

The use of *in vitro* assays to screen for endocrine modulation

by

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Submitted in partial fulfilment of the requirement for the
degree of Magister Scientiae (M.Sc.) in Immunology in the
Department of Medical Bioscience, University of the Western
Cape, South Africa.

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November 2008

Declaration

I, Rahzia Hendricks declare that the thesis entitled ‘The use of *in vitro* assays to screen for endocrine modulation’ is my work and has not been submitted for any degree or examination at any other university and that all sources of my information have been quoted as indicated in the text and/or list of reference.



Rahzia Hendricks

November 2008

ACKNOWLEDGEMENTS

I would like to thank **God, Almighty** for giving me strength for all my accomplishments.

My sincere thanks go to **Prof. EJ. Pool**, for allowing me to further improve my studies and the significant input towards my work.

My sincere appreciation and thanks go to **my parents and brothers** for their unconditional love, support and encouragement. Thanks for giving me the opportunity to prove myself and explore my capabilities.

I would also like to give special thanks to my loving **fiancé** for his unwavering support in all of my endeavours and who has made a major contribution to advancing both my personal growth and my academic career.



My sincere thanks also go to **my family and close friends**, who encouraged me to further my studies and supported me through the struggles of the last two years.

Furthermore, my thanks go to **my colleagues from the Immunology laboratory, Department of Medical Bioscience**, for all their encouragement and support.

Finally, I would like to thank the **National Research Foundation, Canon Collins Trust, DAAD and Ernst and Ethel Eriksen Trust** for financial support of my studies, whom without I would be unable to complete this degree.

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LIST OF ABBREVIATIONS

%	percent
°C	degrees centigrade
µg/ml	microgram per millilitre
2-AAF	2-acetylaminofluorene
<i>A. radix</i>	<i>Astragalus radix</i>
<i>A. sativum</i>	<i>Allium sativum</i>
<i>A.linearis</i>	<i>Aspalathus linearis</i>
A431	human epithelial carcinoma cell line
AFB ₁	aflatoxin B ₁
ANOVA	one way analysis of variance
<i>B. cereus</i>	<i>Bacillus cereus</i>
<i>C. sinensis</i>	<i>Camellia sinensis</i>
CCl ₄	carbon tetrachloride
CD4	helper T cells
CD8	cytotoxic T cells
DHA	docosahexaenoic acid
DMH	1, 2- dimethylhydrazine

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPH	radical diphenylpicrylhydrazyl
E1	Estrone
E2	Estradiol
E3	Estriol
EC	epicatechin
ECG	epicatechin gallate
EGC	epigallocatechin
EGCG	epigallocatechin gallate
ELISA	Enzyme Linked Immunosorbent Assay
EPA	eicosapentaenoic acid
ER	Estrogen Receptor
ER α	Estrogen Receptor alpha
ER β	Estrogen Receptor beta
g	gram
GC	Gas Chromatography
H ₂ SO ₄	sulphuric acid

HPLC	High Performance Liquid Chromatography
HRP	horse radish peroxidase
ICR	Imprinting Control Region
IFN γ	Interferon gamma
Ig	Immunoglobulins
IL-10	Interleukin-10
IL-1 β	Interleukin-1 β
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrophotometry
LDH	Lactate Dehydrogenase
LPS	lipopolysaccharide
M	Molar
<i>M. luteus</i>	<i>Micrococcus luteus</i>
MAT	matairesinol
MCF-7	Human breast adenocarcinoma cell line
mg/ml	milligram per millilitre

MHC	Major Histocompatibility Complex
min	minute
ml	millilitre
NaHCO ₃	Sodium bicarbonate
NIH3T3	mouse embryonic fibroblast cell line
NK cells	natural killer cells
<i>O. niloticus</i>	<i>Oreochromis niloticus</i>
PBMC	peripheral blood mononuclear cells
pg	picograms
pg/ml	picogram per millilitre
PGE ₂	prostaglandin
PHA	phytohemagglutinin
PRR	Pattern Recognition Receptors
R ²	correlation co-efficient
RIA	Radioimmuno assay
RPMI-1640	Roswell Park Memorial Institute 1640
RSC	Radical Scavenging Capacity
SECO-DG	Secoisolariciresinol- Diglucoside

SPE	solid phase extractions
TCR	T cell receptors
TF-3	Theaflavin-3-3' digallate
Th2	T-helper cells 2
TLRs	Toll like receptors
TMB	3,3', 5,5'-tetramethylbenzidine
TNF	Tumour Necrosis Factor
TNF α	Tumour Necrosis Factor alpha
TR	Thearubigin
WBC	whole blood cultures
WHO	UNIVERSITY of the World Health Organisation
x g	gravitational force
XTT	(23-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)
$\gamma\delta$ T cells	gamma delta T cells

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ABSTRACT

Aspalathus linearis (*A. linearis*), commonly known as Rooibos tea or Red bush tea and *Camellia sinensis* (*C. sinensis*) or Black tea are beverages that are consumed throughout the world. These teas possess antioxidant, immunomodulating and anti-cancer actions. The aim of this study was to use *in vitro* assays to screen Rooibos and Black tea for endocrine modulation.

The immune modulating effects of Rooibos and Black tea were investigated using an *in vitro* whole blood culture (WBC) assay. Unstimulated WBCs treated with Rooibos tea secreted higher levels of IL-6, IL-10 and IFN γ than cultures treated with DMSO control. Rooibos treatment of stimulated WBCs resulted in higher IL-6, lower IL-10 and no effect on IFN γ secretion compared to DMSO treated stimulated WBC. Black tea treatment of stimulated WBC resulted in decreased IL-6, IL-10 and IFN γ secretion compared to the DMSO treated stimulated WBC.

Extracts of Rooibos and Black tea were assessed for phytoestrogens using quantitative estrogen ELISAs. Both teas contain phytoestrogens. The quantitative ELISAs showed that Rooibos tea contained significantly lower estrone (E1), estradiol (E2) and estriol (E3) levels than Black tea. The effects of Rooibos and Black tea on proliferation of the estrogen dependant MCF-7 cell line was determined to further characterise the phytoestrogenic properties of the teas. Both Rooibos and Black tea extracts caused a significant inhibition of MCF-7 proliferation.

This study shows that Rooibos tea and Black tea are beverages that can either stimulate or suppress the immune system. Also, both teas contain significant levels of phytoestrogens as determined by quantitative ELISAs. The current study confirms previous reports showing

inhibition of growth in breast cancer cell lines by phytoestrogens. The findings extend related observations on the anti-carcinogenic potential of the two teas.



CHAPTER 1: Literature Review

Overview of Rooibos tea

1.1. Overview of Rooibos tea

1.1.1. Introduction

Aspalathus linearis (*A. linearis*), commonly known as Rooibos tea or Red bush tea, is a legume that grows predominantly and is indigenous to the Cedarberg and neighbouring mountains of South Africa (Nel *et al.*, 2007). There are two kinds of Rooibos, i.e. the domesticated or the 'Nortier' tea and the 'wild' or naturally occurring type. The Nortier variety is cultivated plants. Moreover, the tea has been known to have particular health benefits, such as being caffeine free and possessing compounds that act as anti-oxidants (Nel *et al.*, 2007).



1.1.2. Consumption of Rooibos tea

Consumption of Rooibos tea is increasing in popularity due to its reputation as a health drink, its unique taste, and versatility (Jagani & Wheeler, 2003).

1.1.3. Chemical composition of Rooibos tea

Different flavonoids have been isolated from Rooibos tea. These include flavonols, flavones and dihydrochalcones. Rooibos tea does not contain caffeine (a xanthine alkaloid) and is considered a low tannin beverage (Joubert *et al.*, 2008). A monomeric flavonoid, namely aspalathin is found in Rooibos tea. It is one of the main flavonoids within *A. linearis*. This flavonoid is a C-C linked dihydrochalcone glucoside (Joubert, 1996). Shimamura *et al.*, (2006), isolated another flavonoid aspalalinin, which is a cyclic dihydrochalcone. Rooibos

tea contains another flavone called nothofagin (Joubert, 1996). Flavonoids and non-flavonoids found in Rooibos tea are summarized in Table 1.1.1 and Table 1.1.2, respectively.

Table 1.1.1. Flavonoids found in Rooibos tea

Flavonoid subclass	Found in Rooibos tea	Reference
Monomeric Flavonoids	Aspalathin, Aspalalinin, Nothofagin	Rabe <i>et al.</i> , 1994; Shimamura <i>et al.</i> , 2006
Flavones	Orientin, iso-orientin, isovitexin	Rabe <i>et al.</i> , 1994 ; Joubert <i>et al.</i> , 2008
Flavanones	Dihydro-orientin, dihydro-iso-orientin, hemiphlorin	Bramati <i>et al.</i> , 2002 ; Shimamura <i>et al.</i> , 2006
Flavanols	Quercetin, quercetin-3-robinobioside, hyperoside, isoquercetrin, rutin	Shimamura <i>et al.</i> , 2006 ; Bramati <i>et al.</i> , 2002

Table 1.1.2. Non-flavonoids found in Rooibos tea

Non-flavonoid subclass	Found in Rooibos tea	Reference
Lignans	Vladinol E, secoisolariciresinol, secoisolariciresinol glucoside	Rabe <i>et al.</i> , 1994 ; Shimamura <i>et al.</i> 2006
Phenolic acids	caffeic acid, ferulic acid, <i>p</i> -coumaric acid, <i>p</i> -hydroxybenzoic acid, vanillic acid, protocatechuic acid	Rabe <i>et al.</i> , 1994

1.1.4. Biological properties of Rooibos tea

Several investigators have found that tea is rich in antioxidants. Antioxidant activity is the ability to trap free radicals. These free radicals may oxidize nucleic acids, proteins, lipids or deoxyribonucleic acid (DNA) and can result in degenerative disease. Antioxidant substances such as phenolic acids, polyphenols and flavonoids may be able to scavenge these free radicals, and thereby prevent oxidative damage to cells (Joubert *et al.*, 2008).

Numerous studies have been done to assess the antioxidant levels of Rooibos tea (Lindsey *et al.*, 2002; Inanami *et al.*, 1995; Shimoi *et al.*, 1996). Antioxidant action can be brought about by scavenging of free radicals, thereby preventing oxidation. This can be related to antimicrobial activity in cells and cell membranes (Frei & Higdon, 2003). Unfermented Rooibos tea has a greater antioxidant activity compared to fermented and semi fermented Rooibos tea. Fermentation of Rooibos tea causes a decrease in polyphenols, and this is possibly the reason for the decreased antioxidant potential of fermented Rooibos tea compared to unfermented Rooibos tea (Gadow *et al.*, 1997). Several of the Rooibos tea flavonoids have antioxidant activity. Of these, the flavonoid quercetin has the highest radical scavenging ability. The potency of Rooibos tea flavonoids in decreasing order are quercetin, procyanidin B3, orientin, luteolin, aspalathin, isoquercetrin, iso-orietin, catechin, rutin, vitexin and chrysoeriol (Joubert *et al.*, 2004).

In vivo, antioxidant ability of Rooibos tea has been shown in various studies. The natural antioxidants and scavenging agents of Rooibos tea result in hepatoprotective effects in a rat model of carbon tetrachloride 4 (CCl₄) -induced hepatic fibrosis (Ulćina *et al.*, 2003). A summary of the *in vitro* and *in vivo* effects of the antioxidant activity of Rooibos tea is given in Table 1.1.3.

Table 1.1.3. *In vitro* and *in vivo* studies showing antioxidant activity of Rooibos tea

Description	Reference
<p><i>In vitro</i> studies</p> <p>Inhibition of lipid peroxidation</p> <p>Radical scavenging ability assessed by the radical diphenylpicrylhydrazyl (DPPH) scavenging method</p> <p>Decrease in anti-radical capacity of the aqueous extracts and crude phenolic fractions of Rooibos due to fermentation</p>	<p>Lindsey <i>et al.</i>, 2002</p> <p>Gadow <i>et al.</i>, 1997</p> <p>Joubert <i>et al.</i>, 2004</p>
<p><i>In vivo</i> studies</p> <p>Hepatoprotection in rats treated with Carbon tetrachloride (CCl₄) to induce fibrosis</p> <p>Prevent oxidative stress in streptozocin induced diabetic rats by decreasing glycation end products in plasma and malondialdehyde in plasma</p> <p>Prevention of lipid peroxidation in the brains of mice</p> <p>Radioprotective effects of luteolin from Rooibos tea in mice</p>	<p>Ulćina <i>et al.</i>, 2003</p> <p>Ulćina <i>et al.</i>, 2006</p> <p>Inanami <i>et al.</i>, 1995</p> <p>Shimoi <i>et al.</i>, 1996</p>

Almajano *et al.*, (2008), examined Green tea, Black tea, Rooibos tea, and other commercial teas for its antioxidant and anti-microbial activity. Green and Black teas displayed the highest antioxidant activity. Similarly, the anti-microbial effects of the latter teas were the highest, particularly for *Bacillus cereus* (*B. cereus*) and *Micrococcus luteus* (*M. luteus*). The authors suggest that the highest anti-microbial activity relates to the highest antioxidant activity and less to total polyphenol content only. The Rooibos tea infusion did not exhibit strong anti-microbial activity, against *B. cereus*. Rooibos tea does not contain catechins. However, the polyphenol content of Rooibos tea consists of compounds such as aspalathin,

orientin and rutin. These compounds are postulated to be responsible for the anti-microbial effects (Almajano *et al.*, 2008).

The ability of Rooibos tea to prevent DNA damage has also been extensively studied. The Salmonella anti-mutagenicity assay has been used to screen the mutagenic activity of various natural substances (Weisburger *et al.*, 1997). Aqueous extracts of unfermented and fermented Rooibos tea displayed a strong anti-mutagenic effect against the metabolically active carcinogens, 2-acetylaminofluorene (2-AAF) and aflatoxin B₁ (AFB₁) (Marnewick *et al.*, 2000).

Very few studies have been done on the effects of Rooibos tea on cancer. One study by Marnewick *et al.*, (2005), showed that processed and unprocessed Rooibos tea resulted in a significant ($P < 0.001$) suppression of skin tumorigenesis in Imprinting Control Region (ICR) mice at 75 % and 60 % respectively. These investigators suggest that the various constituents of the teas such as the flavonol/flavone content or the flavonol/proanthocyanidan content may play a role in suppression of tumorigenesis (Marnewick *et al.*, 2005).

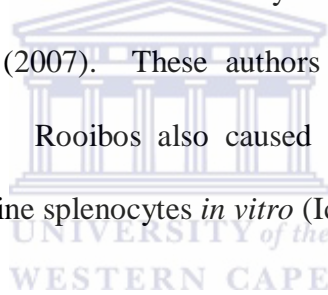
Rooibos also contain phytoestrogens. Phytoestrogens can be defined as compounds with estrogenic activities that are found in plants. These phytoestrogens may activate estrogenic effects on the central nervous system, induce estrus and allow proliferation of the female genital tract. Phytoestrogens may bind to estrogen receptors and thereby activate estrogen response genes. Phytoestrogens may also induce the growth of breast cancer cells (Kurzer and Xu, 1997).

Very few studies have been done to investigate the estrogenic properties of teas. One study showed that Rooibos tea contain three compounds that cross-react with natural estrogens in an Enzyme Linked Immunosorbent Assay (ELISA) (Shimamura *et al.*, 2006). This study

revealed that nothofagin has the highest cross-reactivity with antibodies against the natural estrogens. The other compounds in Rooibos tea with cross-reactivity are iso-vitexin and luteolin-7-glucoside. This study showed that Rooibos tea administration may benefit patients with low estrogen levels, such as patients requiring hormone replacement therapy.

Immuno modulatory activity has also been attributed to Rooibos tea. Kunishiro *et al.*, (2001), showed that Rooibos tea decreased Interleukin-2 (IL-2) production in murine splenocytes. In contrast, Rooibos tea suppressed the production of Interleukin-4 (IL-4). *In vivo* studies showed that Rooibos stimulates antigen specific antibody production, thereby enhancing immune system function.

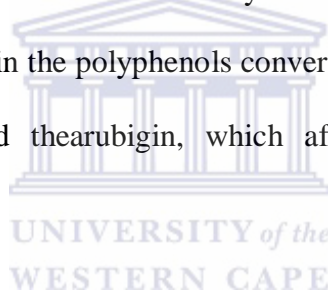
More recently, studies done on the immune modulatory effects of a Rooibos tea fraction was performed by Ichiyama *et al.*, (2007). These authors showed that Rooibos stimulates Interleukin-10 (IL-10) synthesis. Rooibos also caused decreases in IL-2 and Interferon gamma (IFN γ) production in murine splenocytes *in vitro* (Ichiyama *et al.*, 2007).



1.2. Overview of Black tea

1.2.1. Introduction

Black tea is produced from the evergreen tree *Camellia sinensis* (*C. sinensis*), belonging to the family *Theaceae*. The leaves are harvested either by special shears or by machines. After processing and drying of the leaves, the tea is essentially ready for consumption. It is prepared by adding boiling water to the leaves. Different fermentation processes of the leaves produce three main kinds of tea namely Green, Black and Oolong tea. *C. sinensis* leaves have certain polyphenols including an enzyme polyphenol oxidase. The enzyme is activated when the leaves are cut and results in the polyphenols being oxidized. After steaming, the leaves of *C. sinensis* are allowed to dry for 3-6 hours. Thereafter oxidation, of the catechins in the leaves result in the polyphenols converting to other types of polyphenols, such as theaflavine gallate and thearubigin, which after drying results in Black tea (Weisburger, 1997).



1.2.2. Consumption of Black tea

Tea is the second most consumed liquid. Only water has a higher consumption. Black tea is consumed by 73-78 % of the world's population (Krishnan & Maru, 2006).

1.2.3. Chemical Composition of Black tea

Black tea consists of various active compounds that can potentially modulate the immune system. Monomeric flavonoids such as catechins, are the major flavonoids found in tea. The catechins that are most significant are epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC) (Lunder *et al.*, 1989). Epigallocatechin is thought to have health benefits. Black tea contains approximately 10-12 % catechins, 3-6 % theaflavins, 12-18 % thearubigins, 6-8 % flavonols, 10-12 %

phenolic acids and depsides, 13-15 % amino acids, 8-11 % methylxanthines, 15 % carbohydrates, 1 % proteins, and 10 % mineral matter, and <0.1 % volatiles (Harold and Graham, 1992). Table 1.2.1 is a summary of chemicals found in Black tea.

Table 1.2.1. Chemical composition of Black tea

Compounds	Specific chemicals found in Black tea	Percent composition (%)	Reference
Catechins	Catechin, epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin (EC)	10-12	Harold & Graham, 1992 ; Lunder <i>et al.</i> , 1989
Flavonols	Quercetin, kaempferol, rutin	6-8	Dufresne & Farnworth, 2001
Teaflavin	Theaflavins-3-gallate, theaflavin-3'-gallate	3-6	Dufresne & Farnworth, 2001
Thearubigins		12-18	Dufresne & Farnworth, 2001
Amino acid	Theanine		Dufresne & Farnworth, 2001

1.2.4. Biological properties of Black tea

Compared to Green tea, the antioxidant activity of Black tea has not been extensively studied. Black tea contains less polyphenols than Green tea, and is regarded as having a weaker antioxidant activity (Halder & Bhaduri, 1998).

Tea is consumed in various ways. For instance, in the United Kingdom, Canada, Ireland and India, milk is added to tea. Addition of milk is thought to interfere with the antioxidant activity of Black tea. However, Vasundhara *et al.*, (2008), showed that addition of milk does not alter antioxidant activity.

Black tea is a more potent anti-oxidant than Oolong tea or Rooibos tea (Gadow *et al.*, 1997). Furthermore, one or two cups of Black tea contain approximately the same radical scavenging capacity (RSC) as five portions of fruit and vegetables (du Toit *et al.*, 2001).

Black tea may protect against certain diseases. The antioxidants in Black tea protect the prostate from oxidative injury (Siddiqui *et al.*, 2005). Furthermore, thearubigin (TR) and theaflavin result in a significant inhibition of 1, 2- dimethylhydrazine (DMH) - induced oxidative DNA damage in the colon mucosa of rats (Lodovici *et al.*, 2000). Studies showed that Black tea extracts prevent pancreatitis in a rat model by increasing the antioxidant, anti-inflammatory and anti-apoptotic activity (Das *et al.*, 2006). Table 1.2.2 provides a summary of *in vitro* and *in vivo* studies done on the anti-oxidative activity of Black tea.

Table 1.2.2. Antioxidant activity of Black tea

Description	Reference
<p><i>In vitro</i> studies</p> <p>Radical scavenging ability</p> <p>Inhibition of lipid peroxidation</p> <p>Protective role of Black tea against oxidative damage of human red blood cells</p>	<p>Gadow <i>et al.</i>, 1997</p> <p>Cherubini <i>et al.</i>, 1999</p> <p>Halder & Bhaduri, 1998</p>
<p><i>In vivo</i> studies</p> <p>Protection against androgen induced oxidative injury</p> <p>Slow down progression of lens opacity in rats with cataracts</p>	<p>Siddiqui <i>et al.</i>, 2005</p> <p>Thiagarajan <i>et al.</i>, 2001</p>

Black tea exhibits anti-microbial activity against *B. cereus*, *M. luteus* and *Pseudomonas aeruginosa* (Almajano *et al.*, 2008).

Black tea also contains polyphenols that have been associated with an inhibitory effect on tumorigenesis. Liang *et al.*, 1999 found that the Black tea polyphenol theaflavin-3, 3'-digallate (TF-3) strongly inhibits the growth of human epithelial carcinoma cell line (A431) and mouse embryonic fibroblast cell line (NIH3T3). EGCG and TF-3 inhibit epidermal growth factor (EGF) binding to its epidermal growth factor receptor, thereby inhibiting cell proliferation.

Studies on the anti-carcinogenic effects of Black tea and Green tea showed that these teas may facilitate the inhibition of cancer in humans (Steele *et al.*, 2000). Black tea polyphenols have been found to inhibit intestinal carcinogenesis by preventing 1, 2-dimethylhydrazine (DMH) – induced oxidative DNA damage in rat colon mucosa (Lodovici *et al.*, 2000).

It has also been reported that administration of Black tea for 11 weeks decreased skin tumours in mice (Lu *et al.*, 1997).

Not many studies have been done on the phytoestrogenic properties of Black tea. However, in one study investigators found that Black tea administration to oophorectomised rats for 21 days resulted in increased serum estradiol levels (Das *et al.*, 2005). This study also showed that Black tea significantly reduced oophorectomy-induced osteoporosis in these rats.

Tea consists of many compounds that could play a role in immunomodulation. Alkylamine antigens in tea allow gamma delta ($\gamma\delta$) T cells to act more efficiently against invading organisms. *In vitro* and *in vivo* studies showed that Black tea results in production of IFN γ in response to alkylamine antigens (Kamath *et al.*, 2003).

Many studies have shown that Black tea extracts play a role in cytokine secretion. Green and Black tea extracts showed a decrease in release of anti-inflammatory cytokines, Interleukin-6 (IL-6), Interleukin-1 beta (IL-1 β), Interleukin-8 (IL-8), tumour necrosis factor alpha (TNF α)

and prostaglandin (PGE₂) when exposed to lipopolysaccharide (LPS) (Pajonk *et al.*, 2006). Similarly, Black tea results in an inhibitory effect on IL-1 β cytokine production (Crouzier *et al.*, 2000). Black tea had no effect on anti-inflammatory cytokines IL-6, Interleukin-10 (IL-10) and TNF α (Crouzier *et al.*, 2000).

1.3. Safety and Toxicity of teas

Green tea and Black tea infusions showed toxicity to rat hepatocytes (Phillips, 1995). Moreover, 0.46 mg/ml Black tea resulted in a 50 % reduction in rate of protein synthesis in the rat hepatocytes (Phillips, 1995). A comparative study on the toxicity of various commonly consumed beverages such as teas and fruit juices found that black tea has the lowest toxicity (Ekmekcioglu *et al.*, 1999). Extensive literature searches found no toxicity data for Rooibos tea.



1.4. Impact of food-derived substances on the immune system

1.4.1. Introduction

The immune system functions to eliminate foreign micro-organisms (Perdigon *et al.*, 1995). Functions of the immune system may be altered by malnutrition, aging, physical and mental stress or undesirable lifestyle. Men who have poor or undesirable lifestyle have lower Natural Killer (NK) cell activity compared to men with a good lifestyle (Morimoto *et al.*, 2001). Families that suffer from chronic fatigue syndrome have a significantly lower NK cell activity than normal controls (Levine *et al.*, 1998). Moreover, chronic stress depresses the immune system through Toll-like receptors (TLRs) (Zhang *et al.*, 2008). Micronutrient deficiencies may affect immune function by altering the activity of NK cells (Ravaglia *et al.*, 2000). Studies have shown that vitamins and minerals enhance immune function by increasing B-cell activity, enhancing phagocytosis and increasing serum lysozyme (Bendich, 2001; Grimble, 1998; Ballou *et al.*, 1996; Sahoo & Mukherjee, 2003). Regardless of immunosuppressive factors such as, mental and physical stress, a healthy lifestyle can be achieved by consuming foods that enhance the immune system which can lead to a reduction in diseases.

Various substances found in food can modulate immune system responses (Table 1.4.1.). Modulation of the immune system can occur by consumption of foods and thereby, prevent infection, cancer or a declining immune system (Kaminogawa & Nanno, 2004). Food-derived substances may cause immunomodulation indirectly or directly. Intestinal microflora can function in the maintenance of the host immune system. However, consumption of probiotics such as lactic acid bacteria can alter the functioning of the host immune system (Fuller, 1991). Vitamins and minerals or fatty acids may preserve the cell

membrane of lymphocytes (Kaminogawa & Nanno, 2004) and thereby, increase their life-span.

1.4.2. The effect of herbal extracts on the immune system

Herbal products have been used extensively to treat diseases and improve immune system function. The plant of the genus *Echinacea* has been used medicinally for centuries. It has been reported that this plant can result in immune stimulation. These effects include stimulating peripheral blood mononuclear cells (PBMC) to increase IL-10 production (McCann *et al.*, 2007). Moreover, *Echinacea* activates cellular immunity and stimulates phagocytosis of neutrophils *in vitro* and *in vivo* (Jurkstiene *et al.*, 2004). Also, *Echinacea* extracts were able to stimulate splenocytes to produce IL-6 and TNF α (Hwang *et al.*, 2004).

Astragalus radix (*A. radix*) has been used by Chinese herbalists as an immune stimulant. This herb contains polysaccharides, flavonoids, various amino acids, cellulose, zinc and iron, which may all enhance the immune system (Yin *et al.*, 2006). A study showed that feeding *Oreochromis niloticus* (*O. niloticus*) *A. radix* for 1 week stimulated lysozyme activity and phagocytosis (Yin *et al.*, 2006).

Allium sativum (*A. sativum*) or garlic is also often used as a medicinal herbal extract that results in immune modulation. Garlic extracts stimulate the immune system by increasing NK cell activity of spleen cells in Sarcoma-180 bearing mice (Kyo *et al.*, 2001). The constituents of garlic that result in immune stimulation are not known, but could be due to several immunologically active agents (Lamm and Riggs, 2001).

1.4.3. The effect of nutrient supplements on the immune system

Glutamine is an amino acid that influences the immune system. In murine intestinal intraepithelial lymphocytes, glutamine supplementation increased the production of IL-2 and

IFN γ (Horio *et al.*, 2008). Glutamine administration also decreased the incidence of bacteraemia and reduced the length of hospital stay after surgical intervention of cancers (Kaminogawa & Nanno, 2004). The amino acid arginine, improves T-helper cell numbers and acts as a substrate for the production of nitric oxide (Kaminogawa & Nanno, 2004).

Vitamin A deficiency is prevalent in most developing countries. Vitamin A supplementation has been reported to improve antibody titer in response to measles vaccines (Field *et al.*, 2002). Vitamin A was also reported to be essential for immune cells and contributes to lowering the risk of pathogen-mediated disease (Field *et al.*, 2002).

Long-chain n-3 polyunsaturated fatty acids (PUFA) such as, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) enhance NK cell activity, T-cell activation and IFN γ cytokine production (Field *et al.*, 2002). Also, n-3 PUFA has the ability to decrease excessive activation of T cells (Fujikawa *et al.*, 1992).

Soy products contain large amounts of the isoflavone genistein. Exposure to genistein increased the number of splenic B cells and T cells in male and female rats (Guo *et al.*, 2002). Genistein and resveratrol has a moderate inhibitory effect on IFN γ production in murine splenocytes. However, genistein and resveratrol increased the production of IL-10 although the results were not statistically significant (Rachon *et al.*, 2006). Table 1.4.1 is a summary of the effect of food products on the immune system.

Table 1.4.1. A summary of food supplements that modulate the immune system

Nutrients	Immune modulating functions	Reference
<p>Herbal extracts</p> <p><i>Echinacea</i></p> <p><i>Astragalus radix</i></p> <p><i>Allium sativum</i></p>	<p>Increases IL-10 production</p> <p>Stimulates phagocytosis in neutrophils, activate cellular immunity</p> <p>Stimulates lysozyme activity and phagocytosis</p> <p>Stimulates NK cell activity</p>	<p>McCann <i>et al.</i>, 2007</p> <p>Jurkstiene <i>et al.</i>, 2004</p> <p>Yin <i>et al.</i>, 2006</p> <p>Kyo <i>et al.</i>, 2001</p>
<p>Amino acids</p> <p>Glutamine</p> <p>Arginine</p>	<p>Increased production of IL-2 and IFNγ</p> <p>Improves T-cell numbers</p>	<p>Horio <i>et al.</i>, 2008</p> <p>Kaminogawa & Nanno, 2004</p>
<p>Vitamins</p> <p>Vitamin A</p>	<p>Essential for immune cells, decreases pathogen-related diseases</p>	<p>Field <i>et al.</i>, 2002</p>
<p>Fatty acids</p> <p>n-3 PUFAs</p>	<p>Enhance NK cell activity, T-cell activation and IFNγ production</p>	<p>Field <i>et al.</i>, 2002</p>
<p>Isoflavones</p> <p>Genistein</p>	<p>Increased number of splenic B cells and T-cells</p> <p>Increased production of IL-10 and inhibits IFNγ</p>	<p>Guo <i>et al.</i>, 2002</p> <p>Rachon <i>et al.</i>, 2006</p>

1.4.4. Conclusion

Food-derived substances have an impact on the immune system. Herbal extracts, vitamins, amino acids, fatty acids and isoflavones may impact different immune cells. These food-derived substances may enhance the immune system and improve health. The mechanisms by which foods exert immune modulating effects are not fully understood. Therefore, to bridge the gap in knowledge, it is important to scientifically investigate the beneficial and adverse effects of food-derived substances on the immune system.



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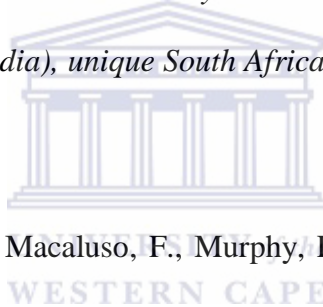
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CHAPTER 2

The Immunomodulatory effects of Rooibos and Black tea using an *in vitro* whole blood culture assay

2.1. Abstract

Aspalathus linearis (*A. linearis*) or Rooibos tea and *Camellia sinensis* (*C. sinensis*) or Black tea are very popular beverages, and approximately 73-78 % of the world's population consume it regularly. Very few studies have been done on the immunomodulatory effects of Rooibos and Black tea. The aim of this study was to determine whether Rooibos and Black tea have an impact on biomarkers of specific immune pathways using an *in vitro* whole blood culture (WBC) assay. Aqueous extracts of Rooibos and Black tea were prepared. After incubating WBCs with extracts, the culture supernatants were screened for specific biomarkers of the immune pathways. Specific biomarkers used in this study were Interleukin-6 (IL-6) for inflammation, Interleukin-10 (IL-10) for humoral immunity and Interferon gamma (IFN γ) for cell mediated immunity. The specific immune biomarkers were assayed using ELISAs. Cytotoxicity tests were also performed using Lactate Dehydrogenase (LDH) leaching from cells as a biomarker. Neither of the teas are cytotoxic. Rooibos addition to unstimulated WBCs induced higher IL-6, IL-10 and IFN γ secretion than control cultures. The addition of Rooibos tea to stimulated WBC induced higher IL-6, lower IL-10 and had no effect on IFN γ secretion compared to control stimulated WBCs. Addition of Black tea to unstimulated WBCs increased IL-6, IL-10 and IFN γ production compared to controls. Stimulated WBC inhibits IL-6, IL-10 and IFN γ production compared to control stimulated WBCs. Data indicate that Rooibos and Black tea modulate the immune system *in vitro* and studies must be conducted to determine the effects of these teas *in vivo*.

2.2. Introduction

The immune system of vertebrates functions to eliminate invasion by non-self antigens. The immune system is made up of several organs and cell types. The organs of the immune system include the bone marrow, thymus, spleen, Peyer's patches and lymph nodes (Perdigon *et al.*, 1995).

The immune system can be subdivided into two types of defence i.e. the innate and adaptive immunity (Medzhitov, 2007). The adaptive and innate immunity differ in function. However, they work in parallel. The innate immunity can also be defined as natural immunity. This pathway is an inborn part of the human immune system. It defends against bacteria, fungi, secreted molecules, waste and transformed cells. Innate immunity is regulated by pattern recognition receptors (PRR), which recognize a wide variety of conserved and molecular regions of micro-organisms (Wolowczuk *et al.*, 2008).

Toll like receptors (TLRs) are a type of PRR. These receptors function to identify viral nucleic acids and bacterial substances such as lipopolysaccharide (LPS). The inflammatory response is brought about by TLRs. Moreover, TLRs stimulate macrophages to produce pro-inflammatory cytokines such as tumour necrosis factor (TNF), interleukin 1 β (IL- β) and interleukin-6 (IL-6). These cytokines further stimulate phagocytosis of pathogens by macrophages and neutrophils (Medzhitov, 2007).

Adaptive immunity is specific for antigens and produces immunological memory. This results in a more pronounced response upon re-exposure to the antigen (Blanco *et al.*, 2008). Adaptive immunity relies on cellular and molecular adaptive mechanisms such as B and T-lymphocytes (Wolowczuk *et al.*, 2008). This type of immunity may generate cellular and humoral immunity. The cellular immunity is regulated by immune cells such as T-

lymphocytes, macrophages, and natural killer cells (NK cells). On the other hand, humoral immunity is characterized by antibody production from B-lymphocytes (Perdigon *et al.*, 1995).

The T-lymphocytes can be divided into CD4 (helper) and CD8 (cytotoxic) T cells. Helper T-cells facilitate the cytotoxic T-cells to kill target cells and allow B cells to produce antibodies. Functions of cytotoxic T cells include killing cells which are infected with intracellular pathogens. B-cells secrete antibodies against pathogens, and thereby destroy them (Weng, 2008).

B and T lymphocytes directly recognize and bind antigens. B cells have antigen receptors called immunoglobulins (Ig) while T cells have T cell receptors (TCR). Ig binds to free antigens, however, TCR bind to processed antigens that are presented on major histocompatibility complex (MHC) molecules. MHC-2 molecules are found on antigen presenting cells (Braciale and Braciale, 1991; Hudson and Ploegh, 2002; Bartl *et al.*, 2003).

The innate and adaptive immunity effector pathways coincide. The molecular mediators of these two pathways are protein molecules called cytokines (Hansson *et al.*, 2002). These cytokines are secreted by various cells and their functions differ. The function of the cytokines depends on the cell they are secreted from. They may act as autocrine, paracrine, or endocrine messengers. The cytokines that are secreted by lymphocytes are known as interleukins (Parkin & Cohen, 2001). Some of the interleukins of the immune system are discussed below.

IL-6 plays an important role in innate immunity. It is both a pro-inflammatory and anti-inflammatory cytokine. Immune cells such as monocytes, macrophages, lymphocytes and mast cells secrete IL-6 (Papanicolaou, 1998). IL-6 functions to promote inflammation by

stimulating expansion and activation of T cells. Moreover, IL-6 causes B-cells to differentiate, and also causes the stimulation of acute phase reactants by hepatocytes (Jones *et al.*, 2001).

IL-10 is a cytokine that is produced by T-helper 2 cells (Th2). This cytokine causes the inhibition of IFN γ synthesis in Th1 cells (Fiorentino *et al.*, 1989). Several cell types may synthesise IL-10 such as Th2 cells, monocytes, macrophages, and B-cells (De Waal Malefyt *et al.*, 1991; Spits and De Waal Malefyt, 1992; Pistoia, 1997). IL-10 functions in stimulating B-cells to proliferate and differentiate. It plays an important role in protecting against intestinal parasites, neutralisation of bacterial toxins, and in local mucosa defense. IL-10 suppresses cellular immunity by inhibiting IFN γ production of T-lymphocytes (Spits and De Waal Malefyt, 1992).

Interferon gamma (IFN γ) is a cytokine that regulates various cellular programs. IFN γ stimulates direct anti-microbial and anti-tumour mechanisms, as well as stimulating antigen processing. This cytokine also attracts leukocytes to the site of infection and regulates the growth, maturation and differentiation of various cell types (Perussia *et al.*, 1983).

Drugs and food can modulate the immune system resulting in immune system activation, immune system sensitization or immune system impairment. Activation of the immune system entails development of inflammation or autoaggressive reactions. Drugs or compounds may also induce naive lymphocytes to differentiate to effector or memory cells resulting in sensitization of the immune system (Langezaal *et al.*, 2001). In addition, impairment of the immune system such as immunosuppression may result due to consumption of drugs or other compounds (Langezaal *et al.*, 2001).

Second to water, tea is the most widely consumed beverage in the world (Krishnan & Maru, 2006). This study focused on two teas namely, *Aspalathus linearis* (*A. linearis*) or Rooibos tea and *Camellia sinensis* (*C. sinensis*) or Black tea respectively. These teas are rich in flavonoids and antioxidants and have been reported to have many beneficial effects for humans.

A. linearis or Rooibos tea is a plant that is indigenous to South Africa (Nel *et al.*, 2007). It has been used as a health beverage and is very popular due to its unique taste and its versatility (Jagani & Wheeler, 2003). Characteristics of this tea include high levels of flavonoids, which result in potent antioxidant activity (Nel *et al.*, 2007). Rooibos tea has several other physiological effects such as anti-microbial activity in cells (Frei & Higdon, 2003), an inhibitory effect on oxidative stress in diabetic rats (Ulćina *et al.*, 2006), and hepatoprotective effects in rats (Ulćina *et al.*, 2003).

C. sinensis or Black tea is a beverage that is consumed regularly by approximately 73-78 % of the world's population (Krishnan & Maru, 2006). One of the main flavonoids found in Black tea was identified as epigallocatechin gallate (Luczaj & Skrydelewska, 2005). Black tea has many physiological effects such as anti-mutagenic and anti-cancer effects (Record & Dreosti, 1998).

Very few studies have been done on the immunomodulatory activity of Rooibos and Black tea. The aim of this study was to screen Rooibos and Black tea extracts for its effects on biomarkers of specific immune pathways using an *in vitro* whole blood culture assay.

2.3. Materials and Methods

2.3.1. Sample preparation for Whole Blood Cultures (WBC)

Samples were prepared by seeping 10 teabags (25g) of Rooibos (Batch no: P 22.11.01 05:03 E19.02.09) and 10 teabags (25g) of Black tea (Batch no: P 28.11.07 13:11 E 26.11.08) in 1 litre of boiling water respectively. The samples were allowed to cool to room temperature. Aliquots of the extracts were stored at -80 °C. For assay, aliquots were thawed and then the teas were sterilised by filtration using a 0.22 µm sterile filter (Lasec, SA). Two-fold dilutions of sterile samples were made with sterilized distilled water.

2.3.2. Preparation of WBC

Blood was collected from healthy male volunteers (24-28 years of age) after informed consent was obtained in line with the South African Ethical Advisory Council. Volunteers were not on medication for at least 1 month before blood collection. Blood samples were collected using endotoxin-free evacuated blood collection tubes (Greiner Bio One GmbH, Austria) containing sodium citrate (3.2 %).

2.3.2.1. The effects of Rooibos tea and Black tea on endotoxin stimulated blood

Stimulated WBC were prepared by mixing blood, Roswell Park Memorial Institute 1640 (RPMI-1640) (Sigma- Aldrich, St. Louis, MO, U.S.A.) medium and 10 µg/ml endotoxin (lipopolysaccharide, LPS) (Sigma-Aldrich, U.S.A.) in dimethyl sulfoxide (DMSO) in the ratio 10:89:1. Unstimulated WBC were prepared by mixing blood, RPMI-1640 medium and DMSO in the ratio 10:89:1. Rooibos tea or Black tea samples (25 µl/well) at different concentrations (250, 125, 62.5, 31.25, 15.625, 7.812 µg/ml) with controls (sterilized distilled water) were dispensed in a 96 well microtiter plate (Nalge Nunc International, Thermo Fisher

Scientific, NY, U.S.A.). Endotoxin stimulated or unstimulated diluted blood was added to all samples and controls (225 μ l/well). The WBC were incubated at 37 °C for 18 hours. After the incubation period, supernatants were collected and assayed for LDH and IL-6.

2.3.2.2. The effects of Rooibos tea and Black tea on phytohemagglutinin (PHA) stimulated blood

Stimulated WBC were prepared by mixing blood with RPMI-1640 medium and 1.6 mg/ml PHA in DMSO in the ratio 10:89:1. Unstimulated WBC were prepared by mixing blood and RPMI-1640 medium in DMSO in the ratio 10:89:1. Rooibos tea or Black tea samples (25 μ l/well) at different concentrations (250, 125, 62.5, 31.25, 15.625, 7.812 μ g/ml) with controls (sterilized distilled water) were dispensed in a 96 well microtiter plate (Nalge Nunc International, Thermo Fisher Scientific, NY, U.S.A.). PHA stimulated or unstimulated diluted blood was added to all samples and controls (225 μ l/well). The WBC were incubated at 37 °C for 48 hours. After the incubation period, supernatants were collected and assayed for LDH, IFN γ and IL-10.

2.3.3. Cytotoxicity assay (Lactate Dehydrogenase Assay)

Lactate Dehydrogenase was used as a biomarker for cytotoxicity. LDH assays were performed on all culture supernatants using a commercially available kit (Biovision Research Products, CA, U.S.A.). Assays were done according to the instructions supplied in the kit manual.

2.3.4. IL-6, IL-10 and IFN γ ELISAs

Cytokine kits were purchased from eBioscience (Human IL-6, IL-10 and IFN γ ELISA Ready-Set-Go, eBioscience, Inc, San Diego, U.S.A.). All the reagents were supplied with the

kit. Ninety six-well microtiter plates (Nalge Nunc International, Thermo Fisher Scientific, NY, U.S.A.) were coated with a 1/250 dilution of capturing antibody (purified anti-human IL-6, IL-10 or IFN γ respectively) in coating buffer (50 μ l/well) and allowed to incubate at 4 °C overnight. All subsequent incubations were performed on a plate shaker (Stuart, Microtiter Plate Shaker, SSMS). Thereafter, the plates were blocked with 200 μ l/well of block solution at room temperature for 1 hour. The plates were then washed five times with wash buffer. Standards (recombinant IL-6, IL-10 or IFN γ , 1 μ g/ml) diluted in assay diluent was prepared. Standards or blood culture supernatants (50 μ l/well) were added to the plates and incubated at room temperature for 2 hours. The washing step was repeated whereafter, 50 μ l of 1/250 dilution detection antibody (Biotin-conjugated anti human IL-6, IL-10 or IFN γ) in assay diluent was added to all wells. The plate was incubated again for 1 hour at room temperature. The washing step was repeated whereafter, 50 μ l of a 1/250 dilution of avidin-horseradish peroxidase (HRP) was added to all wells and incubated for 30 mins at room temperature. The washing step was repeated a final time after which 50 μ l of 3,3', 5,5'-tetramethylbenzidine (TMB) soluble substrate was added to all wells and the plate was incubated in the dark for 20 minutes. The chromogenic reaction was then stopped by addition of 50 μ l of 2 M sulphuric acid (H₂SO₄) to all wells. The plates were then read at 450nm using a microtiter plate reader (Thermo Electron, Original Multiskan Ex).

2.3.5. Statistical analysis

All data is presented as mean \pm standard deviation (SD). One way analysis of variance (ANOVA) was used to compare results with P<0.001 considered as significant.

2.4. Results

2.4.1. Effect of Rooibos tea and Black tea extracts on cellular toxicity

Rooibos tea and Black tea were tested for cellular cytotoxicity using an LDH assay. Results showed that both teas were non-cytotoxic (data not shown).

2.4.2. Effect of Rooibos tea and Black tea extracts on IL-6 production

IL-6 was used as a biomarker to determine the inflammatory response induced by Rooibos and Black tea. The standard curve for the IL-6 ELISA is shown in Figure 2.1. The standard curve was used to calculate the concentrations of IL-6 in samples. The standard curve shows that there is a good correlation ($R^2 = 0.998$) between the absorbance and IL-6 concentration. Results for IL-6 production by unstimulated and stimulated WBC exposed to Rooibos tea extracts are shown in Figure 2.2. Addition of Rooibos tea to unstimulated WBC induced higher IL-6 secretion across all concentrations (7.8125 $\mu\text{g/ml}$ - 250 $\mu\text{g/ml}$) compared to the control ($P < 0.001$). Addition of Rooibos tea extracts at a concentration of 62.5 $\mu\text{g/ml}$ to stimulated WBC resulted in a significant increase of IL-6 production compared to the control ($P < 0.001$).

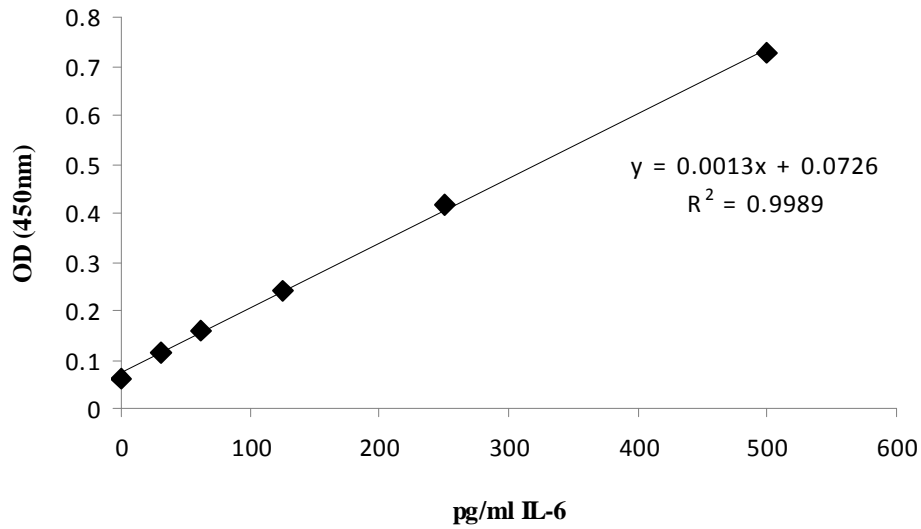


Figure 2.1. Standard curve for IL-6 ELISA. The standard curve obtained shows that there is a good correlation ($R^2 = 0.998$) between absorbance and IL-6 concentration.

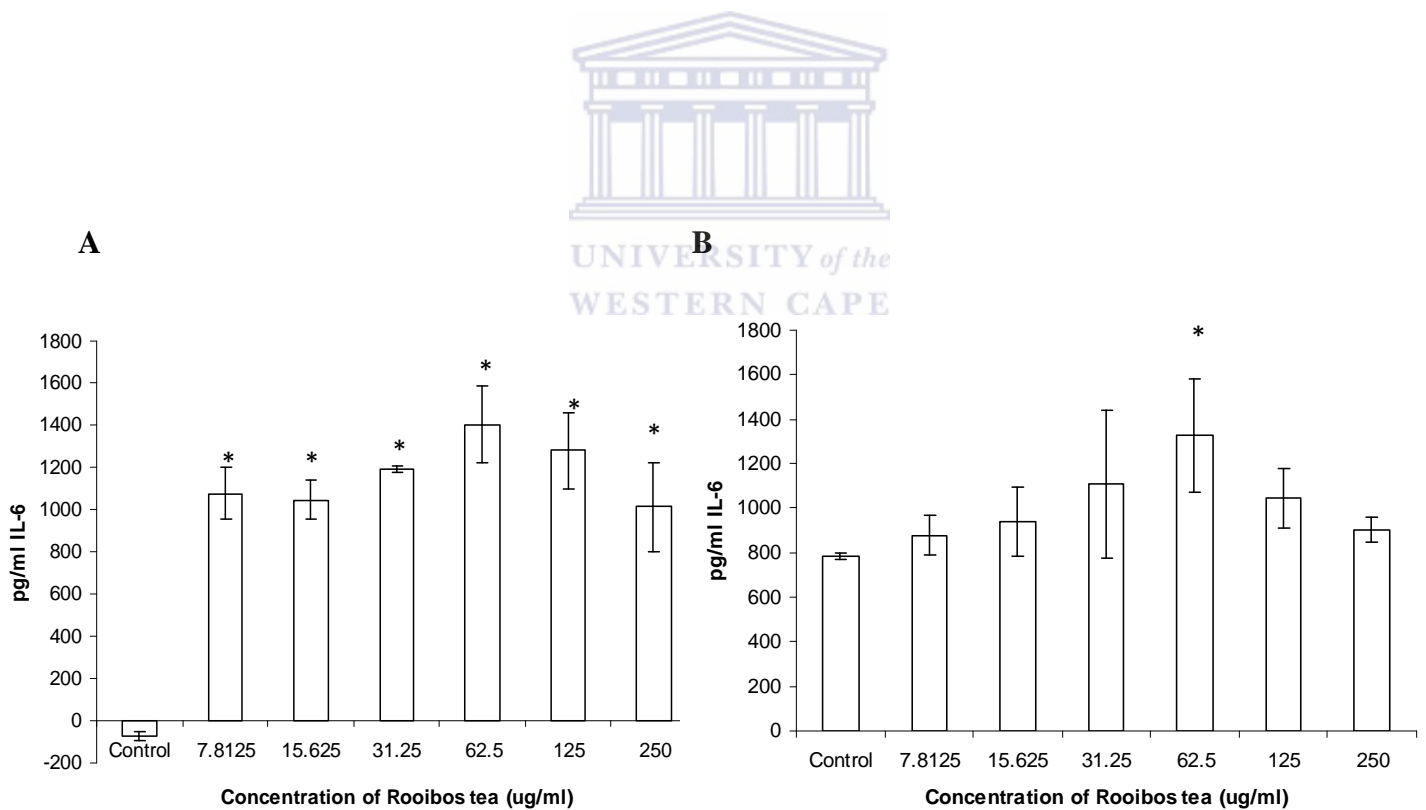


Figure 2.2. IL-6 production (pg/ml) of human whole blood cultures exposed to Rooibos tea. (A) in the absence of a stimulus. (B) in the presence of a stimulus (LPS). * Statistical significance ($P < 0.001$) compared to the control. Bars = standard deviation.

Figure 2.3 shows a graphical illustration of results obtained for IL-6 production for unstimulated and stimulated WBC exposed to Black tea extracts. Addition of Black tea extracts at concentrations from 7.8125 µg/ml - 125 µg/ml induced a higher IL-6 cytokine secretion from unstimulated WBC compared to the control (P<0.001). Addition of 250 µg/ml of Black tea extracts to stimulated WBC resulted in a decrease of IL-6 production compared to the control (P<0.001).

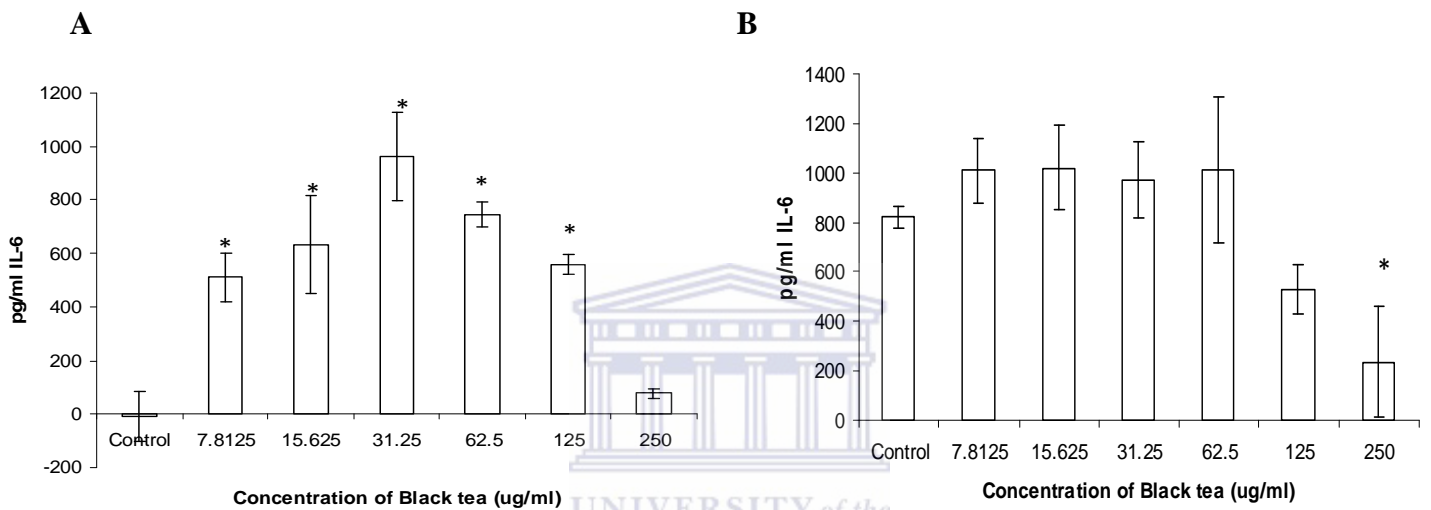


Figure 2.3. IL-6 production (pg/ml) of human whole blood cultures exposed to Black tea. (A) in the absence of a stimulus. (B) in the presence of a stimulus (LPS). * Statistical significance (P<0.001) compared to the control. Bars= Standard deviation.

2.4.3. Effects of Rooibos tea and Black tea extracts on IL-10 production

IL-10 was used as a biomarker for humoral immunity. The standard curve obtained for the IL-10 ELISA is shown in Figure 2.4. The standard curve showed a good correlation ($R^2= 0.999$) and a linear relationship between the absorbance and IL-10 concentration. Standard curves were used to calculate the concentrations of IL-10. Results for IL-10 production by human WBC exposed to Rooibos tea are shown in Figure 2.5. It can be seen that addition of Rooibos tea (7.125 µg/ml - 250 µg/ml) to unstimulated WBC resulted in a significant induction of IL-10 secretion compared to the control (P<0.001). Addition of

Rooibos tea extracts (15.625, 62.5, 250 $\mu\text{g/ml}$) to stimulated WBC resulted in a significant decrease in IL-10 secretion compared to the control ($P < 0.001$).

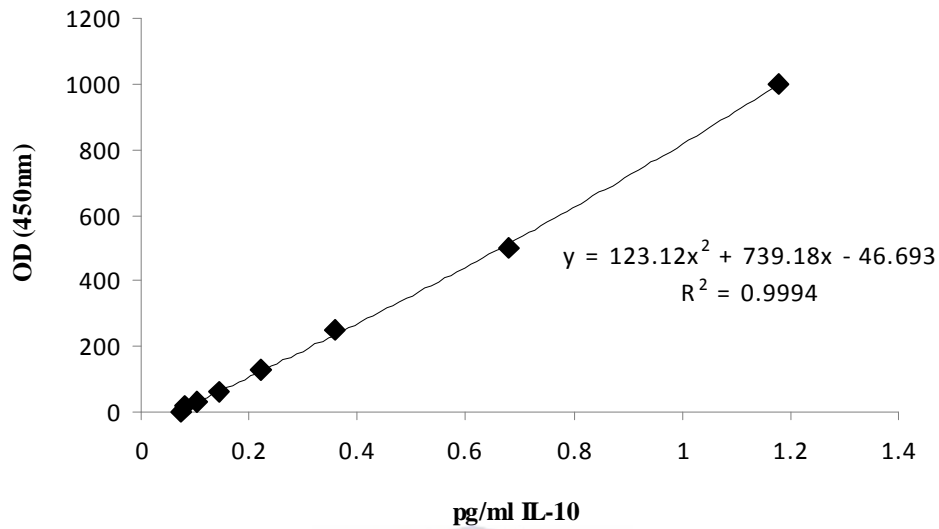


Figure 2.4. Standard curve obtained for IL-10 ELISA. The standard curve showed a good correlation ($R^2 = 0.999$) and a linear relationship between absorbance and IL-10 concentration.

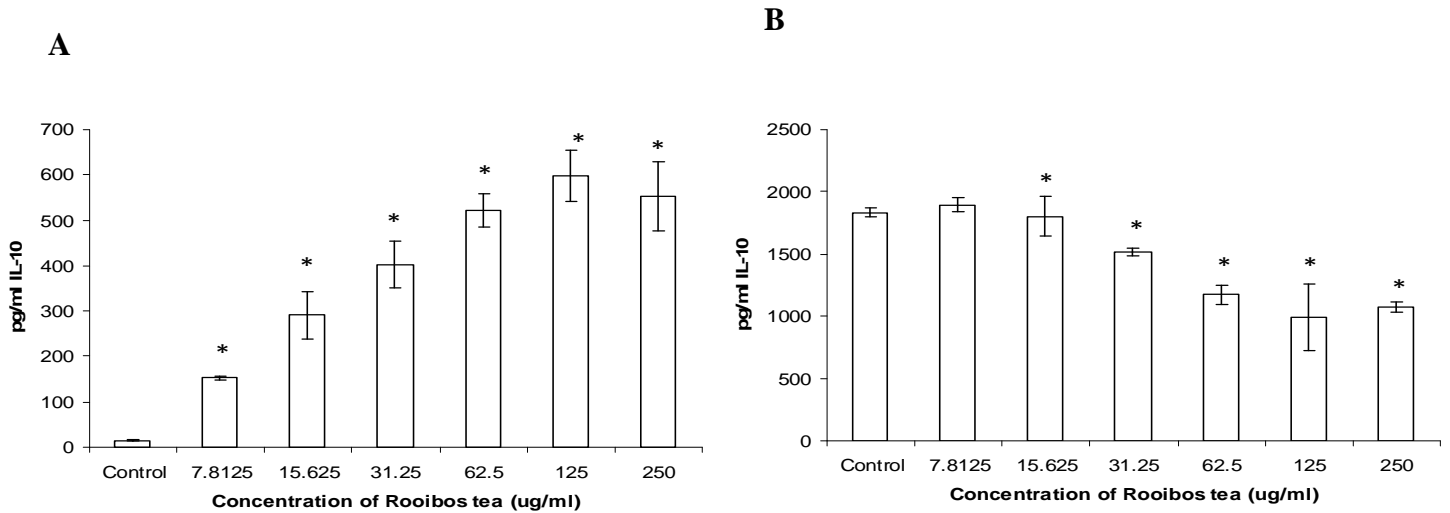
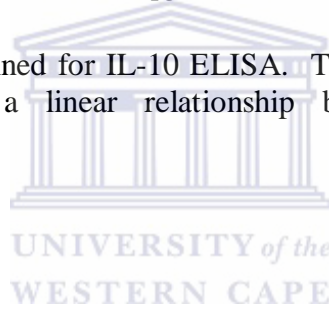


Figure 2.5. IL-10 production (pg/ml) of human whole blood cultures exposed to Rooibos tea. (A) in the absence of a stimulus. (B) in the presence of a stimulus (PHA). * Statistical significance ($P < 0.001$) compared to the control. Bars= Standard deviation.

Results for IL-10 production by human WBC exposed to Black tea extracts are shown in Figure 2.6. Addition of Black tea extracts (7.8125 µg/ml - 62.5µg/ml) to unstimulated WBC induced a higher IL-10 secretion compared to the control (P<0.001). Addition of Black tea extracts (7.8125µg/ml – 250 µg/ml) to stimulated WBC resulted in a statistically significant decrease in IL-10 production compared to the control (P<0.001).

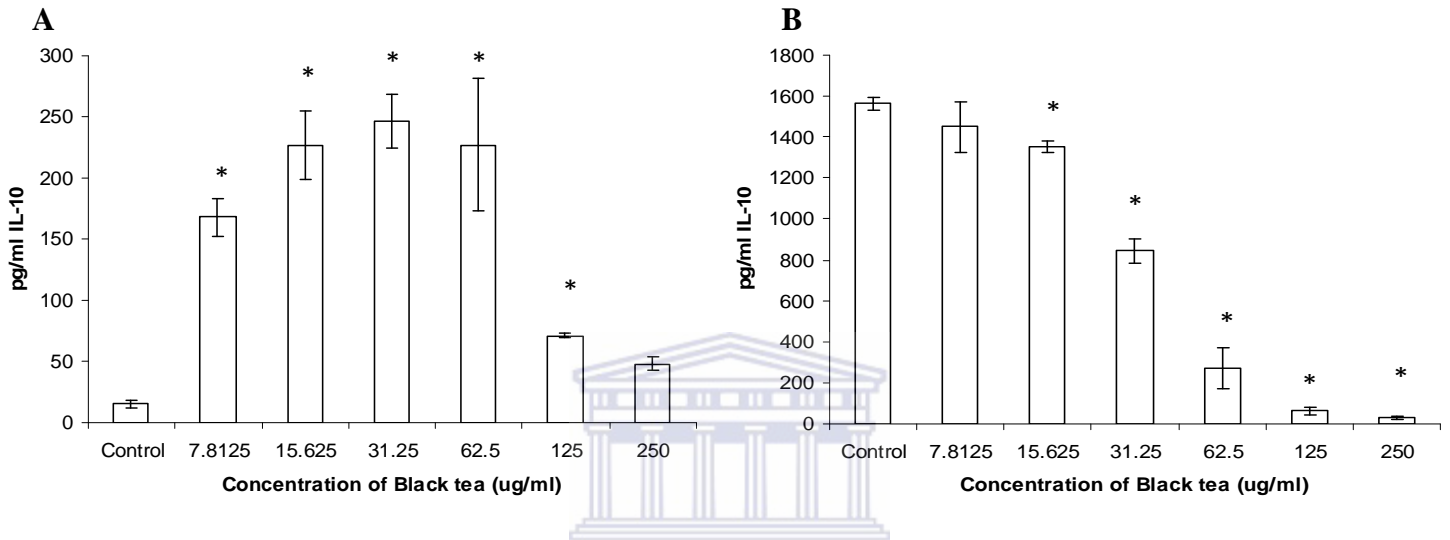


Figure 2.6. IL-10 production (pg/ml) for human whole blood cultures exposed to Black tea. (A) In the absence of a stimulus. (B) In the presence of a stimulus (LPS). * Statistical significance (P<0.001) compared to the control. Bars = standard deviation.

2.4.4. Effects of Rooibos tea and Black tea extracts on IFN γ production

IFN γ was used as a biomarker for cell mediated immunity. The standard curve obtained for the IFN γ ELISA is shown in Figure 2.7. The standard curve showed a good correlation ($R^2= 0.998$) and a linear relationship between the absorbance and IFN γ concentration. Standard curves were used to calculate the concentrations of IFN γ . Figure 2.8 shows a graphical representation for IFN γ production of human unstimulated and stimulated WBC exposed to Rooibos tea. Addition of Rooibos tea extracts (7.8125 µg/ml – 125 µg/ml) to unstimulated WBC showed a stimulatory effect on IFN γ secretion compared to the control

($P < 0.001$). Addition of Rooibos tea extracts (7.8125 $\mu\text{g/ml}$ – 250 $\mu\text{g/ml}$) to stimulated WBC showed no significant difference of $\text{IFN}\gamma$ production compared to the control.

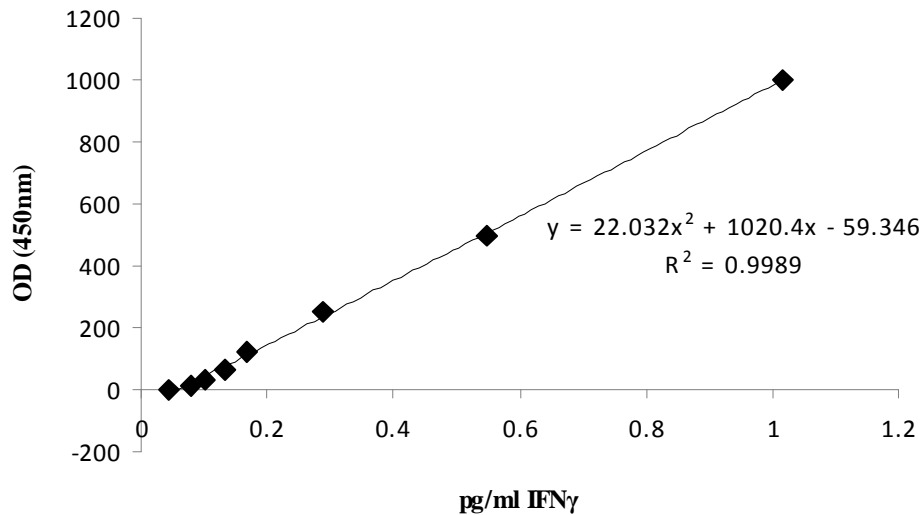


Figure 2.7. The standard curve obtained for $\text{IFN}\gamma$ shows a linear relationship. A good correlation ($R^2 = 0.998$) between absorbance and $\text{IFN}\gamma$ concentration.

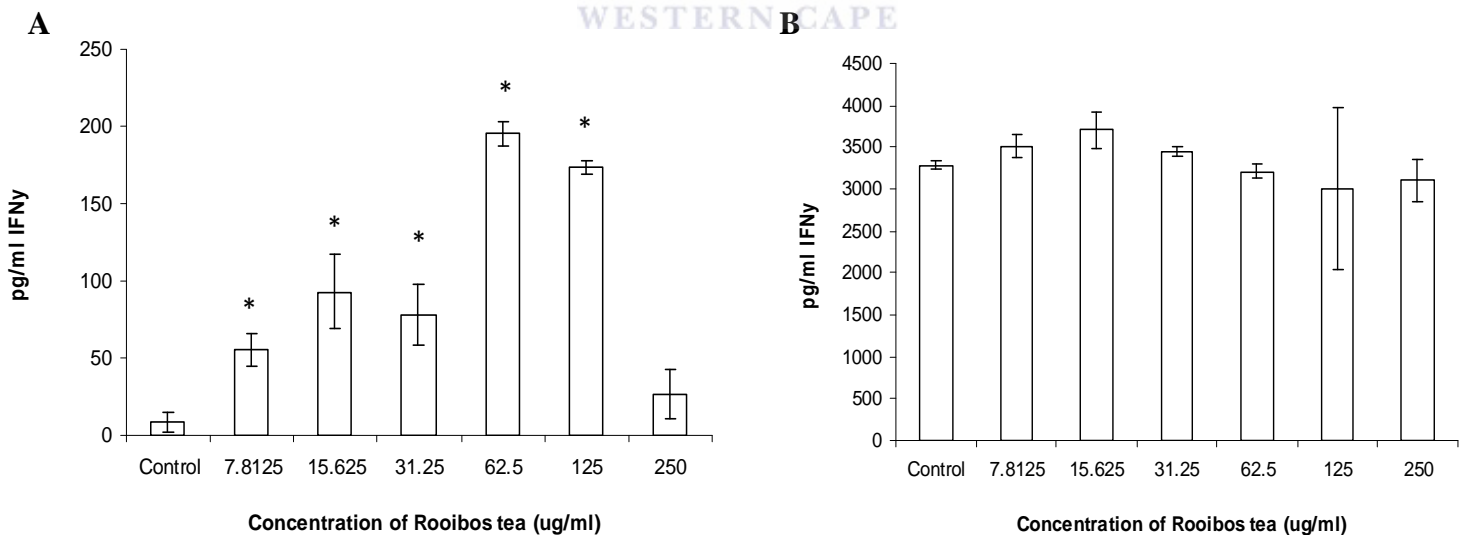
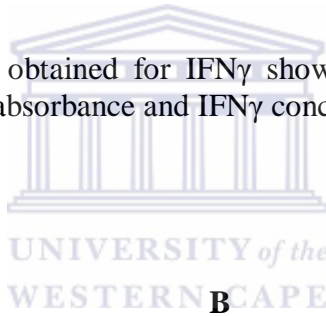


Figure 2.8. $\text{IFN}\gamma$ production (pg/ml) for human whole blood cultures exposed to Rooibos tea. (A) In the absence of a stimulus. (B) In the presence of a stimulus (LPS). * Statistical significance ($P < 0.001$) compared to the control. Bars = standard deviation.

IFN γ production for WBC exposed to Black tea extracts are shown in Figure 2.9. Addition of 15.625 $\mu\text{g/ml}$ Black tea extracts to unstimulated WBC showed a significant increase in induction of IFN γ secretion compared to the control ($P < 0.001$). Addition of Black tea extracts (15.625 $\mu\text{g/ml}$ – 250 $\mu\text{g/ml}$) to stimulated WBC showed a statistically significant decrease in IFN γ production compared to the control ($P < 0.001$). This inhibition is proportional to the concentration of Black tea extracts.

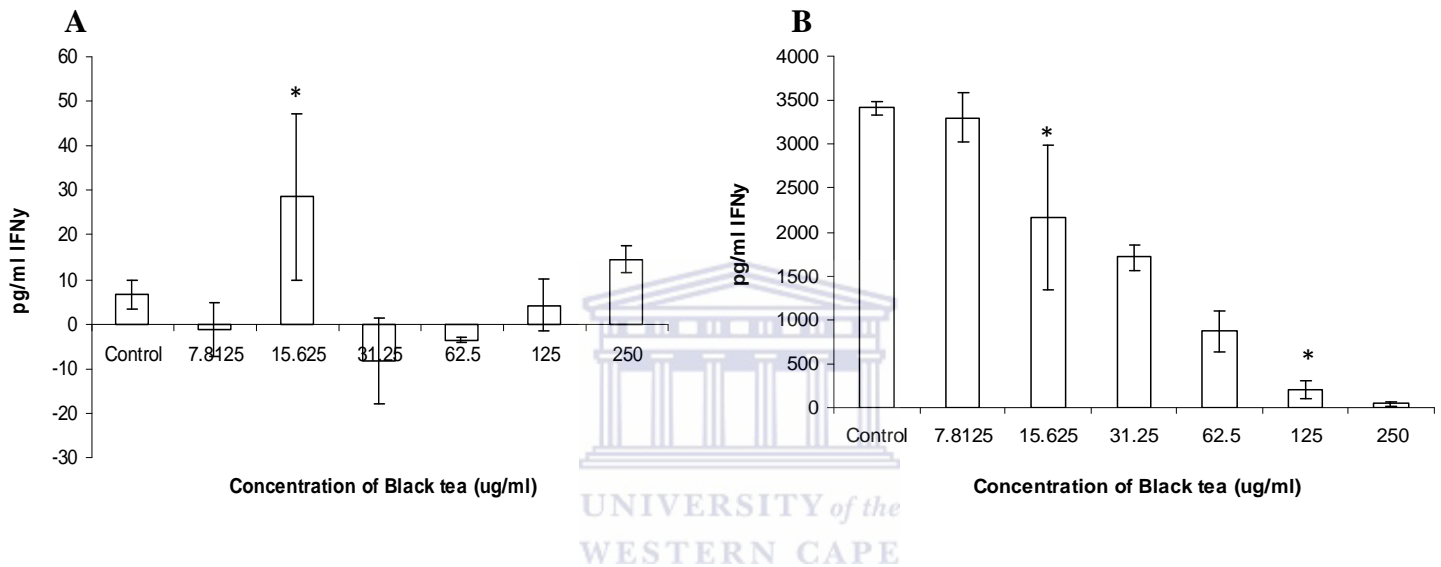


Figure 2.9. IFN γ production (pg/ml) for human whole blood cultures exposed to Black tea. (A) In the absence of a stimulus. (B) In the presence of a stimulus (LPS). * Statistical significance ($P < 0.001$) compared to the control. Bars = standard deviation.

2.5. Discussion

Rooibos tea and Black tea are beverages with a wide range of physiological and pharmacological effects (Frei & Higdon, 2003; Ulćina *et al.*, 2006; Luczaj & Skrydelewska, 2005; Record & Dreosti, 1998). However, very little is known about the immunomodulatory activity of these teas. Tea consists on many compounds that could play a role in immunomodulation. Antioxidant activity of teas, which is very high, may have an effect on immune status (Hamer, 2007).

Rooibos and Black tea showed no cytotoxic effects on WBC (data not shown). The LDH assay is a sensitive assay that determines cytotoxicity, however results gained from this assay do not rule out effect of chemicals on specific cellular pathways. Absence of cytotoxicity of samples does not necessarily indicate that the samples have no effect on the physiological systems (Ganey *et al.*, 1993). Therefore, Rooibos tea and Black tea were further analysed for their effects on the immune system using biomarkers of specific immune pathways.

IL-6 is a cytokine, that is synthesized and secreted from T-lymphocytes, monocytes and macrophages activated by antigens or mitogens. In this study, IL-6 was used as a biomarker for inflammation. As demonstrated in Fig. 2.2 A & B, IL-6 secretion from unstimulated WBC and stimulated WBC was markedly increased by Rooibos tea extract. This study shows that Rooibos tea is capable of inducing inflammatory cytokines *in vitro*. The immunostimulatory effect of Rooibos tea on IL-6 may result in activation of the immune system. This may lead to the activation of hepatocytes to produce acute phase proteins, which in turn activates complement and allows for the phagocytosis of pathogens (Jones *et al.*, 2001). The consumption of Rooibos tea may thus result in better cellular responses to microbial pathogens.

IL-10 is a cytokine that is produced by T-cells, B-cells, and macrophages and was used in this study as a biomarker for humoral immunity. IL-10 stimulates the proliferation and differentiation of B-cells and also regulates Ig synthesis of B cells (Spits and De Waal Malefyt, 1992). In this study, an increase in IL-10 production by unstimulated WBC exposed to Rooibos tea extracts were seen (Figure 2.5 A). This result suggests that the induction of IL-10 by the Rooibos tea extract may contribute to stimulation of B-cells and synthesis of Ig. This study confirms previous reports by Ichiyama *et al.*, 2007, in which a Rooibos fraction resulted in an increase in IL-10 and Immunoglobulin M (IgM) production by murine splenocytes. Rooibos tea extracts resulted in a suppression of IL-10 synthesis from stimulated WBC ($P < 0.001$) (Figure 2.5 B). These results indicate that in the presence of a pathogen, Rooibos tea may have an immunosuppressive effect on the differentiation of T-helper cells to Th2 cells. This may consequently have an impact on cytokines that are needed to mount an effective immune response.

Exposure of unstimulated WBC to Rooibos tea resulted in an increase in IFN γ production (Figure 2.8 A). No significant effect on IFN γ production was observed for stimulated WBC exposed to Rooibos tea (Figure 2.8 B). These results are contrary to those from a study in which a Rooibos fraction decreased IFN γ secretion by murine splenocytes (Ichiyama *et al.*, 2007). Rooibos tea has many constituents such as saccharides and polyphenols, that could play a role in this pleiotropic effect seen in this study.

WBC contain all the cells present in the circulation and the activation of one cell type may have an influence on the functioning of another (Carnaud *et al.*, 2007). Consequently, IL-10 could have inhibited T-cells to synthesise IFN γ . However, other cells present in the WBC may have synthesised it.

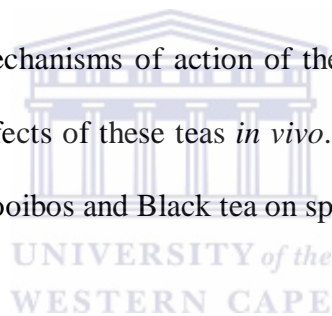
Low concentrations of Black tea induced unstimulated WBC to increase IL-6 production (Figure 2.3.A). Several components in Black tea such as the polyphenols are shown to possess antioxidant activity. This antioxidant activity may play a role in anti-inflammatory processes. However, Maat *et al.*, (2000) , showed that tea consumption in smoking subjects that have increased oxidant stress, does not decrease the plasma levels of IL-6. As a result, these authors disproved the hypothesis that increased antioxidant activity may decrease oxidants and therefore, the inflammatory response (Maat *et al.*, 2000). At high concentrations, Black tea caused a decrease in IL-6 secretion of stimulated WBC (Figure 2.3. A). This result confirms findings from previous studies where stimulated monocytes exposed to Black tea extracts displayed a decrease in IL-6 production (Pajonk *et al.*, 2006).

Addition of Black tea extracts to unstimulated WBC resulted in an increase in IL-10 production compared to the control (Figure 2.6.A). IL-10 production is important in protecting against intestinal parasites, neutralisation of toxins, and in local mucosa defense. The augmentation of IL-10 by Black tea extracts, makes it an ideal dietary component to result in activation and sensitisation of the immune system, and thereby possibly provide protection against infection. The Black tea extracts in this study was found to decrease IL-10 production in stimulated WBC (Figure 2.6 B). In contrast, no effect was seen on LPS-stimulated WBC exposed to Black tea extracts containing approximately 80 % theaflavins (Crouzier *et al.*, 2001).

According to the results of this work, unstimulated WBC exposed to Black tea extracts at a concentration of 15.625 $\mu\text{g/ml}$ caused an increase in $\text{IFN}\gamma$ production ($P < 0.001$) (Figure 2.9 A). Increased $\text{IFN}\gamma$ production could play a role in anti-tumour mechanisms. In this study, stimulated WBC exposed to Black tea extracts resulted in a decrease in $\text{IFN}\gamma$ production

($P < 0.001$) (Figure 2.9 B). In contrast, one *in vitro* study showed that $\gamma\delta$ T cells results in the production of $\text{IFN}\gamma$ in response to alkylamine antigen found in brewed tea (Kamath *et al.*, 2003). Taken together, the results of this study suggest that in the presence of an intracellular pathogen or a tumour, Black tea extracts may inhibit cell mediated immunity and thus increase susceptibility to the host.

The results of this study show that Rooibos and Black tea play a role in modulating the immune system. Specifically Rooibos and Black tea may have an effect on cytokine secretion by human WBC. It is thus possible to use Rooibos and Black tea as a dietary component to either stimulate or suppress immunity. Limitations of this study was only examining *in vitro* effects of Rooibos and Black tea. These *in vitro* studies do not fully elucidate the *in vivo* immune mechanisms of action of these teas. Further work should be done to elucidate the immune effects of these teas *in vivo*. These findings contribute to our understanding of the effects of Rooibos and Black tea on specific immune pathways.



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CHAPTER 3

The detection of phytoestrogens in Rooibos and Black tea

3.1. Abstract

Phytoestrogens can bind to estrogen receptors and exert biological effects. Phytoestrogens may act as mitogens for tumour cells *in vitro* and cause proliferation of cancer cells. The aim of this study was to determine the phytoestrogen levels (estrone (E1), estradiol (E2) and estrone (E3) levels) in Rooibos tea and Black tea using commercially available ELISA kits for the natural estrogens. Rooibos tea contains 1662.8 ± 6.4 pg E1 per g tea, 546.4 ± 24.0 pg E2 per g tea and 575.5 ± 12.7 pg E3 per g tea, while Black tea contains 2265.1 ± 1.2 pg E1 per g tea, 865.5 ± 18.8 pg E2 per g tea, 3961.7 ± 122.8 pg E3 per g tea. The levels of these estrogenic compounds differ significantly between the two teas ($P < 0.001$). These teas could possibly be used as dietary components to increase circulatory phytoestrogen levels. The study also investigated the effects of Rooibos and Black tea on the estrogen dependant mammary cancer cell line, MCF-7. The endpoint assays, LDH and XTT were used to determine cytotoxicity, total cell number and cell metabolic activity of MCF-7 cells exposed to Rooibos and Black tea respectively. No cytotoxic effects of Rooibos and Black tea were observed. MCF-7 cells incubated with $250 \mu\text{g/ml}$ Rooibos tea have lower total cell LDH levels than cells incubated with the DMSO control ($P < 0.001$). MCF-7 cells incubated with $83 \mu\text{g/ml}$ and $250 \mu\text{g/ml}$ of Rooibos tea have lower XTT activity than cells incubated with the DMSO control ($P < 0.001$). MCF-7 cells incubated with $83 \mu\text{g/ml}$ and $250 \mu\text{g/ml}$ of Black tea have lower total cell LDH levels than cells incubated with the DMSO control ($P < 0.001$). MCF-7 cells incubated with 9, 83 and $250 \mu\text{g/ml}$ of Black tea have lower cell XTT activity than cells incubated with the DMSO control ($P < 0.001$). The current study confirms reports showing inhibition of growth in breast cancer cell lines by extracts

containing phytoestrogens. More work has to be done in order to determine the mechanisms of these teas biological activities.



3.2. Introduction

Estrogens are a group of steroid hormones (Gonzalez-Barasso *et al.*, 1999). They control female reproductive function by hormone regulation and signalling. The main endogenous estrogenic hormones include 17- β estradiol, estrone and estriol. These estrogens function to regulate metabolic, behavioural and morphologic changes that occur during different stages of reproduction. Estrogen is also one of the regulatory hormones responsible for bone formation, homeostasis, cardiovascular function, and behaviour. Estrogens are also produced in males but at much lower levels than in females. Estrogens in males regulate production, transport, and concentration of testicular liquid as well as anabolic activity of androgens (Jonošek *et al.*, 2006).

In females, the main source of estradiol are the cells of the ovaries, particularly the theca, granulosa and luteinizing derivatives of these cells. The 'two cell theory' of estrogen synthesis proposes that androgens are secreted by the theca cells and diffuse to the granulosa cells to be aromatized to estrogens (Lieberman, 1996). In contrast, other evidence suggests that both the theca and granulosa cells produce both androgens and estrogens (Lieberman, 1996). Other sources of estrogen are produced by several different tissues of the body and after menopause, this is the main source of estrogen. The extragonadal areas of estrogen biosynthesis include mesenchymal cells of the adipose tissue, osteoclasts in bones, aortic smooth muscle cells and in the brain (Simpson *et al.*, 1999).

Phytoestrogens are natural estrogens which are found in plants, and these display some estrogenic characteristics (Lampe, 2003). Phytoestrogens are made up of 2 major groups, namely isoflavones and lignans. Considerable amounts of phytoestrogens are present in the human diet. Other groups of phytoestrogens include flavones, flavanones, coumestans, and stilbenes (Rice & Whitehead, 2008). Soybeans and soy foods contain high amounts of

isoflavones. Approximately 1.2 - 3.3 mg isoflavones/g dry weight is found in soy products. The isoflavones genistein and daidzein are found mainly in soy products (Reinli & Block, 1996).

Foods such as oilseeds, seaweed, legumes, fruits, and whole grains contain the phytoestrogen lignan (Thompson *et al.*, 1991). Precursors of the mammalian lignans include the plant lignans known as secoisolariciresinol-diglucoside (SECO-DG) and matairesinol (MAT) (Thompson *et al.*, 1997). Mazur *et al.*, (1997) found that teas such as Black, Green and Oolong yielded high amounts of the lignans SECO (561-2890 µg/100 g dry weight) and MAT (56-413 µg/100 g dry weight). The isoflavone content of the teas was minimal.

The action of intestinal microflora allows phytoestrogen metabolism in humans. Other mechanisms, of metabolism in humans include enzymatic modification by P450 cytochromes (Setchell, 1998). The lignans are converted to mammalian lignans enterodiol and enterolactone (Adlercreutz, 2002).

Studies have shown that lignans and isoflavonoids can prevent cancer (Qu *et al.*, 2005; Baylund *et al.*, 2005; Yanagihara *et al.*, 1993; Donghua *et al.*, 1999). On the contrary, other investigators have shown that high intake of phytoestrogens do not significantly reduce cancer (Boker *et al.*, 2004). Phytoestrogens may act as mitogens for tumour cells *in vitro*. Dees *et al.*, (1997) showed that dietary estrogens at low concentrations, do not act as antiestrogens but activate human breast cancer cells to enter the cell cycle and undergo proliferation.

Phytoestrogens lower LDL cholesterol (Anderson *et al.*, 1995) enhancing vascular function (Fortis *et al.*, 1993). They also favourably alter insulin resistance, glycemic control and serum lipoproteins in postmenopausal women with type-2 diabetes (Jayagopal *et al.*, 2002).

The mechanisms of the protective effect of phytoestrogens are not clear. Phytoestrogens may bind to estrogen receptors (ER) and exert its effects. These phytoestrogens may block the effects of endogenous estrogens (Belcher & Zsarnovszky, 2001). Phytoestrogens such as genistein, diadzein and biochanin A stimulate estrogen-related receptors (Suetsugi *et al.*, 2003) such as the estrogen receptor beta (ER β) (Kuiper *et al.*, 1998). Natural phytoestrogens result in neuroprotective responses. A combination of phytoestrogens (genistein, daidzein, equol) also showed that it binds with high affinity to ER β and has the potential to prevent cognitive decline in women with menopause, without affecting the reproductive system (Zhao *et al.*, 2008). Potential phytoestrogens could be used as alternative therapies for neurodegenerative diseases.

To screen for estrogenic activity and quantity, methods that test biological activity are often utilized. Rapid, sensitive and simple quantitative techniques are needed to analyse estrogenic hormones. Enzyme linked immunosorbent assay (ELISA) kits are commercially available and can be used for the quantification of estrogenic hormones. These kits allow large numbers of samples to be analysed simultaneously (Castillo and Barcelo, 1997).

Very few studies have been done to investigate the estrogenic properties of teas. Shimamura *et al.*, (2006), showed that Rooibos tea contain three compounds that cross-reacts with natural estrogens in an ELISA. This study revealed that nothofagin has the highest cross-reactivity with antibodies against the natural estrogens. The other compounds in Rooibos tea with cross-reactivity are iso-vitexin and luteolin-7-glucoside. This study shows that Rooibos tea monitored consumption may potentially benefit patients with low estrogens levels such as patients requiring hormone replacement therapy. The administration of Black tea to oophorectomised rats for 21 days resulted in increased serum estradiol levels (Das *et al.*,

2005). This study showed that Black tea also significantly reduced oophorectomy-induced osteoporosis in these rats.

In 2005, it was estimated by WHO Global Infobase that cancer is the cause of 41,000 deaths in South Africa. In females the second highest incidence of cancer deaths are due to breast cancer (WHO Global Infobase).

Several surgical and chemotherapeutic methods have been used to treat cancer. In general, chemoprevention refers to an agent that is able to block or delay the process of carcinogenesis and thus in turn prevent progression of an invasive cancer (Syed *et al.*, 2008).

For tumours that are estrogen positive, the non-steroidal antiestrogen tamoxifen has been used as a chemotherapeutic. This compound is used worldwide to treat all stages of breast cancer (Jordan, 2007). Tamoxifen competitively antagonizes estrogens at the estrogen receptor and in this way it inhibits the growth of cancer cells (Gielen *et al.*, 2008). Clinical and epidemiological evidence shows a reduction of breast cancer incidence after tamoxifen treatment. However, this treatment has side effects. Some of the side effects include a higher risk of developing endometrial cancer, pulmonary embolism, stroke, and deep vein thrombosis (Mourits *et al.*, 2001). Smoking women who have breast cancer should be encouraged to stop smoking due to increased probability of tamoxifen-induced side-effects such as nausea, depression, and migraines (Zhan *et al.*, 2007).

Research is being pursued on alternative therapies, lacking the adverse effects of conventional anti-cancer drugs, for cancer treatment and prevention. Currently teas are being investigated as potential anti-cancer dietary compounds. The aim of this study was to quantitate levels of phytoestrogens in Rooibos tea and Black tea using commercially

available ELISA kits. This study also investigated the effects of Rooibos and Black tea on the estrogen dependant breast cancer cell line, MCF-7.



3.3. Materials and Methods

3.3.1. Reagents

Reagents were purchased from Sigma (U.S.A.), unless otherwise stated in text.

3.3.2. Sample preparation for estrogen ELISAs and MCF-7 cell cultures

Commercially available Rooibos (Batch no: P 22.11.01 05:03 E19.02.09) and Black tea (Batch no: P 28.11.07 13:11 E 26.11.08) extracts were prepared. Ten tea bags of each tea (25g) were allowed to brew for 30 minutes with 1 litre of boiling water until room temperature was reached. Samples were then subjected to solid phase extractions (SPE) using C-18 columns (Sigma, Aldrich). The SPE columns were conditioned with 4 ml of Phase B mixture (45 % methanol, 40 % hexane and 15 % propanol), then 4 ml ethanol and lastly 4 ml distilled water. After the washing step, 1 litre of sample (Black tea and Rooibos tea respectively) was allowed to run through the column. The column was again washed with 4 ml of water after which it was dried using a vacuum pump (PALL vacuum pump, Life Sciences, 60 Hz, 1.92 Amperes, 220-240 Volts). The hydrophobic molecules attached to the resin were eluted with 3 ml of Phase B mixture and dried under a stream of air. The dried eluate was reconstituted with DMSO to make a 400 times concentrated sample stock solution. Water extracted and reconstituted with DMSO using the same protocol was used as a negative control.

3.3.3. Determination of estrogen in teas using ELISAs

Extracts were diluted with the 0 standard (obtained from the ELISA kits) at a ratio of 1:100 for the Estrone ELISA, and 1:10 for Estradiol and Estriol ELISAs respectively.

3.3.3.1. The determination of estrone (E1) in tea

E1 kits were purchased from IBL Immuno Biological Laboratories, Hamburg. All the reagents required were supplied in the kit. The wells of a microtiter plate were pre-coated with antibody directed towards a unique antigenic site on the E1 molecule. Samples and standards were applied at 50 μ l/well to the anti-estrone coated plate. Thereafter, 100 μ l of enzyme conjugate (Estrone horseradish peroxidase) were added to all wells. The mixture was incubated for 60 minutes at room temperature on a plate shaker (Stuart, Microtiter Plate Shaker, SSMS). After incubation, the wells were washed three times with wash solution and tapped dry. Thereafter, 150 μ l of substrate were added to all wells and incubated for 30 mins at room temperature. The reaction was stopped by adding 50 μ l of stop solution to all wells. The absorbances were then read at 450nm with a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). The 0 pg/ml standard results in maximum binding of the enzyme conjugate. All data was expressed as a percentage of 0 pg/ml standard. A standard curve was drawn using the results obtained for the standards and the concentrations of the samples were read off this curve.

3.3.3.2. The determination of estradiol (E2) in tea

E2 kits were purchased from IBL Immuno Biological Laboratories, Hamburg. All the reagents required were supplied in the kit. The wells of a microtiter plate were pre-coated with antibody directed towards a unique antigenic site on the E2 molecule. Samples and standards were applied at 50 μ l/well to the anti-estradiol coated plate. Thereafter, 200 μ l of enzyme conjugate (Estradiol horseradish peroxidase) were added to all wells. The mixture was incubated for 2 hours at room temperature on a plate shaker (Stuart, Microtiter Plate Shaker, SSMS). After incubation, the wells were washed three times with wash solution and

tapped dry. Thereafter, 100 μ l of substrate was added to all wells and incubated for 15 mins at room temperature. The reaction was stopped by adding 50 μ l of stop solution to all wells. The absorbances were then read at 450nm with a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). The 0 pg/ml standard results in maximum binding of the enzyme conjugate. All data was expressed as a percentage of 0 pg/ml standard. A standard curve was drawn using the results obtained for the standards and the concentrations of the samples were read off this curve.

3.3.3.3. The determination of estriol (E3) in tea

E3 kits were purchased from IBL Immuno Biological Laboratories, Hamburg. All the reagents required were supplied in the kit. The wells of a microtiter plate were pre-coated with antibody directed towards a unique antigenic site on the E3 molecule. Samples and standards were applied at 10 μ l/well to the anti-estriol coated plate. Thereafter, 100 μ l of enzyme conjugate (Estrone horseradish peroxidase) were added to all wells. The mixture was incubated for 60 minutes at room temperature on a plate shaker (Stuart, Microtiter Plate Shaker, SSMS). After incubation, the wells were washed three times with wash solution and tapped dry. Thereafter, 150 μ l of substrate were added to all wells and incubated for 30 mins at room temperature. The reaction was stopped by adding 100 μ l of stop solution to all wells. The absorbances were then read at 450nm with a microtiter plate reader (Thermo Electron Corporation, Original Multiskan Ex). The 0 pg/ml standard results in maximum binding of the enzyme conjugate. All data was expressed as a percentage of 0 pg/ml standard. A standard curve was drawn using the results obtained for the standards and the concentrations of the samples were read off this curve.

3.3.4. MCF-7 cell culture assays

3.3.4.1. Culture of MCF-7 cells

MCF-7 cells obtained from American Type Culture Collection (ATCC) were used for cell culture. For routine maintenance, cells were grown in RPMI-1640 containing glutamine and sodium bicarbonate (NaHCO_3) (Sigma-Aldrich, U.S.A.), supplemented with 10 % fetal bovine serum (Sigma-Aldrich, U.S.A.) and a antibiotic and mycotic mixture (penicillin and streptomycin) in an atmosphere of 5% CO_2 / 95% air under saturating humidity at 37 °C.

3.3.4.2. Effects of tea on MCF-7 cells

Confluent MCF-7 cells were trypsinized with 1 % trypsin solution (Sigma-Aldrich, U.S.A.) and the cell suspension obtained was centrifuged at 1000 x g for 10 minutes. For the cell proliferation assays, cell pellets were mixed with 100 ml phenol red-free RPMI-1640 medium, 1 ml antibiotic- antimycotic mix, 2 ml serum replacement factor and 1 ml glutamax. Cells were counted on a hemocytometer and the cell suspension was diluted to give 10 000 cells per ml. Aliquots of the cell suspension were transferred to a 48-well flat bottom culture plate (Nalge Nunc International, Thermo Fisher Scientific, NY, U.S.A.) (300 μl /well). The cells were then incubated at 37 °C to allow the cells to attach to the plate. A three fold dilution series of the tea extracts were prepared in DMSO. The samples (30 μl /well) were added to medium at a ratio of 1:100. Cells were incubated at 5% CO_2 / 95% air under saturating humidity at 37 °C for 144 hours. The cultures were then assayed for LDH and XTT activity.

3.3.4.3. Lactate Dehydrogenase Assay (LDH) for cytotoxicity

An LDH assay was performed on culture medium to determine cytotoxicity. Briefly, cell culture medium from all wells were removed and used for the assay. A commercially

available LDH kit was used to perform the assay (Biovision Research Products, CA, U.S.A.). Assays were done according to the manufacturers instructions.

3.3.4.4. Total cellular Lactate Dehydrogenase Assay (LDH)

Total cell LDH was used as a biomarker for cell number. Briefly, medium was removed from all wells. For lysis, 200 μ l of cell lysis solution (supplied with the Biovision kit) was added to all wells. Thereafter, the plates were incubated for 30 mins on a plate shaker. LDH assays were performed on cell lysates. Assays were done according to the manufacturers instructions (Biovision kit). Optical densities were measured at 450 nm.

3.3.4.5. XTT (23-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide) assay for cell metabolic activity

XTT was used as a biomarker for cell metabolic activity. An XTT assay was performed according to manufacturer's instructions (Roche, SA). Briefly, 50 μ l of XTT mixture was added to each cell culture. The plate was then incubated at 5% CO₂/ 95% air under saturating humidity at 37 °C for 4 hours. Optical densities were measured at 492 nm at half an hour intervals during the 4 hour incubation period.

3.3.5. Statistical analysis

All data is presented as mean \pm standard deviation (SD). One way analysis of variance (ANOVA) was used to compare results with P<0.001 considered as significant.

3.4. Results

3.4.1. Detection of Estrone (E1), Estradiol (E2) and Estriol (E3) using ELISAs

3.4.1.1. Detection of Estrone (E1) in tea

The standard curve for the Estrone ELISA is shown in Figure 3.1. There is a good inverse correlation ($R^2 = 0.977$) between the percentage of the maximum binding and the log of estrone concentration. The levels of estrone (Table 3.1) found in Rooibos tea was 1662.8 ± 6.4 pg estrone per g tea. Black tea had a level of 2265.1 ± 1.2 pg estrone per g tea. Data analysis showed that estrone concentrations in Rooibos and Black tea were statistically different from one another and to the water control ($P < 0.001$).

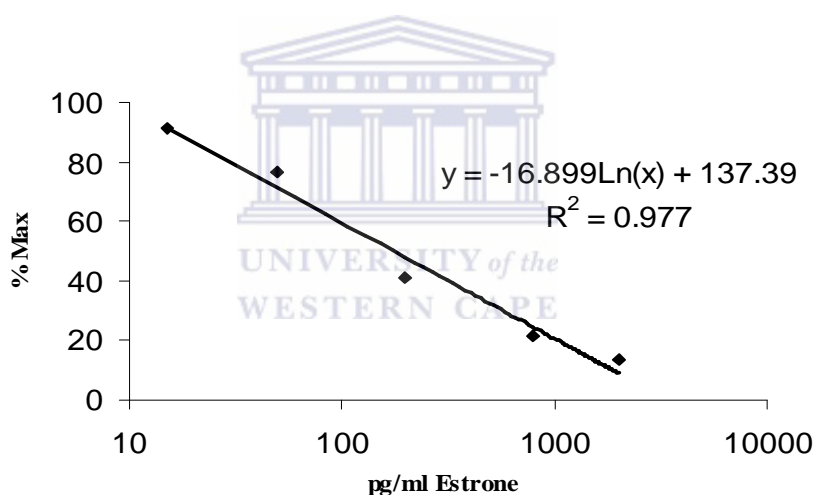


Figure 3.1. Standard curve obtained for the Estrone ELISA.

3.4.1.2. Detection of Estradiol (E2) in tea

The standard curve for the Estradiol ELISA is shown in Figure 3.2. There is a good inverse correlation ($R^2 = 0.9912$) between the percentage of maximum binding and the log of estradiol concentration. Table 3.1 illustrates that 546.4 ± 24.0 pg estradiol per g tea of Estradiol was found in Rooibos tea. A higher amount of Estradiol was found in Black tea

(865.5 ± 18.8 pg estradiol per g tea) (Table 3.1). Data analysis showed that estradiol concentrations in Rooibos and Black tea were statistically different from one another and to the water control (P<0.001).

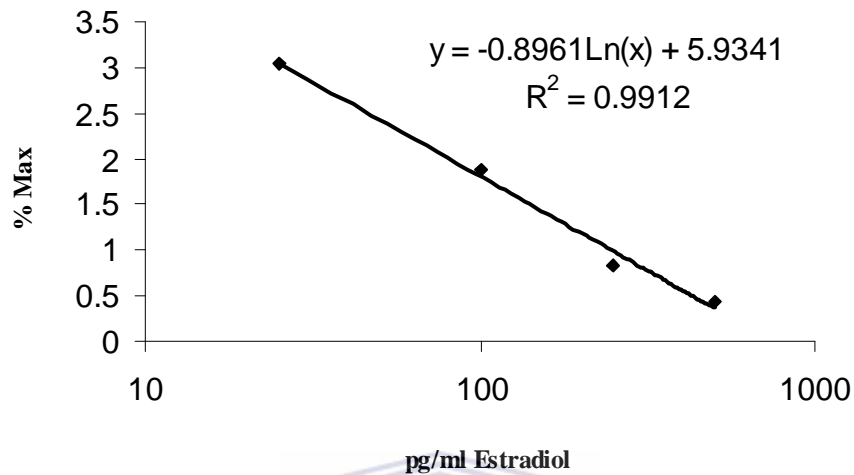


Figure 3.2. Standard curve obtained for Estradiol ELISA.

3.4.1.3. Detection of Estriol (E3)

The standard curve for the Estriol ELISA is shown in Figure 3.3. There is a good inverse correlation ($R^2 = 0.9817$) between the percentage of maximum binding and the log of Estriol concentration. Estriol levels are illustrated in Table 3.1. The amount of Estriol found in Rooibos tea is 575.7 ± 12.7 pg estriol per g tea. Black tea contained a higher amount of Estriol (3961.7 ± 122.8 pg estriol per g tea). Data analysis showed that estriol concentrations in Rooibos and Black tea were statistically different from one another and to the water control (P<0.001).

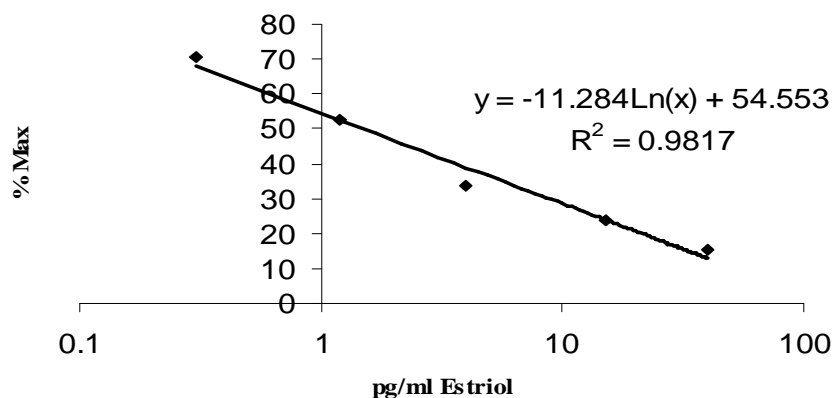


Figure 3.3. Standard curve obtained for the Estriol ELISA.

Table 3.1. Estrone, Estradiol and Estriol levels for Rooibos tea and Black tea (pg/g tea \pm SD) (*significantly lower than Black tea; $P < 0.001$).

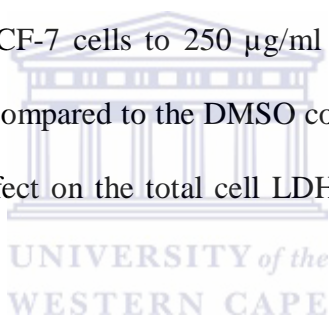
Samples (n=2)	Estrone	Estradiol	Estriol
Rooibos tea	1662.8 \pm 6.4*	546.4 \pm 24.0*	575.5 \pm 12.7*
Black tea	2265.1 \pm 1.2	865.5 \pm 18.8	3961.7 \pm 122.8

3.4.2. The effect of Rooibos tea on MCF-7 cells

MCF-7 cells were exposed to various concentrations of Rooibos tea between 0 – 250 µg/ml. At the end of the exposure period medium LDH, total cell LDH and XTT/ metabolic activity were measured.

Medium LDH was used as a biomarker for cytotoxicity (Ivanova & Uhlig, 2008). Results showed that none of the Rooibos tea concentrations tested were cytotoxic to the MCF-7 cell cultures i.e. medium LDH levels for all the Rooibos exposures were similar to the control (data not shown).

Total cell LDH was used as a biomarker for cell number (Sepp *et al.*, 1996). Results show (Figure 3.4) that exposure of MCF-7 cells to 250 µg/ml Rooibos resulted in a significant decrease in cell LDH ($P < 0.001$) compared to the DMSO control. Rooibos tea concentrations lower than 250 µg/ml had no effect on the total cell LDH compared to the DMSO control (Figure 3.4).



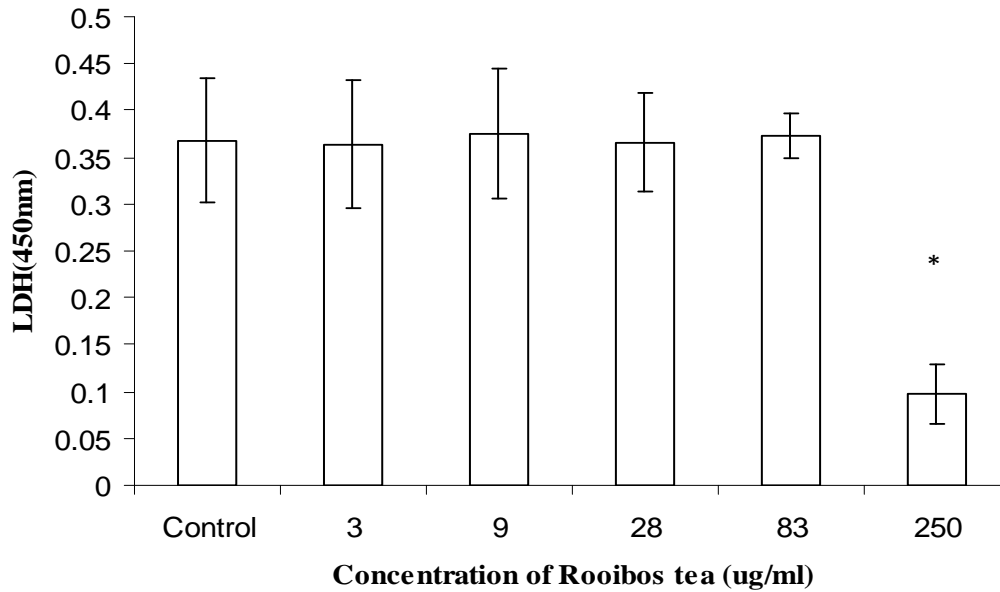


Figure 3.4. The total cell LDH of exposed cultures. MCF-7 cells were incubated with varying concentrations of Rooibos tea (3 µg/ml- 250 µg/ml). DMSO was used as a control. * Statistical significance ($P < 0.001$) compared to the control. Bars = Standard deviation.

XTT was used as a biomarker for cell metabolic activity (Sandasi *et al.*, 2008). Results (Figure 3.5) of the Rooibos tea treatment of MCF-7 cultures show that XTT/metabolic activity was significantly decreased at 83 µg/ml and 250 µg/ml Rooibos tea compared to the DMSO control ($P < 0.001$). None of the other Rooibos tea concentrations tested had an effect on metabolic activity.

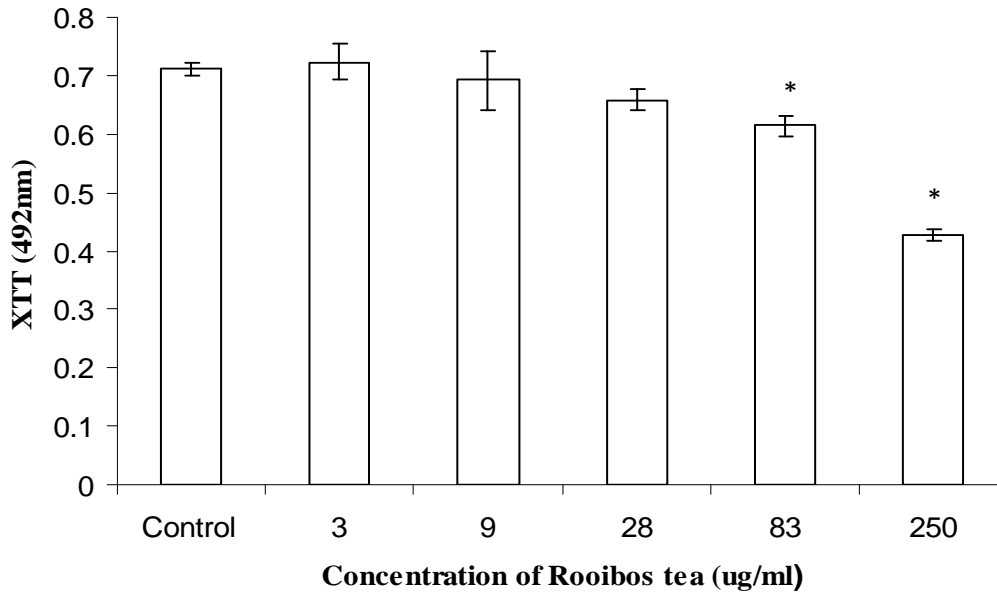


Figure 3.5. The XTT/metabolic activity of exposed cultures. MCF-7 cells were incubated with varying concentrations of Rooibos tea (3 µg/ml- 250 µg/ml). DMSO was used as a control. * Statistical significance ($P < 0.001$) compared to the control. Bars = Standard deviation.

3.4.3. The effect of Black tea on MCF-7 cultures

MCF-7 cells were exposed to various concentrations of Black tea between 0 – 250 µg/ml. At the end of the exposure period cell LDH, XTT metabolic activity and medium LDH were measured.

Medium LDH was used as a biomarker for cytotoxicity (Ivanova & Uhlig, 2008). Results showed that none of the Black tea concentrations tested were cytotoxic to the MCF-7 cell cultures i.e. medium LDH levels for all the Black tea exposures were similar to the control (data not shown).

Total cell LDH was used as a biomarker for cell number (Sepp *et al.*, 1996). Results show (Figure 3.6) that exposure of MCF-7 cells to 83 µg/ml and 250 µg/ml Black tea resulted in a significant decrease in cell LDH ($P < 0.001$) compared to the DMSO control. Black tea

concentrations lower than 83 $\mu\text{g/ml}$ had no effect on the total cell LDH compared to the DMSO control (Figure 3.6).

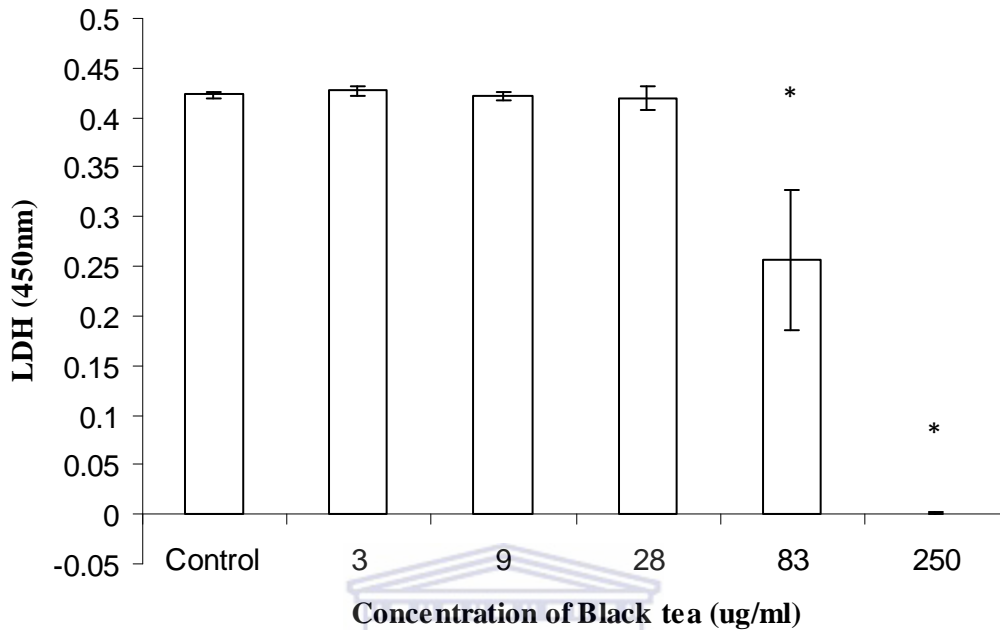


Figure 3.6. The total cell LDH of exposed cultures. MCF-7 cells were incubated with varying concentrations of Black tea (3 $\mu\text{g/ml}$ - 250 $\mu\text{g/ml}$). DMSO was used as a control. * Statistical significance ($P < 0.001$) compared to the control. Bars = Standard deviation.

XTT was used as a biomarker for cell metabolic activity (Sandasi *et al.*, 2008). Results (Figure 3.7) of the Black tea treatments of MCF-7 cultures show that XTT/metabolic activity was significantly decreased at 9, 83 and 250 $\mu\text{g/ml}$ Black tea compared to the DMSO control ($P < 0.001$). None of the other Black tea concentrations tested had an effect on metabolic activity.

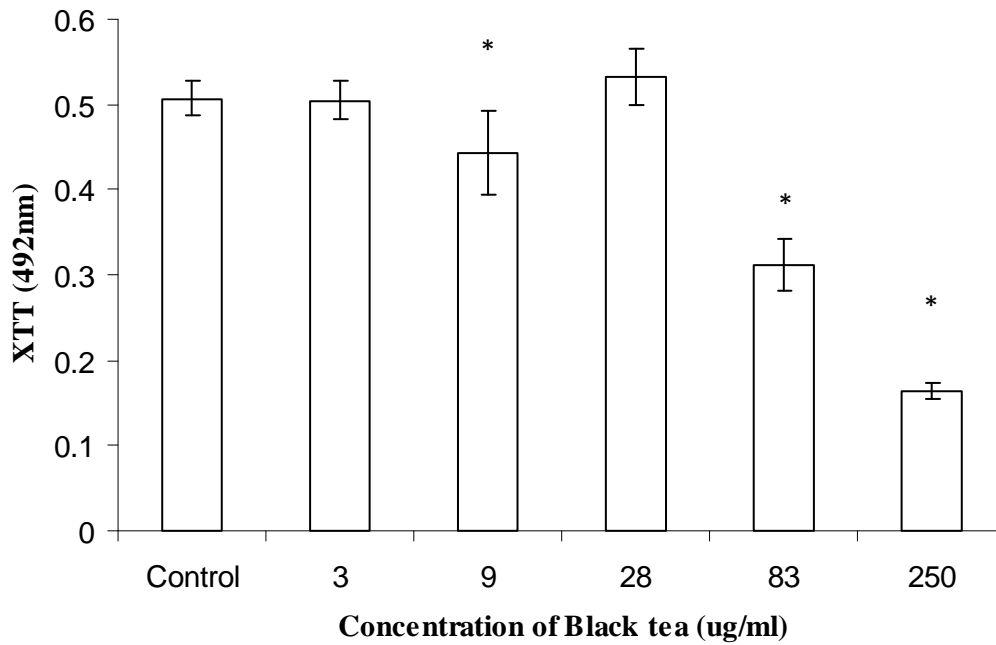
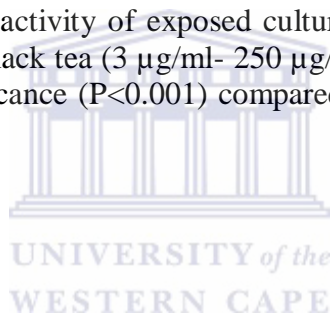


Figure 3.7. The XTT/metabolic activity of exposed cultures. MCF-7 cells were incubated with varying concentrations of Black tea (3 $\mu\text{g/ml}$ - 250 $\mu\text{g/ml}$) and 0 $\mu\text{g/ml}$ DMSO was used as a control. * Statistical significance ($P < 0.001$) compared to the control. Bars = Standard deviation.



3.5. Discussion

Two major groups of phytoestrogens exist in plants, namely isoflavones and lignans. These phytoestrogens can cross react with estrogen receptors and exert estrogenic effects. The phytoestrogen isoflavone has been detected in soybeans and soy products (Reinli & Block, 1996). Several other plant products have been shown to contain phytoestrogens (Thompson *et al.*, 1991). Investigators have shown that tea contain phytoestrogens or compounds that may have estrogenic activity. The lignans SECO and MAT are present in Black, Green and Oolong tea. There is approximately 561-2890 $\mu\text{g}/100\text{ g}$ dry weight and 56-413 $\mu\text{g}/100\text{ g}$ dry weight for SECO and MAT in Black tea, respectively (Mazur *et al.*, 1997). Rooibos tea contains compounds such as nothofagin and isovitexin that are estrogenic (Shimamura *et al.*, 2006). No literature reference could be found that quantitates the specific steroidal estrogens estrone, estradiol and estriol in tea. This study shows that both teas contain compounds that are potentially estrogenic as shown by cross-reactivity in the ELISA for natural estrogens. This study also showed that Rooibos tea contain lower levels of estrone, estradiol and estrone like compounds compared to Black tea (Table 3.1) ($P < 0.001$). Tea can potentially be used as a dietary alternative in a bid to increase circulatory phytoestrogen levels and the type of tea may influence effects (Wu *et al.*, 2005). This study confirms that Rooibos tea and Black tea contain estrogen-like compounds and that there are significant differences between levels of estrogens in these teas.

Rooibos and Black tea showed no cytotoxic effects on MCF-7 cells (data not shown). The LDH assay is a sensitive assay that determines cytotoxicity. However, results gained from this assay do not rule out interference of other specific cellular pathways. Absence of cytotoxicity of samples does not necessarily indicate that the samples have no effect on

physiological systems (Ganey *et al.*, 1993). Therefore, Rooibos tea and Black tea were further analysed for its effects on MCF-7 cells.

A literature search on several electronic databases found no information on the effects of Rooibos tea on the estrogen dependant breast cancer cell line, MCF-7. The results of this study show that Rooibos tea at a high concentration inhibits proliferation (Figure 3.4) and results in a decrease in growth rate (Figure 3.5) of MCF-7 cells ($P < 0.001$). This potential *in vitro* anti-cancer effect confirms previous *in vitro* studies that have shown that Rooibos tea can suppress the growth of skin tumours (Marnewick *et al.*, 2005).

A previous study showed that the amino acid theanine found in Black tea results in cell death of MCF-7 cells (Friedman *et al.*, 2007). This study showed that high concentrations of Black tea inhibit MCF-7 proliferation (Figure 3.6) and result in a decrease in growth rate (Figure 3.7) of MCF-7 cells. Black tea, which contains the higher levels of estrogenic compounds as shown by the estrogen ELISAs, also exhibits a more potent inhibition of MCF-7 cell growth and metabolic activity compared to Rooibos tea.

These results are in line with previous suggestions that phytoestrogens can potentially prevent cancer. This study shows that both Rooibos and Black tea inhibit the growth of the human mammary cell line MCF-7. Further studies are required to determine if these teas have therapeutic activity against mammary cancers *in vivo*.

3.6. References

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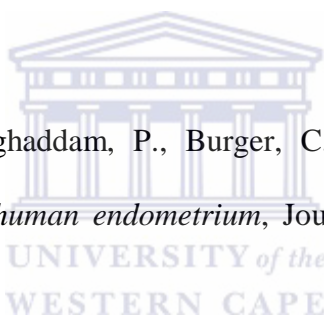
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CHAPTER 4

Conclusion and Future Perspectives

Aspalathus linearis (*A. linearis*), commonly known as Rooibos tea or Red bush tea and *Camellia sinensis* (*C. sinensis*) or Black tea are beverages that are consumed throughout the world. These teas possess physiological and pharmacological properties such as antioxidant, immunomodulating and anti-cancer effects. Although several investigators focussed on the health benefits of these teas, several questions regarding their health benefits still need to be answered. Studies are needed to elucidate the mode of action, biological effects and safety and toxicity levels of the two teas. Human studies on the consumption of Rooibos and Black tea are very limited. Information from human studies may play a critical part in understanding the biological effects of the teas. Appropriate testing methods to determine these effects are also needed. For this reason, the aim of this study was to use *in vitro* assays to screen Rooibos and Black tea for endocrine modulation.

This study used an *in vitro* whole blood culture assay to investigate the immunomodulatory effects of Rooibos and Black tea. Specific biomarkers of the immune system used included the cytokines IL-6, IL-10 and IFN γ . These biomarkers were measured using ELISAs. This study shows that teas modulate the immune system. The study also shows that specific immune pathways can be selectively up or down regulated by the teas.

This knowledge gained through this study has the potential to facilitate individuals with making accurate decisions on whether or not to use the teas as dietary components to stimulate or suppress certain pathways of the immune system. The teas could thus be used as supplements to mount an effective immune response against bacteria, viruses or tumours. However, this study also shows possible adverse immunomodulatory effects of Rooibos and

Black tea such as immune activation or sensitization. Methods to improve this work may include isolating specific active compounds in the teas and elucidating their effect on the specific immune pathways. Further work is required to determine the immunomodulatory effects of these teas in an *in vivo* system.

This study also analysed estrogen-like compounds in Rooibos tea and Black tea using commercially available ELISA kits. Rooibos tea contains 1662.8 ± 6.4 pg estrone per g tea, 546.4 ± 24.0 pg estradiol per g tea and 575.5 ± 12.7 pg estriol per gram tea, while Black tea contains 2265.1 ± 1.2 pg estrone per g tea, 865.5 ± 18.8 pg estradiol per g tea, 3961.7 ± 122.8 pg estriol per g tea. The levels of these estrogenic compounds differed significantly between the two teas. This study showed that ELISAs may successfully be used to quantitate the levels of phytoestrogens in Rooibos and Black tea.

The effects of Rooibos and Black tea on proliferation of the estrogen dependant MCF-7 cell line was determined to further characterise the phytoestrogenic properties of the teas. The endpoint assays, LDH and XTT were used to determine cytotoxicity, total cell number and metabolic activity of MCF-7 cells exposed to Rooibos and Black tea respectively. No cytotoxic effects of Rooibos and Black tea were observed. The LDH and XTT assay showed a decrease in proliferation and metabolic activity of MCF-7 cells exposed to the higher Rooibos and Black tea concentrations, respectively.

This study showed that Black tea contained higher amounts of estrogen-like compounds and that Black tea also displayed a more effective inhibition of MCF-7 cell growth and metabolic activity compared to Rooibos tea. The results of this study confirm previous reports that suggest that phytoestrogens can potentially prevent cancer.

The two teas could be clinically useful as dietary supplements to increase circulatory phytoestrogen levels and anti-cancer dietary agents. *In vivo* studies should be done to confirm these effects.

