antioxidant response element (ARE) mediated induction of antioxidant and cytoprotective genes [4]. Under homeostatic conditions, Nrf-2 is bound to its repressor protein, KEAP1 (Kelch-like ECH-associated protein 1) in the cytoplasm, facilitating its ubiquitination [5]. Elevated ROS levels cause oxidative modification of KEAP1, allowing Nrf-2 to dissociate from KEAP1 and translocate to the nucleus where it binds to ARE and increases antioxidant gene transcription [5]. Antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) provide a protective mechanism against oxidative damage [6]. SOD detoxifies superoxide radicals into hydrogen peroxide and molecular oxygen [7]. Subsequently, GPx and CAT detoxifies hydrogen peroxide into oxygen and water [7]. In the event of increased ROS production and decreased antioxidant capacity, ROS accumulation leads to oxidative stress and apoptosis.

Caspase activity regulates apoptosis through two representative pathways namely the intrinsic (or mitochondrial) and extrinsic (or death receptor) [8]. The mitochondrial pathway leads to apoptosome formation (cytochrome c binding Apaf-1 and pro-caspase-9) and caspase 9 activation [8] whilst the death receptor pathway lead to caspase-8 activation [8]. Caspases -8 and -9 activate executioner caspase-3/7, ultimately activating cytoplasmic endonucleases which degrade nuclear material [9].

In Asian and African countries, about 80% of the population depends on traditional medicine for the treatment and prevention of diseases [10]. Medicinal plants have been shown to be a safe, healthy, effective and inexpensive treatment option for various oxidative stress related diseases [1]. Through reduced ROS levels and enhanced antioxidant capacity, medicinal plants can protected against oxidative renal damage [1]. The tropical medicinal plant *Centella asiatica* (*C. asiatica*) is a part of the Apiaceae family and is native to India, China and South Africa. Common names of *C. asiatica* include Gotu kola, Asiatic/ Indian pennywort, and Tiger herb [11]. *C. asiatica* is frequently used in Ayurvedic and Chinese traditional medicines due to its many pharmacological properties such as hepato-protective, cardio-protective, anti-diabetic, antioxidant, anti-inflammatory and anti-tumour potential [11]. The most prominent group of active compounds isolated from *C. asiatica* is triterpene saponosides (asiatic acid, madecassic acid, asiaticoside, madecassoside and madasiatic

acid). Additionally, *C. asiatica* contains flavonoid derivatives (quercetin, kaempferol, patuletin and rutin), vitamins, minerals, polysaccharides, sterols, and phenolic acids [11]. Previously, toxicity testing has proven that *C. asiatica* dried plant is safe for consumption [12]. In addition, *C. asiatica* standardized extract [ECa 233, (10 g/kg)] showed no sign of toxicity and no lethality [12]. The non toxic nature of *C. asiatica* may be attributed to its high antioxidant activity (84 %) which is comparable to vitamin C (88 %) and grape seed extract (83 %) [13-16]. Although, *C. asiatica* extracts (aqueous and methanolic) have shown anti-proliferative/ cytotoxic activity against a number of cancerous cell lines [17,18]. *C. asiatica* aqueous extract was not cytotoxic in the normal hamster kidney cell line [17].

We hypothesized that an increase in the Nrf-2 antioxidant pathway may protect against oxidant induced cell death. The present study aimed to investigate the effect of *C. asiatica* semi-purified fraction-3 (C3) on antioxidant response signalling and apoptosis induction in human embryonic kidney cells (HEK293).

7.2 Materials and Methods

7.2.1 Materials

C. asiatica leaves were collected in March 2011 (collectors number: Immelman 411) from the Eastern Cape [Langeni forest, roadside (S31°28.135', E28°32.681')], South Africa (SA) and identified by Dr. Kathleen Immelman (Department of Botany at the Walter Sisulu University, SA). Voucher specimens were deposited at the KEI herbarium (13979). HEK293 cells were obtained from Highveld Biologicals (Johannesburg, SA). Solvents [Dichloromethane (DCM), Hexane (HEX), ethyl acetate (EA), methanol, Dimethyl sulphoxide (DMSO)] sulphuric acid, silica gel 60, thin layer chromatography (TLC) silica gel 60 sheets and 4-methoxybenzaldehyde were purchased from Merck (Johannesburg, SA). Dulbecco's minimum essential medium (DMEM), foetal calf serum (FCS), penstrepfungizone (PS) and L-glutamine (L-GLUT) were acquired from Whitehead scientific (Cape Town, SA). The 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) cell proliferation reagent and lactate dehydrogenase (LDH) cytotoxicity detection reagent were purchased from Roche (Mannheim, Germany or Johannesburg, SA). The 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolcarbocyanine iodide (JC-1) kit and the 2', 7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) reagent were purchased from BD Biosciences (Johannesburg, SA). Caspase (-8, -9, -3/7), adenosine triphosphate (ATP) and

glutathione (GSH) kits were acquired from Promega (Madison, USA). Western blot reagents were purchased from Bio-Rad (Johannesburg, SA) and qPCR primers were obtained from Inqaba Biotech.

7.2.2 Plant Extraction and Fractionation

The plant's official name is *Centella asiatica* (L.) Urb and has been confirmed by using the plant list (www.theplantlist.org). The local names are icudwane (Zulu) and varkoortjies (Afrikaans). The English names are tiger herb and pennywort. *C. asiatica* leaves were dried and milled. Ethanol (200–350 ml) was added to milled plant material (10–30 g) and extracted overnight by shaking (4 x g, 37 °C). Ethanol extracts were filtered, rotor evaporated, dried (37 °C) and stored (4°C). Silica column fractionation was used to separate *C. asiatica* compounds based on their polarity. A column (height: 85 cm, diameter: 17 cm) was assembled by adding a tap (25 mm), a cotton wool stop, a thin layer of sand, carefully pouring silica dissolved in DCM: HEX (50: 50) into a column and allowing the silica to set overnight. *C. asiatica* crude extract (17 g) was dissolved in DCM: HEX (70: 30) and a portion of the extract was further dissolved in methanol. Thereafter, *C. asiatica* crude extract was layered onto the silica and 50 ml fractions were collected using a series of solvent systems [DCM: HEX (70: 30), DCM: EA (70: 30), DCM: EA (50: 50), DCM: EA: methanol (50: 40: 10), DCM: EA: methanol (30: 50: 20), EA: methanol (70: 30), EA: methanol (40: 60), EA: methanol (20: 80), methanol (100)]. The 50 ml fractions were boiled (100 °C) to allow evaporation of excess solvent and TLC was used to determine spot patterns and solvent changes. A total of 766 fractions were collected and TLC analysis revealed fractions with similar spot patterns which were combined and pooled into one fraction. Fractionation of *C. asiatica* yielded 13 pooled fractions. For all subsequent experiments, *C. asiatica* crude extract (C) and pooled fraction-3 (C3) were used.

7.2.3 Gas chromatography mass spectrometry (GC-MS)

C. asiatica (C and C3) chemical composition has been subjected to GC-MS (Hewlett-Packard, USA) and the GCD-HP1800A system was used. Ionization energy (70 eV) was used for the detection of GC-MS and the detection was passed under high vacuum (10⁻⁴ to 10⁻⁸ torr). Helium gas was used for the analysis at a constant flow rate (1 mL/min). The mass transfer line and injector

temperature were fixed at 280 and 250 °C. The bioactive compounds which are present in *C. asiatica* (C and C3) were matched with the NIST computer library and reported.

7.2.4 Tissue Culture

HEK293 cells were grown in the appropriate tissue culture conditions in a 25 or 75 cm³ tissue culture flasks (37 °C, 5% CO₂). The HEK293 cells were cultured in DMEM supplemented with 10% FCS, 1% PS and 1% L-GLUT. Cells were seeded, allowed to attach overnight and for all subsequent assays the cells were treated at 80% confluency.

7.2.5 Cell Viability Assay

Cytotoxicity of *C. asiatica* (C and C3) leaf extract to HEK293 cells was measured using the WST-1 assay (Roche, Johannesburg, SA). HEK293 (20,000 cells/well, 96 well plate, in triplicate wells) were allowed to attach overnight (37 °C, 5% CO₂). At 80% confluency, cells were washed twice with 0.1 M phosphate buffer saline (PBS) before exposure to *C. asiatica* [C and C3 (0.2–3 mg/ml)] for 24 hours (h) (37 °C, 5% CO₂). Controls received media containing DMSO (0.750%). Following 24 h treatment, cells were washed twice with 0.1 M PBS, growth media (100 µl/well), WST-1 reagent (10 µl/well) was added and plates incubated (37 °C, 5% CO₂, 3 h). The optical density was measured at 450 nm (620 nm reference wavelength) with a BIO-TEK µQuant spectrophotometer (Analytical and Diagnostic Products, SA). This experiment was conducted on two separate occasions before using GraphPad Prism to calculate the half maximal inhibitory concentration (IC₅₀) [percentage cell viability vs log concentration of *C. asiatica* (C and C3)]. *C. asiatica* crude extract did not yield an IC₅₀ in HEK293 cells. However, an IC₅₀ of 0.984 mg/ml was obtained in C3 treated HEK293 cells and was used in all subsequent experiments. Control treatments were represented by an equal amount of DMSO (0.246%) in growth media without C3.

7.2.6 Treatment of cells

HEK293 cells (2 x 10⁵ cells/ml) were aliquoted into 25 cm³ tissue culture flask and allowed to attach overnight (37 °C, 5% CO₂). At 80% confluency, cells were washed twice with 0.1 M PBS before exposure to C3 (0.984 mg/ml) for 24 h (37 °C, 5% CO₂). Controls received media containing DMSO (0.246%). After 24 h treatment, cells were washed twice with 0.1 M PBS and were used to conduct the JC-1, 20, 70-dichlorofluorescein (DCF), GSH, GSSG, caspase (-8, -9, -3/7) activity,

ATP, comet assays; as well as protein and RNA isolation. The experiments were conducted independently two times for all subsequent assays.

7.2.7 Thiobarbituric acid assay

The thiobarbituric acid assay (TBARS) measures malondialdehyde (MDA), the end product of lipid peroxidation and was conducted as per the method previously described by Phulukdaree *et al* (2010) [19]. Optical density was measured at 532 nm (600 nm reference wavelength) with a BIO-TEK μ Quant spectrophotometer (Analytical and Diagnostic Products, SA). Malondialdehyde concentration was calculated by dividing the optical density (OD) for each sample by the absorption coefficient (156 mM^{-1}).

7.2.8 The DCF assay

The DCF assay measures intracellular ROS (IROS) and was conducted as per the method previously described by Nagiah *et al* (2015) [20]. Events were gated and the fluorescence measured on FL-1 channel (525 nm) of the AccuriTMC6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). A total of 50,000 events were acquired and analyzed using the CFlow Plus Software (BD Biosciences, San Jose, CA, USA).

7.2.9 The GSH assay

The GSH-GloTM assay (Promega, Madison, WI, USA) was used to measure GSH and GSSG levels. The assay was conducted as per the method previously described by Nagiah *et al* (2015) [20]. Luminescence was measured on a ModulusTM microplate luminometer (Turner BioSystems, Sunnyvale, CA, USA). GSH and GSSG concentrations (μM) were determined by extrapolation from a standard curve.

7.2.10 The JC-1 Mitoscreen assay

The JC-1 assay measures percentage mitochondrial depolarization ($\Delta\psi_m$) and was conducted as per the method previously described by Nagiah *et al* (2015) [20]. Scatter plots of 50,000 events per

sample were acquired using the AccuriTMC6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using the CFlow Plus Software (BD Biosciences, San Jose, CA, USA).

7.2.11 The LDH assay

The LDH cytotoxicity detection kit (Roche) was used to measure cell damage by C3. Stress compromises cell membrane integrity and leads to the leakage of LDH from cells. LDH activity in the supernatant (100 μ l/well; triplicate wells) was quantified by addition of LDH reagent (100 μ l/well) and incubation (25 min, RT). Optical density was measured at 500 nm with a BIO-TEK μ Quant spectrophotometer (Analytical and Diagnostic Products, SA). Results are represented as mean OD.

7.2.12 Caspase and ATP assays

Caspase (-8, -9, -3/7) activities and ATP levels were determined using the Caspase-Glo[®]3/7, -8, -9 and ATP assay kits (Promega, Madison, USA). Caspase-Glo[®]3/7, -8, -9 and ATP reagents were reconstituted according to the manufacturer's instructions. Cells (50 μ l/well, 2×10^5 cells/ml) were added into triplicate wells of a microtitre plate for each assay, thereafter caspase -3/7, -8, -9 and ATP reagent (25 μ l/well) was added into appropriate wells. Plates were incubated (30 min, RT) in the dark. Luminescence was measured on a ModulusTM microplate luminometer (Turner BioSystems) and expressed as relative light units (RLU).

7.2.13 Single cell gel electrophoresis (SCGE) or COMET assay

The SCGE assay was performed to assess DNA fragmentation and was conducted as per the method previously described by Singh *et al* (1988) [21]. Cells were stained with GR red (1 μ l, 1000x) instead of ethidium bromide. Slides were viewed using an Olympus IX7I microscope with 510–560 nm excitation and 590 nm emission filters (Wirsam Scientific, Johannesburg, SA). Images from triplicate slides per treatment were captured. Comet tail lengths (CTL's) of 50 comets per treatment were measured using the Soft imaging system (Life Science - ©Olympus Soft Imaging Solutions v5) and average CTL's were calculated (μ m).

7.2.14 Western Blotting

Western Blotting was performed to determine relative protein expressions. Cytobuster (200 µl, Novagen, San Diego, CA, USA) supplemented with protease and phosphatase inhibitors (Roche, Mannheim, Germany) was added to the cells (10 min, 4°C) and centrifuged (13000 x g, 4 °C, 10 min). Protein was quantified using the bicinchoninic acid assay (Sigma, Germany) and standardized to 1 mg/ml. Western blotting was conducted as per the method previously described by Nagiah *et al* (2015) [20]. Membranes were immune-probed with Nrf-2 (ab89443), p53 (CS9289), Bax (CS5023), Bcl-2 (CS2827), heat shock protein (HSP)-70 (CS4872) primary antibodies (1:1000, Cell Signaling, Danvers, USA). Anti-rabbit (sc-2054) or anti-mouse (ab97046) were the secondary antibodies used (1:5,000). Horse radish peroxidase chemiluminescence detector (Bio-Rad) and enhancer solution were used for the antigen-antibody complex and the signal was detected with the Alliance 2.7 image documentation system (UViTech). To correct for loading error and to normalise relative protein expression, β-actin was assessed (A3854; 1:5000). The expression of the proteins were analyzed with UViBand Advanced Image Analysis software v12.14 (UViTech) and data was expressed as relative band density (RBD) and fold change.

7.2.15 Quantitative PCR

Total RNA was isolated using an in-house protocol [22]. RNA was quantified (Nanodrop2000, ThermoScientific, South Africa) and standardized (800 ng/ml). A reaction volume (10 µl) containing RNA template (2 µl), 5X iScript™ reaction mix (2 µl), iScript reverse transcriptase (0.5 µl) and nuclease free water (5.5 µl) was used to synthesize cDNA (iScript™ cDNA Synthesis kit, Bio-Rad, 107e8890). Thermocycler conditions were 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min and a final hold at 4 °C.

Gene expression of *SOD2* [Sense 5'-GAGATGTTACACGCCAG TAGC-3'; Antisense 5-AATCCCCAGCAGTGGGAATAAGG-3'(57 °C)], *CAT* [Sense 5'-TAAGACTGACCAGGGC-3'; Antisense 5'-CAACCTTGGTGAGATCGAA- 3'(58 °C)], *GPx* [Sense 5'-GACTACACCCAGATGAACGAGC- 3'; Antisense 5'-CCCACCAGGAA CTTCTCAAAG- 3'(58 °C)], *Nrf-2* (forward 5'-AGTGGATCTGCCAACTACTC-3'; reverse 5'-CATCTACAAACGGGAATGTCTG-3' (58 °C)), 8-oxoguanine DNA glycosylase (*OGG-1*) (forward 5'-GCATCGTACTCTAGCCTCCAC-3'; reverse 5'-AGGACTTTGCTCCCTCCAC-3' (60 °C)) and *c-myc* (forward 5'-AGCGACTCTGAGGAGGAACAAG-3'; reverse 5'-

GTGGCACCTCTTGAGGACCA-3' (56 °C)) were evaluated using the iQ™ SYBR® Green PCR kit (Bio-Rad; 170e880). The final reaction volume was 10.5 µl [6.25 µl 5X iScript reaction mix, 0.5 µl sense primer, 0.5 µl anti-sense primer, 3.25 µl nuclease-free water, and 2 µl cDNA sample (triplicate wells per sample)] and all assays were carried out using CFX Touch™ Real Time PCR Detection System (Bio-Rad). The reaction included an initial denaturation (95 °C, 4 min), followed by 37 denaturation cycles (95 °C, 15sec), annealing (primer specific temperature, 40sec), extension (72 °C, 30sec) and a plate read for 37 cycles. Under the same conditions, the *β-actin* [Sense 5'-TGACGGGTCACCCACACTGTGCCCAT-3'; Antisense 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'] and *18S*: (Sense: 5'-ACAGGGACAGGATTGACAGA-3'; Antisense: 5'-CAAATCGCTCCACCAACCTAA-3') housekeeping genes were run. Using the method described by Livak and Schmittgen, qPCR results were analyzed, represented as fold change ($2^{-\Delta\Delta CT}$) relative to the house keeping genes (*β-actin* and *18S*) and control [23].

7.2.16 Statistical Analysis

Statistical analysis was performed using the GraphPad Prism (v5) statistical analysis software. Statistical significance was set at 0.05 and comparisons were made using unpaired t tests with Welch correction.

7.3 Results

7.3.1 Chemical composition of C and C3

The GC-MS chromatograms of C and C3 were recorded and presented in Supplementary Figures 4 and 5. Chemical composition of C and C3 constituents were identified after comparison with those available in the NIST computer library. The organic compounds that were identified in *C. asiatica* leaf extract namely caranol, oxymetholone methanol adduct, vitamin E, tocopherol and terpenes (phytosterol and stigmasterol) (Supplementary information: Table 1 and 2).

7.3.2 Cytotoxicity of C3 against HEK293 cells

The WST-1 assay showed that HEK293 cell viability was not significantly altered by C however dose dependently decreased by C3 with an IC_{50} value of 0.984 mg/ml (Figure 7.1). The IC_{50} concentration was used in all subsequent experiments.

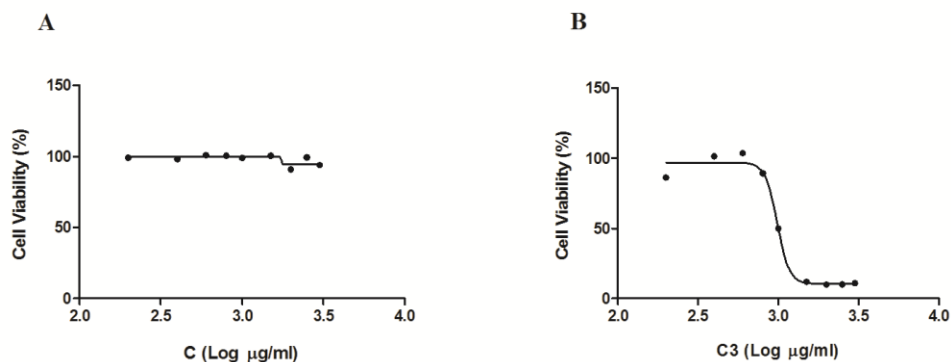


Figure 7.1: *C. asiatica* crude extract [0.2–3 mg/ml] did not influence HEK293 cell viability whereas purified fraction-3 [0.2–3 mg/ml] induced a dose dependent decrease in HEK293 cell viability; IC_{50} value of 0.984 mg/ml.

7.3.3 The effect of C3 on oxidative stress and antioxidant mechanisms in HEK293 cells

The effect of C3 on lipid peroxidation and IROS production was assessed by the TBARS and DCF assays. C3 increased MDA levels 1.319-fold as compared to the control ($p = 0.0003$, Figure 7.2A). Interestingly, IROS levels were not influenced by C3 relative to the control ($p = 0.7423$, Figure 7.2B). Further the overall antioxidant effects of C3 were determined by measuring the concentration of GSH and GSSG, protein expression of Nrf-2 as well as gene expression of *Nrf-2*, *GPx*, *SOD* and *CAT*. Semi-purified fraction-3 significantly increased both GSH (1.885-fold, $p = 0.0002$, Figure 7.2C) and GSSG (3.544-fold, $p < 0.0001$ Figure 7.2D) concentration compared to the control.

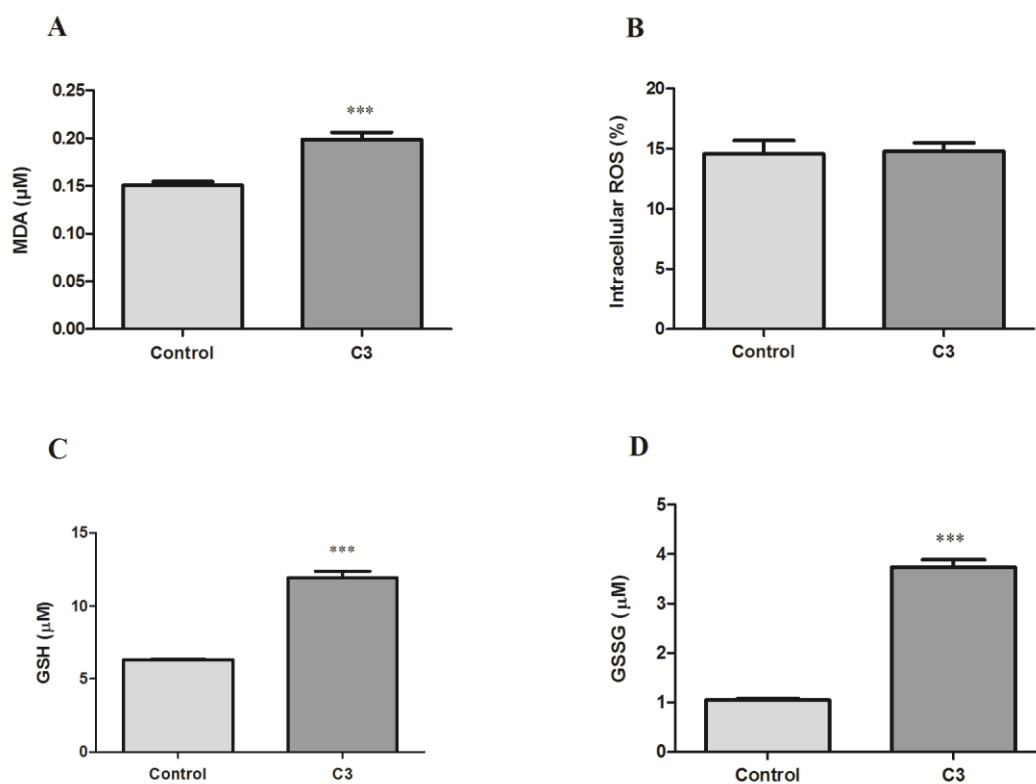


Figure 7.2: Levels of MDA (A), IROS (B), GSH (C) and GSSG (D) in C3 treated HEK293 cells (Values expressed as mean \pm SD, *** $p < 0.0001$, compared to the control).

Nrf-2 protein expression was slightly increased by C3 relative to the control (1.056-fold, $p = 0.0009$ Figure 7.3).

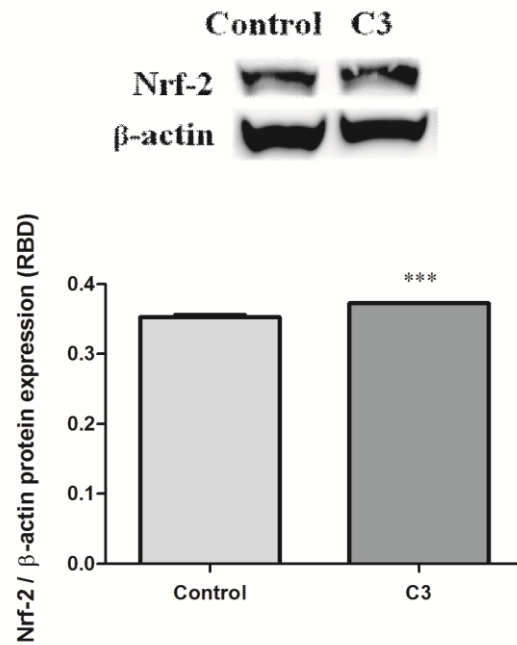


Figure 7.3: Protein expression of Nrf-2 in C3 treated HEK293 cells (Values expressed as mean \pm SD, *** $p < 0.0001$, compared to the control).

Gene expression of *Nrf-2* (2.076-fold, $p < 0.0001$), *GPx* (2.629-fold, $p = 0.0002$), *SOD* (3.621-fold, $p < 0.0001$) and *CAT* (6.425-fold, $p < 0.0001$) were significantly decreased by C3 relative to the control (1.00 fold) (Figure 7.4).

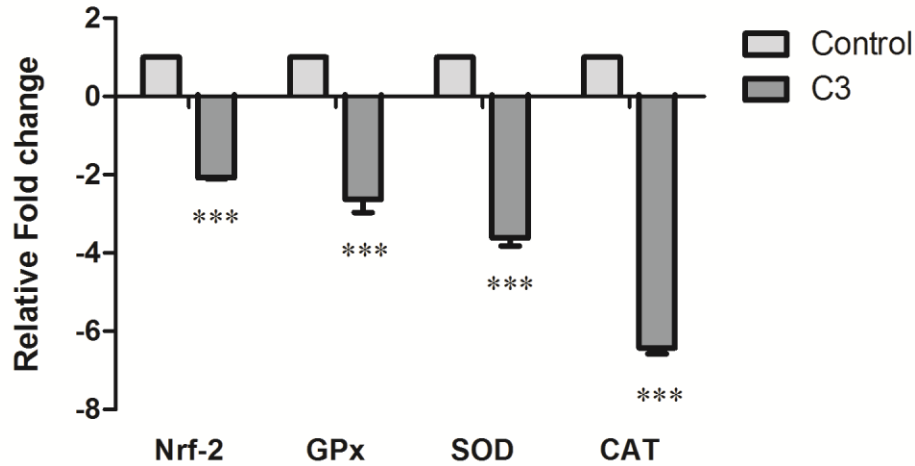


Figure 7.4: Gene expression of *Nrf-2*, *GPx*, *SOD* and *CAT* in C3 treated HEK293 cells (Values expressed as mean \pm SD, *** $p < 0.0001$, compared to the control).

7.3.4 C3 induced cell death pathways in HEK293 cells

The JC-1 and LDH assay were used to determine $\Delta\psi_m$ and cell membrane permeability respectively. C3 significantly increased $\Delta\psi_m$ 3.812-fold compared to the control ($p < 0.0001$, Figure 7.5A). LDH levels were also increased by C3 relative to the control (1.180-fold, $p < 0.0001$, Figure 7.5B).

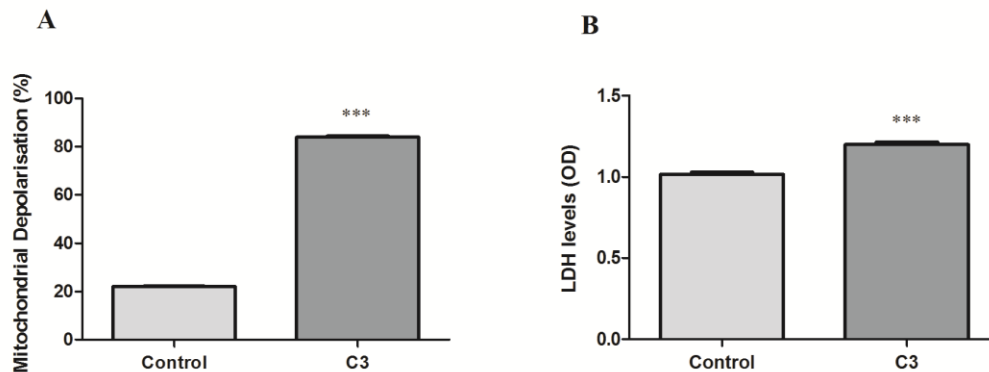


Figure 7.5: Mitochondrial depolarisation (A) and LDH (B) levels in C3 treated HEK293 cells (Values expressed as mean \pm SD, *** $p < 0.0001$, compared to the control).

Caspase activity was measured to evaluate the initiation and execution of apoptosis by C3. Semi-purified fraction-3 significantly increased caspase [-8 (1.576-fold), -9 (2.014-fold) -3/7 (1.391-fold)] activity whilst ATP levels were significantly decreased compared to their respective controls ($p < 0.0001$, Table 7.1).

Table 7.1: Caspase activity (-8, -9, -3/7) and ATP levels in C3 treated HEK293 cells (Values expressed as mean \pm SD, *** $p < 0.0001$, compared to the control).

	Caspase-8 (RLU x 10 ⁵)	Caspase-9 (RLU x 10 ⁵)	Caspase-3/7 (RLU x 10 ⁵)	ATP (RLU x 10 ⁵)
Control	0.759 \pm 0.020	9.610 \pm 0.300	0.274 \pm 0.001	3.075 \pm 0.052
C3	1.195 \pm 0.029 ***	19.35 \pm 0.216 ***	0.381 \pm 0.001 ***	0.467 \pm 0.027 ***

Cell death was further investigated by assessing anti-apoptotic and pro-apoptotic protein expression. Protein expression of *p53* ($p = 0.0775$) and HSP-70 ($p = 0.4693$) were not influenced by C3 relative to their respective controls (Figure 7.6). Bax (1.397-fold, $p = 0.0202$) and Bcl-2 (1.668-fold, $p = 0.0004$) protein expressions were decreased by C3 compared to their respective controls (Figure 7.6).

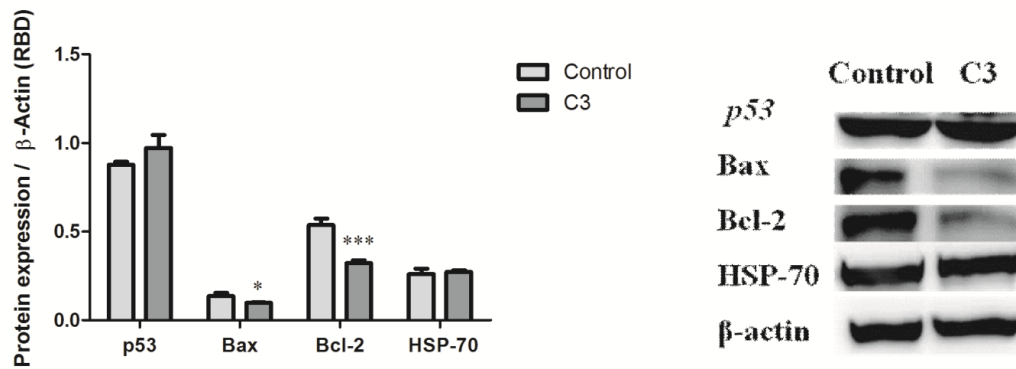


Figure 7.6: Protein expression of *p53*, Bax, Bcl-2 and HSP-70 in C3 treated HEK293 cells (Values expressed as mean \pm SD, * $p < 0.05$, *** $p < 0.0001$, compared to the control).

The SCGE assay was used determine the amount of DNA damage caused by C3. CTL's were significantly increased by C3 relative to the control (1.303-fold, $p < 0.0001$, Figure 7.7).

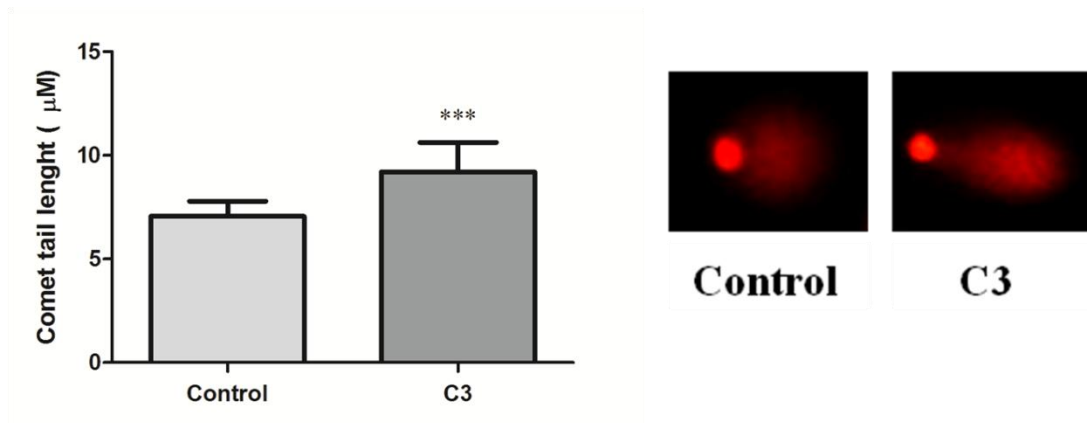


Figure 7.7: DNA fragmentation in C3 treated HEK293 cells (Values expressed as mean \pm SD, *** $p < 0.0001$, compared to the control).

C3 significantly increased *c-myc* gene expression (25.27-fold, $p < 0.0001$, Figure 7.8A) whereas significantly decreased *OGG-1* gene expression (7.650-fold, $p = 0.0004$, Figure 7.8B) compared to their respective controls (1.00 fold).

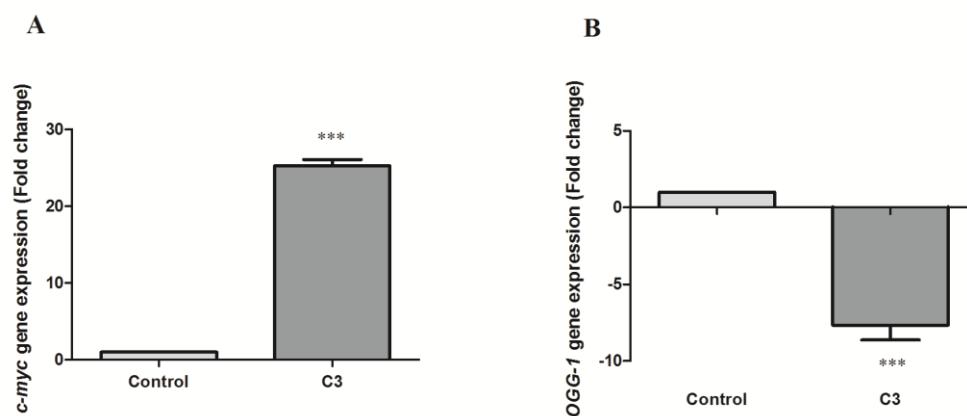


Figure 7.8: Gene expression of *c-myc* (A) and *OGG-1* (B) in C3 treated HEK293 cells (Values expressed as mean \pm SD, *** $p < 0.0001$, compared to the control).

7.4 Discussion

There is an increasing use of medicinal plants in the treatment of various diseases [24]. However, medicinal plant extracts also have the potential to cause renal toxicity [24]. Therefore the toxicity of medicinal plants should be evaluated so that the benefits can be weighed against the risks to determine the safe usage and availability of medicinal plants [24].

Previous studies have indicated that the consumption of *C. asiatica* induced no sign of acute toxicity [12]. Additionally, *C. asiatica* aqueous extract induced no cytotoxic activity in the normal hamster kidney cell line [17]. Our results are consistent with previous studies, demonstrating that C did not significantly influence HEK293 cell viability. C3 on the other hand caused a dose dependant decrease in HEK293 cell viability.

C. asiatica leaf extracts have shown excellent free radical scavenger capabilities [hydroxyl, superoxide (86.4 %) and DPPH (92.7 %)] [14,25] and lipid peroxidation preventive properties. Our findings, however, showed that C3 increased lipid peroxidation but did not influence IROS levels. In eukaryotic cells, GSH is the most abundant antioxidant and is a major player in cellular redox regulation [26]. GSH is oxidized to GSSG during ROS detoxification and the intracellular GSH/GSSG ratio is a vital predictor of the antioxidant capacity [26]. In HEK293 cells, C3 increased GSH and GSSG levels. Our findings indicate that C3 increased the antioxidant capacity in HEK293 cells as evidenced by the increased GSSG levels. The unchanged IROS levels may have been a consequence of the increased antioxidant capacity.

GSH production and utilization is controlled by Nrf-2 mediated expression of glutathione *S*-transferases and GPx [2,27]. Our results demonstrated that C3 slightly increased Nrf-2 protein expression in HEK293, corresponding with the increased GSH and GSSG levels.

Anti-cancerous Nrf-2 activated antioxidant and cytoprotective genes protects normal cells from oxidative damage and cancer initiation [6]. Our results demonstrated that C3 decreased *Nrf-2*, *GPx*, *SOD* and *CAT* gene expression indicating a decrease in the Nrf-2 antioxidant pathway. Interestingly, GSH and GSSG concentrations were increased by C3, suggesting that C3 provides an increased antioxidant capacity independent of the Nrf-2 pathway in HEK293 cells.

A decrease in antioxidant mechanisms such as the Nrf-2 pathway leads to oxidative damage and cell death [2]. C3 significantly increased $\Delta\psi_m$, a possible consequence of a decreased antioxidant gene expression. Increased $\Delta\psi_m$ significantly induced caspase (-8, -9, -3/7) activation, strongly suggesting that both the intrinsic and extrinsic apoptotic pathways were initiated and executed. The mitochondrial protein gradient couples the electron transport chain (ETC) to produce ATP [28]. However, increased $\Delta\psi_m$ disrupts the ETC and uncouples ATP production [28]. Additionally, the activation of ATP dependent caspases (-8, -9, -3/7) may decrease ATP levels. Our findings demonstrated that C3 decreased ATP levels, consistent with the increased $\Delta\psi_m$ and caspase activation. The decrease in ATP levels shifts cell death from apoptosis to necrosis [29]. In HEK293

cells, LDH levels were increased, indicating that the cellular membrane integrity was compromised. Taken together, C3 induced significant apoptosis and possible necrosis in HEK293 cells.

The Bcl-2 family of proteins is responsible for maintaining mitochondrial membrane potential [30]. Anti-apoptotic members such as Bcl-2 stabilizes transmembrane potential, and inhibits pro-apoptotic protein release [30] whereas pro-apoptotic members such as Bax integrates into the outer mitochondrial membrane resulting in membrane permeabilization and pro-apoptotic protein release [31]. In HEK293 cells, protein expression of Bax and Bcl-2 were decreased by C3. Notably, C3 decreased the protein expression of Bcl-2 to a greater extent than Bax, suggesting that Bcl-2 anti-apoptotic activity is decreased which corresponds with the elevated $\Delta\psi_m$ and caspase activation.

Tumor suppressor *p53* has many biological functions including the maintenance of genetic integrity, cell cycle arrest and apoptosis [32]. In normal cells, *p53* expression is maintained at low levels. Our results indicated that C3 did not influence *p53* protein expression in HEK293 cells. In healthy cells, HSP's provide a protective mechanism against apoptotic cell death by inhibiting apoptosome formation and caspase activation [31]. Our findings, however demonstrated that HSP-70 protein expression was not influenced by C3.

Cell death pathways activate cytoplasmic endonucleases [9] which degrade nuclear material [9]. C3 increased DNA fragmentation in HEK293 cells, consistent with the increased cell death.

Oxidative DNA damage leads to DNA adduct formation [33]. The base excision repair enzyme, *OGG-1* excises and repairs DNA adducts [33]. Our results indicated that C3 significantly decreased *OGG-1* gene expression thus decreasing DNA repair.

The *c-myc* transcription factor regulates the expression of genes, involved in cell proliferation, growth and transformation [34]. In normal cells, *c-myc* plays an essential role in proliferation which is linked to growth factor stimulation [35]. Upon growth stimulation, *c-myc* is activated and remains elevated encouraging continuous growth of normal cells [35]. On the other hand, *c-myc* may activate apoptosis via two pathways [36]. The first pathway involves the up-regulation of *p53* and Bax promoting pro-apoptotic protein release [36]. The second pathway is *p53*-independent, involving the disruption of mitochondrial membrane integrity and the release of pro-apoptotic proteins [36]. Both apoptotic pathways converge at the mitochondria resulting in apoptosome formation and caspase-9 activation [36]. Our results demonstrated that C3 significantly increased *c-*

myc gene expression which corresponds with the elevated $\Delta\psi_m$ and caspase activation, suggesting an increase in apoptosis.

Additionally, *c-myc* replaces Nrf-2 at the ARE and decreases antioxidant gene transcription [37]. As a result, *c-myc* over expression decreases intracellular antioxidant expression and induces oxidative DNA damage [38]. In C3 treated HEK293 cells, the decreased antioxidant gene expression may have been a consequence of the substantially elevated *c-myc* gene expression.

7.5 Conclusion

Medicinal plants may exert renal toxicity therefore it is essential to compile scientific information regarding their potential toxicity [1,24]. In HEK293 cells, C3 decreased the antioxidant gene expression however C3 also functions as a natural antioxidant. Purified fraction-3 exerts anti-proliferative activity in HEK293 cells through increased apoptotic cell death which suggests that C3 is potentially toxic to kidney cells.

7.6 Disclosure statement

The author(s) declare that they have no competing interests.

7.7 Role of Funding Source

The National Research Foundation, the South African Medical Research Council and Department of Science and Technology were not involved in study design, collection of samples, analysis of data, interpretation of data, writing of the report and decision to publish. Scientific out-put is a requirement of the National Research Foundation.

7.8 Acknowledgements

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CHAPTER EIGHT

Discussion and Conclusion

Globally, cancer is a leading cause of morbidity and mortality. Cancerous cells facilitate uncontrolled cell proliferation, evasion of apoptosis, invasion, angiogenesis and metastasis [1,2]. Cancer cachexia is a complex condition that occurs in up to one half of all cancer patients [3]. It is characterized by the abnormal loss of body weight [4] through adipose tissue and skeletal muscle depletion [5]. The role of inflammation in carcinogenesis has been extensively documented [6]. Increased pro-inflammatory cytokine levels initiate metabolic events such as increased lipid and protein degradation which contributes to tissue wasting [7,8]. Additionally, increased NF- κ B expression contributes to inflammation-induced carcinogenesis as well as the cachectic syndrome [8,9].

W. somnifera has demonstrated pharmacological value as an antioxidant, anti-inflammatory and anti-tumour agent [10]. The leaves and roots of *W. somnifera* is a good source of antioxidants [11]. Previously in the skin and liver, *W. somnifera* was shown to significantly increase antioxidant enzymes as well as inhibit lipid peroxide levels [12]. Additionally, *W. somnifera* extract suppressed LPS stimulated cytokine (TNF- α and IL-1 β) production in rheumatoid arthritis patients [13].

This study demonstrated that W_{RE} increased DPPH oxidant scavenging activity, but decreased GSH levels in PBMC's and THP-1 cells (72 h). The PBMC TNF- α and IL-10 levels were decreased whereas IL-1 β levels was increased by varying concentrations of W_{RE} . In cancerous THP-1 cells, W_{RE} decreased TNF- α , IL-1 β and IL-6 levels. The potential of W_{RE} to decrease pro-inflammatory cytokine levels in PBMC's and THP-1 cells suggests that it has potential to decrease lipolysis and proteolysis, ultimately decreasing the development and progression of cancer cachexia (Figure 8.1).

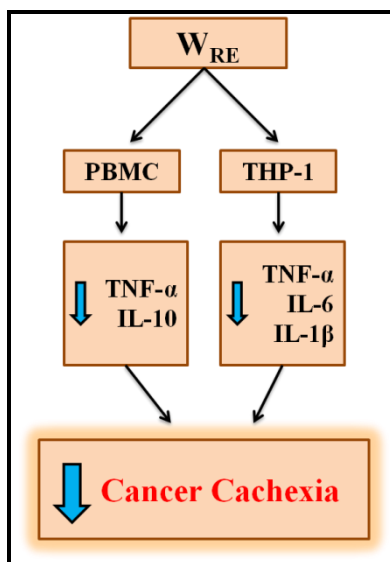


Figure 8.1: *W. somnifera* decreases cancer cachexia associated pro-inflammatory cytokine levels in PBMC's and THP-1 cells (Prepared by author).

W. somnifera root extracts were shown to inhibit tumour growth [14], inhibit skin carcinogenesis in mice [15] and elicit anti-angiogenic effects against human laryngeal carcinoma [16]. Additionally, Malik *et al* (2009) demonstrated that WSF activates both the intrinsic and extrinsic apoptotic pathways in HL-60 cells [17]. Our results showed that at 24 h W_{RE} (0.4–0.8 mg/ml) treatment increased PBMC caspase (-8, -3/7) activities as well as THP-1 caspase (-9, -3/7) activities, suggesting that W_{RE} induced extrinsic apoptosis in normal PBMC's whereas intrinsic apoptosis in THP-1 cells (Figure 8.2). At 72 h, W_{RE} (0.4–0.8 mg/ml) decreased PBMC caspase (-8, -9) activities and THP-1 caspase (-8, -9, -3/7) activities, indicating a dampened initiation and execution of apoptosis (Figure 8.2).

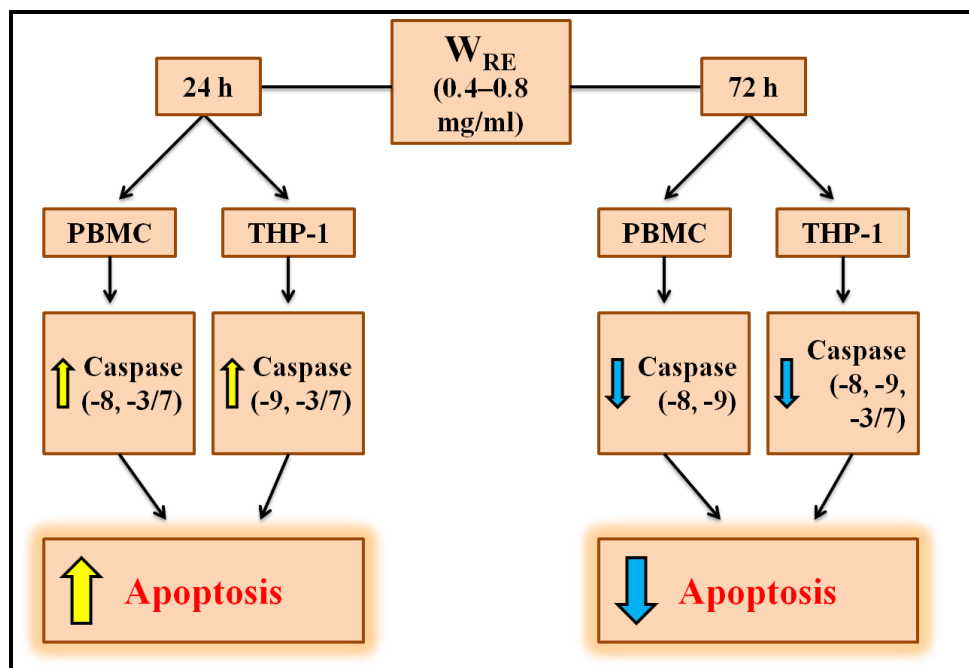


Figure 8.2: *W. somnifera* modulates apoptotic cell death in PBMC's and THP-1 cells (Prepared by author).

The medicinal plant, *C. asiatica* elicits many health benefits including antioxidant [18,19], anti-inflammatory and anti-tumour activity [20,21]. Previous studies have indicated the safe usage, minimal toxicity and effectiveness of *C. asiatica* [22]. Over the years, the usage of *C. asiatica* in food and beverages has increased due to its various health benefits [20]. *C. asiatica* has been used in the treatment of inflammation [23] as well as cancer [24]. The ethanolic *C. asiatica* extract was shown to suppress iNOS and TNF- α expression which correlated with the decrease in NO and TNF- α protein levels [25]. Additionally, TNF- α production was increased by *C. asiatica* aqueous extract but decreased by the ethanolic extract in PBMC's [26].

In this study, C_{LE} increased DPPH oxidant scavenging activity with a concomitant increase in GSH concentrations in PBMC's and THP-1 cells (0.05–0.2 mg/ml, 24 and 72 h). Varying C_{LE} concentrations decreased PBMC TNF- α and IL-10 levels whereas increased and decreased PBMC IL-6 and IL-1 β levels respectively. In THP-1 cells, C_{LE} decreased IL-1 β and IL-6 whereas increased

IL-10 levels. The potential of C_{LE} to decrease pro-inflammatory cytokines levels while increasing anti-inflammatory cytokine levels in THP-1 cells may lead to a decrease in NF- κ B expression, lipolysis and proteolysis, ultimately alleviating the cancer cachectic syndrome (Figure 8.3).

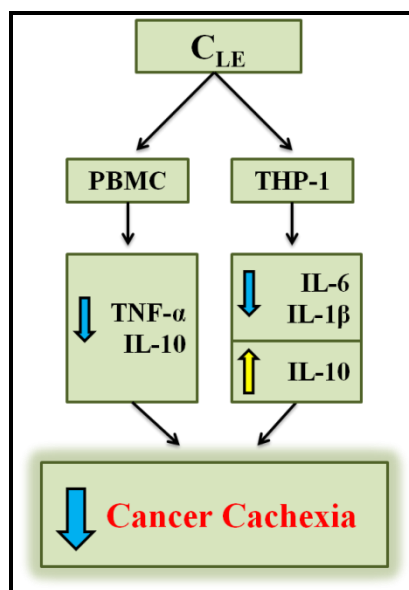


Figure 8.3: *C. asiatica* decreases cancer cachexia associated pro-inflammatory cytokine levels in PBMC's and THP-1 cells (Prepared by author).

In vitro, *C. asiatica* methanolic extract showed anti-proliferative activity in a number of cancer cells [21], with dose dependent inhibition of cell proliferation, DNA damage and induction of apoptosis [21,27,28]. Our findings indicated that at 24 h, varying C_{LE} concentrations increased PBMC caspase (-9, -3/7) activities whereas decreased THP-1 caspase (-9, -3/7) activities, indicating that intrinsic apoptosis was increased in PBMC's whereas decreased in THP-1 cells (Figure 8.4). At 72 h, C_{LE} decreased caspase (-9, -3/7) activities in both PBMC's and THP-1 cells, suggesting a decrease in the intrinsic apoptotic pathway (Figure 8.4).

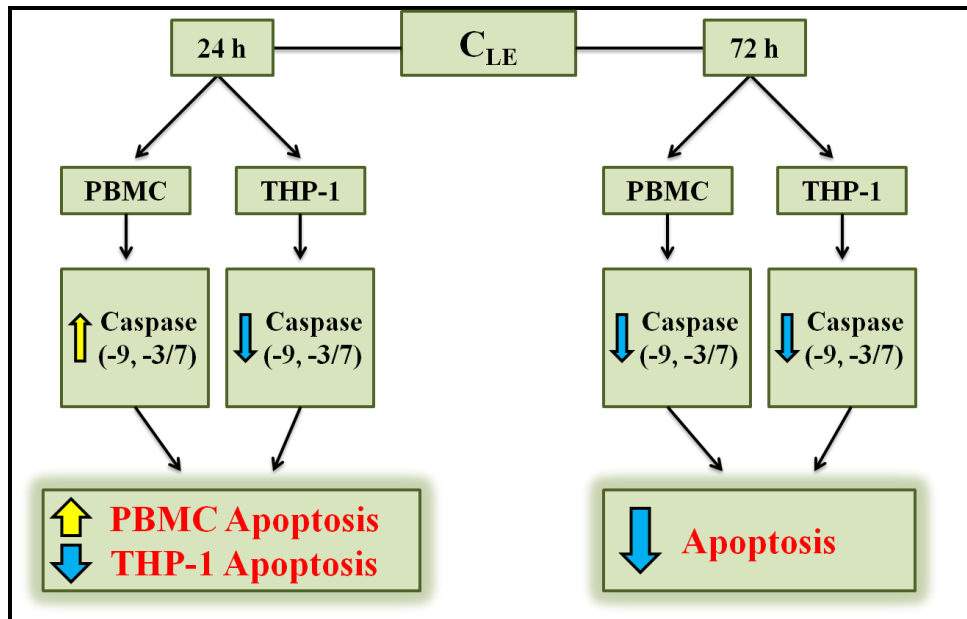


Figure 8.4: *C. asiatica* modulates apoptotic cell death in PBMC's and THP-1 cells (Prepared by author).

In THP-1 cells, C and C3 decreased nitrite, TNF- α , IL-1 β , IL-6 levels and NF- κ B (p50, p65) protein expressions whereas increased IL-10 levels (Figure 8.5). Notably, C3 decrease pro-inflammatory cytokine levels whereas increased anti-inflammatory cytokine levels to a greater extent than C. The potential of C and C3 to down regulate THP-1 nitrite levels, pro-inflammatory cytokine levels and NF- κ B protein expression may decrease pro-inflammatory cytokine mediated LPL inhibition and proteasome pathway activation, ultimately preventing the excessive lipolysis and proteolysis seen in cancer cachexia (Figure 8.5). Additionally, at 24 h, THP-1 caspase (-8, -9, -3/7) activities were increased by both C and C3, suggesting an increase in both the extrinsic and intrinsic apoptotic pathways (Figure 8.5).

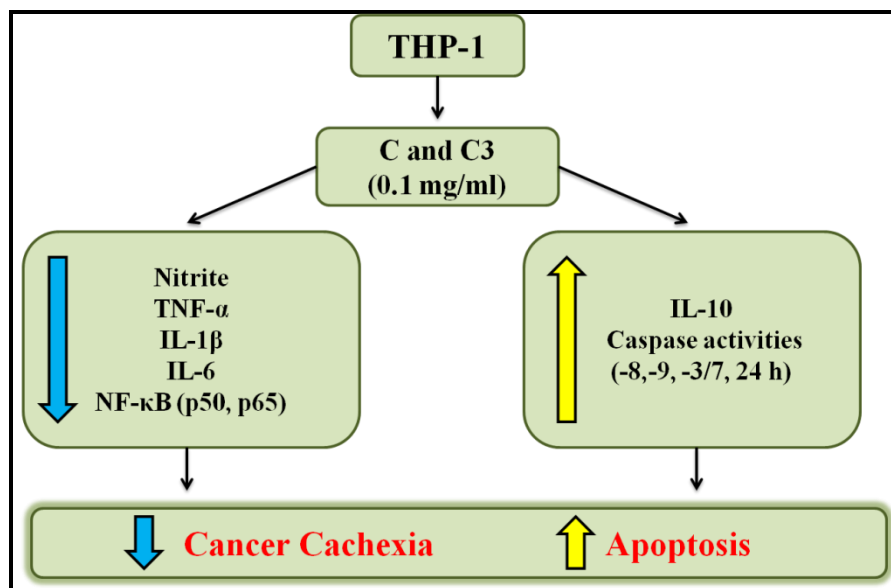


Figure 8.5: C and C3 effectively decreases cancer cachexia associated pro-inflammatory cytokine levels, decreases NF-κB (p50, p65) protein expressions and increases apoptosis in THP-1 cells (Prepared by author).

C. asiatica leaves possess the highest antioxidant activity as compared to other plant parts [29]. Additionally, the antioxidant activity of *C. asiatica* was shown to be higher in the ethanolic leaf extract than the aqueous leaf extract [30]. Previously, *C. asiatica* leaves demonstrated elevated O₂-free radical scavenging activity, DPPH radical scavenging activity and inhibition of linoleic acid peroxidation [31] indicating its free radical scavenging ability and lipid peroxidation preventative property [32]. *C. asiatica* has also been shown to decrease MDA levels and increase CAT activity thereby preventing H₂O₂ accumulation [33]. Oral *C. asiatica* methanol extract treatment significantly increased antioxidant enzymes (SOD, CAT and GPx) in lymphoma-bearing mice [34]. Additionally in rats, exogenous SOD from *C. asiatica* was shown to increase endogenous SOD activities [35]. *C. asiatica* inhibited HepG2 and MCF-7 cell proliferation through apoptosis and DNA damage [21,28]. Interestingly, in human breast cancer (MDA-MB 231) and mouse melanoma (B16F1), *C. asiatica* aqueous extract elicited cytotoxic activity [19]. However, *C. asiatica* was not cytotoxic in A549 and BHK-21 cell lines [19]. In addition, a *C. asiatica* purified fraction inhibited tumour cell growth whereas induced no toxic effects on lymphocytes [36].

In this study, THP-1 antioxidant gene expression, Nrf-2 protein expression and GSSG levels were increased by C but decreased by C3, indicating that the antioxidant responses were increased by C whilst decreased by C3 (Figure 8.6). The IC₅₀ of C and C3 increased THP-1 MDA, IROS and $\Delta\psi_m$ whereas decreased both GPx and Bcl-2 protein expressions (Figure 8.6). Caspase activity and DNA damage were also elevated by both C (-9, -3/7) and C3 (-8, -9, -3/7), indicating that C activated the intrinsic pathway whereas C3 activated both apoptotic pathways (Figure 8.6). Levels of c-PARP and *c-myc* were decreased by C whilst increased by C3. Only C3 reduced *OGG-1* gene expression, suggesting a decrease in DNA repair. Both C and C3 exerted anti-proliferative effects in THP-1 cells by enhancing apoptosis. Notably, C3 more effectively induced apoptosis.

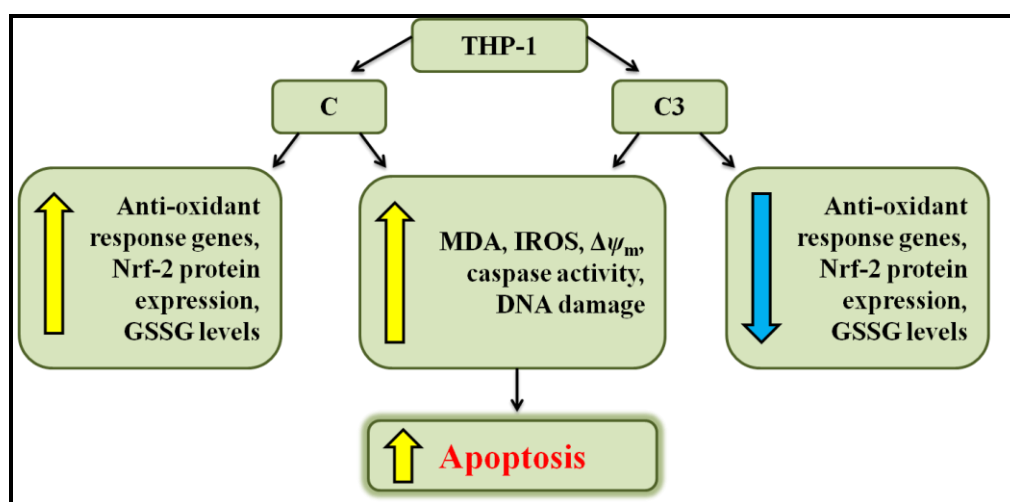


Figure 8.6: The IC₅₀ of C and C3 induces ROS mediated apoptosis in THP-1 cells (Prepared by author).

In this study only C3 dose dependently decreased A549 and HEK293 cell viability. The A549 GSH levels and Nrf-2 protein expression were increase whereas A549 GSSG levels, antioxidant gene expression were decreased by C3 suggesting a decreased antioxidant response (Figure 8.7). In A549 cells, C3 increased MDA, IROS, $\Delta\psi_m$, LDH, caspase (-8, -9, -3/7) activity and DNA damage while decreasing *p53*, Bax and Bcl-2 protein expression, suggesting C3 induced anti-proliferative and cytotoxic effects in A549 cells (Figure 8.7).

In C3 treated HEK293 cells, Nrf-2 protein expression, GSH and GSSG levels were increased whereas antioxidant gene expression was significantly decreased, suggesting that C3 may act as a natural antioxidant but also decreases the Nrf-2 antioxidant response (Figure 8.7). In HEK293 cells, C3 increased MDA, $\Delta\psi_m$, LDH, caspase (-8, -9, -3/7) activity and DNA damage whereas decreased Bax, Bcl-2 protein expression and *OGG-1* gene expression, suggesting that C3 increased cell death whereas decreased DNA repair (Figure 8.7).

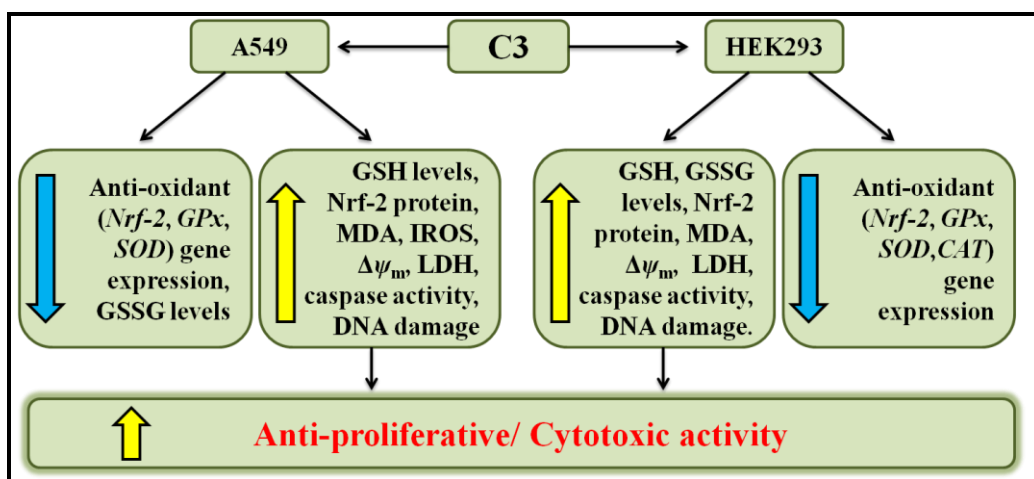


Figure 8.7: C3 decreases anti-oxidant mechanisms and increases ROS mediated apoptosis in A549 and HEK293 cells (Prepared by author).

Future work:

C. asiatica purified fraction-3 should be further fractionated and compounds isolated. These specific compounds should be evaluated to determine which group of compounds are responsible for the anti-cachectic and anti-cancer properties (*in vitro*). Thereafter these compounds should be tested in an *in vivo* model before a clinical study can be conducted.

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APPENDICES

APPENDIX 1

Optimisation for cell viability and inflammatory cytokine production

Aim: To investigate the optimum conditions for the WST-1 cell viability assay and the mitogen most effective at stimulating inflammatory cytokine production (TNF- α , IL-6, IL-1 β and IL-10) in THP-1 cells.

1.1 Methods

1.1.1 Tissue Culture

THP-1 cells were grown in the appropriate tissue culture conditions (75 cm³ flasks, 37°C, 5%). The growth media used was RPMI-1640, FCS (10%), and PSF (2%). The cells were seeded at a concentration of 3×10^5 cells/ml and the growth monitored daily by cell counting. The cells were ready to be split/ diluted once the cell concentration reached 8×10^5 cells/ml.

1.1.2 Plant Extraction

W. somnifera leaves were cut into smaller manageable pieces and milled into a fine powder. Ethanol (200–350 ml) was added to *W. somnifera* leaf powder (10–30 g) and extracted overnight by shaking (4 x g, 37°C). The ethanol extract was filtered, rotor evaporated, dried (37°C) and stored (4°C).

1.1.3 Cell Viability Assay

Cytotoxicity of plant extracts in THP-1 cells was measured using the WST-1 assay (Roche, Johannesburg, SA). The assay was conducted for the number of cells per well (5000 or 10000 or 20000 cells/well of a 96 well plate), WST-1 incubation time (1 or 2 or 3 or 4 h incubation) and whether or not to centrifuge of plates before the WST-1 assay is conducted. Results indicated the following optimum conditions.

Cell density was adjusted (1×10^5 cells/ml) and aliquoted (100 μ l/well) into 96 well plates (U-bottom). Thereafter, prepared *W. somnifera* leaf extract treatments (12–400 μ g/ml) was added (100 μ l/well) into designated wells. Similarly controls received media. Plates were mixed on a micro-plate reader at medium setting for 30s and incubated for 24 h. Thereafter, plates were centrifuged

(162 x g, 10 min). Supernatant was removed, cell pellets were re-suspended in growth media (100 μ l/well), WST-1 reagent (10 μ l/well) was added and incubated (37°C, 5% CO₂, 3 h). The absorbance was read at 450 nm (620 nm reference wavelength) with a Multiskan FC micro-plate reader (Thermo Scientific).

1.1.4 Quantification of Cytokines

Cell density of THP-1 cells were adjusted (2 x 10⁵ cells/ml) and aliquoted (3 ml/well) into 6 well plates. Thereafter PHA (15 μ l/well, 1 mg/ml), Con A (3 μ l/well, 5 mg/ml) and LPS (15 μ l/well, 1 mg/ml) reconstituted in PBS were added and plates were incubated (37°C, 5% CO₂, 4 h) for mitogen stimulation (6, 12, 24, 48 and 72 h). Upon completion of incubation times, plates were centrifuged (162 x g, 10 minutes) and supernatant was stored (-20 °C) for ELISA experimentation. Cytokines levels were estimated using the BD OptEIA ELISA kits (The Scientific group, SA) and the procedure was followed as per the instruction manual. Plates were read at 450nm (570 nm reference wavelength) with a Multiskan FC micro-plate reader (Thermo Scientific). Cytokine concentrations were calculated by extrapolation from a standard curve.

1.1.5 Statistical Analysis

The statistical analysis was performed using the Graph Pad Prism v5 statistical software. Analysis of variance (ANOVA) was used, followed by the Tukey multiple comparison test and linear regressions with $p < 0.05$ chosen as the significant level.

1.2 Results

1.2.1 Cell viability

Results indicated that the ideal conditions were 10000 cells/ well or 1 x 10⁵ cells/ml, centrifugation of plates to pellet THP-1 cells, remove supernatant, re-suspend cells in media, add WST-1 reagent and incubate plates for 3 h. The ethanolic leaf extract of *W.somnifera* decreased THP-1 viability at 25–400 μ g/ml ($p < 0.0001$).

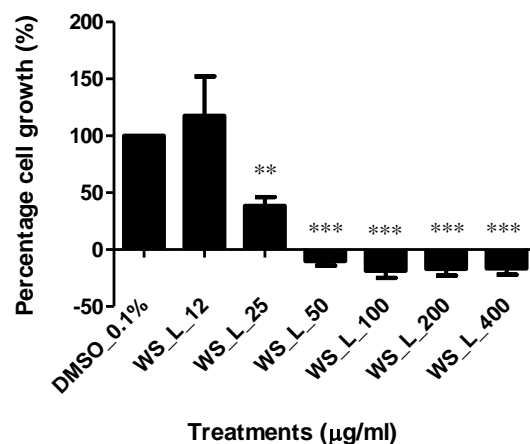


Figure 1.1: Percentage cell growth in *W. somnifera* ethanolic leaf extract treated THP-1 cells, $p < 0.0001$.

1.2.2 Cytokine production by PHA, Con A and LPS stimulation of THP-1 cells

In THP-1 cells, PHA and ConA were unable to adequately stimulate IL-6, IL-1 β , and IL-10 production.

Table 1.1: Production of inflammatory cytokines (IL-6, IL-1 β and IL-10) in PHA stimulated THP-1 cells.

Time (h)	IL-6 (pg/ml) ($p = 0.9152, r^2 = 0.0044$)	IL-1 β (pg/ml) ($p = 0.9990, r^2 < 0.0001$)	IL-10 (pg/ml) ($p = 0.1546, r^2 = 0.5445$)
6	-0.5569 ± 0.3025	1.9781 ± 0.1382	1.3846 ± 0.1163
12	0.0295 ± 0.0697	2.2263 ± 0.1358	1.5085 ± 0.3899
24	-0.3839 ± 0.3758	1.4769 ± 0.0994	1.8376 ± 0.2181
48	0.2658 ± 0.2828	1.6667 ± 0.0553	1.5641 ± 0.2972
72	-0.4768 ± 0.2301	2.1216 ± 0.1352	1.9658 ± 0.4427

Table 1.2: Production of inflammatory cytokines (IL-6, IL-1 β and IL-10) in Con A stimulated THP-1 cells.

Time (h)	IL-6 (pg/ml) ($p = 0.5715, r^2 = 0.1179$)	IL-1 β (pg/ml) ($p = 0.8502, r^2 = 0.0139$)	IL-10 (pg/ml) ($p = 0.4615, r^2 = 0.1912$)
6	-0.9114 \pm 0.3948	0.7080 \pm 0.6292	1.0940 \pm 0.2635
12	-0.3412 \pm 0.2532	0.8199 \pm 0.6183	1.0812 \pm 0.3752
24	-0.9241 \pm 0.2643	0.5426 \pm 0.6044	1.9658 \pm 0.9663
48	-0.2321 \pm 0.3397	0.5402 \pm 0.5107	3.9957 \pm 2.6158
72	-0.5865 \pm 0.5324	0.8443 \pm 0.5861	1.5855 \pm 0.5372

In THP-1 cells, LPS stimulation significantly increased cytokine concentrations at various time points. Concentration of TNF- α was increased between 6 – 24 h while the IL-6, IL-1 β and IL-10 concentrations were highest at 72 h.

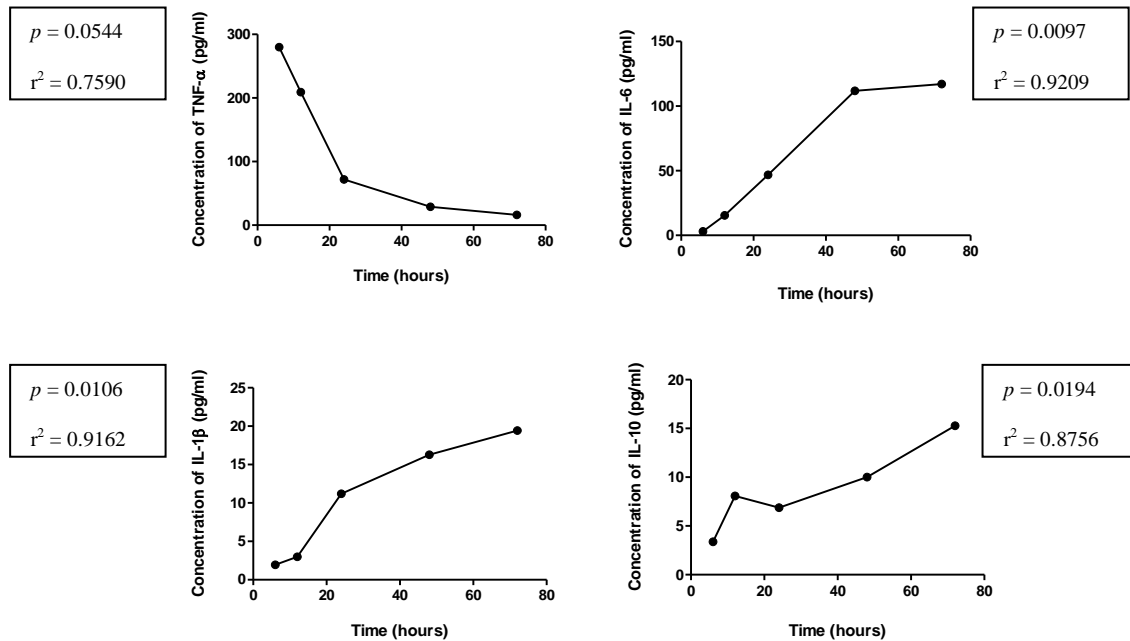


Figure 1.2: Production of inflammatory cytokines (TNF- α , IL-6, IL-1 β and IL-10) in LPS stimulated THP-1 cells (Linear trends).

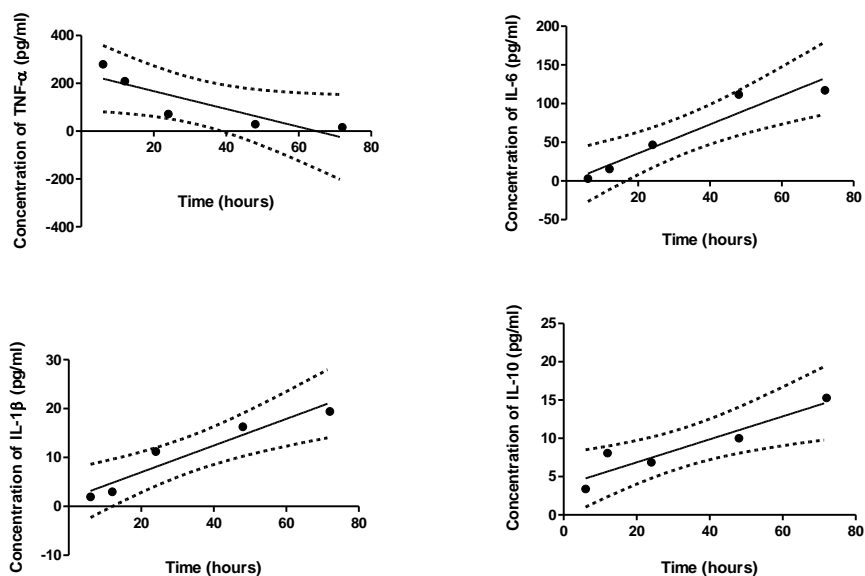


Figure 1.3: Production of inflammatory cytokines (TNF- α , IL-6, IL-1 β and IL-10) in LPS stimulated THP-1 cells (Regressions).

1.3 Discussion

The optimum WST-1 assay conditions were 1×10^5 cells/ml cells, centrifugation of plates, removal of supernatant, re-suspension of cells in complete media, addition of the WST-1 reagent and incubation for 3 h. These conditions lead to the most stable cell viability results. The THP-1 cells were successfully stimulated by LPS to produce inflammatory cytokines (TNF- α , IL-6, IL-1 β , and IL-10) thus LPS was chosen as the preferred stimulant. Maximum production of cytokines was seen at 24 h for TNF- α and 72 h for IL-6, IL-1 β and IL-10. The concentration of LPS will be doubled for further experiments to increase cytokine levels. Cells will be stimulated for an initial 4 h thereafter plant treatments will be added and plates incubated for 24 h (for TNF- α detection) and 72 h (for IL-6, IL-1 β and IL-10 detection). The plates will then be centrifuged, the supernatant collected and stored (-20°C) for ELISA experimentation. These experimental conditions results in an ongoing stimulus of inflammatory cytokines thus creating an environment similarly seen in cancer cachexia.

APPENDIX 2

The potential of South African medicinal plants to modulate inflammatory cytokines associated with cancer cachexia.

The ability of plant extracts to down regulate the expression of pro-inflammatory cytokines and up regulate the expression of anti-inflammatory cytokines may decrease tissue wasting.

Aim: This *in-vitro* study investigates oxidant scavenging activity and the modulation of inflammatory cytokine levels (TNF- α , IL-6, IL-1 β , and IL-10) by *Aloe ferox* (*A. ferox*), *C. asiatica*, *Elytropappus rhinocerotis* (*E. rhinocerotis*), *Tulbaghia violacea* (*T. violacea*) (leaf and root), and *W. somnifera* (leaf and root) extracts (ethanol and water) in LPS stimulated THP-1 cells.

2.1 Material and Methods

2.1.1 Tissue Culture

Please refer to Appendix 1 – Method 1.1.1

2.1.2 Plant Extraction

Plants were sorted into separate parts (root, stem, and leaves), cut into smaller manageable pieces and milled into a fine powder for plant extractions (ethanolic and aqueous). Dried plant material was sequentially extracted in ethanol and distilled water. For the ethanolic extraction, milled plant (10–30 g) was added to ethanol (200–350 ml) and extracted overnight by shaking (4 x g, 37°C). Ethanol extracts were filtered, rotor evaporated, dried (37°C) and stored (4°C). For the sequential aqueous extraction, the remaining plant material from the ethanol extraction was used, distilled water was added (200–350 ml) and extracted for 6 h by shaking (4 x g, 75°C). Water extracts were filtered, dried and stored (4°C).

2.1.3 Cell Viability Assay

Cytotoxicity of plant extracts in THP-1 cells was measured using the WST-1 assay (Roche, Johannesburg, SA). Cell density was adjusted (1×10^5 cells/ml) and aliquoted (100 μ l/ well) into 96 well plates (U-bottom). Thereafter LPS reconstituted in PBS (2 μ l/well, 1 mg/ml) was added and plates were incubated (37°C, 5% CO₂, 4 h) for LPS stimulation. Thereafter, prepared plant extract

treatments (25–1600 µg/ml, *W. somnifera* leaf 0.78–50 µg/ml) was added (100 µl/ well) into designated wells. Similarly controls received media. The plates were mixed on a micro-plate reader at medium setting for 30s and incubated (37°C, 5% CO₂) for 24 h (for TNF-α detection) and 72 h (for IL-1β, IL-6, IL-10 detection). The plates were subsequently centrifuged (162 x g, 10 minutes). The cytokine containing media was collected and transferred to individual wells of a new 96 well plate and stored at -20°C until ELISA experimentation. Cell pellets were re-suspended in growth media (100 µl/well), WST-1 reagent (10 µl/well) was added and plates were incubated (37°C, 5% CO₂, 3 h). The absorbance was read at 450 nm (620 nm reference wavelength) with a Multiskan FC micro-plate reader (Thermo Scientific).

2.1.4 The 2, 2-diphenyl-1 picrylhydrazyl (DPPH) assay

Plant extracts (25–1600 µg/ml, *W. somnifera* leaf 0.78–50 µg/ml) and butylated hydroxytoluene (BHT) (60–300 µM) dilutions were prepared in methanol (99.5% and grade AR). A 50 µM DPPH solution was prepared from a stock solution of 0.135 mM DPPH in methanol. The plant extracts, BHT dilutions and methanol (100 µl) were aliquoted into a 96 well plate, followed by the 50µM DPPH solution (100 µl). Plates were incubated (RT, 30 minutes) in the dark. Absorbance of samples was read at 517nm using a BIO-TEK µQuant spectrophotometer (Analytical and Diagnostic Products, SA).

2.1.5 Quantification of Cytokines

Please refer to Appendix 1 – Method 1.1.4

2.1.6 Statistical Analysis

The statistical analysis was performed using the Graph Pad Prism v5 statistical software. Analysis of variance (ANOVA) was used, followed by the Tukey multiple comparison test, with $p < 0.05$ chosen as the significant level.

2.2 Results

Due to the excessive amount of results and the length of the appendices, the actual results have been excluded.

2.3 Discussion

Antioxidant and anti-inflammatory properties of *A.ferox* [1], *C. asiatica* [2], rhinocerotinoic acid [3], *T. violacea* [4] and *W. somnifera* [5] have been documented. The immunomodulatory capabilities of medicinal plants may prove to be useful in the treatment of cancer cachexia. Majority of the plant extracts increased DPPH oxidant scavenging activity. Plant extracts providing an increased oxidant defense system will be beneficial in preventing excessive oxidative stress, apoptosis and catabolic events. The aqueous *T. violacea* (leaf and root) and *A. ferox* (ethanolic and aqueous) extracts resulted in some increases in pro-inflammatory cytokines and decreased IL-10 levels. Elevated pro-inflammatory cytokines may lead to increased lipid and muscle breakdown thus these plants extracts are not suitable for cachexia treatment. Certain concentrations of *C. asiatica* (aqueous), *E. rhinocerotis* (ethanolic and aqueous), *T. violacea* root (ethanolic), *W. somnifera* leaf (ethanolic and aqueous), and *W. somnifera* root (ethanolic) extracts have decreased pro-inflammatory cytokines however most of these concentrations also substantially decrease cell viability. Excessive decreases in cell viability indicate decreased number of viable cells producing pro-inflammatory cytokines which may contribute to decreased cytokine levels. Ideally, a plant extract should be able to maximally decrease the levels of more than one pro-inflammatory cytokine with minimum variation in cell viability across the concentration range. In THP-1 cells, these plants extracts did not prove to be successful in modulating inflammatory cytokine levels therefore they may not be beneficial in combating the cachectic syndrome. The *C. asiatica* (ethanolic), *T. violacea* (ethanolic) and *W. somnifera* root (aqueous) extracts have shown promising results with regard to anti-oxidant and anti-inflammatory potential which is comparable to previous literature. The experiment was repeated for *C. asiatica* (ethanolic), *T. violacea* leaf (ethanolic) and *W. somnifera* root (aqueous) extracts to verify results. The results proved to be reproducible and experimentation continued on *C. asiatica* (ethanolic) and *W. somnifera* root (aqueous) extracts. The *T. violacea* ethanolic leaf extract was depleted thus experimentation ceased.

APPENDIX 3

Manuscript: *Withania somnifera* modulates cancer cachexia associated inflammatory cytokines and cell death in leukaemic THP-1 cells and peripheral blood mononuclear cells (PBMC's).

Manuscript: *Centella asiatica* modulates cancer cachexia associated inflammatory cytokines and cell death in leukaemic THP-1 cells and peripheral blood mononuclear cells (PBMC's).

SOUTH AFRICAN NATIONAL BLOOD SERVICE
(Association incorporated under section 21)
Human Research Ethics Committee



SANBS
South African National Blood Service

OHRP Number : IORG0006278
FWA Registration Number : IRB00007553
SA NHREC Registration Number : REC-270606-013

Association incorporated under section 21
Registration No. 2007/2028/06

Secretariat: Tel: 011 761 9351 | Fax: 086 6747666 | Cell: 0824072983 | email: allarice.prinsloo@sanbs.org.za

Dr Yen-Ju
Oncology Research Unit
South African Medical Research Council
Yen-ju.shen@mrc.ac.za

Dear Dr Yen-ju

DATE OF COMMITTEE MEETING: 10 May 2011

PROJECT TITLE : The role of Plant-derived Products in the Modulation of Cytokines Implicated in Cancer Induced Cachexia and Immune Modulation

DECISION OF THE COMMITTEE: Approved

CLEARANCE CERTIFICATE NO: 2011/09

- Execution of the study must be compliant with SANBS Research Policy
- Any amendment, extension or other modifications to the protocol must be submitted to This Ethics Committee for approval prior to implementation.
- The Committee must be informed of any serious adverse event, planned and unplanned termination of the study.
- A progress report should be submitted within one year of approval of long-term studies and a final report at completion of both short term and long term studies.
- Kindly refer to the SANBS HREC clearance certificate number on all future correspondence on this study to the HREC secretariat
- Committee guidance documents, Declaration of Helsinki, ICH GCP and MRC Guidelines on Biomedical Research, Clinical Trial Guidelines 2006 Department of Health RSA; Ethics in Health Research: Principles Structure and Processes Department of Health RSA 2004; Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa, Second Edition 2006; Reviewing Clinical trials: a guide for ethics committees 2010

CHAIRPERSON : Prof J.N. Mahlangu

10 May 2011

DATE



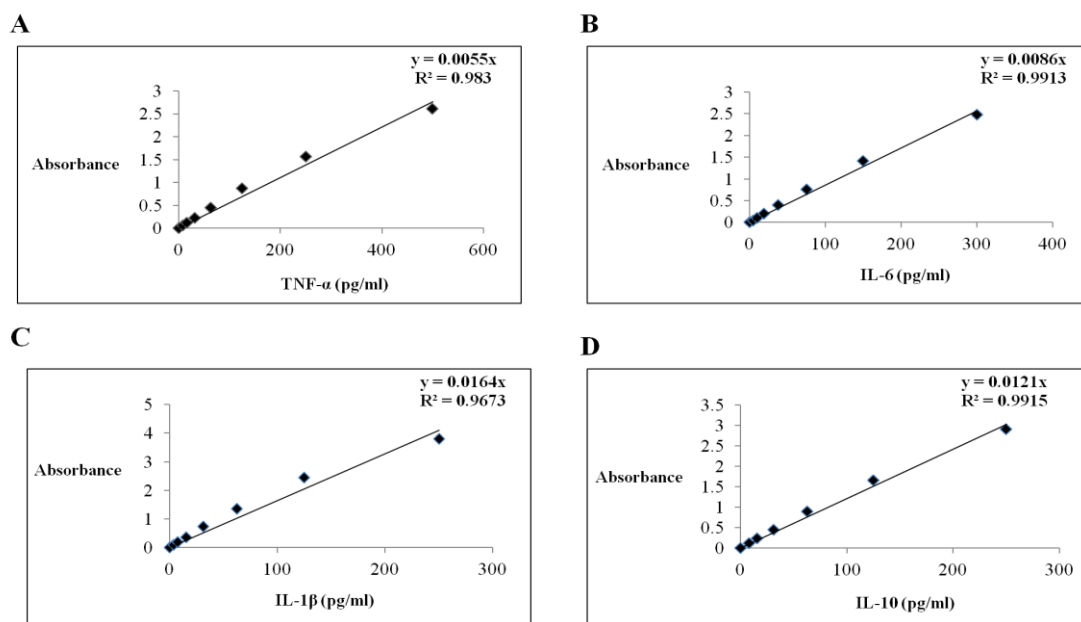


Figure 3.1: TNF-α (A), IL-6 (B), IL-1β (C) and IL-10 (D) standard curves.

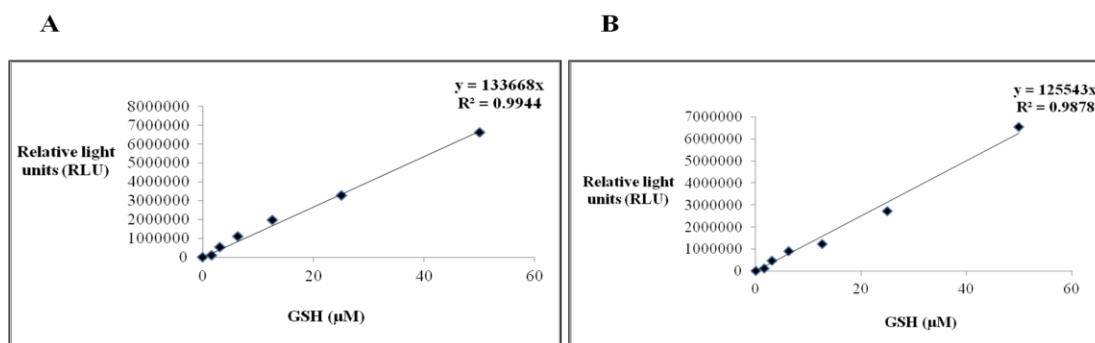


Figure 3.2: GSH standard curves at 24 h (A) and 72 h (B).

APPENDIX 4

Thin layer chromatography.

Silica column fractionation was used to separate *C. asiatica* compounds based on their polarity. A total of 766 fractions were collected and TLC's were performed. TLC analysis revealed fractions with similar spot patterns which were combined into one fraction. However, due to the excessive number of TLC's, only TLC's of fractions combined into pooled fraction-3 were included.

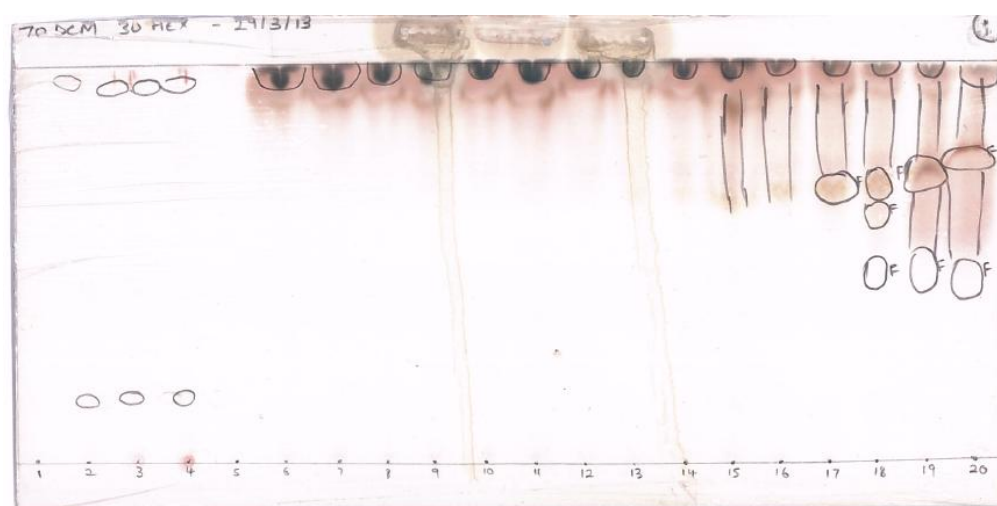


Figure 4.1: TLC analysis of fractions 1 – 20.

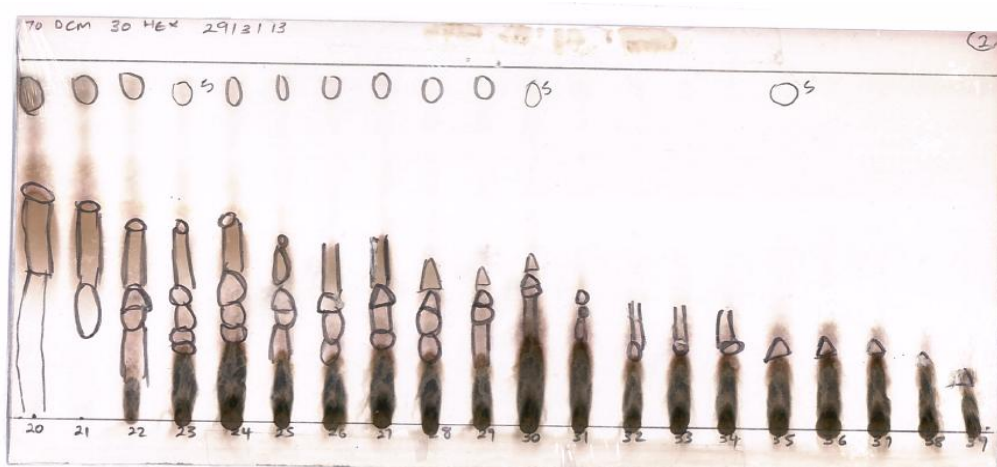


Figure 4.2: TLC analysis of fractions 20 – 39.

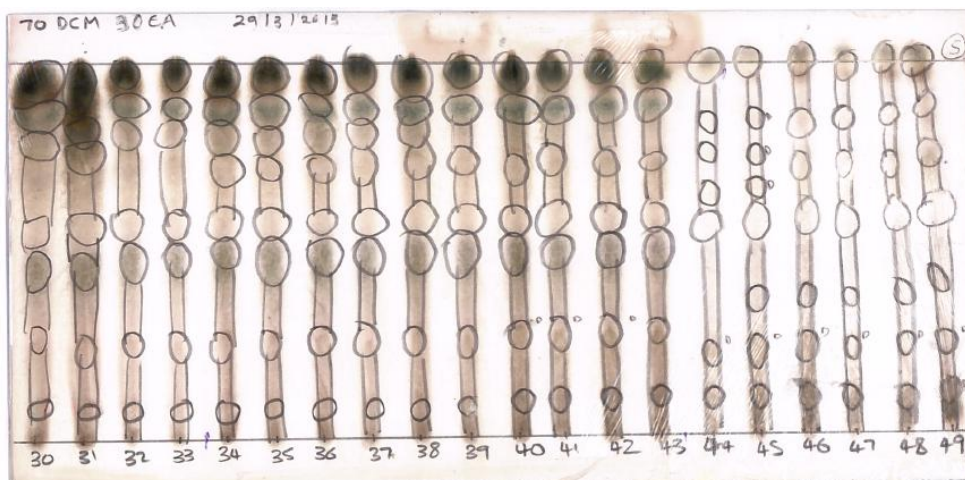


Figure 4.3: TLC analysis of fractions 30 – 49.

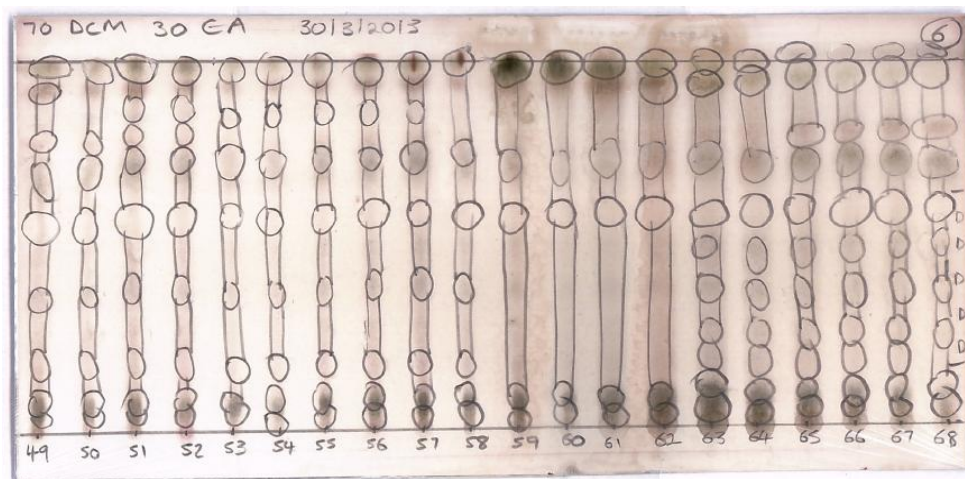


Figure 4.4: TLC analysis of fractions 49 – 68.

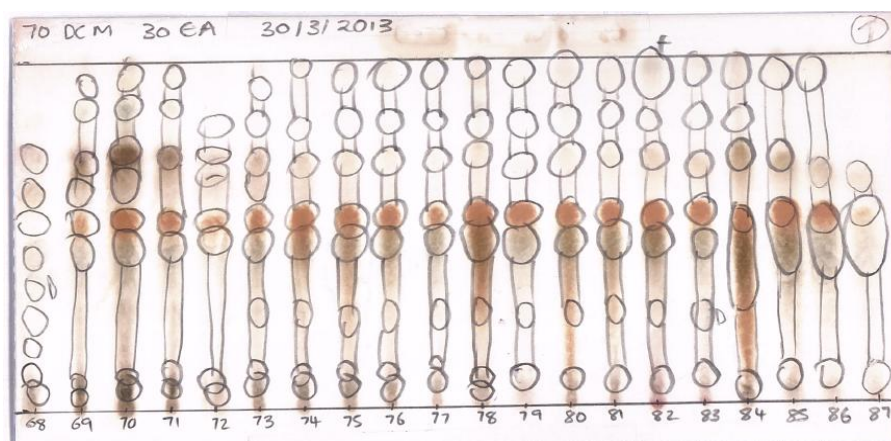


Figure 4.5: TLC analysis of fractions 68 – 87.

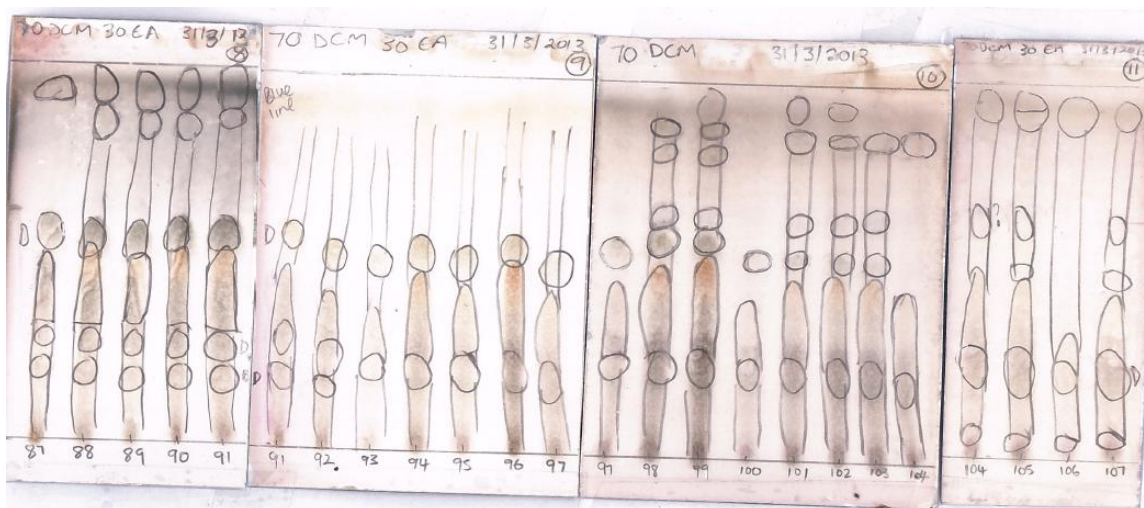


Figure 4.6: TLC analysis of fractions 87 – 107.

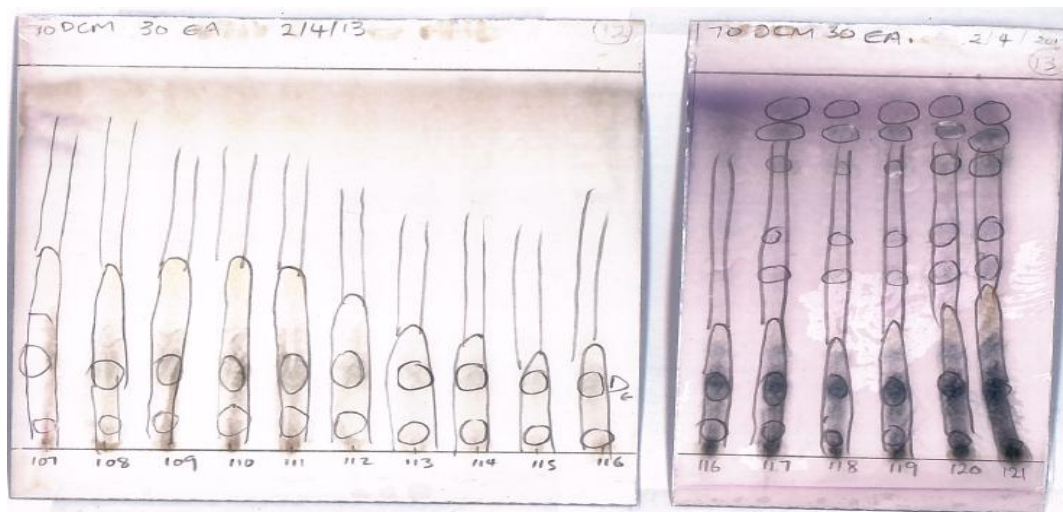


Figure 4.7: TLC analysis of fractions 107 – 121.

Table 4.1: Pooling of fractions based on TLC analysis.

Pooled fraction	Fractions included	Solvent system
1	1 – 14	70 DCM: 30 HEX
2	15 – 21	70 DCM: 30 HEX
3	22 – 116	70 DCM: 30 EA
4	117 – 213	50 DCM: 50 EA
5	214 – 342	50 DCM: 50 EA
6	343 – 361	50 DCM: 40 EA: 10 M
7	362 – 483	50 DCM: 40 EA: 10 M
8	484 – 493	30 DCM: 50 EA: 20 M
9	494 – 503	30 DCM: 50 EA: 20 M
10	504 – 543	70 EA: 30 M
11	544 – 576	70 EA: 30 M
12	577 – 675	40 EA: 60 M
13	676 – 766	20 EA: 80 M

The solvent system is the solvents in the silica column when those fractions were collected.

APPENDIX 5

The potential of *C. asiatica* pooled fractions to modulate inflammatory cytokines associated with cancer cachexia.

Fractionation of *C. asiatica* yielded 13 pooled fractions. The DPPH assay and ELISA were conducted to assess anti-oxidant scavenging activity and the modulation of cytokines by these 13 pooled fractions. Please note there were minimal amounts of pool fraction 6 hence experimentation ceased.

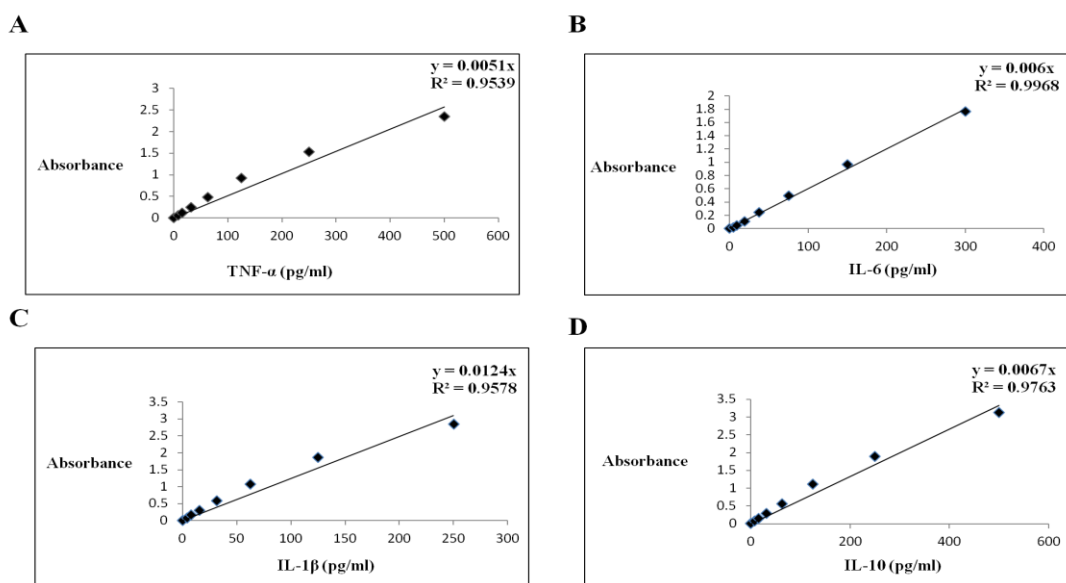


Figure 5.1: TNF- α (A), IL-6 (B), IL-1 β (C) and IL-10 (D) standard curve.

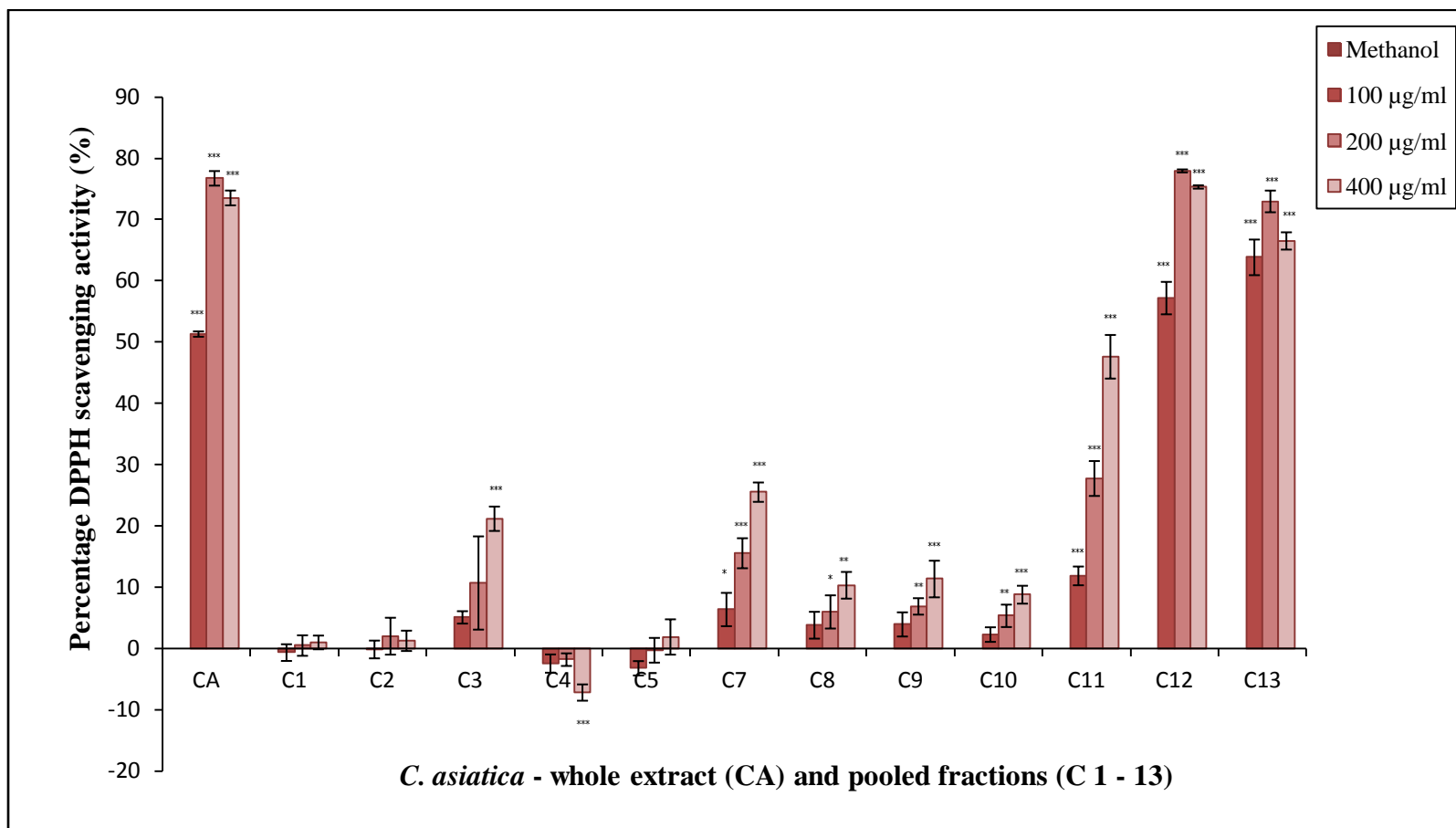


Figure 5.2: Percentage DPPH scavenging activity of *C. asiatica* whole extract (CA) and pooled fractions (C 1 – 13).

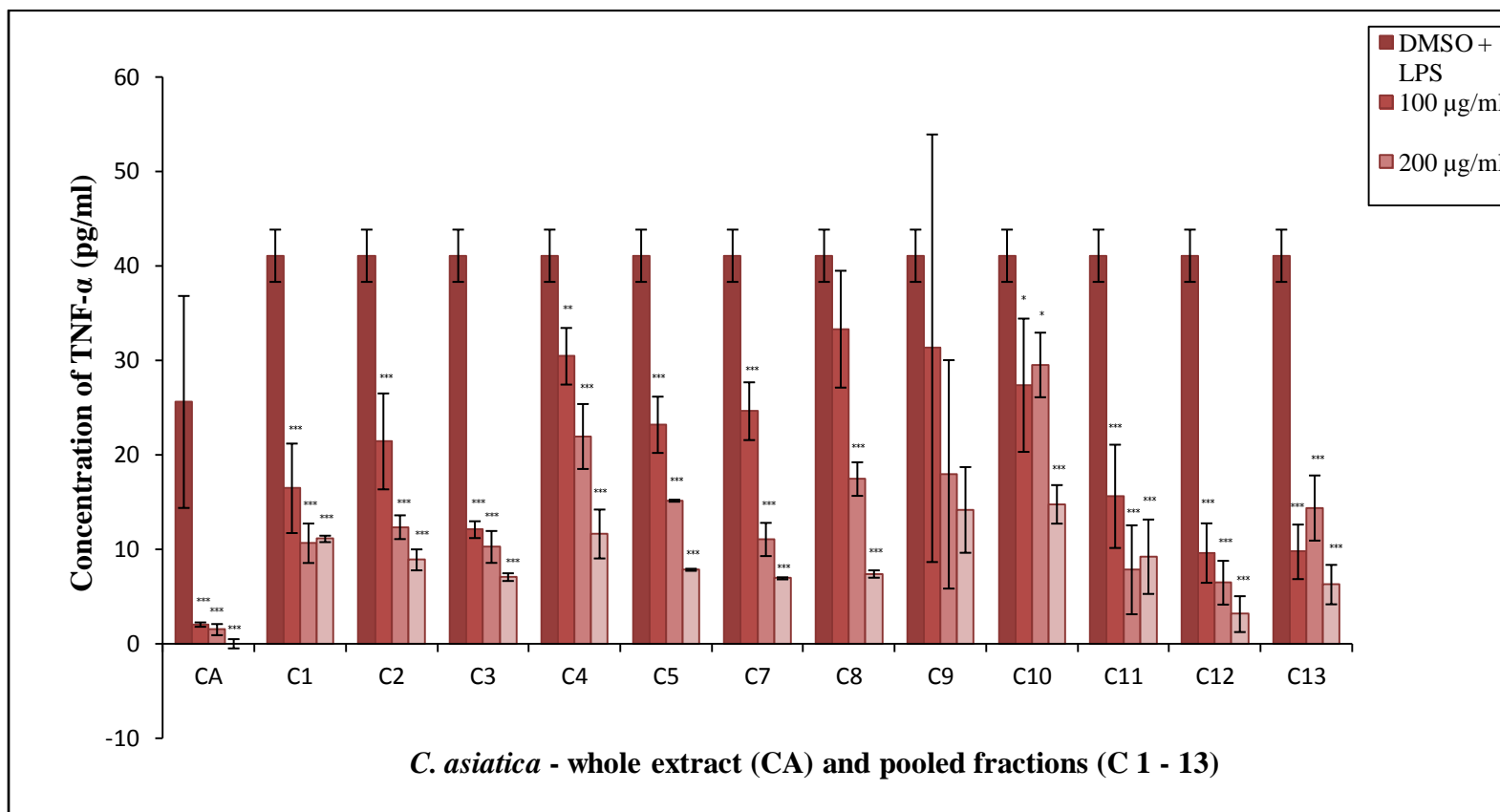


Figure 5.3: Concentration of TNF- α in *C. asiatica* whole extract (CA) and pooled fractions (C 1 – 13) treated THP-1 cells.

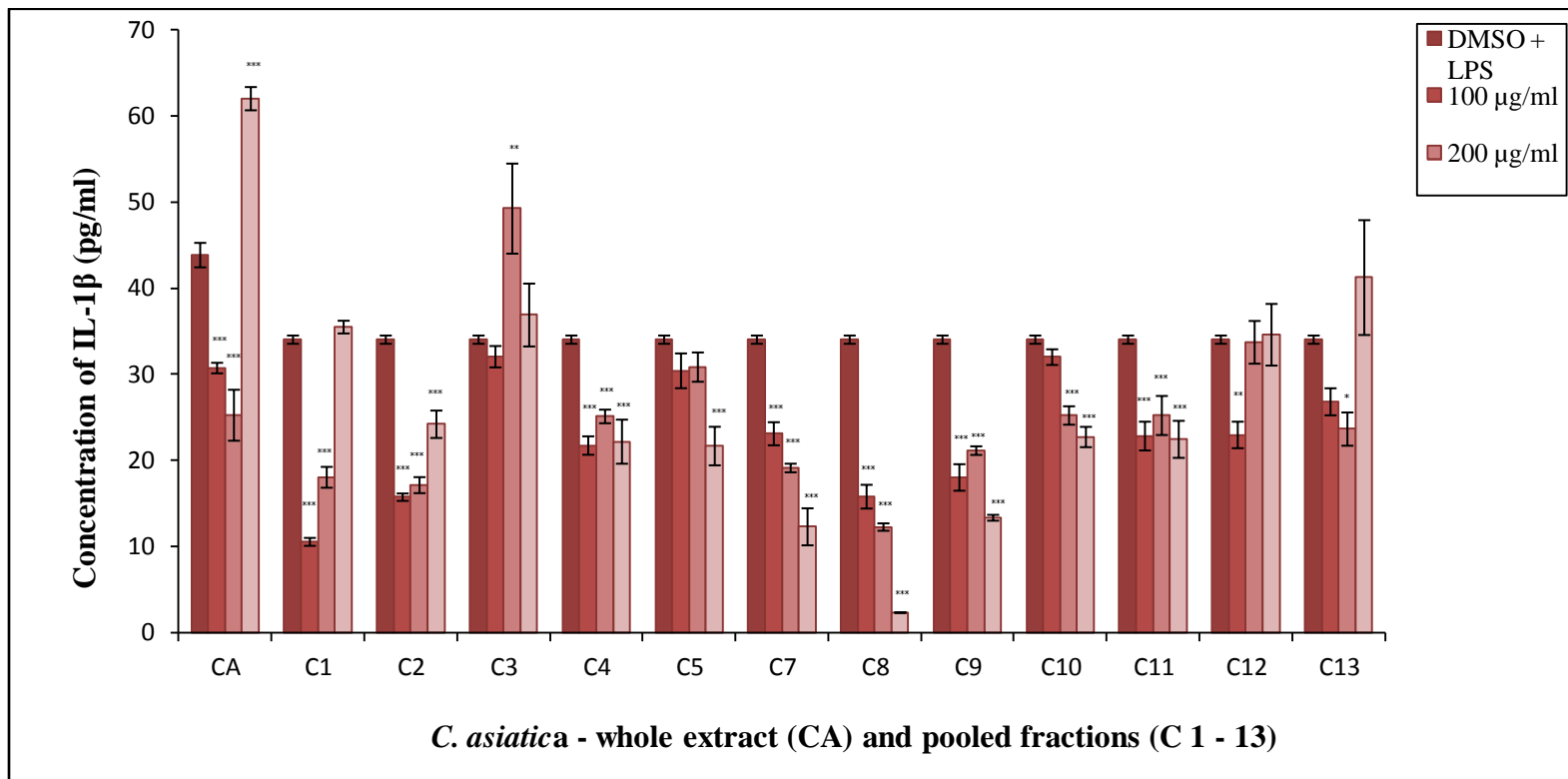


Figure 5.4: Concentration of IL-1 β in *C. asiatica* whole extract (CA) and pooled fractions (C 1 – 13) treated THP-1 cells.

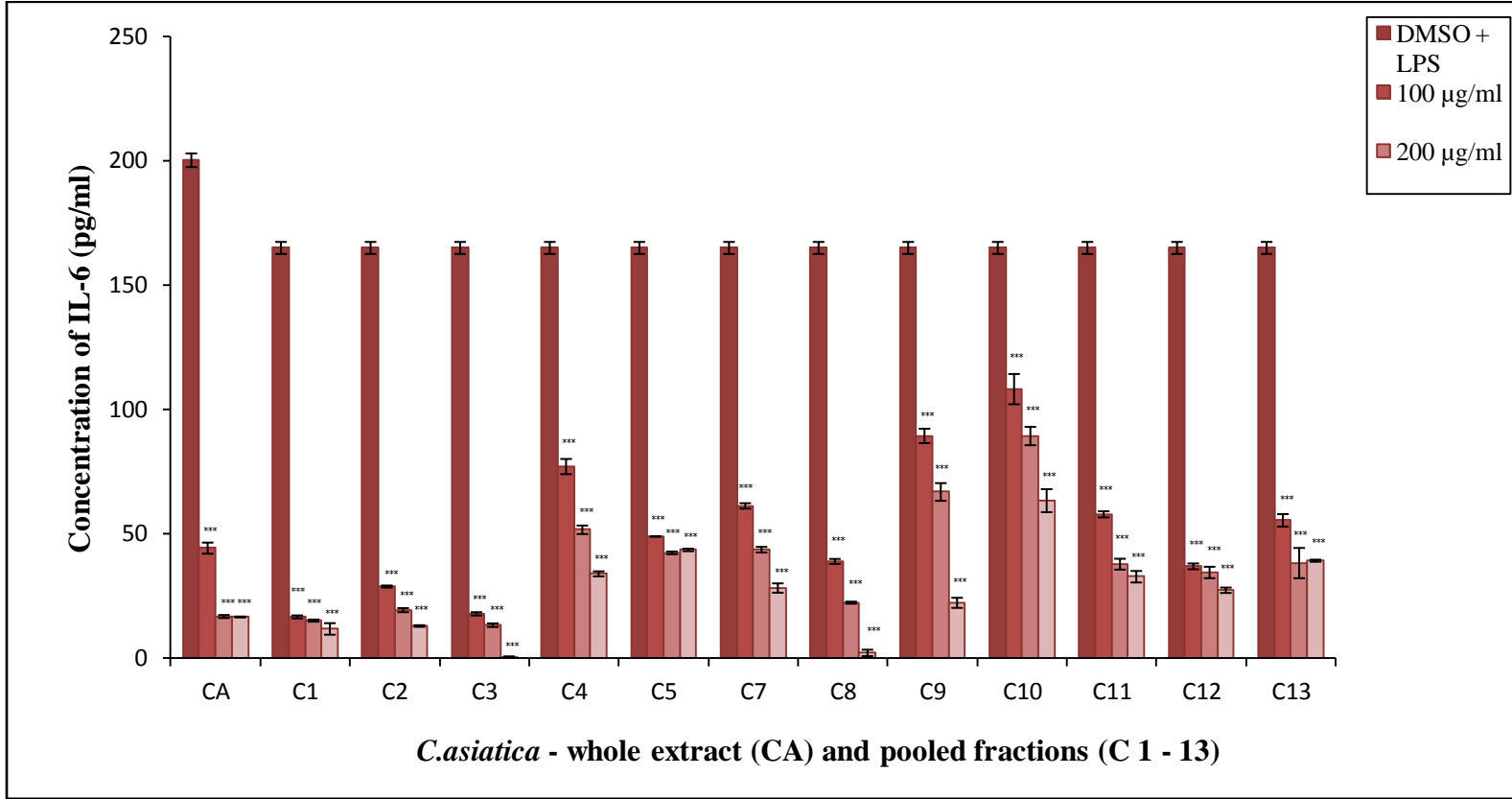


Figure 5.5: Concentration of IL-6 in *C. asiatica* whole extract (CA) and pooled fractions (C 1 – 13) treated THP-1 cells.

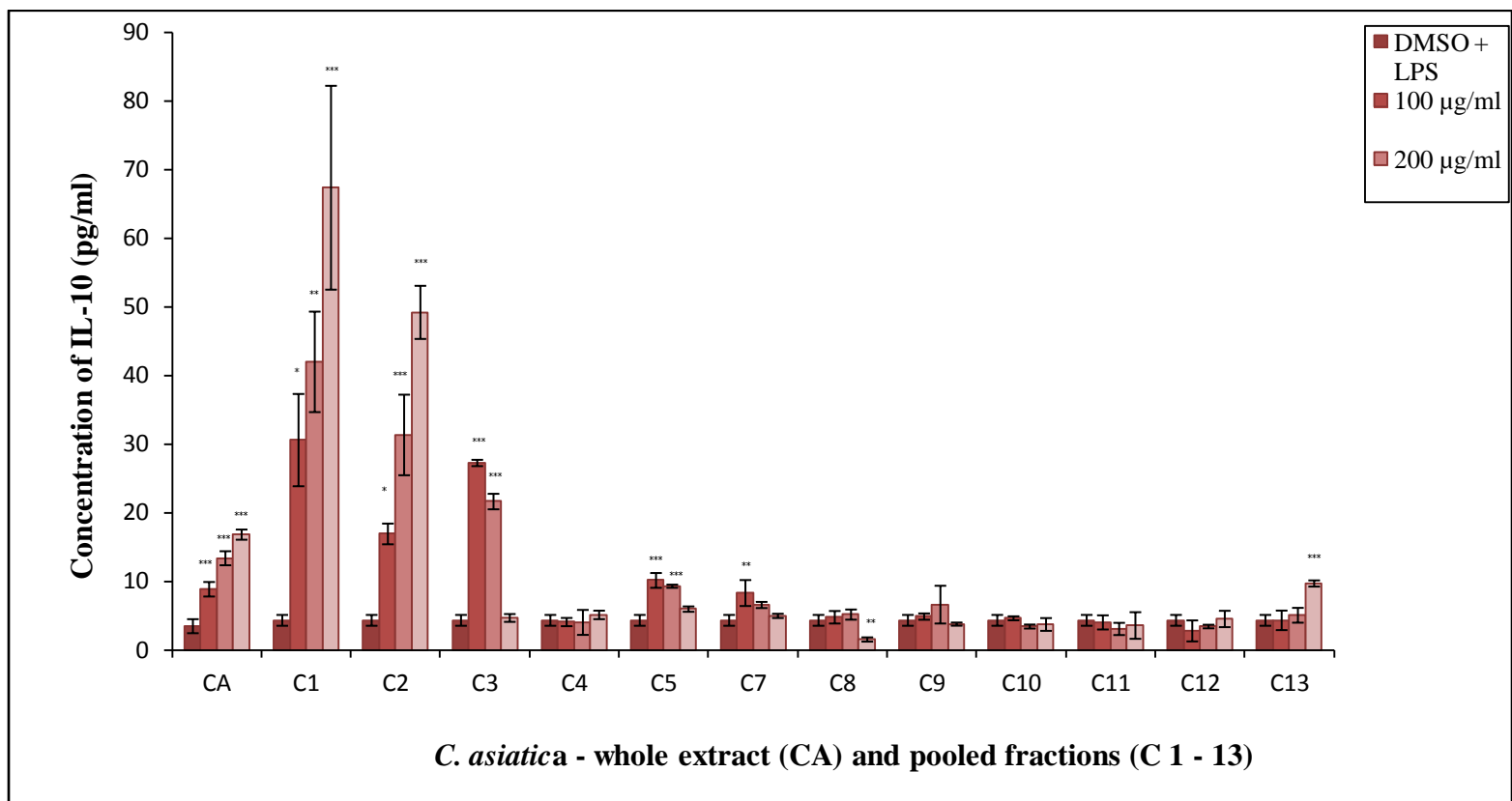


Figure 5.6: Concentration of IL-10 in *C. asiatica* whole extract (CA) and pooled fractions (C 1 – 13) treated THP-1 cells.

The results indicated that pooled fractions 1, 2, and 3 were the most effective in decreasing more than one pro-inflammatory cytokine while increasing the anti-inflammatory cytokine IL-10. Due to the minimal amounts of pooled fraction 1 and 2, experimentation continued on fraction-3.

APPENDIX 6

Gas chromatography mass spectrometry (GC-MS).

6.1 GC-MS Analysis of Bioactive Compounds present in *C. asiatica* crude extract

The GC-MS performance of the crude extract of *Centella asiatica* was recorded. The major chemical constituents which are present in the extract were identified as isoprenoids 1,6,10-Dodecatriene-7,11-dimethyl-3-methylene; 10,10-Dimethyl-2,6-dimethylene bicyclic [7.2.0]undecan; Alpha.-ylangene; Spathulenol. *Centella asiatica* is a rich source for number of non-isoprenoids various classes of non-isoprenoids include steroids stigmasterol-4,7, 22-trien-3.alpha.-ol; 3,26-Dihydroxycholest-5-en-22-one; 2-(Cholest-5-en-3-yl)ethyl acetate; Ergost-25-ene-3,6-dione, 5,12-dihydroxy; 6.alpha.,6.alpha.-Dibromo-6,7-methylenetestosterone; Nitrogen based derivatives like Cycloundecanone-oxime; 5-[3-(4-Methoxyphenyl)oxaziridin-2-yl]pentan-1-ol; 1-(2-Dimethylamino-ethyl)-3,6-dimethyl-1H-pyrimidine-2,4-dione; 1-Propanone, 1-(1-adamantyl)-3-dimethylamino; 3,7-Dihydropurine-2,6-dione, 7-(2-dimethylaminoethyl)-3-methyl [1]. Structures of few selected compounds are shown in Figure 6.2 and 6.3.

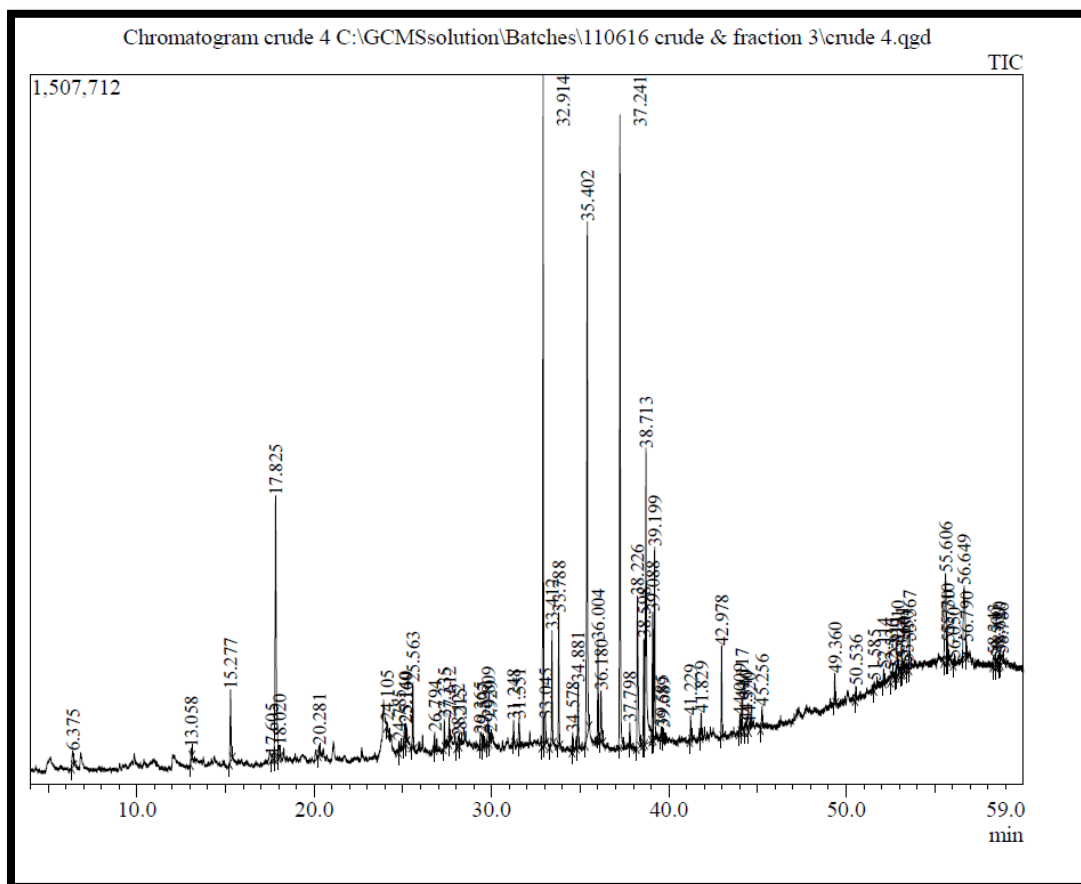


Figure 6.1: GC-MS chromatogram for *C. asiatica* crude extract.

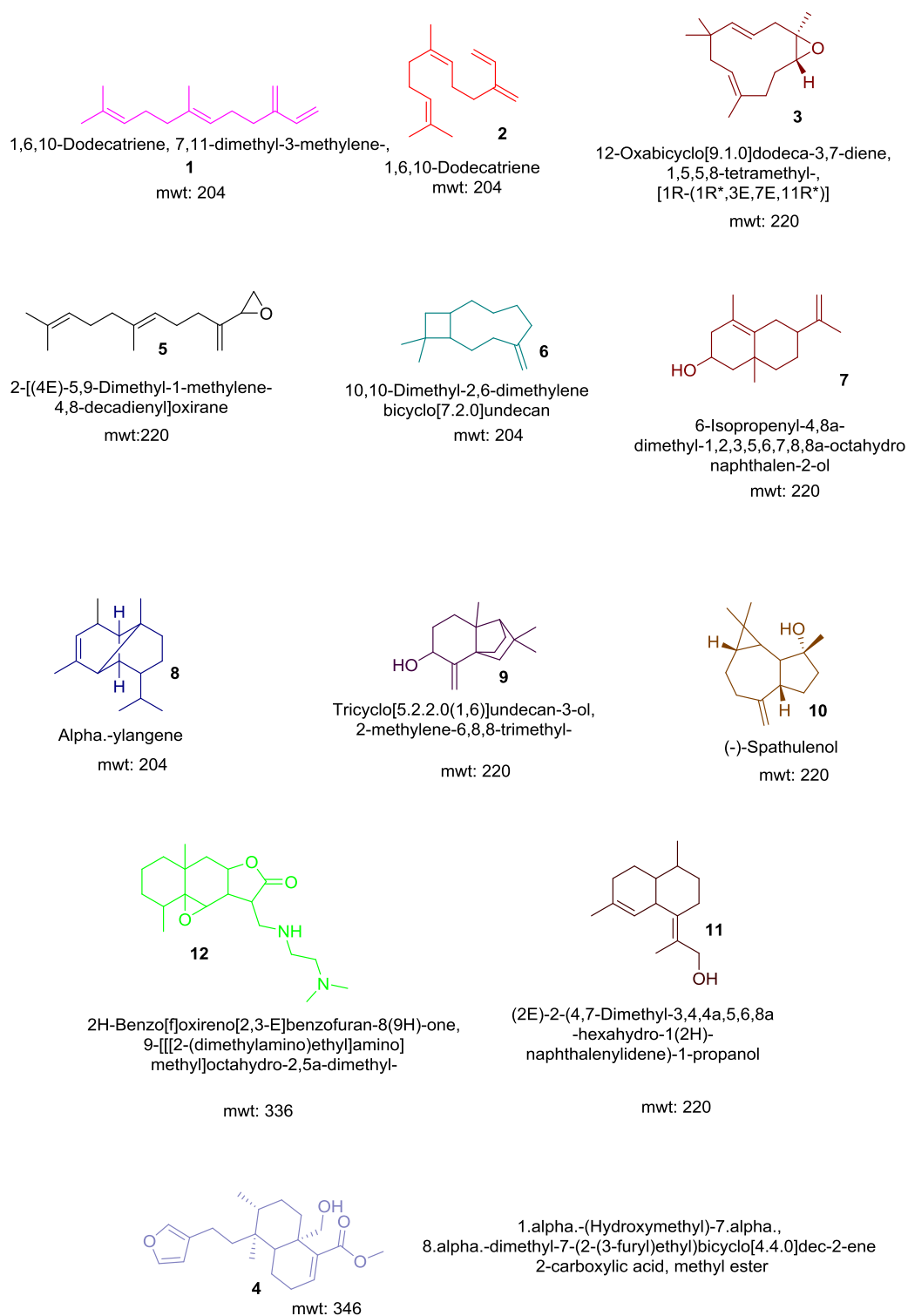


Figure 6.2: Structures of isoprenoid constituents in *C. asiatica* crude extract.

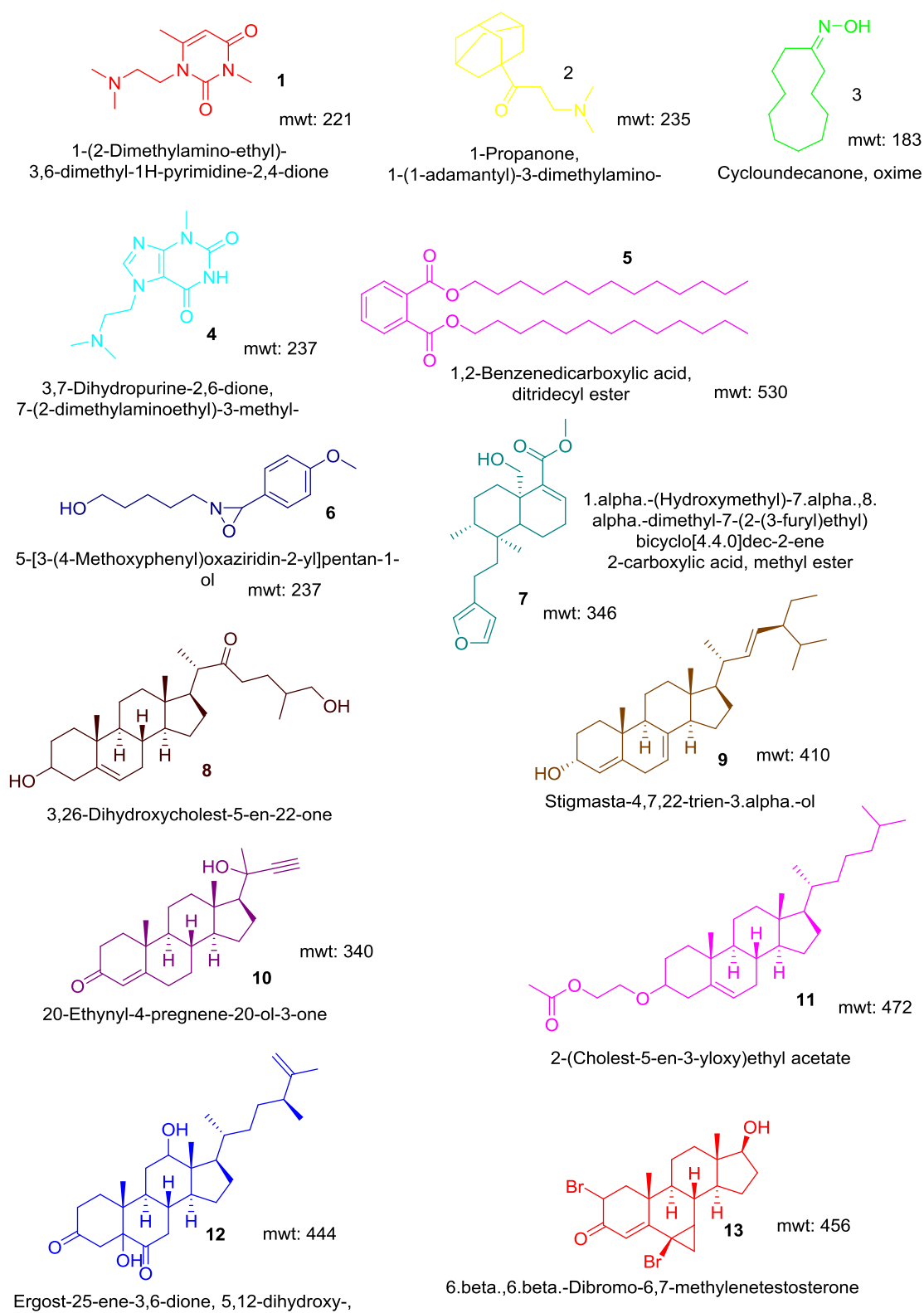


Figure 6.3: Structures of steroids and non-isoprenoid constituents in *C. asiatica* crude extract.

6.2 GC-MS Analysis of Bioactive Compounds present in *C. asiatica* pooled fraction-3

The GC-MS performance of *C. asiatica* pooled fraction-3 was recorded. The major chemical constituents which are present in the extract were identified as isoprenoids (3E)-2,6-Dimethyl-1,3,7-octatriene; Nerolidol; 2E)-2-(4,7-Dimethyl-3,4,4a,5,6,8a-hexahydro- (2H)-naphthalenyldiene)-1-propanol; Nerolidyl acetate; 1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene; Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-[1S-(1.alpha.,4.alpha.,7.alpha.)]-Alpha; Farnesene epoxide; 4,8-Decadienal, 5,9-dimethyl; 1,2-Bis(6,6-dimethylbicyclo[3.1.1] hept-2-en-2-yl)-1,2-ethanediol; Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-trimethyl; 7-(1,3-Dimethylbuta-1,3-dienyl)-1,6,6-trimethyl-3,8-dioxatricyclo[5.1.0.0(2,4)]octane; *C. asiatica* pooled fraction-3 is a rich source for number of non-isoprenoids various classes of non-isoprenoids include steroids 2,2-Dimethylcholest-7-en-3-ol; Cholest-8-en-3-yl acetate; Stigmast-5-en-3.beta.-ol, (24S); 4,4-Dimethylcholest-8(14)-en-3-ol, Cholest-8-en-3-yl acetate; 22,23-Dibromostigmast-5-en-3-yl acetate; Nitrogen based derivatives like 4-Hydroxy-1,3-dimethyl-piperidine-4-carbonitrile; N-[-(3,4-Methylenedioxy)phenylmethyl]-3,4-(methylenedioxy)benzaldimine; Carbohydrates, fatty acid and vitamins 1-O-(10-Undecenoyl)-d-xylitol; 15,17,19-Nonacosatriynoic acid; alpha.-Tocopherol-.beta.-D-mannoside; dl-.alpha.-Tocopherol [1]. Structures of few selected compounds are shown in Figure 6.5 and 6.6.

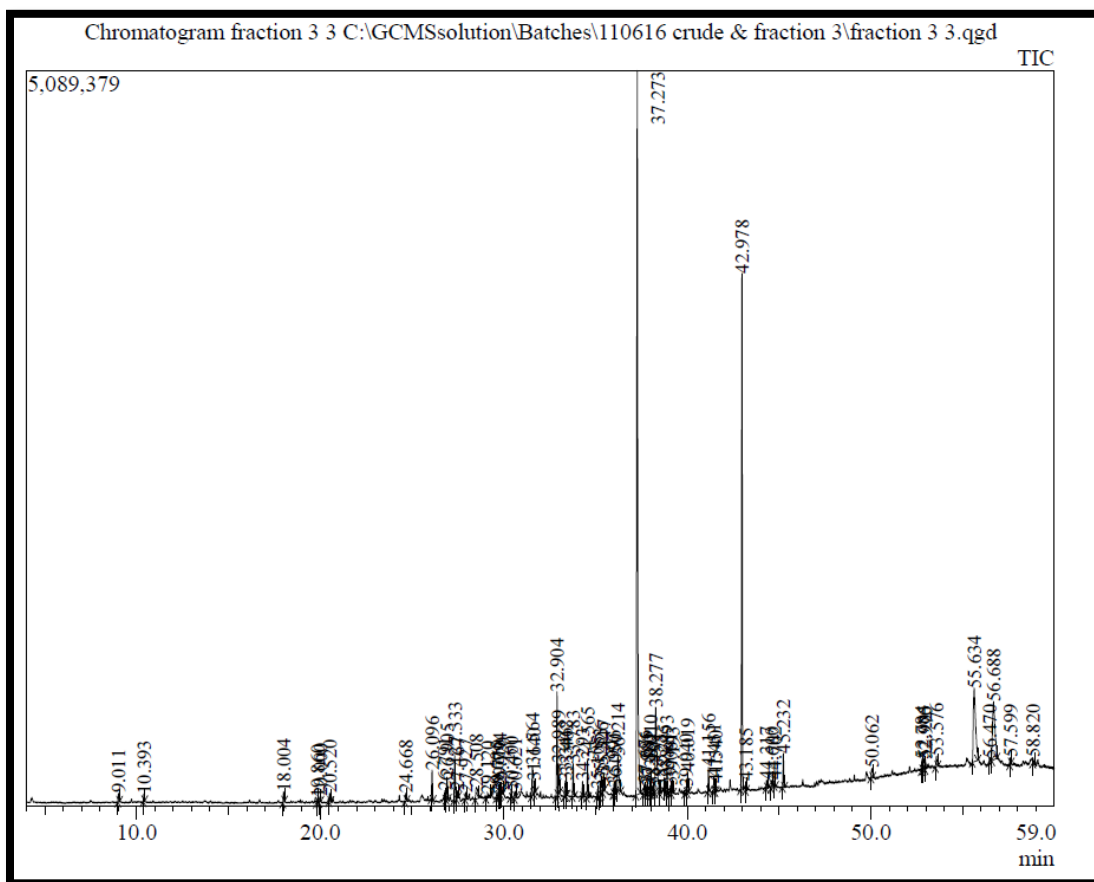


Figure 6.4: GC-MS chromatogram for *C. asiatica* pooled fraction-3.

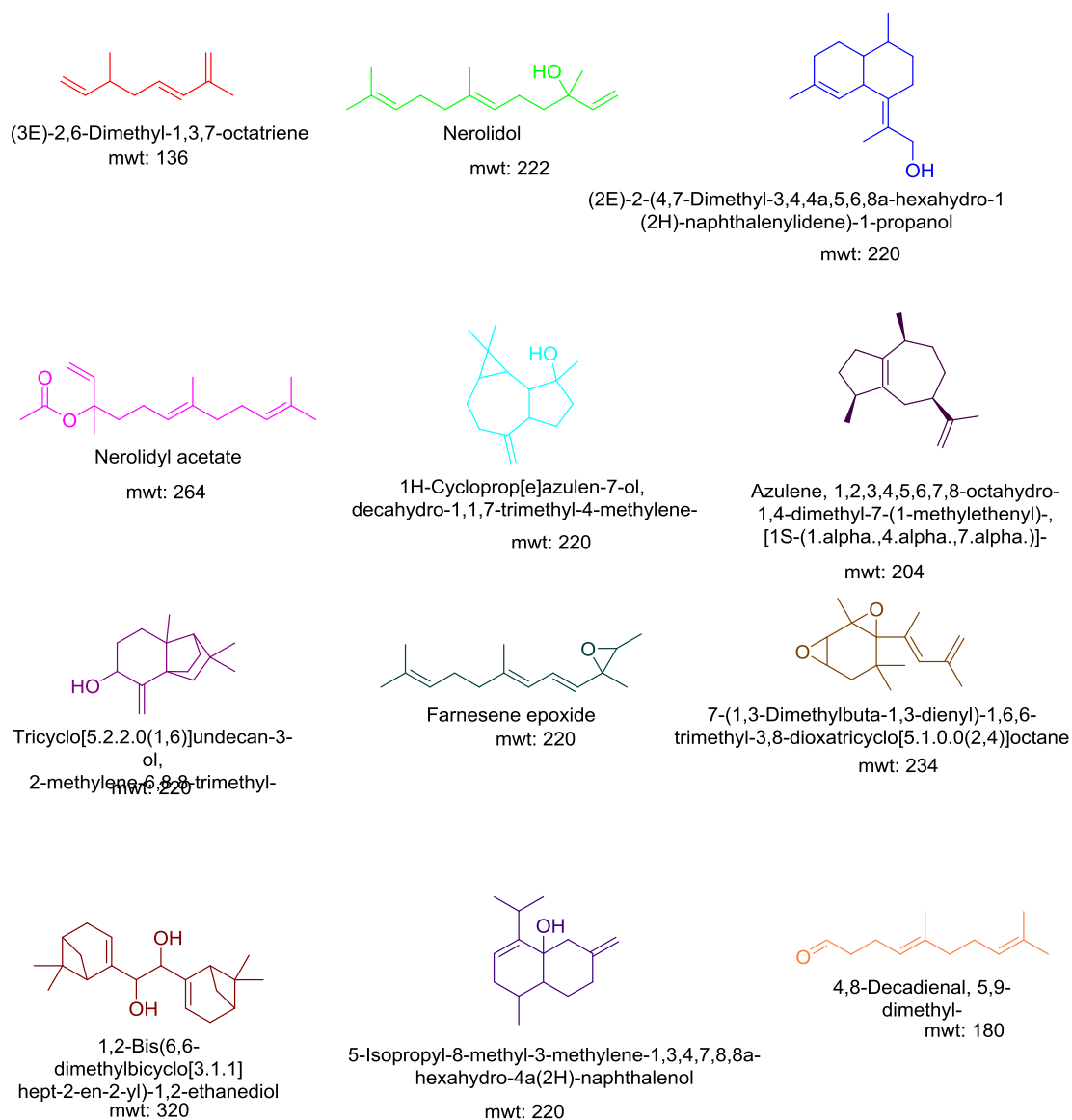


Figure 6.5: Structures of isoprenoid constituents in *C. asiatica* pooled fraction-3.

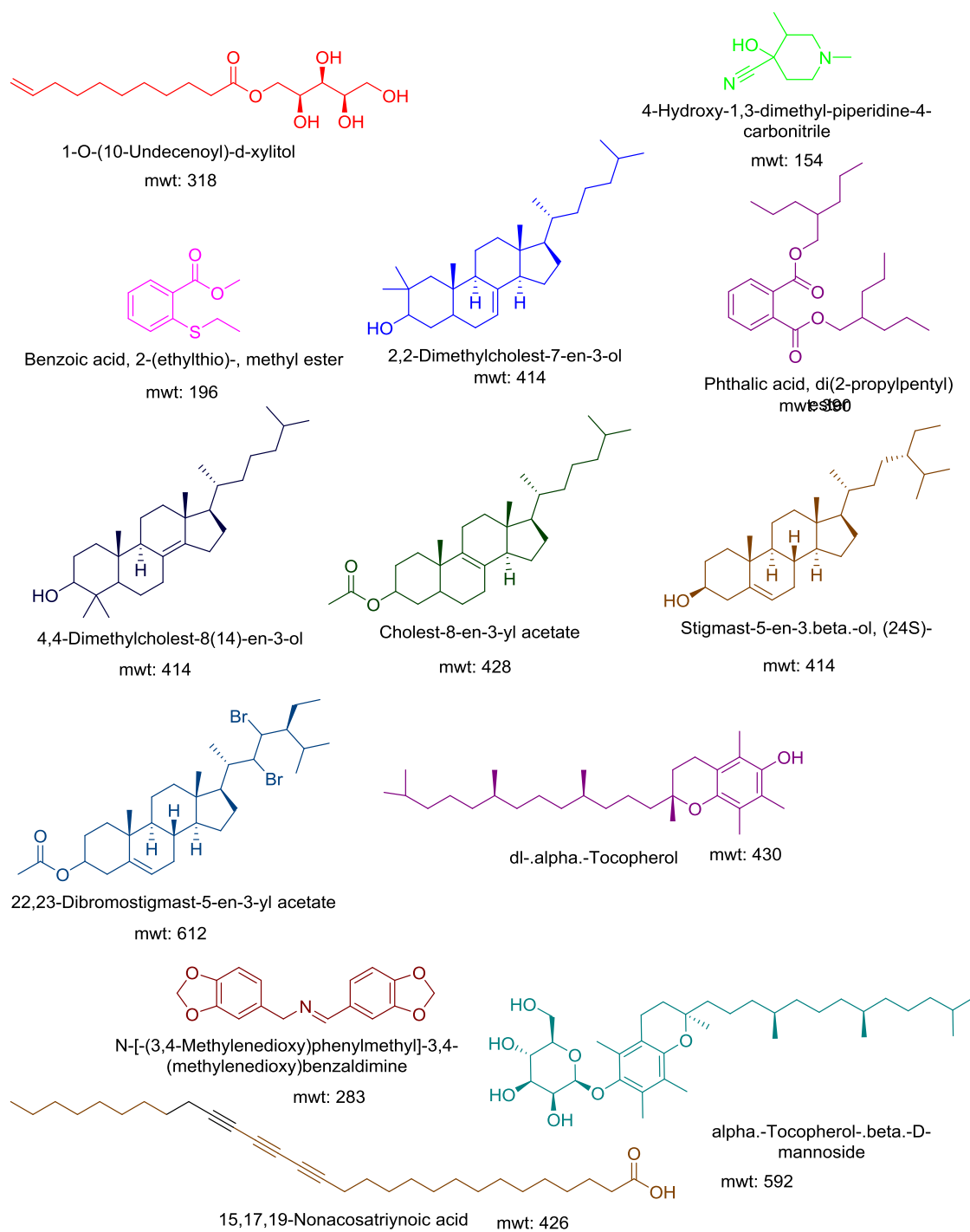


Figure 6.6: Structures of steroids and non-isoprenoid constituents in *C. asiatica* pooled fraction-3.

Reference

1. Duraisamy G; Manokaran K; Ganesan R; Kanakasabapathi D; Chandrasekar U. GC-MS analysis of bioactive compounds from the whole plant ethanolic extract of *Evolvulus alsinoides* (L.) L. *J Food Sci Technol* (2015) 52(2):1212-1217

6.3 Identification of secondary metabolites

The chemical composition of the leaf ethanolic extract of *C. asiatica* constituents were identified after comparison with those available in the computer library (NIST) attached to the GC-MS instrument and reported in Table 7.1 and 7.2. No hit compounds were obtained for *C. asiatica* leaf extract but, were further elucidated in the GC-MS assay. The organic compounds that were identified in *C. asiatica* leaf extract namely: caranol, oxymetholone methanol adduct, vitamin E tocopherol and terpenes such as phytosterol and stigmasterol. Some of the compounds detected in this study were consistent with those of previously published studies in which chemical components were isolated by using various organic solvent extractions (Kashmira *et al.* 2010).

In two phytochemical investigations of the plant examination, the presence of tannins, steroids, terpenoids, flavonoids and glycosides were identified. However, it is interesting to observe that the chemical compounds from *C. asiatica* leaf extracts vary, in earlier International work in places such as India, phytochemical studies reveal the presence of spathulenol, oleic acid, isomycorene and phthalic acid, hex-3-yl isobutyl ester, sitosterol, geranyl-.alpha.-terpinene as the main constituents (Kashmira *et al.* 2010). On the contrary, in other parts of the world, southeast asia the plants grown there showed a higher consistency of 3,4-dihydrocoumarin, nerolidol, Fumaric acid and xylitol. In accordance with previous studies *C. asiatica* variability in chemical composition in the current study are dependent on the selection of extractant, extraction techniques, climatic and geographical conditions (Jayashree *et al.* 2003).

GC-MS results indicating the chemical constituents present in *C. asiatica* crude leaf extract (Table 6.1) and (Table 6.2) *C. asiatica* pooled fraction-3 leaf extract.

Table 6.1: *C. asiatica* crude extract.

Peak no.	Compound	MF	MW (g/mol)	RT	Area (%)
1	4-Hexen-3-one, 4,5-dimethyl- 4,5-Dimethyl-4-hexen-3-one	C ₈ H ₁₄ O	126	17.825	7.53
2	cis-.beta.-Farnesene 1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-, (Z)- (Z)-.beta.-Farnesene (6Z)-7,11-Dimethyl-3-methyle	C ₁₅ H ₂₄	204	24.105	0.24
3	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-, [S-(R*,S*)]- .beta.-Sesquiphellandrene 3-((1S)-1,5-Dimethyl-4-hexenyl)-6	C ₁₅ H ₂₄	204	24.105	0.24
4	1-Cyclohexene-1-methanol, .alpha.,2,6,6-tetramethyl- 1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)ethanol	C ₁₁ H ₂₀ O	168	31.551	0.64
6	5-Caranol, trans,trans-(+)- 4,7,7-Trimethylbicyclo[4.1.0]heptan-2-ol	C ₁₀ H ₁₈ O	154	31.551	0.64
8	2-Isopropyl-5-methyl-1-heptanol	C ₁₁ H ₂₄ O	172	33.045	0.87
9	6.beta.,6.beta.-Dibromo-6,7-methylenetestosterone 1,1-Dibromo-8-hydroxy-5a,7a-dimethyl-1a,4,5,5a,5b,6,7,7a,8,9,10,10a,10b,10c-tetr	C ₂₀ H ₂₆ Br ₂ O ₂	456	36.180	1.14
14	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1.alpha.2.beta.,5.beta.)]- (1S,2R,5R)-(+)-Isomenthol 2-Isopropyl-5-methylcycloh	C ₁₀ H ₂₀ O	156	38.226	4.23

15	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1.alpha.,2.alpha.,5.beta.)- Menthol, trans-1,3,trans-1,4- Neo-Menthol Neomenthol	C ₁₀ H ₂₀ O	156	38.226	4.23
17	2H-Benzo[f]oxireno[2,3-E]benzofuran-8(9H)-one, 9-[[[2-(dimethylamino)ethyl]amino]methyl]octahydro-2,5a-dimethyl-	C ₁₉ H ₃₂ N ₂ O ₃	336	44.009	0.30
19	Oxymetholone Methanol Adduct	C ₂₂ H ₃₄ O ₃	346	44.340	0.27
20	9-(2',2'-Dimethylpropanoilhydrazono)-3,6-dichloro-2,7-bis-[2-(diethylamino)ethoxy]fluorene N'-(3,6-Dichloro-2,7-bis[2-(diethylamin	C ₃₀ H ₄₂ C ₁₂ N ₄ O ₃	576	45.256	0.51
21	gamma.-Tocopherol 2H-1-Benzopyran-6-ol, 3,4-dihydro-2,7,8-trimethyl-2-(4,8,12-trimethyltridecyl)- 6-Chromanol, 2,7,8-trimethy	C ₂₈ H ₄₈ O ₂	416	52.536	0.24
22	Stigmastan-3,5,22-trien (22E)-Stigmasta-3,5,22-triene	C ₂₉ H ₄₆	394	52.910	0.48
23	alpha.-ylangene	C ₁₅ H ₂₄	204	24.785	0.28
24	beta.-Bisabolene Cyclohexene, 1-methyl-4-(5-methyl-1-methylene-4-hexenyl)-, (S)- 1,5-Heptadiene, 6-methyl-2-(4-methyl-3-cyclo	C ₁₅ H ₂₄	204	25.563	1.18
25	cis-.alpha.-Bisabolene 4-[(1Z)-1,5-Dimethyl-1,4-hexadienyl]-1-methyl-1-cyclohexene .alpha.-Bisabolene (Z) (Z)-.alpha.-Bisabo	C ₁₅ H ₂₄	204	25.563	1.18

Table 6.2: *C. asiatica* Pooled fraction-3.

Peak no.	Compound	MF	MW (g/mol)	RT	Area (%)
1	Isomyocorene (3E)-2,6-Dimethyl-1,3,7-octatriene 2,6-Dimethyl 1,3,7-octatriene (alpha menthrene)	C ₁₀ H ₁₆	136	20.520	0.29
2	Oleic Acid 9-Octadecenoic acid (Z)- .DELTA.9-cis-Oleic acid cis-Oleic Acid cis-9-Octadecenoic Acid Emersol 211	C ₁₈ H ₃₄ O ₂	282	24.668	0.29
3	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl- (2,6,6-Trimethyl-2-hydroxycyclohexylidene)acetic acid lactone 4,5,7,	C ₁₁ H ₁₆ O ₂	180	26.096	1.07
4	2-Methoxy-4-methyl-bicyclo[3.2.1]oct-2-ene	C ₁₀ H ₁₆ O	152	26.096	1.07
5	Nerolidol 2 (6E)-3,7,11-Trimethyl-1,6,10-dodecatrien-3	C ₁₅ H ₂₆ O	222	26.791	0.20
6	Fumaric acid, ethyl 2-methylallyl ester	C ₁₀ H ₁₄ O ₄	198	26.905	0.74
7	(-)-Spathulenol 1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1aS-(1a.alpha.,4a.alpha.,7.beta.)] Ent-Spath	C ₁₅ H ₂₄ O	220	27.333	1.65
8	11,11-Dimethyl-spiro[2,9]dodeca-3,7-dien	C ₁₄ H ₂₂	190	27.467	0.22
9	cis (2E)-2-(4,7-Dimethyl-3,4,4a,5,6,8a-hexahydro-1(2H)-naphthalenylylidene)-1-propanol 100	C ₁₅ H ₂₄ O	220	27.927	0.21
10	geranyl-.alpha.-terpinene	C ₂₀ H ₃₂	272	28.508	0.93

11	2-Cyclohexen-1-one, 2,4,4-trimethyl-3-(3-oxo-1-butenyl)- 3-Keto-.beta.-ionone 2,4,4-Trimethyl-3-(3-oxo-1-butenyl)-2-cyclohexen	$C_{13}H_{18}O_2$	206	29.120	0.19
12	Gonane-12,17-dione, 3-(acetyloxy)-5,14-dimethyl-, (3.beta.,5.beta.,8.alpha.,9.beta.,10.alpha.,13.xi.,14.beta.)- 5,14-Dimethyl-12,17-dio	$C_{21}H_{30}O_4$	346	29.710	0.17
13	(p-Hydroxyphenyl)glyoxal Benzeneacetaldehyde, 4-hydroxy-.alpha.-oxo- Glyoxal, (p-hydroxyphenyl)- Glyoxal, p-hydroxyphen	$C_8H_6O_3$	150	29.920	0.18
14	1H-Benzocyclohepten-7-ol, 2,3,4,4a,5,6,7,8-octahydro-1,1,4a,7-tetramethyl-, cis- 1H-Benzocyclohepten-7-ol, 2,3,4,4a,5,6,7,8-octahyd	$C_{15}H_{26}O$	222	30.400	0.28
15	Dehydroxy-isocalamendiol 5-Isopropyl-8-methyl-3-methylene-1,3,4,7,8,8a-hexahydro-4a(2H)-naphthalenol	$C_{15}H_{24}O$	220	31.640	0.33
16	Phthalic acid, hex-3-yl isobutyl ester	$C_{18}H_{26}O_4$	306	33.346	0.43
17	Phthalic acid, 5-methylhex-2-yl isobutyl ester	$C_{19}H_{28}O_4$	320	33.346	0.43
18	3,4-Dihydrocoumarin, 4,4-dimethyl-6-hydroxy- Coumarin-6-ol, 3,4-dihydro-4,4-dimethyl- 6-Hydroxy-4,4-dimethyl-2-chromanone	$C_{11}H_{12}O_3$	192	35.326	0.65
19	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, [1S-(1.alpha.,2.beta.,5.beta.)]- (1S,2R,5R)-(+)-Isomenthol 2-	$C_{10}H_{20}O$	156	38.277	6.08

	Isopropyl-5-methylcycloh				
20	1,1,3,6-tetramethyl-2-(3,6,10,13,14-pentamethyl-3-ethyl-pentadecyl)cyclohexane	C ₃₂ H ₆₄	448	50.062	0.46
21	Cholesta-4,6-dien-3-ol, (3.beta.)-4,6-Cholestadien-3.beta.-ol Cholesta-4,6-dien-3-ol	C ₂₇ H ₄₄ O	384	52.905	0.52
22	(+)-.gamma.-Tocopherol, O-methyl-	C ₂₉ H ₅₀ O ₂	430	53.576	0.52
23	beta.-Sitosterol Stigmast-5-en-3-ol, (3.beta.)- Stigmast-5-en-3.beta.-ol .alpha.-Dihydrofucosterol .beta.-Sitosterin Angelic	C ₂₉ H ₅₀ O	414	56.688	5.90
24	Propiolic acid, 3-(1-hydroxy-2-isopropyl-5-methylcyclohexyl)-, ethyl ester Ethyl 3-(1-hydroxy-2-isopropyl-5-methylcyclohexyl)-2-pro	C ₁₅ H ₂₄ O ₃	252	58.820	0.22
25	Dihydroartemisinin, 6-deshydro-5-deshydroxy-3-desoxy- Dihydroartemisinin, 6-dehydro-5-dehydroxy-3-deoxy	C ₁₅ H ₂₂ O ₃	250	58.820	0.22
26	d,l-Xylitol, 1-O-undec-10-enoyl-1-O-(10-Undecenoyl)-d-xylitol	C ₁₆ H ₃₀ O ₆	318	58.820	0.22

Key: MF = Molecular Formula; MW = Molecular Weight; RT = Retention time

References

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APPENDIX 7

Manuscript: *Centella asiatica* decreases nuclear factor kappa-beta (p50, p65) protein expression, decreases pro-inflammatory cytokine levels and modulates cell death in leukaemic (THP-1) cells.

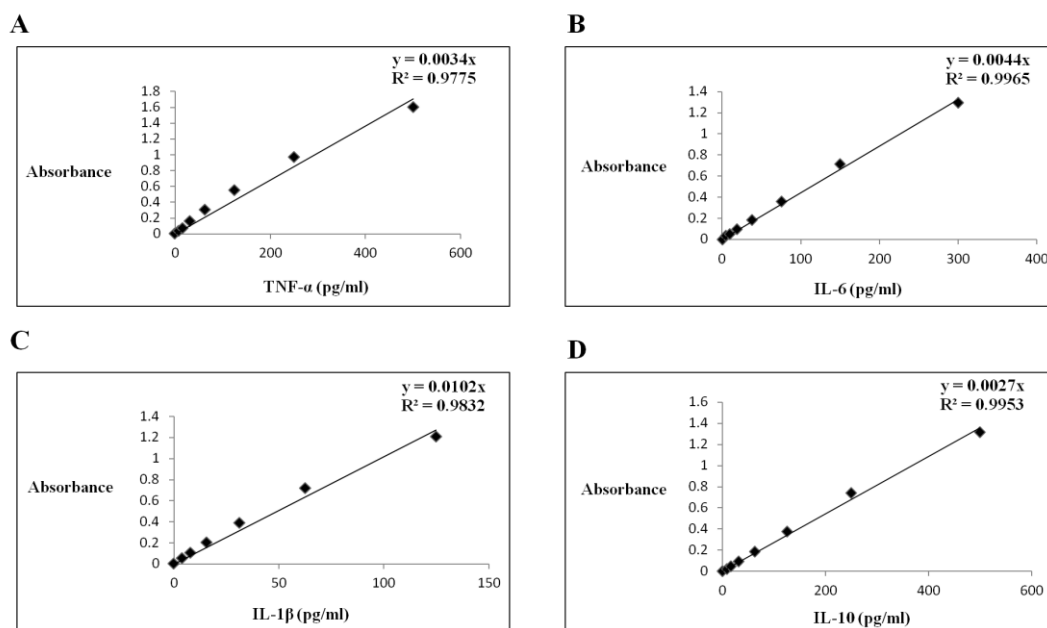


Figure 7.1: TNF- α (A), IL-6 (B), IL-1 β (C) and IL-10 (D) standard curves.

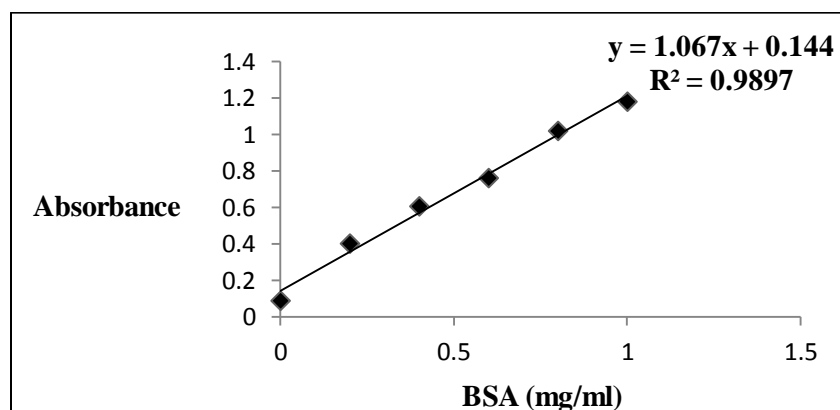


Figure 7.2: BSA standard curve for C and C3 treated THP-1 cells.

Table 7.1: Concentration of inflammatory cytokines (TNF- α , IL-6, IL-1 β and IL-10) in LPS stimulated and C and C3 treated PBMC's.

Extracts (0.1 mg/ml)	TNF- α (pg/ml)	IL-6 (pg/ml)	IL-1 β (pg/ml)	IL-10 (pg/ml)
DMSO+LPS	6.96 \pm 0.45	575.45 \pm 10.46	165.07 \pm 0.79	13.33 \pm 0.64
C	9.80 \pm 0.85 *	520.61 \pm 22.28 **	153.07 \pm 2.08 ***	8.02 \pm 0.77 ***
C3	5.88 \pm 0.59	501.89 \pm 11.96 ***	168.59 \pm 1.01 *	6.42 \pm 0.57 ***
P value	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$

Key - * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$, compared to the control

Table 7.2: Modulation of caspase (-8, -9, -3/7) in LPS stimulated and C and C3 treated PBMC's for 24 h.

Extracts (100 mg/ml)	Caspase-8 (RLU)	Caspase-9 (RLU)	Caspase-3/7 (RLU)
DMSO+LPS	5.58 x 10 ³ \pm 1.85 x 10 ³	8.07 x 10 ⁴ \pm 1.50 x 10 ³	1.07 x 10 ⁵ \pm 4.17 x 10 ³
C_100	5.50 x 10 ³ \pm 1.71 x 10 ²	8.47 x 10 ⁴ \pm 2.24 x 10 ³	9.70 x 10 ⁴ \pm 3.69 x 10 ³
C3_100	5.24 x 10 ³ \pm 1.13 x 10 ⁴	9.43 x 10 ⁴ \pm 2.87 x 10 ³ **	1.06 x 10 ⁵ \pm 1.63 x 10 ³
P value	$p = 0.4930$	$p = 0.0003$	$p = 0.0005$

Key - * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$, compared to the control

Table 7.3: Modulation of caspase (-8, -9, -3/7) in LPS stimulated and C and C3 treated PBMC's for 72 h.

Extracts (100 mg/ml)	Caspase-8 (RLU)	Caspase-9 (RLU)	Caspas-3/7 (RLU)
DMSO+LPS	7.24 x 10 ³ \pm 6.55 x 10 ³	5.65 x 10 ⁴ \pm 4.09 x 10 ³	4.77 x 10 ⁴ \pm 9.97 x 10 ⁰
C_100	2.49 x 10 ³ \pm 1.85 x 10 ³	3.91 x 10 ⁴ \pm 4.61 x 10 ² *	3.07 x 10 ⁴ \pm 2.36 x 10 ³ *
C3_100	2.43 x 10 ³ \pm 3.93 x 10 ²	5.53 x 10 ⁴ \pm 3.99 x 10 ³	4.87 x 10 ⁴ \pm 5.09 x 10 ³
P value	$p = 0.4205$	$p = 0.0010$	$p = 0.0051$

Key - * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$, compared to the control

Due to the minimal decrease in pro-inflammatory cytokines, experimentation only continued on THP-1 cells.

APPENDIX 8

Manuscript: *Centella asiatica* modulates Nrf-2 antioxidant mechanisms and enhances ROS mediated apoptosis in leukaemic THP-1 cells.

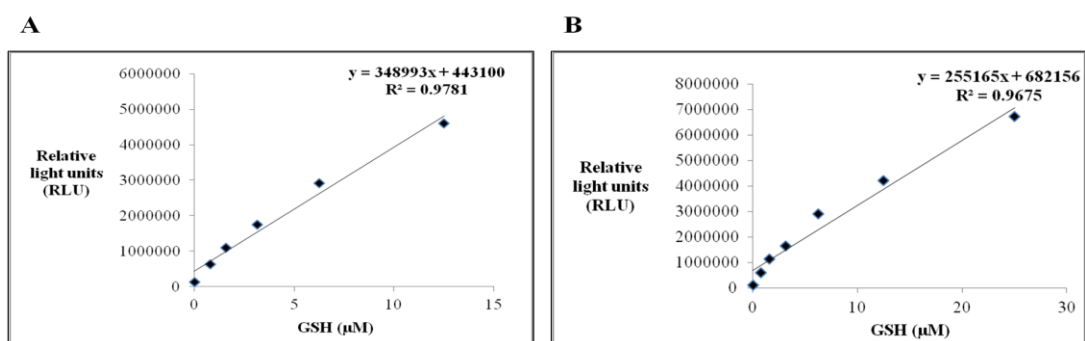


Figure 8.1: GSH standard curves for C (A) and C3 (B) treated THP-1 cells.

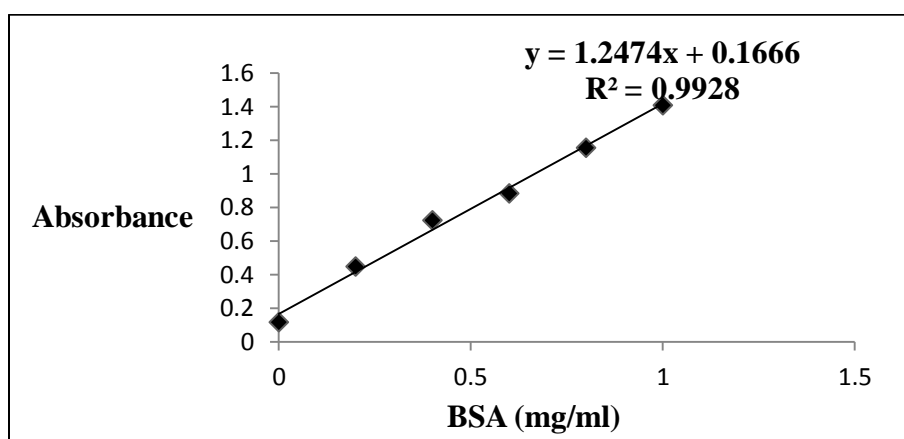


Figure 8.2: BSA standard curve for C and C3 treated THP-1 cells.

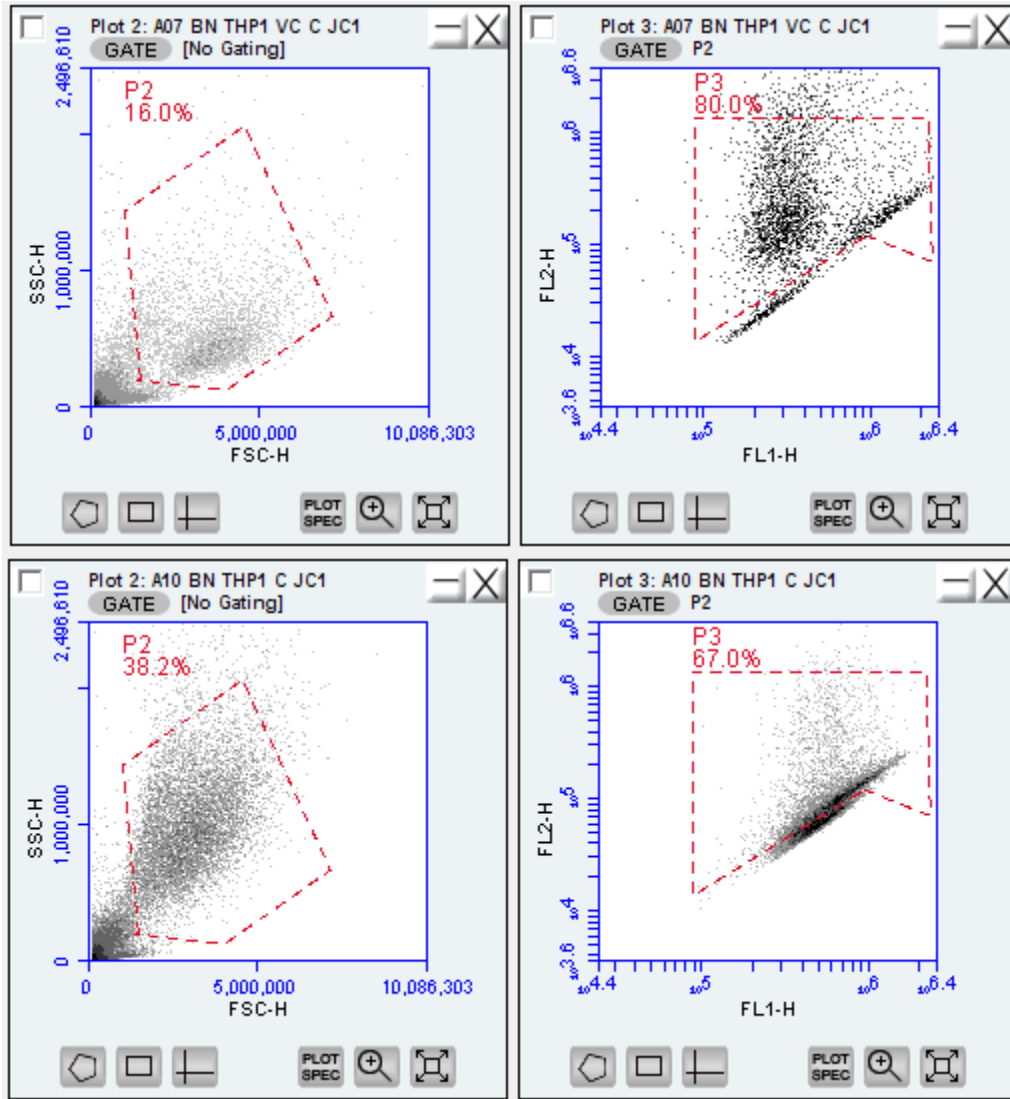


Figure 8.3: JC-1 assay scatter plots for C treated THP-1 cells.

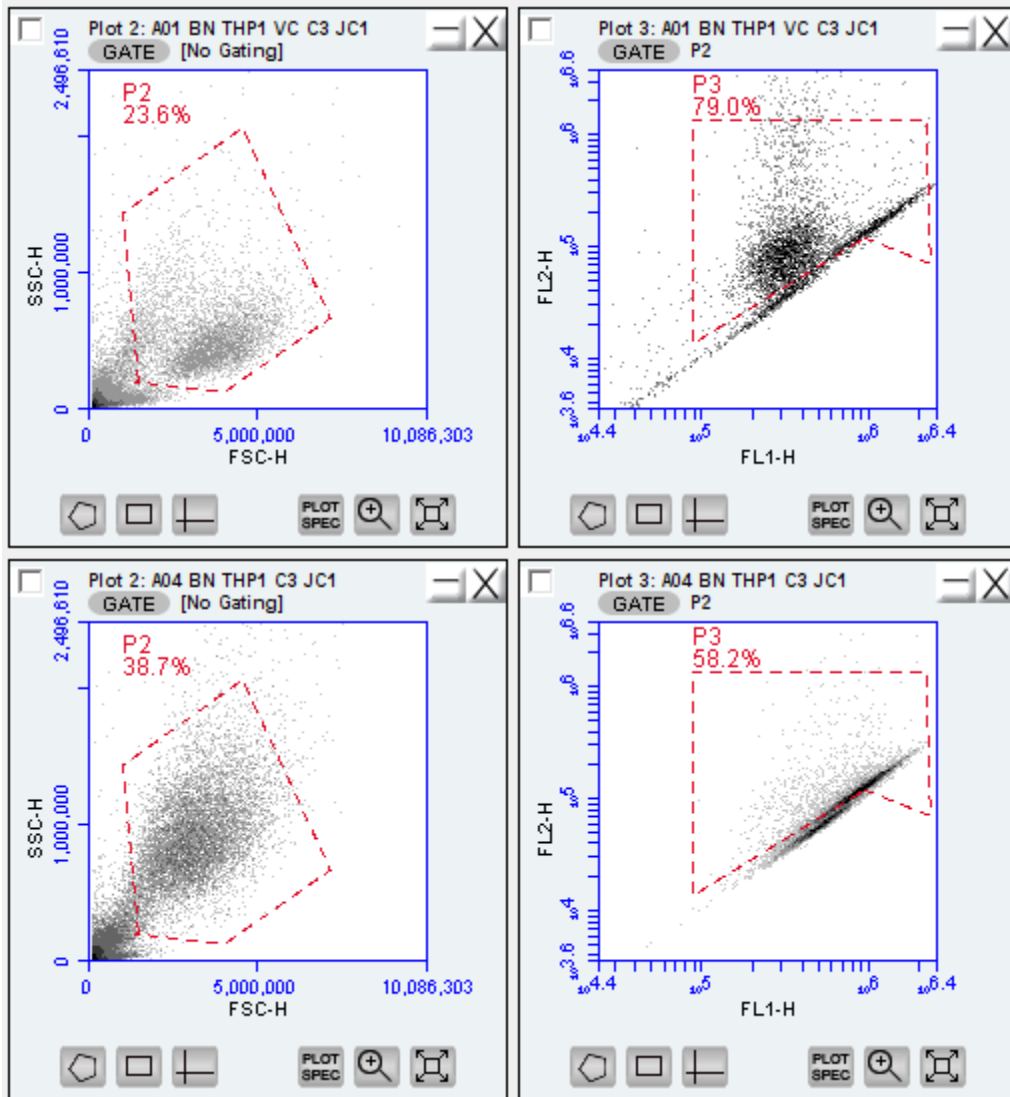


Figure 8.4: JC-1 assay scatter plots for C3 treated THP-1 cells.

APPENDIX 9

Manuscript: *Centella asiatica* purified fraction-3 suppresses the Nrf-2 antioxidant pathway and enhances ROS mediated cell death in cancerous lung (A549) cells.

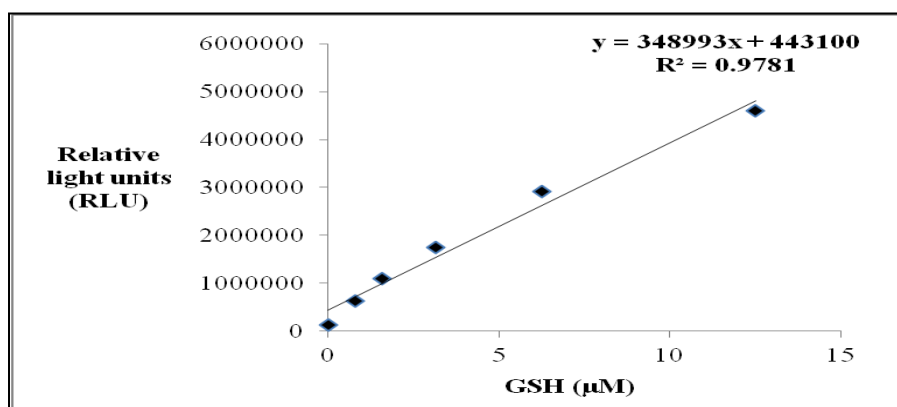


Figure 9.1: GSH standard curve for C3 treated A549 cells.

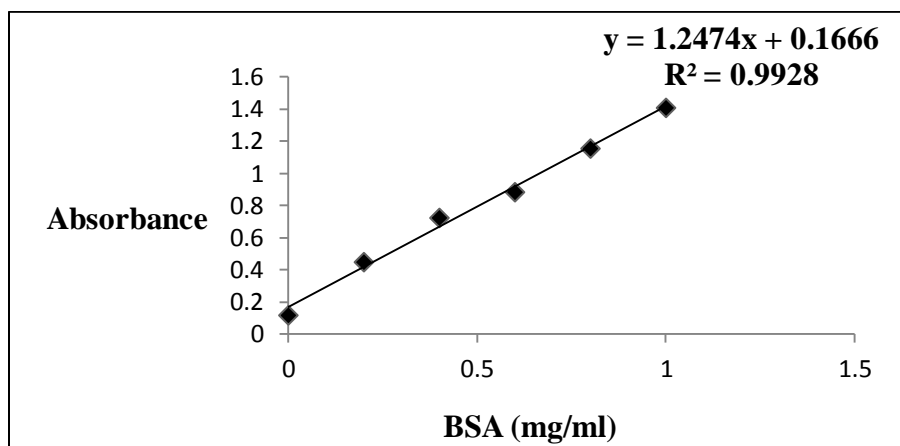


Figure 9.2: BSA standard curve for C3 treated A549 cells.

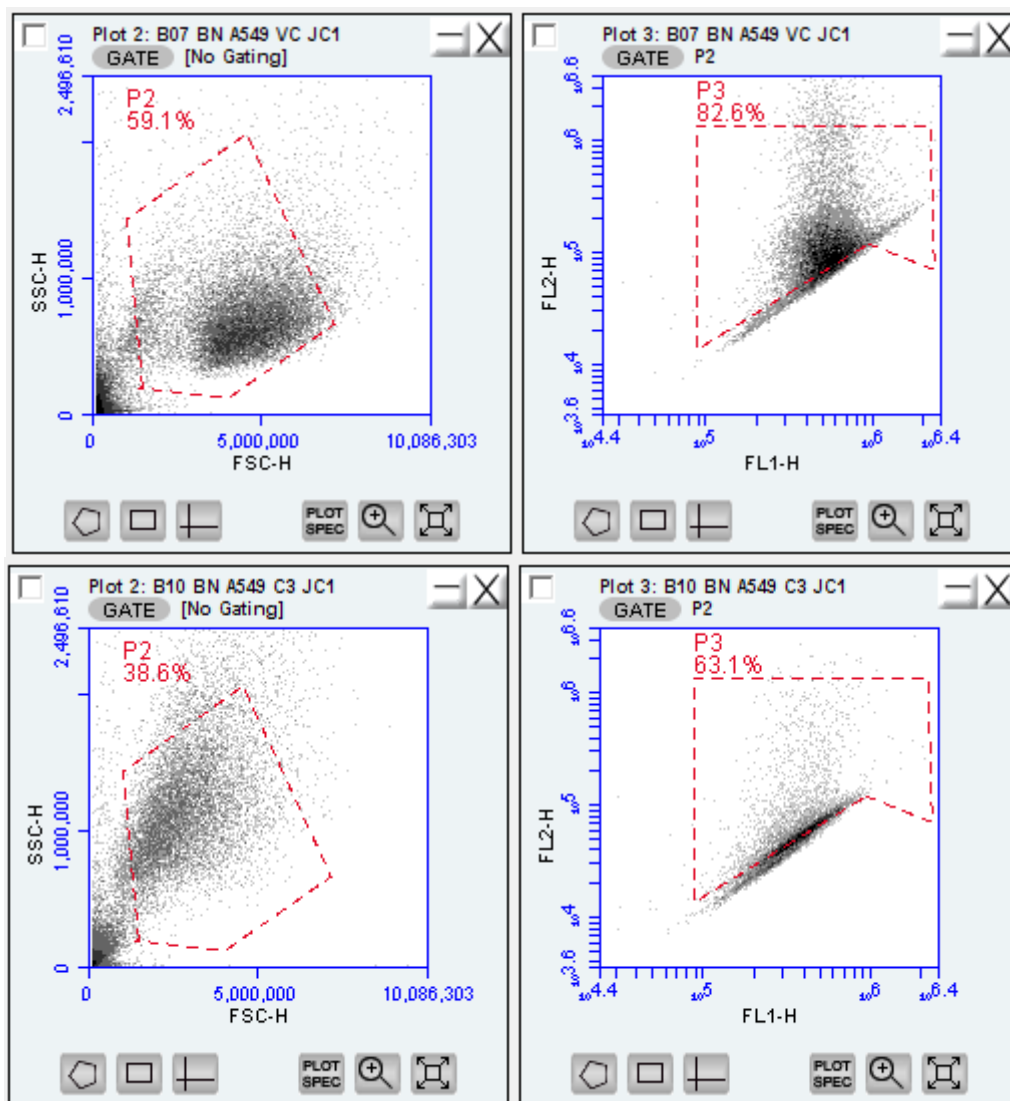


Figure 9.3: JC-1 assay scatter plots for C3 treated A549 cells.

APPENDIX 10

Manuscript: *Centella asiatica* purified fraction-3 suppresses the Nrf-2 antioxidant pathway and enhances apoptotic cell death in human embryonic kidney (HEK293) cells.

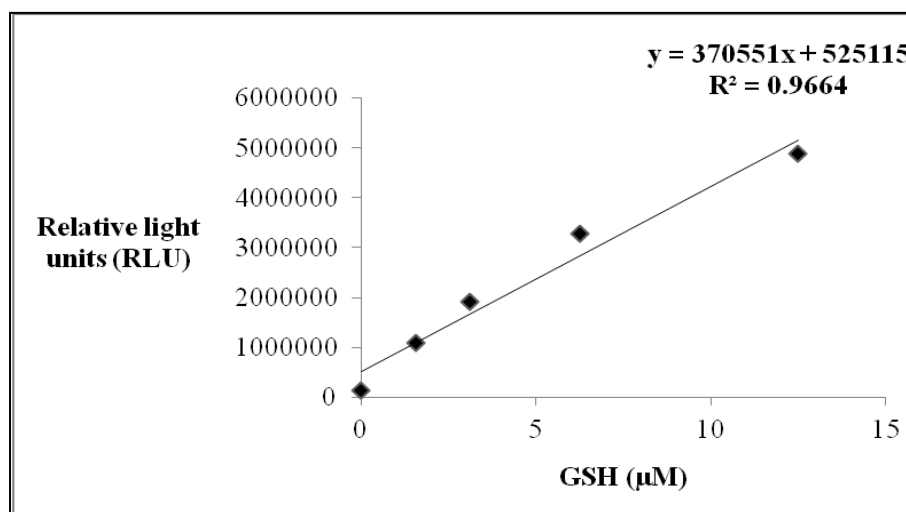


Figure 10.1: GSH standard curve for C3 treated HEK293 cells.

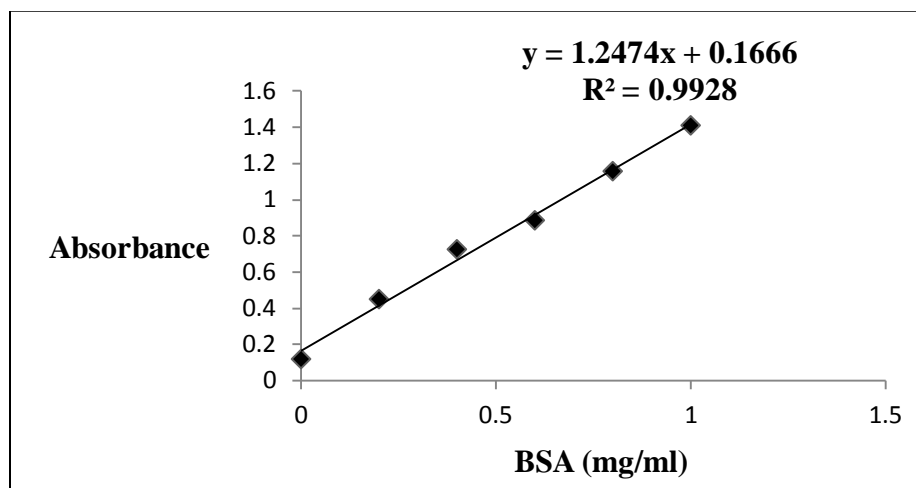


Figure 10.2: BSA standard curve for C3 treated HEK293 cells.

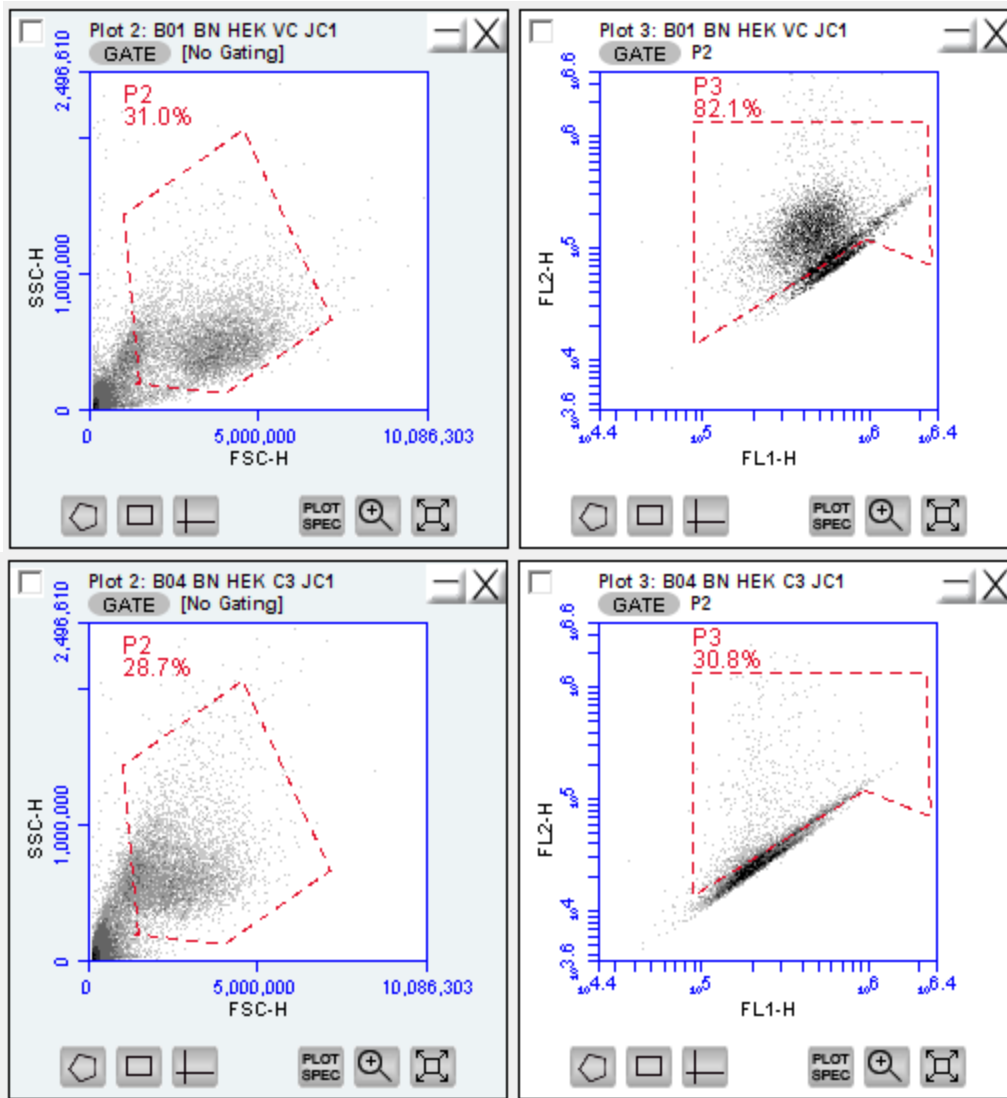


Figure 10.3: JC-1 assay scatter plots for C3 treated HEK293 cells.

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