Protective properties of rooibos (Aspalathus linearis) flavonoids on the prevention of skin cancer

by
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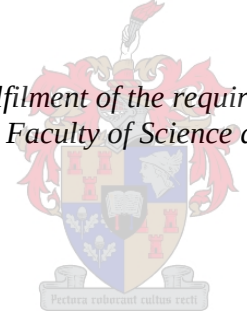

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DECLARATION

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Abstract

Ultraviolet-B (UV-B) radiation is a major cause of skin cancer resulting in an array of events including oxidative damage, DNA damage and inflammation. The keratinocytes and skin macrophages play a pivotal role in inflammation and are known to release a wide range of cytokines in response to UV-B and/or other toxicants such as lipopolysaccharide (LPS). The chronic release of the cytokines, if not controlled, may be detrimental leading to a variety of skin diseases including cancer. Rooibos is well known for its health benefits which include anti-inflammatory effects that are attributed to the anti-oxidant properties of the flavonoids. In the current study the aqueous and methanol extracts of unfermented and fermented rooibos were compared in terms of their polyphenol and flavanol content, while their antioxidant properties were assessed in the FRAP and ABTS assays. The methanol extract of unfermented (MUF) rooibos, which contained the highest levels of total polyphenols and flavanol content as well as the monomeric flavonoids, exhibited the strongest antioxidant properties when compared to its aqueous counterpart (AUF). The fermented rooibos methanol and aqueous (MF and AF) extracts exhibited similar but weaker responses compared to the unfermented extracts. The MUF extract was further fractionated by column chromatography utilising an XAD-4 resin resulting in five major fractions with different polarity. The major rooibos flavonoids were enriched in fractions X-3 and X-4, which also exhibited the highest antioxidant activity although it was similar to the MUF extract. The most polar fractions, X-1 and X-2, contained less flavonoids and exhibited a weaker antioxidant activity.

The anti-inflammatory effects of the rooibos extracts and column fractions were investigated in the UV-B/HaCaT inflammation model monitoring interleukin 1α (IL-1α) production and cell viability indices. In the absence of UV-B exposure the methanol extracts and the flavonoid-enriched fractions, X3 and X-4, increased IL-1α with a decrease in cell viability and increase in apoptosis, suggestive of a pro-inflammatory effect. The most polar fraction X-1 drastically decreased cell viability and apoptosis while IL-1α was increased, which may be attributed to necrotic cell death and a subsequent pro-inflammatory stimulation via an autocrine feedback pathway. A similar effect was noticed with the non-polar fraction X-5, however without adversely affecting the cell growth parameters suggestive of a direct pro-inflammatory effect. The aqueous extracts and the polar fraction X-2 had the opposite effect by decreasing IL-1α with minor effects

on cell viability and apoptosis at low concentrations, suggesting an anti-inflammatory effect. In the presence of UV-B all the extracts and most of the column fractions resulted in a decrease in IL-1α accumulation in comparison to the control, with the methanol unfermented extract and flavonoid enriched fractions being the most active. A further decrease in cell viability and apoptosis was also observed at the higher concentrations. Therefore, rooibos may aid the removal of IL-1α indirectly presumably by inducing cell death although a critical balance appear to exist in the type of cell death, e.g. via apoptosis by which IL-1α is removed or via necrosis where the cytokine is released.

The anti-inflammatory effects of the rooibos extracts and column fractions were also monitored in LPS-induced THP-1 derived macrophages monitoring the release of TNF-α. All the extracts decreased TNF-α release with minor effects on the cell growth parameters. The aqueous fermented extract and the most polar fraction, X-1 were the most active in decreasing TNF-α and fraction X-2 the exhibited the lowest activity at the highest concentrations. The flavonoid enriched column fractions, X-3 and X-4 as well as the non-polar X-5 column fractions reduced the excretion of TNF-α, although cell viability was decreased and apoptosis was increased at higher concentrations. The LPS/macrophage inflammatory model seems to be more resistant to the pro-oxidant effects of the rooibos flavonoids and therefore provides an ideal model to further characterise the anti-inflammatory properties of rooibos. In the UV-B/HaCaT inflammatory model the rooibos-enriched flavonoid extracts seem to remove cytokines through inducing apoptotic cell death thereby indirectly inhibiting inflammation. However, depending on the concentration levels, it could also stimulate inflammation under certain conditions by exhibiting pro-oxidant effects, presumably via iron interactive mechanisms. The anti-inflammatory effects of the more polar rooibos constituents, presumably the tannin-like proanthocyanidins and/or the non-flavonoid constituents of rooibos, exhibiting a lower antioxidant potency, should be further investigated utilising the UV-B/HaCaT keratinocyte inflammatory model. In this regard, the further characterization of fermented rooibos is of interest as the flavonoid enriched MUF and column fractions seems to mask the anti-inflammatory effects due to adverse effects on cell growth indices. The modulation of different cells signalling pathways associated with inflammation need to be characterized to better define the chemopreventive properties of rooibos.

Uitreksel

Ultraviolet B (UV-B) blootstelling is een van die vernaamste oorsake van velkanker en veroorsaak ‘n reeks van veranderings op sellulêre vlak wat insluit effekte soos oksidatiewe skade, DNS beskadiging en inflammasie. Die keratinosiete en makrofage in the vel speel ‘n belangrike role gedurende inflammasie en skei verskillende sitokienes af wanneer die selle blootgestel word aan UV-B insluitende toksiese stowwe soos lipopolisakkariedes (LPS). Die ongekontroleerde en kroniese uitskeiding van die sitokienes kan baie nadelig wees en lei tot verskillende velsiektes in sluitende kanker. Rooibos is bekend vir ‘n verskeidenheid van gesondheids voordele wat onder andere anti-inflammatoriese effekte insluit wat gekoppel word aan die antioksidant eienskappe van die flavonoïede. In die huidige studie is die totale polifenole en flavanol inhoud van water en methanol ekstrakte van onderskeidelik groen en geoksideerde rooibos vergelyk, terwyl the antioksidant eienskappe geëvalueerwas met behulp van die FRAP en ABTS toetse. Verder is die anti-inflammasie van rooibos ondersoek in die UV-B/HaCaT en die LPS/makrofaag inflammatoriese sel modelle. Die methanol ekstrak afkomstig van groen rooibos (MUF), het die hoogste antioksidant aktiwiteitg ehad met ‘n ooreenkomtige hoë vlak van totale polifenole, flavanole, die monomeriese flavonole wanneer dit met die water ekstrak (AUF)vergelyk word. Die water en methanol ekstrakte van die ge-oksideerde rooibos het ‘n laer aktiwiteit wanneer bogenoemde parameters en antioksidant aktiwiteit ter sprake kom. Die MUF ekstrak was verder gefraksioneer met behulp van kolom-chromatografie met XAD-2 as matriks en fraksies met verskillende polariteite is verkry. Die belangrikste rooibos flavonoïede was gekonsentreer in die X-3 en X-4 fraksies wat ook die hoogste antioksidant eienskappe gehad het, alhoewel dit ooreensstem met die MUF ekstrak. Die mees polêre fraksies, X-1 en X-2 bevat minder polifenole en was geassossieer met ‘n lae antioksidant aktiwiteit.

Die anti-inflammatoriese eienskappe van die rooibos ekstrakte en kolom fraksies was getoets in ‘n UV-B/HaCaT keratinosiet inflammasie model waartydens die opeenhoping van interleukin 1 alfa (IL-1α) en sel lewensvatbaarheid getoets is. In die afwesigheid van UV-B lig het die methanol ekstrakte en die flavonoïed verrykte kolom fraksies X-3 en X-4, IL-1α opeenhoping verhoog. Die verhoogte akkumulasie het gepaard gegaan met ‘n verlaging in sel lewensvatbaarheid en‘n verhoging in apoptose (seldood) wat dui op ‘n pro-inflammatoriese effek. Die mees polêre fraksie, X-1 het die sel se oorlewing en

apoptose betekenisvol verlaag terwyl IL-1α akkumulasie verhoog was. Hierdie verhoging in IL- 1α kan moontlik verband hou met ‘n gepaardgaande sel nekrose, IL- 1α uitskeiding en die aktiveering van ‘n outokriene pro-inflammatoriese terugvoer meganismes. ‘n Soortgelyke effek is waargeneem met die mees nie-polêre fraksieX-5, maar sonder ‘n verlies in die lewensvatbaarheid en apoptose wat ‘n pro-inflammatoriese effek aandui. Die water ekstrakte and die polêre fraksie X-2 het egter IL-1α verlaag met minimale effekte op die groei van die selle wat dui op ‘n anti-inflammatoriese reaksie. In die teenwoordigheid van UV-B het al die ekstrakte en kolom fraksies, IL-1α verlaag met die methanol en flavonoïed verrykte ekstrakte die mees effektiefste. Die IL-1α verlaging was verder geassosieer met ‘n verdere daling in beide die sel lewensvatbaarheid en die induksie van apoptose. Dit kom voor as of rooibos IL-1α verlaag deur selle te verwyder met behulp van seldood, alhoewel daar ‘n kritieke balans bestaan wat sal bepaal watter tipe geinduseer word, byvoorbeeld die induksie van apoptose waartydens IL-1α verwyder word of deur nekrose waartydens dit vrygestel word. By hoë konsentrasies en dalende ATP vlakke word induksie van apoptose verhoed en gevolglik vind nekrose plaas wat inflammasie verder kan stimuleer. Hierdie pro-inflammatoriese effekte moet dus verhoed word.

Die anti-inflammatoriese effek van rooibos ekstrakte en kolom fraksies was ook geëvalueer in die LPS-geinduseerde THP-1 makrofaag inflammatoriese model metTNF-α uitskeiding as eindpunt. Al die rooibos ekstrakte het TNF-α verlaag sonder om ‘n noemenswaardige effek te hê op sel lewens vatbaarheid en die induksie van apoptose. Die water ekstrak en die mees polêre kolom fraksie (X-1) was opmerklik die aktiefste by die hoogste konsentrasies terwyl fraksie X-2 die kleinste effek gehad het. Die flavonoied verrykte fraksies X3 and X-4 asook die mees nie-polêrefraksie (X-5) het ook die TNF-α uitskeiding verminder wat gepaarde gegaan het met die induksie van apoptose by die hoogste konsentrasies. Omdat daar minimale effekte op die lewensvatbaarheid van die makrofage by lae konsentrasies verkry is, kandie effek van die water ekstrakte en die meer polêre kolom fraksies aan ‘n tipiese anti-inflammatoriese effek toe geskryf word. Die LPS/makrofaag model is baie meer weerstandbiedend teenoor die pro-oksidatiewe effekte van die rooibos flavonoiede en is ‘n ideale model om die anti-inflammatoriese eienskappe van rooibos verder te ondersoek. In teenstelling hiermee het rooibos ‘n indirekte anti-inflammatoriese effek in die UV-B/HaCaT inflammatoriese model gehad

deur die induksie van apoptose en die verwydering van IL-1α. Afhangende van die konsentrasie vlakke kan dit ook nekrotiese sel dood veroorsaak vanwee die pro-oksidant effekte van die rooibos flavonoiede met moontlike bydra en die rol van yster wat vrygestel word. Dit lei tot ‘n verdere verhoging van die inflammatoriese reaksie in the model vanwee‘n outomatiese terugvoer meganisme. Die anti-inflammatories effekte van die meer polêre rooibos verbindings behoort verder ondersoek te word in die UV-B/HaCaT keratinosiet inflammatoriese model, veral om die moontlike rol van die tannien-agtige proanthosianidiniene en ander nie-flavonoïed verbindings te evalueer. In die geval sal die aandag gevestig moet word op die geoksideerde rooibos omdat die flavonoïede-verrykte methanol en kolom fraksies die anti-inflammatoriese effek verberg as gevolg van hul sitotoksiese effekte. Verder moet die effek van rooibos op die modulering van verskillende sel boodskap stimuli ten opsigte van die induksie van inflammasie beter gedefinieer word ten einde die kankerwerende eienskappe van rooibos verder te ondersoek.

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DEDICATIONS

This Thesis is dedicated to my Daughter, Simamkele Gwashu. Nana your presence in my life gave me the motivation to pursue my dreams and I hope that one day it can inspire you to do the same. Love always!!

ABBREVIATIONS

A.linearis: Aspalathus linearis

AA: Arachidonic acid

ABTS: 2, 2'-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt

AF: Aqueous fermented

$AgNO_3$: Silver nitrate

Ahr: Aryl hydrocarbon receptor

ANOVA: Analysis of variance

Aq: Aqueous

ATP: Adenosine Triphosphate

AUF: Aqueous unfermented

BCC: Basal cell carcinoma

BMZ: Basement membrane zone

CE: Catechin equivalents

$CHCl_3$: Chloroform

CMM: Cutaneous malignant melanoma

COX: Cyclooxynases

CYP: Cytochrome P450

DAC: 4-Dimethylaminocinnamaldehyde

DNA: Deoxyribo nucleic acid

DPBS: Dulbecco's phosphate buffered saline

DPPH: 2,2-diphenylpicrylhydrazyl

EGCG: Epigallocatechin gallate

eNOS: Endothelial Nitric oxide synthase

F1: Fractionation 1

F2: Fractionation 2

FBS: Fetal bovine serum

FRAP: Ferric reducing antioxidant power assay

GAE: Gallic acid equivalents

GER: Genomic excision repair ()

GLM: Generalized linear modeling

GLM: Generalized linear modeling ()

GSH: Glutathione

GSSG: Glutathione disulfide-reduced form

GST: Glutathione-S-transferase

H_2O_2: Hydrogen peroxide

HETEs: Hydroxyl eicosate transnoic acid

HNO_3: Nitric acid

HpETEs: Hydropeoxy eicosate transnoic acids

HPLC: High-perfomance liquid chromatography

IKK: IκB kinases kinase

IL-1α: Interleukin 1 alpha

IL-1β: Interleukin 1 beta

IL-6: Interleukin 6

IL-8: Interleukin 8

iNOS: Inducible nitric oxide synthase

IκB: Inhibitor of κB

LOX: Lipooxygenases

LPS: Lipopolysaccharide

LSM: Least Squares Means

LSM: Least Squares Means

LTs: Leukotrints

MAPK: Mitogen activated protein kinase

MBN: Methyl benzyl nitrosamine

MeOH: Methanol

MF: Methanol fermented

MUF: Methanol unfermented

NADP: *Nicotinamide adenine dinucleotide phosphate*

NADPH: Nicotinamide adenine dinucleotide phosphate-reduced form

NaOH: Sodium hydroxide

NER: Nucleotide excision repair

NFκB: Nuclear factor kappa-B

NLRs: NOD-like receptors

NMSK: Non melanoma skin cancers

nNOS: Neouronal nitrogen oxidase synthase

NO: Nitric oxide

NOD: Nucleotide binding ologomerization

NOS: Nitric oxide synthase

O_2^-: Oxygen radical

OH-8dG: 8-hydroxydeoxy guanosine

ORP: Oxidation-reduction potential

PA_2: Phospholipase A_2

PAL: Phenylalanine ammonia lyase

PAMPs: Pathogen-associated molecular patterns

PKC: Protein Kinase C

PMA: Phorbol 12-myristate 13-acetate

PPAG: Phenylpyruvic acid-2-*O*-glucoside

PRRs: Pattern recognition receptors

RLU: Relative light units

ROS: Reactive oxygen species

RPMI: Roswell Park Memorial Institute medium

SCC: Squamous cell carcinoma

TE: Trolox equivalents

TF: Transcription factor

THP-1: TIB-202™ Homo sapiens peripheral blood cell line

TLC: Thin layer chromatography

TLR: Toll like receptors

TNF-α: Tumor necrosis factor-alpha

TP: Total polyphenol

TPTZ: 2, 4, 6-Tri (2-pyridyl)-s-trazine

Trolox: (±) 6-Hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid

UGT: UDP-glucuronyl transferase

UV-A: Ultraviolet A

UV-B: Ultraviolet B

UV-C: Ultraviolet C

UV-R: Ultraviolet radiation

X-1: XAD-4 fraction 1

X-2: XAD-4 fraction 2

X-3: XAD-4 fraction 3

X-4: XAD-4 fraction 4

X-5: XAD-4 fraction 5

XAD-4: Amberlite XAD-4 polymeric resin

XP: Xeroderma pigmentosum

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intracellular IL-1α production, (f) Fraction X-3- induction of apoptosis. Capital letter Indicates differences between effects in (-) and (+) UV-B, if letters are similar for a specific variable then UV-B radiation had no significant effect.

Chapter 6

1 General introduction

1.1.

1.2. General introduction

The wavelength of ultraviolet (UV) radiation ranges from 200 nm to 400 nm and is further divided into three sub-regions i.e. UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (200-280 nm) (de Gruijl, 1999). The electromagnetic rays reaching the earth consists mostly of UV-A and some UV-B (295-320 nm) wavelengths as all the UV-C wavelengths and much of the UV-B wavelengths (280-295 nm) are absorbed by the ozone layer (Rochette *et al*., 2003). With respect to skin carcinogenesis, the effects of UV-B are limited to the epidermis as the wavelength is not long enough to penetrate to deeper layers (de Gruijl, 1999). Hence most skin cancers arise from keratinocytes, which are the predominant cells of the epidermis (de Gruijl *et al*., 2001; Soehnge *et al*., 1997).

Skin cancers can either be of melanoma or non-melanoma and the latter can be of squamous or basal cell origin. The most common type of skin cancer, basal cell carcinoma (BCC), accounts for up to 80 % of all non-melanoma skin cancers (NMSK), with tumors that are slow growing, locally invasive and that rarely metastasize (Gordon, 2013; Ogden & Telfer, 2009). Untreated actinic keratosis (AK) are precursors for squamous cell carcinoma (SCC), which is the second most common form of skin cancer resulting from a lifetime cumulative or chronic exposure to UV radiation (Gordon, 2013; Lacy & Alwan, 2013; Ogden & Telfer, 2009). Cutaneous malignant melanoma (CMM) is a malignant tumor origination from melanocytes, it is the least common of all skin tumors, accounting for about 4 % of the total number of skin cancers, but the most dangerous as it can metastasis rapidly accounting for about 75% of deaths (Jerant *et al*., 2000; Ogden & Telfer, 2009).

UV-B may lead to tumor development through various mechanisms including DNA damage, oxidative stress and the onset of chronic inflammation (Ichihashi *et al*., 2003). Conjugated double bonds absorb ultraviolet radiation and this increases greatly in ring structures and therefore DNA is highly susceptible to absorbing UV-B (de Gruijl, 1999). Inflammation in the skin is a key determinant of skin carcinogenesis and is associated with the production of various inflammatory mediators such as leukocyte infiltration, release of pro-inflammatory cytokines and expression of inflammatory genes (Suter *et*

al., 2009). Cytokines play a key role in local immune and inflammation responses, which can be either pro-inflammatory such as interleukin-1-alpha (IL-1α), interleukin-1-beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF-α) or anti-inflammatory such as IL-10 (Santangelo *et al.,* 2007). Uncontrolled DNA damage, oxidative stress and inflammatory responses may produce cells with the ability to escape cell cycle check points and an inability to undergo apoptosis, resulting in uncontrolled proliferation and tumor development.

Macrophages play an important role in innate immune inflammatory responses as they are involved in the phagocytosis of microbes that escaped the neutrophils, present antigens to T-helper cells and release a variety of cytokines to initiate inflammation (Fujiwara & Kobayashi, 2005). Macrophages are generated in bone marrow and reach all body tissues through the blood at the onset of inflammation. Chronic activation of macrophage function has been linked to inflammatory diseases and cancer. There are different types of macrophages depending on the form of activation, which can be classical resulting in M1 macrophages or alternate resulting in M2 macrophages (Mosser, 2003). The activation of M1 macrophages occurs via LPS and/or interferon stimulation and this type macrophages is regarded as pro-inflammatory, while M2 macrophages are regarded as anti-inflammatory since they are activated by cytokines such as TNF-α.

Chronic activation of macrophage function has been linked to inflammatory diseases and cancer. The strong link between inflammation and cancer provides a target for the development of anti-inflammatory drugs. However, synthetic drugs currently on the market such as etanercept, ifliximab and thalidomide exert adverse side effects on the cell thus the development of safe anti-inflammatory compounds has been the focus in the field of drug discovery (Kristina *et al.*, 2010). As already established, polyphenols have various health benefits including anti-inflammatory action, which has been attributed in most part to their antioxidant properties (Fraga *et al*., 2010). The anti-inflammatory activity of polyphenols is thought to involve interaction with pro-inflammatory enzymes cyclooxynases (COX), lipooxygenase (LOX) and inducible nitric oxide syntheses (iNOS) (Williams *et al*., 2004). The modulation of signaling molecules

involved in inflammation such as tyrosine kinases and the nuclear factor kappa beta (NFκB), to mention a few, has been reported in the anti-inflammatory activity of polyphenols (Yoon & Baek, 2005).

Rooibos (*Aspalathus linearis*), a South African herbal tea, has a chemical composition rich in flavonoids, which are known to exhibit powerful antioxidant properties involving free radical scavenging, metal chelating and singlet oxygen quenching (Dai & Mumper, 2010; Symonowicz & Kolanek, 2012). The herbal tea has been reported to exhibit health protective properties which include decreasing the damage to the central nervous system in rats by reducing lipid peroxidation through suppressing the accumulation of lipid peroxides (Inanami *et al*., 1995). The antioxidant activity of rooibos has been associated with its major flavonoids, aspalathin and northtofagin, which are abundant in green rooibos but are decreased during fermentation (Joubert *et al.,* 2008). For example, a rooibos hot water extract, prepared from fermented and unfermented plant material, showed a clear decrease in antioxidant activity of rooibos as a result of fermentation associated with decreased total polyphenol (TP) levels (Joubert *et al*., 2008). In another study a strong correlation between the aspalathin content of green rooibos and antioxidant activity in the ABTS assay has been reported (Schulz *et al*, 2003). The evidence of *in vitro* (Joubert *et al*., 2005) and *in vivo* (Marnerwick *et al*., 2010) antioxidant activity of rooibos suggests potential protective effects and warrants further investigations into the association between the antioxidant properties and disease prevention.

Rooibos has been reported to exhibit anti-cancer properties *in vivo,* which was associated with its phenolic composition. One such report indicated that unfermented and fermented rooibos inhibits methylbenzylnitrosamine (MBN)-induced eosophageal squamous cell carcinogenesis in rats (Sissing *et al*., 2011). In another study, ethanol/acetone (E/A) soluble fractions prepared from methanol extracts of processed and unprocessed rooibos inhibited skin tumor development in mice, which was dependant on differences in the flavanol/proanthocyanidin and flavonol/flavone composition (Marnewick *et al*., 2005). However, the challenges of metabolism, bio-availability and contrasting effects on various signaling molecules have produced great

controversy regarding the beneficial health effects of polyphenols (Manach & Donovan, 2004).

The current study will focus on the anti-inflammatory effects of rooibos using previously developed UV-B/HaCaT (Magcwebeba *et al*.,2012) and lipopolysaccharide (LPS)/TPH-1 derived macrophage inflammatory models (Keet *et al*., 2015) with IL-1α and TNF-α as biomarkers of inflammation, respectively. The chemical composition and antioxidant properties of unfermented and fermented rooibos extracts, using methanol and distilled water, are presented in chapter 3. In addition, details on the XAD-4 column chromatography fractionation of the unfermented methanol extract and the subsequent chemical characterization and antioxidant evaluation of the fractions are summarized. The activity of the extracts and the fractions in a UV-B/HaCaT inflammatory model will be presented in chapter 4, where the effect of the extracts on cell viability, apoptosis and the inflammation biomarker, IL-1α, are investigated. In chapter 5, the modulating effects on inflammation of the rooibos extracts and XAD-4 fractions in the LPS/THP-1 derived macrophage inflammatory model with TNF-α as biomarker will be presented. Chapter 6 includes a critical evaluation of the underlying mechanisms likely to be involved in the anti- and/or pro-inflammatory effects of the different rooibos extracts and/or fractions as well as future projections regarding the chemo preventive properties of rooibos flavonoids against skin carcinogenesis.

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2 Literature review

2.1. The skin

2.1.1. Structure

The skin is the largest organ in the human body accounting for approximately 16 % of the total body weight (Venus *et al*, 2010). In morphology, the skin is very complex comprising various tissue types arranged in three layers namely, epidermis, dermis and the hypodermis (Kanitakis, 2002); the epidermis and dermis are separated by the basement membrane zone (Fig 2.1). The skin's main function is to protect against physical and chemical stresses from the external environment as well as having more specialized functions such as sensory, thermoregulation and host defence (Xu *et al*, 2008).

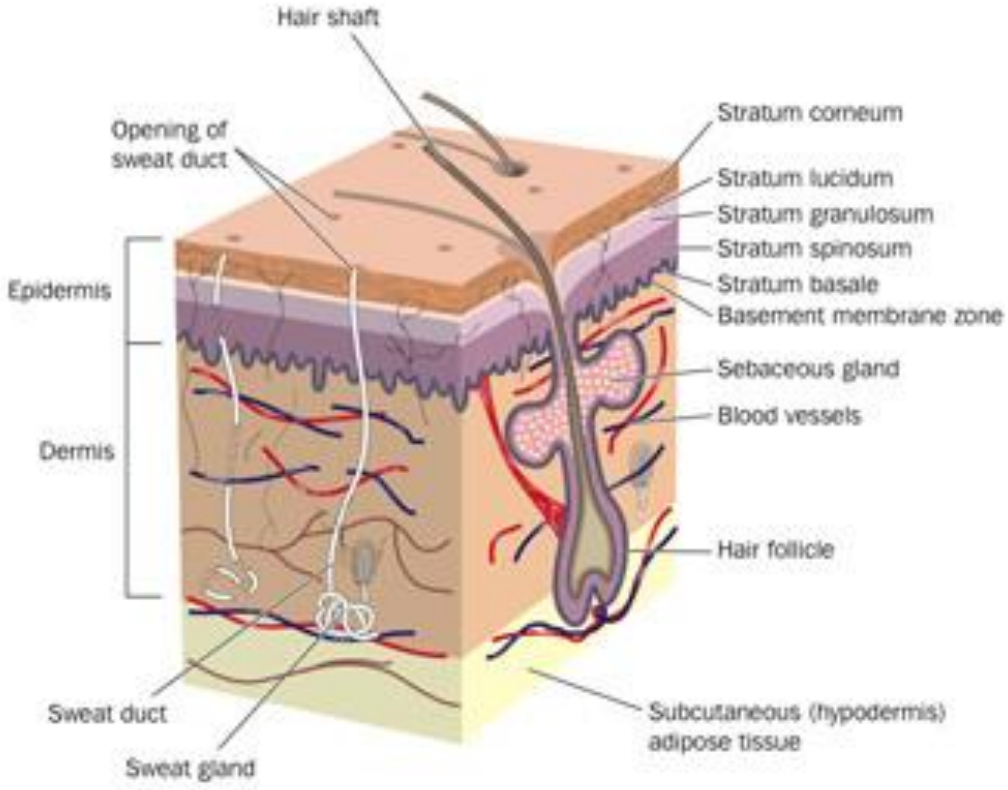

Figure 2.5. Structure of the skin showing the different layers (Adapted from N'Da, 2014)

2.1.1.1. Epidermis

The epidermis is the outermost layer of the skin and the first line of contact with the external environment (Ramose-Silva & Jacques, 2012). Therefore it serves to provide physical, chemical, biological and adaptive immunologic barriers, which protect the skin from external threats (Baroni *et al*., 2012; Lee *et al*., 2006). The epidermis is made up of terminally differentiated stratified squamous epithelium tissue that renews itself continuously (Venus *et al*., 2010). This skin layer is further divided into five sub-layers arranged from inner most to outermost i.e. the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum (Venus *et al*., 2010). Keratinocytes constitute the majority (90-95%) of cells in the epidermal sub layers (Baroni *et al*., 2012) and undergo a specific differentiation process, which results in flattened, enucleate cells called corneocytes (Lee *et al*., 2006). The keratinocytes vary in morphology according to which epidermal layer they are found in. For instance basal layer keratinocytes are columnar possessing a large nucleus; the spinosum layer keratinocytes on the other hand are polygonal, larger and have a vesicular nucleus, while granular layer keratinocytes are flattened and corneum layer keratinocytes are fully differentiated into corneocytes, which appear highly flattened and eventually shed from the skin (Baroni *et al*., 2012). The remaining 5 % of epidermal cells are non-keratinocyte cells, the langerhans cells, melanocytes and merkel cells.

Langerhans cells

Langerhans cells, the most important cells for the immune barrier function of the epidermis, are derived from bone marrow cells and have a dendritic appearance (Baroni*et al*., 2012). These cells are found in their immature state on the mucosal epithelia lining the ocular, oral, and vaginal surfaces and in the skin (Poulin *et al*., 2007). Langerhans cells are mobile, migrating to the dermal lymphatics and to paracortical areas of draining lymph nodes where they present antigens to T-cells (Venus *et al*., 2011).

Melanocytes

Melanocytes are somatic cells found in the neural crest which originate from precursor cells, melanoblasts (Tolleson, 2005; Yamaguchi *et al*., 2007), and are abundant in the

skin and hair follicles (Tolleson, 2005). In the skin, melanocytes form a close association with keratinocytes via their dendrites, enabling the transfer of melanin into the keratinocytes in order to determine skin colour and help protect against the damaging effects of ultraviolet radiation (Tsatmali *et al*., 2002). Melanin production is known to involve the enzymatic activity of tyrosinase in two reactions. Firstly, the hydroxylation of a monophenol and secondly, the conversion of an o-diphenol to the corresponding o-quinone. o-Quinone undergoes several reactions to eventually form melanin (Baroni *et al*., 2012; Plonka *et al.*, 2009). The key function of melanin is to protect the skin against ultra violet radiation (UV-R) (Passeron *et al*., 2007).

Merkel cells

The origin of merkel cells is clouded by great controversy as they have characteristics of both epidermal and neuroendocrine cells (Lucarz & Brand, 2007). Merkel cells are found on the basal layer of the epidermis and in the outer root sheath of hair follicles (Boulais & Misery, 2007). They are oval shaped with a diameter of 10-15 µm. The cells are clustered in touch sensitive zones of glabrous and hairy skin (Van Keymeulen *et al.*, 2009). In addition, merkel cells synthesize neuropeptides inside dense core neurosecretory granules, which are primarily located near low threshold sensory neurons (Misery & Boulais, 2008). The complexes between merkel cells and sensory neurons is termed the merkel cell-neurite complex (Boulais & Misery, 2007) and contains slowly adapting mechanoreceptors, which mediates slowly adapting responses to touch (Boulais & Misery, 2007; Lumpkin & Caterina, 2007).

2.1.1.2. Basement membrane zone

The cutaneous basement membrane zone (BMZ) also known as the dermal epidermal junction is a combination of epidermal basal keratinocytes and the dermal fibroblasts. It comprises an intricate network of macromolecules that link keratin fibres of the basal keratinocytes with collagen fibres of the dermis (Kanitakis, 2002). This structure forms an adhesion complex between two distinct cellular compartments, the dermis and the epidermis and also provides support for proliferation, migration and differentiation of cells (Burgeson & Christiano, 1997; Powell, 2006). The BMZ is important in the

transmission of extracellular signals and growth factors and in the formation of a permeability barrier (Hashmi & Marinkovich, 2011).

The BMZ inhibits passage of molecules between the structures it separates based on size and charge. However, melanocytes and langerhans cells or lymphocytes and tumour cells are allowed to pass through (Burgeson & Christiano, 1997). Morphologically, the BMZ has four distinct layers i.e. the cell membrane consisting of hemidesmosomes, the lamina lucida, which is an electron lucent space, the osminophilic lamina densa, and the sub-basal lamina filamentous zone (Hashmi & Marinkovich, 2011). One unique feature of the cutaneous BMZ is the presence of an anchoring complex comprising the hemidesmosomes, anchoring filaments and the anchoring fibrils (Burgeson & Christiano, 1997). This complex serves as a continuous link between basal keratinocyte and the dermal components (McMillan *et al.*, 2003).

2.1.1.3. Dermis

The dermis is an elastic connective tissue layer, which separates the epidermis and subcutaneous tissues of the skin. It serves to protect the epidermis through providing structural and nutritional support (Kanitakis, 2002 & Powell, 2006). The thickness of the dermis varies depending on the site in which it is found, for example, the dermis on the palms will be thicker than that on more sensitive or delicate skin, like the eye lids and lips (Kanitakis, 2002). Structurally the dermis is divided into two layers, the ‘upper’papillary dermis and the ‘lower’reticular dermis (Tobin, 2006). The dermis possesses interstitial (collagen fibres, elastic tissue and ground substance) and cellular components such as fibroblasts, mast cells, plasma cells, dermal dendritic cells and histiocytes, as well as blood vessels, lymphatic channels and sensory nerves (Lai-Cheong & McGrath, 2009). Collagen constitutes more than 70 % of the dermis interstitial components, while the fibroblasts, which are responsible for the synthesis and renewal of extracellular matrix, is the pre-dominant cell type in the dermis (Baroni *et al*., 2012; Lai-Cheong & McGrath, 2009).

2.1.1.4. Hypodermis

The hypodermis is the innermost and thickest layer of the skin. It enclosesinto and is attached to the dermis by collagen and elastin fibres. This layer is mainly composed of adipocyte cells, which specialize in accumulating and storing fats. Adipocytes are grouped together in lobules separated by connective tissue (Kanitakis, 2002). Adipocytes are arranged in primary and secondary lobules, the morphology of which varies somewhat according to the sex and the body region considered. There are two different types of adipocytes known in mammals i.e. white adipocytes, which store energy as triglycerides and release it according to the organism's needs, and brown adipocytes, which dissipate energy as heat (Himms-Hagen, 1990 & Klaus *et al*., 1991). The fats contained in the adipocytes can be re-directed into the circulation through the veins, during exercise or starvation, and are then transformed into energy (Norgan, 1997). The hypodermis therefore acts as an energy reserve and also passively participates in thermoregulation since fat is a heat insulator.

2.1.2. Skin cancer

The development of cancer is considered to be a result of disturbances in cell signalling pathways controlling the growth of cells such as proliferation, differentiation and apoptosis, to mention a few (de Gruijl *et al*, 2001). These disturbances may be in response to the synthesis of dysfunctional proteins or damaged genes resulting in miscoding during gene expression, hindering translation (de Gruijl, 1999). Failure of normal growth controls and check points may lead to uncontrollable growth of cells that subsequently develop into tumours (Cooper, 2000; de Gruijl *et al*., 2001b & Soehnge *et al*., 1997). Tumours may remain benign for prolonged periods of time and not become malignant and metastasize (Cooper, 2000). The process of skin carcinogenesis, like any other cancer, is divided into three steps namely, initiation, promotion and progression (Klaunig & Kamendulis, 2004).

2.1.2.1. Initiation

Initiation involves damage to DNA, either directly from genotoxic agents or indirectly from epigenetic agents (Klaunig & Kamendulis, 2004). The most potent cause of skin cancer is UV-R, which results in multiple biochemical events such as DNA damage, oxidative stress and inflammation, to be discussed in detail in section 2.1.5, that lead to carcinogenesis (Sarasin, 1999). Initiation is an irreversible process and the extent of damage is dependent of the dose of the carcinogen (Klaunig & Kamendulis, 2004).

2.1.2.2. Promotion

Continual exposure of initiated cells to tumour promoters may lead to clonal expansion where a specific mutation has a selective advantage for the cell, which ultimately grows rapidly resulting in the tumour genotype becoming dominant (Cooper, 2000). In addition, initiated cells may respond differently to cell signalling molecules and this often results in mutated cells escaping cell cycle check points, thereby continuing to divide uncontrollably (de Gruijl *et al*., 2001b). Promotion is a reversible stage dependant on the presence of a tumour promoter (Klaunig & Kamendulis, 2004).

2.1.2.3. Progression

Continuous exposure to a tumour promoter further damages the cell's DNA leading to additional cellular and molecular events that ultimately transforms a benign tumour into a malignant phenotype (Cooper, 2000). Once progression begins it is irreversible, involving genetic instability and disruption of chromosome integrity (Klaunig & Kamendulis, 2004).

2.1.3. Skin cancer types

It has already been established that exposure to the ultraviolet–B (UV-B) wavelength (280-320 nm) of sunlight results in the development of skin cancer (Sarasin, 1999). However, although UV-R is a genotoxic agent, it does not penetrate the skin any deeper than the epidermis, which is located on the upper surface of the skin; therefore most

skin cancers arise from the cells of the epidermis (de Gruijl *et al*., 2001b; Soehnge *et al*., 1997). Only the major skin cancer types will be discussed.

2.1.3.1. Melanoma

Cutaneous malignant melanoma

Cutaneous malignant melanoma (CMM) is a malignant tumour of melanocytes (Ogden & Telfer, 2009). CMM are the least common of all skin tumours, accounting for about 4 % of the total skin cancers, but the most dangerous as they can metastasise rapidly accounting for about 75 % of deaths associated with skin cancer (Jerant *et al.*, 2000). Melanoma malignancies are divided into four subtypes according to clinic-pathological grounds (MacKie, 2006) namely; superficial spreading, nodular, acral and lentigo. The superficial spreading melanoma is the most common and in clinical features it is similar to the nodular melanoma. About 2-8 % of melanomas are amelanotic (without melanin, no pigmentation) making these very difficult to diagnose (Gordon, 2013). Due to the vast variety in the clinical features, the ABCDE rule has been developed, which outlines the expected clinical features and warning signals for most melanomas (Gordon, 2013). The “A” is for the asymmetry of the of the mole (is one half bigger than the other), “B” is for the border of the mole (are they blurred or ragged?), the “C” is for colour (does the mole have an uneven colour, a mixture of brown and black), the “D” is for diameter (is the mole larger than 6mm) and “E” is for the evolution, enlargement or elevation of the lesion.

2.1.3.2. Non-melanomma

Squamous cell carcinoma

Squamous cell carcinoma (SCC) is the cancer of the squamous layer (stratum spinosum) of the epidermis (Lacy & Alwan, 2013) and in most cases, SCC develops as a result of untreated actinic keratosis (AK), a precursor of SCC. It is the second most common type of skin cancer accounting for about 16 % of skin cancers and results from a lifetime cumulative or chronic exposure to UV radiation (Gordon, 2013; Ogden & Telfer, 2009). Other factors that may predispose individuals to the development of SCC include ionizing radiation, chronic exposure to industrial carcinogens, post transplant

immune suppression, chronic wounds and human papilloma virus infection. SCC is locally invasive causing tissue destruction and is characterized by the ability to metastasize via lymphatic or haematogenous spread (Gordon, 2013; Ogden & Telfer, 2009). These types of malignancies occur mainly on sun exposed parts of the body i.e. face, back of hands and neck.

Basal cell carcinoma

Basal cell carcinoma (BCC) originates in the basal layer of the epidermis (stratum basale) and is the most common skin cancer type constituting 80 % of all non melanoma skin cancer (NMSK). Unlike SCC, basal cell carcinoma does not have premalignant lesions (Gordon, 2013; Ogden & Telfer, 2009). This type of tumour is characterized by slow growth, is locally invasive and rarely metastasize. The most potent predisposing factor for BCC is chronic UV exposure (de Gruijl *et al*., 2001b). There are six different subtypes of BCC i.e. nodular (most common), pigmented, cystic, sclerosing (most difficult to treat), superficial and nevoid (Jerant *et al*., 2000).

2.1.4. Skin cancer incidence

When investigating the incidence of skin cancer it is important to note that although there is an overall rise in total number of reported cases, the incident rates vary amongst the different skin cancer types. Non-melanoma skin cancer, which includes BCC and SCC, are the most reported skin cancer types (Jerant *et al*., 2000). However, CMM has been reported to have the highest mortality rate (Giblin & Thomas, 2007) accounting for approximately 75 % of deaths associated with skin cancer (Jerant *et al*., 2000). Although NMSC is associated with old age, CMM has been reported to peak between the ages of 20-45. Skin cancer incidence has been on the rise since the 1970s when it was considered a rare disease with approximately 6 cases of mortality per 100 000 reported cases in the United States (Garbe & Leiter, 2009). In three decades, the mortality rates grew by three fold from 6 to 18 per 100 000 cases in the United States and this trend was also observed in Central Europe. However, in Australia and New Zealand incident rates are said to be the highest at 30-60 per 100 000 inhabitants per

year (Leiter & Garbe, 2008). This is due to the correlation between CMM incidence and geographical location in terms of the proximity to the equator of the earth (Garbe & Leiter, 2009). Changing societal activities, which include more sun exposure and the depletion of the ozone layer causing more UV rays to penetrate the atmosphere, are the main causes of the increase in skin cancer incidence for the last four decades (Jerant *et al*., 2000). Education regarding the risk of sun exposure is therefore an alternative method to manage the rise in skin cancer incidence, specifically, CMM which is most likely to result in death.

2.1.5. Role of Ultraviolet radiation in skin carcinogenesis

Ultraviolet radiation forms part of the electromagnetic spectrum of sunlight and can initiate a cascade of complex events that lead to skin carcinogenesis, specifically by affecting initiation and promotion (Sarasin, 1999). Such events include DNA damage leading to mutations in genes that control the cell cycle and promote tumour development (Mouret *et al*., 2006). Other events include the induction of gene expression through signal transduction pathways that may contribute to tumour promotion, and suppression of immune response, which induces tolerance to antigens (Ichihashi *et al*., 2003; Sarasin, 1999; Xu & Fisher, 2005). Sunlight consists of electromagnetic radiation of various wavelengths that is divided into three main regions namely, ultraviolet, visible light and infrared (Soehnge *et al*., 1997). Ultraviolet radiation (200-400 nm) is further divided into three sub-regions i.e. UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (200-280 nm) (de Gruijl, 1999). UV-C and most of the UV-B spectrum (280-295 nm) are absorbed by the ozone layer; therefore the UV that reaches the earth consists mostly of UV-A and some UV-B (295-320 nm) (Rochette *et al*., 2003). UV-B is considered to be the most carcinogenic and its effects are limited to the epidermis (de Gruijl, 1999). The effects of each component of UV-R on the skin are dependent on the depth of penetration and the energy it carries (Clydesdale *et al*., 2001). For instance, UV-A has longer wavelengths and thus carries less energy and UV-B has shorter wavelengths and therefore caries more energy.

2.1.5.1. DNA damage

Conjugated bonds can absorb ultraviolet light and this absorption increases greatly in ring structures. Ultraviolet light can directly or indirectly cause damage to organic molecules including DNA depending on the wavelength of the photo incident (de Gruijl, 1999 & Mouret *et al*., 2006). UV-B is directly absorbed by DNA, resulting in the transformation of adjacent pyrimidine bases to the highly genotoxic dipyrimidine photo products i.e cyclobutane pyrimidine dimers and pyrimidine-pyrimidome photo products (Fitch *et al*., 2003; Ichihashi *et al*., 2003). On the other hand, UV-A has a much lower phototoxic effect as DNA is not a chromophore for UV-A and therefore does not absorb UV-A. However, UV-A indirectly causes damage to DNA through absorption by non-DNA chromophores resulting in oxidative stress, which subsequently damages DNA (Ichihashi *et al*., 2003; Rochette *et al*., 2003). Although the pyrimidine dimer is the most carcinogenic it is important to note that UV-R also causes a wide range of other forms of damage to DNA such as protein-DNA crosslinks, oxidative base damage and single strand breaks. However, these can also be caused by other factors and cannot be attributed solely to UV induced damage (de Gruijl, 1999). Nevertheless, the pyrimidine dimers result in point mutations that are uniquely UV-B induced such as C→T and CC→TT from CT and CC dimers. These occur as a result of the "A" rule which states that DNA polymerase inserts and an "A" wherever there is an un-instructional lesion (Ichihashi *et al*., 2003; Melnikova & Ananthaswamy, 2005). This is further supported by the frequent recovery of these point mutations on UV-induced skin cancer cells (Ichihashi *et al*., 2003). In the case of the TT dimer the A inserted results in a A→T mutation, which repairs the original lesion; the TT dimer is therefore not highly mutagenic.

Cells have in place repair mechanisms to avoid UV-induced skin cancers or tumorigenesis, such as nucleotide excision repair (NER), which is concerned with repairing most of the UV-induced DNA damage such as bulky chemical adducts (Ichihashi *et al*., 2003). The NER process consist of five steps requiring at least 30 gene products i.e. the recognition of the DNA lesion, single strand incision at the flanking free sides of the lesion, excision of a single stranded DNA nucleotide, DNA repair synthesis

to replace the excised DNA lesion and ligation of the remaining single stranded nick (Aboussekhra *et al*., 1995). The process is subdivided into two pathways, the transcription-coupled repair (TCR), which repairs the transcribed DNA from the damaged strand, and genomic excision repair (GER), which repairs the damaged nucleotide strand. The importance of NER in skin cancer prevention is supported by the increased incidence of skin cancer on sun exposed areas in xeroderma pigmentosum (XP) patients, which are defective in NER (Fitch *et al*., 2003; Ichihashi *et al*., 2003; Sarasin, 1999).

There are two types of genes that are frequently affected by UV-B damage and these include the tumour suppressor genes (e.g. p53) and oncogenes (e.g. ras). These genes are crucial for cell cycle control, maintenance of gene integrity, proliferation and differentiation and mutations may result in transformation of cells and subsequent tumour development. The p53 genes code for a transcription factor (TF) protein, which is involved in DNA repair through two pathways i.e. cell cycle arrest to allow time for repair of damaged DNA or NER and GER. Chronic UV-B exposure leads to signature mutations on the p53 gene, which disables the TF's ability to induce cell cycle arrest, DNA repair and apoptosis. Subsequently, the mutated cells gain clonal expansion advantage leading to uncontrolled proliferation with the mutations and subsequent tumorigenesis. Mutations on the p53 gene are an indication of the early stages of cancer initiation and have been identified in UV-induced cancers. In addition, mutated p53 is reported as the most common mutation present in skin cancers with point mutations found on pre-tumour lesions such as keratoacanthomas and actinic keratosis (Sarasin, 1999). Other events such as activation of oncogenes or inactivation of other tumour suppressor genes are needed for the progression of cancer (Melnikova & Ananthaswamy, 2005).

2.1.5.2. Oxidative stress

Various reports have linked reactive oxygen species (ROS) to age related diseases including cancer (Kovacic & Jacintho, 2001). Reactive oxygen species can be produced by endogenous and exogenous sources related to mitochondrial activity, inflammatory related cell activation, cytochrome P450 cycle and peroxisomes. Oxidative metabolism

in the mitochondria converts 4-5 % of molecular oxygen to the superoxide radical through single electron reduction, which can be subsequently converted to hydrogen peroxide through the activity of superoxide dismutase (Barber & Harris, 1994). In the presence of metal ions, specifically iron, hydrogen peroxide can generate the highly reactive hydroxyl radical through the Fenton reaction (Betteridge, 2000). Activated inflammatory cells, neutrophils, eosinophils and macrophages release oxygen radicals (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide (NO_2) during respiratory burst. During the formation of peroxisomes by the activity of peroxisome proliferators H_2O_2 escapes, and together with the former radicals, these shift the redox balance towards oxidative stress (Rao & Reddy, 1991). The cytochrome (CYP) cycle can result in the release of ROS through the uncoupling of certain CYP450 enzymes, i.e. CYP450 2E1 as well as metabolism of phenobarbital by CYP450 2B (Parke & Sapota, 1996; Rice *et al*., 1994). Environmental carcinogens such as UV-R, chlorinated compounds, metal ions, barbiturates, phorbol esters and peroxisome proliferating compounds form part of the exogenous factors that generate ROS in cells (Klaunig *et al*., 1997; Rice-Evans & Burdon, 1993). Under normal physiological conditions the cells oxidant to antioxidant ratio is kept in balance by the cell's endogenous antioxidant defence mechanisms, a shift in this balance towards more oxidants will result in oxidative stress and damage to macro-molecules (Sies, 1985). Antioxidant defence mechanism of cells can be enzymatic including superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) or non-enzymatic via the involvement of vitamins E, C, β-carotene, glutathione and co-enzyme Q (Abuja & Albertini, 2001; Betteridge, 2000). Superoxide dismutase and GPx are located in the cytosol and mitochondria with the former being involved in reduction of superoxide to form hydrogen peroxide and water, while the latter removes hydrogen peroxide.

The cell's antioxidant mechanisms are controlled by the redox state of the cell and there are four major redox systems namely, NADP/NADPH, thioredoxin, glutaredoxin and glutathione, which is the most important in maintaining redox balance due to its high levels (Schafer & Buettner, 2001).Glutathione is present in oxidized (GSH) and reduced (GSSG) form. The oxidized form is 10-100 fold more than the reduced form thus even small changes in GSH will result in significant shift in the GSH/GSSG ratio and therefore

redox balance (Schafer & Buettner, 2001). The GSH/GSSG ratio has been implicated in signal transduction and cell cycle control and should be closely regulated (Herrlich & Böhmer, 2000). There are various factors involved in regulating the GSH/GSSG ratio such as glutathione disulphide or glutathione reductase and by removing excess GSSG or increasing glutathione reductase activity, the latter can maintain a good GSH/GSSG ratio even under oxidative stress conditions (Schafer & Buettner, 2001). A decrease in GSH concentration has been linked to an induction in mitochondria dependant apoptosis. This has been validated by the block in apoptosis induction following restored GSH concentration (Ghibelli *et al*., 1995; Ghibelli *et al*., 1999).

Reactive oxygen species can directly form DNA lesions such as double or single strand breaks, purine, pyrimidine or deoxyribose modifications and DNA cross-links. Many types of ROS can form oxidized DNA bases, however the hydroxyl radical needs to be generated in close vicinity of the nucleus in order to oxidize DNA (Sies, 1985). In contrast, hydrogen peroxide and peroxy nitrite are less reactive and therefore easily diffuse into cells and are more likely to oxidize DNA bases (Radi, 1998). Interestingly, activated macrophages readily release superoxide and nitric oxide, which can react to form peroxy nitrite. This may subsequently oxidize DNA, explaining the association between inflammation and mutations leading to carcinogenesis (Marnett, 2000). The most common oxidative DNA adduct is the 8-hydroxydeoxy guanosine (OH-8dG), which results in G-T transversions that occur widely on mutated oncogenes and tumour suppressor genes (Hussain & Harris, 1998; Moriya, 1993).

2.1.5.3. Inflammation

Inflammation is a physiological response, involving both innate and adaptive immunity, following exposure to various insults such as tissue injury, microbial pathogen infection, chemical toxins or ultraviolet light radiation. The process of inflammation is very complex involving signalling cascades, activation of transcription factors, increase in inflammatory enzymes, release of various oxidants and pro-inflammatory molecules (Bengmark, 2004). Acute inflammation is short term, self-limiting and is meant to be therapeutic to the host organism; whereas an excessive, prolonged inflammatory response can lead to chronic inflammation. The latter involves the generation of various

oxidants, i.e. superoxide anion, hydroxyl radical, hydrogen peroxide and nitric oxide by inflammatory cells, which can damage normal tissue resulting in induction of signalling molecules and enzymes leading to the development of degenerative diseases such as neurological diseases, metabolic disorder, cardiovascular disease and cancer (Santangelo *et al*., 2007).

The initial stages of inflammation involve leukocyte infiltration, release of pro inflammatory cytokines and expression of inflammatory genes. Activation of inflammation occurs as a result of recognitions of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) e.g. toll like receptors (TLR) and nucleotide binding oligomerization domain-like (NOD-like) receptors, (NLRs) (Chen *et al*., 2007; Gordon, 2013; Li *et al*., 2010, Miller & Modlin, 2007). The stimulus is then carried into the cell through activation of signalling molecules and enzymes that bring about the inflammatory response. Cytokines play a key role in local immune and inflammation responses in cells (Santangelo *et al.,* 2007). Various cytokines are involved in the inflammatory response which are either pro- (interleukin 1β. 6, 8 and TNF-α) or anti-inflammatory (IL-10). The balance between pro- and anti-inflammatory cytokines should be tightly regulated in order to prevent the development of chronic inflammation (Santangelo *et al*., 2007). Specific cytokines are released by different cells of the skin layers and there are some that are suggested to be key role players in the inflammation process. These are the pro-inflammatory cytokines, interleukin 1 alpha (IL-1α), tumour necrosis factor alpha (TNF-α) and IL-6. Other pro-inflammatory cytokines include IL-17, IL-12 and IL-23. The process of inflammation related cancer involves the stimulation of chemotactic cytokines followed by the recruitment of mast cells resulting in the activation of pro-inflammatory cytokines such as IL-1, TNF-α and chemokines. The next step is the recruitment of monocytes differentiating into macrophages. The use of anti-inflammatory compounds has been reported to protect against various tumours, which validates the role of chronic inflammation in cancer development (Gerhäuser *et al*., 2003; Lin & Karin, 2007; Naugler & Karin, 2008). Of importance in this connection is the involvement of transcription factors, cytokines such as IL-1, TNF-α and IL-6, chemokines and leukocyte infiltration.

The transcription factor (TF), nuclear factor kappa B (NFκB), is amongst the key role players in inflammation related cancer as it is involved in the expression of various pro-inflammatory cytokines. NFκB is activated by a variety of pro-inflammatory cytokines and results in the activation of anti-apoptotic genes as well as increasing angiogenesis (Karin & Greten, 2005; Naugler & Karin, 2008). Specifically, continual phosphorylation of the NFκB subunits and factors involved in its activation is required for the oncogenicity. In addition, the aberrant regulation of this TF has been reported in most cancers (Naugler & Karin, 2008). Another factor STAT3, which is also an important factor along with NFκB, is thought to be closely related to the link between inflammation and cancer (Grivennikov & Karin, 2010). Specific pro-inflammatory cytokines have been reported to play important roles in cancer development i.e. TNF-α mutant mice were reported to be protected against the development of skin carcinogenesis (Arnott *et al*., 2003; Moore *et al*., 1999). TNF-α has also been linked to increased tumour growth and invasion as well as increasing leukocyte recruitment which all favour tumorigenesis. Another cytokine with a key role in inflammation, IL-1α has also been linked to carcinogenesis (Gabay *et al*., 2010).

The most predominant cells of the epidermis, keratinocytes, were reported to increase their expression of IL-1α after exposure to UV-B (Kupper *et al*., 1987). Keratinocytes are known to constitutively produce IL-1α, which is only released upon cell rupture during disease states or severe stress (Ansel *et al*., 1988; Sauder *et al*, 1982; Schmitt *et al*., 1985; Wood, 1996). IL-1α production in response to UV-B was shown to be highly dependent on the UV-B dose resulting in differential expressions indicating the activation of a cascade of signalling molecules (Chung *et al*., 1996). UV-B is known to induce IL-1α expression through its ability to induce c-jun and c-fos, which encodes proteins involved in the AP-1 complex formation, activating NFκB or by inducing the synthesis and release of various cytokines such as IL-1α, TNF-α and IL-6, which may in turn stimulate IL-1α production via an autocrine regulatory pathway (Devary *et al.*, 1991 & Luo *et al*., 2004).

Macrophages play an important role in the onset of inflammation and have three main functions namely phagocytosis of apoptotic bodies of neutrophils and the remaining

microbes, presenting the microbes to T-helper cells and the release of cytokines (Fujiwara & Kobayashi, 2005). There are two types of macrophages depending on their metabolism, M1 or M2 (Mosser, 2003). The former can be activated by LPS and results in a pro-inflammatory response, whereas the latter is activated by cytokines resulting in an anti-inflammatory response (Mosser, 2003). LPS activates the innate immunity and inflammation through toll like receptor (TLR) signalling, specifically TLR4, which subsequently induces gene expression via the activation of signalling pathways including MAPK, which subsequently activates nuclear factor kappa B (NFκB) (Akira, 2003; Wright, 1999). Two proteins are responsible for the recognition of LPS by host organisms and these are the LPS binding protein (LBP) and CD14; the latter serves as a membrane receptor for the LPS-LBP complex (Pugin *et al*., 1993). LPS activated microphages (M1) can result in pro-oxidant effects as well as sequestering intracellular iron as a mechanism to withhold iron from invading pathogens (Cairo *et al*., 2011). Various anti-inflammatory studies on macrophages by different natural products have suggested that these products inhibit the expression of inflammatory mediators through inhibiting key steps in the activation on NFκB. For example, iNOS expressed in LPS or cytokine activated raw 264.7 macrophages was found to be inhibited by quercetin (Hämäläinen *et al*., 2007), while treatment of raw 264.7 macrophages with ferulaldehyde, a phenolic degradation end product, decreased the phosphorylation of MAPK enzymes and therefore decreased the expression of NFκB regulated genes (Tucsek *et al*., 2011).

2.2. Polyphenols

2.2.1. Origin and biosynthesis

Phytochemicals are a group of non-nutritive compounds produced by plants, which possess various protective and/or health benefit properties (Arts & Hollman, 2005). One of the largest group of phytochemicals, the polyphenols, are secondary plant metabolites which are essential to plants and which display diverse biological functions namely, antioxidant, anti-microbial, antiviral and anti-inflammatory properties (Ignat, *et al*, 2011). Polyphenols are widely distributed and can be found naturally occurring in

plant based foods such as fruits, vegetables and beverages (teas, fruit juices and wine) (Archivio *et al*., 2007). In addition, polyphenols can be found in the by-products of agricultural and industrial processes, which include remainders of juice and wine making (berry skins), olive mill waste waters, olive leaves from the olive industry, peel and seed residues from the citrus industry as well as peels of several fruits (Ignat *et al*., 2011).

Plants produce primary metabolites for normal growth and survival namely, carbohydrates, amino acids and proteins, from these, secondary metabolites are derived. It is thought that the role of secondary metabolites is to defend against pathogens and to protect plants against various stressors i.e. UV radiation (Korkina *et al*., 2008). The biosynthesis of polyphenols therefore includes the processing of a primary metabolite through various methylation, hydroxylation and glycosylation pathways. More specifically, polyphenols are derived from metabolites of the shikimic acid pathway. Shikimic acid undergoes metabolic transformation yielding indol, tryptophan, phenylalanine and tyrosine; phenylalanine is catalytically de-aminated by phenylalanine ammonia lyase (PAL) forming cinnamic acid and its derivatives i.e. hydroxycinnamic acid, 4-coumaric acid and 4-coumaroyl-CoA (Quideau *et al*., 2011). Cinnamic acid and its close derivatives form phenolic acids, which are simple polyphenols; these are further transformed through catalytic reactions to form the more complex secondary polyphenols i.e. flavanoids and isoflavanoids, stillbenoids, coumarines, tannins, suberins and Lignans (Korkina *et al*., 2009).

2.2.2. Structure and classification

The core structure of polyphenols comprises aromatic rings with one or more hydroxyl groups (Ignat *et al*., 2011). This can range from simple to very complex structures depending of the number of aromatic rings as well the structural elements that bind the rings together. Polyphenols can be classified into three main groups: flavonoids, non-flavonoids and phenolic acids (Câmara *et al*., 2013). To date there are over 8000 identified polyphenols, 4000 of those are flavonoids and represent the largest group of

polyphenols and also the most studied (Câmara *et al*., 2013). Flavonoids are of simple phenolic nature since they have a small molecular weight with a structure including two aromatic rings, rings A and B, joined by a 3-carbon hydrocyclic ring C (Fig 2.2) (Ignat *et al*., 2011). The biosynthesis of the flavonoid core can be traced to the deamination reaction of phenylalanine by PAL, which results in the production of rings B and C. Synthesis of ring A occurs from three condensation reactions of three malonyl-CoA units (Tsao, 2010). The classification of flavanoids is dependent on the substitutions on the heterocyclic ring C that binds rings A and B, while differing substitutions in rings A and B results in different molecules within each sub-class. Flavonoids can be further divided into eight sub-classes namely, flavones, isoflavones, flavanones, flavonols, flavanols, flavanonols, and chalcones. Of these, flavones and flavanols are the most structurally diverse and widely occurring.

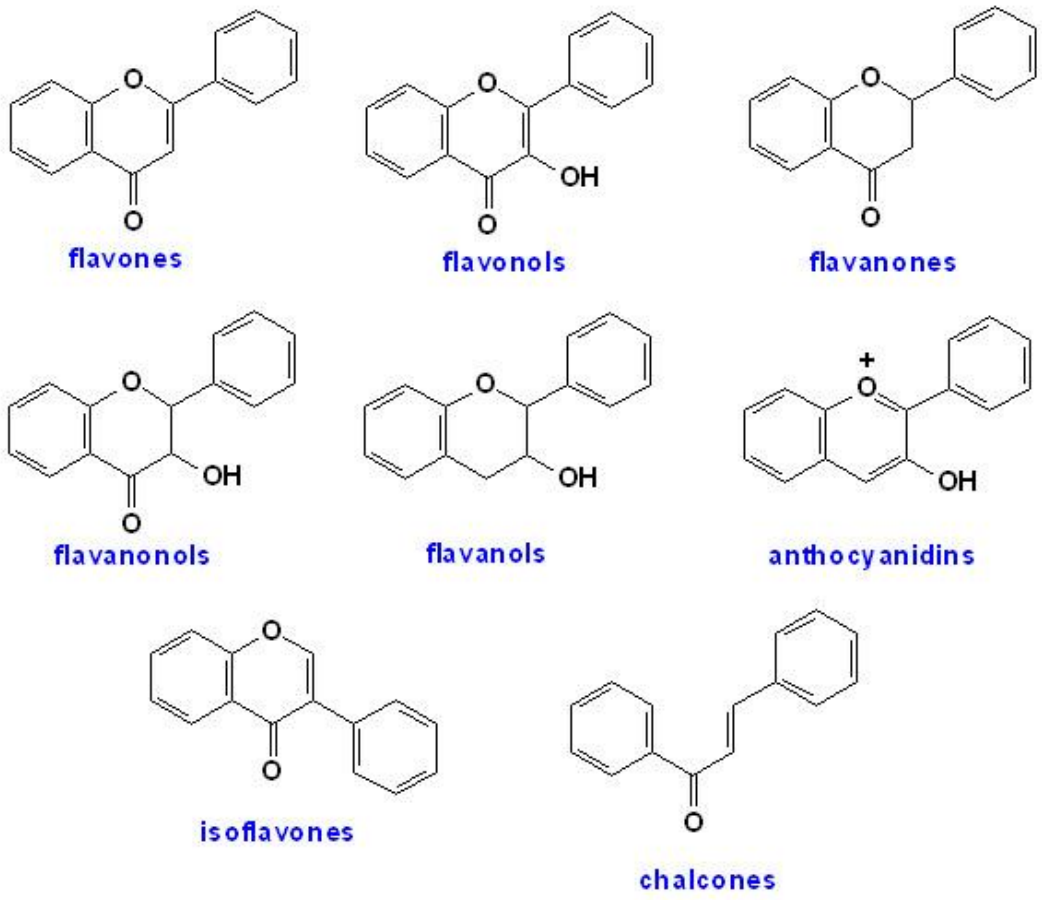

Figure 2.2. The basic structures of flavonoids and sub-classes. (Adapted from Tsao, 2010)

2.2.3. Chemical properties and biological activity

The oxidation-reduction potential (ORP) of the molecule can be used as a measure of ease with which a molecule can transfer or accept electrons to/from another molecule, which is referred to as the electron transfer potential of a molecule. ORP is measured in volts or milli volts (mV);in the standard ORP table the values range from highest to lowest ORP (-0.667 to +0.867V). Compounds with very low ORP equals a high electron transfer potential and molecules with high ORP equals low electron transfer potential and are therefore likely to accept electrons from other molecules. Some polyphenols possess very low ORP (E^o 0.25 – E^o 0.75 volts) and are therefore likely to donate $1e^-$ easily to compounds with higher ORP (Han *et al*., 2012; Nijveldt *et al*., 2001).

2.2.3.1. Antioxidant action

Radical scavenging

Free radicals are produced from enzymatic and non-enzymatic reactions in biological systems and have both beneficial and deleterious effects (Valko *et al*., 2006). Generally, free radicals are identified as atoms or molecules with one or more unpaired electrons in orbitals. The free radicals are extremely unstable, therefore highly reactive species and can be found as anionic, cationic or neutral forms. Furthermore, reactions involving free radicals are usually branched chain reactions, which can be deleterious to biological structures (Bergendi *et al.*, 1999). An example of such a reaction is that of lipid peroxidation which comprises of six reactions (Fig 2.3) (Fraga *et al*., 2010).

$$LH \rightarrow L^{\bullet} \quad (1)$$
$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (2)$$
$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \quad (3)$$
$$L^{\bullet} + L^{\bullet} \rightarrow nonradicalproduct \quad (4)$$
$$LOO^{\bullet} + LOO^{\bullet} \rightarrow nonradicalproduct \quad (5)$$
$$L^{\bullet} + LOO^{\bullet} \rightarrow nonradicalproduct \quad (6)$$

Figure 2.3. Branched chain reaction during lipid peroxidation. (Adapted from Fraga *et al*., 2010)

Therefore scavenging the radical produced in reaction 1, 2 and 3 would either prevent or retard the chain reaction. In biological systems redox reactions involve a pair consisting of a strong reducing agent (molecule with high electron transfer potential) and a weak oxidizing agent (molecule which will readily accept electrons from another molecule) or *vice versa*. In the case of polyphenols and free radicals, the free radical can act as a relatively strong oxidizing agent that can accept an electron or more from the polyphenol/s present. Therefore the ability of polyphenols to donate electrons to other molecules forms the basis for its free radical scavenging characteristic, which subsequently breaks the chain reactions that could possibly be harmful to biological structures (Korkina *et al*., 2009).

Metal chelating action

Specific coordination sites in the core structure of flavonoids allow for complexes to form between flavonoids and metal ions and this process is called metal chelating. These coordination sites are between the 5-hydroxy and 4-carbonyl group, and the 3-hydroxy and 4-carbonyl group in the A-ring and between the 3', 4'-hydroxy group in the B-ring (Symonowicz & Kolanek, 2012). Studies have shown that the newly formed flavonoid-metal complexes have higher radical scavenging ability (Kostyuk *et al*., 2001). However, it is important to note that formation of a complex between a metal and a flavonoid does not automatically render higher antioxidant activity, rather, the resulting electron transfer potential or ORP of the complex will determine its activity (Fraga *et al*., 2010).

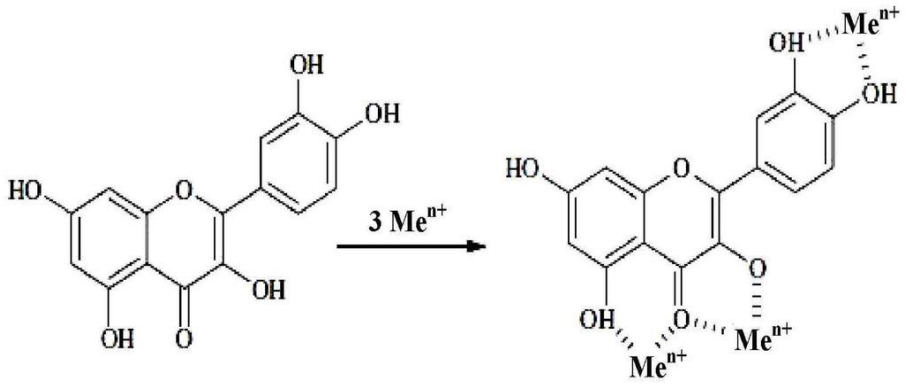

Figure 2.4. Metal (Me^{+}) binding sites of flavonoids (Adapted from Kurek-Górecka *et al*., 2013)

Interacting with antioxidant enzymes and membranes

In addition, flavonoids also bind specifically to enzymes, competing for the active sites with the natural ligand (Fraga *et al* 2010). Some of the antioxidant enzymes affected by flavonoids include oxido-reductases, lipoxygenases, nitric oxide synthase, xanthine oxidase and NADPH-oxidase, which all produce nitrogen and oxygen intermediates. Therefore, inhibition of these enzymes contributes to the antioxidant activity of polyphenols (Korkina *et al* 2009). Polyphenols can also interact with cell membranes and for this function the hydroxyl group is most important for the interaction. However, too many hydroxyl groups will increase the hydrophilicity of the flavonoids and subsequently decrease chances of interacting with membranes. Structural dimensions of flavonoids also increase their chances of inserting into the lipid bilayer resulting in structural changes that modify association with membrane enzymes and ligand-receptor interaction thereby modifying signal transduction. Flavonoids have also been known to prevent the passage of free radicals when embed into cell membrane and also neutralize lipid soluble radicals that can lead to lipid oxidation (Fraga *et al* 2010).

2.3. Rooibos

2.3.1. Origin and development of rooibos industry

Rooibos is produced from the plant *Aspalathus linearis* which belongs to the genus *Aspalathus* consisting of more than 270 species most of which are endemic to the Cape floristic region. *A. linearis* is a shrub like leguminous bush originating from the Cederberg Mountains in the Western Cape of South Africa. In 1772 Carl Thunburga a botanist, visited Africa and reported that the indigenous khoi people were using a rooibos plant to make a beverage (Joubert & Schulz, 2006). It was only at the beginning of the 20th century, 1904 when Benjamin Ginsberg also observed this phenomena and bought the rooibos plant from the khoi and started marketing the tea. In 1930 P. Le Fras Nortier, a medical practitioner and nature lover of Clanwilliam recognized the agricultural value of rooibos through his experiments together with local farmers, O. Berg and H. Rion. These first experiments and the participation of other farmers in the Clanwilliam area laid foundation for the rooibos industry (Joubert & De Beer, 2011).

Initially different species of *Aspalathus,* all naturally occurring in the Cederberg area, were used for making rooibos tea but nowadays only types from *A. linearis* are commonly used for tea production (Joubert & De Beer, 2011). During the Second World War rooibos tea demand increased drastically due to the shortage of oriental tea and this was a major boost for the rooibos industry leading to the establishment of the Clanwilliam tea co-operative company to improve marketing conditions. However, after the Second World War the rooibos market declined and collapsed by 1953-4 leading to the establishment of the rooibos tea control board in 1954 in order to regulate marketing of the herbal tea and price stabilization. The board was abolished on 1stOctober 1993 leading to the establishment of the South African Rooibos Council in 2005 to coordinate activities relating to rooibos tea marketing research and development as well as natural resource management.

The rooibos industry has grown tremendously over the past five decades with just 524 tons of tea sold locally per year in 1955 to over 4000 tons by 1993 and 750 tons exported per year in 1955 to 5633 tons exported per year by 2010. Germany is the major international market which started importing the tea from South Africa in 1961; by 2003 exports to Germany peaked by 73 %. Exports to the United States of America increased by 5 fold from 67 tons to 346 tons of tea per year in a period of 10 years. Exports to the United Kingdom increased by 10 fold over the same period from 75 tons to 772 tons. The rooibos industry comprised 12 % of the total tea market share in 1984 and this value had almost doubled by 2010 to 23 %.

2.3.2. Cultivation

Although cultivation of rooibos was initiated in the early 1930s, it was only after World War Two that commercial cultivation of rooibos took off. Cultivation takes place mainly in the Cedarberg mountain region but can also occur at places as far as Darling and Nieuwoudtville. Planting of seedlings of about 100 mm-150 mm in height occurs in the winter months resulting in 8000-10000 plants per hectare (Joubert & Schulz, 2006). The rooibos plant grows in deep, well drained, sandy soil with well nodulated roots that

enable fixing of element nitrogen from soil water (Nolte, 1968). Branching is stimulated eight months later by topping the plant to about 30 cm .The first harvest can occur 18 months after planting, between the hot summer months and early autumn with full production only reached after three years (Joubert *et al*., 2008). Harvest is initiated by toping the whole bush to an average length of 45 cm as the active growth of the plant should not exceed 50 cm or the result will be a tea with poor quality (Joubert *et al*., 2008). Flowers are avoided during harvest as they also negatively affect the quality of the tea product (Joubert *et al*., 2008; Joubert & Schulz, 2006). After harvest the branches are bound in bundles to be transported to the processing yard or fermentation heaps.

2.3.3. Processing

Manufacturing of rooibos produces two forms, the traditional/fermented form and the green/unfermented form of rooibos. Most common in the market is the traditional rooibos, which has been on the market since the initiation of the rooibos industry. Green rooibos has been newly introduced to the market, with the first production only in 2001, as a result of an increased market for a tea with higher antioxidant value (De Beer *et al*.,2002). Processing of traditional rooibos involves extensive oxidation, resulting in leaves with a red-brown like colour and sweet, honey-like aroma. Oxidation is initiated by shredding the tea shoots to about 3-4 mm. The shreds are placed in fermentation heaps and bruised, followed by addition of water, which facilitates the extraction of polyphenols from the plant which are later absorbed; these are thought to be responsible for the characteristic colour of traditional rooibos (Joubert & De Beer, 2011; Joubert *et al*., 2008). Fermentation is allowed to occur over-night with the average time usually between 12 and 14 hrs; thereafter shreds are spread thinly and allowed to dry in the sun throughout the day. Certain factors may affect fermentation time namely, the presence of young growth, the age of the bush and the cultivation area (Joubert, 1994). There are various stages of fermentation, which can be identified by the aroma of the shreds; the different aromas include hay-like or grassy, sweet apple or caramel like and sour. The sour like aroma is indicative of over fermentation (Joubert & Schulz, 2006).

Oxidation inside the heaps requires adequate aeration and this is accomplished through turning the heap over a few time during fermentation; inadequate aeration results in a tea product with low quality *(Joubert et al*., 2008). Shreds may also be brushed to remove lumps during the drying process and when drying is completed the tea is sieved and steam pasteurized before packaging to remove any microbial species that may be present.

Figure 6.5. Difference in colour between fermented and unfermented rooibos (Adapted from Cape point press, 1 August 2012, Rooibos tea)

Fermentation of rooibos usually results in a tea with less phenolic content as most of the polyphenolic compounds are oxidized (this will be discussed in more detail in subsequent sections) green rooibos undergoes minimal oxidation in order to retain the phenolic content (Erickson, 2003). Minimizing oxidation can be achieved by either inactivating enzymes responsible for the oxidation by means of a steaming process or drying the shoots at lower temperatures and air humidity to critical moisture before shredding.

2.3.4. Phenolic composition

Rooibos is well known as a caffeine free herbal tea and although previously reported to contain low tannin levels, ca 50 % of the hot water soluble solids are tannin like substances (Joubert *et al*., 2008). Unique to rooibos are the dihydrochalcone glucosides

aspalanin (cyclic) and aspalathin (c-c linked) (Koeppen & Roux, 1966; Shimamura *et al*., 2006) as well as the dehydroxy dihydrochacone glucoside, nothofagin, which has only been identified in the heartwood of *Nothofagus fusa* (Hillis & Inoue, 1967). Other rooibos flavonoids comprise of the flavones (orientin, isoorientin, vitexin, isovitexin, luteolin, luteolin-7-O-glycosideand chrysoeriol), flavanones (dihydro-isoorientin and dihydro-orientin, heiphlorin) and flavonols (hyperoside, quercetin, quercetin-3-robinobioside, rutin and iso-quecertrin) (Ferreira *et al*., 1995.; Koeppen *et al*., 1962; Marais *et al*., 2000; Rabe *et al*., 1994; Shimamura *et al*., 2006). Fermentation of rooibos causes substantial quantitative changes to its phenolic composition such as the drastic decrease in aspalathin levels; less than 7 % is left in the fermented rooibos due to oxidation to the flavonone analogues, dihydro-isoorientin and dihydro-orientin (Bramati *et al.*, 2003; Joubert, 1996; Marais *et al*., 2000). During the breakdown of aspalathindiastereomeric mixtures of dihydro-isoorientin and dihydro-orientin are formed as the major and minor products, respectively, with the highest concentrations of orientin and isoorientin occurring after six hours (Krafczyk & Glomb, 2008). The flavones (orientin, isoorientin , vitexin, isovitexin) and flavanones (dihydro-isoorientin and dihydro-orientin, heiphlorin) are also degraded during fermentation but to a lesser extent than the dihydrochalcones (Marais *et al*., 1998). Rooibos also contains additional flavonoids which are not affected by fermentation such as the phenolic acids, caffeic acid, ferulic acid, p-coumaric acid, p-hydroxybenzoic acid, vanillic acid and proto catechuric acid have also been identified in rooibos (Rabe *et al.,* 1994). The decrease in the major flavonoids following fermentation results in lower antioxidant levels. It has been found that the addition of fungal hydrolyzing enzymes in green rooibos prior to fermentation results in a semi-fermented plant material with increased antioxidant levels (Pengilly *et al*., 2008).

2.3.5 Metabolism of flavonoids on the skin

The skin contains physical, biological and chemical barriers in order to protect the body from the external environment and this limits the permeability of certain compounds. Specifically, the stratum corneum forms stacks of lipid bilayes that are rich in fibrous

proteins such as keratins, ceramides, cholesterol, and free fatty acids which prevent penetration of hydrophilic compounds (Elias, 2005). The structure of polyphenols ranges from simple to very complex compounds and need assistance to penetrate the physical barrier of the skin and for their transdermal delivery (Korkina *et al*., 2008). Skin penetration of polyphenols can be induced by coupling them to liposomes or lipogels (Casagrande *et al*., 2007; Cevc, 2004; Sinico *et al*., 2008), electrically through iontophoresis or skin permeation enhancers (Fang *et al*., 2006; Touitou, 2002). The polyphenols can then be distributed equally across all skin layers or concentrated in one depending on the specific polyphenol (Marti-Mestres *et al*., 2007). The distribution of polyphenols on skin layers has been validated by the topical application of EGCG as a transdermal gel on SKH-1 mice which resulted in its fast penetration through all skin layers but later the compound was mostly concentrated in the epidermal layer at three times more than was found in the dermal layer (Yang *et al*., 2007).

Oral administration has been preferred over topical application due to the easier absorption of polyphenols through the intestine compared to the cutaneous skin and their low stability due to possible auto oxidation by atmospheric oxygen (Akagawa *et al*., 2003; Kostyuk & Potapovich, 1989; Laughton *et al*., 1989; Sang *et al*., 2007). However, topical application has been reported to be most beneficial for protection against the effects UV radiation as oral administration raises questions about the number of transporters and metabolic obstacles involved in their delivery from the gastro-intestinal tract to cutaneous skin (Myriam *et al*., 2006). Beside the role of the stratum corneum as the most critical structure for epidermal barrier function there is increasing evidence indicating that enzymatic and non-enzymatic compounds on cutaneous skin are able to react with low molecular weight compounds and facilitate their metabolism on the skin (Elias, 2005). Foreign compounds such as topically applied dermatological drugs, skin care products as well as compounds arising from contact with plants and/or toxins may be activated or inactivated by xenobiotic metabolizing enzymes of the skin resulting in health or adverse effects (Korkina *et al*., 2008). The skin contains a variety of phase I and phase II metabolizing enzymes that are distributed differently across the layers of the skin; most abundant in the keratinocyte (Elias, 2005). Some of these enzymes may interact with polyphenols thus facilitating their metabolism after cutaneous absorption.

Phase I enzymes are involved in the oxidation and reduction of small molecular weight foreign compounds on the skin after penetration and cytochrome P450 enzymes (CYP) comprise the first class of these enzymes. The CYP1A1, CYP2B6/7, CYP2E1 and CYP3A5 are few enzymes shown to be present in mammalian skin (Swanson, 2004). Interestingly, the CYP1 family enzymes have been reported to interact with various carcinogens including UV-B as they were induced in keratinocytes and HaCaT cell cultures exposed to UV-B (Katiyar *et al*., 2000; Villard *et al*., 2002; Wei, Rannug, & Rannug, 1999). Therefore, CYP1 enzymes (CYP1A1 and 1B1) up-regulated or activated in response to UV-B could enhance the bio-activation of environmental carcinogens leading to adverse photo-reactions on the skin that could result in carcinogenesis (Katiyar *et al*., 2000). Flavonoid compounds that can inhibit CYP1 enzymes have been reported to exhibit protective effects on the skin. Synthetic and plant phenols have been reported to inhibit CYP1 enzymes which reduced the content of carcinogenic compounds in the skin by inhibiting aryl hydrocarbon hydroxylase (Das, Khan, Asokan, Bickers, & Mukhtar, 1987). A second class of phase I enzymes has been identified on the skin that can metabolize polyphenols namely the peroxidises (Strohm & Kulkarni, 1986). This enzyme class oxidizes polyphenols to quinine or phenoxyl radicals that can interfere with the structure and/or function of a number of biological molecules and modify their function. The third class of phase I enzymes important for the metabolism of polyphenols on the skin is the lipooxygenases. These are non-heme iron containing enzymes found in both plants and animals. Three isoforms have been characterized in animal cells and include 5-LOX in leukocytes, 12-LOX in monocytes and platelets and 15-LOX reticulocytes during their maturation to erythrocytes. In the skin the 15-LOX is expressed as two isoforms 15-LOX1 and 15-LOX2 and play a critical role in barrier permeability properties. The plant derived phenolic acid (urushiol) has been reported to be polymerized under oxidative conditions by LOXs in soybean (Brash *et al*., 2007).

Phase two enzymes are detoxification enzymes involved in the protection against endogenous toxins and xenobiotics. Two classes of phase II enzymes have been identified in cutaneous skin which interacts with polyphenols (Wilkinson & Clapper,

1997). These are the glutathione-S-transferase (GST) and UDP-glucuronyl transferase (UGT). (See section 2.3.6 for more details).

2.3.6 Bioactivity and anti-carcinogenic properties flavonoids

Flavonoids have been identified to exhibit protective effects in cancerous cells by inducing apoptosis and inhibiting proliferation through their interaction with phase I (CYP450) and phase II enzymes (GST,UGT) (Walle *et al*., 2001). The interaction of flavonoids and CYPs can be either through stimulating the biosynthesis of some CYPs, modulating the activities of certain CYP enzymes and the flavoniods can also be metabolized by several CYPs. Specifically, the CYP1 enzymes are involved in activation of carcinogens such as UV-B, benzo[a]pyrene, aflatoxin B_1 and 7,12-dimethybenze[a]anthracene (DMBA) and flavonoids have been shown to reduce the activation of these CYPs (Omiecinski *et al*, 1999). Quercetin which is found in small amounts in rooibos has been reported to exhibit antagonist activity to the aryl hydrocarbon receptor (Ahr), a ligand activated transcription factor involved in activation of the CYP1 family enzymes. This subsequently prevents cancer by inhibiting activities of several drug metabolising enzymes (Kang *et al*, 1999).

Polyphenols are also known to interact with phase II enzymes specifically UGT and GST which protect the cells against endogenous and exogenous carcinomas through glucuronidation and nucleophillic addition of glutathione to a variety of carcinogens (Fisher *et al*., 2001). The majority of green tea polyphenols have been found to have alleviating effects on phase II enzymes by inducing mutagen activated protein kinases through antioxidant response elements (Yu *et al*., 1997). The most active green tea polyphenol EGCG was found to increase apoptosis and cell cycle arrest in human epidermoid carcinoma cells A431 (Ahmad *et al*., 1997). Furthermore, EGCG has also been found to suppress proliferation by binding the epidermal growth factor (Liang *et al*., 1997). More evidence for green tea anti-proliferative capacity was found with EGCG exhibiting the highest activity in inhibiting MCF-7 breast cancer cells, HT-29 colon cancer cells and UACC-375 melanoma cells (Valcic *et al*., 1996).

Rooibos tea was initially popular for its ability to alleviate allergies, insomnia and anti-depressant properties. Scientist from Japan and South African were the first to investigate the biological properties of rooibos scientifically leading to the discovery of a long list of protective effects (Inanami *et al*., 1995; Ito *et al*., 1991; Komatsu, Kator *et al*., 1994; Sasaki *et al*, 1993; Yoshikawa *et al*., 1990). The chemical composition of rooibos is rich in flavonoids which are known to exhibit powerful antioxidant activities involving free radical scavenging, metal chelating and singlet oxygen quenching as well as enzyme activity inhibition mechanism. Studies have reported on the antioxidant activity of rooibos extracts, one such study discovered that aqueous extracts of rooibos scavenge the $O2^{\bullet-}$, $^{\bullet}OH$ radicals when using electron spin resonance spectrometry (Yoshikawa *et al*., 1990). In another study rooibos hot water extract from fermented and unfermented plant material using the ABTS and FRAP assays found that the was a clear decrease in antioxidant activity of rooibos as a result of fermentation as a result of the decreased TP levels (Joubert *et al*., 2008). Furthermore, a strong correlation between the aspalathin content of green rooibos and antioxidant activity from the ABTS assays has been reported (Schulz *et al*, 2003). In addition, the aspalathin content in green rooibos also correlated with the pro-oxidant activity of rooibos but that correlation was not observed with the TP content (Galati & O'Brien, 2004). Rooibos was found to have protective properties as is decreased the damage to the central nervous system by decreasing lower levels of TBARS and suppressed accumulation of lipid peroxides in rats following chronic administration of traditional rooibos. The herbal tea was found to enhance the activity of phase II enzymes and thereby protecting against adverse effects of oxidative damage**.**

2.3.7 Anti-inflammatory activity of flavonoids and their chemoprevention properties

The link between chronic inflammation and cancer provides a target for the development of anti-inflammatory drugs (Karin *et al*., 2004). However, synthetic drugs already on the market exert other adverse side effects thus the development of safe anti-inflammatory compounds has been the focus of research into the field of drug

discovery (Tweedie *et al*., 2007). As already established, polyphenols have various benefits including anti-inflammation. The anti-inflammatory activity of polyphenols is thought to involve interaction with pro-inflammatory enzymes such as cyclo-oxynases (COX), lipo-oxygenase (LOX) and inducible nitric oxide synthase (iNOS) as well as enhancement of the activity of peroxisome proliferators (Williams *et al*., 2004). Polyphenols may also inhibit the activity of phospholipase kinases, tyrosine kinases and the nuclear factor kappa beta (Yoon & Baek, 2005) as well as enhance the activity of phase II enzymes i.e. GST and UGT (Kim *et al*., 2004). Polyphenols have been found to inhibit the activity of phospholipase A_2 (PA_2), COX and LOX thereby reducing the release of AA, PGs and LTs which have anti-inflammatory implications (Aviram & Fuhrman, 1998; Baumann, Bruchhausen, & Wurm, 1980; Laughton, Evans, Moroney, Hoult, & Halliwell, 1991; Welton et al., 1986; Yoon & Baek, 2005). The arachidonic acid (AA) pathway involves the release of AA from membrane phosphoslipids through the activity of PA_2. AA can be metabolized by COX to prostaglandins (PGs) and thromboxa A2 or by lipooxygenase into hydropeoxyeicosatetransnoic acids (HpETEs), hydroxyl eicosatetransnoic acid (HETEs) and leukotrints (LTs).

Some of the green tea polyphenols, pro-delphidin B-4 3'-O-gallate, and pro-delphidin B2 3,3' di-O-gallate suppressed mRNA and protein expression of COX-2 and ultimately blocking PGE_2 release in LPS stimulate Raw 264.7 macrophages (Hou *et al*., 2007;). Other green tea flavonoids including epigallocatechin (EGC), gallocatechin, epicatechingallate (GCEG), catechingallate (CG), epigallocatechingallate (EGCG) also exhibit inhibitory effects on the activities of COX-1/2 in different human and mouse cell lines (Hong *et al*, 2001; Kundu *et al*., 2003; Seeram *et al*, 2003; Gerhäuser *et al*., 2003). Another enzyme reported to be inhibited by polyphenols is iNOS which is involved in producing nitric oxide from L-arganine. The NOS enzyme family consist of three isoforms the endothelial (eNOS), neouronal (nNOS) and the inducible (iNOS). The latter is only expressed in response to inflammatory stimuli thus further escalating the inflammatory condition through the release of NO. iNOS expressed in LPS or cytokine activated RAW 264.7 macrophages was found to be inhibited by quercetin (Chen *et al*., 2001). The nuclear factor kappa-B (NFκB) is a transcription factor (TF) involved in

immune, inflammatory, apoptotic, proliferative responses and more. This TF is stimulated by various sources and binds on promoter regions of many genes that code for cytokines (TNF-α, IL-6, IL-1β), inducible enzymes (COX-2, iNOS). These molecules are involved in proliferation, angiogenesis, adhesion and tumour invasion and are therefore key role players in the process of carcinogenesis (Santangelo *et al*., 2007).

The activation process for NFκB is a multistep process requiring the activation of other factors such as induction of specific IκB kinases which phosphorylate IκB. Phosphorylated IκB leads to rapid ubiquination and subsequent degradation of IκB in the proteasome; degradation of IκB releases NFκB in the cytosol, resulting in rapid translocation to the nucleus where it binds to specific NFκB recognition elements in the promoter region of target genes (Epstein *et al*., 1997). The cascade of events involved in the activation of NFκB serves as a target for possible inhibition in studies aimed at controlling inflammatory diseases including cancer (Karin *et al*., 2004). Polyphenols have been found to inhibit NFκB at multiple steps in its activation pathway. The main flavonoid in green tea was reported to reduce the degradation of IκB which blocks the release of NFκB in the cytosol and the expression of the iNOS promoter in LPS activated macrophages (Lin & Lin, 1997).

2.3.8 Controversy of polyphenols

Various studies have suggested the ability of polyphenols to protect against deleterious diseases including cancer (Curtis *et al*., 2004; Kuriyama *et al*., 2006; Lambert *et al*., 2005; Surh, 2003). The beneficial effects of polyphenols in most part have been attributed to their antioxidant properties (Lambert & Yang, 2003; Sang *et al*, 2005). These antioxidant effects can either be direct where the polyphenols act as radical scavengers or indirect where they can induce the activities of various antioxidants inside the cell (Dinkova-Kostova & Talalay, 2008). Nonetheless, polyphenols are known to have a very low plasma bioavailability, less than 1 μM in subjects consuming high doses of supplements, therefore it is not clear how the *in vitro* antioxidant effects are relevant *in vivo* (Manach & Donovan, 2004). Studies *in vitro* have shown the biological effects of

the major green tea polyphenol, EGCG, used concentrations far above those found in plasma (10-100 fold) thus questioning the relevance of these results (Yang *et al*., 2008; Ju, Lu *et al*., 2007; Yang *et al*., 2006). In the direct antioxidant reactions polyphenols are consumed in the reaction and therefore continual replenishment is needed implying that these reactions are almost impossible considering the level of polyphenols in plasma (Yang *et al*., 2007). In addition, many other antioxidants (in the cell) are present at much higher levels than the polyphenols. For example vitamin C averages at 50 μM, vitamin E ranges from 20-30 μM (Lee *et al*., 1997; Ness *et al*., 1999), albumin and urate are normally above several hundred μM (Dieber-Rotheneder *et al*., 1991; Wen *et al*., 1996). These are therefore more likely to interact with antioxidants than the polyphenols with very low bioavailability in serum.

The ability of polyphenols specifically green tea polyphenols to act as anti-oxidants have long been reported to be the underlying causes of their health benefits (Lambert & Elias, 2010). However, the recent reports on the pro-oxidant effects of the tea polyphenols raise some concerns (Joubert *et al.*, 2005). High levels of polyphenols are required for these pro-oxidant reactions to occur ,however, there is little evidence showing that these effects actually happen *in vivo* due to lack of careful dose response studies (Hou *et al*., 2006; Lambert *et al*., 2010). It is not known at what point the effects of polyphenols shift from being beneficial to being detrimental to the cell. The green tea polyphenol, EGCG, has been shown to have pro-oxidant effects which, at high doses have been reported to be deleterious (Galati *et al*, 2006; Isbrucker *et al*, 2006). For instance, 10-20 mg/kg per day of tea based dietary supplements resulted in herpatoxity (Bonkovsky, 2006). Furthermore, oral administration of the green tea polyphenol, teavigo, containing 90 % EGCG, to beagle dogs resulted in a dose depended toxicity accompanied by vomiting, diarrhea, liver necrosis and death (Isbrucker *et al*., 2006).

Polyphenols have also been reported to have diverse effects on signalling pathways at different concentrations. NFκB is a TF known to regulate the expression of genes involved in cellular differentiation, proliferation, apoptosis, oxidative response, inflammation and immune response. Chronic activation of NFκB has been implicated in many diseases including cancer (Karin, 2006; Karin & Greten, 2005). This TF has been

reported to be induced by oxidative stress and therefore inhibited by various antioxidants including polyphenols (Wadsworth, 1999). Therefore, the relevance of the antioxidant effects of polyphenols is limited as they have various other effects on NFκB. No correlation was found between the antioxidant effects of 34 dietary plants and their ability to inhibit LPS induced NFκB activation (Paur *et al*, 2008).

Summary

Polyphenols have been suggested to poses various beneficial effects including the ability to act as anticancer agents. This ability has been attributed in most part to its antioxidant function. However, the challenges of metabolism, bio-availability and contrasting effects of various signalling molecules have rendered great controversy in the beneficial effects of polyphenols. Further, evidence has also emerged of the toxicity of polyphenols at high concentrations which are required to fulfil their role as radical scavengers. It is therefore more likely for polyphenols to acts as antioxidants through activating antioxidant enzymes in the cells. Therefore, clear effects of different polyphenols need to be investigated with the consideration of their plasma levels and their individual biological activities.

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3 Antioxidant activity of rooibos extracts and flavonoid and non-flavonoid fractions of unfermented rooibos.

Abstract

Rooibos, a South African herbal tea, has been reported to contain a number of polyphenols, specifically flavonoids, known to exhibit antioxidant effects that are thought to form the basis of their health beneficial effects *in vivo*. The aim of the current study was to compare the chemical composition evaluated by HPLC analyses, and antioxidant activities utilising the 2, 2'-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) and the ferric reducing antioxidant power (FRAP) assays of methanol and aqueous extracts of unfermented and fermented rooibos. The methanol extract of unfermented (MUF) rooibos, containing the highest levels of total polyphenols and flavanols, exhibited the highest antioxidant activity. In contrast the aqueous extracts of both unfermented (AUF) and fermented (AF) rooibos exhibited a far lower anti-oxidant potency, presumably associated with the extraction of the more polymeric tanning-like flavanol/proanthocyanidin type of compounds. Column fractionation of the MUF extract yielded five fractions of decreasing polarity with the most polar fractions, X-1 and X-2, containing the lowest levels of polyphenols and antioxidant activity. Most of the rooibos polyphenols were enriched in fractions X-3 and X-4 exhibiting the highest antioxidant activities. Methanol extraction was more effective in extracting the major rooibos polyphenols, aspalathin and nothofagin, and fractionation of the unfermented extract confirmed that these flavonoids are associated with high antioxidant properties. The selective extraction and recovering of specific bioactive polyphenols can be effected by utilising different extraction solvent systems likely to have important implications when focussing on specific biological properties of interest.

3.1 Introduction

Polyphenols are secondary plant metabolites with diverse biological functions namely antioxidant, antimicrobial, antiviral and anti-inflammatory activity (Ignat *et.al*, 2011). These compounds are widely distributed and occur naturally in plant based foods such as fruits, vegetables and beverages, including teas, fruit juices and wine (Manach *et al*., 2004). Polyphenols are divided into different classes i.e. flavonoids, non-flavonoids and neo-flavonoids according to their chemical structure (Vladimir-Knežević *et al*., 2012) with flavonoids being the largest class and most widely studied (Ignat *et a*l., 2011). The potential beneficial effects of flavonoids *in vivo* have been attributed to their strong *in vitro* antioxidant properties (Kovacic & Jacintho, 2001). As various reports link oxidative stress to chronic diseases, including cancer, the protective effects of tea (*Camellia sinensis*) against these diseases have been extensively reported (Arts & Hollman, 2005; Manach *et al*., 2005; Vauzour *et al*., 2010). In this regard, the South African herbal teas gained vast popularity due to their health benefits, which are attributed to their high flavonoid content (Joubert *et al*., 2008; Joubert *et al*., 2009). *In vitro* and *in vivo* antioxidant activity of rooibos suggests potential protective effects in disease and warrants further investigations into the role of these antioxidant properties for disease prevention or treatment.

Rooibos contains a number of flavonoids including the two unique antioxidants the dihydrochalcone-*C*-glucosides, aspalathin and nothofagin, (Snijman *et al*., 2009). Other phenolic compounds in rooibos include the flavones, orientin, isoorientin, vitexin and isovitexin as well as the flavonols, rutin and isoquercitrin, to mention a few (Joubert & De Beer., 2012). The herbal tea has been extensively used as a value added product in foods, beverages and cosmetics mainly due to its antioxidant properties. Several studies have linked the antioxidant activities of rooibos with its polyphenol content e.g the aspalathin content in rooibos was found to correlate with antioxidant properties (Schulz *et al*., 2003). During fermentation, enzymatic oxidation of the phenolic compounds occurs leading to degradation of the major flavonoids in rooibos, such as aspalathin and nothofagin, which coincided with a reduction in antioxidant properties (Joubert *et al*., 2004). Aspalathin and nothofagin constitutes up to 11 % and 2 % of the

soluble solids in unfermented rooibos, respectively, while fermentation decreases the by 10 to 20 fold (Joubert, 1996).

This aim of the current study was to investigate (i) the antioxidant properties of extracts of fermented and unfermented rooibos using two different extraction solvents i.e. methanol and water and (ii) to further fractionate the methanol extract of unfermented rooibos by column chromatography utilising a XAD-4 reverse phase matrix. Detailed chemical analyses and assessment of the antioxidant properties were conducted using the different extracts and column fractions.

3.2 Materials and methods

3.2.1 Chemicals

Amberlite XAD-4 was obtained from Sigma-Aldrich, St Louis, USA. Thin later chromatography plates (TLC silica gel 60 F_{254}) were obtained from Merck KGaA, Darmstadt, Germany. Methanol CHROMASOLV (HPLC grade), gallic acid, (+)-catechin hydrate, p-anisaldehyde and hexane were purchased from Sigma-Aldrich (St Louis, USA). Anhydrous sodium carbonate, folin-Ciocalteu reagent, anhydrous sodium acetate, glacial acetic acid, and hydrochloric acid were obtained from Merck Chemicals (KGaA, Darmstadt, Germany). 2,4,6-Tri (2-pyridyl)-s-trazine (TPTZ), (±) 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), 4-dimethylamino cinnamaldehyde (DAC), potassium peroxodisulfate, 2,2'-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich (St Louis, USA).

3.2.2 Preparation of extracts

Fermented and unfermented rooibos plant material (*Aspalathus linearis*) were purchased from Rooibos Ltd, Clanwilliam, South Africa.

3.2.2.1 Methanol extraction

Fermented and unfermented finely ground dried plant material (50 g) was stirred in chloroform (300 ml) for 24 hrs to remove lipophilic constituents such as chlorophyll. The plant material was vacuum filtered through a Whatman No 4 filter paper using a Buchner funnel. This step was repeated twice (3 times in total) and the chloroform filtrate discarded. The residual plant material was extracted by blending with a polytron (Kinematica, Lasec, SA) in methanol (300 ml) for five minutes between 10 000 -13 000 rpm. The mixture was vacuum filtered through a Whatman No 4 filter paper using a Buchner funnel. This step was repeated twice (3 times in total). All filtrates were collected, combined and filtered through a Whatman No 1filter paper. The methanol was evaporated using a rotary evaporator (Rotavapor® R II, BUCHI, Flawil, Switzerland) at 40 °C *in vacuo*. The dried extracts were weighed to calculate the yield, transferred to 50 ml amber bottles and stored in a desiccator at room temperature. The methanol extract was prepared in duplicate (extraction 1 and extraction 2).

3.2.2.2 Aqueous extraction

The fermented and unfermented plant material, (50 g) was steeped in boiled de-ionised water (500 ml) for 30 min. Extracts were filtered through a single layer of cheese cloth and subsequently vacuum filtered through a Whatman No 4 and a Whatman No 1 filter paper utilising a Buchner funnel. The final filtrate was transferred into a one litre round bottomed flask and frozen in a methanol bath for 30 min. The extract was freeze dried in the dark using an Alpha I-6 freeze dryer (Christ Gefriertrocknungsanlagen, Ostetrode, Germany). The extraction yield was determined and the samples transferred into 50 ml amber bottles and stored in a desiccator at room temperature. Two independent extractions were conducted (extraction 1 and extraction 2).

3.2.3 Column fractionation of the unfermented Methanol extract

3.2.3.1 Extract preparation

Unfermented rooibos plant material, 500 g, was extracted with methanol (MeOH) as described in section 3.2.2.1 by using 3 l of $CHCl_3$ and MeOH, respectively. The

chloroform ($CHCl_3$) extraction was conducted by stirring the mixture using a magnetic stirrer in a glass beaker (5 l), while the methanol extraction step was conducted by blending the plant material in a polytron. The filtrate from the methanol extraction was dried and stored in a desiccator at 4 °C until use.

3.2.3.2 XAD-4 column chromatography

Amberlite XAD-4 matrix was rinsed extensively with distilled water until free of chloride ions using 1 % $AgNO_3$/1N HNO_3 solution to test wash water for the presence of chloride ions. The XAD-4 matrix was regenerated by successively stirring the matrix in 4 l of hexane, acetonitrile and methanol overnight, respectively. The matrix was filtered and allowed to dry in between each solvent exchange. The XAD-4 matrix was re-suspended in 15 % methanol and packed in a glass column (75x1180 mm). The methanol unfermented (MUF) extract (20 g) was dissolved in 50 ml of 15 % MeOH: H_2O (v/v) and quantitatively applied to the column. Elution was with a stepwise gradient of decreasing polarity as shown below (Table 3.1) at a flow rate of approximately 20 ml/min. A pre-fraction of 500 ml was collected followed by 250 ml fractions during the stepwise increase of the MeOH with MeOH:$CHCl_3$ (1:1 v/v; 1.5) as the final eluting solvent. The column fractionation of the MeOH extract was conducted in duplicate, with duplicates assigned as fractionations F1 and F2.

3.2.3.3 Thin layer chromatography

Thin layer chromatography (TLC) was used to analyse the column fractions in order to determine the presence of aspalathin and nothofagin, the major flavonoids of rooibos. The samples were spotted (20 μl) and the TLC plate, which was developed using a running solvent containing chloroform:methanol:aceticacid:distilled water (55:36:1:8 v/v/v/v). The plates were viewed under ultraviolet light (312 nm) followed by spraying with methanol:sulphuricacid:aceticacid:p-anisaldehyde 85:5:10:0.5 (v/v/v/v) solution and plates heated at 120 °C for 15 min for colour development. Fractions containing compounds with similar R_f values were combined, yielding five main fractions consisting of two pre-aspalathin fractions (X-1 and X-2), the major aspalathin fraction (X-3), aspalathin/nothofagin fraction (X-4) and a post aspalathin and nothofagin fraction (X-5). Fractions were dried *in vacuo* using a rotary evaporator (40 °C), weighed (Table 3.5)

and stored in sealed round bottom flasks at 4 °C. Dried fractions (30 mg) were dissolved in methanol and spotted on TLC plates for the final comparison.

Table 3.1 Stepwise increase of the Methanol (%) utilised for the XAD-4 fractionation of unfermented rooibos Methanol extract

Solvent composition	Volume (ml)
15 % MeOH*	2000
25 % MeOH	2000
50 % MeOH	5000
75 % MeOH	4500
100 % MeOH	2000
MeOH:CH_3Cl_3 (1:1 v/v)	1500

Abbreviation: MeOH-methanol, CHCl3–chloroform.

3.2.4 Chemical characterisation of extracts/fractions

3.2.4.1 HPLC analyses

HPLC analysis was kindly conducted by the Agricultural Research Council, Stellenbosch, South Africa, using the method as described by Beelders *et al*. (2012). Quantification of aspalathin, nothofagin, orientin, isoorientin, vitexin, isovitexin, hyperoside, isoquercitrin, rutin, quercetin-3-*O*-robinobioside, phenylpyruvic acid-2-*O*-glucoside (PPAG), and luteolin-7-*O*-glucoside in rooibos extracts were conducted using authentic standards. Quantification of aspalathin, nothofagin and PPAG was monitored at 288 nm while the other flavonoids were monitored at 350 nm. Quercetin-3-*O*-robinobioside was quantified as rutin equivalents due to unavailability of an authentic standard.

3.2.4.2 Total polyphenol determination

Total polyphenols (TP) of extracts and column fractions were quantified based on the Folin-Ciocalteu reagent method (Singleton & Rossi, 1965). The extracts were diluted to

0.15 and 0.25 mg/ml, while the fractions were diluted to 0.15, 0.2 and 0.3 mg/ml. The Folin-Ciocalteu reagent (100 µl, diluted 5 times) was prepared in distilled water and Na_2CO_3 (7.5 % w/v) to a final volume of 1l. Gallic acid stock solution (1 mg/ml) was prepared in distilled water and diluted to a concentration range of 10, 20, 40, 60, 80 and 100 µg/ml to generate a standard curve. Folin-Ciocalteu reagent (100 µl), Na_2CO_3 (80 µl), and the standards and/or samples (20 µl) were added to the microtiter plate in duplicate and incubated for 2 hrs at 30 °C. Absorbance was determined spectrophotometrically at 765 nm using a Biotek Synergy HT microplate reader (Winooski, Vermont, USA) and TP expressed as mg gallic acid equivalents (GAE)/g of the extract and/or column fractions.

3.2.4.3 Flavanol/proanthocyanidin (FLAVA) determination

The FLAVA content of extracts and XAD-4 column fractions was determined using the 4-Dimethylaminocinnamaldehyde (DAC) assay based on the method by Mc Murrough and McDowell (1978). The extracts were diluted with distilled water to concentrations of 0.4 and 0.5 mg/ml and the column fractions to 0.3, 0.4 and 0.5 mg/ml. 4-Dimethylaminocinnamaldehyde (DAC) reagent was prepared fresh daily in HCl (40 m M):MeOH (1:3 v/v) at a concentration of 0.5 mg/ml. The standard, (+) catechin, stock solution (0.5 mg/ml) was prepared in methanol and diluted to 2, 5, 10, 15, 20, and 25 µg/ml with distilled water to generate a standard curve. Standards and/or samples (35 µl) were pipetted into a 96 well plate in duplicate and incubated with DAC reagent (175 µl) for 2 min at 37 °C. The absorbance of the samples was determined at 640 nm with the Biotek Synergy HT microplate reader (Winooski, Vermont, USA). The flavanol concentration was expressed as mg (+) catechin equivalents (CE)/g extract and/or column fraction.

3.2.5 Antioxidant assays

All antioxidant assays were performed on a Biotek Synergy HT microplate reader (Winooski, Vermont, USA). Stock solutions of 1 mg/ml extracts and column fractions were prepared in distilled water and stored at 4 °C for no longer than 4 days.

3.2.5.1 Ferric reducing antioxidant power (FRAP) assay

The FRAP activity of the extracts and column fractions was determined according to the method of Benzie & Stein (1996). Extracts were diluted to 0.15 and 0.25 mg/ml and the column fractions to 0.3, 0.4 and 0.5 mg/ml. Acetate buffer (300 mM, pH3.6) was prepared by adding 3.1g of sodium acetate and 16ml of glacial acetic acid into 1l distilled water and the buffer stored at 4 °C for no longer than 3 months. The FRAP reagent was prepared by adding 10 ml of 10 mM TPTZ in 40 mM HCl, 10 ml of 20 mM $FeCl_3$ in distilled water and 100 ml of the acetate buffer just before use and kept in the dark. A standard curve was generated by using a 5 mM stock of (±)-6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox) dissolved in ethanol and diluted to different concentrations (50, 100, 200, 300, 400 and 500 µM). The FRAP reagent (180 µl) was added to 20 µl of trolox standard and rooibos samples, incubated at 37 °C for four min and the absorbance determined at 592 nm. FRAP was expressed as mmol trolox equivalents (TE)/g of extract and/or column fraction. The FRAP determination was repeated once.

3.2.5.2 2, 2'-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) assay

The ABTS radical scavenging activity of the extracts and column fractions was determined according to the method of Re *et al* (1999). The extracts were diluted to concentrations ranging between 0.15 and 0.25 mg/ml and the column fractions to 0.05, 0.1 and 0.15 mg/ml. The ABTS reagent, dissolved in deionised water to yield a 7 mM solution, was prepared 12-16 hrs before use and diluted to yield an absorbance between 0.68 and 0.72 before use. Trolox stock solution (1 mM) was prepared to generate a standard curve with a concentration series of 50, 100, 150, 200, 250 and 300 µM. The ABTS reagent (180 µl) was added to standards and rooibos samples (20 µl) and incubated at 30 °C for 4 min and the absorbance determined at 734 nm. The percentage inhibition for the standards was calculated by using the blank as 100 % and radical scavenging expressed as mmol TE/ g of extract and/or column fraction.

3.2.6 Statistical analysis

Comparisons between extraction solvents and fermentation type of rooibos extracts were analysed by the 2X2 factorial model using the two-way ANOVA and deemed significant when $p<0.05$. For the column fractions, t-test analysis was used to compare variations between the first and the second fractionation. Statistical differences between the column fractions and the methanol unfermented extract were investigated using a one-way ANOVA with the GLM procedure also at $p<0.05$.

3.3 Results

3.3.1 Solvent extraction and yields

Methanol extraction of the unfermented (MUF) rooibos plant material resulted in a two-fold higher soluble solid recovery compared to the fermented (MF) plant material (Table 3.2). However, recovery from the aqueous extraction of both the unfermented (AUF) fermented (AF) rooibos plant material was comparable and similar to the MUF extract.

Table 3.2 Soluble solid recoveries during extraction of fermented and unfermented rooibos with water and methanol.

	Soluble solids(g)/50 g plant material*		
	1st extraction	**2nd extraction**	**Average**
MeOH/unfermented (MUF)	6.8	6.2	6.5±0.42
MeOH/fermented (MF)	3.4	2.8	3.1±0.42
Aqueous/unfermented (AUF)	7.4	6.7	7.0±0.49
Aqueous/Fermented (AF)	6.7	6.7	6.7±0.00

*The extraction was repeated twice and values shown represent the extract yield from 50 g starting plant material. Abbreviations: MeOH–methanol

3.3.2 Chemical analysis: rooibos extracts

HPLC analysis: Aspalathin and nothofagin were more abundant in the unfermented rooibos extracts, while isoorientin and orientin were the most abundant polyphenols in the fermented extracts (Table 3.3). The MUF and MF rooibos extracts contained comparable amounts of luteolin, rutin and isoquercitrin. Higher amounts of hyperoside and quecertin-3-O-robinobioside were recovered in the MF extracts. Fermentation resulted in a 10-fold decrease in the aspalathin and nothofagin levels in the methanol extracts. This resulted in a concomitant 2-fold increase in the levels of the oxidised products of aspalathin, orientin and isoorientin, and of nothofagin, vitexin and isovitexin. In the aqueous extracts, fermentation resulted in a 20 to 15 fold lower levels of aspalathin and nothofagin while the levels of the oxidised products were similar. Methanol extraction was more efficient in recovering monomeric polyphenol compounds compared to aqueous extraction as it contained higher amounts of all the compounds detected as lower levels of the rooibos flavonoids were detected in the aqueous extracts

Total polyphenol (TP) content: Both MUF ($p<0.0001$) and AUF ($p=0.0017$) extracts contained significantly higher TP levels when compared to their fermented counterparts (Table 3.4). Methanol extraction of unfermented plant material was significantly more efficient in extracting TP when compared to Aq ($p<0.0001$), while no difference in TP content was noticed between the Aq and MeOH extracts of the fermented plant material.

FLAVA content: Both MUF AUF rooibos extracts contained significantly ($p<0.0001$) higher levels of FLAVA compared to the corresponding fermented rooibos extracts (Table 3.4). Methanol extraction of unfermented plant material was significantly more efficient in extracting FLAVA constituents when compared to the aqueous extraction ($p<0.0001$), while no significant difference was noticed between the AF and MF extracts.

Table 3.3 Concentration of monomeric compounds in fermented and unfermented rooibos extracts prepared with methanol and water.

Flavonoid classes	Flavonoid (g/100g extract)	MUF	MF	AUF	AF
Dihydrochalcones	Aspalathin	11.21±0.69	0.98±0.02	9.04±0.11	0.46±0.01
	Nothofagin	1.99±0.08	0.19±0.00	1.4±0.03	0.10±0.00
Flavones	Isoorientin	1.48±0.03	3.13±0.05	0.89±0.03	1.05±0.02
	Orientin	1.10±0.02	2.30±0.02	0.73±0.03	0.97±0.03
	Vitexin	0.19±0.00	0.48±0.01	0.12±0.00	0.17±0.01
	Isovitexin	0.27±0.00	0.60±0.01	0.16±0.00	0.18±0.00
	Luteolin	0.24±0.00	0.25±0.02	Nd	0.03±0.00
Flavonols	Rutin	0.44±0.00	0.43±0.01	0.37±0.01	0.23±0.00
	Isoquecitrin	0.41±0.01	0.48±0.00	0.27±0.01	0.18±0.00
	Hyperoside	0.36±0.01	0.58±0.01	0.23±0.01	0.21±0.00
	Quecertin-3-O-robinobioside	1.00±0.02	1.66±0.03	0.83±0.00	0.89±0.01
	PPAG	0.43±0.00	0.71±0.01	0.47±0.00	0.54±0.01
	Ferrulic Acid	nd	0.11±0.00	Nd	0.06±0.00

Values represent means ± standard deviation of duplicate determinations. Abbreviations: MUF-methanol unfermented, MF-methanol fermented, AUF–aqueous unfermented, AF–aqueous fermented. PPAG-phenylpyruvic acid-2-*O*-glucoside

3.3.3. Antioxidant properties

FRAP assay: Both MUF ($p<0.0001$) and AUF ($p<0.0001$) extracts exhibited significantly higher antioxidant activity compared to the fermented rooibos extracts (Table 3.4). The MUF extract displayed significantly higher ($p<0.0001$) antioxidant activity when compared to the AUF extract. However, no difference in antioxidant activity was noticed between the MF and AF extracts.

ABTS assay: The radical scavenging activity of the MUF ($p<0.0001$) and AUF ($p=0.001$) extracts were significantly higher when compared to their fermented counterparts (Table 3.6). The MUF extract exhibited significantly ($p=0.0178$) higher

radical scavenging activity compared to the AUF, while no difference in antioxidant activity was noticed between the MF and AF rooibos extracts.

Table 3.4 Polyphenol content and antioxidant activity of rooibos extracts from unfermented and fermented plant material.

		Unfermented	Fermented
TP	MeOH	$344.1\pm21.7_{A}^{a}$	$244.7\pm15.6_{A}^{b}$
(mg GAE/g extract)	Aqueous	$285.9\pm10.4_{B}^{a}$	$258.7\pm12.4_{A}^{b}$
FLAVA	MeOH	$34.3\pm6.3_{A}^{a}$	$8.6\pm1.9_{A}^{b}$
(mg CE per g extract)	Aqueous	$22.5\pm2.0_{B}^{a}$	$10.7\pm1.9_{A}^{b}$
FRAP	MeOH	$2.18\pm0.16_{A}^{a}$	$1.27\pm0.08_{A}^{b}$
(mmol TE/g extract)	Aqueous	$1.65\pm0.16_{B}^{a}$	$1.31\pm0.09_{A}^{b}$
ABTS	MeOH	$1.93\pm0.38_{A}^{a}$	$1.03\pm0.10_{A}^{b}$
(mmol TE/g extract)	Aqueous	$1.63\pm0.23_{B}^{a}$	$1.19\pm0.13_{A}^{b}$

Values represent means ± standard deviations of duplicate determinations from two different experiments. Comparisons between extracts were analysed by the 2X2 factorial model using the GLM procedure and significant differenced indicated by $p<0.05$. Different letters indicate $p<0.05$, Subscript capital letters indicate difference between extraction type (MeOH vs Aqueous) and superscript (small caps) indicates difference between extract type (fermented vs unfermented). Abbreviations: TP - Total polyphenols, FRAP-Ferric reducing antioxidant power assay, ABTS-2, 2'-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt assay; FLAVA – flavanol/proanthocyanidin; CE = (+) catechol equivalents, MeOH – methanol, TE – Trolox equivalents

3.3.4 XAD-4 column fractionation of the unfermented MeOH extract and fraction yields

The MUF extract, containing the highest levels of polyphenols (Table 3.3, Table 3.4) and antioxidant activity (Table 3.4), was selected to be fractionation by chromatography on an XAD-4 column. The collected fractions were also chemically characterised by HPLC, and TP and FLAVA content as well as antioxidant properties were determined. Thin layer chromatography analysis of the 60 fractions collected from the XAD-4 column resulted in combination of fractions 1-6 (X-1), 7-21 (X-2), 22-36 (X-3) and 37-60 (X-4) and the remainder fraction (X-5) from the MeOH:$CHCl_3$ (1:1) elution (Table 3.5). Group separation during the stepwise increase in the methanol content resulted in fractions containing the major groups of non-flavonoid (X-1 to X-2) and flavonoid constituents (X-3 to X-5) of rooibos. Both column fractionations (F1 and F2) provided similar group separations, although the second fractionation showed low levels of aspalathin in fraction X-1 (Fig 3.1).

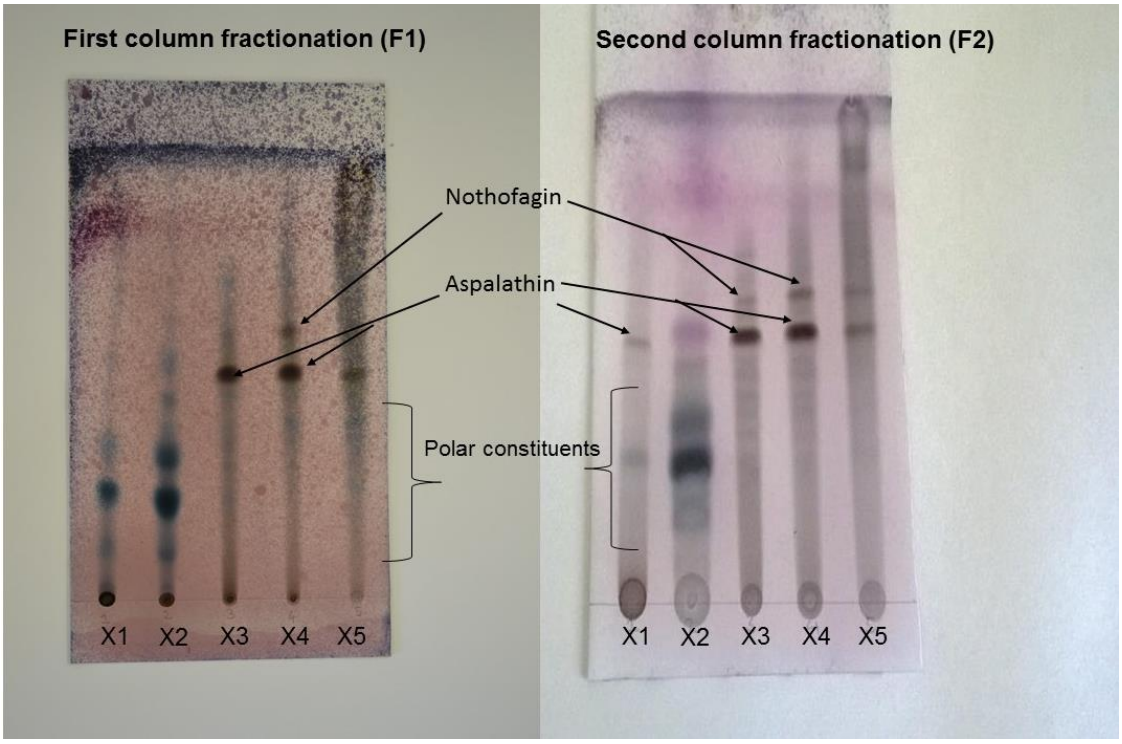

Figure 3.7. TLC profile of the different fraction form XAD-4 chromatography showing the different compounds found on each fraction. Dried fractions (30 mg) were dissolved in methanol (100µl) and spotted on TLC plates and developed for two hrs.

Overall the two fractionations resulted in a high recovery of over 85 % of the original MUF extract (20 g) although a lower recovery was obtained during the second column fractionation (F2). Fraction X-5 had the lowest yield while fractions X-2 and X-4 gave the highest yield from both fractions. An approximate 50 % lower yield was obtained for fraction X-1 during the second (F2) column fractionation (Table 3.5).

Table 3.5 Fraction yield (g) of the XAD-4 fractionation of the MUF rooibos extract (g).

Solvent	Combined fractions**	Fraction	F1	F2
15 % (MeOH/H_20)	Pre-fraction (500 ml) + 1-6	X-1	4.0	2.4
25-50 % (MeOH/H_20	7-21	X-2	5.2	5.2
50 – 75 % (MeOH/H_20)	22-36	X-3	3.2	2.8
75-100 % (MeOH/H_20)	37-60	X-4	5.9	5.7
MeOH:MeOH/$CHCl_3$(1:1)	61+ (2l)	X-5	1.2	1.0
Total			19.5	17.1
%Recovery*			97.5	85.5

Abbreviations: X-XAD-4 fractions (250 ml), F1-1st fractionation, F2-2nd fractionation. *Fraction recovery was expressed as a percentage of 20 g MeOH extract applied to the column. **Combination of the fractions was slightly different during the 2nd fractionation.

3.3.4.1 Chemical characterisation of XAD-4 column fractions

HPLC analysis (Table 3.6): None of the known rooibos flavonoids found in the MUF extract were detected in fractions X-1 and X-2 from the first fractionation (F1), which contained the more polar constituents. However, low levels of all the flavonoids, were detected in fraction X-1 and to some extend in fraction X-2 from the second fractionation (F2), which could be related to weak column equilibration prior to the fractionation of the

MUF extract. The major rooibos flavonoids detected in the MUF extract were enriched in fraction X-3 and X-4, with aspalathin, orientin and isoorientin detected in equal amounts in both fractions while nothofagin was more concentrated in fraction X-4. Vitexin and isovitexin, oxidised products of nothofagin and luteolin, rutin, hyperoxide, isoquecetrin and quercitrin-3-O-robinobioside were enriched in fraction X-4. Fractionation F2 provided similar results with respect to the distribution of these rooibos flavonoids in X-3 and X-4. Fraction X-5, which contained the least polar constituents, had far less rooibos flavonoids with F2 yielding slightly higher flavonoid levels.

Total Polyphenol content (Table 3.7): Fractions X-1 and X-5 of F1 contained similar levels of polyphenols, which were significantly lower than the MUF extract. Fraction X-2 contained the lowest TP levels, while the polyphenols were enriched ($P<0.05$) in fractions X-3 and X-4. Significantly ($p<0.05$) higher TP levels were detected in X-1 when compared to the MUF extract during F2, which as mentioned above, is related to inadequate column equilibration. Fraction X-2 again exhibited the lowest TP levels in F2 with fraction X-5 containing similar levels of TP to fraction X-5 of F1. The TP levels were again enriched in fraction X-3 and X-4 for F2 with fractions X-3 containing the highest TP levels ($P<0.05$).

FLAVA content: Fractions X-1 and X-4 from F1 contained comparable FLAVA levels, which were significantly lower than the MUF extract. The lowest FLAVA levels were detected in the X-2 and X-5, while fraction X-3 contained similar levels when compared to the MUF extract. During the F2, fraction X-1 contained significantly higher FLAVA levels than the MUF extract. A similar trend is noticed for fractions X-2 and X-5, containing the lowest FLAVA levels although it was significantly higher than the corresponding fractions from F1. Fractions X-3 and X-4 from F2 contained similar FLAVA levels when compared to the MUF extract.

Table 3.6 Concentrations of monomeric compounds in fractions from an XAD-4 column chromatography of the Methanol unfermented extract

Flavonoid classes	Flavonoids (g/100g extract/fraction)	MUF	X-1		X-2		X-3		X-4		X-5	
			F1	F2	F1	F2	F1	F2	F1	F2	F1	F2
Dihydrochalcones	Aspalathin	14.1	Nd	3.39	0.09	0.33	30.59	29.64	27.95	31.83	1.49	2.23
	Nothofagin	1.89	Nd	0.40	nd	nd	1.55	1.40	5.06	5.19	0.74	1.87
Flavones	Orientin	1.31	Nd	0.72	nd	0.07	2.76	2.41	2.61	2.92	0.35	0.47
	Isoorientin	1.75	Nd	1.07	nd	0.07	3.36	2.62	3.68	4.09	0.41	0.70
	Vitexin	0.25	Nd	0.10	nd	nd	0.34	0.28	0.59	0.65	2.13	0.20
	Isovitexin	0.33	Nd	0.17	nd	nd	0.24	0.17	0.85	0.81	0.45	0.78
	Luteolin	0.30	Nd	0.18	nd	nd	0.23	0.16	0.81	0.79	0.51	0.66
Flavonols	Rutin	0.51	Nd	0.42	nd	nd	0.57	0.44	1.20	1.13	0.52	0.90
	Hyperoxide	0.41	Nd	0.21	nd	nd	0.32	0.25	1.04	1.00	0.54	1.00
	Isoquecetrin	0.51	Nd	0.24	nd	nd	0.35	0.25	1.32	1.28	0.62	1.18
	Quecertin-3-O-robinobioside	1.15	Nd	1.03	nd	nd	0.87	0.66	2.82	2.37	2.13	3.42
	PPAG	0.51	Nd	0.17	0.10	0.39	nd	1.10	1.19	0.79	0.50	0.14

Values in a column are from two different XAD-4 fractionation runs (F1 and F2). Abbreviations: MUF-methanol unfermented, X1 to X5 – XAD-4 column fractions. PPAG -phenylpyruvic acid-2-*O*-glucoside

3.3.5 Antioxidant activity of the XAD-4 fractions

FRAP assay: With the F1, fractions X-1 and X-5 exhibited similar and significantly lower than the MUF extract iron reducing activities with fraction X-2 showing the lowest antioxidant activity (Table 3.7). The highest antioxidant activity in F1 was obtained in fraction X-3 followed by X-4 both of which did not differ significantly from the MUF extract. F-2 provided similar results except for fraction X-1 and X-3, which exhibited an increased FRAP activity in relation to F1 and X-5, which exhibited a lower FRAP activity. No enrichment of the FRAP activity of the MUF extract was achieved.

ABTS assay: In contrast to the FRAP assays, fractions X-1, X-3 and X-4 from F-1 exhibited the highest activity and did not differ significantly from each other and from the MUF extract. Fractions X-2 and X-5 exhibited comparable and significantly lower ($P<0.05$) radical scavenging activity compared to MUF. No significant difference ($P<0.05$) in the antioxidant activity was noticed between the fractions X-3, X-4 and MUF from F2, while the activity of fraction X-1 was significantly lower than the MUF extract ($P<0.05$). Fraction X-2 from F2 exhibited the lowest activity while fraction X-5 was also significantly ($P<0.05$) lower than the MUF extract.

Overall the highest antioxidant activity across both column fractionations was associated with fractions X-3 and X-4 for both FRAP and ABTS assays while no enrichment of the antioxidant activity was obtained.

Table 3.7 Chemical characterization and antioxidant activity of XAD-4 fractions from a methanol unfermented extract

	MUF	X-1		X-2		X-3		X-4		X-5	
		F-1	F-2	F-1	F-2	F-1	F-2	F-1	F-2	F-1	F-2
TP (mg GAE/g extract)	178.9 (8.8)$_{c}$C	99.9 (4.7)$^{*}_{d}$	230.8 (21.2)*B	30.7 (4.8)$_{f}$	40.2 (7.8)E	257.3 (9.6)$^{*}_{a}$	278.5 (11.4)*A	213.2 (5.5)$_{b}$	224.3 (34.7)B	80.9 (3.3)$_{e}$	89.7 (11.5)D
Flavanol (mg CE/g extract	35.6 (7.6)$_{a}$B	18.9 (3.4)$^{*}_{b}$	55.0 (4.0)*A	0.8 (0.3)$^{*}_{c}$	2.0 (0.6)*C	38.2 (1.7)$_{a}$	38.1 (4.2)B	15.0 (2.6)$^{*}_{b}$	29.1 (0.5)*B	1.4 (0.5)$^{*}_{c}$	5.9 (0.5)*C
FRAP (mmol TE/g extract)	2.23 (0.35)$_{b}$AB	0.93 (0.07)$^{*}_{c}$	1.67 (0.21)*B	0.21 (0.03)$_{d}$	0.24 (0.02) C	2.41 (0.34)$^{*}_{a}$	3.22 (0.17)*A	2.17 (0.48)$_{b}$	2.47 (0.10)AB	0.86 (0.04)$^{*}_{c}$	0.75 (0.04)*C
ABTS (mmol TE/g extract)	2.80 (0.32)$_{a}$A	2.26 (0.49)$_{a}$	1.93 (0.26)B	0.25 (0.01)$^{*}_{b}$	0.23 (0.01)*C	2.94 (0.51)$_{a}$	2.93 (0.19)A	2.72 (0.47)$_{a}$	2.8 (0.46)A	0.79 (0.12)$_{b}$	1.01 (0.19)D

The values represent duplicates from two different experiments. Subscript – ONE WAY ANOVA testing differences between means of all the fractions from F1 including the MUF extract. Uppercase- ONE WAY ANOVA testing differences between means of all the fractions from F2 including the MUF extract. Means that do not differ significantly are indicated by the same letter and if letters differ then means are significantly different. *Indicates significant differences ($P<0.05$) between fractionation 1 and 2. Abbreviations: MUF-methanol unfermented, X-XAD fraction, TP-Total polyphenols DAC -4-Dimethylaminocinnamaldehyde, FRAP-Ferric reducing antioxidant power assay, ABTS-2, 2'-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt assay, F1 – fractionation 1, F2- fractionation 2, GAE – gallic acid equivalent, CE – catechin equivalent, TE – Trolox equivalent.

3.4 Discussion

The use of herbal teas has received increased attention due to their proposed health benefits associated with their antioxidant properties (Joubert *et al*., 2009; Joubert & De Beer, 2011). As a result, rooibos has developed from being consumed as a household beverage to preparation of extracts as a value added product for use in foods, dietary supplements, and in pharmaceutical and cosmetic products (Joubert & De Beer, 2011). Rooibos tea is known for its low tannin levels, although it has been reported that 50 % of the hot water extract soluble solids is tannin-like substances (Joubert *et al*., 2008). The main constituents consist of proanthocyanidin type of heteropolymers, containing (+)-catechin and (−)-epicatechin chain extending units and (+)-catechin as terminal unit (Marais *et al*., 1998). Of the monomeric polyphenolic constituents, rooibos is known to contain the unique dihydrochalcones, aspalathin and nothofagin (Joubert, 1996; Joubert & De Beer, 2012). Other flavonoids present in rooibos include the flavones, orientin, isoorientin, vitexin, isovitexin, luteolin, the flavonols, rutin, isoquercitrin, quecertin, hyperoside, luteolin-7-O-glucoside and the flavanol, catechin (Ferreira *et al*., 1995.; Koeppen *et al*., 1962; Marais *et al*., 2000; Rabe *et al*., 1994; Shimamura *et al*., 2006).

The chemical structures of some of these rooibos flavonoids are illustrated in Fig 3.2. The bioactive polyphenolic compounds can be selectively extracted with different organic solvents (Perva-Uzunalić *et al*., 2006) and the polarity of the extraction solvent is important in determining the yield of the recovered soluble solids (Franco *et al*., 2008). Other factors influencing the yield and efficiency of extraction include the extraction time and temperature as well as chemical and physical characteristics of the sample (Dai & Mumper, 2010). Solvents commonly used in extracting polyphenols include methanol, ethanol, ethyl acetate, acetonitrile and water (Franco *et al*., 2008). Methanol is found to be a more efficient extraction solvent for simple, low molecular weight polyphenols, while more polar solvents, like water, are more efficient in extracting the more complex polyphenols (Franco *et al*., 2008; Metivier *et al*., 1980). In support of this, major polyphenols from leaves of persimmon (*Diospyros kaki*), which were mainly proanthocyanidins, were found to be water soluble (Kayoko *et al*., 2010)

while extraction of polyphenols and proanthocyanidins from pomegranate's peel was found to be more effective with water as the extraction solvent (Wissam *et al*., 2012).

Results obtained from the current study are in accordance with literature in that methanol extraction is more efficient in extracting major monomeric flavonoids from the rooibos plant material. This was evident as the methanol extracts, both MUF and MF, contained more polyphenols compared to the aqueous extracts, AUF and AF, according to the HPLC, TP and FLAVA analyses. However, the extraction yield from the fermented plant material was about 50 % lower compared to the unfermented plant material when using methanol as the extraction solvent. The low yield is likely as a result of the oxidation of monomeric flavonoids as well as other rooibos constituents. In support of this suspicion, aspalathin and nothofagin were reduced by approximately 10-fold, with a concomitant increase in the oxidised products including orientin, isoorientin, vitexin and isovitexin after fermentation. In contrast, aqueous extraction resulted in similar yields from both the fermented and unfermented plant material. The fermented rooibos plant material is known to contain more complex flavonoids as a result of the oxidation, which are likely to be more soluble in water as mentioned above. Therefore, the aqueous solvent seems to more effectively extract these complex polyphenols as the AF extract contained slightly higher TP and FLAVA (flavanol/proantocynidins) levels compared to the MF extract. This data is in accordance with reports that polyphenols of the proanthocyanidin type are more water soluble as mentioned above (Kayoko *et al*., 2010).

Extracts prepared from the unfermented rooibos exhibited significantly higher antioxidant activity in both the ABTS and FRAP assays compared to the corresponding fermented extracts due to the far higher levels of the rooibos flavonoids. As expected the antioxidant levels of rooibos extracts were reduced during fermentation, which is in agreement with studies indicating that a decrease in TP content of fermented rooibos is associated with a reduction in radical scavenging properties (Standley *et al*., 2001; Joubert *et al*., 2004).

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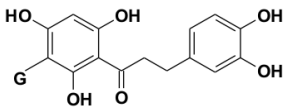
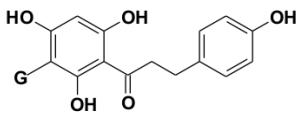
DIHYDROCHALCONES



ASPALATHIN



NOTHOFAGIN

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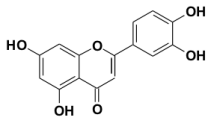
FIAVONES



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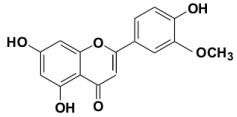

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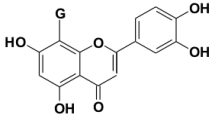

ORIENTIN

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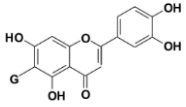
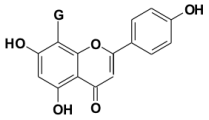


ISOORIENTIN



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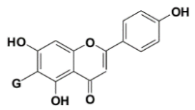

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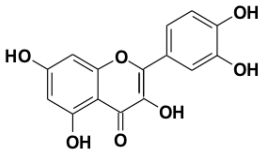
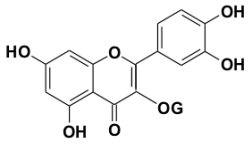
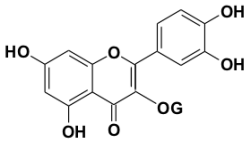


QUERCETIN



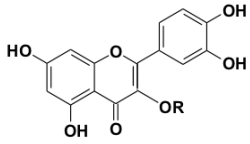
ISOQUERCITRIN

G = GLUCOSE



HYPEROSIDE

G = GALACTOSE



RUTIN

Figure 3.8 Chemical structures of major flavonoids of rooibos (Adapted from Snijman, 2007)

The reduction in antioxidant activity is related to the oxidation of aspalathin and nothofagin, as mentioned above and the formation of their oxidised flavones constituents. It has been shown that these oxidised compounds exhibit reduced antioxidant activity in the ABTS and lipid peroxidation assays (Snijman *et al*, 2009). Therefore, solvent selection for the extraction of rooibos polyphenols and the recovery of optimal antioxidant activity seems to be critical in providing the desired outcome of extracts. Depending on the specific outcome needed, a specific solvent system can be selected, e.g. extraction of rooibos with a less polar solvents, such as ethyl acetate, increased the antioxidant potency of the extract, although the recovery of polyphenols was much lower (Joubert *et al*., 2004). Our study, however, suggests that for optimal recovery of polyphenols (TP and FLAVA) and increased antioxidant activity (FRAP and ABTS) methanol should be used for extraction. A specific solvent system should therefore be selected to obtain an extract for investigating a specific biological effect as in certain biological systems high antioxidant potency would not be of value

In order to further identify the most active flavonoid and/or flavonoid combinations in the rooibos extract, chromatographic fractionation of the unfermented rooibos methanol extract was conducted using an XAD-4 resin. Column chromatography utilising specific matrixes has been reported to be an effective method of separating polyphenols with the XAD resins generally used for the adsorption of organic compounds, specifically for hydrophobic compounds up to MW 20000 (Ku & Lee, 2000). Amberlite XAD-2, and XAD-4, are macro-reticular, hydrophobic and poly-aromatic resins and have been widely used for the separation of low molecular weight compounds. XAD was reported to be more efficient in adsorbing organic compounds compared to silica gels, alumina and activated carbon columns due to their wide range of pore structures and physiochemical characteristics, chemical stability as well as high selectivity for aromatic solutes (Li *et al*., 2001). Flavonoids adsorb to the resin through van de Waals forces, which is dependent on the pH of the elution solvent, number of benzene rings and the functional groups on the benzene ring (Ku & Lee, 2000). The main aim of the current study was to separate the major flavonoid and/or non-flavonoid constituents utilising a solvent eluting system of aqueous and methanol stepwise gradient and to characterise these in terms of their polyphenol content as well as antioxidant properties.

Five fractions of decreasing polarity were collected with major rooibos flavonoids enriched in fractions X-3 and X-4, which also exhibited the highest antioxidant activities. These fractions were collected during fractionation when using the 50 to 100 % methanol:aqueous eluting solvent. The more polar fractions (X-1 and X-2), eluting in the 15 to 50 % aqueous:Methanol solvent, contained low levels of polyphenols. The most non-polar fraction, X-5, collected during the MeOH:$CHCl_3$ (1:1 vol/vol) elution step, contained low TP and flavanol levels and also exhibited weak anti-oxidant activity. F-2, however, provided different results from that of F1 for fraction X-1, when considering the TP and flavanol constituents, presumably due to weak column equilibration. This became evident from the HPLC analyses of fraction X-1 as low levels of all the rooibos flavonoids were detected suggesting possible sample spill-over into the solvent front peak.

The antioxidant activity of the 5 fractions (X-1 – X-5) was consistent with the chemical analysis with fractions X-3 and X-4, which contained the bulk of the rooibos flavonoids, exhibiting the highest activity. Fractions X-1 (F1) and X-2 (F1 and F2), containing almost no flavonoids, exhibited the lowest antioxidant activity, while X-5 (F1 and F2) exhibited the second lowest antioxidant activity. It appears that the antioxidant activity of rooibos is mainly dependant on the dihydrochalcones and their oxidation flavones and flavonol products. The FLAVA content does not seem to be associated with anti-oxidant response as the high FLAVA content of fraction X-1 (F2) does not result in high antioxidant activity. This became apparent as X-1 (F-2) exhibited a weaker activity than fractions X-3 and X-4 in both the FRAP and ABTS assays (Table 3.7). This has interesting implications for the biological activities of FLAVA and the interaction with iron as will be discussed in the subsequent chapters in the current thesis. Of interest was that the iron reduction power (FRAP) of fraction X-3 from F2 was significantly higher when compared to X-4 while the radical scavenging activity (ABTS) was similar. Thus the high nothofagin content of fraction X-4 seems to decrease the iron reducing capacity of the fraction, presumably due to the lack of the catechol group on the B-ring (Fig 3.2) known to be important in the iron chelating properties of rooibos flavonoids (Snijman *et al*., 2009). Aspalathin and nothofagin have been shown to exhibit similar ABTS scavenging properties while the iron chelating properties differed vastly when

considering the inhibition of iron induced microsomal lipid peroxidation (Snijman *et al*., 2009).

In summary, methanol appears to be a more efficient extraction solvent than water for rooibos unfermented plant material to obtain extracts with a high yield of rooibos flavonoids with a maximum antioxidant capacity. Although, fractionation of the MUF extract resulted in enrichment of the dihydrochalcones content and TP levels in fractions X-3 and X-4, the FLAVA content and antioxidant activity was similar to the original MUF extract. This would imply that specific combinations of polyphenols are required to sustain a specific antioxidant response. The antioxidant properties of rooibos however are thought to be important when considering the health benefits such as anti-inflammatory activity. Therefore, the differential antioxidant activity of the rooibos extracts and XAD-4 fractions could be valuable in assessing specific biological properties e.g. the anti-inflammatory and possible anti-carcinogenic properties of rooibos.

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4. Anti-inflammatory effects of unfermented and fermented rooibos extracts, flavonoid and non-flavonoid enriched column fractions utilising the UV-B/HaCaT keratinocyte inflammatory model

Abstract

Rooibos (*Aspalathus linearis*), a South African herbal tea known to contain numerous polyphenols, specifically the flavonoids, known to exhibit anti-inflammatory properties *in vivo*. In the current study the anti- and pro-inflammatory effects of methanol and aqueous fermented (MF and AF) and unfermented (MUF and AUF) rooibos extracts and flavonoid and non-flavonoid enriched column fractions of the MUF extract were monitored in a UV-B/HaCaT keratinocyte inflammatory model utilising IL-1α and cell survival indices as endpoints. In the absence of UV-B the methanol extracts were the most active in increasing IL-1α production reflecting a pro-inflammatory effect, which was associated with a decrease in cell viability and an increase in apoptosis at the highest concentrations. In contrast, the aqueous extracts decreased IL-1α production at all concentrations with a similar but weaker effect on the cell growth parameters at the higher concentrations. The MUF extract and flavonoid-enriched column fractions X-3 and X-4 and the more non-polar fraction X-5, showed similar effects regarding the induction of IL-1α and a decrease in cell growth parameters at the highest concentrations. The non-flavonoid fraction X-1 also increased IL-1α associated with a drastic decrease in both cell viability and apoptosis, presumably due to the induction of cell necrosis. Fraction X-2 resulted in a decrease in IL-1α at high concentrations only slightly affected cell viability and induction in apoptosis suggesting an anti-inflammatory effect. In the presence of UV-B all the rooibos extracts decreased IL-1α production with a further decrease in the cell growth parameters with the MUF extract exhibiting the highest activity. Both MUF and AUF extracts also decrease apoptosis at the highest concentration again suggesting the induction of cell necrosis. The MF and AF extracts decrease IL-1 in the absence of any effect on cell viability and apoptosis at low concentrations suggesting a direct anti-inflammatory effect. Of the column fractions, X-1, X-3 and X-4 decreased both IL-1α and apoptosis with a dramatic decrease in cell viability simulating the effect of the MUF and AUF. In contrast fraction X-5 mimicked the pro-inflammatory effect obtained in the absence of UV-B irradiation although both cell viability and apoptosis decreased. Fraction X-2 seems not exhibited any effect on IL-1αand/or apoptosis although cell viability was slightly decreased at the highest concentrations. Flavonoids are known to exhibit pro-oxidant effects at high concentrations which could be enhanced in the presence of UV-B leading to a

decrease in cell survival indices by enhancing apoptosis and presumably cell necrosis, the latter which could further enhance inflammation. Critical dose response effects therefore seem to exist whereby rooibos flavonoid and non-flavonoid constituents exhibit either anti- or pro-inflammatory effects in the absence and presence of UV-B irradiation which complicate the utilisation of polyphenol-enriched extracts as a chemopreventive tool in UV-B-induced skin carcinogenesis.

4.1. Introduction

Skin damage occurs in response to various stimuli such as bacteria, fungi, chemical irritants or exposure to ultraviolet radiation (Suter *et al*., 2009). The latter is the most common cause of skin damage and can initiate a cascade of complex events that lead to skin carcinogenesis (Sarasin, 1999). Such events include oxidative stress, DNA damage, an inability to initiate apoptosis and inflammation (Mouret *et al*., 2006). Inflammation in the skin encompasses the stimulation of various inflammatory mediators such as release of pro-inflammatory cytokines, expression of inflammatory genes and leukocyte infiltration. Cytokines play a key role in local immune and inflammation responses in cells and the balance between pro- and anti-inflammatory cytokines should be tightly regulated in order to prevent the development of chronic inflammation (Santangelo *et al*., 2007). Specific cytokines are released by different skin cells with the pro-inflammatory cytokines, interleukin 1 alpha (IL-1α), tumor necrosis factor alpha (TNF-α) and IL-6, as key role players in inflammation. In the keratinocytes, IL-1α is constitutively produced and is retained inside the cell. The cytokine is only released during disease states making it a good measure of the extent of damage to the cell (Luo *et al*., 2004). During apoptosis, however IL-1α is retained in the nuclei and subsequently degraded without affecting inflammation (Cohen *et al.,* 2010).

Acute inflammation is short term, self-limiting and is anticipated to be therapeutic to the host organism; whereas an excessive, prolonged inflammatory response can lead to chronic inflammation. The latter involves the generation of various oxidants, i.e. superoxide anions, hydroxyl radicals, hydrogen peroxide and nitric oxide by inflammatory cells, which damage normal tissue resulting in induction of signalling molecules and enzymes leading to the development of degenerative diseases i.e. neurological diseases, metabolic disorders, cardiovascular disease and cancer (Santangelo *et al*., 2007). The strong association between chronic inflammation and cancer has resulted in the investigation of anti-inflammatory drugs as a possible tool in chemoprevention. The focus has been on the development of anti-inflammatory drugs from naturally occurring compounds such as the flavonoids found in plants since synthetic anti-inflammatory drugs currently utilised exert other adverse side effects (Kristina *et al*, 2010).

The anti-inflammatory activity of polyphenols is thought to inactivate the transcription of pro-inflammatory enzymes such as cyclooxygenases (COX), lipooxygenase (LOX) and inducible nitric oxide syntheses (iNOS) as well as to enhance the activity of peroxisome proliferators (Williams *et al*., 2004). Polyphenols may also inhibit the activity of phospholipase kinases, tyrosine kinases and the nuclear factor kappa beta (Yoon & Baek, 2005). In addition, polyphenols have been found to inhibit the activity of phospholipase A_2 (PLA_2), COX and LOX thereby reducing the release of arachidonic acid (AA) and subsequently its eicosanoid products, prostaglandins (PGs) and leukotrienes (LTs), which have anti-inflammatory implications (Aviram & Fuhrman., 1998; Baumann *et al*., 1980; Laughton *et al*., 1991; Weltone*t al*., 1986; Yoon & Baek, 2005).

The South African herbal tea, rooibos, is known to exhibit antioxidant and anti-inflammatory effects associated with its polyphenol content (Joubert *et al*., 2008). The anti-cancer properties of rooibos have been reported to include the inhibition of methyl-benzyl-nitrosamine (MBN)-induced oesophageal squamous cell carcinogenesis in rats as well as modulating skin tumor promotion in a dimethylbenzanthracene initiated and phorbol ester cancer promotion mouse skin carcinogenesis model, implying disruption of growth regulatory parameters in pre-cancerous lesions (Marnewick *et al.*, 2005; Sissing *et al*., 2011). However, at present very little is known about the anti-inflammatory effects of rooibos and the role in modulating skin carcinogenesis.

The current investigation initiated studies on the anti-inflammatory effects of rooibos in post exposure UV-B/HaCaT keratinocyte cell model (Magcwebeba *et al*., 2012) using IL-1α as biomarker for inflammation. The modulation of IL-1α accumulation by methanol and aqueous extracts of fermented and unfermented rooibos as well as flavonoid and non-flavonoid enriched fractions, prepared from the unfermented methanol rooibos extract, were investigated. In addition, cell viability and apoptosis were evaluated.

4.2. Materials and methods

4.2.1. Chemicals and reagents

RPMI-1640, Dulbecco's phosphate buffered saline (DPBS), L-glutamine, trypsin-versene (EDTA), Hank's buffered salt solution (HBSS) and heat inactivated fetal bovine serum (FBS) were obtained from Lonza (Braine-l'Alleud, Belgium). Human recombinant IL-1α ELISA kit was purchased from R&D systems (Minneapolis, USA). CellTiter-Glo luminescent cell viability and caspase-3/7 assay kits were purchased from Promega (Madison, USA). Triton X-100 for membrane research was obtained from Roche (Indianapolis, USA).

4.2.2. Plant material, preparation of extracts and column fractionation

Unfermented and fermented rooibos plant material was purchased from Rooibos Ltd (Clanwilliam, South Africa). Extracts were prepared as described in chapter 3; briefly, methanol extracts were prepared by stirring plant material first in chloroform for 24 hrs and filtering; this step was repeated two times. The residual plant material was homogenised in methanol using a polytron and filtering, this step was also repeated two times. The resulting filtrate was evaporated at 40 °C using a rotary evaporator. The aqueous extracts were prepared by steeping the plant material in boiled water for 30 min, filtered and the resulting filtrate freeze dried. All the extracts were stored desiccated in amber bottles at room temperature.

The methanol unfermented (MUF) extract was fractionated by column chromatography using XAD-4 amberlite resin. Briefly, the column was prepared in 15 % methanol and the extract applied in 15 % methanol. The column was eluted using a step wise gradient of decreasing polarity as described in chapter 3 and fractions were pooled following thin layer chromatography analysis, concentrated by evaporation *in vacuo* at 40 ºC and stored at 4 °C.

4.2.3. Cell maintenance

Immortalized keratinocytes, HaCaT cells, were a gift from the Department of Human Biology at the University of Cape Town, South Africa. The HaCaT cell model is a spontaneously transformed immortal keratinocyte cell line from adult human skin which is commonly used in research related to skin keratinocytes. The cells have a high capacity to differentiate and proliferate in vitro. The cells were grown in RPMI-1640 supplemented with 10 % FBS and 2 mM L-Glutamine and cultured for 24 hrs at 37 °C in 5 % CO_2/95 % air. Cells were detached with trypsin-EDTA and seeded at a density of 180 000 cells/well in clear 24 well plates (cytokine and caspase-3/7 determinations) or 30000 cells/well in solid white 96 well plates for cell viability.

4.2.4. Experimental conditions

4.2.4.1. UV-B irradiation

Prior to UV-B irradiation, the culture medium was removed and DPBS (100 µl) added to 96 well plates and for 24 well plates (600 µl) prior to UV-B irradiation. Non irradiated plates were washed with the same volume of DPBS. A UV crosslinker (UVitek Limited, Aberdeen, UK) fitted with six 8 watt UV tubes emitting an average wavelength of 312 nm (Vilberlourmat, Marne La Vallée, France) was used for irradiation at 80 mj/cm^2 24 hrs after seeding.

4.2.4.2. Treatment with extracts and/or column fractions

Stock solutions of tea extracts and column fractions were prepared in RPMI-1640 supplemented with 0.5 % FBS, 2 mM L-GLUT to a concentration of 2 mg/ml with final DMSO concentration of 0.5 % and filtered using Minisart syringe filters (Sartorius Stedim Biotech GmbH, Goettingen, Germany). These stock solutions were diluted to obtain rooibos concentrations varying between 0.025 mg/ml to 0.8 mg/ml to obtain specific dose response effects. After irradiation, DPBS was removed and the cells were incubated with the extracts and/or column fraction containing culture medium for an additional 24 hrs.

4.2.5. Experimental end points

4.2.5.1. Cell viability assay

Cell viability was determined in 96 well plates using a CellTitre-Glo Luminescent kit to monitor ATP content according to the manufacturer's instructions. After incubation with extracts or column fractions, 100 µl of the ATP reagent was added to the wells containing 100 µl medium with cells. The plate was then shaken for 2 min and incubated at room temperature for 10 min. Luminescence was measured using a Veritas microtitre luminometer (Promega, Madison, USA) and the luminescence signal determined as relative light units (RLU) and expressed as percentage of the negative control (cell not exposed to UV-B and containing no treatments).

$$\%\ \text{ATP change} = \frac{\text{RLU [treated cells]}}{\text{RLU [control]}} X\ 100$$

4.2.5.2. Intracellular IL-1α determination

Intracellular IL-1α was determined in 24 well plates with cell seeded at a density of 180 000 cells/well. Cells were washed with 600 µl DPBS and lysed with 600 µl of 0.5 % Triton X-100 in phosphate buffered saline (pH 7.4) along with one freeze-thawing-shaking cycle to facilitate lyses. Cell lysates were stored at -80 ºC until analysed. IL-1α was quantified using a human recombinant IL-1α ELISA kit according to the manufactures instructions. A standard curve was generated using human recombinant IL-1α prepared in 1 % BSA in DPBS (w/v) using concentrations ranging from 7.8 to 500 pg/ml. Absorbance was measured at 450 nm using the Biotek Synergy HT microplate reader (Winooski, Vermont, USA). Data were analysed using the standard curve generated from Gen5™ Data Analysis Software (version 2 for Windows). Intracellular IL-1α was expressed as pg/ml of cell lysate and as the fold increase of the untreated control (samples not irradiated). The following formula was used:

$$\text{IL-1α Fold Increase} = \frac{\text{IL-1α (sample)}}{\text{IL-1α (control)}}$$

4.2.5.3. Caspase-3/7 assay

Caspase 3/7 activity was determined in 24 well plates. The cells were washed with 600 µl DPBS and lysed with 600 µl of 0.5 % Triton X-100 in phosphate buffered saline (pH 7.4) along with one freeze-thawing-shaking cycle to facilitate lyses. Cell lysates were stored at -80 °C until analysed. Activity of caspase-3/7 was determined using the caspase-Glo 3/7 assay according to the manufactures instructions. The assay is based on the cleavage of a luminogenic caspase-3/7 substrate generating a "glow-type" luminescent signal. Luminescence is proportional to the amount of caspase activity present. Lysates were assayed in white 96 well microtitre plates and the assay substrate was added at a ratio of 1:1 and incubated for 30 min. Luminescence was determined using the Veritas microtitre plate luminometer (Promega, Madison, USA) and the luminescence signal was measured in relative light units (RLU). Induction of apoptosis was expressed as fold increase of the samples not exposed to UV-B.

$$\text{Fold Increase} = \frac{\text{RLU [treated cells (+UV)]}}{\text{RLU [treated cells (-UV)]}}$$

4.2.6. Statistical analysis

This data was analysed using a 1-way ANOVA where there were 6 levels and the post hoc Tukey's Studentized Range Test was used for multiple pairwise comparisons between the means of the different levels. For the 1-way ANOVA's where only two levels were present, Student's *t*-test was used. Statistical significance was considered at $p < 0.05$.

4.3. Results

The modulating effects of the different rooibos extracts (MUF, MF, AUF and AF) and XAD-4 column fractions (X-1 – X-5) on intracellular accumulation of IL-1α were compared in relation to their effects on cell viability and the induction of apoptosis. Comparisons of the treatments were conducted separately in the absence or presence of irradiation as well as between the UV-B untreated and treated samples.

4.3.1. Modulation of cell viability, IL-1α and apoptosis by aqueous and methanol extracts

Effects in the absence of UV-B exposure

(i) Modulation of cell viability

The methanol unfermented (MUF), aqueous unfermented (AUF) and aqueous fermented (AF) rooibos extracts resulted in a significant ($p<0.05$) decrease in cell viability (ATP production) with clear dose response effects. No significant differences were observed between the extracts at the highest concentration. In contrast, a far weaker dose response effect was noticed from treatment with the methanol fermented (MF) extract at similar extract concentrations (Table 4.1).

(ii) Modulation of IL-1α accumulation

The MUF and MF extracts significantly ($P<0.05$) increased IL-1α (pg/ml) production at the highest concentrations exhibiting a pro-inflammatory effect with the MUF the most active (Table 4.1). In contrast, treatment with aqueous extracts significantly ($P<0.05$) decreased the IL-1α production in an inverse dose dependant manner when compared to the control exhibiting anti-inflammatory effects, with the two lowest concentrations of the AUF and AF extracts exhibiting the highest effect.

(iii) Modulation of apoptosis

At the highest concentration all the extracts significantly ($P<0.05$) increased apoptosis in comparison to the control, with the MUF extract exhibiting the biggest effect ($P<0.05$) and the MF extract only slightly increasing apoptosis (Table 4.1). The AUF extract exhibited a similar activity when compared to the AF extract at the highest concentration.

Effects in the presence of UV-B exposure

(i) Modulation of cell viability

Cell viability was significantly decreased in HaCaT cells exposed to UV-B (Fig 4.1a and Fig 4.1d). When compared to the effects of the extracts in the absence of UV-B exposure, the MUF(Fig 4.1a), MF (Fig 4.1b) and AUF (Fig 4.2a) extracts further enhanced the reduction in cell viability caused by UV-B in a dose response manner. The AF extract however, exhibited similar effects in cell viability in the absence and presence of UV-B except at the highest concentrations (Fig 4.2b).

When comparing the effects of the rooibos extracts in the presence of UV-B, the MUF extract was the most active in reducing cell viability when compared MF, AUF and AF extracts (Table 4.1). The AUF was significantly more active than the AF extract at the highest concentration.

(ii) Modulation of IL-1α accumulation

Exposure to UV-B significantly increased IL-1α concentration by 3-fold (Fig 4.1b and Fig 4.1e). When compared to the effects in the absence of UV-B, the MUF (Fig 4.1c) extract significantly ($P<0.05$) decreased IL-1α in the presence of UV-B at higher concentrations. The other extracts, however, showed a significant ($P<0.05$) increase in IL-1α concentration compared to the samples not UV-B irradiated although the difference between the (-) UV-B and (+) UV-B effects was lower with the MF (Fig 4.1d) and AUF (Fig 4.2C) extracts.

All the rooibos extracts significantly ($P<0.05$) decreased IL-1α production in UV-B treated HaCaT cells (Table 4.1). In the presence of UV-B the MUF extract was more active in reducing IL-1α when compared to the MF and AUF, which exhibited similar activities. The AF extract exhibited the weakest response with the highest effect at the lowest concentration.

(iii) Modulation of apoptosis

UV-B exposure significantly ($P<0.05$) increased apoptosis by 3-fold (Fig 4.1c and Fig 4.1f). When compared to the effects in the absence of UV-B, the MUF extract (Fig 4.1e) significantly decreased apoptosis in the presence of UV-B at the highest concentration while the MF extract (Fig 4.1f) increased apoptosis in the presence of UV-B at all concentrations. However, the MF extract did not alter the induction of apoptosis by UV-B. The AUF (Fig 4.2e) and AF (Fig 4.2f) extracts decreased apoptosis at the highest concentrations in the presence of UV-B when compared to effects in the absence of UV-B.

Treatment with the MUF and AUF extracts in the presence of UV-B resulted in a significant ($P<0.05$) decrease in apoptosis at higher concentrations when compared to the control (Table 4.1). The MF and AF extracts also significantly ($P<0.05$) decreased apoptosis in the presence of UV-B in a dose dependent manner but to a lesser extent.

Table 4.1: Modulation of icIL-1α accumulation by the different methanol extracts of rooibos in relation to their effect on cell viability (ATP content) and apoptosis.

		Absence of UV-B				Presence of UV-B			
	[extract] mg/ml	**ATP production % viability**	**IL-1α (pg/ml)**	**IL-1αpg/ml) Fold**	**Caspase-3/7 Fold**	**ATP production % viability**	**IL-1α (pg/ml)**	**IL-1α (pg/ml) Fold**	**Caspase 3/7 Fold**
Control		100±1.4$_a$	57.7±5.8$_a$	1.00±0.10$_a$	1.01±0.16$_a$	81.7±7.9$_a$	172.8±16.6$_a$	3.01±0.30$_a$	3.74±0.53$_a$
MUF	**0.05**	96.3±1.5$_b$	59.6±8.0$_a$	1.03±0.15$_a$	1.56±0.23$_b$	63.9±2.2$_b$	124.4±10.5$_b$	2.08±0.30$_b$	4.00±0.40$_a$
	0.1	88.4±1.9$_c$	65.6±8.4$_b$	1.12±0.16$_a$	1.70±0.08$_c$	57.2±2.5$_c$	73.9±3.3$_c$	0.92±0.13$_c$	1.87±0.28$_c$
	0.2	67.0±2.0$_e$	100.4±7.4$_c$	1.71±0.16$_b$	4.06±0.23$_d$	41.3±4.9$_d$	59.3±2.5$_d$	0.51±0.07$_d$	0.46±0.06$_d$
MF	**0.05**	98.3±2.0$_b$	59.8±3.9$_a$	1.02±0.09$_a$	1.13±0.12$_a$	86.8±3.2$_a$	120.2±17.5$_b$	1.82±0.59$_b$	3.87±0.29$_a$
	0.1	94.8±1.4$_b$	57.0±7.0$_a$	0.97±0.07$_a$	1.17±0.19$_a$	73.0±3.3$_e$	117.4±14.4$_b$	1.77±0.50$_b$	3.56±0.22$_a$
	0.2	85.6±1.4$_c$	74.1±11.1$_b$	1.28±0.20$_a$	1.44±0.19$_b$	65.8±3.6$_b$	97.3±13.6$_b$	1.59±0.44$_b$	3.02±0.28$_b$
AUF	**0.05**	89.9±1.5$_c$	32.8±2.5$_d$	0.56±0.05$_c$	1.43±0.27$_b$	78.2±3.3$_a$	102.0± 4.0$_b$	2.69±0.41$_a$	3.16±0.36$_a$
	0.1	79.0±1.6$_d$	35.7±5.5$_d$	0.63±0.08$_c$	1.86±0.31$_b$	71.9±4.1$_b$	105.9±4.0$_b$	2.35±0.01$_b$	2.72±0.22$_b$
	0.2	62.8±1.9$_f$	46.8±3.6$_e$	0.80±0.06$_a$	3.66±0.29$_d$	57.8±2.1$_c$	94.9±1.4$_c$	1.72±0.13$_b$	1.95±0.78$_c$
AF	**0.05**	82.5±2.2$_c$	36.3±3.6$_d$	0.61±0.04$_c$	1.36±0.26$_b$	80.7±2.6$_a$	122.2±14.5$_b$	3.60±0.28$_a$	3.31±0.38$_a$
	0.1	69.5±1.3$_e$	39.2±5.7$_d$	0.66±0.11$_c$	1.63±0.10$_c$	73.9±3.7$_e$	143.5±9.3$_d$	3.15±0.14$_a$	2.93±0.48$_a$
	0.2	59.1±1.2$_f$	46.0±6.3$_e$	0.69±0.02$_c$	2.85±0.58$_d$	68.4±1.6$_b$	143.2±11.5$_d$	2.70±0.26$_b$	2.13±0.39$_c$

All values represent means from at least two independent experiments. The % ATP production is calculated using the control in the negative UV as 100%., IL-1α fold increase in the negative UV-B is calculated against the control whereas in positive UV-B the IL-1α fold is calculated against the corresponding treatment IL-1α (pg/ml) in the non UV-B exposed cells. The same is applicable for tor the caspase-3/7 fold. Subscript: Indicates differences between values in a column, if letters differ then the values differs significantly ($P<0.05$). Abbreviations: icIL-1α- intracellular IL-1α, MUF-methanol unfermented, MF-Methanol fermented, AUF-Aqueous unfermented, AF-Aqueous fermented, IL-1α-Interleukin 1-alpha.

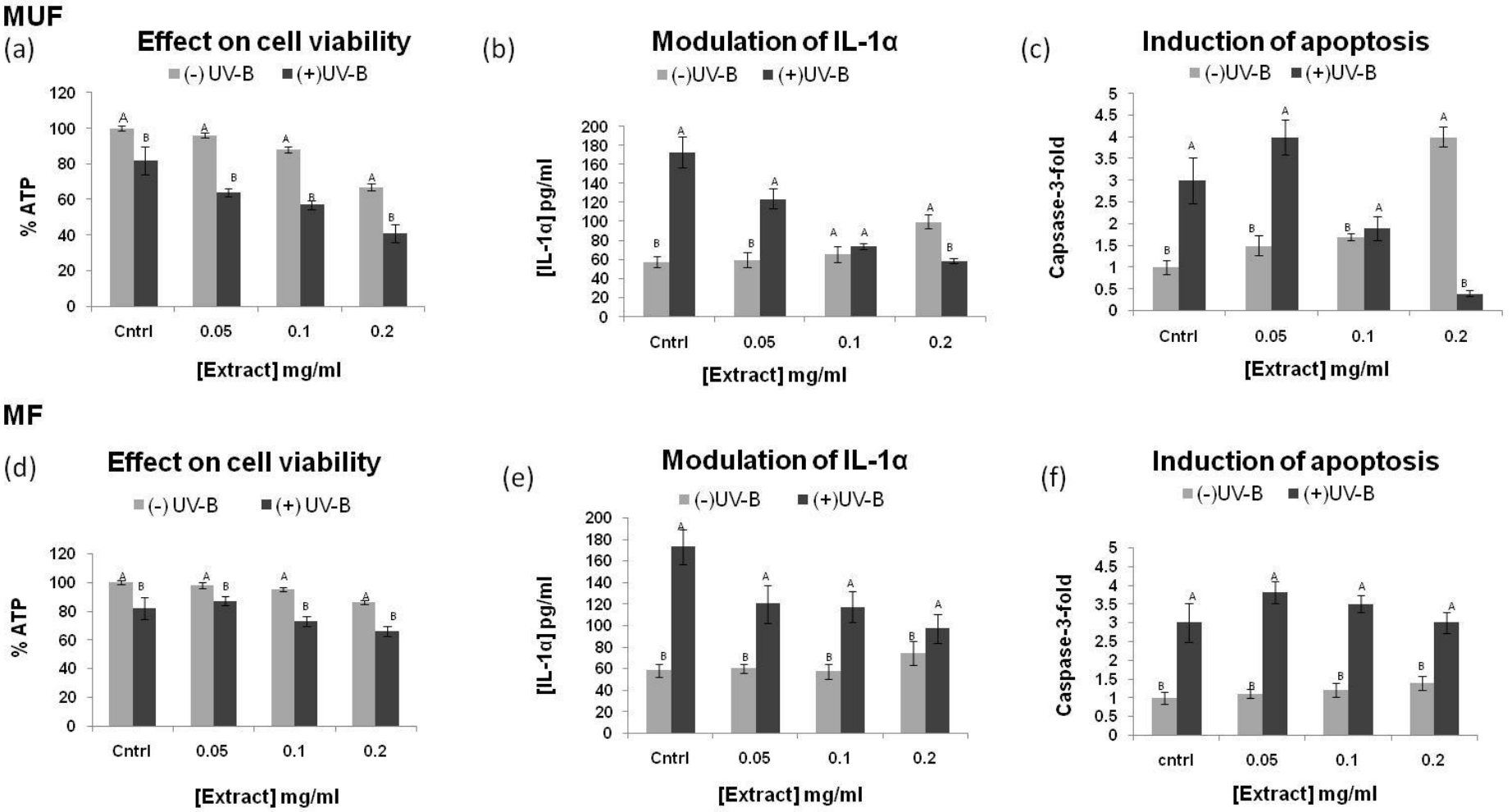

Figure 4.1 Differences between effects on inflammation and cell growth parameters by the different rooibos extracts in the absence and presence of UV-B. (a) MUF-effect on cell viability measured in % ATP content, (b) MUF-modulation of intracellular IL-1α production, (c) MUF-induction of apoptosis, (d) MF-effect cell viability (e) MF- modulation of intracellular IL-1α production, (f) MF- induction of apoptosis. Capital letter Indicates differences between effects in (-) and (+) UV-B, if letters are similar for a specific variable then UV-B radiation had no significant effect.

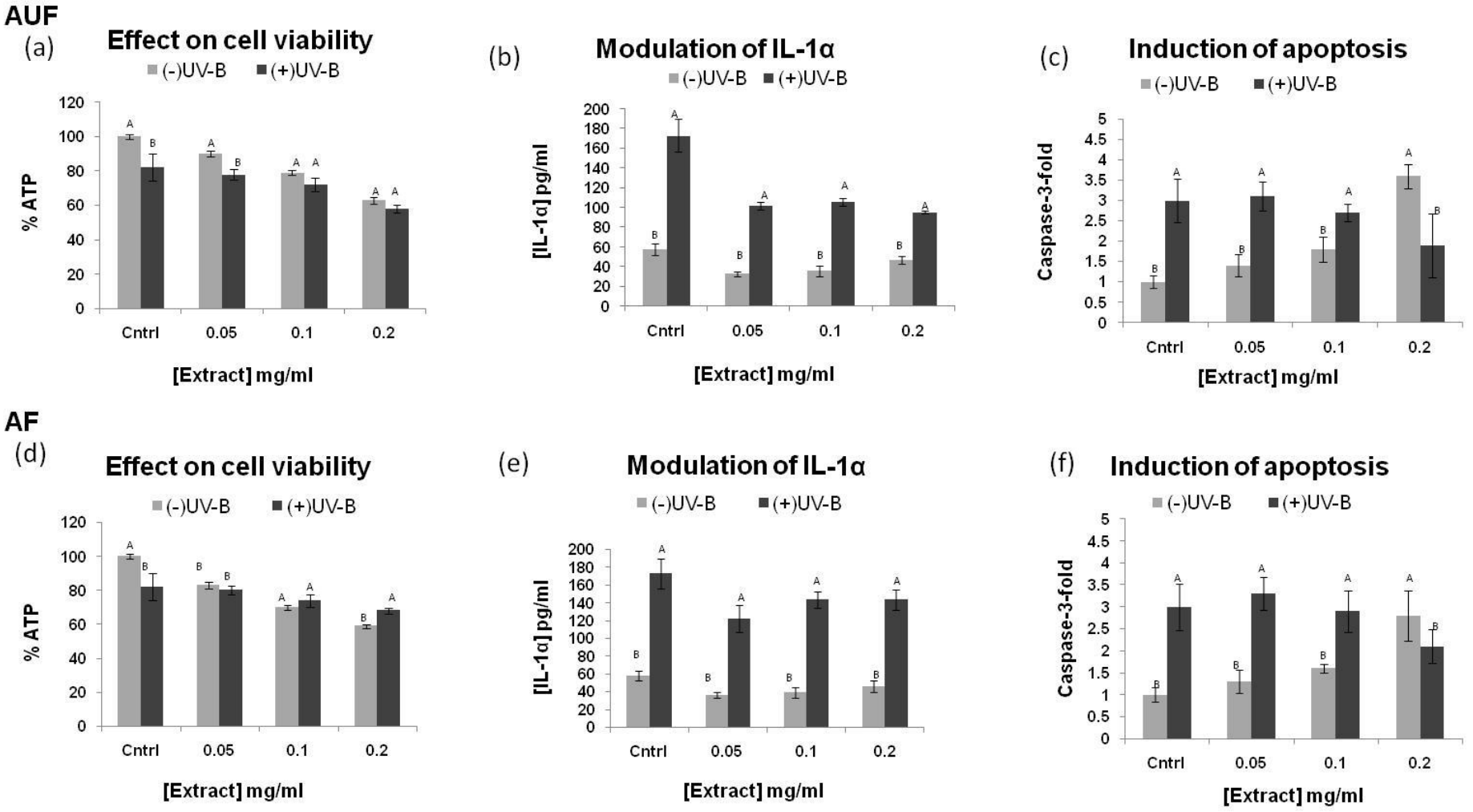

Figure 4.2 Differences between effects on inflammation and cell growth parameters by the different rooibos extracts in the absence and presence of UV-B. (a) AUF-effect on cell viability measured in % ATP content, (b) AUF-modulation of intracellular IL-1α production, (c) AUF-induction of apoptosis, (d) AF-effect cell viability (e) AF- modulation of intracellular IL-1α production, (f) AF- induction of apoptosis. Capital letter Indicates differences between effects in (-) and (+) UV-B, if letters are similar for a specific variable then UV-B radiation had no significant effect.

4.3.2. Modulation of cell viability, IL-1α accumulation and apoptosis by XAD-4 column fractions of MUF rooibos extract

Effects in the absence of UV-B exposure

(i) Modulation of cell viability

The MUF significantly decreased cell viability at the concentration of 0.2 mg/ml (Table 4.2). Fraction X-1 exhibited a similar effect to the MUF at the same concentration (0.2 mg/ml) while the other fractions had lower effects when compared to the MUF extract. The column fractions decreased cell viability dose dependently with fraction X-1 as the most effective at the 3 highest concentrations. Fractions X-3 and X-4 exhibited a similar effect on cell viability, except at the highest concentration where fraction X-3 was more effective. Fraction X-5 was the least effective in decreasing cell viability followed by fraction X-2.

(ii) Modulation of IL-1α accumulation

The MUF extract significantly ($P<0.05$) increased IL-1α at a concentration of 0.2 mg/ml (Table 4.2) and at this concentration was most effective in decreasing IL-1α compared to all the fractions except for fraction X-5, which exhibited a similar effect. Fractions X-1, X-3 and X-4 also increased IL-1α dose dependently at 0.2mg/ml but to a lesser extent. In contrast, fraction X-2 decreased ($P<0.05$) the IL-1α concentration at the two highest concentrations when compared to the treatment control.

(iii) Modulation of apoptosis

The MUF extract showed the highest apoptotic response, which was significantly higher when compared to the control and to all the fractions at comparative concentrations (Table 4.2). Fractions X-3 and X-4 resulted in significantly higher apoptosis at all the concentrations in a dose response manner when compared to the other fractions. Fractions X-2 and X-5 exhibited a similar but weaker effect in inducing apoptosis with a significant ($P<0.05$) increase at the higher concentrations. In contrast, fraction X-1 significantly ($P<0.05$) decreased apoptosis at high concentrations.

Table 4.2 Modulation of icIL-1α accumulation in HaCaT cells exposed to UV-B by different XAD-4 fractions from a MUF rooibos extract in relation to their effect on cell viability

		Negative UV-B				Positive UV-B			
		%ATP production	IL-1α (pg/ml)	IL-1αfold increase	Caspase-3/7 fold increase	%ATP production	IL-1α (pg/ml)	IL-1α fold increase	Caspase-3/7 fold increase
	Control	100.0±6.2$_a$	56.5±8.0$_a$	1.00±0.09$_a$	1.00±0.11$_a$	78.3±8.8$_a$	130.1±20.3$_a$	2.12±0.38$_a$	3.35±0.44$_a$
MUF	**0.05**	93.6±3.6$_a$	61.3±2.7$_a$	1.03±0.16$_a$	2.38±0.18$_b$	72.3±5.2$_a$	133.4±16.8$_a$	2.24±0.37$_a$	3.27±0.66$_a$
	0.1	80.1±4.0$_b$	72.5±10.3$_c$	1.20±0.05$_c$	4.67±0.85$_c$	57.7±5.9$_b$	75.5±11.7$_c$	0.89±0.08$_b$	1.48±0.21$_b$
	0.2	52.0±9.7$_d$	130.5±24.6$_b$	2.00±0.07$_b$	5.26±0.72$_c$	25.1±7.4$_c$	42.4±5.1$_b$	0.32±0.04$_c$	1.09±0.11$_c$
X1	**0.05**	76.4±12.6$_b$	41.8±3.8$_e$	0.58±0.04$_d$	1.01±0.13$_a$	71.2±9.5$_a$	128.6±11.8$_a$	3.15±0.39$_a$	3.46±0.19$_a$
	0.1	70.2±11.4$_b$	41.8±2.3$_e$	0.62±0.05$_d$	1.01±0.03$_a$	62.0±9.9$_a$	102.6±29.4$_a$	3.02±0.36$_a$	3.70±0.60$_a$
	0.2	54.5±7.2$_d$	69.6±0.7$_e$	1.2±0.55$_a$	0.78±0.05$_a$	40.5±5.4$_b$	62.8±10.7$_c$	1.20±0.99$_b$	3.71±0.53$_a$
	0.4	20.7±2.5$_f$	103.3±13.4$_d$	1.84±0.48$_e$	0.63±0.05$_d$	10.9±3.9$_d$	25.0±4.9$_b$	0.26±0.07$_c$	0.47±0.17$_d$
	0.8	6.4±1.8$_g$	186.2±4.1$_c$	2.53±0.56$_e$	0.51±0.07$_d$	3.3±1.3$_e$	23.2±3.3$_b$	0.16±0.08$_c$	0.66±0.16$_d$
X2	**0.05**	90.8±4.0$_a$	57.8±6.8$_a$	1.02±0.12$_a$	1.08±0.19$_a$	73.3±6.0$_a$	122.4±6.3$_a$	1.97±0.08$_a$	2.91±0.24$_a$
	0.1	90.6±6.2$_a$	61.9±9.6$_a$	1.01±0.15$_a$	1.13±0.20$_a$	71.7±9.0$_a$	127.0±11.8$_a$	2.19 ±0.22$_a$	2.97±0.57$_a$
	0.2	84.2±5.0$_b$	55.0±2.6$_a$	0.95±0.16$_a$	1.47±0.34$_a$	70.5±3.8$_a$	131.5±13.1$_a$	2.43±0.24$_a$	4.15±0.45$_a$
	0.4	80.0±7.3$_b$	36.5±2.8$_e$	0.52±0.04$_f$	1.55±0. 20$_e$	58.9±6.1$_b$	137.4±7.2$_a$	3.64±0.51$_d$	3.71±0.46$_a$
	0.8	65.1±7.6$_c$	30.6±7.9$_e$	0.62±0.11$_f$	1.71±0.50$_e$	57.5±12.1$_g$	119.4±19.0$_a$	2.80±0.79$_a$	3.86±0.51$_a$
X3	**0.05**	82.4±8.3$_b$	66.3±3.6$_a$	1.17±0.16$_a$	1.39±0.28$_a$	67.9±2.0$_b$	142.5±14.1$_a$	2.15±0.25$_a$	4.46±0.76$_a$
	0.1	80.7±9.2$_b$	62.7±9.2$_a$	1.03±0.11$_a$	1.94±0.64$_e$	61.7±7.8$_b$	135.9±9.3$_a$	2.22±0.36$_a$	3.13±0.43$_a$
	0.2	80.0±3.3$_b$	64.4±7.4$_a$	1.10±0.27$_a$	2.50±0.82$_e$	52.4±2.3$_b$	98.2±15.2$_f$	1.45±0.35$_b$	1.94±0.32$_b$
	0.4	66.0±5.6$_c$	98.6±10.9$_d$	2.07±0.57$_e$	3.20±0.73$_f$	22.4±3.0$_c$	64.2±6.0$_c$	0.61±0.08	0.89±0.08$_d$
	0.8	34.7±7.3e	127.8±12.5$_b$	2.34±0.51$_e$	3.11±0.12$_f$	9.3±1.7$_d$	44.7±8.2$_b$	0.36±0.07$_c$	0.52±0.05$_e$
X4	**0.05**	88.2±11.8$_b$	62.0±4.6$_a$	1.12±0.10$_a$	1.14±0.27$_a$	70.1±4.8$_a$	164.3±23.1$_a$	2.55±0.47$_a$	4.38±0.47$_a$
	0.1	84.2±10.8$_b$	63.3±6.4$_a$	1.08±0.06$_a$	1.57±0.57$_a$	58.7±6.8$_b$	158.5±11.0$_a$	2.39±0.41$_a$	2.93±0.34$_a$
	0.2	77.0±6.2$_b$	76.0±6.6$_c$	1.28±0.20$_a$	1.83±0.68$_b$	52.1±6.0$_b$	136.5±27.9$_a$	1.82±0.35$_a$	2.08±0.56$_b$
	0.4	66.3±8.6$_c$	81.7±8.0$_c$	1.42±0.40$_a$	3.12±0.23$_e$	25.5±2.3$_c$	101.0±20.7$_a$	1.42±0.37$_b$	1.29±0.21$_c$
	0.8	48.5±8.6	106.8±16.0$_d$	2.11±0.66$_e$	3.04±0.44$_e$	15.2±3.0$_d$	87.5±11.1$_g$	0.84±0.27$_b$	0.92±0.08$_c$
X5*	**0.025**	93.2±6.1$_a$	76.9±1.7$_c$	1.13±0.06$_a$	1.64±0.44$_e$	69.2±9.2$_a$	108.3±21.8$_a$	1.97±0.39$_a$	1.28±0.85$_b$
	0.05	92.4±6.3$_a$	85.5±1.8$_d$	1.27±0.06$_2$	1.27±0.04$_a$	68.1±9.4$_a$	123.5±11.4$_a$	1.77±0.45$_a$	1.82±0.83$_b$
	0.1	87.6±8.1$_a$	108.4±12.7$_d$	1.50±0.07$_e$	1.12±0.63$_a$	60.4±3.7$_b$	135.6±7.9$_a$	1.58±0.60$_a$	1.82±0.82$_b$
	0.2	82.8±1.9$_b$	158.7±32.5$_b$	2.24±0.52$_e$	1.48±0.81$_a$	57.2±3.8$_b$	167.2±13.7$_a$	0.97±0.27$_b$	1.70±0.44$_b$
	0.4	82.7±7.1$_b$	198.1±22.9$_f$	3.00±0.30$_g$	2.36±0.13$_b$	59.0±5.7$_b$	246.7±35.9$_e$	1.10±0.36$_b$	2.15±0.81$_a$

All values represent means from at least two independent experiments. % ATP production is calculated against by setting the (-) UV-B control as 100 %. For the negative UV, IL-1α fold is calculated against the IL-1α (pg/ml) control whereas in the IL-1α fold for the positive UV-B is calculated against the IL-1α (pg/ml) for the corresponding treatment dose in the cells not exposed to UV-B. The same applies for the Caspase-3/7 fold increase. Subscript: Indicates differences between values in a column, if at least one letter is similar between values then the values do not differ significantly. Abbreviations: MUF-methanol unfermented, X–XAD-4 fraction, IL-1α- interleukin-1alpha. Due to the insolubility of factions X-5 a higher concentration could not be evaluated.

Effects in the presence of UV-B exposure

(i) Modulation of cell viability

UV-B significantly ($p<0.05$) decreased cell viability in HaCaT cells (Fig 4.2a and Fig 4.2d). When compared to the effects in the absence of UV-B, the MUF extract (Fig 4.3a), fractions X-3 (Fig 4.5d) and X-4 (Fig 4.5a) further decreased ($p<0.05$) cell viability. Fractions X-2 (Fig 4.4a) and X-5 (Fig 4.5d) exhibited a similar but reduced effect on cell viability when compared to that in the presence of UV-B. Fractions X-1 (Fig 4.3d), however did not show additional effects on cell viability.

At similar concentrations, the MUF extract exhibited the highest activity in reducing cell viability in the presence of UV-B (Table 4.2). All the fractions also decreased cell viability in the presence of UV-B with typical dose response effects, with fraction X-1 exhibiting the highest activity followed by fractions X-3 and X-4, while X-2 and X-5 showed the weakest response.

(ii) Modulation of IL-1α accumulation

IL-1α production increased significantly ($p<0.05$) by 3-fold after UV-B exposure (Table 4.2). When compared to the effect in the absence of UV-B, the MUF extract (Fig 4.3b) and fractions X-1 (Fig 4.3e) and X-3 (Fig 4.4e) decreased IL-1α in the presence of UV-B at high concentrations in a dose dependent manner. Fraction X-4 (Fig 4.5b) did not have a further effect on IL-1α in the presence of UV-B at the highest concentration. Fractions X-2 (Fig 4.4b) resulted in an increase in IL-1α in the presence of UV-B at all concentrations although that effect was comparable to that of the UV-B treated control. In contrast, fraction X-5 further increased IL-1α accumulation at all the concentrations (Fig 4.5e) in presence of UV-B exposure.

In the presence of UV-B the MUF extract significantly ($P<0.05$) decreased IL-1α at high concentrations and was the most effective at decreasing IL-1α at the concentration of 0.2 mg/ml (Table 4.2). Fractions X-1, X-3 and X-4 also decreased IL-1α at the concentration of 0.2 mg/ml while fraction X-2 had no effect on IL-1α accumulation and X-5 resulted in increased IL-1α at this concentration. When considering dose response effects, IL-1α accumulation was significantly reduced in the presence of UV-B by fractions X-1, X-3 and X-4 at the highest doses, with X-1 exhibiting the highest activity.

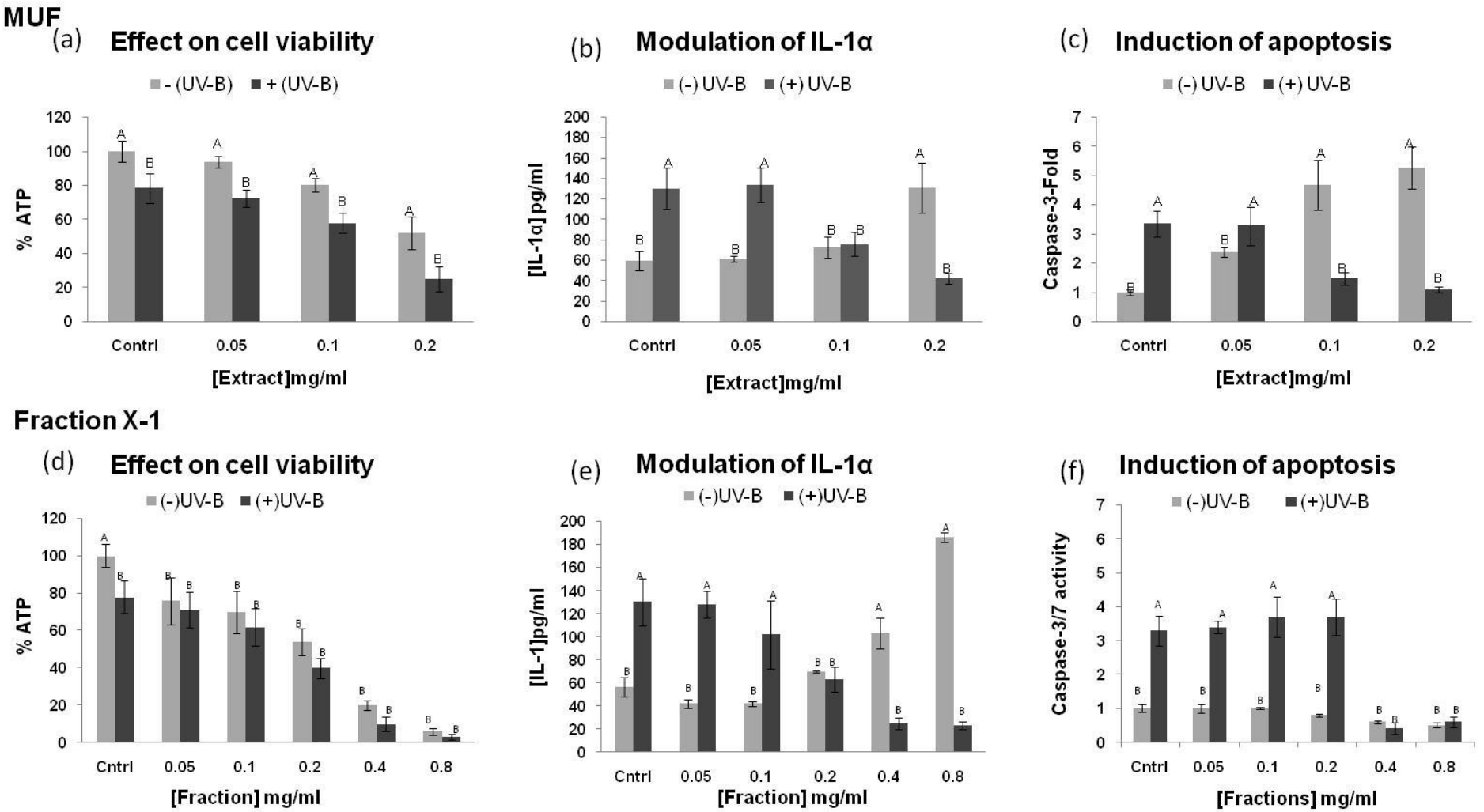

Figure 4.3 Differences between effects on inflammation and cell growth parameters by the MUF rooibos extract and fraction X-1 in the absence and presence of UV-B. (a) MUF-effect on cell viability measured in % ATP content, (b) MUF-modulation of intracellular IL-1α production, (c) MUF-induction of apoptosis, (d) Fraction X-1-effect cell viability (e) Fraction X-1- modulation of intracellular IL-1α production, (f) Fraction X-1- induction of apoptosis. Capital letter Indicates differences between effects in (-) and (+) UV-B, if letters are similar for a specific variable then UV-B radiation had no significant effect.

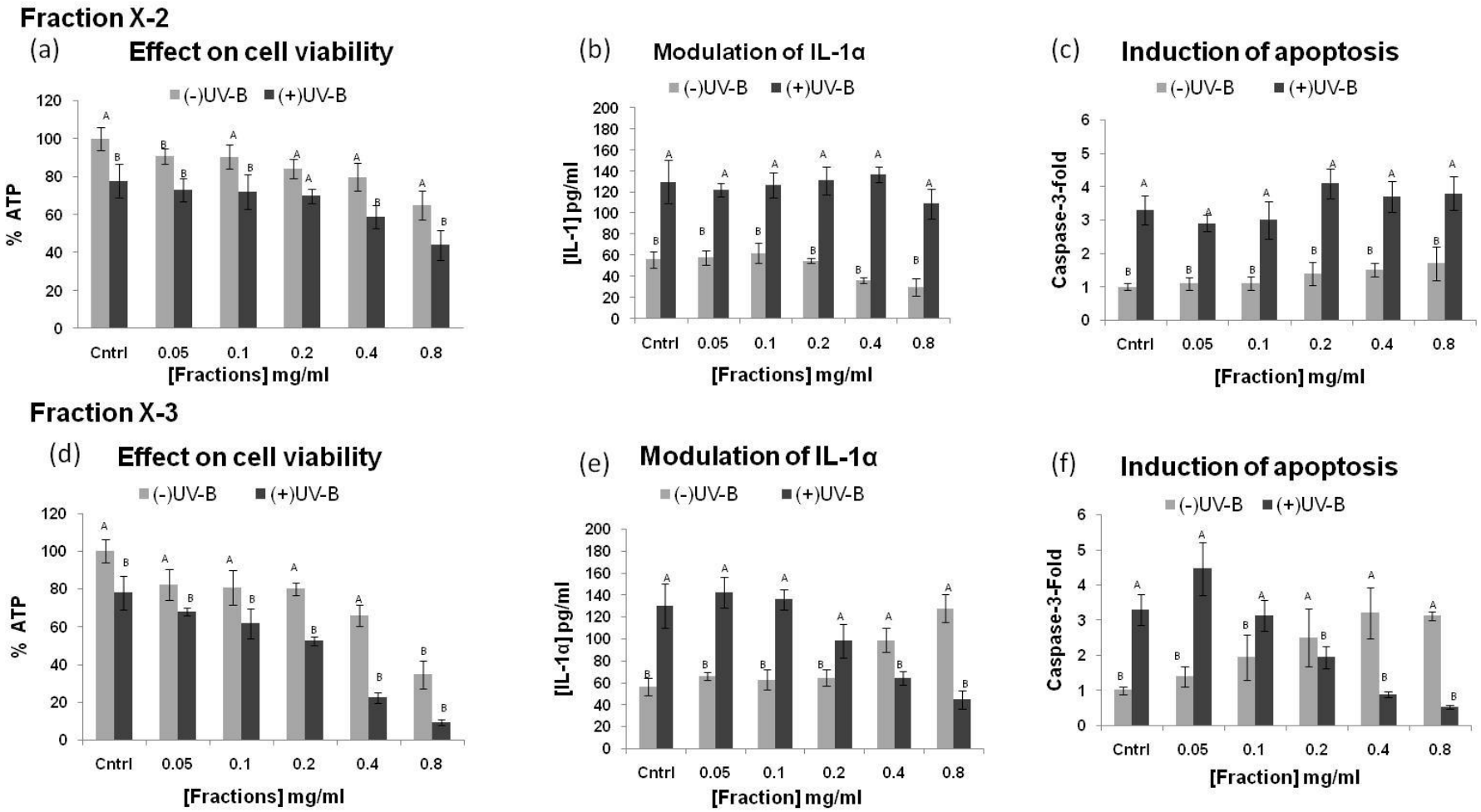

Figure 4.4 Differences between effects on inflammation and cell growth parameters by the different rooibos fractions in the absence and presence of UV-B. (a) Fraction X-4-effect on cell viability measured in % ATP content, (b) Fraction X-4-modulation of intracellular IL-1α production, (c) Fraction X-4-induction of apoptosis, (d) Fraction X-5-effect cell viability (e) Fraction X-5- modulation of intracellular IL-1α production, (f) Fraction X-5- induction of apoptosis. Capital letter Indicates differences between effects in (-) and (+) UV-B, if letters are similar for a specific variable then UV-B radiation had no significant effect.

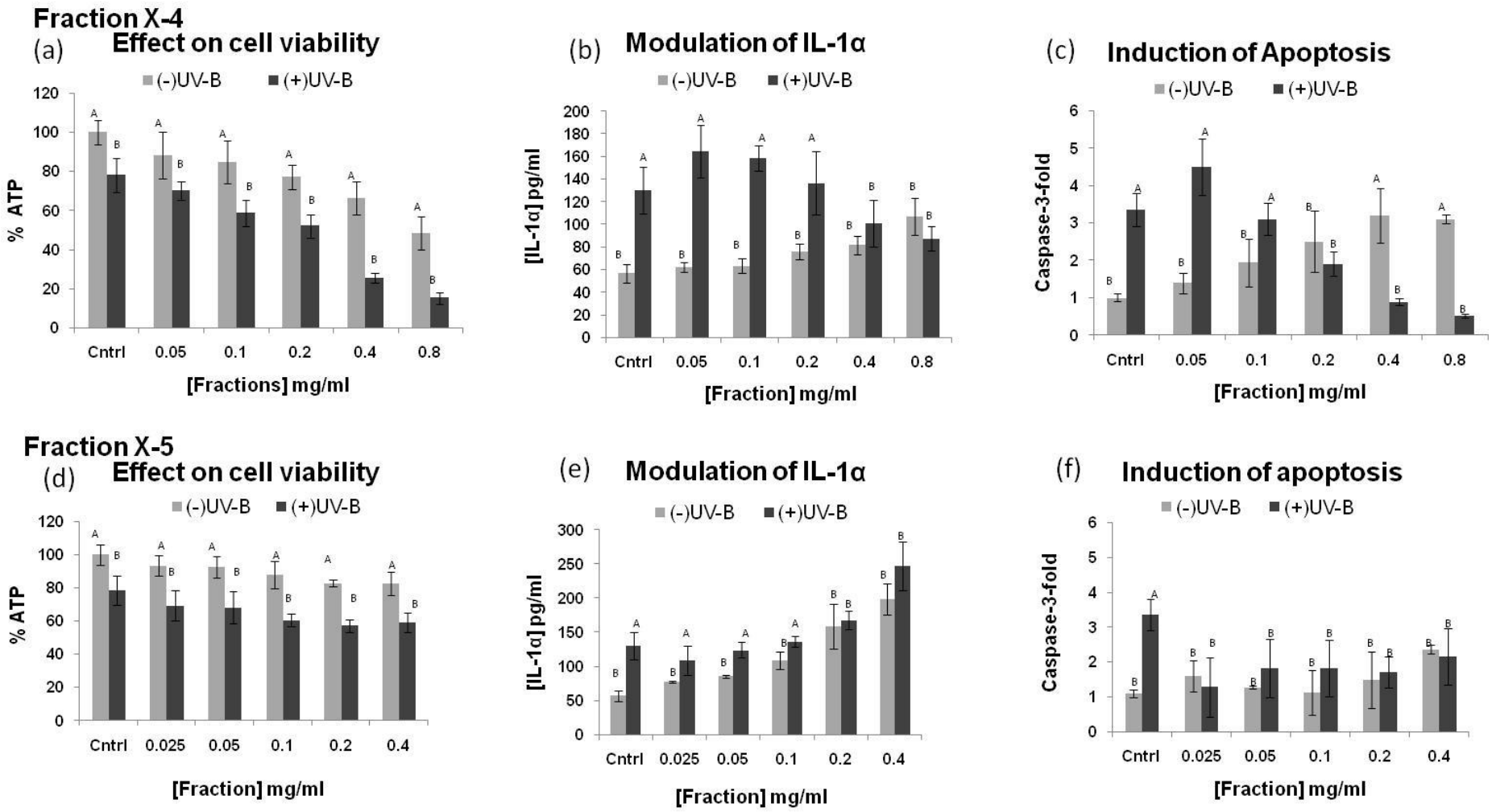

Figure 4.5 Differences between effects on inflammation and cell growth parameters by the different rooibos fractions in the absence and presence of UV-B. (a) Fraction X-2-effect on cell viability measured in % ATP content, (b) Fraction X-2-modulation of intracellular IL-1α production, (c) Fraction X-2-induction of apoptosis, (d) Fraction X-3-effect cell viability (e) Fraction X-3- modulation of intracellular IL-1α production, (f) Fraction X-3- induction of apoptosis. Capital letter Indicates differences between effects in (-) and (+) UV-B, if letters are similar for a specific variable then UV-B radiation had no significant effect.

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(iii) Modulation of apoptosis

A significant ($p<0.05$) 3-fold increase in apoptosis was observed following UV-B exposure (Table 4.2). When compared to the effect in the absence of UV-B the MUF extract (Fig 4.3c), fractions X-3 (Fig 4.4f) and X-4 (Fig 4.5c) decreased apoptosis in the presence of UV-B, at the concentration of 0.2 mg/ml, with MUF exhibiting the highest effect. Fraction X-2 increase apoptosis in the presence of UV-B compared to the effect in the absence of UV-B at all the concentrations tested (Fig 4.4c) at any of the concentrations utilised. Fractions X-1 (Fig 4.3f) and X-5 (Fig 4.5f) did not alter apoptosis at any of the concentrations tested when compared to the UV-B untreated cells.

In the presence of UV-B, the MUF extract significantly decreased apoptosis at high concentrations in comparison to the UV-B treated cells control (Table 4.2). At the concentration of 0.2 mg/ml fractions X-3, X-4 and X-5 also decreased apoptosis but to a lesser extent compared to the MUF extract at this concentration, while fractions X-1 and X-2 had no effect of apoptosis compared to the control. When considering dose response effects, fractions X-1, X-3, X-4 and X-5 significantly ($P<0.05$) decreased apoptosis at high concentrations with X-1 and X-3 exhibiting the highest effects.

4.4. Discussion

Exposure to UV-B increases the production of the primary cytokines, IL-1α and TNF-α in the skin which, in addition to oxidative stress, may result in a cutaneous inflammatory response and apoptosis (Xu & Fisher, 2005). The modulation of the production of these primary cytokines by naturally occurring plant polyphenols has been suggested to be important prevention tools to alleviate inflammation in the skin following UV-B exposure (Nicols & Katiyar, 2010). At present very little is known about the protective effects of the rooibos flavonoids against UV-B induced skin inflammation. A recent study showed that rooibos extracts indirectly exhibited anti-inflammatory properties by removing keratinocytes following UV-B exposure through apoptosis (Magcwebeba, 2013). The current study further elucidate the possible anti-inflammatory properties of extracts of fermented and unfermented rooibos as well as specific flavonoid and non-flavonoid enriched fractions.

In the absence of UV-B exposure, treatment of HaCaT cells with the MUF and MF rooibos extracts resulted in an increased IL-1α production suggesting pro-inflammatory effects. Of interest is that MUF extract, unlike the MF extract, resulted in a 4-fold increase in apoptosis, which was also associated with a decrease in cell viability. Both the decreased cell viability and increased apoptosis are presumably due to pro-oxidant effects of the rooibos flavonoids which may be responsible for the pro-inflammatory effect by the extracts. The MUF extract contained high levels of flavonoids (Chapter 3), exhibiting a high antioxidant capacity when compared to the MF extract. Therefore, the MUF seems to result in an increased oxidative stress which may be due to mitochondrial membrane depolarisation known to be affected by the rooibos flavonoids (Magcwebeba, 2013). It has been reported that methanol and aqueous extracts from unfermented rooibos exhibited pro-oxidant properties (Joubert *et al.*, 2005), while cytotoxicity towards HaCaT cells has also been associated with increased oxidative stress (Magcwebeba, 2013). These findings are in agreement with studies utilising the green tea polyphenol EGCG reported to exhibit pro-oxidant effects at high doses (Galati *et al*, 2006; Isbrucker *et al*, 2006). Furthermore, the pro-oxidant effects of polyphenols have been suggested to be responsible for an increase in oxidative stress and the

potential toxicity in cells (Lambert & Elias, 2010). This increased oxidative environment can in turn activate transcriptional mechanisms and signalling pathways involved in immune response and inflammation leading to the accumulation of IL-1α. Hence the observable pro-inflammatory effects by the methanol extract. The reduced effect of MF extract on IL-1α accumulation may be associated with the decrease in the monomeric flavonoids and a subsequent decrease in anti-oxidant properties associated with fermentation (Chapter 3).

The AUF and AF extracts like the MUF extract increased apoptosis at the higher concentrations which was associated with decreased cell viability, however IL-1α production was decreased significantly ($P<0.05$) at all the concentrations tested. Of interest is that the AUF extract contained lower polyphenol levels and exhibited a lower antioxidant activity when compared to the MUF (Chapter 3). Hence the lower pro-oxidant activity by the aqueous extracts as reflected in their reduced effects on cell viability. Therefore, the extracts sustain cell viability at lower concentrations possibly related to their antioxidant properties, whilst at higher concentrations pro-oxidant effects occur resulting in a decrease in cell viability. The lack of overt effects on cell viability and apoptosis as observed at the lower concentrations of both AUF and AF extracts coupled to a decrease in IL-1α may be indicative of an anti-inflammatory effect. However, at higher doses of the AUF and AF extracts, the decrease in IL-1α may be associated with the decrease in viable cells and increased apoptosis suggesting an indirect anti-inflammatory effect. Therefore, depending on the dose of flavonoids, the extracts may exhibit direct or indirect pro-inflammatory effects as in the case of the aqueous extracts or pro-inflammatory effects as in the case of the methanol extracts. In addition, a critical balance between pro- and anti-oxidant effects seem to exist, which will determine either an anti- or pro-inflammatory responses in HaCaT cells.

UV-B irradiation of HaCaT cells resulted in a 3-fold increase in IL-1α production, which was associated with a similar increase in apoptosis and a reduction in cell viability as reported previously (Magcwebeba *et al*., 2012). In the presence of UV-B, treatment with the methanol extracts resulted in a dose dependent decrease in IL-1α production accompanied by a further decrease in cell viability, while apoptosis was significantly

reduced when compared to the control. UV-B is known to result in the generation of free radicals which could lead to excessive ROS production providing an environment favourable for cell necrosis (Svobodová *et al*., 2011). This was observed when the cells were exposed to the MUF extract at the higher concentration where cell viability was reduced by up to 60 %, presumably due to synergistic pro-oxidant effects from the high levels of monomeric rooibos flavonoids and that affected by UV-B. Cells need energy for the induction of apoptosis, therefore at high extract concentrations and low ATP levels a subset of the cell culture population may instead undergo necrotic cell death, hence the lower level of apoptosis. The reduction in the intracellular IL-1α may therefore reflect cell death though both apoptosis and necrosis. Therefore, the methanol extracts seem to have no direct anti-inflammatory effect but rather result in decreased IL-1α which may be reflective of the decrease in cell viability.

Treatment with the aqueous extracts in the presence of UV-B also resulted in a decrease in IL-1α production accompanied by a decrease in apoptosis and a decrease in cell viability at the highest concentrations. As mentioned above, the decrease in apoptosis could result from necrotic cell death. At the lower concentrations, all the extracts decreased IL-1α production in the presence of UV-B exposure without affecting apoptosis, which was associated with a minimal effect on cell viability suggesting an anti-inflammatory effect. A typical dual effect seems to prevail where at higher extract concentrations IL-1α is removed by apoptosis and/or cell necrosis based on the availability of ATP. It has been reported that depletion of ATP resulted in necrotic cell death while apoptosis is induced under ATP-supplying conditions (Cohen *et al*., 2010). However, care needs to be taken as at excessively high extract concentrations, necrotic cell death may occur and subsequently enhance IL-1α release thereby exhibiting a pro-inflammatory effect. The reduction of intracellular IL-1α levels in HaCaT cells exposed to cytotoxic doses of ionomycin has been reported (Magcwebeba *et al*., 2012). Cell cytotoxicity could not be assessed under the current experimental conditions due to interference of the polyphenols in cytotoxicity assays (Wisman *et al*., 2008). Alternative determination of IL-1α release in the cell culture medium should be monitored in the future to assess the role of the extracts on cell necrosis and cytokine release.

As the MUF extract exhibited the highest anti-oxidant effects and was the most effective in reducing IL-1α following UV-B irradiation it was therefore selected for fractionation. The goal for fractionation was to:(i) separate the major rooibos flavonoids from the non-flavonoid constituents (ii) Determine whether polyphenol enrichment promotes or counteracts the stimulation of IL-1α by UV-B compared to the MUF extract (iii) Assess whether fractionation of the MUF extract is an applicable approach to further characterise the anti-inflammatory effects of rooibos.

In the absence UV-B irradiation, fractions X-1, X-3, X-4 and X-5 resulted in increased IL-1α production at high concentrations similar to the MUF extract, which in the case of fractions X-3 and X-4 was accompanied by a decrease in cell viability and an increase in apoptosis. In contrast, fraction X-1, decreased apoptosis and cell viability at high concentrations. Fractions X-3 and X-4 containing the highest levels of rooibos flavonoids may result in pro-oxidant effects leading to ROS production, as mentioned above, known to be closely associated with the induction of apoptosis and cytotoxicity (Galati *et al.*, 2002). However, fraction X-5, containing lower flavonoid concentrations, increased IL-1α in the absence of any effect on cell viability and apoptosis, suggesting a clear pro-inflammatory response. As mentioned in the previous section, low ATP levels, render cells un-able to undergo apoptosis but rather results in necrotic cell death. An increase in cell necrosis is associated with IL-1α release into the extracellular environment which, may augment intracellular accumulation of IL-1α in the viable HaCaT cells via positive feedback loop (Lee *et al*., 1991; Cohen *et al.*, 2010). However, this aspect should be further investigated under the current experimental conditions as mentioned above. Fraction X-2 only marginally affected cell viability and apoptosis but decreased IL-1α production at high concentrations, suggesting direct anti-inflammatory activity. Based on these results fractionation of the MUF extract successfully separated the rooibos flavonoid and non-flavonoids resulting in fractions that exhibited (i) cytotoxicity (fraction X-1), (ii) decrease cell viability and increased apoptosis (fractions X3, and X-4), (iii) pro-inflammatory (fraction X-5) and (iv) anti-inflammatory (fraction X-2) effects in the absence of UV-B irradiation.

In the presence of UV-B irradiation, the MUF extract, fractions X-1, X-3 and X-4 decreased IL-1α production at high concentrations accompanied by a further decrease in cell viability and apoptosis. UV-B is known to induce oxidative stress by generating ROS (Martinez *et al*., 2015) and thus the HaCaT cells were probably more susceptible to the cytotoxicity of MUF extract and column fractions resulting in a further decrease in cell viability and apoptosis. The latter maybe due to an increase in necrotic cell death as suggested above. The resultant reduction of UV-B induced IL1α accumulation may therefore be as a result of cell removal either by apoptosis, necrosis or a combined effect of the two processes. Of interest, fraction X-1 was very toxic and closely mimicked the effects of MUF extract despite the absence of the rooibos flavonoids. Fraction X-2 only slightly reduced IL-1α production with no effects on apoptosis while cell viability was significantly decreased in the absence of UV-B at the highest concentrations. However, unlike in the absence of UV-B, a clear anti-inflammatory effect was not evident in the presence of UV-B irradiation as IL-1α levels were not changed after treatment with fraction X-2. Fraction X-5 further increased the accumulation of IL-1α with no overt effects on cell viability and a slight increase in apoptosis, indicative of a pro-inflammatory effect by the non polar constituents in the fraction. It became evident from the above mentioned results that rooibos extracts and the column fractions of the MUF extract (X-1, X-3 and X-4) could result in a decrease in intracellular IL-1α production in HaCaT cells following UV-B exposure. This decrease could be a result of the modulation of cell growth parameters including apoptosis and/or cell necrosis.

The high cytotoxicity of the MUF extracts and some of the column fractions may result in excessive apoptosis and subsequent cell necrosis due to high levels of flavonoids thereby indirectly reducing intracellular IL-1α. Therefore, the MUF extract as well as the rooibos enriched column fractions X-3 and X-4 did not exhibit clear anti-inflammatory effects in the UV-B/HaCaT cell model. However, in the absence of UV-B their high anti-oxidant levels may be associated with pro-oxidant effects resulting in cell death as observed by the increased apoptosis and decreased cell viability. Fraction X-1 which contained no flavonoids also exhibited similar effects in relation to IL-1α both in the presence and absence of UV-B although it appeared to be more toxic compared to the flavonoid enriched fractions. Therefore, further characterisation of this fraction should be

considered in order to identify the compounds possibly responsible for the anti-oxidant effects and high toxicity. Future studies should also consider the effects of non-flavonoid and more complex oligomeric flavonoids of rooibos such as the proanthocyanidin type constituents in inhibiting inflammatory response following exposure to UV-B, as the AUF and AF rooibos extracts and fraction X-2 exhibited anti-inflammatory effects in the absence of excessive cytotoxic effects in the UV-B/HaCaT cell model. Although the fractionation of the MUF extract may have enriched the rooibos flavonoids in some fractions, these higher levels were not associated with higher activity in the UV-B/HaCaT model. Therefore, it appears as though there may be synergistic effects in the anti-oxidant activity of the rooibos flavonoids thus the use of the while extract is more beneficial. In conclusion, the anti-inflammatory properties of rooibos flavonoids and/or non-flavonoid constituents depends on the dose and subsequent effects on cell survival indices, which are important in futuristic endeavours related to the underlying mechanisms involved in the anti-inflammatory and chemopreventive properties of rooibos.

4.5. References

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5. Modulation of lipopolysaccharide-induced tumor necrosis factor alpha (TNF-α) in macrophages by different unfermented and fermented rooibos extracts and flavonoid and non-flavonoid enriched rooibos fractions.

Abstract

TNF-α is one of the primary cytokines to be released by LPS activated macrophages and subsequently drives the inflammatory response mediated through various signaling pathways. An uncontrolled inflammatory response is known to lead to chronic inflammation, which has been implicated in various diseases, including cancer. Therefore, development of anti-inflammatory products that can specifically inhibit TNF-α release may be beneficial in attenuating inflammatory diseases. The modulation of LPS-induced TNF-α release by methanol and aqueous extracts from unfermented and fermented rooibos plant material and flavonoid and non-flavonoid enriched chromatographic fractions of the unfermented rooibos methanol extract were monitored in macrophages derived from THP-1 monocytes. The rooibos extracts exhibited no overt effects on apoptosis, while cell viability was decreased at the highest concentrations tested. TNF-α release was significantly ($P<0.05$) decreased with the methanol extracts exhibiting the highest activity. However, fractionation of the methanol extract of unfermented rooibos resulting in enrichment of the major flavonoids in fractions X-3 and X-4 resulted in a decrease in cell viability and increased apoptosis, likely associated with pro-oxidant effects. Fraction X-1 which contained very low levels of rooibos flavonoids was the most effective in decreasing TNF-α followed by the flavonoid enriched fractions X-3 and X-4 as well as the non polar fraction, X-5. Fraction X-2 was the least effective as it modulated TNF- α only at the highest concentration and with no effect on cell growth indices. Since the rooibos extracts decreased TNF-α without overtly affecting cell growth indices, the study showed, for the first time that rooibos flavonoids may attenuate the LPS-induced inflammatory response in macrophages. Underlying mechanism may include the modulation of the oxidative status by the rooibos flavonoids which may involve interaction with intracellular iron known to be retained during macrophage activation, among other parameters associated with the induction of inflammation.

5.1. Introduction

Macrophages, derived from monocytes, are phagocytic cells of the innate immune system located in various tissue types including skin (Linton & Fazio, 2003). These cells are important mediators of inflammation and are recruited early during the initiation and progression of atherosclerosis, one of the main inflammatory diseases. In order to fulfill their immune modulatory function, macrophages produce a wide range of cytokines, reactive oxygen species and proteases (Linton & Fazio, 2003). Cytokines released by macrophages in response to an inflammatory stimuli include tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), IL-6, IL-8, and IL-12, which lead to the recruitment of other inflammatory cells (Duque & Descoteaux, 2014). Although macrophages are the main source of these cytokines, they are also produced by activated lymphocytes, endothelial cells, and fibroblasts. TNF-α is one of the first cytokines to be released by activated macrophages, effecting vasodilatation and increasing of vascular permeability, thereby providing favorable conditions for lymphocyte, neutrophil, and monocyte infiltration (Beutler, 1999).

NF-κB is a transcription factor important in the regulation of inflammatory genes and is activated by a wide variety of inflammatory stimuli, including TNF-α, IL-1, lipopolysaccharide (LPS) and hydrogen peroxide (H_2O_2) (Karin, 2006; Karin *et al*., 2002; Karin & Greten, 2005; Naugler & Karin, 2008). Activation occurs as a result of a cascade of events following stimulation of cells with pro-inflammatory agents. These include the activation of the inhibitor of κB (IκB), the IκB kinase (IKK), which in turn phosphorylates and degrades IκB-α. Degradation of IκB-α leads to NF-κB translocation to the nucleus and binding to a specific DNA consensus sequence. The transcriptional activation of NF-κB regulated genes subsequently results in the onset of inflammation (Naugler & Karin, 2008).

Since TNF-α release is one of the key factors in skin inflammation, the inhibition thereof may aid in the prevention of skin cancer as inflammation is one of the major causes of squamous cell carcinoma (SCC) (Scott *et al*., 2003). However, inhibiting the expression of TNF-α in TNF-α knockout mice or by using TNF-α inhibition antibodies exhibited several potentially serious side effects, hence the need to discover natural and safe

inhibitors of TNF-α (Sethi *et al*., 2009). There have been numerous studies demonstrating the modulation of key elements in cellular signal transduction pathways by polyphenols resulting in the inhibition of pro-inflammatory mediators through the inactivation of NF-κB (García-Mediavilla *et al*., 2007; Martínez-Flórez *et al*., 2005; Musonda & Chipman, 1998; Ramiro *et al*., 2005; Umesalma & Sudhandiran, 2010). For example, quercetin and other phenolic compounds have been reported to exhibit anti-inflammatory effects *in vitro* by attenuating the activation of NF-κB (García-Mediavilla *et al*., 2007; Martínez-Flórez *et al*., 2005; Musonda & Chipman, 1998; Ramiro *et al*., 2005). However, the detailed molecular mechanism of action of the NF-κB signaling pathway during an inflammatory process is still poorly understood.

The purpose of the current study was to investigate the anti-inflammatory effects of rooibos flavonoids in macrophages. The anti-inflammatory properties of different rooibos extracts prepared from fermented and unfermented plant material were investigated utilizing an LPS-induced inflammatory macrophage model with TNF-α release and the modulation of cell viability and apoptosis as endpoints. Subsequently, the potential anti-inflammatory effects of different flavonoid and non-flavonoid fractions of a methanol extract, prepared from unfermented rooibos, were assessed.

5.2. Materials and methods

5.2.1. Chemicals

RPMI-1640, Dulbecco's phosphate buffered saline (DPBS), Ultra-glutamine (UGlut), trypsin-versene, Hank's buffered salt solution (HBSS) and heat inactivated fetal bovine serum (FBS) were obtained from Lonza, Braine-l'Alleud, Belgium. Human Recombinant TNF-α ELISA kit was purchased from R&D systems, Minneapolis, USA. CellTiter-Glo luminescent cell viability and caspase-3/7 assay were purchased from Promega, Madison, USA. Triton-x100 for membrane research was obtained from Roche, Indianapolis, USA. Phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharides (LPS) were purchased from Sigma-Aldrich, St Louis, USA.

5.2.2. Plant material and preparation of extracts and XAD-4 column fractions

Unfermented and fermented rooibos plant material was purchased from Rooibos Ltd, Clanwilliam, South Africa. Methanol (MUF and MF) and aqueous (AUF and AF) extracts were prepared as described in detail (Section 3.2.2) in Chapter 3. In short, plant material was extracted by chloroform and subsequently with methanol and solvents evaporated at 40 °C. The aqueous extracts were prepared by steeping the plant material in boiled water and filtrates freeze dried. All the samples were stored in desiccator at room temperature. The MUF extract, prepared as described in section 3.2.3.1, was fractionated by column chromatography in an XAD-4 amberlite resin, as described in Chapter 3, using a stepwise methanol water gradient. Thin layer chromatography resulted in combination of fractions with similar non-flavonoid and flavonoid constituents yielding five main fractions, which were subsequently dried *in vacuo* at 40 ºC and stored in airtight containers at 4 °C.

5.2.3. Cell maintenance

Human monocytic leukaemia (THP-1) cells were purchased from the European collection of cell cultures (ECACC). Cells were grown in RPMI-1640 supplemented with 10 % fetal bovine serum (FBS) and 2 mM Ultra-Glutamine (U-GLUT) at a density between 300 000 and 900 000 cells/ml. Cells were incubated in the presence of 100 ng/ml of PMA for 72 hrs to stimulate differentiation, subsequently rinsed with 100 µl of HBSS and equilibrated for 24 hrs in fresh RPMI supplemented with 0.5% FBS and 2 mM U-GLUT. For experiments, cells were seeded at a density of 20 000 cells/well in 96 well microtiter plates for the cell viability assay (solid white plates) as well as TNF-α and apoptosis determinations (clear tissue culture plates).

5.2.4. Experimental conditions

5.2.4.1. LPS stimulation and extract and/or column fraction treatments

LPS (10 ng/ml) was added in the presence of different concentrations of the rooibos extracts and/or column fractions to stimulate TNF-α release and monitor the modulating effect of the rooibos samples on TNF-α release. Rooibos extracts and the column fractions were prepared in RPMI-1640 supplemented with 0.5 % FBS, U-GLUT (2 mM) to a concentration of 2 mg/ml and final DMSO concentration of 0.5 %. Samples were sterilized using minisart syringe filters (0.2 µm) (Sartorius Stedim Biotech GmbH, Goettingen, Germany) prior to conducting the experiment. The extracts and/or column fraction preparations were diluted to concentrations ranging from 0.025 mg/ml to 0.8 mg/ml in the culture medium containing 0.5 % DMSO. Cells treated with the culture medium (0.5% DMSO) in the presence of LPS (10 ng/ml) were used as the positive control.

5.2.5. Experimental End points

After 6 hrs of incubation in the presence of 10 ng/ml LPS and the different rooibos samples, the supernatant was transferred into different 96 well plates for TNF-α determination. The remaining cells were washed with 150 µl of DPBS and stored in 150 µl of 0.5 % triton at -80 °C for caspase-3/7 activity determination. Samples were allowed to thaw at 37 °C on a heating block and vortexed for 30 sec to facilitate lyses. For the determination of cell viability of the extracts and/or column fractions separate plates were used. The positive control was used as a bench mark and set at 100 % for ATP production and the fold increase of TNF-α and caspase-3/7 activities set at 1.

5.2.5.1. Cell viability assay

Cell viability was determined using a CellTitre-Glo luminescent kit to monitor ATP content according to the manufacturer's instructions. After incubation with extracts and fractions, 100 µl of the ATP reagent was added to the wells containing 100 µl medium

as described in Chapter 4 and the ATP content was determined using the equation below.

$$\% \text{ ATP content} = \frac{\text{Relative light units (treatment samples)}}{\text{Relative light units (+LPS)}}$$

5.2.5.2. TNF-α assay

TNF-α was quantified using human recombinant TNF-α ELISA kit according to the manufacturer's instructions. TNF-α standards and cell lysates were prepared (Section 5.4.2.1) and assayed in duplicate in 96 well ELISA plates. Absorbance was measured at 450 nm using the biotek synergy HT microplate reader (Winooski, Vermont, USA). Data was analysed using the standard curve generated from Gen5™ Data Analysis Software (version 2 for Windows). Extracellular TNF-α was expressed as pg/ml of cell lysate and as the fold increase compared to the untreated control (-LPS).

$$\text{TNF-α release} = \frac{\text{TNF-α (treatement sample)}}{\text{TNF-α (+LPS)}}$$

5.2.5.3. Caspase 3/7 assay

Caspase 3/7 activity was determined using the Caspase-Glo 3/7 assay as described in Chapter 4 with minor modifications. The lysates and the assay substrate were incubated for one hour instead of 30 min to obtain optimal luminescence development in the macrophages. Induction of apoptosis was expressed as fold increase compared to the positive control, i.e. cells exposed to LPS in the absence of the rooibos extracts and/or column fractions.

$$\text{Fold change} = \frac{\text{Relative light units (treatment samples)}}{\text{Relative light units (+LPS)}}$$

5.2.6 Statistical analysis

Details of the statistical analyses performed are described in Chap 4 section 4.2.6

5.3. Results

5.3.1. LPS modulation of TNF-α and cell viability parameters

Exposure of THP-1 monocytes to LPS resulted in a 17.3% reduction in cell viability following the 6-hrs treatment. The TNF-α release was significantly ($P<0.05$) increased (approximately 7-fold), which was associated with a 2-fold increase in apoptosis (Table 5.1).

Table 5.1 LPS-induced TNF-α accumulation in THP-1 macrophages in relation to the effect on cell viability and apoptosis.

Treatments	Cell viability	TNF-α release		Caspase-3/7 activity
	(% ATP production)	(pg/ml)	Fold	Fold
(-) LPS	117.3±9.8A	67.0±13.5A	0.14±0.02A	0.59±0.08A
(+) LPS	100±9.7B	431.0±53.1B	1.00±0.03B	1.00±0.18B

(-) LPS represents the cells not exposed to LPS representing the negative control; (+) LPS represents cells treated with LPS representing the positive control. The (-) LPS values are calculated using the positive control as a bench mark set at 100 % for ATP production with the fold increase of TNF-α and caspase-3/7 activities set at 1.

Table 5.2 Modulation of TNF-α accumulation by different extracts of rooibos in relation to their effect on cell viability (% ATP content) in THP-1 macrophages exposed to LPS.

Extract	Concentration (mg/ml)	Cell viability	TNF-α		Caspase-3/7 activity
		% ATP production	(pg/ml)	Fold	Fold
		100±9.7^{A}	431.0±53.1^{A}	1.00±0.03^{A}	1.00±0.18^{A}
MUF	0.05	88.1±9.1^{B}	98.1±13.1^{B}	0.27±0.06^{C}	1.11±0.10^{A}
	0.1	81.4±10.2^{B}	74.1±10.0^{C}	0.21±0.06^{C}	1.15±0.24^{A}
	0.2	67.8±9.5^{C}	73.1±10.8^{C}	0.30±0.05^{C}	1.56±0.66^{A}
MF	0.05	92.8±14.0^{A}	72.5±7.9^{c}	0.15±0.02^{D}	1.06±0.05^{A}
	0.1	90.2±12.6^{A}	60.3±16.5^{C}	0.12±0.03^{D}	1.22±0.28^{A}
	0.2	79.6±13.6^{B}	73.4±11.6^{C}	0.16±0.03^{D}	1.13±0.25^{A}
AUF	0.05	83.0±11.4^{B}	171.1±13.7^{D}	0.42±0.07^{B}	1.12±0.15^{A}
	0.1	77.8±10.7^{B}	177.2±22.8^{D}	0.47±0.15^{B}	1.24±0.26^{A}
	0.2	69.9±12.2^{C}	145.1±29.1^{D}	0.36±0.08^{B}	1.86±0.89^{A}
AF	0.05	92.3±11.5^{A}	254.3±21.6^{E}	0.54±0.09^{B}	1.00±0.27^{A}
	0.1	82.1±5.6^{B}	121.1±16.0^{F}	0.27±0.05^{C}	1.17±0.29^{A}
	0.2	77.9±9.3^{B}	65.4±23.2^{C}	0.16±0.05^{D}	1.02±0.00^{A}

% ATP production was calculated using the LPS treated cells as a positive control set at 100%. TNF-α and caspase-3/7 fold was calculated against the same positive control set to 1. Values with differing letters in a column are significantly different from each other, if at least one letter is the same between values then there is no significant difference. Abbreviations: TNF-α-tumor necrosis factor alpha; MUF-methanol unfermented; MF-methanol fermented, AUF-aqueous unfermented; AF-aqueous fermented

5.3.2. Modulation of TNF-α release and cell growth parameters by rooibos extracts

(i) Effect on cell viability

The MUF and AUF extracts decreased ($P<0.05$) cell viability at all the concentrations when compared to the LPS treated macrophages (Table 5.2). The MF and AF extracts, however only decreased cell viability at the highest and two highest concentrations.

(ii) Effect on TNF-α release

Exposure to all the different rooibos extracts resulted in a significant ($P<0.05$) decrease in TNF-α release. The methanol extracts were more effective in decreasing TNF-α release when compared to the aqueous extracts, except for the highest concentration of the AF extract. Both the MF and AF extracts were more effective ($P<0.05$) than their unfermented counter parts when considering the fold decrease. Of interest is that the highest concentration of the AF exhibited a similar fold decrease as the MF extract.

(iii) Effect on apoptosis induction

None of the extracts significantly increased apoptosis, although a slight non-significant increase was noticed at the highest concentrations for the MUF and AUF extracts.

5.3.3. Modulation of TNF-α release and cell growth parameters by unfermented rooibos XAD-4 column fractions (Table 5.3).

(i) Effect on cell viability

The MUF extract and fraction X-2 at all concentrations tested had no significant effect ($p<0.05$) on cell viability, while fraction X-1 resulted in a significant decrease in cell viability only at the highest concentration (0.8 mg/ml). When compared to the MUF extract, fraction X-3 significantly ($P<0.0$) decreased cell viability at a concentration level above 0.4 mg/ml, while fractions X-4 and X-5 decreased cell viability at concentrations greater than 0.2 mg/ml. The non-polar fraction X-5 was the most active in reducing cell viability and became insoluble in the culture medium at concentrations above 0.4 mg/ml.

Table 5.3 Modulation of TNF-α accumulation by the unfermented methanol extract and different column fractions in relation to their effect on cell growth parameters in the presence of LPS

Treatments	Extract/Fractions (mg/ml)	Cell viability	TNF-α		Caspase-3/7 activity
		% ATP production	pg/ml	Fold	fold increase
		100,0±6.1^{A}	352.0±44.0^{A}	1.00±0.08^{A}	1,00±0,11^{A}
MUF	**0.1**	97.6±10.9^{A}	295.2±54.9^{C}	0.88±0.16^{A}	1,08±0,19^{A}
	0.2	103.4±5.6^{A}	96.7±16.4^{E}	0.18±0,05^{D}	0,75±0,15^{B}
	0.4	95.2±7.8^{A}	<10*	-	0,75±0,16^{B}
X-1	**0.05**	110.2±9.4^{A}	280.0±37.2^{C}	0.88±0.15^{A}	1,02±0,11^{A}
	0.1	113.2±10.8^{A}	209.4±51.5^{C}	0.34±0.09^{C}	1,06±0,16^{A}
	0.2	119.8±13.5^{A}	13.7±1.8^{F}	-	0,84±0,14^{A}
	0.4	113.5±15.6^{A}	<10	-	0,75±0,06^{B}
	0.8	82.3±8.7^{B}	<10	-	0,97±0,13^{A}
X-2	**0.05**	93.4±14.4^{A}	426.4±66.8^{A}	1.28±0.31^{A}	1,03±0,13^{A}
	0.1	96.3±11,2^{A}	415.8±47.7^{A}	1.20±0.27^{A}	0.94±0,17^{A}
	0.2	96.2±12.3^{A}	382.5±45.4^{A}	1.19±0.20^{A}	0,94±0,12^{A}
	0.4	97.0±10.6^{A}	306.7±71.8^{B}	0.90±0.19^{A}	0,94±0,18^{A}
	0.8	98.7±14.9^{A}	268.4±46.2^{C}	0.99±0.11^{A}	0,87±0,11^{A}
X-3	**0.05**	98.5±9.8^{A}	372.6±44.4^{A}	1.10±0.22^{A}	0,89±0,17^{A}
	0.1	94.1±6.4^{BA}	341.4±53.5^{A}	1.05±0.24^{A}	0,97±0,13^{A}
	0.2	90.7±7.1^{A}	306.4±37.7^{B}	0.95±0.06^{A}	1,26±0,11^{C}
	0.4	86.4±8,6^{B}	243.4±44.9^{C}	0.79±0.09^{B}	1,38±0,24^{C}
	0.8	82.6±5.7^{B}	<10	-	1,40±0,25^{C}
X-4	**0.05**	89.2±16.7^{A}	291.6±74.6^{A}	1.04±0.16^{A}	0,71±0,13^{B}
	0.1	85.5±15,3^{A}	385.8±83.5^{A}	0.91±0.10^{A}	0,96±0,17^{AB}
	0.2	82.3±8.7^{B}	358.0±54.7^{A}	1.16±0.25^{A}	1,01±0,16^{A}
	0.4	76.9±8.8^{B}	245.5±61.5^{C}	0.97±0.15^{A}	1,20±0,13^{C}
	0.8	69,6±6,7^{C}	<10	-	2.02±0,54^{D}
X-5	**0.025**	99.1±13.5^{A}	410.2±60.5^{A}	0.92±0.10^{A}	1,03±0,17^{A}
	0.05	97.5±11.4^{A}	343.3±77.1^{A}	1.05±0.16^{A}	1,08±0,23^{A}
	0.1	82.7±6.1^{B}	276.7±36.9^{IC}	0.95±0.09^{A}	1,26±0,24^{C}
	0.2	76.6±9.9^{B}	233.1±42.7^{C}	0.92±0.07^{A}	1,68±0,06^{D}
	0.4**	60.6±7.4^{C}	181.0±37.5	0.98±0.11^{A}	1,71±0,19^{D}

% ATP production was calculated using the LPS treated cells as a positive control set to 100%. TNF-αandcaspase-3/7 fold was calculated against the same positive control set to 1. Values with differing letters in a column are significantly different from each other, if at least one letter is the same between values then there is no significant difference. *Not detected. **X-5 was not soluble beyond 0.4 mg/ml. Abbreviations: MUF-Methanol unfermented rooibos extract, X-XAD-4 fraction, LPS-lipopolysaccharide, TNF-α-tumor necrosis factor alpha.

(i) Effect on TNF-α release

The MUF extract was more active in reducing TNF-α than all fractions except X-1 at similar dose (0.2mg/ml). Fraction X-1 was the most effective in decreasing TNF-α at all concentrations tested, exhibiting a typical dose response effect, while fraction X-2 was the least effective in decreasing TNF-α when considering concentrations above 0.4mg/ml. The MUF extract and fractions X-3 and X-4 decreased TNF-α release beyond the detection limit at the highest concentration as well as fraction X-1 at the two highest concentrations.

(ii) Effect on apoptosis induction

The MUF extract significantly ($p<0.05$) decreased apoptosis at higher concentrations, while fraction X-1 and X-2 had no significant effect. Fractions X-3, X-4 and X-5 significantly ($p<0.05$) induced apoptosis at the two highest concentrations.

5.4. Discussion

The pro-inflammatory effects of TNF-α are mainly manifested through the activity of TNF-α receptors, specifically TNF-R1 stimulating the release of other pro-inflammatory cytokines such as interleukin-1, (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and platelet derived growth factor, to name a few (Köck *et al*., 1990; Wu & Zhou, 2010). The expression of TNF-α, which is predominantly released from LPS activated microphages, has been reported to be increased in various diseases and is also implicated in all stages of tumor development (Arnott *et al*., 200). Several studies have reported the resistance of TNF-α knockout mice to developing tumors and the inability of cell to develop tumors when treated with TNF-α antibody which validates its role in tumorigenesis (Arnott *et al*., 2003; Baer *et al*., 1998; Moore *et al*., 1999; Wu & Zhou, 2010). Therefore, the inhibition of TNF-α expression is essential to block early stages of carcinogenesis.

In the current study, PMA differentiated THP-1 monocytes had a relatively low expression of TNF-α, which upon stimulation with LPS for 6 hours resulted in an approximately 7-fold increase. This activation of the innate immunity and inflammation is

thought to involve toll like receptor (TLR) signaling, specifically TLR4, which subsequently induces gene expression via the activation of NFκB (Akira, 2003; Wright, 1999). There are numerous inhibitors of TNF-α that have been reported for treatment of diseases such as inflamed bowel disease, psoriasis and rheumatoid arthritis (Sethi *et al*., 2009). However, these drugs exhibit severe side effects leading to the need to develop natural inhibitors of TNF-αthat can be as effective (Sethi *et al*., 2009). For example, resveratrol, an antioxidant component of grape, has been reported to exert anti-inflammatory activities in macrophages through its ability to inhibit LPS-induced expression of pro-inflammatory markers including TNF-α (Bi *et al*., 2005). Quercetin, found in small quantities in rooibos, has also been reported to decrease LPS-stimulated release of TNF-α in raw 264.7 macrophages (Wadsworth & Koop, 1999). In addition, another rooibos polyphenol, luteolin was reported to decease serum levels of TNF-α in mice treated with LPS (Kotanidou *et al*., 2002) and was shown to suppress LPS-induced expression of various pro-inflammatory mediators, including TNF-α (Chen *et al*., 2007). The above reports on the anti-inflammatory effects of rooibos flavanoids highlight the importance of the current study to further elucidate the anti-inflammatory, which have been lacking in literature.

Co-exposure of THP-1 derived macrophages to LPS and rooibos extracts decreased TNF-α release accompanied by a slight decrease in cell viability, while apoptosis was not affected. The methanol extracts were generally more effective in decreasing TNF-α levels compared to the aqueous extracts implying a role of anti-oxidant properties of the flavonoid constituents (chapter 3). However, in contrast to the HaCaT cells (Chapter 4) the MF extract was more active than the MUF extract at the lowest concentration in the absence of any effects on the cell viability parameters. A similar effect was noticed with the aqueous extracts with AF exhibiting a higher TNF-α inhibitory activity than the AUF at higher concentrations. This suggests that during fermentation of rooibos, the oxidation of the major monomeric flavonoid constituents with an associated decrease in antioxidant activity may be associated with an increased inhibition of TNF-α release inhibitory activity. Due to the absence of apoptosis and with no significant decrease in cell viability, when compared to keratinocytes (Chapter 4), all the rooibos extracts (MUF,

MF, AUF and AF) seem to exhibit direct anti-inflammatory properties on the LPS/microphage inflammatory model.

Of the XAD-4 fractions, X-1, the most polar fraction, was the most effective in decreasing TNF-α release even exceeding the activity of the MUF extract, without affecting apoptosis and cell viability. Of interest is that, at the two highest concentrations the TNF-α level was reduced below the detection limit. In contrast, fraction X-2 only significantly ($P<0.05$) reduced TNF-α at the 2 highest concentrations. Fractions X-3 and X-4 completely inhibited TNF-α release at the highest concentration accompanied by a slight decrease in cell viability and a significant increase in apoptosis. Fraction X-5, the less polar fraction, exhibited the highest decrease in cell viability and significantly ($P<0.05$) induced apoptosis, while TNF-α release was only decreased at the highest concentrations similar to fraction X-2. With respect to the current model, no clear relationship seems to exist between the anti-oxidant potency of the rooibos flavonoids and anti-inflammatory effects. In this regard the MUF extract and the flavonoid enriched rooibos column fractions X-3 and X-4 exhibited the highest antioxidant properties as well as contained the bulk of the rooibos flavonoids which include the highly active anti-oxidant, aspalathin. Fermentation is known to reduce the anti-oxidant and the aspalathin levels which provide interesting opportunities to further elucidate the anti-inflammatory components of fermented rooibos.

The high TNF-α inhibitory activity of X-1, the most polar fraction, and the AF extract suggests that more polar constituents in rooibos seems to play an important role in the proposed anti-inflammatory effects. High levels of non-flavonoid “tannin-like” and “sugar-like” compounds in aqueous fermented rooibos have been reported and need to be taken into account when evaluating the effects of fraction X-1 and the AF extract (Joubert *et al.*, 2008). In the present study the flavonoid-enriched fractions (X-3 and X-4) were less effective at decreasing TNF-α but more effective at reducing cell viability and inducing apoptosis when compared to the MUF extract and fraction X-1 at the same concentration levels. It is therefore likely that upon fractionation, the enriched flavonoids in the fractions resulted in an increase pro-oxidant effects, which subsequently increased the induction of apoptosis and decreased cell viability, masking the direct

anti-inflammatory effects of the rooibos MUF extract. Therefore, fractionation separated the rooibos flavonoids to groups exhibiting direct anti-inflammatory properties (X-1) and indirect anti-inflammatory properties (X-3, X-4 and X-5).

The interaction of activated macrophages and polyphenolic constituents also need to be considered from a different perspective. LPS activated microphages have been reported to increase intracellular iron. Apart from antioxidant effects, rooibos flavonoids also are known to exhibit iron chelating properties (Snijman *et al*., 2009) and may interact with intracellular iron altering the redox status as well as iron homeostasis in the macrophages following LPS activation. These effects could inhibit NFκβ since the activation of the latter has been reported to occur at two levels of redox regulation, one in the nucleus and the other in the cytoplasm (Toledano & Leonard, 1991, Xiong *et al*., 2003). The result would be a subsequent decrease in the transcription of pro-inflammatory genes. Of interest is that transcription factors such as NFκB and AP-1, have been reported to be activated by LPS through various signaling pathways (Lu *et al*., 2008). Redox regulation in the cytoplasm is likely to involve the mitochondria and the interactive role of Fe and rooibos flavonoids during inflammation should be further explored.

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6. General discussion

General Discussion

Increased oxidative stress and inflammation have been identified as key events during cancer promotion (Federico *et al*., 2007; Lu *et al*., 2006; Reuter *et al*., 2010). Inflammation in the skin can be in response to various stimuli i.e. infection by bacteria or fungi, chemical irritants or exposure to ultraviolet light (Suter *et al*., 2009). Chronic inflammatory responses involving generation of oxidants such as superoxide anions, hydroxyl radicals, hydrogen peroxide and nitric oxide may damage normal tissue resulting in induction of signalling molecules and enzymes leading to the development of degenerative diseases (Santangelo *et al*., 2007). These include neurological diseases, metabolic disorder, cardiovascular disease and cancer.

The South African herbal tea, rooibos, has long been reported to exhibit beneficial health properties including anti-microbial, anti-diabetic, anti-inflammatory and anti-carcinogenic effects (Joubert *et al.*, 2008). Studies investigating the anticancer effects of rooibos have reported the ability of rooibos to inhibit oesophageal squamous cell carcinogenesis in rats as well as skin tumor promotion in mice, implying the disruption of growth regulatory parameters in pre-cancerous lesions (Marnewick *et al*., 2005; Sissing *et al*., 2011; Petrova *et al*., 2011). However, studies on the anti-inflammatory properties of rooibos are limited.

The aim of the current study was to use *in vitro* inflammatory models, the UV-B/HaCaT (Magcwebeba *et al*., 2012) and the LPS/Macrophage (Keet, 2015) cell models to evaluate the anti-inflammatory properties of rooibos methanol and aqueous extracts as well as flavonoid and non-flavonoid enriched fractions prepared from an unfermented methanol extract.

6.1. Role of extraction solvent on chemical characteristics and antioxidant activities

The total polyphenol (TP) and flavanol (FLAVA) content of methanol and aqueous extracts, prepared from unfermented and fermented rooibos, were determined and the concentration of the monomeric flavonoids quantified by HPLC. The methanol extract of

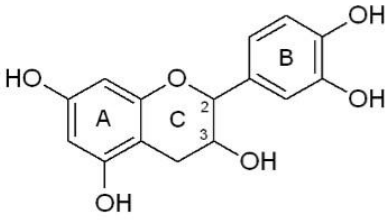
unfermented rooibos, MUF, containing the highest concentration of monomeric polyphenolic compounds, exhibited the highest antioxidant activity in the FRAP and ABTS assays.

The major rooibos flavonoids, predominantly found in the unfermented plant material, were mostly recovered in methanol, which is in accordance with the notion that polyphenols are more soluble in non polar solvents (Franco *et al*., 2008; Metivier *et al*., 1980). The aqueous extract contained mainly the oxidised flavone products of aspalathin and nothofagin and it is also known to contain polymeric tannin-like substances. It has been reported that an aqueous fermented rooibos extract consists of up to 50 % complex tannin-like substances, compared to the 14% in a methanol extract of unfermented rooibos (Joubert *et al*., 2008). Hence the significant reduction in the TP and FLAVA content and anti-oxidant activity were observed in the MF and AF extracts with no significant difference between them. It would appear that fermentation slightly favours the solubility of the fermented constituents, presumably the tannin-like compounds, in an aqueous solvent. The polarity of the extraction solvent therefore, determines the nature of the polyphenols extracted and the resultant antioxidant properties of the extract.

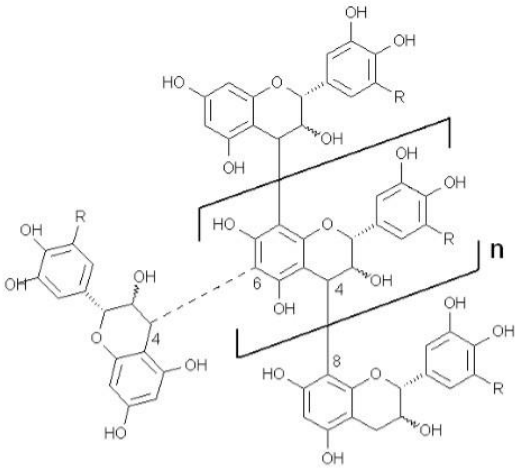
In summary the high anti-oxidant properties of rooibos were associated with a high TP and FLAVA content as well as high monomeric flavonoids while fermentation resulted in a significant decrease in all these parameters. Methanol as a solvent seemed to be important in determining the antioxidant properties of the extract in the unfermented rooibos. However, the more water soluble non flavonoid constituents in rooibos i.e. polymeric tannin-like compounds (Fig 6.1) became more prominent role players in the fermented rooibos. In support of this, the presence of several non flavonoid carboxylic acids compounds such as the hydroxyl cinnamates or cinnamic acids and hydroxybenzoic acid, mainly found in red wine (Cheynier *et al*., 2006) has been previously reported in rooibos (Rabe *et al*., 1994). In addition, oligosaccharides and polysaccharides constituents have also been reported present in rooibos even though these compounds have received little research attention and hence have not been fully characterised (Nakano *et al*., 1997).

6.2. Structure activity relationships of flavonoids

Based on the high levels of rooibos monomeric flavonoids and the high antioxidant properties, the MUF extract was further fractionated by XAD-4 column chromatography using a stepwise methanol/water gradient (Chapter 3). Five fractions of decreasing polarity were collected with the major rooibos flavonoids enriched in fractions X-3 and X-4 HPLC analysis indicated that aspalathin eluted prior to nothofagin while aspalathin and the oxidised products, orientin and iso-orientin, were equally distributed between fractions X-3 and X-4 (Fig 6.2). The majority of the oxidised products of nothofagin eluted with the parent compound in fraction X-4 together with the rooibos flavonols. The flavonoid enriched fractions also exhibited the highest antioxidant activities. The orto position of hydroxyl groups in the B ring allows aspalathin to readily form an enol, which increases the polarity of aspalathin compared to nothofagin (Rabe *et al*., 1994) and therefore facilitating its elution prior to nothofagin, lacking the catechol arrangement.



Catechin



Condensed tannins
(pro-anthocyanindins)

Figure 6.1. Chemical structure of condensed tannins consisting of basic catechin moieties (Adapted from Schofield *et al*., 2010)

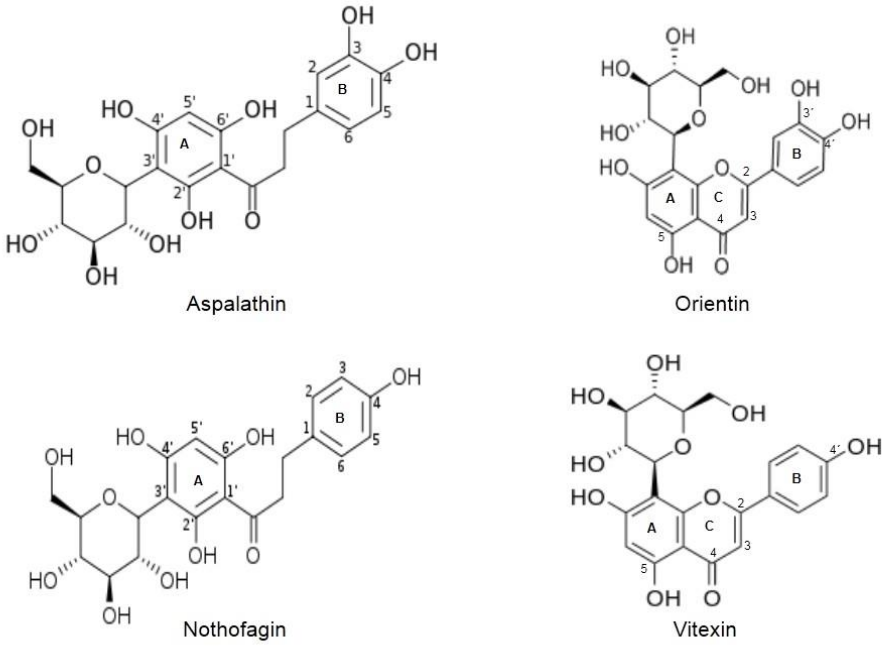


Figure 6.2 Chemical structure of the main rooibos flavonoids and some of their respective oxidised products. (Adapted from Snijman*et al*., 2009)

Structural features known to play an important role in the antioxidant properties of rooibos flavonoids (Fig 6.3A) include the C3'-C4' dihydroxy configuration (ortho-dihydroxy functionality) in the B-ring, the C2=C3 double bond and 4-keto group in the C-ring and the C5-OH group in the A-ring (Dai & Mumper, 2010; Snijman *et al.*, 2009; Joubert & Ferreira, 1996). In addition, flavonoids are also known to exhibit metal chelation properties as they possess a catechol in the B ring, the C-4 keto group (C-ring) and the C-5 hydroxy group (A-ring), which are recognised binding sites for metal ions (Fig 6.3B). Metal chelating could play a significant role in the relative antioxidant potency of the rooibos flavonoids in membrane environments (Snijman *et al*., 2009).

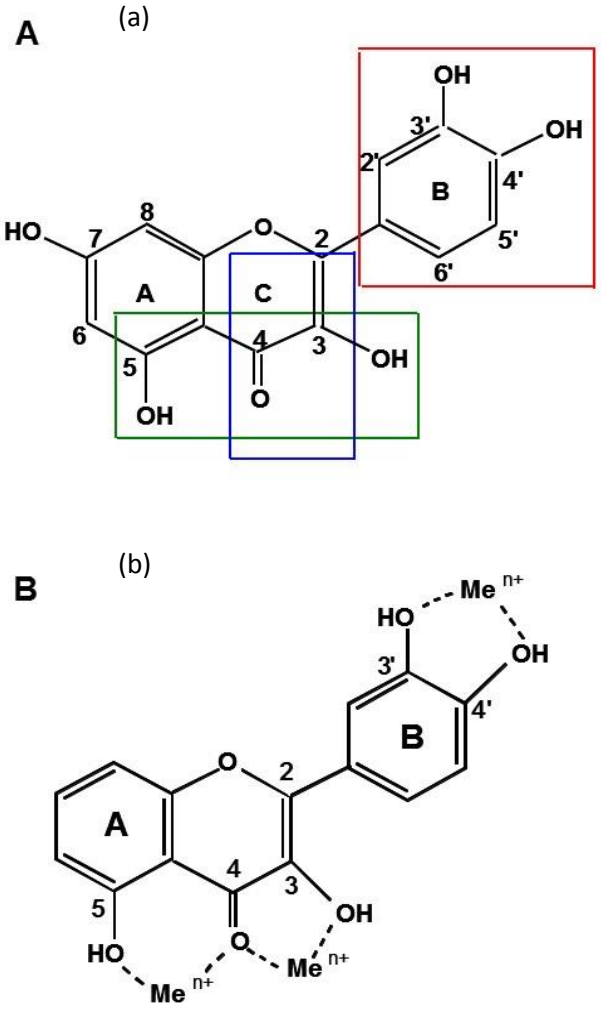


Figure 6.3. (a) Key flavonoid structural requirements for effective radical scavenging (Bors et al., 1990). (b). Trace metals (Me^{n+}) binding sites in flavonoids (Pietta, 2000)

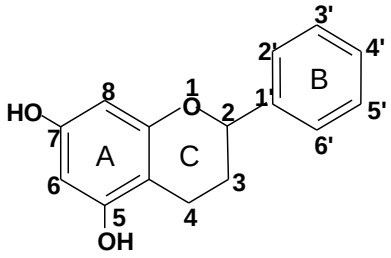
The potent antioxidant properties of aspalathin has been associated with the 2', 6' dihydroxy groups (A-ring), keto-enoltautomerisation of the carbonyl and α-methylene groups as well as the 3, 4 hydroxyl groups (B ring), which are important for the high potency to scavenge free radicals by the dihydrochalcones (Rezk *et al*., 2002). This would explain the high antioxidant properties of fractions X-3 and X-4 containing high levels of aspalathin and nothofagin. In addition, the corresponding flavones of

aspalathin, orientin and iso-orientin, as well as the other rooibos flavonoids are less effective antioxidants (von Gadow*et al*., 1997) as they lack the 6´-OH group in the A ring, although they retain the 3',4' dihydroxy group (B ring) and the C2=C3 double bond (C ring) (Table 6.1). The antioxidant activity of the flavones derived from nothofagin, the flavones vitexin and isovitexin, was also less active as they lack the 3'-hydroxy on the A ring (Krafczyk *et al.*, 2009; Snijman *et al*., 2009). Hence, fractions containing more aspalathin and nothofagin exhibited the highest antioxidant activity compared to the more non-polar X-5 containing far less of the rooibos flavonoids. The polar fractions, X-1 and X-2, contained low levels of the major rooibos flavonoids and are likely to contain the more water soluble, non-flavonoid, constituents of rooibos such as the tannin-like comounds, hydroxy cinnamates and hydroxy benzoic acid constituents. Although rooibos extracts have been reported to exhibit anti-inflammatory activity (Joubert *et al*., 2008) it is not known whether the rooibos flavonoid and non-flavonoid constituents are involved. Against this background the anti-inflammatory effects of the extracts and XAD-4 fractions were evaluated in UV-B/HaCaT keratinocyte and the LPS/macrophages cell inflammatory models utilising IL-1α accumulation and TNF-α release as endpoints, respectively.

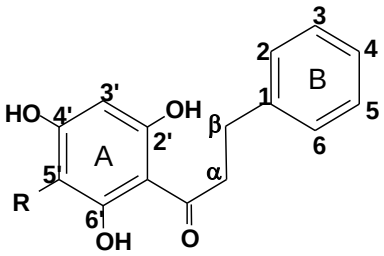
6.3. Inhibition of UV-B induced IL-1α in HaCaT cells

Chronic UV-B exposure is one of the key risk factors predisposing to skin cancer development, possibly as a result of prolonged inflammatory responses (Aggarwal & Gehlot, 2009). Specific cytokines have been identified as key role players of inflammation. These include the pro-inflammatory cytokines interleukin 1 alpha (IL-1α), tumor necrosis factor alpha (TNF-α) and IL-6 (Shaikh, 2011). In the keratinocytes, IL-1α is constitutively produced and only released during disease states making it a good candidate to measure the extent of damage as upon damage the released IL-1α could further enhance an inflammatory response leading to chronic inflammation (Luo *et al.,* 2004).

Table 6.1. Structural differences amongst flavonoids



Flavonoid



Dihydrochalcone

	C_2=C_3	C_3	C_4C=O	C_6	C_8	B ring substitution
Dihydrochalcone						
Aspalathin						3, 4-dihydroxy
Nothofagin						4-hydroxy
Flavone						
Luteolin	+	-	+	-	-	3´, 4´-dihydroxy
Chrysoeriol	+	-	+	-	-	4´-hydroxy-3´-methoxy
Orientin	+	-	+	-	CG	3´, 4´-dihydroxy
Isoorientin	+	-	+	CG	-	3´, 4´-dihydroxy
Vitexin	+	-	+	-	CG	4´-hydroxy
Isovitexin	+	-	+	CG	-	4´-hydroxy
Flavanol						
(+)-Catechin	-	OH	-	-	-	3´, 4´-dihydroxy
Flavonol						
Quercetin	+	OH	+	-	-	3´, 4´-dihydroxy
Isoquercitrin	+	OG	+	-	-	3´, 4´-dihydroxy
Hyperoside	+	OGa	+	-	-	3´, 4´-dihydroxy
Rutin	+	ORu	+	-	-	3´, 4´-dihydroxy

ORu: O-rutinosyl; OG: O-glucopyranosyl; OGa: *O*-galactosyl; CG/R: C-glucopyranosyl. (Adapted from Snijman *et al*., 2009).

In the present study, in the absence of UV-B, the methanol extracts of both fermented and unfermented rooibos increased IL-1α, which was accompanied by a decrease in cell viability and an increase in apoptosis at the highest concentrations, with the unfermented extract (MUF) exhibiting the highest activity. At higher concentrations all the fractions, except for X-2, increased IL-1α, which was also associated with decreased cell viability and increased apoptosis. This may be regarded as a pro-inflammatory effect. Treatment with the column fractions indicated that, at an equal concentration, the non-polar fraction, X-5, exhibited similar responses to the MUF extract as it also significantly increased IL-1α. The polyphenol enriched fractions X-3 and X-4 also increased IL-1α, although to a far lesser extent. Furthermore, the high levels of rooibos flavonoids in the unfermented extracts and the flavonoid enriched column fractions, which have high anti-oxidant activity and a major effect on cell viability, could mask their effect on the inflammatory response. The high anti-oxidant levels in the MUF extract as well as flavonoid enriched fractions may lead to changes in the cellular redox resulting in pro-oxidant effects and subsequent induction of apoptosis. It appears that the rooibos flavonoids may act as “double edged swords” when considering the cellular redox state. The type of flavonoids, with respect to its antioxidant potency and the dosage, may be determining factors impacting the balance between beneficial or deleterious effects as previously suggested (Bouayed & Bohn, 2010).

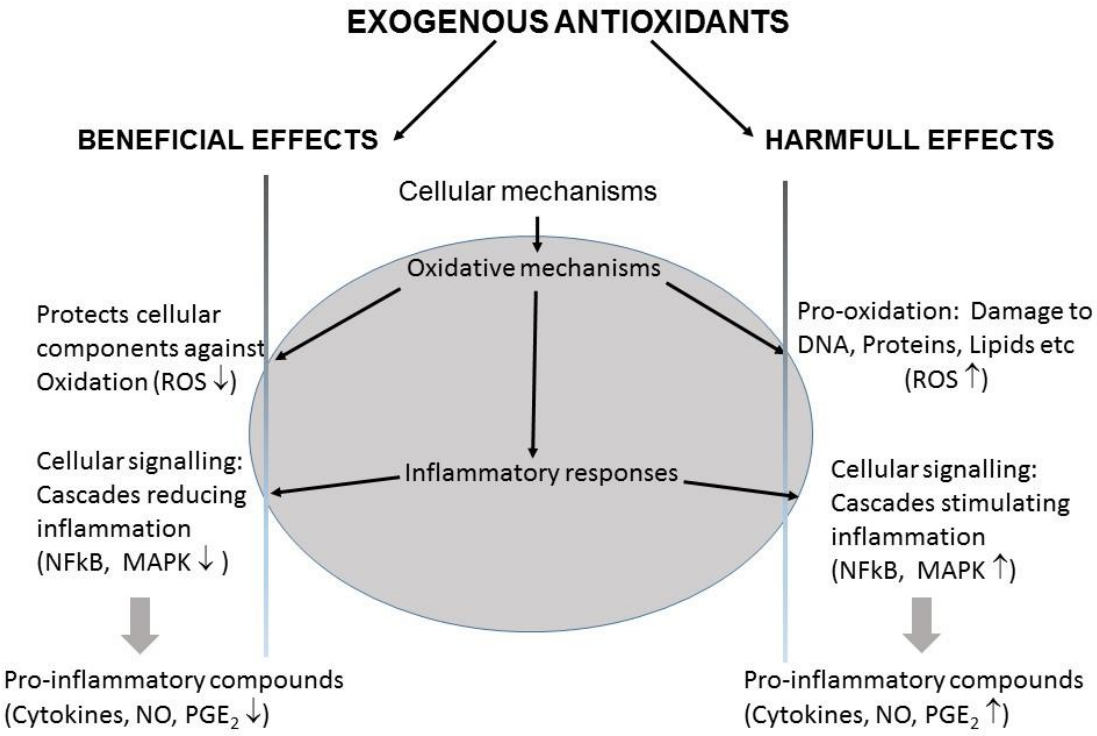


Figure 6.4 Schematic diagram illustrating the “double edged sword” concept with respect to beneficial antioxidant and anti-inflammatory effects contrasting by the dose dependent adverse effects on these parameters (Adapted from Bouayed & Bohn, 2010)

The MF and AF extracts as well as the polar column fractions were less effective in reducing cell viability presumably due to the lower flavonoid content in comparison to the fermented extracts and the flavonoid enriched fractions. Specifically, fraction X-2 closely mimicked the effect of the AUF and AF rooibos extracts as IL-1α was significantly reduced and minimal effects on cell viability and apoptosis were observed. The decrease in IL-1α by the aqueous extracts and fraction X-2 could be indicative of a direct inhibition on IL-1α suggesting an anti-inflammatory effect. Since fraction X-2 is non-polar containing low levels of the known rooibos flavonoids (Chapter 3) it possibly contains anti-inflammatory compounds similar to those in the aqueous extracts of

rooibos. It has been reported that a rooibos aqueous extract increased the anti-OVA IgM levels in sera of BALB/c mice *in vivo* (Ichiyama *et al*., 2007). This effect has been reported to suppress the formation of IL-2 and IFN-γ by T cells (Ichiyama *et a*l., 2007). These immunological effects were not associated with the presence of the major rooibos flavonoids such as aspalathin, orientin and rutin, but rather with the presence of oligosaccharides and polysaccharides, the polar constituents in fermented rooibos. Therefore, the involvement of the polar rooibos constituents on inflammatory responses should be further investigated.

Of interest are the effects of fraction X-1 (polar) and X-5 (non-polar) where X-1 increased IL-1α accompanied by a drastic decrease in cell viability and apoptosis, while fraction X-5 also increased IL-1α but with a reduced effect on the cell growth indices. Therefore, fraction X-5 seems to contain typical pro-inflammatory constituents, while the drastic decrease in cell viability by X-1 may be related to cell necrosis and the release of IL-1α into the culture media. The latter could result in stimulation of the cytokine production in the remaining viable cells via positive feedback (Ninomiya-Tsuji *et al*., 1999; Cohen *et al*., 2010). It has been reported that, when ATP levels fall below a certain level, apoptosis cannot be induced and cell death occurs by necrosis (Leist *et al*., 1997; Nicotera *et al*., 1998). During necrosis the intracellular cytokines are released into the extracellular medium thereby further enhancing the inflammatory response through different signally pathways including the stimulation of IL-1α production in the remaining viable cells as mentioned above.

In the presence of UV-B all the rooibos extracts decreased IL-1α production in comparison to the control, with the MUF extract exhibiting the highest activity. Cell viability was further decreased, while apoptosis was decreased at the higher extract concentrations. Since apoptosis was decreased, accompanied by a further decrease in cell viability, specifically by the MUF and AUF extracts, the pro-oxidant effects of the rooibos flavonoids appear to be enhanced in the presence of UV-B, which is known to induce free radicals in cells (Aitken *et al.*, 2007). The observed decrease in IL-1α could be a result of the removal of the damaged cells via a combination of excessive apoptosis and the additional role of necrotic cell death, which also needs to be

considered as mentioned above. The column fractions X-1, X-3 and X-4 all exhibited similar effects to the MUF extract in decreasing the concentration and fold of IL-1α at the highest concentration. The column fractions X-3 and X-4 further decreased cell viability, which is in accordance with the high flavonoid levels and increased anti-oxidant levels presumably leading to pro-oxidant effects. Fraction X-1 also had similar effects although it contained non-flavonoid constituents, while fraction X-2 decreasedIL-1α production only at the highest concentration. This decrease was associated with a decrease in cell viability, while apoptosis was not significantly increased. As this fraction seems to exhibit anti-inflammatory effects in cells in the absence of UV-B exposure, the therapeutic potential should be further explored. Fraction X-5, however, increased IL-1α ($P<0.05$) without a dramatic decrease in cell viability, while apoptosis was decreased significantly, once again implying a pro-inflammatory effect as suggested in the absence of UV-B irradiation.

From these results it is evident that rooibos extracts and the XAD-4 fractions, except for fraction X-5, decreased intracellular IL-1α production in HaCaT cells following UV-B exposure through a further reduction of cell viability. However, a dominating role of apoptosis due to an increased pro-oxidant activity of the methanol extracts and XAD-4 fractions seems to prevail, although the effect of subsequent necrosis tended to become more important at higher concentrations. As mentioned above the necrotic release of IL-1α may augment the inflammatory response. Therefore the effects of the rooibos flavonoids and/or non-flavonoid constituents on inflammation depends on the anti-oxidant properties, polarity indices and dose of the flavonoids, which are important for future studies related to the underlying mechanisms involved in the chemo-preventive properties of rooibos.

Schematic diagram of the formation of phenoxy radicals involving quercetin and (-) epigallocatechin (EGC) illustrated the potential pro-oxidant intermediates likely to be involved in the reduction in cell viability and the induction of apoptosisin the HaCaT keratinocytes (Fig 6.5). The antioxidant and possible pro-oxidant properties of polyphenols are related to the redox potential of phenoxy radicals with quercetin having a very low E_7/V (pH = 7) of 0.3, compared to that of 0.43 and 0.57 of EGC and (+)-

catechin, respectively. The E_7/V of the major rooibos flavonoid is not known but a study showed that it exhibited a similar IC50 concentration to EGCG and

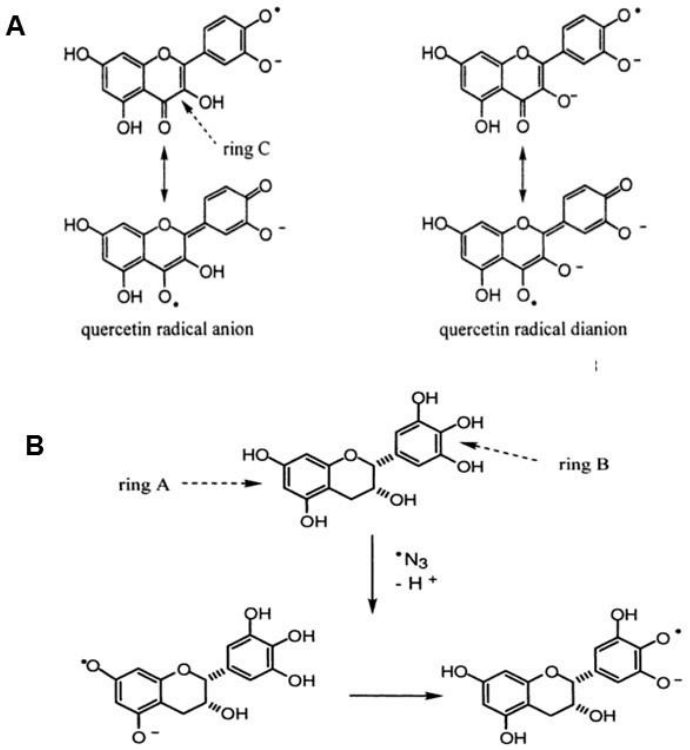


Figure 6.5 (A) The quercetin radical anion and quercitindianion, (B) one-electron oxidation of (-) epigallocathecin (Adapted from Haslam, 1999)

quercetin in the ABTS´ radical scavenging assay, implying a comparable redox potential of the phenoxy radical and hence pro-oxidant properties (Snijman *et al*., 2009). Of interest, the phenoxy radical of rooibos flavone, rutin exhibited an E7/V of 0.6 which is known to exhibit a far weaker radical scavenging activity than aspalathin in the ABTS assay.

As rooibos is known to inhibit cancer promotion in skin using different carcinogenesis models (Marnewick *et al.*, 2005; Petrova *et al.,* 2011), the current study provides interesting clues to further elucidate the anti-inflammatory properties of rooibos in the quest to develop a pharmaceutical product against skin cancer. Green tea polyphenols

have been shown to provide protection against UV-B skin carcinogenesis as oral and topical application of polyphenols before UV-B exposure decreased tumour development *in vivo* (Wang et al., 1994). Another study reported an inhibitory effect of orally administered black tea; decaffeinated green tea and decaffeinated black tea on UV-B induced benign and malignant tumor formation (Wang *et al.,* 1991). With respect to the UV-B/HaCaT cell model, the anti-inflammatory effects of other plant phenolic extracts cells have also been investigated (Saliou *et al*., 1999). Wine polyphenols and grape wine extracts were reported to exhibit anti-inflammatory effects in HaCaT cells exposed to UV-B (Tomaino *et al*., 2006). These studies strengthened the use of the UV-B/HaCaT model to further elucidate the effects of rooibos on inflammation.

6.4. Inhibition of LPS stimulated TNF-α in macrophages

TNF-α is also released by keratinocytes following UV-B exposure, however, the levels are far less than those released by macrophages following activation by LPS (Köck *et al*., 1990), which was the motivation behind the use of the LPS/macrophage model in his study. Macrophages are key inflammatory cells recruited during the early stages of inflammation (Fujiwara & Kobayashi, 2005) and are produced in bone marrow and transported through the blood to all tissues upon injury. Upon stimulation by UV-B and/or LPS, macrophages contributes to the inflammatory response by producing and releasing pro-inflammatory cytokines such as TNF-α and IL-1α (Duque & Descoteaux, 2014). Most studies identifying TNF-α anti-bodies to modulate the adverse effects of its release, have been associated with severe side effects giving rise to a need to develop safer alternatives (Sethi *et al*., 2009). There are a number of plant derived products identified that can inhibit TNF-α release and/or expression. Quercetin, found in rooibos in small quantities, has been reported to decrease LPS-stimulated release of TNF-α in Raw 264.7 macrophages (Wadsworth & Koop, 1999), while In another study, luteolin suppressed LPS-induced expression of various pro-inflammatory mediators including TNF-α (Chen *et al*., 2007).

In the current study the rooibos extracts decreased TNF-α release in LPS-stimulated macrophages without overtly affecting cell viability and apoptosis, with the methanol extracts exhibiting the most prominent effects at the lowest concentrations levels tested. However, the MUF and AF exhibited the highest inhibitory effect at the highest concentration in the absence of apoptosis, which is associated with a direct anti-inflammatory mechanism. This effect is in contrast to the keratinocytes where cell survival parameters also play a determining modulating role, as mentioned above.

The XAD-4 derived column factions prepared from the MeOH extract of unfermented rooibos also decreased TNF-α with the most polar fraction, X-1, exhibiting the highest activity. The flavonoid-enriched fractions (X-3 and X-4) in addition slightly decreased cell viability and induced apoptosis at high concentrations similar to the MUF extract; however, the fractions were less effective in inhibiting TNF-α release. A similar effect was noticed with the non-polar fraction X-5 although it was less active, presumably due to lower level of rooibos flavonoids. The reduction in cell viability and the marginal increase in apoptosis could, as mentioned above, be related to pro-oxidant effects induced by the rooibos flavonoids while the non-polar constituents appear to be less effective in decreasing in the cell survival indices. In this regard fraction X-1 exhibited the highest activity in reducing TNF-α further suggesting that the more polar constituents of rooibos exhibited more direct anti-inflammatory properties, implying the more complex tannin-like proanthocyanidins or the more water soluble non-flavonoid constituents

6.5. The role of nuclear factor kappa B (NF-κB)

The production of pro-inflammatory cytokines is known to be associated with the activation of transcription factors including nuclear factor kappa B (NFκB) and the activator protein-1 (AP-1) among others (Karin & Greten, 2005). The expression of the TNF-α cytokine in response to LPS by macrophages has been reported to involve NFκB activation through toll-type receptors (TLR) signalling. The importance of this transcription factor is highlighted, as it is central in the link between inflammation and

cancer. Aberrant regulation of NFκB signalling has been reported in many tumours (Pikarsky *et al*., 2004) and the ability of a number of polyphenol compounds to inhibit TNF-α have been associated to decreased NFκB activation (Baer *et al*., 1998). NF-κB regulates inflammation and immune responses of host organism as it is involved in the expression of about 27 cytokines and chemokines as well as the expression of iNOS and COX-2, enzymes that may elevate the inflammatory response (Surh, 2003). Cytokines released via NF-κB signalling can also induce its translocation to the nucleus serving as a positive feedback regulation on the transcription factor which may further elevate the inflammatory response. The transcription factor has been found to be constitutively activated in a variety of diseases such as asthma, rheumatoid arthritis and inflammatory bowel disease as well as human cancers (Baldwin, 2001; Chen *et al*., 1999; Epstein *et al*., 1997; Tak & Firestein, 2001). Therefore, modulation of NFκB activation has been a key target in the development of anti-inflammatory drugs for its pivotal role in inflammation regulation.

The ability of both UV-B and LPS to stimulate cytokine production through the involvement of the NFκB serves as a possible model to investigate the direct anti-inflammatory activity by the rooibos flavonoids. However, the modulation of this transcription factor are masked by the major rooibos flavonoids adversely affected the cell growth parameters in HaCaT cells at high concentrations, presumably due to pro-oxidant effects (Fig 6.4). However, the less polar rooibos constituents present in the aqueous fermented extract and fraction X-2 (HaCaT) and fraction X-1 (macrophages) had minor effects on cell viability and the induction of apoptosis, could therefore be involved in the modulation of the down regulation of NFκB and the induction of pro-inflammatory cytokines.

6.6. The role of iron in the anti-inflammatory activity of polyphenols

A second mechanism that could be involved in the anti-inflammatory effects of the rooibos flavonoids is related to their iron chelating properties. It is known that iron is released from ferritin associated with the increased oxidative stress in keratinocytes

following UV-B exposure which contribute to ROS production via the Fenton reaction thereby enhancing the inflammatory response (Imlay *et al*., 1988). In macrophages, stimulation by LPS retains iron in order to kill the invading pathogens, resulting in a change the oxidative status thereby resulting in the inflammatory response. The reduction of oxidative stress by sequestering iron would attenuate the inflammatory response reactions involving free radicals (Bergendi *et al.*, 1999).

6.7. Tolerance to pro-oxidant effects of polyphenols in HaCaT cells verses THP-1 macrophages

The rooibos extracts had no overt effects on cell viability and apoptosis in the LPS/macrophage model whilst attenuating TNF-α release with the methanol extracts showing highest activity. Macrophages produce a wide variety of free radicals in order to kill invading pathogens and are equipped with multiple defence mechanisms against these oxidants (Ishii *et al*., 1999). Therefore, the response of macrophages to treatment with the rooibos extracts and/or column fractions is suggestive of the resistance of the cells to the pro-oxidant effects of the polyphenol compounds resulting in lower response in reduction cell viability and the induction of apoptosis. The HaCaT cells appears to more readily undergo apoptotic cell death, as they are considered to be pre-neoplastic cells known to be more sensitive towards suicidal cell death (Schulte-Hermann *et al*., 1993). Therefore, the combined effect of UV-B and rooibos flavonoids exhibited adverse effects on cell integrity as observed in UV-B/HaCaT inflammatory model in the presence of the MUF and AUF as well as the flavonoid enriched rooibos column fractions. It is therefore evident, that with increased anti-oxidant levels the polyphenols could result in pro-oxidant effects (Fig 6.5) which could result in a reduction in cell viability and even into necrotic cell death in more susceptible HaCaT cell model. In comparison to the macrophages, the HaCaT cell has less capacity to accommodate oxidative stress and subsequently resort to apoptosis and necrotic cell death which masked the inflammatory response.

6.8. Summary and benefits of current investigation

The ability of rooibos extracts and column fractions to inhibit the key primary cytokines governing inflammation in the LPS/macrophage model is likely to be indicative of their anti-cancer properties in skin. In addition, the major rooibos flavonoids were shown to exhibit no anti-inflammatory effects in the post UV-B/HaCaT models although protection against skin cancer may be accomplished through the induction of cell death. However, exposure to excessive levels of the rooibos flavonoids, specifically in the HaCaT model, may induce pro-oxidant effects and subsequently increase cell toxicity by reducing cell viability, induction of apoptosis and/or necrotic cell death. The current study also indicated that no clear relationship exist between the anti-oxidant potency and/or hydrophobicity of the rooibos flavonoids and the anti-inflammatory effects as the non-flavonoid constituents also seem to play an important role. Therefore, effects of rooibos on inflammation cannot solely be attributed to the major flavonoids and their anti-oxidant properties as the fermented aqueous extract and non-flavonoid column fractions also exhibited high anti-inflammatory action in both the HaCaT and macrophage cell models. Furthermore, the fractionation of the MUF extract resulted in fractions with enriched polyphenols levels but this did not result in higher anti-oxidant activity and/or inflammatory response. Thus the use of pure compounds of the major polyphenols may not be beneficial as the extract is more active.

6.9. Proposed future studies

The aqueous extracts from fermented rooibos as well as the more polar column fractions inhibited inflammation more efficiently than the more flavonoid containing extracts and column fractions. Thus, it seemed that more polar rooibos constituents as well as well as the complex flavonoids found in fermented tea also exhibited antioxidant activity which resulted in the inhibition of the pro-inflammatory cytokines without adverse cytotoxic effects in the cells, especially in the LPS/macrophage inflammatory model. Future studies should therefore focus on aqueous extracts of fermented rooibos and the polar rooibos column fractions to further characterise their anti-inflammatory potency in

the *in vitro* models. With regards to the unfermented extracts and the flavonoid enriched column fractions, high doses exhibited detrimental toxicity in the HaCaT cell which were further increased in the presence of UV-B. Therefore, experiments should be conducted utilising specific doses to avoid the drastic decrease in cell viability and the presumed increase in necrosis. Effects on cellular oxidative status by monitoring the activation if transcription factors nrf2, NFκB, AP1 among others should also be investigated as the induction oxidative stress and inflammation are known to be closely connected to these transcription factors. The modulation of these transcription factors could be in essence very good biomarkers to select optimal doses to minimise adverse effects related to the redox status of cells and to further characterise the underlying anti-inflammatory mechanisms of rooibos.

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