CHEMOPREVENTIVE PROPERTIES OF SOUTH AFRICAN HERBAL TEAS, ROOIBOS (*ASPALATHUS LINEARIS*) AND HONEYBUSH (*CYCLOPIA* SPP.): MECHANISMS AGAINST SKIN CARCINOGENESIS

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DECLARATION

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Abstract

The present study employed a two-phased approach to investigate the possible mechanisms involved in the chemopreventive properties of rooibos (*Aspalathus linearis*) and different honeybush species (*Cyclopia* spp.) *in vitro*. In the first phase, the effect of unfermented methanol and aqueous herbal tea extracts against the growth parameters (cell viability, proliferation and apoptosis) of normal (CRL 7761); premalignant (HaCaT); and malignant (CRL 7762) skin cells was evaluated and compared to green tea extracts. The predictive potential of polyphenol content (total polyphenol and flavanol/proanthocyanidins) and antioxidant properties (ABTS; ORAC; FRAP and LPO) in the biological activity of extracts in cells was also assessed. Of the herbal teas, the methanol extract of rooibos was the most active and it inhibited the growth of skin cells presumably by inducing mitochondrial dysfunction via membrane depolarisation. At lower concentrations, this activity was associated with inhibition of cell proliferation that was selective for cancer cells whilst higher concentrations induced apoptosis that was more prominent in premalignant cells. The strong antioxidant properties of the extracts implicated the role of pro-oxidative polyphenol/iron interactions involving monomeric flavonoids and polymeric proanthocyanidins in the cytotoxic effects of rooibos. The strong relationship between total polyphenolic and flavanol/proanthocyanidins content, antioxidant properties and reduction of cell viability indicated that these parameters (polyphenols and antioxidant properties) can serve as predictive tools for the cytotoxic effects of rooibos *in vitro*. The aqueous extracts of honeybush species, although weaker, displayed similar effects to rooibos extracts in cells with *C. genistoides* being the most effective at selectively inhibiting the proliferation of cancer cells whilst the pro-apoptotic activity of *C. subternata* and *C. intermedia* was more prominent in premalignant cells. The underlying mechanisms are also likely to result from pro-oxidative mechanisms resulting from polyphenol/iron interactions that mainly involve polymeric flavanol-like proanthocyanidin compounds in honeybush. In contrast, the methanol extracts exhibited weaker cytotoxic effects and protected cancer cells from going into apoptosis. The cytoprotective effects of honeybush species are possibly mediated by the major monomeric compounds such as mangiferin and hesperidin through antioxidant mechanisms that result in reduction of oxidative stress. Due to the possible dual role of the monomeric and polymeric compounds in the honeybush

extracts, the total polyphenolic content of these herbal teas may not be a good indicator of biological activity *in vitro*. However, as aqueous extracts displayed high flavanol/proanthocyanidins content and exceptional activity in the ABTS assay, these parameters may be considered as indicators of cytotoxicity. On the other hand, methanol extracts, particularly from the xanthone-rich species (*C. genistoides* and *C. longifolia*) which exhibited the weakest cytotoxic effects, were more active in the ORAC thus this assay may be a useful predictor for cytoprotective activity. In the second phase, an *in vitro* UVB/HaCaT model which used IL-1α as a biomarker for early inflammation was developed and validated with known anti-inflammatory compounds, dexamethasone and ibuprofen. It was used to determine the specific mechanisms involved in the modulatory effects of the herbal tea extracts against inflammation. Rooibos extracts and the aqueous extract of honeybush enhanced the cytotoxic effects of UVB in the model and exhibited indirect anti-inflammatory effects as they removed icIL-1α containing cells via apoptosis. In contrast, methanol extracts of honeybush exacerbated icIL-1α by protecting UVB stimulated cells from undergoing apoptosis. In conclusion, methanol extract of rooibos and aqueous extracts of honeybush species may be useful in protecting the skin after UVB exposure. These herbal tea extracts may block initiation and delay the promotion stage during skin carcinogenesis by removing premalignant cells via apoptosis and preventing onset of inflammation. In contrast, due to their cytoprotective effects, methanol extracts of honeybush may be more effective at preventing oxidative stress in skin before UVB exposure. Future studies should focus on the effects of extracts and polyphenolic fractions on the oxidative status of the cells and development of biomarkers of chemoprevention that can be utilised *in vivo* and in human skin.

Uittreksel

In hierdie studie word moontlike velkankerwerende eienskappe van rooibos (*Aspalathus linearis*) en ‘n aantal heuningbos (*Cyclopi*a spp.) spesies deur twee afsonderlike benaderings bestudeer. Die eerste benadering ondersoek die effek van die kruietee op groeiparameters van velselle [lewensvatbaarheid, groei en dood van normale selle (CRL 7761), vroeë kankerselle (HaCaT) en kankerselle (CRL 7762)]. Tydens eksperimente is die moontlikheid om polifenoolinhoud (totale polifenole, en flavanol/proantosianidiene verhouding) en antioksidant-eienskappe te gebruik om die biologiese funksies van die ekstrakte in die selle te voorspel, geevalueer. Die metanolekstrak van rooibos het die groei van selle die effektiefste gestop, moontlik deur depolarisasie van die mitokondriale membraan. By lae konsentrasies van die ekstrak is die groei van kankerselle selektief gestop, terwyl vroeë kankerselle die sensitiefste by hoër konsentrasies was. Die hoë antioksidant-aktiwiteit van die rooibosekstrak kan moontlik ‘n rol speel in die indusering van sitotoksiese effekte in die selle en kan toegeskryf word aan die pro-antioksidant aktiwiteit van die polifenole weens hul interaksie met yster. ‘n Spesifieke funksie word vir die monomeriese flavonoïede en die polimeriese proantosianidiene geïmpliseer. Die sterk verwantskap tussen die totale polifenoolinhoud, flavanol/proantosianidien inhoud en antioksidant aktiwiteit met die verlaging in selgroei, maak hul relevante parameters van die voorspellingsmodel. Die waterekstrakte van heuningbos induseer ook soortgelyke maar swakker effekte met die induksie van kankersel dood, met *C. genistoides* die selektiefste en *C. subternata* en *C. intermedia* die aktiefste spesies wat die groei van die vroeë kanker selle inhibeer. Die onderliggende meganismes betrokke blyk ook aan ‘n pro-oksidant effek toe geskryf te wees, waartydens spesifieke polifenool/yster interaksies betrokke is. In teenstelling met rooibos, beskerm die metanolekstrak van heuningbos kankerselle teen seldood, wat moontlik verband hou met die antioksidant-eienskappe van die hoof monomeriese polifenole, mangiferien/isomangiferien en hesperidien. Vanweë die dubbele rol van die monomeriese polifenole en polimeriese verbindings in heuninghbosekstrakte is die totale polifenol inhoud nie ‘n goeie indikator van die biologiese aktiwiteit *in vitro* nie. Daarenteen is die flavanol/proantosianien inhoud en die hoë aktiwiteit in die ABTS antioksidanttoets goeie indikators om seldood te voorspel. In teenstelling hiermee het die metanolekstrakte van die xantoon-ryke spesies (*C. genistoides* en *C.*

longifolia) ‘n baie lae effek op seldood, maar ‘n hoë aktiwitiet in die ORAC toets getoon, wat ‘n goeie rigtingwyser is om die beskermende effek in selle te voorspel.

Met die tweede benadering is die anti-inflammatoriese eienskappe en die onderliggende meganismes van die kruietee ondersoek in ‘n UVB/HaCaT selmodel. Intrasellulêre interleukin 1α (IL-1α) is as merker gebruik en die model is geëvalueer deur bekende anti-inflammatoriese verbindings soos dexamethasone en ibuprofin te gebruik. Die metanolekstrak van rooibos en die waterekstrak van heuningbos het die toksiese effek van UVB in die model verhoog deur selle met verhoogde vlakke,van icIL-1α te verwyder deur middel van die induksie van seldood. Die metanolekstrak beskerm die selle teen die oksidatiewe skade wat deur UVB geïnduseer word en verwyder nie selle met hoë IL-1α vlakke nie. Ter opsomming blyk dit dat die metanolekstrak van rooibos en die waterekstrak van heuningbos moontlik gebuik kan word om die vel te beskerm teen die induksie van icIL-1α en sodoende die inisiëring van kanker te blokkeer en ook die promosie van kanker te vertraag. Die beskermende effek van die metanolekstrak kan moontlik aangewend word om die oksidatiewe skade wat deur UVB veroorsaak word teen te werk deur dit aan te wend voordat blootstelling plaasvind. Toekomstige studies behoort verdere karakterisering van die polifenoolsamestelling van die ekstrakte in te sluit en hul effek op die oksidatiewe status en anti-inflammoriese effekte van selle te bepaal ten einde sekere merkers te identifiseer vir vel studies *in vivo*.

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and brother Vuyani Magcwebeba.

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Abbreviations

A	Adenine
AAPH	2,2'-azobis(2-amidinopropane) dihydro-chloride
AHH	Arylhydrocarbon hydroxylase
ABTS	2,2'-azinobis 3-ethyl-benzothizoline-6-sulfonic acid
ATP	Adenosine triphosphate
ARC	Agricultural Research Council
B [*a*] P	Benzo [*a*] pyrene
BCC	Basal Cell Carcinoma
BrDU	BrdU - 5-bromo-2'-deoxyuridine
BTP	Black tea polyphenols
C	Cytosine
CPD	Cyclobutane pyrimidine dimers
COX-2	Cyclo-oxygenase-2
CYP450	Cytochrome P450
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
CYP1A2	Cytochrome P450, family 1, subfamily A, polypeptide 2
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DMBA	12-dimethylbenz [*a*] anthracene
DPBS	Dulbeco's phosphate buffered saline
DNA	Deoxyribonucleic acid
EC	(-)-epicatechin
ECG	(-)-epicatechin-3-gallate
EGCG	(-)-epigallocatechin-3-gallate
EGC	(-)-epigallocatechin
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
Fe^{2+}	Iron (II)-Ferric iron
Fe^{3+}	Iron(III)-Ferrous iron
FRAP	Ferric reducing antioxidant power
G	Guanine

GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
GTP	Green tea polyphenols
H_2O_2	Hydrogen peroxide
HBSS	Hank's buffered salt solution
HOCl	Hypochlorus acid
IL-1α	Interleukin-1 alpha
icIL-1α	Intracellular interleukin-1alpha
exIL-1α	Extracellular interleukin-1 alpha
sIL-1α	Secreted interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
iNos	Inducible nitric oxide synthase
JC-1	1^{st} J-aggregate-forming cationic dye
JNK	c-Jun-N-terminal kinase
LPO	Lipid peroxidation
LPS	Lipopolypolysaccharide
MAPK	Mitogen-activated protein kinases
NADPH	Nicotinamide adenine dinucleotide phosphate
NCI	National Cancer Institute
NHEK	Normal Human Epidermal keratinocytes
NMSC	Non-melanoma skin cancer
NO	Nitric oxide
O_2	Molecular oxygen
$O_2^{\bullet-}$	Superoxide anion
$^1O_2$	Singlet oxygen
$OH^{\bullet}$	Hydroxyl radical
ORAC	Oxygen Radical Absorbance Capacity
PAH	Polycyclic aromatic hydrocarbons
PGE_2	Prostaglandin E2
PMA	Phorbol 12-myristate 13-acetate
$R^{\bullet}$	Radical
RLU	Relative light units
$ROO^{\bullet}$	Peroxyl radical
ROOR	Peroxide
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute

SANBI	South African National Biodiversity Institute
SCC	Squamous cell carcinoma
SOD	Superoxide dismutase
T	Thymine
TBA	Thiobarbituric acid
TNF-α	Tumor necrosis factor-α
TRAIL	TNF-related apoptosis-inducing ligand
TPA	12-O-tetradecanoylphorbol-13-acetate
UVB	Ultraviolet B

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Therefore I tell you, do not worry about your life,
what you will eat or drink; or about your body, what you will wear.
Is not life more than food and the body more than clothes?
Look at the birds of the air; they do not sow or reap or store away in barns,
and yet your heavenly Father feeds them.
Are you not much more valuable than they?
Can any one of you by worrying add a single hour to your life? (Matthew 6: 25-27)

Chapter 1

General Introduction

General introduction

Skin cancer caused by chronic exposure to the sun's ultraviolet-B (UVB) rays has become a major global concern as its incidence has increased to levels that are considered to be of epidemic proportions (Diepgen and Mahlar, 2002; Aceituno-Madera et al., 2010; Kim and Armstrong, 2012). The high incidence rate of skin cancer has been attributed to the sun-seeking behaviour of individuals as well as greater UVB exposure resulting from ozone depletion. Due to its latituditional position which is in close proximity to the ozone hole over Antarctica, South Africa and other similarly placed countries such as Australia and Chile have the highest incidences of skin cancers in the world and these are associated with high UV indices (Abarca and Casiccia, 2002; Human and Bajic, 2002; Mqoqi et al., 2004). The primary target for UVB-induced damage is the keratinocytes and thus as a result the most commonly occurring human skin cancer arises from the epidermal layer (Soehnge et al., 1997). These include non-melanoma skin cancers and cutaneous melanoma; the latter has a lower incidence rate but accounts for a higher mortality rate (Armstrong and Kricker, 2001; Aceituno-Madera et al., 2010). Non-melanoma skin cancers are comprised of the frequently diagnosed basal cell carcinoma associated with facial disfigurement (Kwasniak and Garcia-Zuazaga, 2010; Lomas et al., 2012) and the metastasizing squamous cell carcinoma which has potential to cause death (Shulstad and Proper, 2010). The development of cancers in skin, like in any other organ, is a multistage process that involves initiation, promotion and progression (DiGiovani, 1992, Vincent and Gatenby, 2008). The irreversible stage of initiation involves exposure of normal cells to DNA damage and the accumulation of mutations that mainly target the p53 gene (Erb et al., 2005). Promotion, which is a reversible stage, is characterised by oxidative stress, chronic inflammation and hyperproliferation leading to hyperplasia and formation of benign tumors (Rundhaug and Fischer, 2010). The complex stage of progression is characterised by hypoxia, acidosis and malignant transformation of benign tumors. The phenotypic properties exhibited by tumors in this stage include angiogenesis, invasiveness and metastasis (Digiovani, 1992; Vincent and Gatenby, 2008).

Skin cancer is the most preventable disease, and if diagnosed early, it can be treated effectively (Cummings et al., 1997), but the prevention strategies and treatment options that have been put in place have limitations and draw-backs. There is low compliance to the primary prevention strategy entailing sun-avoidance, use of sun-screens and wearing of protective clothing (Dobbinson et al., 2008). Treatment is highly expensive, invasive with the cancers having a high recurrence rate (Anthony, 2000; Ceilley and Rosso, 2006). Therefore, there is a need for an alternative approach that is easily accessible, less invasive and cost-effective. Emphasis for effective control of cancer incidence has been placed on an intervention strategy that can delay malignant transformation primarily by targeting the reversible stage of promotion and this has been identified as chemoprevention (Shureiqi et al., 2000). The latter utilises subcytotoxic levels of natural or synthetic agents to retard the process of carcinogenesis in skin (Stratton et al., 2000)

Epidemiological and laboratory studies have indicated a positive association between cancer reduction and intake of dietary plant foods and this has been attributed to the presence of phytochemicals (Greenwald et al., 2001). Due to their safety, low toxicity and general acceptance, these natural compounds have been targeted for use in chemoprevention (Amin et al., 2009). One group of phytochemicals that has shown potential in chemoprevention studies are the polyphenols (Youn and Yang, 2011); these compounds are also abundantly present in South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.). The major polyphenolic constituents in the unfermented plant material are the dihydrochalcones, aspalathin and nothofagin, in rooibos and in honeybush it is the xanthones, mangiferin and iso-mangiferin, as well as the flavanone, hesperidin (Joubert et al., 2008). These polyphenolic compounds have been associated with antioxidant, anti-mutagenic, anti-proliferative and anti-inflammatory properties exhibited by extracts *in vitro*, *in vivo* and in human studies (as reviewed by Joubert et al., 2008; Joubert and de Beer, 2011; Joubert et al., 2011). These biological properties have been associated with prevention of chronic disorders such as cancer.

The anti-cancer properties of these herbal teas in skin have been demonstrated as rooibos and honeybush (*C. intermedia*) extracts exhibited anti-tumor activity in

mouse skin (Marnewick et al., 2005; Petrova et al., 2011). These studies indicated the potential of these herbal teas as chemopreventive agents that could be used topically against skin carcinogenesis. However, the specific mechanisms underlying the activity of the herbal teas against tumor promotion still remain elusive. The biological effects of extracts of these herbal teas against the growth parameters of skin cancer cells, as well as their effects against pro-inflammatory cytokines responsible for initiating inflammation and mediating this process during carcinogenesis, are still not known. In addition, the relationship between the chemical constituents, antioxidant properties and their biological activity in skin cells still needs to be determined. Thus the objective of this study was to use different skin cell culture systems to determine the chemopreventive properties of the extracts in a dual approach. The first aim was to conduct a comparative evaluation on the effects of the herbal tea extracts against different cell growth parameters, including cell viability, proliferation and pro-apoptotic indices. The extracts were chemically characterised to determine the role of polyphenolic constituents or groups of constituents in the biological activity of the herbal teas in skin. In addition, several in *vitro* assays were used to determine the predictive potential of the polyphenolic content and antioxidant properties in the cytotoxicity of the extracts in premalignant, malignant and non-malignant skin cells. The second aim was to develop an *in vitro* model which could be used to determine the specific mechanisms involved in the anti-inflammatory effects of the extracts of the herbal teas.

Aspects of the skin, skin carcinogenesis, chemoprevention and the role of polyphenolic compounds in this process, as well as the chemical, biological properties of tea (*Camellia sinensis*), rooibos and honeybush were reviewed in detail in Chapter 2. The predictive potential of the polyphenolic composition and antioxidant properties of rooibos and honeybush extracts (of several *Cyclopia* species) in the modulation of skin cell viability *in vitro* were discussed in detail in Chapter 3 whilst Chapter 4 focuses on the anti-proliferative and pro-apoptotic properties of rooibos and different honeybush species in skin cells. In Chapter 5, interleukin-1α induction in human keratinocytes (HaCaT) as an *in vitro* model for chemoprevention in skin was developed and different mechanisms for modulation of the onset of inflammation presented. The UVB/HaCaT model was then utilised in

Chapter 6 to determine the *in vitro* effects of herbal tea extracts (rooibos and honeybush) on UVB-induced cell growth parameters and interleukin-1α accumulation in keratinocytes. The outcomes of chemopreventive properties of the herbal extracts *in vitro,* as well as the implication for skin carcinogenesis, are discussed in detail in Chapter 7 and conclusions were drawn. Gaps in our current knowledge as highlighted by the current research study formed the basis for recommendations for future studies.

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Chapter 2

Literature review

What's new under the sun?.....polyphenols: food for thought

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Contents **Pages**

1. Skin anatomy and function

The skin, which is the largest organ in the body, serves as a protective barrier that shields internal organs from the adverse effects of the external environment (Lai-Cheong and McGrath, 2009; Venus et al., 2011; Baroni et al., 2012). Morphologically, it is composed of three different layers namely the epidermis, dermis and hypodermis (Fig. 2.1); each of these layers have their own properties and function. The barrier function of preventing dehydration, microbial invasion, physical and chemical damage is mostly confined to the epidermis. This outer most layer of the skin is comprised of specialised cells that are adapted to various functions involving: formation of an impermeable barrier, photoprotection against ultraviolet (UV) damage, immunological defense against pathogens and toxic substances including repair of damaged tissue (Wickett and Visscher, 2006; Hwa et al., 2011).

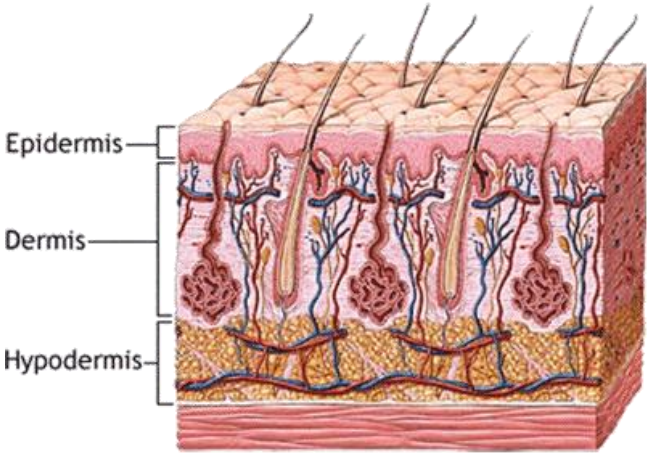


Fig. 2.1. Different skin layers (Adapted from Brannon, 2007)

1.1. Epidermis

The principal cell type in the epidermis is the keratinocyte, constituting more than 95% of this layer while Merkel cells, Langerhans cells and melanocytes account for the remaining 5% (Lai-Cheong and McGrath, 2009). Structurally, the epidermis may be divided into five different layers/strata mainly based on the state of cellular differentiation (Fig. 2.2). These layers include stratum basale/basal layer, stratum spinosum/spinous layer, stratum granulosum/granulous layer, stratum lucideum

/translucent layer (only found in palms of hand and soles of feet) and stratum corneum/cornified layer (Venus et al., 2011).

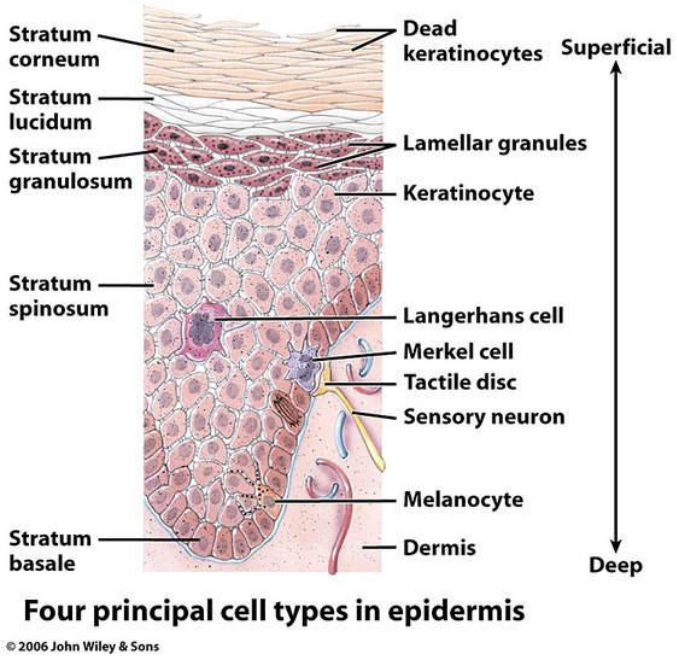


Fig. 2.2. The different epidermal layers and cell types (Adapted from Tortora and Grabowski, 2008).

1.1.1. Cell differentiation, barrier formation and immunological defense

Since the epidermal layer is continuously exposed to the adverse effects of the external environment, it has in place, a set of first line defense mechanisms which protect the skin against damage (Alberts et al., 2002; Baroni et al., 2012). One of these mechanisms involves the formation a physical barrier that is constantly renewed through a process of cellular differentiation and desquamation. During this process, differentiating progenitor cells from the stratum basale, acquire biochemical and morphological changes as they migrate up the different epidermal layers to form lipid-enriched corneocytes (Candi et al., 2005; Wickett and Visscher, 2006; Powell, 2006). Corneocytes in the stratum corneum, form a lipid-rich barrier that is selectively impermeable to toxic chemicals and microorganisms while it prevents dehydration by retaining essential bodily fluids (Fuchs, 1990). These cells are eventually shed off and replaced by underlying differentiated keratinocytes from the stratum basale through a process of desquamation.

When the physical barrier is breached, the skin has in place a second line of defense through a chemical barrier. This involves the production of anti-microbial

peptides, surface receptors and soluble factors such as cytokines and chemokines from keratinocytes. These defense molecules protect the skin against infection and physical damage (Suter et al., 2009; Venus et al., 2011). The protective function also extends to other epidermal cells sparsely distributed between keratinocytes. Melanocytes in the basal layer protect the nuclei of basal cell keratinocytes from harmful effects of ultraviolet radiation by producing the photoprotective pigment known as melanin (DeLeo et al., 1998; Wickett and Visscher, 2006). Langerhan's cells, present throughout the epidermal and dermal layer, form part of the immunosurveilance system against viruses and tumour cells (Lai-Cheong and McGrath, 2009). Merkel cells, situated near hair follicles and unmyelinated nerve endings in the basal layer, are associated with the transduction of fine touch.

1.2. Dermis

The dermal layer is a complex structure of connective tissue, blood vessels and lymphatic vessels, collagen and elastic fibres, nerve endings, ground substance and various cell types which include the predominant fibroblasts, mast cells, dendritic cells, and endothelial cells (Kanitakis, 2002). These dermal components protect the body against mechanical injury as well as providing structural support and nutrient supply to the epidermis. They also function in immune defense, thermoregulation and wound healing (Sorrell and Caplan, 2004; Venus et al., 2011).

1.3. Hypodermis

The hypodermis which lies beneath the dermis is a layer of adipose tissue comprised of adipocytes that produce fatty acids and triglycerides. This layer mainly functions in thermoregulation, energy storage and in mechanical resistance (Kanitakis, 2002).

2. Skin cancers

The protective function of skin against injury and its activity in the repair of damaged tissue is mainly facilitated through a complex signalling network between epidermal and dermal cells. The chronic dysregulation of this network e.g. due to a persistent infection or long-term exposure to toxic chemicals and UV irradiation, can

provide conditions that lead to the development of various skin disorders including cancer (Marks and Furstenberger, 1993).

In skin, the most commonly diagnosed cancers affect the epidermal layer and these include the non-melanoma skin cancers (NMSCs) and the rare but fatal melanoma skin cancer (Jhappan et al., 2003, Kim and Armstrong, 2012). Non-melanoma skin cancers consist of the frequently diagnosed basal cell carcinoma and the metastasising squamous cell carcinoma.

2.1. Basal cell carcinoma

Basal cell carcinoma (BCC) is thought to develop from the basal stem cells in the bulges of hair follicles in the epidermis (Kwasniak and Garcia-Zuazaga, 2011). Although it rarely metastasizes, it can however, cause significant tissue destruction and facial disfigurement resulting in considerable morbidity mainly affecting the head and neck area. Clinically, BCC does not have a detectable precursor lesion but rather develops de *novo* and its manifestation has been described as a “palpable, localised, translucent tumour with overlying teleangiectasias” (Göppner and Leverkus, 2011). Histologically, this malignancy may be divided into three different phenotypes: the nodular type with pearly borders, central crust and ulceration; the superficial scaly erythrematous patch and the infiltrative sclerosing morpheaform that presents as a scar-like centrally atrophic tumour. The development of basal cell carcinoma in skin is attributed to various risk factors including the following: exposure to certain environmental agents, prevalence of certain skin lesions, occurrence of genetic and hereditary disorders as well as immunosuppresion (Brooke, 2005). The predisposing risk factors of basal cell carcinoma as well as those of squamous cell carcinoma and melanoma skin cancer are summarised in Table 2.1.

2.2. Squamous cell carcinoma

Squamous cell carcinoma (SCC) is a malignancy of keratinocytes from the epidermal layer (Shulstad and Proper, 2010). The development of this neoplasm is mostly associated with the presence of its principal precursor lesion, actinic keratosis. Similar, to basal cell carcinoma, SCC also affects the head, neck and trunk area. Squamous cell carcinoma can be histologically classified into two categories; SCC *in*

situ or invasive SCC. Squamous cell carcinoma *in situ* affects the entire thickness of the epidermis and exhibits keratinocytes of multiple sizes and shapes. Invasive SCC on the other hand, is more aggressive as it can penetrate the dermis and metastasize to other organs. Clinically, SCC presents as a scaly lesion that may appear pink or brown and exists in multiple variants which include spindle, acantholytic, verrucous, lymphepithelioma-like, desmoplastic, adenosquamous, cystic and keratoacanthoma squamous cell carcinomas.

2.3. Melanoma

The malignancy of cutaneous melanoma is one of the most aggressive forms of skin cancer and it arises from the transformation of melanocytes situated in the basal layer of the epidermis (Jhappan et al., 2003). Melanoma, based on its location and state of progression, may be histologically classified either into melanoma *in situ* or metastatic melanoma. Melanoma *in situ*, only affecting the epidermis, consists of variably sized nests of atypical melanocytes that exhibit a radial growth pattern. Metastatic melanoma, on the other hand, displays a vertical growth phase that penetrates both the epidermal and dermal layers. This malignancy, depending on its appearance and growth, may further be subdivided into six morphological subtypes which include superficial spreading, nodular, lentigo maligna, acral lentiginous, desmoplastic and mucosal (Shashanka and Smitha, 2012).

3. Incidence of skin cancers

The magnitude of the incidence of skin cancers, particularly non-melanoma skin cancers, is difficult to gauge due to a lack of standardised registries and underreporting (Donaldson and Coldiron, 2011; Kim and Armstrong, 2012). Nevertheless, epidemiological studies have indicated a worldwide increase in the incidence of skin cancer. Since the second half of the 20th century their incidence has increased at a rate of 3 to 8% per year mainly affecting fair skinned individuals. It has further been estimated that rates will continue to double every 10 to 20 years (Garbe and Leiter, 2009; Aceituno-Madera et al., 2010; Lomas et al., 2012). Non-melanoma skin cancers have the highest incidence rates with basal cell carcinoma followed by squamous cell carcinoma being the most commonly diagnosed skin cancers in men.

Table 2.1. The various risk factors that contribute to the development of non-melanoma and melanoma skin cancers

Predisposing factors to skin cancer	Basal Cell Carcinoma	Squamous Cell Carcinoma	Melanoma
Environmental agents			
Ultraviolet radiation	Yes	Yes	Yes
Ionizing radiation	Yes	Yes	Yes
Arsenic	Yes	Yes	-
Polycyclic aromatic hydrocarbons	-	Yes	Yes
Phorbol esters	-	Yes	Yes
Tobacco	Yes	Yes	-
Psoralen+ UVA (PUVA)	Yes	Yes	-
Injuries and skin disorders			
Dermatofibromas	Yes	-	-
Nevus sebaceous	Yes	-	-
Burn scars, wounds and ulcers	Yes	Yes	Yes
Unilateral Basal cell nevus	Yes	-	-
Human papilloma virus	-	Yes	-
Inflammatory disorders		Yes	-
Actinic keratoses	-	Yes	-
Bowen's disease	-	Yes	-
Nevoid Basal cell carcinoma syndrome	Yes	-	-
Bazex-dupre-christol syndrome	Yes	-	-
Xeroderma pigmentosum	Yes	Yes	-
Albinism	Yes	Yes	Yes
Freckles	-	-	Yes
Moles	-	-	Yes
Family history	Yes	Yes	Yes
Immunosuppression			
HIV	Yes	Yes	-
Lymphoma	Yes	Yes	-
Solid-organ transplants	Yes	Yes	-

Adapted with modification from Alam and Ratner, 2001; Mackie et al., 2009; Göppner and Leverkus, 2011).

In contrast, melanoma has a lower incidence but accounts for a higher mortality rate; the overall number of deaths from melanoma is reported to be three times higher than all other cutaneous malignancies combined (Boi et al., 2003; Geller and Annas, 2003). This disease is generally more common in women but differences exist between populations.

Geographical latitude plays a significant role in the prevalence of skin cancer as the incidence of this disease increases with close proximity to the equator and reduction of latitude (Giblin and Thomas, 2007). Consequently, countries like Australia, New Zealand, and USA have higher incidence rates than the UK. A high prevalence of skin cancer has also been detected from countries in the Southern hemisphere such as Australia, Zimbabwe, New Zealand and South Africa (Jones et al., 1999; Mqoqi et al., 2004; Lomas et al., 2012). Australia has the highest levels of both NMSCs and melanoma cancers whilst South Africa has been reported to have the second highest levels of melanoma skin cancer in the world. South African NMSC incidence has been rated the second highest in Sub-Saharan Africa.

The increase in the incidence of skin cancer across the world is attributed to various factors, which include an increase in life expectancy and higher frequency of early diagnosis. However, the main predisposing risk factor that has been identified, is increased exposure to UVB radiation and is associated with sun-seeking behaviour such as use of sunbeds, wearing of less protective clothing and outdoor occupation (Giblin and Thomas, 2007; Kim and Armstrong, 2012). Climatic changes such as ozone depletion have also been suggested to play a significant role in the incidence of skin cancer (Diffey, 2004; Norval et al., 2011). Studies have indicated that Southern countries in close proximity to the Antarctic ozone hole, such as Australia, Chile and South Africa, have high UV indices which predispose them to a high prevalence of skin cancers (Saxe et al., 1998; Abarca et al., 2002; Human and Bajic, 2002).

4. The role of ultraviolet radiation in skin cancer development

Ultraviolet radiation (UVR) forms part of a continuous spectrum of electromagnetic rays emitted from the sun and its wavelength is subdivided into three categories: UVA (320-400nm), UVB (280-320nm) and UVC (200-280nm) (de Gruijl, 1999).

Most of the solar UV radiation, including all of the genotoxic UVC and part of UVB, is absorbed by the atmospheric ozone layer and approximately 90 to 99% of the amount that reaches the earth mainly results from UVA while the remaining range of 1 to 10% is from UVB (Hussein, 2005; Narayanan et al., 2010). Ultraviolet radiation, consisting of UVA and UVB, can penetrate the skin and initiate the carcinogenic

process presumably through DNA damage; the damage also affects proteins and lipids (Heck et al., 2004). Due to its low energy and poor absorption by DNA, UVA is considered a weaker carcinogen and its mutagenic and carcinogenic effects in cells is thought to occur indirectly via oxidative damage resulting from the generation of reactive oxygen species (Rünger, 1999). The longer wavelength of UVA confers to it a stronger penetrating power that reaches the dermal layer; consequently, its effects in skin are mostly associated with photo-aging resulting from oxidative damage in the dermis (Krutmann, 2001; Bachelor and Bowden, 2004). In contrast, UVB is regarded as a stronger carcinogen as it is effectively absorbed by DNA and proteins (Heck et al., 2004). Due to its high energy, it can excite the DNA molecule and cause damage directly as well as indirectly via the generation of reactive oxygen species. Since UVB has a shorter wavelength, it has less penetrating capacity and thus its carcinogenic effects mainly affect the epidermis.

The role of ultraviolet radiation, particularly UVB, as the major causative agent in the development of non-melanoma and melanoma skin cancers has been established in various experimental models and epidemiological studies (de Gruijl, 1999; Chang et al., 2010). Epidemiological studies have indicated a high prevalence of these skin cancers in the following populations: fair-skinned and sun-sensitive individuals, workers with outdoor occupations and people living closer to the equator (Mackie et al., 2009; Kim and Armstrong, 2012). In addition, most of these skin tumours develop in sun exposed body areas and SCC, which mainly affects the head and neck region, is linked to chronic cumulative UVB exposure (Markey, 1995).

In contrast, melanoma skin cancer, which mostly develops in the trunk and lower limbs, is associated with intermittent exposure (Boi et al., 2003). Although most BCCs develop in the head and neck area, its incidence has rather been associated with intermittent sun-exposure (Kwasniak and Garcia-Zuazaga, 2011).

4.1. UVB-induced DNA damage

Absorption of the UVB wavelength by DNA bases activates them to an excited state resulting in direct DNA damage with cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) photoproducts being the major lesions that form at dipyrimidine sites while others include cytosine photohydrates and purine photoproducts (Ikehata and

Ono, 2011). Although these photoproducts can be removed through the nuclear excision repair (NER) mechanism but improper removal/misrepair of these products can accumulate signature mutations involving incorrect base substitution of C to T at dipyrimidine sites and CC to TT in neighbouring bases upon DNA replication.

UVR radiation, particularly UVA, can also damage DNA indirectly as it can be absorbed by cellular photosensitisers resulting in altered energy. This can either cause DNA damage directly through type I photosensitisation reaction or indirectly via generation of reactive oxygen species (Bergamanson and Sheldon, 1997; Rünger, 2008). One of the ROS formed by UVA is singlet oxygen and it induces DNA base modifications via type II photosensitisation reactions with guanine, due to its lowest ionizing potential amongst the DNA components, being the hotspot for oxidative damage (Bachelor and Bowden, 2004; Cadet et al., 2010). Consequently, the primary DNA lesion for UVA oxidative damage is 8-hydroxy-deoxyguanosine but strand breaks as well as oxidised pyrimidines are also generated at lower levels (Rastogi et al., 2010). Frequent mutations resulting from the accumulation of oxidative DNA lesions associated with 8-hydroxy-deoxyguanosine mainly involve G to T or A to C transversions.

Although UVA is weakly absorbed by DNA, it has been reported to form cyclo pyrimidine dimers (CPD) at much higher levels than 8-hydroxy-deoxyguanosine but the yield is still far lower than that of UVB (Rünger, 2008). Hence, it is thought that the mutagenic and carcinogenic properties of UVA may result from the weak ability of this wavelength to form a few CPD dimers but this is still debatable.

Mutations in DNA can lead to cancer development as they can affect the regulation of cell growth through the inactivation of tumour suppressor genes, as well as activation and conversion of proto-oncogenes to oncogenes (Soehnge et al., 1997; Hussein, 2005). One of the key responsive genes to UVR is the tumour suppressor p53 gene, which is responsible for cell cycle arrest leading to DNA repair and apoptosis in cells with irreparable damage. Mutations in the p53 gene and its related proteins, dysregulates cell proliferation and this is presumably the initiating step in skin carcinogenesis.

5. Mechanisms of skin carcinogenesis

Skin cancer development, like most neoplasms, is a multistage process involving the following three distinct stages: initiation, promotion and progression.

5.1. Initiation

In the initiation stage, exposure of normal cells to a single genotoxic agent causes irreversible DNA damage that results in mutations, which reduce the cells sensitivity to tissue growth constraints thus generating precancerous cells (Vincent and Gatenby, 2008). This population of transformed cells exhibits no phenotypical changes and requires additional stimuli to form a tumour.

5.2. Promotion

Tumour development in the promotion stage is a long-term process involving repeated exposure of initiated cells to specific agents, which can either be of exogenous origin such as UVB light and phorbol esters or endogenous origin like growth factors (Rundhaug and Fischer, 2010). These promoters enhance the clonal expansion of initiated cells via modulation of signalling pathways that mediate inflammation, cell proliferation and apoptosis (Vincent and Gatenby, 2008). In addition, these agents exacerbate genomic instability by inducing oxidative stress via increased production of reactive oxygen species from activated inflammatory cells and other sources. Promotion is reversible in the early stages as seen when tumours regress after removal of the promoter, however, prolonged epigenetic events lead to irreversible genetic alteration that progress to malignant tumours in the late stage of carcinogenesis.

5.3. Progression

Tumour progression in skin is a process whereby benign and other premalignant lesions formed during tumour promotion are converted to malignant tumours (Digiovanni, 1992). Cellular changes involved in this stage include the accumulation of additional genetic alterations that occur independently of tumour promoters, adaptation to acidic environments resulting from upregulation of glycolysis, this

confers a potent proliferative advantage over normal healthy cells. Progression stage is phenotypically characterised by invasiveness, metastasis and angiogenesis.

6. Cellular events promoting tumour development

6.1. Cell proliferation and hyperplastic transformation

Cell proliferation and maintenance of sustained hyperplasia is one of the crucial cellular processes of cancer promotion in skin (Rundhaug and Fischer, 2010). This cellular event, which is stimulated by tumour promoters/mitogenic stimuli such as UVB irradiation, mechanical damage or chemical irritants, overcomes the latency of a hyperproliferative state caused by mutations in the tumour suppressor and oncogenes during the initiation stage (Marks and Furstenberger, 1993; Hussein, 2005). Mutations in these genes, dysregulate termination mechanisms causing damaged cells to evade cell-cycle arrest and apoptosis and generate hyperproliferative cells. The endless growth of these cells contributes to epidermal hyperplasia which is sustained by repeated exposure to the tumour promoter. Although sustained hyperplasia is a necessary event for tumour development, it is however not a sufficient condition for malignant transformation. In order for hyperplastic transformation to be effective in carcinogenesis, cell proliferation must be induced on an already transformed cell population, or in concurrence with a genotoxic agent that damages cellular DNA (Strauss, 1992; Tomatis, 1993). As a result, hyperplastic transformation in skin is often accompanied by cellular and molecular events that ensure malignant transformation and promote tumourigenesis. Inflammatory reactions play an important role in this process, as they modulate mitogenic signalling pathways that promote cell survival and/or inhibit apoptosis as well as induce genomic instability, immunosuppresion and angiogenesis (Collota et al., 2009). The critical role of inflammation during cancer development has been established and extensively reviewed (Lu et al., 2006; Kundu and Surh, 2008).

6.2. Inflammation and skin cancer development

6.2.1. Inflammation

Inflammation is a physiological response that functions as the body's defense mechanism against tissue damage and it is caused by various pathogenic, physical

and chemical agents (Tortora and Grabowski, 1996). An acute inflammatory response is beneficial to the body and mainly involves the recruitment of immune cells which aid in the eradication of insults and repair of damaged tissue. Chronic exposure to injurious or infectious agents can prolong inflammation into a pathophysiological process that contributes to the development of chronic diseases such as cancer (Aggarwal et al., 2006).

In skin, an inflammatory response is mostly triggered by a range of environmental stimuli which include UVB irradiation and toxic chemicals such as phorbol esters (Suter et al., 2009). An acute inflammatory response is characterised by erythrema, oedema, leukocyte infiltration and hyperplasia; and it is initiated by keratinocytes in the epidermal layer (Barker et al., 1991; Clydesdale et al., 2001). Although the inflammatory response is a multifactorial process that is mediated by various signalling molecules, cytokines are considered as one of the key role players in initiating and sustaining this process (Williams and Kupper, 1996; Werner and Grose, 2003).

6.2.2. Cytokines in the inflammatory process

The early signalling events in the inflammatory process in skin are mediated by primary cytokines produced, either constitutively as precursor proteins (proIL-1α and β) or upon induction (TNF-α) by various stimuli in keratinocytes (Barker et al., 1991; Grone, 2002). ProIL-1β exists as an inactive molecule in keratinocytes while proIL-1α which is predominant in keratinocytes is produced as a biologically active cytosolic protein and also exists as a myristoylated membrane-bound protein (mIL-1α) (Mizutani et al., 1991; Dinarello, 1997). Activation of keratinocytes by a pro-inflammatory stimulus, enhances IL-1α production in these cells (Kupper et al., 1987). This results in the translocation of cytosolic pro-IL-1α to the nucleus where it acts synergistically with the activating agent to induce TNF-α production and other pro-inflammatory mediators in keratinocytes. Due to the lack of a signalling peptide that facilitates extracellular transport, proIL-1α remains cell-associated. However, upon cell injury, both IL-1α and TNF-α are released into the external environment where they activate pro-inflammatory signalling via their respective surface receptors expressed on epidermal and dermal cells. Activation of these cells enhances the

production of primary cytokines (TNF-α, IL-1α/β), secondary cytokines (IL-8, and IL-6) and other pro-inflammatory mediators (Kock et al., 1990; Cohen et al., 1991). Production of IL-8 results in the recruitment of ROS producing neutrophils in the early stages of inflammation (Freedberg et al., 2001) while IL-6 is a paracrine growth factor that recruits immune cells at the later stages of inflammation (Turksen et al., 1992; Kaplanski et al., 2003). Persistent activation of these pro-inflammatory mediators causes chronic inflammation leading to carcinogenesis in skin (Dinarello, 2006). The role of cytokine-mediated inflammation in skin carcinogenesis has been demonstrated in various *in vitro* and *in vivo* inflammatory models mostly induced by UVB irradiation and phorbol esters (Lee et al., 1994; Marks et al., 1995; Clydesdale et al., 2001).

6.2.3. Cytokines in skin carcinogenesis

The accumulation of the primary cytokines, TNF-α, IL-1α and β, in tumour sites in response to local inflammatory responses has been linked to the different stages of tumourigenesis, which include cell transformation, proliferation, survival, angiogenesis, invasion and metastasis (Aggarwal et al., 2006; Apte et al., 2006). TNF-α is reported to enhance cellular transformation initiated with 3-methylcholanthrene in fibroblasts and furthermore it acts as an endogenous tumour promoter by selectively inducing the growth of transformed cells. Lastly, this cytokine induces the production of angiogenic factors associated with tumour progression in malignant cells. Interleukin-1β at low doses is associated with a limited inflammatory response while at higher doses it is associated with tissue-damage and tumour invasiveness. Interleukin-1α on the other hand, demonstrates dual activity, the membrane bound IL-1α is associated with anti-tumour activity as it activates immune cells that kill malignant cells while the cytosolic pro-IL-1α is involved in cell proliferation associated with carcinogenesis. It has also been postulated that IL-1α allows transformed cells to escape the cytotoxic effects by blocking TNF-related apoptosis inducing ligand (TRAIL) induced apoptosis in transformed cells but not normal keratinocytes (Kothny-Wilkes et al., 1999). The role of IL-1α in leukocytes infiltration and epidermal hyperplasia in tumour development has also been demonstrated on mouse skin treated with PMA (Lee et al., 1994). Due to the strong link between cytokine-mediated chronic inflammation and carcinogenesis, cytokines

have been used as biomarkers in mechanistic studies that monitor molecular mechanisms involved in carcinogenesis (Moore et al., 1999; Murphy et al., 2003). They also serve as useful tools in pharmaceutical studies concerned with skin toxicity and chemopreventive efficacy of novel anti-inflammatory compounds during drug development (Katiyar et al., 1995; Luster et al., 1995; Katiyar and Mukhtar, 1997a; Ishida and Shakaguchi, 2007; Kim et al., 2012). Chronic upregulation of cytokine production also contributes to cancer development through excessive production of ROS from immune cells as these molecules act as effectors of malignant transformation in cells (Kundu and Surh, 2008).

6.3. Oxidative stress and carcinogenesis

6.3.1. Reactive oxygen species

Reactive oxygen species (ROS) in cells are mainly produced from molecular oxygen which exists as a non-reactive biradical in its ground state (McKersie, 1996; Valko et al., 2006; 2007). Molecular oxygen may be activated to generate reactive oxygen species in two different mechanisms: that is either through energy absorption from physical irradiation leading to the formation of singlet oxygen ($^1O_2$) or via the one-electron incomplete reduction of oxygen in metabolic processes to form ROS in the following manner: superoxide anion ($O_2^{-\bullet}$) → non-radical hydrogen peroxide (H_2O_2) → hydroxyl radical ($OH^\bullet$) → water (H_2O).

The majority of reactive oxygen species is generated from electron transport chain in the mitochondria and NADPH oxidase system from inflammatory cells. Other sources include cytochrome P450, xanthine oxidase and peroxisomes (Nagata, 2005; Leonarduzzi et al., 2010). The production of these radicals in cells may be exacerbated by exogenous sources which include UV radiation, phorbol 12-myristate 13-acetate (PMA) and calcium.

6.3.2. Sources of reactive oxygen species (ROS)

Superoxide anion is the primary oxygen radical that accumulates in cells and it is generated from the mitochondrial respiratory chain in aerobically growing cells (Valko et al., 2007). The respiratory chain (Fig. 2.3), which is the major site for ATP synthesis, is composed of four complexes consisting of complex I (or NADH-

ubiquinone reductase (NQR), complex II (succinate-ubiquinone reductase (SQR), complex III (ubiquinol-cytochrome c reductase) and complex IV (and cytochrome c oxidase). It also includes complex V which is also known as F_1F_0 ATP synthase (Saraste, 1999).

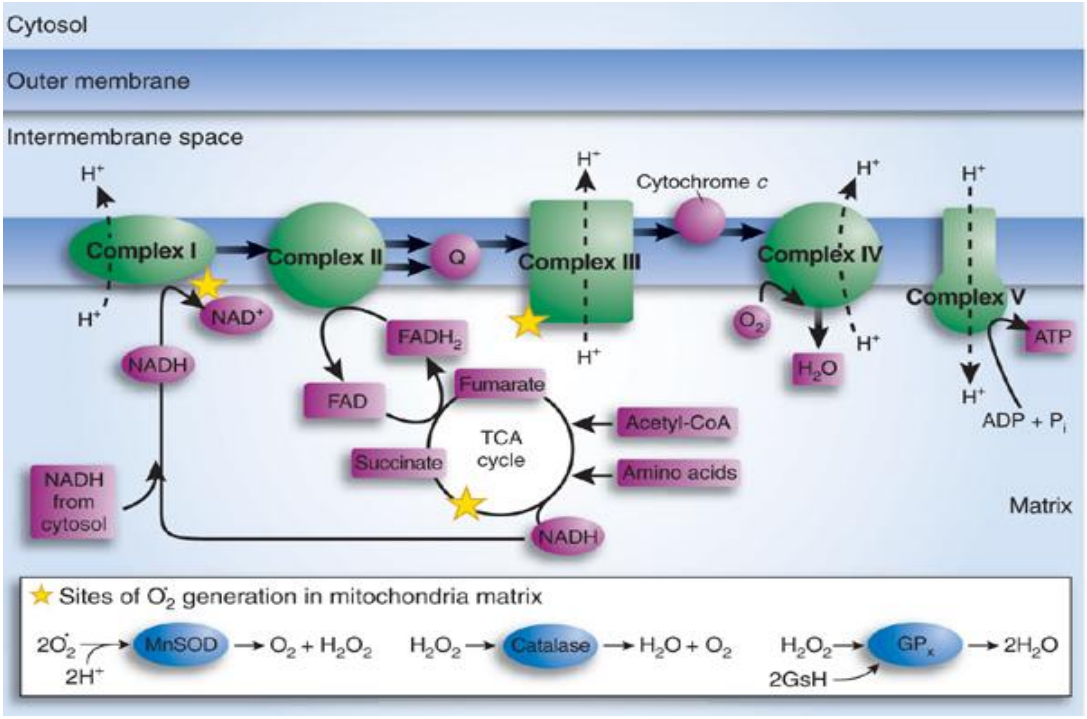


Fig. 2.3. Mitochondrial electron transport chain involved in ATP synthesis (adapted from Beal, 2005)

During oxidative phosphorylation, electrons from NADH and succinate are transferred through the complexes to oxygen which is then reduced to water. The resulting energy from electron transfer, pumps protons out into the interstitial membrane creating an electrochemical proton gradient that is used by ATP synthase to generate ATP from ADP and inorganic phosphate (Brown, 1992). During energy transduction in the respiratory chain, some electrons “leak” out prematurely from complex I and complex III resulting in the incomplete reduction of oxygen to superoxide anion. During inflammation, there is an increased amount of ROS resulting from the activation of an oxidative respiratory burst in immune cells via activation of the electrogenic NADPH oxidase system (van Heerebeek et al., 2002). NADPH oxidase enzyme consists of a membrane-bound flavocytochrome b_{558} comprising of catalytic subunit pg91 and p22 phox. The other regulatory subunits p47phox, p67phox, p22phox, GTPases and Rac are localized in the cytosol in

resting cells but upon activation (Fig. 2.4), these components translocate to the flavocytochome b_{558} molecule in the membrane to form the active NADPH oxidase complex (Leonarduzzi et al., 2010).

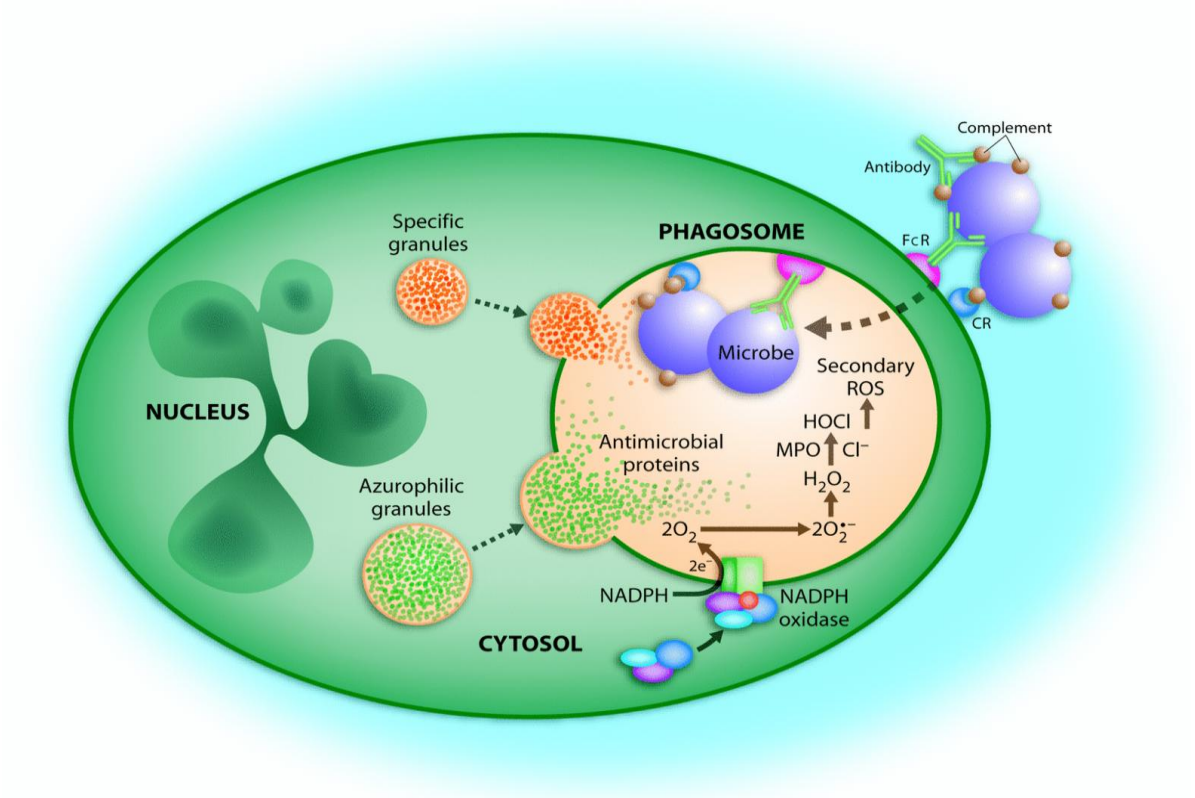


Fig. 2.4.Generation of reactive oxygen species (ROS) by the NADPH oxidase system via the formation of superoxide anion, hypochlorous acid and secondary ROS (Adapted from Quinn et al., 2006)

The activated electrogenic NADPH complex transfers electrons across the membrane and reduce oxygen to the bactericidal superoxide anion. Superoxide anion can be dismutated, either spontaneously or by the superoxide dismutase antioxidant enzyme, to hydrogen peroxide which is then converted to a hydroxyl radical and singlet oxygen. In addition, the release of myeloperoxidase from the azurophilic granules in phagocytes, catalyses the formation of the anti-microbial hypochlorus acid (HOCl) from hydrogen peroxide and chloride (Winterbourn et al., 2006).

Additional endogenous sources of ROS include xanthone oxidase, which, during purine catabolism reaction, reduces molecular oxygen to superoxide anion and hydrogen peroxide (Valko et al., 2006; Sharma et al., 2012). Peroxisomes are considered to be the major site of hydrogen peroxide, the latter is produced by these organelles during oxidative metabolic processes involving the fatty acid β-oxidation, glycolate oxidase reaction, the enzymatic reaction of flavin oxidases, and the disproportionation of $O_2^{\bullet-}$.

Cytochrome p450 enzymes also generate superoxide anion and hydrogen peroxide following the breakdown or uncoupling of the P450 catalytic cycle (Zangar et al., 2004; Bae et al., 2011). The reduction of cytochrome P450 in the endoplasmic reticulum via the NAD (P) H-dependent pathway also yields superoxide anion and hydrogen peroxide. At lower concentrations, ROS defends against invading pathogens and activates redox-sensitive signalling pathways involved in inflammation and cell proliferation (Valko et al., 2007; Leonarduzzi et al., 2010). The activity of ROS in cells is regulated by intracellular antioxidant mechanisms involving enzymatic (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic (glutathione (GSH), vitamins (A, E and C) and carotenoids) antioxidant molecules. The activity of ROS in cells is regulated by intracellular antioxidant mechanisms involving enzymatic (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic (glutathione (GSH), vitamins (A, E and C) and carotenoids) antioxidant molecules. Chronic exposure to exogenous agents such as UVB irradiation, PMA and calcium ionophore can exacerbate ROS production thus overwhelming endogenous antioxidant mechanisms leading to oxidative stress (Nagata, 2005). Under these conditions ROS can damage cellular bio-macromolecules such as DNA, proteins and lipids.

6.3.3. Oxidative damage

6.3.3.1. DNA damage

Reactive oxygen species can cause DNA damage either directly (hydroxyl radical and singlet oxygen) or indirectly (superoxide anion and hydrogen peroxide) by reacting with other molecules/transition metals present in cells to generate highly reactive molecules that can react with DNA (McKersie, 1996; Cadet et al., 2010).

The hydroxyl radical is the most reactive species against all components of the DNA molecule. In purine and pyrimidine bases, it causes oxidative damage via addition reactions to double bonds while it abstracts hydrogen atom from the backbone of the deoxyribose sugar (Cooke et al., 2003). Singlet oxygen on the other hand, is only able to react with guanine via one-electron reduction reactions.

Superoxide anion and hydrogen peroxide can only cause damage by generating hydroxyl radical in the presence of metals such as iron (Valko et al., 2007). Superoxide can reduce aqueous Fe^{3+} (Table 2.2: Equation 5) and also facilitate the release of Fe^{2+} from molecules containing iron-sulfur clusters (Equation 3 and 4) by yielding an unstable cluster via reduction of Fe^{3+}. The released Fe^{2+} can generate the highly reactive hydroxyl radical by participating in Fenton type reaction with hydrogen peroxide (Equation 1). The latter is formed upon protonation of superoxide anion in an aqueous solution (Equation 2). The hydroxyl radical may also be formed via the Haber-Weiss reaction (Equation 6) which combines the Fenton reaction and reduction of Fe^{3+} by superoxide anion. However, there is controversy on the feasibility of this reaction *in vivo* as it is mostly demonstrated *in vitro* (Koppenol, 2001). The hydroxyl radical causes damage that affect bases and generates numerous DNA adducts with 8-oxo-7,8 dehydro-2'-deoxyguanosis being the most commonly occurring and often used as a biomarker for oxidative DNA damage (Cooke et al., 2003; Cadet et al., 2010). Other DNA adducts include cytosine and thymine glycol and its redox and deamination products as well as ring-opened structures, base free sites and DNA-protein cross-links. Abstraction reactions in deoxyribose sugar also yield modifications and strand breaks. Even though they are less frequent, DNA-protein cross-links are not readily repaired and can be lethal if replication or transcription precedes repair (Sharma et al., 2012).

Table 2.2. Reactions involved in the generation of radical by superoxide anion

Equation 1	Fe^{2+} or $Cu^{+}+H_2O_2+ H^{+}$	→	Fe^{3+} or Cu^{2+} + $OH^{\bullet}+H_2O$
Equation 2	$2O_2^{\bullet-} + 2H^{+}$	→	$H_2O_2 + O_2$
Equation 3	$[2Fe^{2+}+2Fe^{3+}-4S] + O_2^{\bullet-}+ 2H^{+}$	→	$[Fe^{2+}+3Fe^{3+} + 4S] + H_2O_2$
Equation 4	$[Fe^{2+}+3Fe^{3+}- 4S] + H_2O_2$	→	$[3Fe^{3+}- 4S] +Fe^{2+}$
Equation 5	$O_2^{\bullet-}+Fe^{3+}$or Cu^{2+}	→	$O_2 +Fe^{2+}$or Cu^{+}
Equation 6	$O_2^{\bullet-}+H_2O_2+H^{+}$	→	$O_2 + OH^{\bullet}+H_2O$

Adapted from Perron et al., 2009

If oxidative products are left unrepaired, they can lead to mutations which play an essential role in tumourigenesis, the most common mutation arise from the oxidation of guanine and involve to G:T to T:A transversions (Valko et al., 2006). The role of nuclear DNA damage in the process of carcinogenesis has been studied extensively and is well established. It has been indicated that mitochondrial DNA is more susceptible to oxidation than nuclear DNA and this is ascribed to the following: close proximity to the ROS producing system, limited DNA repair capacity (NER) and lack of the protective protein histones. Subsequently, mitochondrial oxidative DNA damage has been demonstrated in the carcinogenesis process and mutations identified in human cancers found to affect the respiratory complexes in the electron transport chain (Inoue et al., 2003; Chatterjee et al., 2006).

6.3.3.2. Protein damage

Oxygen radicals cause direct and indirect modifications in proteins and these results from hydrogen abstraction and oxidation reactions affecting the side chains of all amino acid residues, with cysteine and methionine residues being the major targets (Berlett and Stadtmann, 1997; Sharma et al., 2012). Direct modifications involve introduction of carbon-centred radicals yielding alkoxyl and hydroxyl radicals, formation of protein cross-links resulting in disulphide bridges; as well as induction of methionine sulphoxide (McKersie, 1996; Valko et al., 2007). Metals may catalyse site-specific damage to the amino acid residue via Fenton type reactions (Valko et al., 2006). Oxidative action by ROS may also cause modification indirectly via aggregation of proteins with lipid peroxidation breakdown products.

Modifications resulting from oxidation inactivate protein function and predispose them to degradation by proteases. Extensively oxidised proteins that have formed cross-links and aggregates are resistant to degradation (Dean et al., 1997).

6.3.3.3. Lipid peroxidation

Excessive ROS production can cause damage to both cellular and organelle biological lipid membranes by enhancing lipid peroxidation (Sharma et al., 2012). The lipid membrane is a bilayer that is composed of a mixture of phospholipids and glycolipids that have fatty acid chains attached to carbon 1 and 2 of the glycerol backbone by an ester linkage (McKersie, 1996). The unsaturated bond between two carbon atoms and the ester linkage are the most common sites for oxidation in fatty acids. The overall process of lipid peroxidation involves three distinct steps: initiation, propagation and termination. Initiation involves hydrogen abstraction from the methylvinyl group of an unsaturated fatty and formation of a carbon centred radical. During propagation, the resonance structure is involved in chain reactions that generate lipid hydroperoxides and other carbon centred free radicals which can participate in a second H abstraction. In the presence of iron, the chain reaction is amplified via Fenton type reaction below.

$ROOH + Fe^{2+} \rightarrow OH^{-} + RO^{\bullet} + Fe^{3+}$

Lipid hydroperoxides may be converted to conjugated dienes or decomposed to various other products including aldehydes, such as MDA, and hydrocarbons such ethane and ethylene. These products are commonly used as biomarkers of lipid peroxidation in tissue. Lipid peroxidation reactions in membranes are terminated when the carbon or peroxyl radicals cross-link to form conjugated products that are not radicals as depicted in the following reactions:

$R^{\bullet} + R^{\bullet}$ to R-R

$R^{\bullet} + ROO^{\bullet}$ to ROOR

$ROO^{\bullet} + ROO^{\bullet}$ to $ROOR + O_2$

Typically high molecular weight, cross-linked fatty acids and phospholipids accumulate in peroxidised membrane lipid samples. Lipid peroxidation may in turn lead to changes in the fluidity of the plasma membrane and may cause leakage of molecules and subsequent dysfunction in its essential role.

7. Antioxidant defense mechanisms in the skin

Oxidative stress in skin results from endogenous and exogenous sources and, like other tissues, this organ has adapted several mechanisms to cope with the increasing oxygen concentrations (Kohen, 1999). These include the repair systems, prevention, and physical and antioxidant defense mechanisms. One of the most important defense mechanisms in skin is the antioxidant system comprised of enzymatic and non-enzymatic antioxidant molecules. The enzymatic group includes superoxide dismutase (SOD), catalase and the glutathione system consisting of glutathione (GSH), gluathione peroxidise (GPx) and glutathione reductase (Pugliese, 1998; Chen et al., 2012). These molecules function in the following mechanisms: SOD catalyzes the dismutation of the superoxide radical to hydrogen peroxide and oxygen. Hydrogen peroxide is then detoxified to water by the membrane-bound catalase and cytosolic GPx. The enzymatic degradation of hydrogen peroxide occurs in the presence of GSH, which is necessary for hydrogen donation. Upon this reaction (Equation 1), two GSH molecules combine to form a disulfide molecule (GSSG). In order for GSH to participate in hydrogen donation again, it requires regeneration by glutathione reductase and NADPH as depicted below.

1. H_2O_2 + GPx + 2GSH→ GSSG + H_2O or ROH

2. GSSG +glutathione reductase +NADPH→ 2 GSH +NADP

Glutathione forms part of the low molecular weight non-enzymatic group comprised of hydrophilic (cytosol) and lipophilic (lipid membranes) antioxidant compounds. These include vitamins such as L-ascorbic acid (vitamin C) and α-tocopherol (vitamin E). These molecules levels which are much higher in the epidermis, prevent damage either directly by scavenging radicals via electron/hydrogen donation reactions or indirectly through metal chelation. Although non-enzymatic molecules posses several advantages over the enzymatic system,

they are however only effective if their concentration is sufficient to compete with the biological target (Steiling et al., 1999). Glutathione is regarded as the major antioxidant and redox buffer of the cells, the activity of this molecule is dependent on the high ratio of GSH/GSSG, which is maintained by GSH reductase (Pugliese, 1998; Valko et al., 2007).

Apart from acting as cofactor to detoxifying enzymes, GSH scavenges radicals directly or indirectly via activation of vitamin C and E (Chen et al., 2012). Vitamin C is a water soluble antioxidant that neutralises free radicals in the cytosolic compartments and also plays a role in regenerating oxidised vitamin E in sites of lipid peroxidation. In skin, α-tocopherol, the most abundant form of vitamin E, is a lipid soluble antioxidant that protects cell membranes from oxidative stress and is delivered to the deepest layer of the straturm corneum by sebaceous gland secretion.

Another antioxidant defense system is the thioredoxin reductase system consisting of thioredoxin, thioredoxin reductase and NADPH (Pugliese, 1998). It is found particularly in high levels in the epithelial tissue. This antioxidant system plays an important role in protecting the cells from hydrogen peroxides by facilitating the reduction of proteins in redox reactions involving dithiol cystein-disulfide groups and NADPH. Although this antioxidant system is absent from the straturm corneum, thioredoxin is highly active in keratinizing cells while both thiredoxin and thioredoxin reductase are present in Langerhan's cells and melanocytes.

Despite the skin's innate defenses', increased oxidative stress resulting from ageing and exposure to UVR, can overwhelm the skin's antioxidant reserves and enzymatic machinery leading to the development of diseases such as skin cancer (Valko et al., 2006; Chen et al., 2012).

8. Prevention strategies and treatment plans

In an effort to reduce the incidence of skin cancer, countries have designed national intervention strategies involving primary and secondary prevention (Cummings et al., 1997; Schulz, 1998; Edlich et al., 2004). Primary prevention promotes sun-awareness and behavioural life-style changes such as sun avoidance,

wearing of protective clothing and use of sunscreens. Secondary prevention strategies involve screening and early detection in combination with sun-education; these factors ensure the effective treatment of skin cancer. Available treatment options fall into three categories: surgical treatments, radiation therapy and superficial therapy. Surgical treatment is the most commonly used in the treatment of high risk tumours, but it is highly invasive, costly and can cause disfigurement. An alternative method of therapy with excellent cosmesis is radiation but it can predispose patients to development of additional skin cancer. Although the use of pharmacological agents in superficial therapy offers an alternative for patients avoiding other treatment methods, it is only effective for low-risk tumours (Ceilley and Rosso, 2006).

The limitations and drawbacks of prevention strategies and treatment options contribute to morbidity and mortality rate of skin cancer as there is low-compliance to behavioural changes while treatment methods are costly, highly invasive and cause disfigurement and have high a recurrence rate (Anthony, 2000; Kim and Armstrong, 2012). Consequently, emphasis for skin cancer management has been placed on intervention strategies that delay malignant transformation (Shureiqi et al., 2000). Since reversal of genetic mutations in the inititation stage is not a feasible option, the focus has been on the modulation of critical events in the promotion stage. One promising alternative strategy that has been targeted for use against cancer promotion is chemoprevention.

9. Chemoprevention as a novel approach

Chemoprevention is a strategy that utilises dietary or pharmacological agents to inhibit or reverse the development of cancer before cellular invasion across the basement membrane (Wright et al., 2006; Wu et al., 2011). However, before novel compounds are applied for clinical use in humans, they are subjected to safety and chemopreventive efficacy testing in preclinical models and clinical trials. Most chemoprevention studies follow the strategy of the National Cancer Institute (NCI) drug development screening program system which utilises different phases of *in vitro* and in *vivo* efficacy testing in the following preclinical models: biochemical pre-screening assays, *in vitro* efficacy models, *in vivo* short-term screening assays and

animal efficacy models (Steele et al., 1996; 1998; 2010). The most active compounds in animal models, with acceptable toxicity profiles, progress to phase IIb (modulation of selected biomarkers in humans) and phase III (cancer reduction in large populations) in clinical trials (Stratton et al., 2000). Novel chemopreventive agents are considered effective if they adhere to the following criteria: afford protection in high risk healthy individuals, prevent against cancer development and secondary forms of cancer in individuals with existing pre-malignant lesions and those treated for primary cancers, respectively (Russo, 2007).

9.1. Preclinical chemoprevention models

9.1.1. Pre-screening biochemical assays

Pre-screening assays are a series of short-term, mechanistic based assays developed to assess the efficacy of novel chemopreventive compounds against biochemical events linked to cancer development (Steele et al., 1998). These pre-screening assays are cost-and time-effective and allow for rapid biological characterisation, provide mechanism of action and classify compounds before *in vitro* efficacy testing (Shureiqi et al., 2000). Classification of prospective chemopreventive compounds into a single category is considered difficult, as compounds possess a range of anti-carcinogenic properties. Consequently, chemopreventive compounds are categorised according to the following biological properties: anti-hormones, anti-inflammatory, antioxidants, glutathione inducers and inhibitors of arachidonic acid metabolism, ornithine decarboxylase and protein kinases. In general, the anti-carcinogenic activity of chemopreventive compounds may be divided into three categories: deactivation of carcinogen, antioxidant activity and anti-proliferative activity. The most commonly used biochemical assays for each category are depicted below in Table 2.3. Further development of standard protocols to assess other relevant mechanisms, relating to cellular differentiation, immunosuppresion and intercellular communication were reported to be still in progress (Steele et al., 1998).

Table 2.3 Biochemical pre-screening assays commonly used by NCI drug development program for novel chemopreventive agents

Biochemical assay
Cancer blocking properties
Carcinogen-DNA binding assay
GSH induction
GST induction
NAD (P) H-quinone reductase induction
Antioxidant properties
GST inhibition
Peroxisomal enzyme inhibition
Cu/Zn superoxide dismutase
Xanthine oxidase
Prostaglandin synthase hydroperoxidase
5-lipoxygenase
Anti-proliferative properties
Tyrosine kinase
Cyclic AMP phosphodiesterase
Protein Kinase C
ODC inhibition
Poly (ADP-ribose) polymerase activity
EGRF/tyrosine kinase
5-alpha reductase inhibition
Aromatase inhibition
Endonuclease inhibition
Protease and collagenase inhibition
Ras-farnesylation inhibition

Adapted from Steele et al., 1998

9.1.2. In vitro efficacy models

In vitro models are a series of short-term bioassays used to select and rank the chemopreventive efficacy of compounds for subsequent testing in animal models, as well as provide insight into the potential mechanism of action (Steele et al., 1998). The Chemoprevention Branch of NCI drug development programme has developed a series of *in vitro* models employing the use of organ-specific human primary epithelial cells. The five commonly used *in vitro* models which measure the efficacy of test compounds by monitoring inhibitory effects against the following: (1) benzo [*a*] pyrene (B [*a*] P) induced transformation in primary rat epithelial cells (2) anchorage-independent growth in human lung tumour (A427) cells (3) carcinogen-induced hyperplastic alveolar nodule formation in mouse mammary gland organ cultures (4) anchorage dependent growth of JB6 epidermal cells and (5) carcinogen-induced cell growth and differentiation in human foreskin epithelial cells.

In vitro models have been shown to be highly effective in predicting the chemopreventive efficacy of compounds in an *in vivo* model (Steele et al., 1996; 1998). However, it was suggested that application of *in vitro* models commonly used by NCI chemoprevention branch, as well as any single assay, may not be inclusive of all the possible mechanisms of cancer chemoprevention. Therefore, a battery of *in vitro* assays need to be applied when testing the chemopreventive efficacy of novel compounds. It has also been indicated that new *in vitro* models utilising relevant tissue, primary or immortalised cells is urgently needed to rapidly and efficiently screen chemical agents for potential chemopreventive activity (Steele et al., 1998). Various factors have been recommended for consideration during selection of *in vitro* models and these involve: inclusion of epithelial cell when possible as most cancers are of epithelia origin, organ specificity, and human primary cells due to their intact drug metabolising system and possession of a normal genome.

9.1.3. In vivo efficacy models

In vivo models are used to prioritise and monitor the efficacy of chemopreventive agents that will proceed to human clinical trials. They employ the use of well established rodent carcinogenesis models which are either carcinogen-induced, spontaneous or transgenic (Steele et al., 1998; 2010; Wu et al., 2011). The intention of chemoprevention studies is to utilise organ-specific experimental models that would determine efficacy of compounds against specific forms of cancer as well as provide valuable dose-response, toxicity and pharmacokinetic data required for phase I clinical trials. Ideally the experimental models should have short-term duration, be target specific, indicate high tumour incidence showing human relevance and be considerate of mechanistic data and toxicity profiles generated *in vitro* and correspond with existing literature.

Carcinogenesis animal models utilised to measure the chemopreventive efficacy of compounds in skin include the classical two-stage chemically and UVB-induced mouse skin cancer models. In the two-stage chemical model, tumours are induced with a single topical application of 7,12-dimethylbenz [α] anthracene (DMBA) or (B [*a*] P) and promotion is effected with multiple doses of 12-O-tetradecanolyphorbol-13-acetate (TPA) applied to the back skin of SENCAR or CD-1 mice. The UVB

model is considered extremely relevant to the etiology of human skin cancer (Steel et al., 2010). Skin lesions in this model are effected after multiple exposure of SKH-1 hairless or PTCH gene knocked mice to UVB-light. They develop tumours exhibiting characteristics similar to squamous cell carcinoma and basal cell carcinoma, respectively.

A number of synthetic and natural test chemopreventive agents, administered in the diet or applied topically, have proven effective in both chemically and UVB-induced skin carcinogenesis models (Marnewick et al., 2005; Steele et al., 2010; Petrova et al., 2011). Natural compounds from dietary components such as fruits, vegetables and tea, have shown potential as prospective chemopreventive agents (Steele et al., 1998; Stratton et al., 2000; Afaq, 2011). Due to their safety, low toxicity and general acceptance, these compounds have been targeted for use as an inexpensive and easily accessible intervention strategy in cancer management (Swan and Ford, 1997; Amin et al., 2009; Dai and Mumper, 2010).

10. Phytochemicals and their role in chemoprevention

The intake of natural compounds from dietary plant foods and products such as fruits, vegetable, wine and tea has been associated with the prevention of various diseases including cancer (Greenwald et al., 2001; Boeing et al., 2012). Studies have provided strong scientific evidence between cancer reduction and high fruit and vegetable consumption and this has been attributed to additive and/or synergistic interaction of the complex mixtures of phytochemicals present in whole foods (Liu, 2003; Amin et al., 2009). One group of natural compounds that has been studied in cancer prevention is the polyphenols. *In vitro* studies have been useful in providing various mechanisms involved in their anti-carcinogenic properties while clinical trials have determined their relevance against various types of cancers in humans (Russo, 2007; Korkina et al., 2009; Vauzour et al., 2010). However, several clinical studies have failed to find a connection between intake of polyphenols and cancer reduction. This has been attributed to challenges of assessing the real impact of these compounds due to the lack of knowledge on the exact composition in foods and the bioavailability of active compounds. Nevertheless, most *in vitro* and *in vivo* studies have indicated the beneficial effects of these compounds on human health and their

potential use as additives in products such as supplements, sunscreens and skin care products for cancer management (Bogdan and Baumann, 2008; Ramos, 2008; Afaq, 2011). Consequently, in recent years, identification, chemical and biological characterisation of polyphenolic compounds and their chemopreventive properties has become a major area of cancer research (F'guyer, 2003; Youn and Yang, 2011).

10.1. Distribution of polyphenolic compounds

Polyphenolic compounds are one of the most commonly occurring groups of phytochemicals and their occurrence, chemistry and physiological significance has been reviewed extensively (Bravo, 1998; Dai and Mumper, 2010). These bioactive compounds are produced as secondary metabolites in medicinal and dietary plants which include, fruits, vegetables and teas where they function in defense against pathogens, predators and environmental stresses. They also play a significant role in regulating plant growth and reproduction (Ignat et al., 2011). Plant polyphenols are mostly derived from trans-cinammic acid in a process involving primary and secondary metabolism of L-phenylalanine via the shikimic acid and phenylpropanoid pathway, respectively (Vladimir-Knežević et al., 2012). Phenolic compounds range from simple structures with a single phenolic ring to complex polyphenolic structures with hydroxyl groups on aromatic rings (Ignat et al., 2011). Based on the number and structural components of the phenolic rings, the polyphenolic compounds may be divided into several classes but the main groups include flavonoids, phenolic acids, tannins (hydrolysable and condensed), stillbenes and lignans.

10.2. Flavonoids

One of the most commonly occurring groups of phytochemicals is the low molecular weight flavonoids which have over 4000 identified species. These compounds are characterised by a C6-C3-C6 structure (Fig. 2.5), consisting of two aromatic rings A and B joined by a heterocyclic ring C (Ignat et al., 2011).

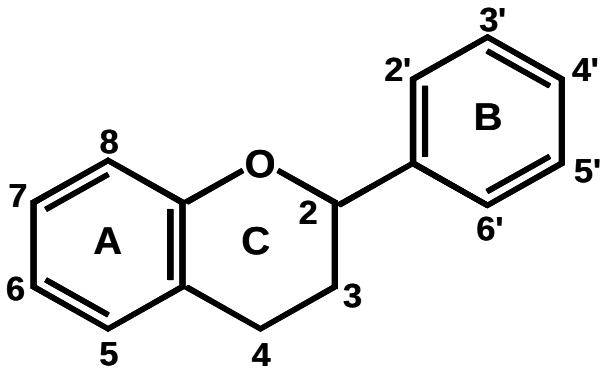


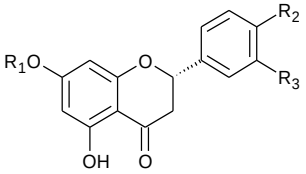
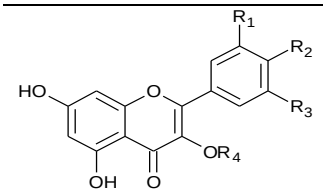
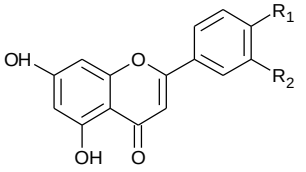
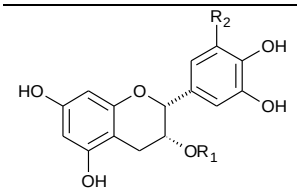
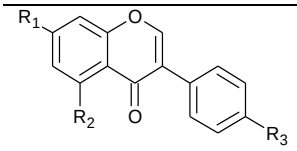
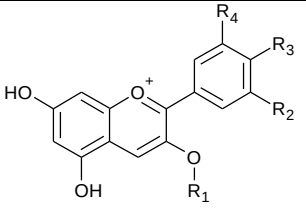
Fig. 2.5. Flavone backbone structure and numbering pattern common for flavonoids. Adapted from Harbone and Baxter, 1999.

Flavonoids are subdivided into different subclasses based on their structural characteristics relating to unsaturation, oxidation and variation in number and arrangement of hydroxyl groups, as well as the nature of alkylation and glycosylation (Peterson and Dwyer, 1998; Dai and Mumper, 2010). These subclasses include anthocyanins, flavones, isoflavones, flavanones, flavonols and flavanols and are found in various dietary sources (Table 2.4.)

10.3. Absorption, bioavailability and metabolism of flavonoids in skin

Absorption of topically applied polyphenols is challenging as the highly effective barrier properties of the skin complicates the penetration of large exogenous molecules with high molecular weight (> 500 Dalton), or low lipophilicity through the epidermal layer of undamaged skin (Korkina et al., 2008). It has been shown that the transdermal delivery of topically applied plant polyphenols can be improved by incorporating these compounds into liposomes or lipogel (Fang et al., 2006a; Casagrande et al., 2006; Sinico et al., 2008); or utilising electrically assisted methods such as electroporation and iontophoresis (Fang et al., 2006b). Once absorbed, polyphenolic compounds can either be distributed through all layers or concentrated in a specific layer and this is dependent on the physicochemical properties of the polyphenol and nature of the delivery system.

Table 2.4. Flavonoid subclasses and their various dietary sources

Classes	Example (compound name and substitution)	Dietary source
 Flavanone	Hesperidin: R_1= *O*-rutinosyl; R_2=OCH_3; R_3=OH	Lemons, oranges, cumin and peppermint
	Naringerin: R_1=H; R_2= OH; R_3=H	Grape fruit, berries, citrus fruit
 Flavonol	Quercetin: R_1=R_2=OH; R3=H	Apples, berries, onions, grapes, olives, raisins, chilli pepper, Oregano, Sage and thyme
	Myricetin: R_1=R_2=R_3; R_4=H	Apple, berries and tea
	Kaempferol: R_1=R_3=R_4=H; R_2=OH	Onion, broccoli, apples, berries, black grapes and tea
 Flavone	Luteolin: R_1=H; R_2=OH	Lemon, olives, celery, red pepper apples, parsley, cereals and herbs
	Apigenin: R_1=OH	Berries, parsley, cereal grains and celery
 Flavanol	Catechin: R_1=H; R_2=OH	Apples, apricots, berries, black grapes, beans, chocolate, red wine
	Epigallocatechin gallate: R_1=gallate; R_2=OH	Beans, teas
 Isoflavone	Genistein: R_1=OH; R_2=OH; R_3=OH	Soybeans, black beans, peas, sprouts
	Daidzein: R_1=OH; R_2=H; R_3=OH	Soybeans, black beans, peas, sprouts
 Anthocyanidin	Cyanidin: R_1=OH; R_3=OH; R_4=OH	Black grapes, red wine
	Delphinidin: R_1= R_2=R_3=R_4=OH	Berries, aubergine skin

Adapted from Rice-Evans et al., 1996; Peterson and Dwyer, 1998.

For instance, the phenylproponoids caffeic acid and chlorogenic acid penetrated all the skin layers with a flux value of 0.32 and 0.48 µg cm^{-2} h^{-1}(Marti-Mestres et al., 2007). Caffeic acid (molecular weight of 354.3kDa) displayed superior percutaneous absorption than chlorogenic acid (molecular weight of 180.2kDa) and this was attributed to the solubility of this compound with an optimal log of $K_{o/w}$ -1 while chlorogenic acid was 1.1. The absorption of both these compounds was more efficient than oraposide with a molecular weight of 624.61kDa and a suboptimal log of $K_{o/w}$ -1.3, however this phenylpropanoid was more concentrated in the stratum corneum. It has been found that application of EGCG from green tea as a transdermal gel in SKH-1 mice has an efficient penetrating capacity as it quickly permeated all the skin layers with majority of this compound being concentrated in the epidermal layer.Traces of EGCG have also been reported in the plasma, liver and small intestine, thus transdermal EGCG was regarded an effective way to deliver prolonged levels to plasma and tissues. Lesional skin allows for more efficient absorption of compounds than intact skin thus plant polyphenols presumably penetrate faster in lesional skin as has been reported for the anti-psoriasis drug, anthralin (1,8-dihydroxy-10H-anthracen-9-one) (Geilen and Orfanos, 2002).

Although topical application of polyphenols has poor absorption, it is regarded as the more effective route of administration as it offers direct pharmacological intervention, increases the levels of the parent compound in skin and minimises potential systemic toxicity (Korkina et al., 2008; OyetakinWhite et al., 2012). Even though systemic application is associated with poor bioavailability of polyphenols but it does show advantage with the easier intestinal absorption when compared to cutaneous absorption (Korkina et al., 2008). The human skin contains numerous enzymatic and non-enzymatic factors capable of reacting with polyphenols and facilitating their metabolism. Interaction of polyphenols with some of these molecules may interfere with their activity and cause cytotoxic effect. Consequently, topical application of polyphenolic compounds in skin has some adverse and beneficial effects. On the one hand these compounds may incur non-enzymatic transformation resulting from exposure to atmospheric oxygen and UV as well as interaction with the skins' redox system. Interaction of polyphenols with these factors may result in their autoxidation leading to the generation of reactive oxygen species that induce cytotoxic effects associated with mutagenicity, sensitisation and carcinogenesis in

skin. On the other hand, polyphenolic compounds can protect against chemical and photo carcinogenesis either by modulating the activity of phase I and II drug metabolising enzymes or the signalling molecules involved in inflammation, cell proliferation and apoptosis.

10.4. The biological properties of flavonoids in chemoprevention

The anti-carcinogenic properties of the flavonoids have been demonstrated in various animal models and cell culture systems (Kuo, 1997; Yao et al., 2011). These studies have indicated that chemopreventive properties of these compounds are mediated by the following mechanisms: antioxidant/pro-oxidant properties, modulation of metabolising enzymes, anti-inflammatory activity and modulation of proliferation and apoptosis.

10.4.1. Antioxidant activity

Flavonoids protect normal cells against oxidative damage, caused by free radicals or carcinogens, by exerting antioxidant effects involving the following mechanisms: (i) direct radical scavenging in hydrogen or electron donation reactions (ii) chelating metal ion involved in Fenton reactions and (iii) modulation of enzymes related to oxidative stress (GPx, GR and SOD) (Vayalil et al., 2003; Ramos and Aller, 2008; Lambert and Elias, 2010; Procházková et al., 2011).

The efficiency of flavonoids as antioxidants is determined by their ability to delay or inhibit the oxidation of macromolecules at lower concentrations than the oxidizable substrate. The chemical structure also plays an important role in this activity as these compounds must be able to stabilise the radicals after scavenging reactive species to prevent chain reactions that cause oxidative damage in cells (Rice-Evans et al., 1996; Apak et al., 2007). The structural requirement for effective antioxidant activity includes the following: catechol group on the B ring; 2, 3 unsaturation with the 4-oxo function in the C ring; and 3, 5 hydroxyl groups with 4-oxo function in the A and C rings. These components function in radical scavenging, stabilisation of the quinone radical and electron delocalisation.

10.4.2. Pro-oxidant activity in chemoprevention

Depending on the cell type, dose and time of treatment, flavonoids can also induce selective toxicity towards tumour cells by enhancing their persistent pro-oxidative state causing oxidative damage that sensitises these cells to ROS-induced apoptosis (Korkina et al., 2008; Ramos, 2008). The pro-oxidant effects of flavonoids are associated with increased ROS levels and/or modulation of endogenous antioxidant molecules such as GSH. Radicals formed by flavonoid compounds include semiquinone or phenoxyl radicals and ROS (hydroxyl radical). These reactive molecules are generated from the following processes: auto-oxidation catalysed by redox active polyphenol-metal complexes, radical scavenging, as well as the Fenton type reaction (Procházková et al., 2011). In chemoprevention, these radicals are associated with the induction of programmed cell death in cancer cells. For instance, quercetin has been shown to generate sufficient ROS in cancer cells for the induction of apoptosis via activation of p38 signalling pathway and recruitment of caspases (Lee et al., 2009). Apoptotic activity of flavonoids can also be mediated independently of the caspases and mitochondria through DNA damage (Jeong et al., 2009). Due to their chelating activity, flavonoids can also interfere with metal-containing enzymes in cells causing mutation that modifies the activity of cancer cells leading to cell death (Korkina et al., 2008). On the other hand, these natural compounds can also modulate endogenous antioxidant defense system such as GSH. For instance, the flavones, chrysin and apigenin, are reported to efficiently deplete GSH levels in cancer cells and this was associated with augmentation of toxic effects from pro-oxidants (Gibellini et al., 2010). It has also been indicated that quercetin generates semi-quinone and quinone radicals that reduce GSH levels and activity leading to activation of apoptosis via the mitochondrial pathway.

11. Tea (*Camellia sinensis*) in chemoprevention

One of the most studied and promising groups of natural compounds against skin cancer are the catechins, particularly EGCG, from green tea as well as the theaflavins and thearubigins from black tea (Yang et al., 2002; Nichols and Katiyar, 2010; Youn and Yang, 2011). The beneficial health effects of these tea polyphenols has been demonstrated in various cell culture systems and human studies, which

indicated the potential use of these compounds as chemopreventive agents that could be used with other strategies to prevent cancer development in skin (Katiyar and Mukhtar, 1997b; 2000).

11.1 Background and processing

Tea produced from the plant of *Camellia sinensis* is, next to water, the most consumed beverage in the world. It is produced either from dried fresh or enzymatically oxidized young buds and leaves of *Camellia sinensis* L. (Theaceae) (Lin et al., 2008). This plant originates from South China but is also cultivated in many other countries such as Japan, India, Sri Lanka, Indonesia, Australia, Kenya and Chile. In general, more than 300 types of teas are produced from this plant and, based on their processing procedure and degree of oxidation, they are divided into: green tea (unfermented), oolong tea (partially fermented) and black tea (fully fermented) (Sang et al., 2011). The processing of green tea involves steaming or pan-frying of plant leaves which inactivates enzymes and prevents the oxidation of the tea constituents, while for oolong tea, the remaining enzymes are used for the partial oxidation of fresh leaves. In black tea, leaves are crushed to catalyse enzymatic oxidation of catechins, which subsequently leads to their polymerisation and formation of complex molecules. The fermentation process has been shown not only to affect the chemical composition of the teas but also their biological activity. Consequently, green tea which is known to have a higher polyphenolic content is considered to be more effective. However, other studies have also focused on polyphenolic compounds of black tea as they have found to also have protective effects (Shukla, 2007; Yang et al., 2011).

11.2. Chemical composition

Chemical analysis has indicated the presence of purine (xanthine) alkaloids, polyphenolic compounds, terpenoids and other compounds in teas (Yang et al., 2000; Sang et al., 2011). In brewed green tea, tea polyphenols, which mainly consists of catechins, account for 30 to 42% of soluble solids with (-)-epigallocatechin gallate (EGCG) being the major polyphenolic constituent that makes up 50 to 80% of total catechins (Balentine et al., 1997; Harbowy et al., 1997).

The other catechins include (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epicatechin (EC). Other flavonoids such as quercetin, myricetin and kaempferol and their glycosides exist as minor compounds and 19 O-glycosylated flavonols, 7 C-glycosylated flavones, 28 acylated glycosylated flavonols, and 3 flavonols have also been identified from green and black teas (Lin et al., 2008). The alkaloid, caffeine accounts for 2 to 6% of the soluble solids and there's reportedly no difference regarding its content between green and fermented teas thus indicating its stability during the fermentation process (Sang et al., 2011). During fermentation, catechins are oxidized and polymerised to theaflavins and thearubigins in a process that is catalysed by polyphenol oxidase. Theaflavins and thearubigins are mainly found in black and oolong teas (Sang et al., 2011). The theaflavins make up 2 to 6% of the soluble solids in brewed black tea and are key constituents in the properties of colour and taste and also play a major role in the formation of thearubigins (Balentine et al., 1997; Haslam, 2003; Kuhnert, 2010; Kuhnert et al., 2010a). These polyhydroxylated oligomers, thearubigins, are the major compounds in black tea and account for 60 to 70% of the tea solids in the infusion (Drynan et al., 2012).

11.3. Chemopreventive properties

11.3.1. Anti-carcinogenic properties

Studies employing the use of chemical and UVB animal skin carcinogenesis models have indicated that polyphenolic extracts from green and black teas can protect against initiation, promotion and progression stages during cancer development; by reducing the size and number of carcinogen-induced tumours on mouse skin (Wang et al., 1989; 1992; 1994; Huang et al., 1992). The protective effects of these teas against tumourigenesis have been ascribed to their polyphenolic constituents although caffeine has also been indicated as a biologically important constituent in the activity of the teas (Huang et al., 1997; Lu et al., 1997). The anti-tumour activity of the following compounds, EGCG, caffeine and black tea polyphenols, has been demonstrated on mouse skin (Lu et al., 2002; Patel et al., 2008). Conflicting results have been reported between the protective activity of tea polyphenols, with some studies reporting a higher activity from black tea polyphenols (Record and Dreosti, 1998; Roy et al., 2009) while the extensively studied green tea

polyphenols were found to have stronger protective effects in other studies (Huang et al., 1997; Shukla, 2007). The protective effects of tea polyphenols applied systemically and topically have been demonstrated in various studies with the topical application found to be more effective in protecting the skin against damage (Bickers et al., 2000; OyetakinWhite et al., 2012). The relatively lower activity of systemically applied tea polyphenolic constituents when compared to topical application has been attributed to the limited bioavailability of these compounds in the targeted cells skin cells. The poor bioavailability of catechins, particularly EGCG, has been related to their high molecular weight and the presence of a large number of hydroxyl groups in their aromatic rings (Lee et al., 2002; Sang et al., 2011).

In addition, some of the systemically applied catechins undergo considerable biotransformation to form conjugated metabolites mainly through the following pathways: glucuronidation, methylation and ring-fission metabolism (Lambert and Yang, 2003). Metabolites from catechins are reported to either have reduced or similar activity to the parent compound (Lambert et al., 2007). Apart from poor bioavailability and biotransformation, oral consumption of tea polyphenols is also associated with systemic toxicity. In one study, oral ingestion of capsules containing green tea extracts by beagle dogs caused an unexpected morbidity and mortality that resulted in the early termination of the treatment group (Kapetanovic et al., 2009). In contrast, a safety study profiling the toxicity of green tea extracts indicated that topical application has the least harmful effects and only exhibited minor dermal irritations (Isbrucker et al., 2006).

Tea and its polyphenolic compounds have been reported to prevent cancer by interfering with the process of carcinogenesis at different stages (Fig. 2.6). Although the underlying mechanisms involved seem to be complex, the following events have however been implicated in their biological activity: anti-mutagenicity, modulation of detoxifying enzymes, oxidative stress, inflammation, cell growth parameters and immune activity (Kuroda and Hara, 1999).

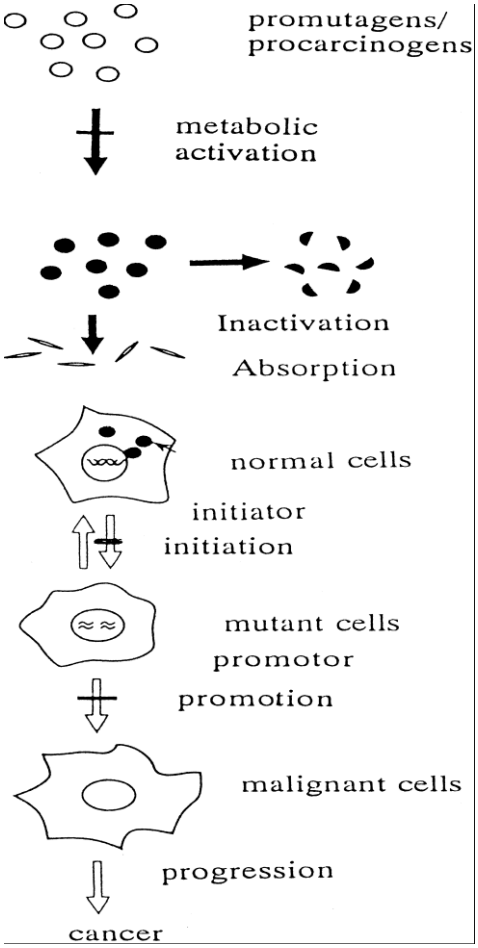


Fig. 2.6. Inhibition of multistep carcinogenesis by tea polyphenol at various stages, i.e. during the metabolic activation, initiation and promotion (Kuroda and Hara, 1999)

11.4. Possible mechanisms in chemopreventive activity

11.4.1. Anti-mutagenic and anti-genotoxic activity

Studies have indicated that green and black teas and their constituents can protect against cancer development through their ability to modulate the mutagenicity of various dietary, environmental and occupational mutagens/and or carcinogens (Bu Abbas et al., 1996; Weisburger et al., 1996; Gupta et al., 2002). The mechanisms that underlie the anti-mutagenic activity of green and black tea extracts involve protection of cells against DNA damage via direct deactivation of the ultimate carcinogen and/or modulation of CYP450 dependent bio-activation of pro-mutagens (Catterall et al., 1998; Morley et al., 2005). In skin, oral consumption and topical application of green tea polyphenols (GTP) protected against polycyclic aromatic hydrocarbons (PAHs)-induced tumourigenicity in mice (Wang et al., 1989). The

protection of mouse skin and human epidermal and inflammatory cells against carcinogen-induced DNA damage by green tea polyphenolic fractions and EGCG has been attributed to the radical scavenging activity of these compounds (Wang et al., 1991; Morley et al., 2005). However at higher concentrations, EGCG has been associated with pro-oxidant activity that would promote DNA damage (Morley et al., 2005). Polymeric black tea polyphenols have also been shown to possess similar anti-initiating properties to monomeric green tea polyphenols as they inhibited the *in vitro* formation of [^{3}H] - B [*a*] P-derived DNA adducts and cytochrome P-450 isozymes CYP 1A1 and 1A2 activity in rat liver microsomes (Wang et al., 1994). In mouse skin, topical application of these polyphenolic compounds reduced B [*a*] P induced DNA adducts (Krishnan and Maru, 2005).

11.4.2. Modulation of detoxifying enzymes and antioxidant activity

Tea and its polyphenolic constituents have been suggested to prevent the onset of cancer development either by inhibiting phase I enzymes or inducing phase II enzymes involved in the metabolic activity of carcinogens (Katiyar and Mukhtar, 1997a). For instance, green tea catechins inhibited CYP 450 dependent arylhydrocarbon hydroxylase (AHH), 7-ethoxycoumarin-O-deethylase and 7-ethoxyresorufin-O-de-ethylase activities in liver microsomes (Wang et al., 1988). These polyphenols also inhibited epidermal AHH activity and enzyme mediated carcinogen (B [*a*] P and DMBA) binding to DNA. It was then proposed that the anti-mutagenic activity of green tea polyphenols result from blocking the metabolic activation of a carcinogen (PAH) and DNA binding via inhibition of CYP 450 activity and this was attributed to impairment of the electron flow from NADPH to the cytochrome (Wang et al., 1989; Kuroda and Hara, 1999). Green tea polyphenols also stimulate the induction of phase II enzymes (UDP-glucuronosyltransferase, and NADP-quinone oxidoreductase) and antioxidant enzymes associated with the elimination of carcinogens and reactive oxygen species (Yang et al., 2002). For instance, oral administration of green tea polyphenols in mice increased the activity of antioxidant and phase II enzymes (glutathione peroxidase, catalase, quinone reductase and glutathione S-transferase) in various organs (Khan et al., 1992). Topical application and oral administration of EGCG to mice before UVB exposure

restored the activity of glutathione peroxidise, catalase and glutathione levels with topical application exhibiting higher protective effects (Vayalil et al., 2003).

11.4.3. Antioxidant/pro-oxidant activity

Antioxidant activity is one of the most extensively studied aspects of tea, particularly green tea and its polyphenols, however, the *in vitro* effects related to chemoprevention have been difficult to establish *in vivo* (Rice-Evans, 1999; Lambert and Elias, 2010). Several studies employing the use of *in vitro* assays have indicated the electron and hydrogen donating properties which underlie the antioxidant mechanism of tea polyphenols. Tea polyphenols have been shown to protect cells against oxidative damage either directly via radical scavenging or indirectly through the modulation of redox-sensitive signaling molecules such as transcription factors, kinases and pro-oxidant enzymes (Shukla, 2007; Yusuf et al., 2007). Studies utilizing mouse epidermal microsomes, cell lines and human skin, indicated that catechins, particularly EGCG, inhibits UV-induced lipid peroxidation, DNA damage and ROS-mediated phosphorylation of mitogen-activated protein kinase (MAPK) signalling pathways associated with tumor promotion (Katiyar et al., 1994; Katiyar et al., 2001a; Katiyar et al., 2001b; Tobi et al., 2002). This activity was attributed to the radical scavenging activity of catechins, consequently, it was shown that pretreatment of mouse skin with EGCG reduces the number of H_2O_2 and inducible nitric oxide synthase (iNOS) expressing cells (Katiyar and Mukhtar, 2001c). Pretreatment of UVB exposed NHEK epidermal cells with EGCG inhibited the intracellular release of H_2O_2 and this was concomitant with the deactivation of ROS-mediated ERK1/2, JNK, and p38 signalling pathways (Katiyar et al., 2001a).

Topical treatment of human skin with EGCG before UVB exposure was also found to inhibit UV-induced H_2O_2 and NO production in both the epidermis and dermis, the infiltration of ROS producing leukocytes was also suppressed (Katiyar et al., 2001b). This study also indicated that EGCG protects the human skin from UVB induced oxidative stress by enhancing and maintaining the activity of intracellular antioxidants which included superoxide dismutase (SOD), catalase, glutathione peroxidise (GPx). The radical scavenging activity of black tea and its polyphenols have also been demonstrated *in vitro*, where black tea extracts and theaflavins inhibited lipopolysaccharide (LPS)-induced iNOS gene expression and activity as well as O_2^-

(Lin et al., 1999; Sarka and Badhuri, 2001). Black tea extracts and the theaflavins were found to have more efficient radical scavenging activity than green tea. However, another study that investigated the effects of tea polyphenols against lipid peroxidation found that black tea polyphenols possess at least the same antioxidant potency as catechins present in green tea (Leung et al., 2001). Apart from the radical scavenging activity demonstrated *in vitro*, consumption of black tea extracts has also been found to increase the activity of SOD and GPx as well as prevent carbon-tetrachloride-induced liver depletion of GSH in mice (Sur-Altiner and Yenice, 2000; Sun et al., 2012).

On the other hand, green tea polyphenols have been associated with pro-oxidant activity that results in cytotoxic effects and this has been attributed to their low redox potential which confers to them high reactivity. Upon interaction of catechins with oxygen, autoxidation catalysed by complex formation with transition metals such as iron, occurs, resulting in the formation of reactive oxygen species, semiquinone and phenoxyl radicals that enhance the oxidative stress in cells (Korkina et al., 2008; Lambert and Elias, 2010). The pro-oxidant activity of catechins is associated with promotion of oxidative stress that leads to cell cycle arrest and apoptosis in cancer cells (Lambert and Elias, 2010).

11.4.4. Anti-inflammatory and immunomodulatory activity

The anti-inflammatory properties of tea extracts and their polyphenolic constituents have been evaluated in various cell culture systems, animal models and human skin (Katiyar et al., 1999; Zhao et al., 1999; Das et al., 2002; Chatterjee et al., 2012). Topical application or oral consumption of green tea polyphenols have been shown to protect against carcinogen-induced inflammation by significantly inhibiting cutaneous edema, hyperplasia and leukocyte recruitment as well as the gene expression and production of various pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α and IL-12) and other mediators such as COX-2 and PGE_2 in mouse skin (Katiyar et al., 1995; Katiyar and Mukhtar, 1997a; Meeran et al., 2009). In human skin, topical application of the major polyphenolic constituent of green tea, EGCG, before UV exposure inhibited leukocyte recruitment, erythrema, myeloperoxidase and PGE_2 production (Katiyar et al., 1999). The anti-inflammatory activity of theaflavin-2 (TF-2),

a major component of black tea extract, has been demonstrated *in vivo* where its topical application on mouse skin prior to TPA exposure reduced edema, erythrema hyperplasia and leukocyte infiltration (Gosslau et al., 2011). This study also showed that TF-2 inhibits the gene expression and production of various pro-inflammatory markers such as cytokines, adhesion molecules, ornithine decarboxylase (ODC), cyclo-oxygenase- 2 (COX-2) and transcription factors. Studies have either reported comparable activity between the extracts of these teas *in vivo* (Das et al., 2002) or higher activity from green tea extracts *in vitro* (Chatterjee et al., 2012).

11.4.5. Anti-proliferative and pro-apoptotic activity

Studies have indicated that the polyphenolic/non-polyphenolic constituents of green and black teas prevent tumour development in skin by inhibiting cell proliferation and inducing apoptosis in malignant cells without adversely affecting normal cells (Lu et al., 2002). For instance, topical application of EGCG and caffeine reduced tumour incidence on UVB exposed mouse skin and this was associated with increased apoptotic activity in both non-malignant and malignant tumours whilst there was slight inhibition of cell proliferation. An *in vitro* study that characterised induction of cancer cell-specific apoptosis by green tea polyphenolic fraction and EGCG indicated that this process involves cell-cycle arrest at lower concentrations and induction of apoptosis at higher concentrations (Ahmad et al., 1997). The anti-proliferative and pro-apoptotic activity of orally administered black tea polyphenols has been demonstrated against mouse skin tumours (Lu et al., 1997). The induction of apoptotic activity by black tea polyphenols has been associated with modulation of signalling pathways involved in cell proliferation leading to cell-cycle arrest at G0-G1 phase (Halder et al., 2008). The molecular mechanisms involved in the induction of apoptosis by green tea and black tea polyphenols is presumably via the mitochondrial pathway. This is attributed to the fact that these compounds up-regulate p53 and Bax in mouse skin and further induced of cytochrome *c* release, caspase activation, apoptotic protease activating factor and poly (ADP-ribose) (Roy et al., 2009).

Tea polyphenols, due to their health promoting properties, have gained considerable popularity and form part of the group natural compounds that have been suggested for use as supplement in skin care products or sunscreens as an

effective in the management of skin cancer (Katiyar et al., 2000; F'guyer et al., 2003; Afaq, 2011). Consequently, there has been a surge of cosmeceutical formulations containing green tea extracts (Stallings and Lupo, 2009; Mukta and Adam, 2010). However, it has been reported that most cosmeceutical products containing tea extracts may still require adequate testing in controlled clinical trials to ensure their efficacy. In addition, the phenolic content in these products needs to be standardised as some products may have little-to-no therapeutic effect (Stallings and Lupo, 2009).

12. Rooibos (*Aspalathus linearis*)

12.1. Background

Rooibos is a South African herbal tea with a use that spans over more than 230 years (Joubert and Schulz, 2006) and is still enjoyed by an ever-increasing market and appears to be headed towards becoming the second most consumed beverage in the world after ordinary tea (*Camellia sinensis*) (Joubert and de Beer, 2011). It is prepared from the indigenous plant of *Aspalathus linearis* (Fig. 2.7), which grows naturally in the Cedarberg mountain region in South Africa, encompassing Citrusdal, Clanwilliam and Nieuwoudtville in the Western and Northern Cape Province of South Africa (Joubert et al., 2008).

Fig 2.7. The rooibos plant (*Aspalathus linearis*) (photo courtesy of www.bushmanskloof.co.za

Various species and ecotypes of *Aspalathus linearis* have reportedly been used to produce rooibos tea in the past, but today the species that is of commercial importance is only the red type that is categorised into cultivated - Nortier type and the wild - growing Cederberg type (Joubert and de Beer, 2011). Over the years, there has been an increasing demand of rooibos in the local and international market where, due to its low tannin content and absence of caffeine, this herbal tea has been mostly enjoyed as a healthy alternative to oriental tea (Joubert et al., 2008; Joubert and de Beer, 2011). Rooibos is exported to 31 countries with the major importers being Germany, Japan, the Netherlands, England, Malaysia, South Korea, Poland, China and the United States (Joubert and de Beer, 2011).

A range of cosmetic care products containing rooibos extracts are also available in the South African market and are distributed to 34 other countries (Joubert and Schulz, 2006; Joubert and de Beer, 2011). Other final products of rooibos include iced-teas, flavoured drinks and extracts and it is also found as an active ingredient in functional foods (Joubert and de Beer, 2011).

12.2. Processing

Depending on the processing method, rooibos is either prepared as fermented “oxidised” herbal tea or unfermented “unoxidised” “green” tea (Joubert et al., 2008a). In the fermented tea, the enzymatic oxidation of plant material leading to rapid browning is initiated by shredding the leaves and stems, and then accelerated by bruising and wetting. During the oxidation process, polyphenols responsible for inducing the typical red-brown colour of rooibos tea are released and there is also development of the characteristic sweet aroma and flavour. After the oxidation process, the plant material is sun-dried and processing is accomplished by sieving and steam pasteurisation, this ensures a product of high microbial quality. The fermentation process affects phenolic composition and biological activity of this herbal tea (Standley et al., 2001; Schulz et al., 2003), consequently there has been an increasing demand for unfermented green rooibos in the market due to its higher antioxidant activity associated with health promotion (De Beer and Joubert, 2002). Preparation of unfermented rooibos requires minimal oxidative changes to ensure retention of the green colour and its major polyphenolic compound, aspalathin, and this can be achieved by inactivation of enzymes via the rapid steaming process

(Joubert and De Beer, 2011). Another alternative discussed in this review is to slow down the rate of chemical reactions by using low temperatures and water, as well as exclusion of oxygen during drying. Although a vacuum drying process to produce a good quality product with a long shelf life was patented (De Beer and Joubert, 2002), it was not feasible as vacuum drying is a batch-wise and expensive procedure (Joubert and de Beer, 2011). A technique that is utilised in the industry involves sun-drying of plant material spread out in thin layer but in this process, production of good quality rooibos is reported to be a challenge (De Beer and Joubert and, 2002).

12.3 Phenolic composition

The two phenolic compounds that are unique only to rooibos are the dihydrochalcones *C*-glucoside, aspalathin (Koeppen and Roux, 1965), and aspalanin which is a cyclic dihydrochalcone (Shimamura et al., 2006). However, the major flavonoids contained predominantly in unfermented rooibos are the dihydrochalcones, aspalathin and nothofagin (Krafczyk and Glomb, 2008; Beelders et al., 2012). Nothofagin is a rare dihydrochalcone *C*-glucoside that has been previously identified in the heartwood of *Nothofagus fusca* (Hillis and Inoue, 1967) and bark of *Schoepfia chinensis* (Huang et al., 2008). Other compounds found in high levels in green rooibos include the flavones (orientin, isoorientin, vitexin, isovitexin, luteolin and chrysoeriol), flavonols (quercetin, iso-quercitrin, hyperoside, luteolin-7-*O*-glucoside, quercetin-3-*O*-robinobioside and rutin), flavanones (dihydroorientin and hemiphlorin) as well as the alpha-hydroxy acid, phenylpyruvic acid glucoside (PPAG), which is a non-flavonoid (Joubert and De Beer, 2011; Beelders et al., 2012).

A recent study indicated that the composition of monomeric phenolic compounds in extracts may be affected by seasonal variation and quality grade of plant material (Joubert et al., 2012). However, most importantly, the levels of the flavonoids are known to be significantly reduced by fermentation where there is also oxidative degradation of aspalathin which substantially decreases the amount of this dihydrochalcone almost by 98% (Schulz et al., 2003). The two mechanisms described for oxidation of aspalathin, lead to its conversion to iso-orientin and orientin and dimerisation (Marais et al., 2000; Krafczyk and Glomb, 2008; Krafczyk et al., 2009). The two dimers formed during oxidation have been established as

atropisomers that stem from oxidative A to B ring coupling (Krafczyk et al., 2009). More recently, a third colourless dimer and two coloured structures with dibenzofuran skeleton [(S)- and(R)-3-(7,9-dihydroxy-2,3-dioxo-6-β-D-glucopyranosyl-3,dihydrodi-benzo[b,d]furan-4a(2H)-yl) propionic acid] have been identified and found to be key chromophores that play an important role in colour formation during fermentation of rooibos (Heinreich et al., 2012). It was also found that these chromophores are ultimately degraded to unknown more stable tannin-like structures in fermented rooibos.

The aqueous extracts of fermented rooibos have more (50%) complex tannin-like substance than the methanol extract of unfermented (14%) tea (Joubert et al., 2008). Although there is limited information on the structure of tannins, the dimer procyanidin B3, trimer bis-fisetinidol-(4β, 6: 4β, 8)-catechin and the pentamer have been identified (Ferreira et al., 1995). However, these compounds are reportedly present in extremely low concentration thus giving credence to the low-tannin characteristic of rooibos, which is even lower than that of *Camellia sinensis* teas (Blommaert and Steenkamp, 1978).

Although rooibos is known to be caffeine free traces of the alkaloid, sparteine, have been reported (Van Wyk and Verdoorn, 1989). Other compounds that have been isolated from fermented rooibos tea extracts include phenolic acids such as benzoic acid, p-hydroxybenzoic acid, protocatechuic acid, vanillic acid and syringic acid, and the cinnamic acids, p-coumaric acid, ferulic acid and caffeic acid (Rabe et al., 1994; Ferreira et al., 1995; Shimamura et al., 2006; Krafczyk and Glomb, 2008).

12.4. Biological properties

12.4.1. Anecdotal health properties

Rooibos tea has traditionally been used in folk medicine to alleviate the symptoms of various digestive, respiratory, sleep and skin disorders (Van Niekerk and Viljoen, 2008; Joubert et al., 20008a). In 1968, the medicinal properties of rooibos tea were discovered by Annetjie Theron who noticed the ability of this herbal tea to relieve chronic restlessness, vomiting and stomach cramps in her colicky baby (Annique, 2011). This instigated her investigation into the other medicinal properties of rooibos where she found that ingestion of this herbal tea or topical application of its extracts

could alleviate the symptoms of a wide range of ailments including dermatological problems such as eczema, acne, nappy rash and ageing. Consequently, this led to the development of cosmetic skin care products and toiletries containing the rooibos extract (Joubert et al., 2008). Today, rooibos extracts are incorporated into a whole range of skin care products to help combat ageing and protect against the adverse effects of UV radiation (Tiedke and Marks, 2002; Bogdan and Baumann, 2008). A range of some of the skin care products containing rooibos extracts are presented in Fig. 2.8. Although there is limited information on the scientific validation of rooibos extracts in skin, the proposed health effects of these cosmetic products has been related to the biological properties of rooibos demonstrated in various *in vitro* assays, cell culture systems, animal models and human skin (Joubert and de Beer, 2011).

Fig. 2.8. Range of some of the rooibos skin care products available in the market (images taken from local pharmacy stores)

12.4.2. Antioxidant/pro-oxidant properties

Since rooibos has traditionally been used in the fermented form for drinking as herbal tea, most of its antioxidant activity has been evaluated from aqueous extracts of the fermented plant material (Joubert et al., 2008a). As reviewed, studies employing the use of various antioxidant assays and test systems have demonstrated the radical scavenging activity of rooibos extracts against physiologically relevant reactive oxygen species (Joubert et al., 2008a). The radical scavenging activity of rooibos extracts in these studies has been associated with

protection against lipid peroxidation (Joubert et al., 2004; 2005), DNA damage (Lee and Jang, 2004) and reduction of cell viability as well as cytotoxicity associated with gap-junction intercellular communication (Yoo et al., 2008; 2009). The protective effects against oxidative stress in cells also involved induction of intracellular antioxidant enzymes (SOD and catalase) (Yoo et al., 2008), this effect has also been demonstrated in animal models and humans. For instance, in rat liver, rooibos aqueous extracts enhanced the antioxidant status by reducing oxidised glutathione (GSSG) and increased reduced glutathione (GSH) levels (Marnewick et al., 2003; 2009). Similar effects were reported in humans, as consumption of rooibos tea increased GSH as well as the GSSG/GSSH ratio in plasma (Marnewick et al., 2011).

Selective extraction has been shown to enhance the antioxidant activity of rooibos extracts, as plant material extracted with an organic solvent exhibited stronger antioxidant activity ascribed to high levels of polyphenols in the fraction (Lee and Jang, 2004). The role of monomeric flavonoids and polymeric extracts in the antioxidant properties of rooibos has been demonstrated (Joubert et al., 2004; Snijman et al., 2009). Due to the strong radical scavenging activity exhibited by extracts containing high levels of monomeric compounds, the antioxidant activity of rooibos has mainly been ascribed to the monomeric flavonoids, more specifically the dihydrochalcones, aspalathin and nothofagin. Consequently, aspalathin content was found to be a good indicator of antioxidant activity and shown to contribute 43% of the total antioxidant capacity of rooibos (Schulz et al., 2003; Joubert et al., 2008c). Aspalathin was further shown to have similar antioxidant potency as the highly reactive EGCG and quercetin (Snijman et al., 2009), both known to also act as pro-oxidants in the presence of iron in the Fenton reaction model (Azam et al., 2004; Joubert et al., 2005). Therefore, dihydrochalcones are thought to play a significant role in the pro-oxidant activity of rooibos extracts demonstrated in the Fenton-reaction model system involving regeneration of Fe^{2+} from Fe^{3+} (Joubert et al., 2005). However, due to the similar activity between a crude aspalathin fraction and ethyl acetate extracts containing higher monomeric polyphenolic content, the antioxidant/pro-oxidant properties of rooibos has been suggested to result from synergistic interactions between the dihydrochalcones and other flavonoids present in this herbal tea. The majority of rooibos monomeric compounds are known to have poor efficacy in the lipid environment and this has been ascribed to their

physicochemical properties (Snijman et al., 2009), thus they are likely to exert their effect at the membrane interphase. The polymeric compounds on the other hand, have been shown to be more effective in the protection of lipid environment (Joubert et al., 2004).

Studies have indicated that the antioxidant activity of rooibos can either be higher, comparable, or less effective than green tea (Von Gadow et al., 1997; Joubert et al., 2008c). The protective effects are reduced during fermentation process as oxidation lowers the polyphenolic content of the plant material (Standley et al., 2001).

12.4.3. Anti-inflammatory and immunomodulatory activity

The ability of rooibos tea to prevent inflammation and protect against immunosuppresion which is associated with the development of various diseases including cancer has been reported (Joubert et al., 2008a; Joubert and de Beer, 2011). The immunomodulatory activity of rooibos extract on antibody and cytokine production has been demonstrated in murine splenocytes as aqueous extracts improved antibody production and cell survival via the stimulation of IL-2 and inhibition of IL-4 (Kunishiro et al., 2001). In whole blood cultures, rooibos extracts enhanced the immune response by increasing the release of IL-6, IL-10 and IFN-γ in unstimulated cells but decreased IL-10 in stimulated cells (Hendricks and Pool, 2010). A study also reported an augmentation of antibody and IL-10 generation in murine splenocytes (Ichiyama and Yamamoto, 2007). Consumption of rooibos exhibited anti-inflammatory activity in the skin by inhibiting leukocyte infiltration (Shindo and Kato, 1991) while the topically applied extract reduced TPA-induced COX-2 on mouse skin associated with prevention of skin carcinogenesis (Na et al., 2004).

12.5. Protective effects in skin

The antioxidant activity of rooibos was found to stabilise sunscreens by quenching radicals inside the formulation, thereby improving their efficacy in skin (Jung et al., 2012). Rooibos extracts blended with *Camellia sinensis* have been shown to have anti-wrinkling effects in human skin (Chuarienthong et al., 2010). Consumption of rooibos tea has also been reported to provide beneficial activity in patients with

dermatological problems as it exhibited anti-viral activity by decreasing the incidence of recurrent herpes simplex, incurable human papilloma infection and prurigo nodularis on human skin. Alleviation of Behcets disease, psoriasis vulgaris and acne pustulosa was associated with the ability of rooibos to inhibit leukocyte infiltration (Shindo and Kato, 1991).

12.6. Anti-carcinogenic properties in skin

The chemopreventive properties of rooibos against the development of skin cancer have been demonstrated in mouse models (Marnewick et al., 2005; Petrova, 2009). Topical application of unfermented and fermented rooibos methanol extracts in a two-stage chemical carcinogenesis model inhibited tumour promotion by reducing the size and number of tumours on mouse skin (Marnewick et al., 2005). This study indicated that the fermented rooibos extracts are more effective than the unfermented extracts. Similar effects were demonstrated in the UVB skin carcinogenesis model as topically applied fermented rooibos extracts exhibited stronger activity against tumour incidence and volume on mouse skin (Petrova, 2009). The antioxidant and anti-inflammatory activity of the polyphenolic constituents have been implicated in the anti-tumour activity. In addition, rooibos has been suggested to prevent cancer development by reducing the proliferative capacity of tumours (Joubert et al., 2008a)

13. Honeybush (*Cyclopia* spp.)

13.1. Introduction

Honeybush is one of the South African herbal teas that has a long history of traditional use by the local inhabitants in the Cape dating back to the 18th century (Joubert et al., 2011). It is prepared from the plants of *Cyclopia* spp (Family: Fabacea:Tribe:Podalrieae) some of which are presented in Fig 2.9. The honeybush species is comprised of more than 20 leguminous shrubs that grow endemically in the coastal planes and mountain regions of the Western and Eastern Cape region of South Africa (Joubert et al., 2011).

Cyclopia intermedia ***Cyclopia genistoides***

Cyclopia subternata

Fig. 2.9. The flowering shoots and bushes of some of the *Cyclopia* spp. (http://www.arc.agric.za/honeybushbotanical information)

Due to the frequent fires that occur in their natural habitats, these plants have developed fire survival strategies which categorises them into sprouters and non-sprouters (Schutte et al., 1995). Sprouters such as *C. intermedia*, *C. sessiliflora* and *C. genistoides* have a lignotuber from which, new shoots resprout to form the base of the main stem after fire or harvesting (Joubert et al., 2011). On the other hand, non-sprouters such as *C. maculata* and *C. subternata* do not have a lignotuber thus their fire survival strategy depends on efficient seedling which involves regeneration of seeds in the soil and seed bank. The honeybush species are generally characterised by woody stems, sweet smelling pale yellow flowers with a honey-like aroma and trifoliate leaves which vary from long needle- like to short leaves (Schutte, 1997). The leafy shoots and flowers of the honeybush plant have traditionally been fermented to prepare tea which has a long history of regional use that has been regarded as an indication of their prevalence in those areas (Joubert et al., 2011).

One of the earliest honeybush species used, in the 1920s, as common substitute for tea in the Cape Peninsula was *C. genistoides* while *C. subternata* was mostly used in Caledon (Overberg) and George (Southern Cape) areas (Joubert et al., 2008). Other species that have traditionally been used for tea by colonists are *C. latifolia*, *C. longifolia* and *C. intermedia.* Trading of honeybush tea was initiated with *C. intermedia* in the 1930's and was first commercialised in the 1960's (Joubert et al., 2011). However, honeybush tea had limited commercial activity which mainly centred in the Langkloof area and remained largely unknown outside its natural habitat until its rediscovery by the South African National Biodiversity Institute (SANBI) and ARC-Infruitec/Nietvoorbij in the early 1990s. Since then, local and global markets have grown substantially with the Netherlands, Germany, United Kingdom and United States of America being the major importers of this herbal tea (Joubert et al., 2011).

13.2. Processing

The most commonly used *Cyclopia* spp. for commercial cultivation of honeybush are *C. subternata* and *C. genistoides*, while *C. intermedia,* which makes up most of the production of honeybush, is still largely harvested from the wild (Joubert et al., 2011). The plant material is processed to give either fermented or unfermented honeybush tea. Manufacturing of fermented honeybush involves cutting, wetting and fermentation at high temperatures (>60°C) for 18-60 h, depending on the temperature, thereafter plant material is dried in the sun or under controlled conditions and then sieved (Joubert et al., 2011).

The detrimentral effect of fermentation on phenolic composition of honeybush species has led to the commercial production of unfermented honeybush. Although the patented process of vacuum drying has been developed (De Beer and Joubert, 2002), unprocessed honeybush is prepared by immediate normal hot air drying of cut and steamed plant material. The major polyphenolic compounds of honeybush, i.e. mangiferin and hesperidin, are relatively stable during production of green honeybush (Joubert et al., 2010). Processed plant material is packaged for local and international markets for consumption as a beverage or further processed by preparing extracts for food products, cosmetics and toiletries (Joubert et al., 2011).

13.3. Phenolic composition

The major compounds present in all of the *Cyclopia* spp. that have been analysed are the xanthones mangiferin and iso-mangiferin and the flavanone, hesperidin (Joubert et al., 2011). Other compounds that have been identified from the different *Cyclopia* spp include flavanones (hesperitin, eriodicytol, naringerin, eriocitrin, narirutin, naringerin-5-*O*-β-D-glucopyranoside, eriodicytol-7-*O*-β-D-glucopyranoside; eriodicytol-7-*O*-β-D-glucopyranoside), flavones (luteolin, 5-deoxyluteolin, scolymoside and diosmetin), isoflavones (formonetin, a formonetin-diglucoside, afromosin, calycosin, wistin, orobol, pseudobaptigenin, fujikinetin and isosakuranetin), flavonols (kampferol glucosides), dihydrochalcones (phloretin-3',5'-di-*C*-glucoside), benzophenones (iriflophenone-di-*O,C*-hexoside; iriflophenone-3-*C*-β-glucoside) coumestans and other compounds such as epigallocatechin gallate and *p*-coumaric acid (Joubert et al., 2003; Joubert et al., 2011). More compounds have recently been identified for the first time in the aqueous extracts of *C. subternata* and these include, a benzophenone (iriflophenone-di-*O*,*C*-hexoside), flavanone ((*R*)- and (*S*)-eriodictyol-di-*C*-hexoside), flavone (vicenin-2) and the dihydrochalcone 3-hydroxyphloretin-3',5'-di-*C*-hexoside (De Beer et al., 2012).

Similarly to rooibos, honeybush is also known to be caffeine free and has low tannin content (Joubert et al., 2011). The tannins in honeybush have been reported to be of the proanthocyanidins type and have been estimated to constitute 4.34% of fermented honeybush hot water extracts. Fermentation, similar to other teas, also reduces phenolic content in honeybush; consequently unfermented extracts with high levels of polyphenolic compounds, particularly mangiferin in *C. genistoides*, have been identified as a better alternative, due to their potential health promoting properties (Joubert et al., 2008a; Joubert et al., 2011).

13.4. Biological properties

13.4.1. Anecdotal health properties

Due to its medicinal properties, honeybush tea has been used by indigenous people for many centuries in folk medicine (Joubert et al., 2008a). Honeybush tea has been reported to treat various ailments such as respiratory infections, digestive

problems as well as soothing the central nervous system and boosting the immune system. In women, it was claimed to provide menopausal relief and stimulate breast-milk production. Anecdotal evidence has further indicated that topically applied honeybush can relieve dermatological problems such as eczema, nappy rash and also improve skin healing (Cape honeybush, 2003). However the cosmetic industry is not as developed as that of rooibos (Joubert et al., 2008a; Joubert et al., 2011). Their protective effects in skin are based on their biological properties which include antioxidant and anti-inflammatory activity as well as the health promoting properties of their polyphenolic compounds. More recent studies have indicated the potential of honeybush extracts to prevent cancer development in skin (Marnewick et al., 2005; Petrova, 2009).

13.4.2. Antioxidant activity

Studies have indicated that honeybush extracts can protect against oxidative stress via radical scavenging activity, iron-reducing potential, and inhibition of lipid peroxidation as well as protection of intracellular antioxidant defense system (Joubert et al., 2008a). The radical scavenging activity of aqueous extracts from honeybush (*C. intermedia*) has been demonstrated against superoxide anion and hydroxyl radical (Steenkamp et al., 2004) and ABTS (Ivanova et al., 2005). Oral consumption of aqueous extracts of unfermented and fermented *C. intermedia*, protected against oxidative status in rats by reducing oxidised glutathione and increasing GSH levels (Marnewick et al., 2003). Methanol extracts of unfermented and fermented methanol aqueous extracts also inhibited iron-induced lipid peroxidation in rat liver microsomes (Marnewick et al., 2005). Both studies indicated that fermented honeybush contains less total polyphenols and have lower antioxidant activity. The reducing effect of fermentation on antioxidant activity has also been demonstrated with aqueous extracts of different honeybush species (Joubert et al., 2008b). However, for *C. genistoides*, fermentation did not affect activity against lipid peroxidation and this was attributed to pro-oxidant activity which may result from high mangiferin content. Although, the antioxidant activity of known polyphenols found in honeybush has been demonstrated, the pro-oxidant properties still remain elusive.

Selective extraction of honeybush plants with methanol has been shown to increase total polyphenol content of honeybush extracts. However, one study

indicated that solvent extraction with methanol reduced antioxidant activity of *C. intermedia* as aqueous extracts exhibited higher radical scavenging activity (Hubbe and Joubert, 2000). Contrary to rooibos, studies have also shown that the total polyphenolic content of honeybush species is not a good indicator for total antioxidant activity (TAA) (Joubert et al., 2008b; 2008c). The antioxidant activity of honeybush extracts appears to be assay and species dependent, therefore no individual species can be singled out as having the strongest antioxidant activity (Joubert et al., 2008a). In addition, in most of the studies conducted, the antioxidant activity of honeybush extracts was less effective than rooibos and *Camellia sinensis*, except for one study where some of the honeybush species exhibited higher or similar activity to fermented rooibos extracts and black tea (Joubert et al., 2008b). The radical scavenging activity of honeybush extracts in a recent study was also associated with photoprotective effects in skin (Petrova et al., 2011).

13.4.3. Anti-inflammatory activity

Topical application of honeybush (*C. intermedia)* methanol extract exhibited anti-inflammatory properties in mouse skin by inhibiting the effects of well known pro-inflammatory agent, TPA (Marnewick et al., 2005). Honeybush modulated the inflammatory response on UVB exposed mouse skin by reducing erythrema and edema as well as COX-2 expression. This activity was affected by fermentation as unfermented extracts exhibited stronger activity presumably attributed to higher levels of polyphenolic compounds (Petrova et al., 2011). Although the major polyphenolic compounds (mangiferin and hesperidin) were not as effective as the extracts, they also exhibited anti-inflammatory activity against edema and erythrema while hesperidin also inhibited cyclo-oxgenase-2 (COX-2) expression. Mangiferin has also been reported to modulate the inflammatory response by suppresing various pro-inflammatory mediators which include cytokines, adhesion molecules and prostaglandins via inhibition of transcription factor activity (Garrido et al., 2004; Leiro et al., 2004). The anti-inflammatory activity of hesperidin against cytokine production and HIF-1 activity has been demonstrated against mast-cell mediated inflammatory response (Choi et al., 2007).

13.4.4 Anti-carcinogenic properties

In a two-stage chemical skin carcinogenesis model, topical application of the methanol extracts of unfermented and fermented *C. intermedia* exhibited anti-carcinogenic activity against DMBA- initiated and TPA- promoted tumours by reducing their incidence and volume (Marnewick et al., 2005). In this study, honeybush extracts exhibited a higher protective activity than rooibos extracts, and this activity was lower for the fermented extract. The antitumour activity of honeybush in UVB carcinogenesis model has also been reported (Petrova et al., 2011), where an unfermented honeybush extract, having a higher polyphenolic content, was more effective at reducing tumour incidence. However, the fermented extract exhibited higher activity against tumour volume. This study further showed, the protective effect of the major polyphenols, mangiferin and hesperidin, against tumour promotion on mouse skin, however these compounds exhibited less activity effects than the extracts. On the other hand, the unfermented extract of honeybush was found to have stronger protective effects against tumour incidence than rooibos but was less effective than this herbal tea against against tumour volume (Petrova, 2009).

14. Conclusion

The skin, which is the largest organ in the body, continuously interfaces with the external environment which may have adverse effects on its normal functions. Chronic exposure and dysregulation of physiological processes can lead to cancer development. The most common cancers include the frequently diagnosed basal cell carcinoma and metastasising squamous cells; these malignancies arise from the epidermal layer. These cancers are mainly caused by chronic exposure to UVB light resulting in oxidative stress, inflammation and hyper-proliferation in initiated cells. Due to climatic changes, low-compliance with prevention strategies and inefficiency of current treatment; the incidence rate of skin cancer has increased to epidemic proportions over the years. To curb the morbidity rate of this disease, the focus for cancer management has been placed on chemoprevention as an intervention strategy that could directly alter the process of carcinogenesis by primarily targeting the reversible stage of promotion. The National Cancer Institute, USA, has devised a drug screening programme which utilises different preclinical *in vitro* and *in vivo* models to test the chemopreventive efficacy of potential compounds before their

clinical application in humans. The set of epithelial *in vitro* models developed for chemoprevention have been considered useful tools in predicting efficacy of novel compounds *in vivo* as well as provide insight into their mechanism of action. However, for accuracy and inclusion of various possible mechanisms, the use of a battery of *in vitro* assays has been recommended as well as the development of new *in vitro* models utilising primary and immortalised cell lines from relevant epithelial cells with normal genome.

Natural compounds found in plants have shown potential as chemopreventive agents in these studies and due to their safety, low toxicity and general acceptance; they have been targeted for cancer management in skin. Polyphenols are one of the most extensively studied groups of compounds which possess health properties that are associated with protective effects in skin. One of the most studied and effective polyphenols in skin are those from the plant of *Camellia sinensis.* EGCG which is the major polyphenolic compound found in green tea, has been incorporated into skin care products due to its efficiency in chemoprevention studies, however clinical studies are still needed to validate the health claims in humans. Comprehensive literature exists on anti-carcinogenic properties of this tea in skin and although the mechanisms involved are complex, they have been proposed to involve anti-mutagenic, anti-inflammatory and anti-proliferative activity. The South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.), have been used in skin care products because of their health promoting properties. Studies have substantiated their protective properties against skin carcinogenesis in animal models but the underlying mechanisms involved are still not clear. Although the biological properties of the polyphenolic compounds of the herbal teas have been implicated in their anti-carcinogenic activity, their chemical and biological characteristics in skin cells still need to be evaluated. The relationship between the chemical constituents and biological properties of the extracts also needs to be evaluated so as to determine the predictive role of chemical constituents and antioxidant properties in the biological activity of the extracts. This would assist in establishing quality control measures that could be utilised as tools to ensure the efficacy of extracts *in vitro.*

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Chapter 3

The predictive potential of the polyphenolic composition and antioxidant properties of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) extracts in the modulation of skin cell viability *in vitro*

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The predictive potential of the polyphenolic composition and antioxidant properties of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) extracts in the modulation of skin cell viability *in vitro*

ABSTRACT

The polyphenolic constituents and antioxidant properties of rooibos (*Aspalathus linearis)* and honeybush (*Cyclopia intermedia)* extracts have been associated with the chemopreventive properties of these herbal teas in mouse skin. The current study investigated the relationship between the polyphenolic content, antioxidant properties and the effect of different herbal tea extracts on the viability of different skin cell cultures, using green tea (*Camellia sinensis*) as a benchmark. The methanol extracts of rooibos and green tea exhibiting high polyphenolic content, radical scavenging (ABTS; ORAC) activity, iron reducing (FRAP) and chelating capacity (LPO), were the most effective in reducing viability of skin cells. Methanol extracts of the different honeybush species, containing high levels of monomeric polyphenols, strong hydrogen donating capacity in the ORAC assay and increased activity in iron-related (FRAP; LPO) assays, exhibited weaker effects against cell viability than the green tea and rooibos extracts. In contrast, the aqueous extracts of honeybush exhibited higher inhibitory activity against viability of skin cells than the methanol extracts and this activity correlated with high flavanol/proanthocyanidin content and strong electron donating properties in the ABTS assay. The highly reactive rooibos polyphenols and green tea catechins seem to disrupt mitochondrial function through pro-oxidant effects that are likely to involve auto-oxidation and polyphenol/iron interactions. The polyphenolic content and antioxidant properties could be good predictors for the reduction of cell viability by the herbal extracts in skin cells. However, the respective antioxidant and pro-oxidant effects and the relative ratio of the monomeric and polymeric constituents complicates the predictive role of these parameters in the biological activity of honeybush extracts *in vitro*.

Key words: polyphenols, antioxidant properties, skin cell viability and chemoprevention

1. Introduction

Polyphenolic compounds, present in a variety of dietary sources such as fruit, vegetables and tea, posses potent antioxidant properties that are associated with the prevention of chronic diseases including cancer (Dai and Mumper, 2010). Several *in vitro* and *in vivo* studies, targeting the reversible stage of cancer promotion, demonstrated the chemopreventive properties of these natural compounds in skin (F'Guyer et al., 2003; Baliga and Katiyar, 2006). Consequently, polyphenols have gained considerable popularity as more topical skin care formulations are utilising compounds such as the green tea catechins (Allemann and Baumann, 2008; Afaq, 2011). Rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) are South African herbal teas that possess chemopreventive properties and these have been ascribed to their polyphenolic constituents (Joubert et al., 2008a). In rooibos, the major polyphenolic compounds are the dihydrochalcones, aspalathin and nothofagin, while the major honeybush polyphenols are the xanthones, mangiferin and iso-mangiferin and the flavanone, hesperidin (De Beer et al., 2010; Beelders et al., 2012). The anti-cancer properties of unfermented rooibos and honeybush in mouse skin have recently been substantiated, as topical application of polyphenol enriched extracts resulted in anti-tumor and photoprotective activities (Marnewick et al., 2005; Petrova et al., 2011). Although the polyphenolic constituents have been implicated in the anti-cancer properties of these herbal teas, the underlying mechanisms are still unclear.

The anticancer properties of tea (*Camellia sinensis*) in skin have been studied extensively with the protective effects against several stages of carcinogenesis attributed to the green tea catechins as well as the theaflavins and thearubigins of black tea (Katiyar et al., 1997; Nomura et al., 2000; Roy et al., 2009). *In vitro* assays have been useful in demonstrating the protective properties of these tea polyphenols against cancer development and in predicting their potential chemopreventive efficacy *in vivo* (Steele et al., 1996). Different cell culture systems have also been used to determine the mechanisms of standardized or purified tea extracts in chemoprevention and these have suggested that tea polyphenols modulate the cancer transformation processes by exhibiting anti-mutagenic, anti-proliferative, pro-

apoptotic and anti-neoplastic activities (Steele et al., 2000, Han et al., 2009). The antioxidant properties of tea polyphenols also seem to play a protective role against the adverse effects of reactive oxygen and nitrogen species during carcinogenesis (Rice-Evans, 1999). Although *in vitro* assays have also defined a strong relationship between polyphenolic compounds and the antioxidant properties of rooibos and honeybush (Joubert et al., 2008b; Snijman et al., 2009), information on their role in disrupting the growth and/or survival of cancer cells is limited.

In the present study, the polyphenolic content and antioxidant properties of rooibos and different honeybush extracts were investigated in order to determine their predictive potential in the modulation of skin cell viability *in vitro*, using green tea as benchmark.

2. Materials and methods

2.1. Chemicals

2,2'-azinobis (3-ethyl-benzothizoline-6-sulfonic acid) (ABTS), thiobarbituric acid (TBA), 6-hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2, 4, 5-tri (2-pyridyl)-S-triazine (TPTZ), DMSO, gallic acid, (+)-catechin (>96%), (-)-epigallocatechin-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-gallate (ECG), caffeine, mangiferin and hesperidin were obtained from (Sigma-Aldrich, USA). Aspalathin and nothofagin were purified from unfermented rooibos to a purity of >95% by HPLC at the PROMEC Unit in the (Medical Research Council, South Africa). Phenyl pyruvic acid-2-*O*-glucoside and isomangiferin were purified from unfermented rooibos to a purity of >95% at the (Agricultural Research Council, South Africa). Orientin and isoorientin were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Vitexin, isovitexin, hyperoside, isoquercitrin, luteolin, luteolin-7-*O*-glucoside and rutin were from Extrasynthese (Genay, France). Folin-Ciocalteau reagent, *p*-dimethylaminocinnam-aldehyde (DMACA), [2,2'-azobis(2-amidinopropane) dihydro-chloride] (AAPH) and all other analytical reagents used were purchased from Merck, (Darmstadt, Germany).

2.2. Plant material.

Green tea (*C. sinensis*), imported from China, was a gift from Vital Health Foods (Kuilsriver, South Africa). Unfermented ("green" or "unoxidized") rooibos (*A. linearis*) was obtained from Rooibos Ltd (Clanwilliam, South Africa), while honeybush (*C. intermedia, C. subternata, C. genistoides* and *C. longifolia*) herbal teas were provided by the Agricultural Research Council, Infruitec-Nietvoorbij, Stellenbosch, South Africa.

2.3. Preparation of extracts.

The aqueous extracts of the different plant materials were prepared by steeping plant material (100 g) in freshly boiled deionised water (1000 mL) for 30 min. Extracts were filtered through a double cheese cloth to remove the majority of the plant material, followed by filtration through a Whatman No. 4 and subsequently through Whatman No.1 filter paper. The filtrates were freeze-dried and stored desiccated in amber vials at room temperature until used. Methanol extracts were prepared in triplicate from the plant material (50 g) by extracting with chloroform (3 x 300 mL) for 24 h, followed by filtration through Whatman No. 4 filter paper. The residual plant material was subsequently extracted with methanol (3 x 300 mL) for 1 h, and the resulting extracts pooled and dried *in vacuo* at 40˚C. The extraction yields were determined prior to pulverization wherafter it was stored desiccated in amber vials at room temperature. The soluble solid content of the aqueous extracts was determined by drying sample aliquots (3 x 4 mL) at 110°C until a constant weight to calculate the yield.

2.4. Chemical characterisation.

Total polyphenol (TP) content. The total polyphenol content of the methanol and aqueous extracts was determined according to the standard method of Singleton and Rossi, (1965) with minor modifications using gallic acid as standard. Methanol and aqueous dried extracts were diluted with deionised H_2O to a required concentration of 0.05% m/v. Briefly, 7.5% Na_2CO_3 solution (4 mL) and 10% Folin-Ciocalteu reagent (5 mL) were added to the sample (1 mL) and the mixture was

incubated at 37°C for 2 h. The TP content was determined spectrophotometrically at 765 nm and the results were expressed as mg gallic acid equivalents/100 mg extract.

Flavanol/proanthocyanidin. Extracts were dissolved in distilled water (0.05% m/v) and different dilutions were prepared. Flavanol/proanthocyanidins were quantified according to the method of McMurrough and McDowell, (1978) with minor modifications and catechin (1.72 mM) prepared in methanol was used as a standard. The p-dimethylaminocinnamaldehyde (DMACA) reagent (0.05% m/v) was prepared immediately before use in methanol:hydrochloric acid (32 %) (3:1 v/v). The absorbance was determined spectrophotometrically at 640 nm and the flavanol/proanthocyanidins content was expressed as mg catechin equivalents/100 mg extract

HPLC analyses. An Agilent 1200 HPLC system, consisting of a quaternary pump, autosampler, in-line degasser, column oven, fluorescence and diode-array detector (Agilent Technologies Inc., Santa Clara, USA) with Chemstation 3D LC software was used for HPLC-DAD analysis. Stock solutions of all standards were prepared in DMSO and aliquots frozen at -20°C. Extracts were dissolved in deionised water (6 mg/mL). Standard calibration mixtures were prepared in a range of concentrations by dilution with HPLC grade water. Ascorbic acid (final concentration 10 mg/mL) was added to standard calibration mixtures and samples before filtering to protect phenolic compounds from degradation during preparation and analysis. UV-Vis spectra were recorded for all analyses from 220 to 400 nm. Peaks were identified by comparing retention times and UV-Vis spectra with that of authentic standards.

Green tea: Analyses were conducted using a modified version of Lin et al., (2008). Separation was performed on a Gemini C18 column (150 × 4.6 mm, 5 µm particle size, 110 Å pore size) (Phenomenex) protected by a guard column containing the same stationary phase at 30°C and a flow rate of 1 mL/min. Solvent A, 0.1% formic acid (v/v), and B, acetonitrile, were used in the following solvent gradient: 0–6 min (12% B), 6-7 min (12-18% B), 7-14 min (18-25% B), 14-19 min (25-40% B), 19-24 min (40-50% B), 24-29 min (50-12% B), 29-40 min (12% B). Catechin and

epicatechin was quantified using fluorescence detection (excitation = 275 nm; emission = 315 nm) using authentic standards. Epigallocatechin gallate (275 nm), epigallocatechin (275 nm), epicatechin gallate (275 nm) and caffeine (270 nm) were quantified using UV-Vis detection and authentic standards.

Herbal teas: Quantification of aspalathin, nothofagin, phenylpyruvic acid-2-*O*-glucoside (PPAG), orientin, isoorientin, vitexin, isovitexin, hyperoside, isoquercitrin, rutin, quercetin-3-*O*-robinobioside and luteolin-7-*O*-glucoside in rooibos was conducted using the method described by Beelders et al., (2012). All compounds were quantified using authentic standards from a chromatogram monitored at 288 (aspalathin, nothofagin, PPAG) and 350 nm (other compounds), except quercetin-3-*O*-robinobioside which was quantified as rutin equivalents due to unavailability of an authentic standard. Honeybush herbal teas were analysed according to the method described by (de Beer and Joubert, 2010). Mangiferin (320 nm), isomangiferin (320 nm), eriocitrin (288 nm) and hesperidin (288 nm) were quantified using authentic standards. In addition, eriodictyol-glucoside (288 nm), phloretin-3′, 5′-di-*C*-glucoside (288 nm), iriflophenone-3-*C*-glucoside (288 nm) and scolymoside (320 nm) were quantified as eriocitrin, phloretin-3′-*C*-glucoside (nothofagin), hesperidin and luteolin equivalents, respectively, as no authentic reference standards were available.

2.5. Antioxidant assays

2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS). The radical scavenging activity of the aqueous and methanol extracts was determined according to the method of Re et al., (1999) with minor modifications. Briefly, Trolox standards and samples were prepared in PBS and distilled water, respectively. $ABTS^{\bullet+}$ solution, consisting of K_2 S_2O_8 (88 µL, 140 mM) and ABTS (5 mL, 7 mM), was prepared and kept in the dark for 16 h prior to use. The $ABTS^{\bullet+}$ solution (1 mL) was mixed with the sample or Trolox standard (50 µL) and incubated at 37 °C for 4 min. Absorbance was measured spectrophotometrically at 734 nm and the µmoles of ABTS scavenged calculated using the molar extinction coefficient 1.5 X 10^4 mol^{-1} L cm^{-1}. The radical scavenging activity was expressed as mmol Trolox equivalents/g extract.

Ferric reducing antioxidant power (FRAP). The ferric reducing antioxidant power of the aqueous and methanol extracts was determined based on the method of Benzie and Strain, (1996). Trolox was used as a standard and the iron reducing capacity was expressed as mmol Trolox equivalents/g extract.

Oxygen radical absorbance capacity (ORAC). Determination of the oxygen radical absorbance capacity of the extracts was perfomed according to the method of Huang et al., (2005) using Trolox as a standard. Fluorescence measurement was performed on a Biotek Synergy HT microplate reader (Winooski, Vermont, USA). Antioxidant activity was expressed as mmol Trolox equivalents/g extract.

Lipid peroxidation (LPO). Liver microsomes were prepared from male Fischer 344 rats. Rat livers were homogenized in 0.15 M KCl containing 10 mM potassium phosphate buffer (pH 7.4), centrifuged at 9000g and the supernatants (S9) stored at -80°C until used. Microsomes were purified on a Sepharose 2B column as described previously by Gelderblom et al., (1984). Inhibition of iron-induced microsomal lipid peroxidation by the aqueous and methanol extracts was determined by the method described by Snijman et al., (2009). Assays were conducted in duplicate and IC_{50} values calculated using the 4-parameter logistic curve (Sigmoidal variable slope) in GraphPad Prism version 5.04 for Windows (GraphPad Software, La Jolla, USA). All the antioxidant assays were the mean of triplicate determinations for each extract.

2.6. Studies in cell cultures

Spontaneously immortalised keratinocytes (HaCaT) were a gift from the Department of Human Biology of the (University of Cape Town, South Africa). The HaCaT keratinocyte cell line has various features of premalignant or cancerous cells and it has been utilised as a premalignant cell model (Adhami et al., 2003; Han et al., 2011). Non-malignant normal fibroblast-like skin cells (CRL 7761) and basal carcinoma cell line (CRL 7762), collected from the same patient were purchased from the American Tissue Culture Collection (ATCC). The HaCaT cell line was grown in RPMI-1640 (Lonza, Walkersville, USA), while Dulbecco's Modified Eagle's medium (DMEM, Lonza, Walkersville, USA) was used for the normal and skin cancer cells. The media was supplemented with 10% heat inactivated fetal bovine serum

(FBS, Gibco, USA) and L-glutamine (2 mM), while for the skin cancer cells, DMEM was also supplemented with HCl to a final concentration of 0.12 mM. Cells were incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C and grown as monolayer cultures to a confluency of 70 to 80% prior to commencing with the experiments. Tests to determine mycoplasma contamination in the different cell lines were conducted regularly. In experiments, the following passage were used: HaCaT (p70 to p80), normal cells (p16 to p20) and cancer cells (p15 to p22). Cells were plated in a 96-well microtiter plate at a density of 5 x 10^3 per well in the respective media (100 µL) containing 10% FBS and cultured for 24 h as described above.

Treatment with extracts and pure compounds. Tea extracts, EGCG and aspalathin were dissolved in DMSO and the dilutions prepared in the culture media containing 0.5% FBS. Mangiferin and hesperidin were first solubilised in DMSO by sonication before dilution in the medium. All samples were filter sterilized (0.22 µm), and control wells contained media with the respective DMSO concentrations. The final concentration did not exceed 2% DMSO for skin cancer cells and 0.5% DMSO for HaCaT cells and normal skin cells. Cells were incubated for 24 h prior to determining cell viability.

Cell viability assay. As plant extracts interfere with the redox chemistry of the routinely used standard LDH and MTT assays Wisman et al., (2008); a chemiluminescence assay measuring ATP content in cells was used to determine cell viability by following the CellTiter-Glo®Luminescent manufacturer's protocol from Promega (Madison, USA). Microplates were first equilibrated at room temperature for 30 min, before the luciferase agent was added. Plates were rotated for 2 min followed by a 10 min incubation at room temperature in the dark prior to determining the luminescence signal which was expressed as relative light units. IC_{50} values were calculated using the 4-parameter logistic curve (Sigmoidal variable slope) in GraphPad Prism version 5.04 for Windows (GraphPad Software, La Jolla California USA). Four to five replicates of the different dilutions were used for each extract and the experiment was repeated at least three times.

Statistical analyses. An analysis of variance (ANOVA) was used to test for significant group effects when more than two groups were present, using the SAS's

GLM procedure (Cary, NC, USA). For unbalanced data Tukey-Cramer adjustments were automatically made. Levene's Test was used to test for the homogeneity of the variances and Tukey's Test as the post-hoc test. Where only two groups were compared, the T-tests were used. Statistical significance was measured at $P<0.05$. Spearman's rank correlation coefficients were determined for measuring the strength of the relationship between the different variables.

3. Results

3.1. Extraction yields

Aqueous extraction resulted in a higher ($P<0.05$) yield of soluble solids when compared to methanol extraction with the exception of rooibos where no significant difference was noticed between the extracts (Table 3.1). The yield of aqueous extracts was in the following order: *C. longifolia* > green tea > *C. genistoides* > *C. intermedia* ≅ *C. subternata* > rooibos. With respect to methanol extraction, green tea ≅ *C. longifolia* ≅ *C. genistoides* exhibited significantly ($P<0.05$) higher yields compared to *C. intermedia* ≅ *C. subternata* ≅ rooibos.

3.2. Chemical analysis

Total polyphenol (TP) content. Methanol extracts contained higher ($P<0.0001$) levels of TP than the aqueous extracts except for *C. intermedia* where the content was similar between extracts (Table 3.1). For the aqueous extracts, the content decreased in the following order: rooibos ≅ *C. longifolia* > *C genistoides* ≥ *C. subternata* ≥ *C. intermedia* ≅ green tea. The TP content of the methanol extracts prepared from rooibos was the highest followed by *C. longifolia* ≅ green tea > *C. subternata* ≅ *C. genistoides* > *C. intermedia.*

Flavanol/proanthocyanidin content. The highest levels were found in the methanol and aqueous extracts of green tea followed by the methanol extract of rooibos. The opposite was noticed for the honeybush species as the flavanol/proanthocyanidin content of the aqueous extracts was significantly higher than the corresponding methanol extracts. The flavanol/proanthocyanidin content of

aqueous extracts of the herbal teas decreased in the following order *C. subternata* > *C. intermedia* ≅ rooibos > *C. genistoides* ≅ *C. longifolia.* For the methanol extracts of honeybush species *C subternata* had the highest levels followed by *C. genistoides* ≅ *C. intermedia* ≅ *C. longifolia* with similar levels.

HPLC analysis of monomeric polyphenols. The level of the individual polyphenols was significantly higher ($P<0.05$) in the methanol extracts when compared to the aqueous extract of green tea and the herbal teas (Table 3.2). The methanol extract of green tea contained the highest level of EGCG followed by the alkaloid, caffeine. Lower levels of the other catechins were present in the following order: EGC > ECG > EC > catechin. In the aqueous extract the levels of EGCG and ECG were 3-fold lower than in the methanol extract.

The dihydrochalcones (DHC), aspalathin and to a lesser extent, nothofagin, were the major polyphenols in rooibos methanol extract followed by the flavones, isoorientin> orientin > isovitexin > luteolin > vitexin. The flavonols decreased as follows: isoquercitrin > rutin > hyperoside > quercetin robionobioside (QROB). In the aqueous extract, the concentration of the polyphenols was lower but aspalathin and nothofagin were still the major compounds. The flavone content decreased in the following manner: isoorientin > orientin > isovitexin > vitexin > luteolin. The flavonols decreased as follows: rutin > isoquercitrin > hyperoside > QROB. The levels of the non-flavonoid, phenylpyruvic acid glucoside (PPAG), were 0.39 ± 0.02 and 0.42 ± 0.01 mg/100mg extract in the methanol and aqueous extract, respectively.

The major polyphenols in the methanol extracts of honeybush were the xanthones, mangiferin and isomangiferin, and the flavanone, hesperidin (Table 3.2). *Cyclopia longifolia* and *C. genistoides* extracts contained the highest ($P<0.05$) xanthone levels (xanthone-rich) while *C. intermedia* and *C. subternata* extracts contained the highest flavanone levels (flavanone-rich), mainly hesperidin. In the xanthone-rich species, the mean xanthone to flavanone ratios varied between 6:1 and 8:1 in the methanol extracts for *C. genistoides* and *C. longifolia*, respectively. In the aqueous extracts the respective mean ratios increased to 17:1 and 19:1, mainly due to the significant reduction in the concentration of hesperidin.

In the flavanone-rich extracts, the xanthone to flavanone ratio was approximately 1:1 in the methanol extracts while in the aqueous extracts it was 6:1 and 2:1 for *C. intermedia* and *C. subternata*, respectively. Of the other compounds, the highest dihydrochalcone, phloretin-3,5′-di-*C*-glucoside (PDG), concentration was found in *C. genistoides* while *C. subternata* followed by *C. longifolia* had highest levels of the benzophenone, iriflophenone-3-*C*-glucoside (IPG). *Cyclopia subternata* also exhibited the highest levels of the flavone, scolymoside, and the flavanones, eriocitrin and eriodictyol-glucoside (EDG). The methanol extract of *C. intermedia* exhibited the highest concentration of luteolin while EDG and scolymoside were not detected in the aqueous extract.

3.3. Antioxidant properties (Table 3.1).

ABTS assay. The radical scavenging activity of the methanol extract of rooibos and green tea was significantly ($P<0.05$) higher than the aqueous extract while the opposite was noticed for the honeybush species. The activity of the different methanol extracts decreased as follows: rooibos > green tea > *C. subternata* ≥ *C. longifolia* ≥ *C. genistoides* > *C. intermedia*. *Cyclopia subternata* exhibited the highest radical scavenging activity of all the aqueous extracts followed by *C. longifolia* ≅ rooibos ≅ *C. genistoides* > *C. intermedia* with green tea having the lowest response.

FRAP assay. The ferric iron reducing potential of the methanol extracts of rooibos and green tea exhibited similar activity and was significantly higher ($P<0.05$) when compared to the aqueous extracts. Of the honeybush methanol extracts, *C. longifolia* exhibited the highest activity ($P<0.05$) followed by *C. genistoides* > *C. subternata* > *C. intermedia*.

Table 3.1. Comparative antioxidant and cytotoxic effects of different extracts of green tea, rooibos and honeybush herbal teas in relation to the chemical composition.

	Extract type**	Tea and herbal tea groups*					
		Camellia sinensis	***Aspalathus linearis***	***Cyclopia genistoides***	***Cyclopia longifolia***	***Cyclopia intermedia***	***Cyclopia subternata***
Extract yield (%)*	MeOH	$29.47\pm1.55^{a}_{A}$	$15.07\pm1.50^{b}_{A}$	$24.89\pm0.17^{a}_{A}$	$25.00\pm1.39^{a}_{A}$	$16.25\pm2.27^{b}_{A}$	$15.39\pm0.35^{b}_{A}$
	Aq	$33.20\pm0.48^{b}_{B}$	$16.33\pm0.40^{e}_{A}$	$29.72\pm0.90^{c}_{B}$	$36.917\pm0.48^{a}_{B}$	$23.03\pm0.40^{d}_{B}$	$22.97\pm0.92^{d}_{B}$
Total polyphenols (mg GAE/100 mg extract)	MeOH	$25.65\pm3.27^{b}_{A}$	$35.07\pm3.44^{a}_{A}$	$21.60\pm2.43^{c}_{A}$	$26.10\pm2.40^{b}_{A}$	$17.21\pm1.82^{d}_{A}$	$22.05\pm1.51^{c}_{A}$
	Aq	$16.10\pm2.16^{c}_{B}$	$25.05\pm2.84^{a}_{B}$	$19.39\pm1.39^{b}_{B}$	$23.95\pm2.46^{a}_{B}$	$16.45\pm2.06^{c}_{A}$	$17.50\pm2.25^{bc}_{B}$
Flavanols/proanthocyanidins (mg CE/100 mg extract)	MeOH	$13.23\pm0.37^{a}_{A}$	$2.71\pm0.16^{b}_{A}$	$1.22\pm0.16^{d}_{A}$	$1.20\pm0.16^{d}_{A}$	$1.13\pm0.10^{d}_{A}$	$1.40\pm0.15^{c}_{A}$
	Aq	$7.76\pm0.31^{a}_{B}$	$1.80\pm0.15^{c}_{B}$	$1.62\pm0.21^{d}_{B}$	$1.45\pm0.16^{d}_{B}$	$1.79\pm0.14^{c}_{B}$	$2.25\pm0.20^{b}_{B}$
ABTS (mmol TE/g extract)	MeOH	$10.90\pm0.75^{b}_{A}$	$11.49\pm0.81^{a}_{A}$	$8.08\pm0.21^{d}_{A}$	$8.41\pm0.42^{cd}_{A}$	$6.68\pm0.29^{e}_{A}$	$8.70\pm0.31^{c}_{A}$
	Aq	$6.77\pm0.65^{d}_{B}$	$9.08\pm0.45^{b}_{B}$	$8.87\pm0.54^{b}_{B}$	$9.37\pm0.33^{b}_{B}$	$8.12\pm0.20^{c}_{B}$	$10.09\pm0.39^{a}_{B}$
FRAP (mmol TE/g extract)	MeOH	$3.08\pm0.34^{a}_{A}$	$3.04\pm0.19^{a}_{A}$	$1.77\pm0.16^{c}_{A}$	$2.01\pm0.14^{b}_{A}$	$1.30\pm0.07^{e}_{A}$	$1.56\pm0.08^{d}_{A}$
	Aq	$1.81\pm0.14^{bc}_{B}$	$2.24\pm0.18^{a}_{B}$	$1.68\pm0.16^{cd}_{A}$	$1.88\pm0.23^{b}_{A}$	$1.61\pm0.11^{d}_{B}$	$1.67\pm0.11^{cd}_{B}$
ORAC (mmol TE/g soluble solids	MeOH	$7.77\pm0.09^{e}_{A}$	$14.02\pm1.01^{a}_{A}$	$10.46\pm0.75^{c}_{A}$	$11.91\pm0.38^{b}_{A}$	$8.92\pm0.22^{d}_{A}$	$10.53\pm0.57^{c}_{A}$
	Aq	$4.37\pm0.10^{e}_{B}$	$9.12\pm0.53^{a}_{B}$	$7.22\pm0.32^{c}_{B}$	$8.55\pm0.09^{b}_{B}$	$6.57\pm0.29^{d}_{B}$	$7.27\pm0.23^{c}_{B}$
LPO (IC_{50} - mg/ml)	MeOH	$0.23\pm0.01^{c}_{A}$	$0.24\pm0.01^{c}_{A}$	$0.79\pm0.01^{a}_{A}$	$0.75\pm0.01^{ab}_{A}$	$0.82\pm0.01^{a}_{A}$	$0.66\pm0.005^{b}_{A}$
	Aq	$0.34\pm0.01^{c}_{B}$	$0.33\pm0.00^{c}_{B}$	$0.89\pm0.02^{b}_{A}$	$0.98\pm0.01^{ab}_{B}$	$1.14\pm0.01^{a}_{B}$	$0.81\pm0.011^{b}_{B}$
ATP IC_{50} for HaCaT cells (mg/ml)	MeOH	$0.08\pm0.01^{c}_{A}$	$0.13\pm0.02^{c}_{A}$	$0.72\pm0.10^{a}_{A}$	$0.72\pm0.10^{a}_{A}$	$0.53\pm0.07^{b}_{A}$	$0.47\pm0.06^{b}_{A}$
	Aq	$0.17\pm0.03^{d}_{B}$	$0.15\pm0.02^{d}_{A}$	$0.68\pm0.11^{a}_{A}$	$0.51\pm0.08^{b}_{B}$	$0.48\pm0.08^{bc}_{A}$	$0.41\pm0.09^{c}_{A}$
ATP IC_{50} for normal cells (mg/ml)	MeOH	$0.23\pm0.06^{d}_{A}$	$0.26\pm0.05^{d}_{A}$	$1.85\pm0.17^{a}_{A}$	$1.14\pm0.13^{c}_{A}$	$1.37\pm0.17^{b}_{A}$	$1.08\pm0.20^{c}_{A}$
	Aq	$0.34\pm0.06^{c}_{B}$	$0.29\pm0.05^{c}_{B}$	$0.88\pm0.19^{a}_{B}$	$0.53\pm0.04^{b}_{B}$	$0.50\pm0.13^{b}_{B}$	$0.37\pm0.07^{c}_{B}$
ATP IC_{50} for cancer cells (mg/ml)	MeOH	$0.21\pm0.06^{d}_{A}$	$0.24\pm0.03^{d}_{A}$	$2.29\pm0.61^{a}_{A}$	$0.80\pm0.16^{c}_{A}$	$1.29\pm0.23^{b}_{A}$	$1.14\pm0.15^{b}_{A}$
	Aq	$0.41\pm0.07^{c}_{B}$	$0.29\pm0.05^{d}_{B}$	$0.94\pm0.24^{a}_{B}$	$0.52\pm0.09^{b}_{B}$	$0.44\pm0.10^{bc}_{B}$	$0.43\pm0.14^{bc}_{B}$

Values represent means ± standard deviations of three (chemical and antioxidant assays) to five (cell viability assay) replication of at least two experiments. *Statistical analyses for significant group effects (more than 2 groups) were assessed by either the One-way ANOVA F-test or the Welch's test, depending if homogeneity of group variances were present (Levene's test). If significant group differences were present, the post hoc Tukey's Studentized Range Test was used for between group comparisons at 5% significance level. **Statistical comparisons for only two groups, i.e. MeOH vs Aq for each tea, the Student's T-test were used. Means in a row (Tea and herbal tea groups) or column (Extract type) followed by the same letter (lower and upper case) do not differ significantly, if letters differ then $P<0.05$. Abbreviations: GAE – gallic acid equivalents; CE – catechin equivalents; TE – Trolox equivalents; IC_{50} – concentration yielding 50% inhibition; ATP – adenosine triphosphate, MeOH – methanol; Aq – aqueous. *Extract yields obtained from 100 g of dry tea material. HaCaT - keratinocytes; normal skin cells- CRL 7761 -; skin cancer cells- CRL 7762 -. FRAP - ferric reducing antioxidant potential; ABTS - 2,2'-azinobis (3-ethyl-benzthiazoline-6-sulphonic acid) LPO - lipid peroxidation; ORAC – Oxygen radical scavenging capacity.

The aqueous extracts of *C. intermedia* and *C. subternata* were more active than the corresponding methanol extracts while no significant difference was found between the methanol and aqueous extracts of *C. genistoides* and *C. longifolia*.

ORAC assay. Similar to the other antioxidant assays, the methanol extracts of rooibos and green tea were more active ($P<0.05$) scavengers of peroxyl radicals than the aqueous extracts. Contrary to their activity in the ABTS and FRAP assays, the methanol extracts of the honeybush species were more active than their aqueous counterparts in ORAC. For both the methanol and aqueous extracts the descending order of activity was rooibos > *C. longifolia* > *C. subternata* ≅ *C. genistoides* > *C. intermedia* > green tea.

LPO assay. For the inhibition of iron-induced lipid peroxidation (LPO), all the methanol extracts exhibited a higher ($P<0.05$) protective effect than their respective aqueous extracts. The methanol extracts inhibited lipid peroxidation in a decreasing order of: green tea ≅ rooibos > *C. subternata* ≥ *C. longifolia* ≅ *C. genistoides* ≅ *C. intermedia.* Aqueous extracts of rooibos and green tea displayed a similar protective activity followed by *C. subternata* ≅ *C. genistoides* ≥ *C. longifolia* ≥ *C. intermedia*.

3.4. Modulation of cell viability (cellular ATP content).

Methanol extracts of green tea and rooibos were more ($P<0.05$) active than their corresponding aqueous extracts in disrupting cellular ATP production in the different skin cell cultures, except in the HaCaT cell line where no difference was noticed between the rooibos extracts (Table 3.1). In contrast, the aqueous extracts of honeybush exhibited higher activity in normal and cancer cells. However, in HaCaT cells the methanol and aqueous extracts displayed similar activities, except for *C. longifolia,* where the aqueous extract of the displayed higher activity than methanol extract.

Table 3.2. Concentration of monomeric compounds in extracts prepared from green tea, rooibos and different honeybush species.

Compounds/phenolic subgroups		Concentration (µg/ mg extract) MeOH	Aq	Polyphenolic subgroups		Concentration (µg/mg extract) MeOH	Aq
		Camellia sinensis				***Aspalathus linearis***	
Catechins	EGCG	111.93±3.01$_A$	46.10±1.49$_B$	DHC:	Aspalathin	124.24±1.44$_A$	83.87±2.08$_B$
	ECG	20.37±3.53$_A$	7.48±4.31$_B$		Nothofagin	27.59±0.38$_A$	16.68±0.27$_B$
	EGC	42.25±1.79$_A$	31.98±3.20$_B$		**Total**	**151.82±1.66$_A$**	**100.55±1.81$_B$**
	EC	14.92±1.06$_A$	11.28±1.16$_B$	Flavones	Isoorientin	15.79±0.10$_A$	10.94±1.95$_B$
	Catechin	1.32±0.78$_A$	1.13±1.15$_B$		Orientin	11.60±0.06$_A$	8.88±1.45$_B$
	Total	**190.79.1±6.23$_A$**	**97.97±6.29$_B$**		Vitexin	1.60±0.00$_A$	1.20±0.00$_B$
					Isovitexin	2.62±0.02$_A$	1.51±0.16$_B$
Alkaloid	Caffeine	57.54±1.59$_A$	40.10±0.30$_B$		Luteolin	1.78±0.09$_A$	0.45±0.11$_B$
					Total	**33.39±0.09$_A$**	**22.98±3.47$_B$**
				Flavonols	Rutin	4.26±0.01$_A$	3.60±0.01$_B$
					Hyperoside	3.53±0.07$_A$	1.48±0.77$_B$
					Isoquercitrin	4.51±0.03$_A$	2.00±0.89$_B$
					QROB	1.16±0.00$_A$	0.75±0.00$_B$
					Total	**13.46±1.53$_A$**	**7.83±1.21$_B$**

***Cyclopia* spp. (Xanthone-rich)**

Polyphenolic subgroups		Concentration (ug/mg extract) MeOH	Aq	MeOH	Aq
		Cyclopia genistoides		***Cyclopia longifolia***	
Xanthones	Mangiferin	156.07±4.31$_A$	77.48±0.25$_B$	180.04±6.18$_A$	126.99±3.36$_B$
	Isomangiferin	39.99±0.93$_A$	30.11±0.84$_B$	48.99±1.94$_A$	33.09±0.78$_B$
	Total	**196.06±4.66$_A$**	**107.59±0.65$_B$**	**229.04±5.61$_A$**	**160.08±4.11$_B$**
Flavanones	Eriocitrin	1.61±0.00$_A$	1.11±0.03$_B$	2.83±0.09$_A$	1.82±0.08$_B$
	Hesperidin	32.94±1.03$_A$	4.12±0.10$_B$	24.60±0.65$_A$	6.73±0.38$_B$
	EDG	1.04±0.02$_A$	1.15±0.02$_B$	-	-
	Total	**35.58±1.02$_A$**	**6.38±0.10$_B$**	**27.43±0.73$_A$**	**8.54±0.46$_B$**
Flavones	Luteolin	0.84±0.02$_A$	0.16±0.01$_B$	0.77±0.02$_A$	0.17±0.01$_B$
	Scolymoside	-`	-	4.30±0.10$_A$	3.30±0.11$_B$
	Total	**0.84±0.02$_A$**	**0.16±0.01$_B$**	**5.07±0.11$_A$**	**3.47±0.12$_B$**
DHC	PDG	20.76±0.35$_A$	2.30±0.02$_B$	1.31±0.25$_A$	0.83±0.08$_B$
Benzophenone	IPG	2.54±0.08$_A$	16.95±0.25$_B$	12.55±0.98$_A$	8.81±0.61$_B$

***Cyclopia* spp. (Flavanone-rich)**

		Cyclopia intermedia		***Cyclopia subternata***	
Xanthones	Mangiferin	67.7±2.16$_A$	39.77±0.41$_B$	62.57±1.46$_A$	22.02±2.99$_B$
	Isomangiferin	20.02±0.71$_A$	14.26±0.40$_B$	15.95±0.26$_A$	8.70±1.46$_B$
	Total	**87.77±2.87$_A$**	**54.04±0.75$_B$**	**78.52±1.26$_A$**	**30.72±4.45$_B$**
Flavanones	Eriocitrin	2.93±0.20$_A$	1.25±0.06$_B$	5.25±0.25$_A$	3.27±0.25$_B$
	Hesperidin	88.77±11.55$_A$	7.32±0.56$_B$	63.15±8.62$_A$	7.98±0.21$_B$
	EDG	0.39±0.00A	-	3.14±0.21$_B$	3.87±0.20$_A$
	Total	**92.10±11.74$_A$**	**8.57±0.62$_B$**	**71.54±9.07$_A$**	**15.12±0.23$_B$**
Flavones	Luteolin	3.62±0.27$_A$	0.23±0.01$_B$	1.10±0.06$_A$	0.13±0.05$_B$
	Scolymoside	0.96±0.14	-	9.16±0.31$_A$	4.03±0.13$_B$
	Total	**4.58±0.26$_A$**	**0.23±0.01$_B$**	**10.26±0.37$_A$**	**4.16±0.09$_B$**
DHC	PDG	0.65±0.03$_A$	0.68±0.01$_A$	14.13±0.88$_A$	12.53±1.42$_B$
Benzophenone	IPG	3.91±0.27$_A$	3.63±0.11$_A$	13.69±0.35$_A$	9.32±0.16$_B$

Values represent means ± standard deviations of triplicate determinations. Comparison between aqueous and methanolic extracts was analysed with the student t-test and significant differences (P<0.05) are indicated with. Means for each tea or herbal tea (in a row) followed by the same upper case letters in subscript do not differ significantly, if letters differ then P<0.05. Abbreviations: MeOH – methanol; Aq – aqueous, SS – soluble solids; EGCG - epigallocatechin gallate; EGC - epigallocatechin; ECG - epicatechin gallate; EC – epicatechin; DHC – dihydrochalchones; QROB - quercetin-3-*O*-robinobioside; PDG - phloretin-3',5'-di-*C*-glucoside; IPG - iriflophenone-3-*C*-glucoside; EDG - eriodictyol-glucoside.

Inter-plant comparisons between the methanol extracts indicated that rooibos and green tea were the most active against all three cell lines. The activity of the different honeybush species was dependent on the cell type. In the HaCaT cell line, the activity of the honeybush teas decreased in the following order: *C. subternata* ≅ *C. intermedia* > *C. longifolia* ≅ *C. genistoides*. In the normal cell line the order was *C. subternata* ≅ *C. longifolia* > *C. intermedia* > *C. genistoides* while in the cancer cells the order was *C. longifolia* > *C. subternata* ≅ *C. intermedia* > *C. genistoides*. The aqueous extracts of rooibos and green tea were also the most active against the HaCaT cells followed by *C. subternata* ≥ *C. intermedia* ≅ *C. longifolia* > *C. genistoides*. In the normal cell line, rooibos, green tea and *C. subternata* exhibited a similar activity followed by *C. intermedia* ≅ *C. longifolia* > *C. genistoides.* In the cancer cell line, the aqueous extract of rooibos exhibited the highest activity followed by green tea ≥ *C. subternata* ≥ *C. intermedia* ≅ *C. longifolia* > *C. genistoides.* Irrespective of the cell type or extract used, *C. genistoides* exhibited the lowest activity of the honeybush species.

The HaCaT cells were the most ($P<0.05$) sensitive cell type, when assessing the activity of the different extracts, except for the aqueous extracts of *C. intermedia*, *C. longifolia* and *C. subternata* where all three skin cell lines exhibited a similar response (Fig. 3.1). The cancer cells were more sensitive ($P<0.05$) than the normal cell line for the methanol extract of *C. longifolia* while the opposite was noticed for the methanol extracts of rooibos and *C. genistoides*. The remainder of the extracts exhibited a similar activity against both cell types.

3.5. Inter-relationships between chemical composition, antioxidant activity and cell viability.

Green tea. The high levels of TP and flavanol/proanthocyanidins content in methanol extracts co-incided with a high antioxidant activity in the ABTS, FRAP, ORAC and LPO assays (Table 3.1). This was also related to a significant reduction in cell viability as determined by the decrease in ATP production (IC_{50}). The reduced antioxidant activity recorded for the aqueous extracts was associated with a lower TP and a 50% lower total catechin levels (Table 3.2) which co-incided with an almost 2-fold increase in the IC_{50} values in the different skin cell types.

Rooibos: A similar pattern was observed for rooibos as the TP content in methanol extracts was closely related to strong antioxidant properties and reduction of ATP production in cells (Table 3.1). The DHC, flavones and flavonols were the major constituent in the methanol extract (Table 3.2). However, a clear difference between the aqueous and methanol extract regarding the effect on cell viability was not evident (Table 3.1).

No significant difference was noticed between IC_{50} values of the methanol and aqueous extract in HaCaT cells whilst differences in IC_{50} values for normal and cancer cell lines was, although significant, not as prominent as that observed for green tea. A 30% lower amount of the rooibos flavonoids was noticed in the aqueous extract which was verified by a reduced antioxidant activity.

Honeybush: There was a weak negative correlation between TP content of extracts and flavanol/proanthocyanidins content (Table 3.3). Evaluation of the relationship between polyphenolic content and the antioxidant assays indicated a moderate positive correlation between TP content, FRAP and ORAC whilst a negative moderate correlation was observed between TP content and LPO. There was no correlation between TP content and the activity of the extracts in the ABTS assay.

In contrast, flavanol/proanthocyanidins content correlated positively with ABTS and negatively with ORAC whilst the relationship with iron related assays was either weak (LPO) or absent (FRAP). Except for HaCaT cells, activity of extracts against cell viability negatively correlated with FLAVA, ABTS (moderate) and LPO (weak) whilst a positive correlation was observed with ORAC. There was no relationship between TP content, FRAP and reduction of cell viability. The correlations were reflective of the different trends in the chemical constituents, antioxidant properties and biological activity of honeybush species. For instance, the aqueous extract of *C. subternata*, exhibiting the highest flavanol/proanthocyanidin content and activity in the ABTS and LPO assays was the most active against cell viability, as discussed above (Table 3.1). On the other hand, the methanol extracts of the xanthone-rich species, particularly *C. genistoides*, displayed the highest TP content and activity in the ORAC and FRAP assay but exhibited the weakest activity against cell viability.

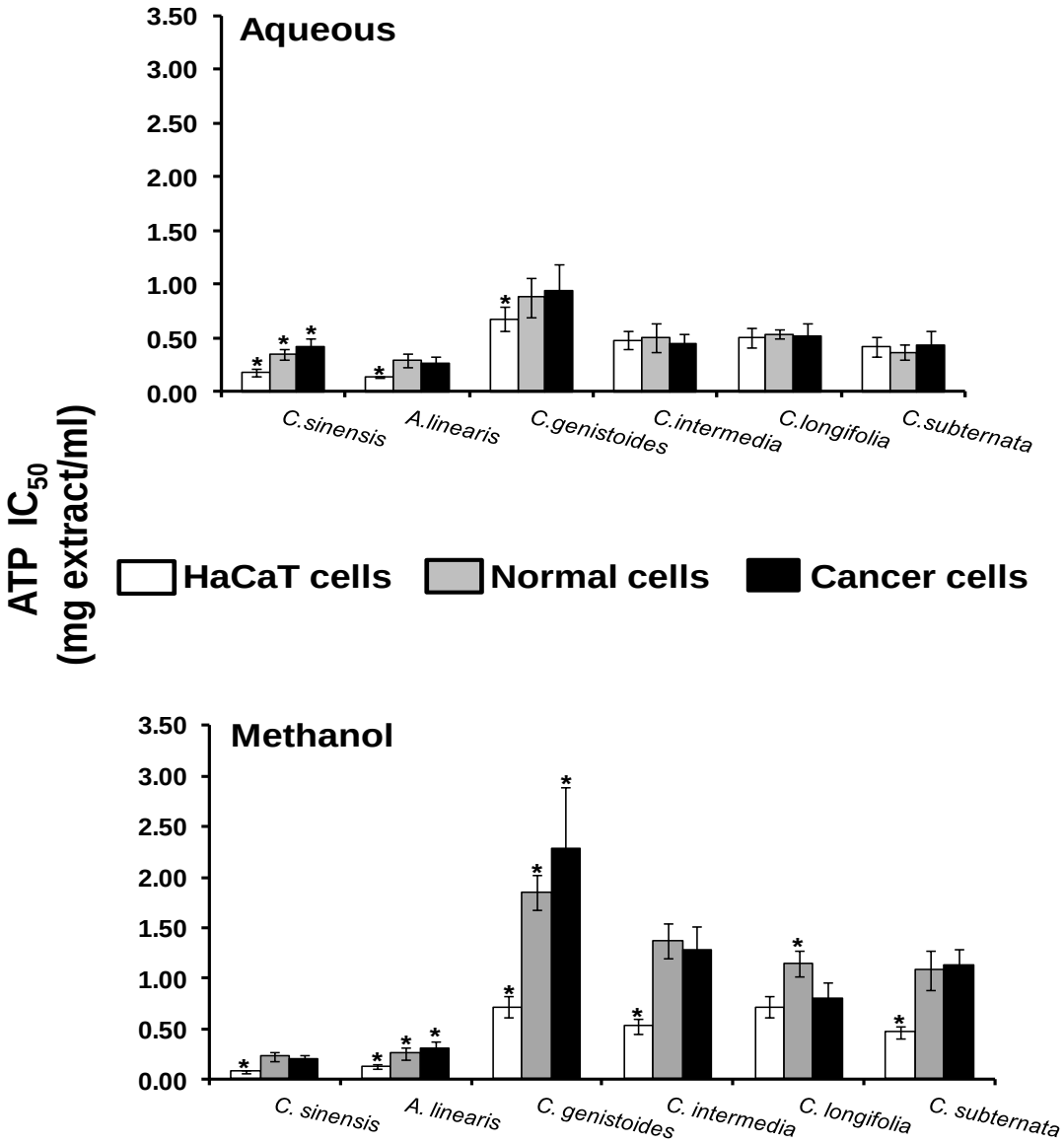


Fig. 3.1. Comparative susceptibility of the different cell line in response to the green tea, rooibos and different honeybush extracts. Values represent mean±standard deviation of five replicates Statistically analysis between the different cell lines for each tea/herbal tea included either an ANOVA F-test or the Welch's test, depending if homogeneity of group variances are present (Levene's test). If significant group differences were present, the post hoc Tukey's Studentized Range Test was used for between group comparisons at 5% significance level. *indicates a significant ($P<0.05$) difference) between the different cell lines.

Table 3.3. Correlation analyses between chemical parameters, antioxidant properties and the modulation of cell viability of methanol and aqueous extracts of the honeybush species.

Chemical/antioxidant parameters	FLAVA	ABTS	FRAP	LPO	ORAC	Cell viability		
						HaCaT cells	Normal Cells	Cancer Cells
TP	r -0.334 P=0.0041	-	r 0.517 P<0.0001	r -0.412 P=0.0037	r 0.544 P<0.0001	-	-	-
FLAVA	-	r 0.504 P<0.0001	-	r 0.365 P=0.0105	R -0.775 P<0.0001	r -0.539 P<0.0001	r -0.539 P<0.0001	r -0.538 P<0.0001
ABTS	-	-	r 0.332 P <0.0001	-	-	-	r -0.641 P<0.0001	r -0.499 P<0.0001
LPO	-	-	-	-	r -0.672 P<0.0001	-	r -0.317 P=0.0382	r -0.396 P=0.0053
ORAC	-	-	-	-	-	r 0.421 P=0.0003	r 0.628 P<0.0001	r 0.472 P<0.0001

Spearman correlations were used to calculate correlation coefficients (r values). P<0.05 was considered statistically significant. Abbreviations: TP - total polyphenols; FLAVA - flavanol/proanthocyanidins; FRAP - ferric reducing antioxidant potential; ABTS - 2,2'-azinobis (3-ethyl-benzthiazoline-6-sulphonic acid) LPO - lipid peroxidation; ORAC – Oxygen radical scavenging capacity.

3.6. Effect of pure compounds and their relative levels in corresponding extracts associated with the IC_{50} for the reduction in cell viability.

Of the pure polyphenolic compounds tested, EGCG was up to seven fold more active (P<0.05) than aspalathin (Table 3.4). As also observed for green tea and rooibos extracts, HaCaT cells were the most sensitive cell line to the pure compounds. The cancer cell line showed an increased sensitivity to EGCG as compared to the normal cells whilst both cell lines showed similiar sensitivity to aspalathin. When considering the IC_{50} values for pure EGCG and aspalathin, the corresponding levels of the monomeric compounds associated with the IC_{50} in the respective methanol and aqueous extracts were much lower (Table 3.2). Due to the weak solubility of mangiferin and hesperidin no IC_{50} values could be obtained although, as discussed for EGCG and aspalathin, the levels associated with the IC_{50} values of the respective extracts were far lower. The methanol extract of green tea and rooibos were, as expected, higher than the aqueous extracts. However, when considering the mangiferin, isomangiferin and hesperidin levels, the opposite was

Table 3.4. Disruption of ATP production (IC_{50}) in the different skin cells by pure compounds and their corresponding levels in the green and herbal tea extracts.

	Pure Compounds	IC_{50} for ATP (µg/mL)					
		HaCaT cells		Normal cells		Cancer cells	
Teas and polyphenol subgroups	EGCG**	$39.2 \pm 5.0^{a}_{A}$		$79.4 \pm 5.0^{b}_{A}$		$59.1 \pm 4.9^{c}_{A}$	
	Aspalathin**	$230.3 \pm 45.1^{a}_{B}$		$385.6 \pm 132.7^{b}_{B}$		$419.1 \pm 114.6^{b}_{B}$	
	Mangiferin	> 300		> 300		> 300	
	Hesperidin	> 436		> 436		> 436	
		Monomeric polyphenol levels in IC_{50} for ATP (µg/ml)*					
	Monomeric polyphenols in extracts	**MeOH**	**Aq**	**MeOH**	**Aq**	**MeOH**	**Aq**
C. sinensis	EGCG	$8.6\pm1.3_{A}$	$8.0\pm1.6_{A}$	$25.7\pm5.0_{A}$	$15.8\pm2.3_{B}$	$23.4\pm3.8_{A}$	$19.1\pm3.9_{B}$
linearis	Aspalathin	$16.3\pm2.7_{A}$	$11.4\pm0.9_{B}$	$31.8\pm7.0_{A}$	$24.7\pm5.4_{B}$	$38.5\pm7.6_{A}$	$22.12\pm5.8_{B}$
C. genistoides							
Xanthones:	Mangiferin	$112.1\pm16.0_{A}$	$52.6\pm8.7_{A}$	$288.8\pm26.4_{A}$	$68.0\pm14.5_{B}$	$357.1\pm96.1_{A}$	$72.8\pm19.0_{B}$
	Isomangiferin	$28.7\pm4.1_{A}$	$20.4\pm3.4_{B}$	$74.0\pm6.8_{A}$	$26.4\pm5.6_{B}$	$91.5\pm24.6_{A}$	$28.3\pm7.4_{B}$
Flavanone	Hesperidin	$23.7\pm3.4_{A}$	$2.8\pm0.5_{B}$	$61.0\pm5.6_{A}$	$3.6\pm0.8_{B}$	$75.4\pm20.3_{A}$	$3.9\pm1.0_{B}$
C. longifolia							
Xanthones:	Mangiferin	$128.9\pm18.7_{A}$	$64.6\pm11.7_{B}$	$205.4\pm22.7_{A}$	$68.0\pm5.3_{B}$	$143.2\pm28.6_{A}$	$66.1\pm14.0_{B}$
	Isomangiferin	$35.1\pm5.09_{A}$	$16.8\pm3.1_{B}$	$55.9\pm6.2_{A}$	$17.7\pm1.4_{B}$	$39.0\pm7.8_{A}$	$17.2\pm3.7_{B}$
Flavanone:	Hesperidin	$17.6\pm2.6_{A}$	$3.4\pm0.6_{B}$	$28.1\pm3.1_{A}$	$3.6\pm0.3_{B}$	$19.6\pm3.9_{A}$	$3.5\pm0.7_{B}$
C. intermedia							
Xanthones:	Mangiferin	$35.9\pm5.0_{A}$	$19.0\pm3.3_{B}$	$92.8\pm11.5_{A}$	$20.09\pm5.3_{B}$	$87.1\pm15.5_{A}$	$17.6\pm3.9_{B}$
	Isomangiferin	$10.6\pm1.5_{A}$	$6.8\pm1.2_{B}$	$27.4\pm3.4_{A}$	$7.20\pm1.9_{B}$	$25.7\pm4.6_{A}$	$6.3\pm1.4_{B}$
Flavanone:	Hesperidin	$47.1\pm6.0_{A}$	$3.5\pm0.6_{B}$	$121.6\pm15.0_{A}$	$3.73\pm1.0_{B}$	$114.1\pm20.3_{A}$	$3.3\pm0.7_{B}$
C. subternata							
Xanthones:	Mangiferin	$29.4\pm3.9_{A}$	$9.2\pm1.9_{B}$	$67.6\pm12.3_{A}$	$8.13\pm1.6_{B}$	$71.2\pm9.5_{A}$	$9.6\pm3.1_{B}$
	Isomangiferin	$7.5\pm1.0_{A}$	$3.6\pm0.8_{B}$	$17.2\pm3.1_{A}$	$3.2\pm0.6_{B}$	$18.2\pm2.4_{A}$	$3.8\pm1.2_{B}$
Flavanone	Hesperidin	$29.7\pm4.0_{A}$	$3.3\pm0.7_{B}$	$68.2\pm12.4_{A}$	$3.0\pm0.6_{B}$	$70.96\pm9.8_{A}$	$3.47\pm1.1_{B}$

Values represent means±standard deviations of triplicate determinations. *Statistical analyses for significant group effects (more than 2 groups) were assessed by either the One-way ANOVA F-test or the Welch's test, depending if homogeneity of group variances were present (Levene's test). If significant group differences were present, the post hoc Tukey's Studentized Range Test was used for between group comparisons at 5% significance level. **For statistical comparisons between pure compounds, the Student's T-test were used. Means in a row followed by the same letter (lower case superscript) or in a column (upper case subscript) do not differ significantly, if letters differ then $P<0.05$. Abbreviations: IC_{50} – concentration yielding 50% inhibition of ATP content. MeOH – methanol; Aq – aqueous. *C. sinensis- Camellia sinensis* EGCG - epigallocatechin gallate; *A. linearis- Aspalathus linearis*; *C. genistoides*; *Cyclopia genistoides*; *C. intermedia*; *Cyclopia intermedia*; *C. longifolia- Cyclopia longifolia*; *C. subternata-Cyclopia subternata*.

noticed for honeybush with far lower levels associated with the aqueous extracts exhibiting the highest activity against cell viability.

4. Discussion

Green tea catechins possess electron and hydrogen donating properties, which underlie their free radical scavenging and chain-breaking antioxidant mechanisms (Lambert and Elias, 2010). The electron donating properties of these compounds are also associated with the chelation and reduction of metals such as copper and iron (Huang et al., 2005; Apak et al., 2007; Korkina et al., 2008; Lambert and Elias, 2010). Although metal chelation is regarded as a secondary antioxidant mechanism, it may also lead to pro-oxidant effects that result from the highly reactive iron (II) which is generated from reduction of iron (III) (Perron and Brumaghim, 2009). The antioxidant and pro-oxidant properties of catechins have been associated with the protection of normal cells and the selective killing of tumor cells, respectively (Srichairatanakool et al., 2006; Chen et al., 2010a).

In the current study, the methanol extract of green tea, exhibiting high polyphenolic content and strong antioxidant properties, effectively reduced the viability of the different skin cells. The strong electron (ABTS, FRAP) and hydrogen donating (ORAC) properties of the methanol extract in the present study implicated the role of catechins and their redox properties in the biological activity of extracts as redox reactions are known to underlie the activity of green tea polyphenols (Lambert and Elias, 2010). Of interest is that the hydrogen donating capacity of green tea extracts in the present study was significantly lower than the herbal tea extracts. This may be attributed to the saturation of the heretocylic ring in catechins (Fig. 3.2) which has been associated with a weak hydrogen capacity and iron related pro-oxidant effects (Rice-Evans et al., 1996; Sugihara et al., 2001). As reduction in cell viability by green tea extracts was closely related to their strong antioxidant properties, it is likely that the effect in cells results from pro-oxidant effects that are possibly mediated by the redox properties of the catechins. The pro-oxidant effects of catechins have been ascribed to their low redox potential which confers to them high reactivity that leads to autoxidation, a process that generates semi-quinone phenolic

radicals and reactive oxygen species in the presence of an iron catalyst (Korkina et al., 2008; Perron and Brumaghim, 2009; Lambert and Elias, 2010).

The accumulation of these free radicals in cells causes oxidative stress that leads to cytotoxic effects associated with mitochondrial dysfunction (Galati et al., 2006). In the present study, extracts reduced cell viability by decreasing ATP content in cells. Reduction of ATP content *in vitro* has been associated with cell cycle arrest, membrane depolarisation and mitochondrial defects that primarily targets complex II in the electron transport chain in cells exposed to an iron chelator, desferoxamine (DFO) (Yoon et al., 2003). Complex II defects were found to induce oxidative stress that leads to cell cycle arrest (Byun et al., 2008). It was reported that the underlying mechanisms in DFO-induced complex II defects involve down-regulation of iron-sulfur subunits via iron depletion that results from iron chelation (Yoon et al., 2003).

Since catechins are known iron-chelators and studies have indicated the ability of EGCG, to accumulate in the mitochondria and exert modulatory changes in the respiratory chain (Zheng and Ramirez, 2000; Lagoa et al., 2011), it is likely that the reduction of cell viability effected by green tea extracts in the present study may also involve modulation of respiratory chain complexes in a similar iron interaction mechanism(s) as those described for DFO. The strong relation between iron-reduction assay (FRAP), iron chelating (inhibition of LPO) and the reduction in cell viability further emphasised the possible involvement of specific pro-oxidative polyphenol-iron interactions in the reduction of skin cell viability by green tea extracts. Although EGCG was the major compound in green tea extract it may, however, not be the only reactive constituent in the green tea as its relative levels in the extract associated with reduction of cell viability (IC_{50} values) was lower than those required for the pure compound. This indicated that EGCG in the extract is more effective at reducing cell viability than the isolated compound thus implicating a synergistic or additive effect between the monomeric flavanols and other polyphenolic compounds of green tea. The strong link between the green tea total polyphenolic and flavanol/proanthocyanidins content, catechin levels and antioxidant properties indicated that these parameters could be used as good indicators to predict the cytotoxic activity of green tea extracts *in vitro*. This is in agreement with existing

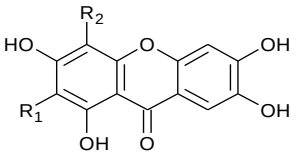
literature as green tea extracts containing high levels of catechins, particularly EGCG, have been found to induce cytotoxic effects in rat hepatocytes *in vitro* (Schmidt et al., 2005).

It was shown that the pro-oxidant activity mediates the cytotoxic effects of catechins demonstrated *in vitro* and *in vivo* (Galati et al., 2006). Cytotoxic effects of catechins, as mentioned earlier, are known to mostly be selective for cancer cells (Chen et al., 2010a) and this was also evident in the present study as EGCG targeted the viability of cancer cells and HaCaT cell line. Caffeine was also one of the major compounds found in green tea in the present study and high levels of this alkaloid in extracts was associated with reduction of cell viability (IC_{50} values). Caffeine is known to synergistically enhance the cytotoxic effects of chemotherapeutic agents in cell lines (Aida and Bodell, 1987; Goth-Goldstein, 1982) and may therefore also play a role in the reduction of the cell viability in skin cells.

The instability of the polyphenols in the culture media during the 24h incubation period may underestimate the activity of the pure compounds such as EGCG and aspalathin (see below) as compared to that of tea and herbal tea extracts against cell viability *in vitro*. Thus pure compounds are likely to be more susceptible to auto-oxidation as when they do form part of complex mixtures. In this regard it was shown that the activity of tannins can be altered by spontaneous oxidation resulting in the reduction in their cytotoxic effects (Wisman et al., 2008).

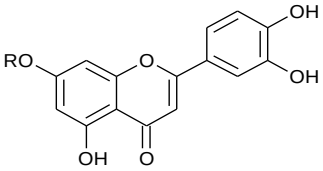
In the present study rooibos displayed similar properties to green tea, as the highest activity was exhibited by the methanol extract, which also displayed the the highest polyphenolic content. The major monomeric polyphenolic constituents in rooibos extracts were the dihydrochalcones (aspalathin and and nothofagin).Both rooibos extracts exhibited a significantly ($P<0.05$) higher hydrogen donating activity (ORAC) when compared to green tea and the different honeybush species. This could be related to the structural characterisitics (Fig. 3.2) of the rooibos flavonoids which include the presence of the unsaturated 2, 3 bond and the 4-oxo function in the C ring involved in radical stabilisation (Rice-Evans et al., 1996).

Honeybush polyphenols



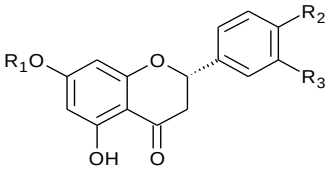
Xanthones

Mangiferin: R_1=*C*-β-D-glucopyranosyl, R_2=H
Isoangiferin: R_1=H, R_2=*C*-β-D-glucopyranosyl



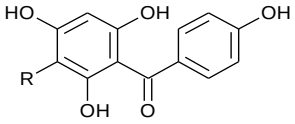
Flavones

Luteolin: R=OH
Scolymoside: R=rutinosyl



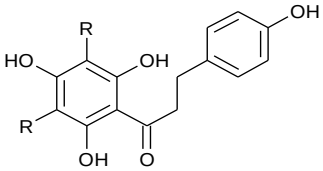
Flavanones

Hesperidin: R_1=rutinosyl, R_2=OH, R_3=OCH_3
Eriocitrin: R_1=rutinosyl, R_2, R_3=OH
Eriodictyol glycoside: R_1=H, R_2,R_3=OH



Benzophenone

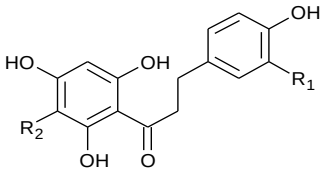
Iriflophenone-3-*C*-β-glucoside:
R=*C*-β-D-glucopyranosyl



Dihydrochalcone

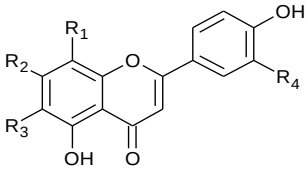
Phloretin-3',5'-di-*C*-β-glucoside:
R=*C*-β-D-glucopyranosyl

Rooibos flavonoids



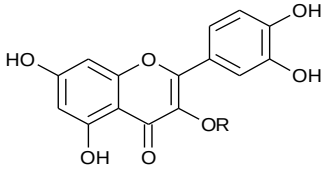
Dihydrochalcones

Aspalathin: R_1=OH, R_2=*C*-β-D-glucopyranosyl
Nothofagin: R_1=H, R_2=*C*-β-D-glucopyranosyl



Flavones

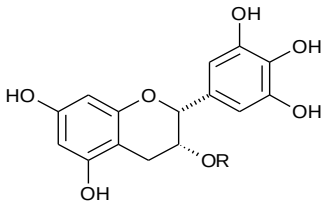
Isoorientin: R_1, R_2, R_4=OH, R_3=*C*-β-D-glucopyranosyl
Orientin: R_1=*C*-β-D-glucopyranosyl, R_2, R_3=H, R_4=OH
Vitexin: R_1=*C*-β-D-glucopyranosyl, R_2, R_3=H, R_4=H
Isovitexin: R_1, R_2, R_4=H, R_3=*C*-β-D-glucopyranosyl
Luteolin: R_1, R_2, R_3, R_4=H
Luteolin-7-*O*-glucoside: R_1, R_3=H, R_2=*C*-β-D-glucopyranosyl, R_4=OH
Chrysoeriol: R_1, R_2, R_3, R_4=OCH_3



Flavonols

Quercetin-3-*O*-robinobioside: R=robinobiosyl
Isoquercitrin: R=β-D-glucopyranosyl
Rutin: R=rutinosyl
Hyperoside: R=galactopyranosyl

Green tea flavanol



Epigallocatechin gallate: R = gallate

Fig 3.2. Chemical structures of the major green tea and herbal tea polyphenolic constituents

Since rooibos extracts exhibited similar IC_{50} values to green tea in the reduction of cell viability this suggested a common mechanism seems to prevail. Therefore specific pro-oxidative reactions involving iron, as discussed for green tea, may also prevail for rooibos extracts. The iron-associated pro-oxidant properties of rooibos monomeric and polymeric fractions have been demonstrated in a Fenton-type reaction *in vitro* (Joubert et al., 2005) and also implicated in the cytotoxic activity of rooibos extracts evaluatedin a liver carcinogenesis model *in vivo* (Marnewick et al., 2009). The close relationship between activity of rooibos extracts in the iron related assay (LPO and FRAP) and reduction of cell viability in the current study, also further emphasised the relevance of the proposed role of polyphenol/iron interactions in the cytotoxic effects of rooibos extracts in the mitochondria. Modulatory effect on complex I and II by flavonoids such quercetin and luteolin, which are found in rooibos have been reported (Hodnick et al., 1987), thus modulation of mitochondrial respiratory chain by rooibos extracts is plausible.

Aspalathin is known to exhibit a similar antioxidant potency to EGCG and quercetin when utilising the ABTS antioxidant assay, however, its acitivity is less effective in a lipid environment (Snijman et al., 2009). The limited efficacy of rooibos flavonoids has been attributed to their physicochemical properties which preferentially position these compounds at the lipid/aqueous interphase or in the aqueous phase. In the current study, aspalathin was far less (approximately 5 fold) effective in reducing cell viability than EGCG, yet its relative high levels in the extracts were associated with reduction of cell viability (IC_{50}) which indicates that aspalathin may not be the main active constituent. Thus synergistic and/or additive effect between rooibos polyphenolic compounds, such the flavone and flavonols, in the extracts may also prevail in the reduction of skin cell viability. Since rooibos monomeric compounds are known to have limited efficacy in the lipid environment it is possible that polymeric proanthocyanidins may also play a significant role in the cytotoxic effects of this herbal tea. Crude polymeric fractions from rooibos extracts have been reported to be more effective in a lipid environment than fractions exhibiting higher levels of monomeric rooibos compounds (Joubert et al., 2005).

The four different honeybush species varied with respect to their polyphenolic composition, antioxidant activities and the reduction of cell viability amongst each other and when compared to green tea and rooibos extracts. The methanol extracts

of the honeybush species contained high levels of TP, mainly due to the high concentrations of the xanthones, mangiferin and isomangiferin and flavanone, hesperidin. In contrast, the aqueous extracts, contained far less of these polyphenols especially hesperidin, whilst the flavanol/proanthocyanidin content increased which can be ascribed to the presence of the polymeric proanthocyanidins (Joubert et al., 2008a). These differences may be attributed to the higher efficiency of methanol and aqueous mixtures of organic solvents, in extracting low (monomeric) and high (polymeric) molecular weight polyphenolic constituents, respectively (Dai and Mumper, 2010). There was no correlation between the total polyphenolic content and reduction in cell viability when considering the honeybush species as the aqueous extracts, having a lower TP content, exhibited a higher activity than the methanol extracts. The xanthone-rich (*C. genistoides* and *C. longifolia*) and flavanone-rich extracts (*C. subternata and C. intermedia)* subgroups, exhibited varying effects in the antioxidant and cell viability assays suggesting different roles for the polyphenolic constituents. For instance, a high TP content of the methanol extracts derived from the xanthone rich species, co-incided with high levels of monomeric polyphenols and high antioxidant activity in the FRAP, LPO and ORAC assays. The honeybush methanolic extracts, particularly that of *C. genistoides*, were less toxic to the cell cultures than the aqueous extracts, suggesting that the iron reducing, chelating and hydrogen donating properties of the monophenolic compounds are associated with cytoprotection against oxidative stress.

The protective effects of these compounds may be attributed to their structural and redox properties. The redox potential of the major polyphenols of honeybush, mangiferin and hesperidin, is higher and thus these compounds are not as reactive as the catechins and quercetin (Pardo-Andreu et al., 2006a, Korkina et al., 2008). Most of the honeybush polyphenols conform to the structural requirement (Fig. 3.2) needed for the formation of stable radicals upon hydrogen donation and iron-chelation i.e. a catechol structure in the B ring, the number and position of hydroxyl groups in the A-ring in conjugation with a 4-oxo-functional group in the C-ring (Apak et al., 2007; Rice-Evans et al., 1996). The iron-chelating activity of mangiferin in lipid membranes has been studied extensively and its dual iron interactive mechanisms involve stimulation of iron (II) autoxidation to iron (III) and the formation of a stable complexes with iron (III) (Pardo Andreu et al., 2005, Pardo-Andreu et al., 2006). It

has been proposed that the high redox potential of mangiferin as well as its co-ordination with iron (III) prevents mangiferin from undergoing pro-oxidation and causing dysfunction in the mitochondria (Pardo-Andreu et al., 2007). However, mangiferin may not be the only polyphenol involved in protection as in the present study, one of the flavanone- rich species, *C. subternata*, exhibiting the highest activity against lipid peroxidation, contained high levels of eriocitrin, a flavanone suggested to be more or less effective as mangiferin in the protection of lipid membranes (Joubert et al., 2008b). In addition, high levels of scolymoside in *C. subternata* and *C. longifolia* co-incided with strong protective activity of these species against lipid peroxidation. Scolymoside is known to have strong radical scavenging activity that protect the cells from oxidative stress and this is attributed to its chemical structure that allows for stabilization of the formed radicals (Kim et al., 2000). Thus, the major monomeric compounds could be acting synergistically with other minor polyphenolic constituents such as eriocitrin and scolymoside in protecting the cells by preventing damage of lipid membranes.

When considering the methanol extracts of the flavanone-rich species, *C. subternata* and *C. intermedia*, the latter species exhibited the lowest activity in the antioxidant assays. The xanthone and flavanone content of all the species was reduced in the aqueous extracts due to a low extraction efficiency of mangiferin and specifically hesperidin. This resulted in a marked increase in the xanthone to flavanone ratio which was associated with a reduced protective effect against cell viability. In this regard the cytoprotective activity of mangiferin has been demonstrated *in vitro* and was associated with a reduced metal–induced oxidative stress and apoptosis in HepG2 cells exposed to cadmium (Satish Rao et al., 2009). Hesperidin has also been shown to be protective against oxidative stress in primary hepatocytes (Chen et al., 2010b). In the current study mangiferin and hesperidin did not exhibit any effect on cell viability at the concentrations tested, however the levels of these compounds associated with the IC_{50} for the aqueous extracts was far below when compared to the methanol extracts, suggesting thay they play a minor role, if any to the cytotoxic effects of the honeybush extracts.

The flavanol/proanthocyanidin content of the aqueous honeybush extracts co-incided with a high radical scavenging activity (ABTS) as compared to the methanol extracts. The aqueous extracts of flavanone-rich species (*C. subternata* and *C.*

intermedia), containing the highest flavanol/proanthocyanidin levels, exhibited the highest activity against cell viability. The activity of the aqueous extracts in cells, similarly to rooibos and green tea, may result from pro-oxidant effects that involve flavanol-like proanthocyanidin. However, the weak or absence of correlation between iron-related assays and reduction of cell viability suggested that iron-chelation may not be a key role player in the pro-oxidant activity of the honeybush extracts as it is associated with monomeric polyphenols and cytoprotection. In addition, the activity of the most active species against cell viability, *C.subternata*, in LPO was similar to *C. genistoides* which displayed the lowest effects in cells, thus further indicating the irrelevance of iron-chelation in the cytotoxic effects of honeybush. Therefore, the cytotoxic effects of the aqueous extracts of the honeybush species, the role of polymeric proanthocyanidins and the proposed pro-oxidative mechanisms still need to be characterised. As it is known that compounds with a relatively high molecular weight and low lipophilicity have limited membrane penetration capacity (Korkina et al., 2008), the transportation of honeybush pro-anthocyanidin into cells needs to be further elucidated

The HaCaT cell line, which is regarded as a premalignant cell model (Adhami et al., 2003; Han et al., 2011), was the most responsive to green tea and rooibos extracts. A similar response was noticed with the methanol extracts of honeybush whilst very little differentiation between the three different cell lines was noticed when using the aqueous extracts. This would imply that, depending on the extract, premalignant cells are targeted by rooibos and honeybush extracts to undergo cell death but this should be characterized in more detail, especially as the HaCaT keratinocytes and the normal/cancer cells have different cellular backgrounds.

In summary, green tea and the herbal teas reduced viability of skin cells that is likely to result from disruption of mitochondrial function possibly resulting from the pro-oxidative polyphenol/iron interactions. However, the polyphenolic compounds in honeybush extracts seem to exhibit a dual role, polymeric flavanol/proanthocyanidin compounds appear to reduce cell viability by inducing a pro-oxidant effect while the monomeric polyphenols, particularly the xanthones and flavanones, seem to exert a protective anti-oxidant effect. The polyphenolic constituents and antioxidant/pro-oxidant properties of green tea and rooibos seem to play an important role in the biological activity of this tea/herbal tea extracts *in vitro*. Therefore these parameters

may be useful biomarkers to predict cytotoxic activity of green tea and rooibos but their predictive potential *in vivo* still needs to be further investigated. On the other hand, a different set of predictive criteria such as flavanols/proanthocyanidin content and ABTS for strong cytotoxic effects and ORAC for weaker activity that is most likely to involve cytoprotection need to be considered for honeybush species. This is of importance as these biomarkers could be used as quality control parameters to evaluate herbal extracts that can be utilized in the genesis of pharma- and/or nutraceutical products against skin carcinogenesis. Since reduction of cell viability involving a decrease in ATP content is likely to result from mitochondrial dysfunction, aspects of membrane depolarization, cell cycle arrest and apoptosis need to be investigated.

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Chapter 4

Anti-proliferative and pro-apoptotic properties of rooibos (*Aspalathus linearis*) and different honeybush species (*Cyclopia* spp.) in skin cells

Anti-proliferative and pro-apoptotic properties of rooibos (*Aspalathus linearis*) and different honeybush species (*Cyclopia* spp.) in skin cells

Abstract

One of the hallmarks of cancer promotion is the induction of cellular hyperproliferation and apoptotic resistance, which confers a growth advantage in initiated cells. These events have been the focus area for chemoprevention studies indicating that polyphenolic compounds can prevent cancer development by modulating cell proliferation and apoptosis in cancer cells. The aim of the current study was to determine the anti-proliferative and pro-apoptotic activity of methanol and aqueous extracts of rooibos (*Aspalathus linearis*) and several honeybush species (*C. genistoides, C. intermedia, C. longifolia and C. subternata*) in different skin cells, using green tea (*Camellia sinensis*) as benchmark. The effect of the extracts was monitored on different cell growth indices including cell viability and proliferation, as well as the induction of apoptosis as defined by caspase-3 activity, Hoechst staining and confirmation by flow cytometry. At low concentrations the extracts inhibited cell proliferation in skin cells, with the methanol extract of rooibos and green tea being the most active. At higher concentrations, rooibos, green tea and the aqueous extracts of *C. intermedia and C. subternata* induced apoptosis with HaCaT cells being the most sensitive cell line. Methanol extracts of honeybush were far less effective against the induction of apoptosis, presumably due to the antioxidant properties of the major polyphenolic constituents. The lower concentrations of rooibos and *C. genistoides* extracts were more effective at selectively inhibiting the proliferation of cancer cells. Specific polyphenol to flavanol/proanthocyanidin ratio appear to exist in extracts when effecting the modulation of the growth parameters of skin cells. In rooibos, the dihydrochalcones and flavonol/flavones are likely to dominate but they may interact synergistically or additively with the proanthocyanidin tannin-type constituents. For honeybush, the xanthone/flavanone constituents are likely to play a protective role against apoptosis while the flavanol-like proanthocyanidins may be more prominent against cell viability, proliferation and induction of apoptosis. Although the herbal teas may be useful tools in modulating skin cancer development, specific ratios of the herbal tea constituents and the underlying mechanisms involved, still needs to be elucidated.

Keywords: Skin cells, anti-proliferative, pro-apoptotic, caspase-3, rooibos, honeybush

1. Introduction

The incidence of skin cancer, resulting from exposure to UVB irradiation, has become a global concern as it continues to rise rapidly with the non-melanoma skin cancers, comprised of basal cell carcinoma and squamous cell carcinoma, being the most frequently diagnosed in Caucasian populations (Kim and Armstrong, 2012). The development of these non-melanoma skin cancers, like any other cancer, is a multistage process involving three distinct stages, namely initiation, promotion and progression (Digiovani, 1992; Melnikova and Anathaswamy, 2005). One of the hallmarks of cancer promotion, caused by chronic exposure to UVB-irradiation and other known tumor promoters, is the induction of cellular hyper-proliferation. This hyper-proliferative state overcomes the latency of initiated cells leading to hyperplastic transformation during cancer development (Marks and Fursenberger, 1993; Rundhaug and Fischer, 2010). The activation of cell survival mechanisms and dysregulation of apoptosis caused by loss of fas-fas ligand interactions and mutations in the p53 genes are key determinants regulating this process in damaged cells. The apoptotic-resistance conferred by these events allows for the clonal expansion of transformed cells with DNA mutations to progress into malignant skin tumours. Consequently, induction of apoptosis to eliminate transformed cells has been identified as an important phase in the prevention of carcinogenesis (Melnikova and Ananthaswamy, 2005).

Some dietary components have been shown to have the ability to interfere with the process of cancer development thereby reducing the risk of different types of cancers (Fresco et al., 2010). Much of the research focus on chemoprevention in the skin has been placed on plant polyphenolic compounds (Afaq, 2011; Youn and Yang, 2011). One of the mechanisms suggested to play an important role in the chemopreventive properties of dietary compounds is the elimination of potentially damaged/mutated cells via apoptosis. Studies indicated that the mechanisms involved in the suppression of cancer development by polyphenols include the modulation of signal transduction pathways leading to growth arrest and induction of apoptosis in cancer cells (Ahmad et al., 1997; Roy et al., 2009; Chilampali et al., 2011; Pihie et al., 2012). Numerous chemopreventive studies on the protective role

of green and black teas (*Camellia sinensis*) and their polyphenolic constituents against skin carcinogenesis in mice have been conducted (Yang et al., 2002). A study using green tea, black tea and decaffeinated tea indicated that tea drinking or topical application inhibited different stages of skin cancer development (Huang, et al., 1997). Although the inhibitory activity of decaffeinated green tea and black teas, administered orally, was substantially weaker than green tea, the addition of caffeine to decaffeinated green tea restored the inhibition of UVB-induced carcinogenesis. Various protective mechanisms have been proposed for the anti-carcinogenic activity of tea (Yang et al., 2002). These include the elimination of carcinogens via detoxifying enzymes, antioxidant activity, modulation of signal transduction pathways as well as inhibition of cell proliferation resulting in cell cycle arrest and stimulation of apoptosis. The biological activity of green and black teas against carcinogenesis has been attributed to their respective phenolic constituents, epigallocathecin-3-gallate (EGCG) and theaflavin-3-3-digallate, as well as caffeine. These polyphenolic compounds of green and black tea have been shown to induce stress signals and suppress cell proliferation in cell cultures (Liang et al., 1999; Chen et al., 2003). EGCG was also found to cause mitochondrial dysfunction that leads to apoptosis via pro-oxidant activity as it induced mitochondrial membrane depolarisation that was associated with formation of hydrogen peroxide and induction of caspase-dependent apoptosis in pancreatic cells (Qanungo et al., 2005).

Rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) are South African herbal teas that possess health-properties that are associated with the prevention of cancer (Joubert et al., 2008). The anti-cancer properties of these herbal teas have been demonstrated in different animal carcinogenesis models (Marnewick et al., 2005; 2009; Sissing et al., 2011). In the two-stage mouse skin carcinogenesis models, utilising 7,12-dimethylbenz [*a*] anthracene (DMBA) as an initiator and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and UVB as promoters, polyphenol-enriched extracts of the herbal teas exhibited anti-tumor promoting and photoprotective effects, respectively (Marnewick et al., 2005; Petrova, 2009; Petrova et al., 2011). The anti-tumor and photoprotective properties of the herbal tea extracts demonstrated in these models were attributed to their polyphenolic constituents. The protective effects of the major honeybush polyphenolic constituents, mangiferin and

hesperidin against UVB-induced skin damage has been demonstrated but information on the role of rooibos flavonoids is still lacking (Petrova, 2009; Petrova et al., 2011). Although it has been suggested that the herbal tea extracts protect the skin against carcinogenesis by modulating cell proliferation, the effect of the extracts against different growth parameters that influence survival of skin cancer cells still remains to be elucidated.

The aim of the present study was to determine the effect of unfermented rooibos and honeybush extracts on cell proliferation and apoptosis in different skin cell culture systems using green tea as benchmark.

2. Materials and methods

2.1 Reagents.

Heat inactivated fetal bovine serum (FBS) was purchased from Invitrogen, USA. RPMI-1640, Dulbecco's Modified Eagle's medium (DMEM), L-glutamine, trypsin-versene and Hank's buffered salt solution (HBSS) were obtained from Lonza, Belgium. Dulbeco's phosphate buffered saline (DPBS), dimethyl sulfoxide (DMSO), Hoechst 33342 and staurosporine were purchased from (Sigma-Aldrich, USA). The cell proliferation ELISA, BrdU chemiluminescent kit was obtained from (Roche, Germany). The CellTiter-Glo®Luminescent cell viability assay kit and the caspase-3/7 assay kit were purchased from (Promega, USA). The BD^{TM} mitoscreen (JC-1) kit (BD Bioscience, USA), PE active caspase-3 apoptosis kit and APO-DIRECT^{TM} kit were from BD^{TM} Pharmingen, USA. Cells were analysed by flow cytometry using the FACSCalibur$^{TM.}$ (BD Biosciences, USA)

2.2 Plant material and preparation of extracts.

Green tea (*Camellia sinensis*), imported from China, was a gift from Vital Health Foods (Kuilsriver, South Africa). Unfermented ("green" or "unoxidized") rooibos (*Aspalthus linearis*) and honeybush (*C. intermedia, C. subternata, C. genistoides* and *C. longifolia*) herbal teas were obtained from Rooibos Ltd (Clanwilliam, South Africa) and the Agriculture Research Council, Infruitec-Nietvoorbij, Stellenbosch, South

Africa, respectively. The aqueous and methanol extracts of the different plant materials were prepared in triplicate, as previously described in Chapter 3. In short, the aqueous extracts of the different plant materials were prepared by steeping plant material in freshly boiled deionised water for 30 min, followed by filtering and freeze-drying of the filtrates. Methanol extracts were prepared following extraction with chloroform to remove lipid soluble material. The methanol extracts were dried *in vacuo* at 40 ˚C. The dried extracts were stored desiccated in amber vials at room temperature.

2.2 Cell culture.

Spontaneously immortalised keratinocytes (HaCaT) were a gift from the Department of Human Biology of the University of Cape Town (Cape Town, South Africa). Non-malignant normal fibroblast-like skin cells (CRL 7761) and basal carcinoma cell line (CRL 7762) skin cells obtained from the same patients were purchased from the American Tissue Cell Culture Collection (ATCC, USA). Cells were maintained in their respective media supplemented with 10% FBS and L-glutamine (2 mM); HaCaT (RPMI-1640), normal cells (DMEM) and skin cancer cells (DMEM containing 0.12 mM of HCL) up until confluency. The following passages were utilised in experiments HaCaT (p70 to p80), normal cells (p16 to p20) and cancer cells (p15 to p22).

2.3 Modulation of cell proliferation and apoptosis

Cells were seeded in black solid (Porvair Sciences, UK) and clear tissue culture 96-well microtiter plates for BrdU and caspase-3 assays, respectively, at a density of 5 x 10^3 per well in their respective media (100 µL) containing 10% FBS. Cells were cultured for 24 h at 37 °C in 5% CO_2/95% air to a confluency of 70 to 80%. Thereafter, media was decanted and replaced with the fresh media containing 0.5% FBS, and the different dilutions of the various extracts. The plates were incubated for another 24 h and subjected to the different treatment protocols outlined below.

Cell proliferation assay. Cell proliferation was determined with the BrdU chemiluminescent immunoassay kit following the manufacturer's prescribed

instructions. This assay quantifies cell proliferation based on the measurement of BrDU incorporation during DNA synthesis (Roche, Germany). Briefly, after 24 h incubation in the presence of the extracts the cells were labelled by adding the BrdU solution (10 µL) for 2 h at 37 °C. After incubation, media was decanted and cells fixed with a denaturing solution (200 µL) and incubated at room temperature for 30 min. After removal of the denaturing solution, cells were incubated with the BrdU antibody (100 µL) for 90 min. Plates were washed with saline (3 x 250 µL), treated with the substrate (100 µL), covered with foil and shaked for 3 min before quantification in the Veritas^{TM} microplate luminometer (Promega, USA). The luminescent signal was measured in relative light units (RLU) and the extent of cell proliferation expressed as a percentage (%) of the control treatment as follows:

$$\% \text{ cell proliferation} = RLU_{\text{treated cells}}/RLU_{\text{control cells}} \times 100$$

IC_{50} values for % inhibition was calculated on the basis of the best fit for dose-response data using the 4-parameter logistic curve (sigmoidal variable slope) in GraphPad Prism version 5.04 for Windows (GraphPad Software, USA). The BrdU assay was conducted using four to five replicates of the different dilutions for each extract and the experiment was repeated at least twice.

Cell viability and apoptosis assays. The concentrations range of the different extracts utilised for apoptosis encompassed the IC_{50} values generated in the cell viability (Chapter 3) and proliferation assays. From the honeybush, only two (*C. intermedia* and *C. subternata*) of the most active species against cell viability were selected. The CellTiter-Glo®Luminescent assay was used for the determination of cell viability by monitoring the ATP content following the manufacturer's instruction. IC_{50} values were calculated as described for cell proliferation. Apoptosis was determined in the cell lysates following treatment with a cell lysis buffer (20 µL) in combination with one freeze-thaw cycle. Cell lysates were transferred (25 µL) into a white solid plate and incubated with the caspase 3/7 reagent (25 µL) for 1 h in the dark at room temperature. The RLU were determined in the Veritas microplate luminometer. Induction of apoptosis was calculated as a fold increase compared to the control as well as expressed as a percentage of the viable cells. Staurosporine

was used as the positive control and different concentrations were used for the HaCaT (75 nM), normal (100 nM) and cancer (200 nM) cells. The negative control contained an equal volume of the buffer.

Hoechst stain. Cells were seeded (30 x 10^4) in DMEM (1 mL) onto heat-sterilised cover-slips in small petri dishes (35 mm) and exposed to the different extracts at concentrations equalling the IC_{50} concentrations effecting reduction of cell viability (Chapter 3) and cell proliferation as obtained in the current study. Staurosporine was used as the positive control whilst cells exposed to an equal volume of buffer served as the negative control. The growth medium was discarded and cells washed with DPBS and incubated with 2 mL of 1 µg/mL Hoechst 33342 (prepared in medium) for 30 min at 37 °C and the cells viewed under UV light using a Axoivert, Zeiss microscope (Göttingen, GERMANY) fitted with a blue filter (exclusion 358 nm, emission 461 nm). Magnification of 40 x was used to record photographs.

2.4 Characterization of pro-apoptotic activity of rooibos by flow cytometry

Of the herbal teas, the pro-apoptotic activity of the methanol extract of rooibos, exhibiting the highest activity against cell viability (Chapter 3), was further characterised utilising normal cells in order to investigate the underlying cellular mechanisms of apoptosis. Cells were seeded in a 75 cm^2 tissue culture flask (15 mL DMEM) at a density of 1 x 10^6 and incubated for 24 h. The medium was discarded and cells incubated for 24 h with the methanol extract representing the IC_{50} concentration as determined for the reduction of cell viability (Chapter 3). Cells were washed with HBSS, removed from the flask with a cell scraper and re-suspended in DPBS at a concentration of 1 x 10^6/mL in 15 mL polystyrene sterile centrifuge tubes. Cells were then analysed by flow cytometry for caspase-3 activity, membrane depolarisation (JC-1) and DNA damage.

Caspase-3 activity. The induction of caspase-3 activity was determined with the PE active caspase-3 apoptosis kit according to the manufacturer's instructions. The assay utilises a rabbit caspase-3 antibody to detect the active form of caspase-3 in cells (BD Pharmingen ^{TM}, USA). Cells were washed twice with cold 1 x PBS (500

µL), pelleted by centrifugation for 5 min (300 x g) and fixed with a BD cytofix / cytoperm solution ™ (500 µL) for 20 min on ice. The supernatant was discarded, cells washed with 1 x PBS (500 µL), re-suspended in 1 x BD Perm/Wash™ buffer (100 µL) plus antibody (20 µL) and incubated for 30 min at room temperature. After incubation cells were pelleted (300 x g), washed with 1 x BD Perm/Wash™ buffer (1 mL) and re-suspended in the 1 x BD Perm/Wash™ buffer (500 µL) for analysis by flow cytometry.

Membrane depolarisation. The effect of the rooibos methanol extract on mitochondrial integrity was assessed with the BD™ mitoscreen (JC-1) kit (BD Bioscience, USA), following the manufacturer's instructions. The kit utilises JC-1 (1st J aggregate forming cationic dye) which is sensitive to membrane potential changes. Incorporation of JC-1 monomers into the mitochondria is dependent on a polarised membrane potential and their accumulation results in the formation of concentration dependent red fluorescent aggregates. In depolarised membranes, JC-1 remains as monomers in the cytoplasm and exhibits a lowered red fluorescence.

Treated cells were re-suspended in DPBS (1 mL), were centrifuged at 400 x g for 5 min at room temperature. The supernatant was discarded, the cells re-suspended in JC-1 working solution and incubated for 15 min at 37 °C in 5% CO_2/95% air. Cells were pelleted by centrifugation (300 x g), washed twice with "1 x assay buffer (1 mL), re-suspended in the 1 x assay buffer" (500 µL) and analysed by flow cytometry for JC-1 uptake in cells.

DNA fragmentation. The ability of rooibos methanol extract to induce DNA damage during apoptosis was monitored, using the APO-Direct kit™ by following the manufacturer's instructions. The Apo-direct assay is a single step staining method that labels fragmented DNA to monitor apoptotic cells (BD Pharmingen™, USA). Cells were re-suspended in PBS and centrifuged for 5 min at 300 x g, the supernatant was discarded, and cells were fixed by re-suspending the pellet in 1% paraformaldehyde in PBS and incubated for 60 min on ice. The cells were pelleted by centrifugation and the supernatant was removed and cells washed twice with PBS and centrifuged. The pellet in the residual PBS was gently vortexed and cells were

stored overnight in 1 mL of 70% (v/v) ethanol at -20 °C. The ethanol was then aspirated and cells washed twice with wash buffer (1 mL) and centrifuged, the pellet was re-suspended in DNA labelling solution (50 µL) and incubated for 60 min at 37 °C. The cells were rinsed twice with the rinsing buffer (1 mL) and centrifuged, pellet was re-suspended in PI/RNase solution and analysed for DNA fragmentation by flow cytometry.

2.5 Statistical analysis

Significant group differences (independent classification variable) were evaluated with an ANOVA F1 test or the Welch's test, depending if homogeneity of group variances were present when more than two groups were present, using SAS's GLM procedure. Levene's Test was used to test for the homogeneity of the variances and Tukey's Test as the post-hoc test. When only two groups were compared the student T-test was used. When the original variables had non-parametric distributions, they were individually transformed to become parametric. The following transformations were conducted for the methanol extracts: the square root for *C. sinensis*; 1/squared root for *C. genistoides* and the inverse transformation for *C. intermedia* and *C. subternata* (Fig 4.1A and B). The Kruskal Wallis and Post hoc Tukey type test was used for the analyses for the aqueous extracts for *C. sinensis* and *A. linearis* (non parametric analyses). Statistical significance was measured at $P<0.05$. Spearman's rank correlation coefficients were determined for measuring the strength of the relationship between the different variables.

3. Results

3.1 Effect of tea/herbal tea extracts on cell proliferation in skin cells

Green tea and rooibos extracts exhibited the highest activity against the proliferation of different skin cells with the methanol extract being significantly ($P<0.05$) more effective than the aqueous extract (Table 4.1). Both green tea and rooibos extracts inhibited the proliferation of HaCaT cells and cancer cells at significantly ($P<0.05$) lower concentrations than the normal cells with the rooibos

Table 4.1. Anti-proliferative activity of aqueous and methanol extracts of green tea and different herbal teas in skin cells

Cell type	Extract Type**	Tea and herbal tea groups*					
		Camellia. sinensis	*Aspalathus linearis*	*C. genistoides*	*C. intermedia*	*C. longifolia*	*C. subternata*
HaCaT cells BrDU IC_{50} (mg/ml)	MeOH	$0.035\pm0.003^{c}_{A}$	$0.037\pm0.005^{c}_{A}$	$0.339\pm0.043^{a}_{A}$	$0.350\pm0.077^{a}_{A}$	$0.196\pm0.034^{b}_{A}$	$0.190\pm0.019^{b}_{A}$
	Aq	$0.045\pm0.007^{d}_{B}$	$0.068\pm0.011^{d}_{B}$	$0.385\pm0.063^{a}_{A}$	$0.202\pm0.037^{b}_{B}$	$0.172\pm0.018^{c}_{B}$	$0.164\pm0.039^{c}_{A}$
Normal cells BrDU IC_{50} (mg/ml)	MeOH	$0.063\pm0.009^{d}_{A}$	$0.058\pm0.014^{d}_{A}$	$0.837\pm0.109^{a}_{A}$	$0.151\pm0.013^{c}_{A}$	$0.131\pm0.023^{c}_{A}$	$0.200\pm0.025^{b}_{A}$
	Aq	$0.154\pm0.021^{c}_{B}$	$0.208\pm0.038^{b}_{B}$	$0.843\pm0.098^{a}_{A}$	$0.091\pm0.022^{d}_{B}$	$0.101\pm0.016^{d}_{B}$	$0.098\pm0.019^{d}_{B}$
Cancer cells BrDU IC_{50} (mg/ml)	MeOH	$0.035\pm0.012^{d}_{A}$	$0.016\pm0.003^{e}_{A}$	$0.373\pm0.080^{a}_{A}$	$0.150\pm0.046^{c}_{A}$	$0.381\pm0.069^{a}_{A}$	$0.223\pm0.030^{b}_{A}$
	Aq	$0.124\pm0.025^{a}_{B}$	$0.048\pm0.022^{b}_{B}$	$0.136\pm0.041^{a}_{B}$	$0.143\pm0.037^{a}_{B}$	$0.168\pm0.049^{a}_{B}$	$0.158\pm0.039^{a}_{B}$

Values represent means ± standard deviations of five replication of at least two experiments. * The original variables had non-parametric distributions, but were individually transformed to become parametric. Statistical analyses for significant group effects (more than 2 groups) were assessed by either the One-way ANOVA F-test or the Welch's test, depending if homogeneity of group variances were present (Levene's test). If significant group differences were present, the post hoc Tukey's Studentized Range Test was used for between group comparisons at 5% significance level. **Statistical comparisons for only two groups, i.e. MeOH vs Aq for each tea, the Student's T-test were used. Means in a row (Tea and herbal tea groups) or column (Extract type) followed by the same letter (lower super scrip and upper t case subscrip) do not differ significantly, if letters differ then $P<0.05$. Abbreviations: IC_{50} – concentration yielding 50% inhibition of DNA synthesis; BrdU - 5-bromo-2'-deoxyuridine; MeOH – methanol; Aq – aqueous.

methanol extract displaying the highest activity against the cancer cell line (CRL 7762) (Fig. 4.1). The methanol extract of green tea exhibited similar activity between the HaCaT and cancer cell line whilst the aqueous extract was more active against HaCaT cells. In contrast to green tea, rooibos extracts, the aqueous extracts of honeybush exhibited higher activity than the methanol extracts, except for *C. genistoides* that demonstrated similar activity between extracts in HaCaT and normal cells (Table 4.1). The aqueous extracts of the different honeybush species inhibited the proliferation of normal cells at concentration lower than those required for HaCaT and the cancer cells, except for *C. genistoides*. The aqueous extracts of the *C. genistoides* displayed the highest inhibitory activity against the proliferation of cancer (CRL 7762) and HaCaT cells whilst exhibiting the least activity against normal cells (Fig. 4.1). The activity of the methanol extracts differed between the species with *C. genistoides* being the least active against normal cells whilst exhibiting higher activity against the proliferation of HaCaT and cancer cells. *Cyclopia subternata* was more active against HaCaT, whilst *C. longifolia* was more active against normal cells and *C. intermedia* targeted normal and cancer cells at similar concentrations.

3.2 Modulation of apoptotic activity in skin cells

Green tea and rooibos induced caspase-3 activity in a dose-dependent manner in the different skin cell lines (Figs. 4.2 A and B). Depending on the dose, the methanol extracts exhibited the highest activity when compared to the aqueous extracts. HaCaT cells were the most sensitive cell line while cancer cells exhibited the weakest response for both green tea and rooibos extracts. However, the aqueous extract of rooibos displayed similar activity between normal and cancer cells. A similar effect was noticed for both green tea and rooibos extracts when the data was normalised against viable cells (ATP content; Figs 4.2 C and 4.2D). The induction of apoptosis by both extracts of green tea and rooibos was closely related to the reduction of cell viability as an inverse correlation existed between induction of caspase-3 (fold increase) and the ATP content (Table 4.2). The aqueous extracts of the honeybush species (*C. intermedia and C. subternata*) also induced a dose-dependent caspase-3 fold increase. (Figs. 4.3 A and 4.3B) but this effect was far

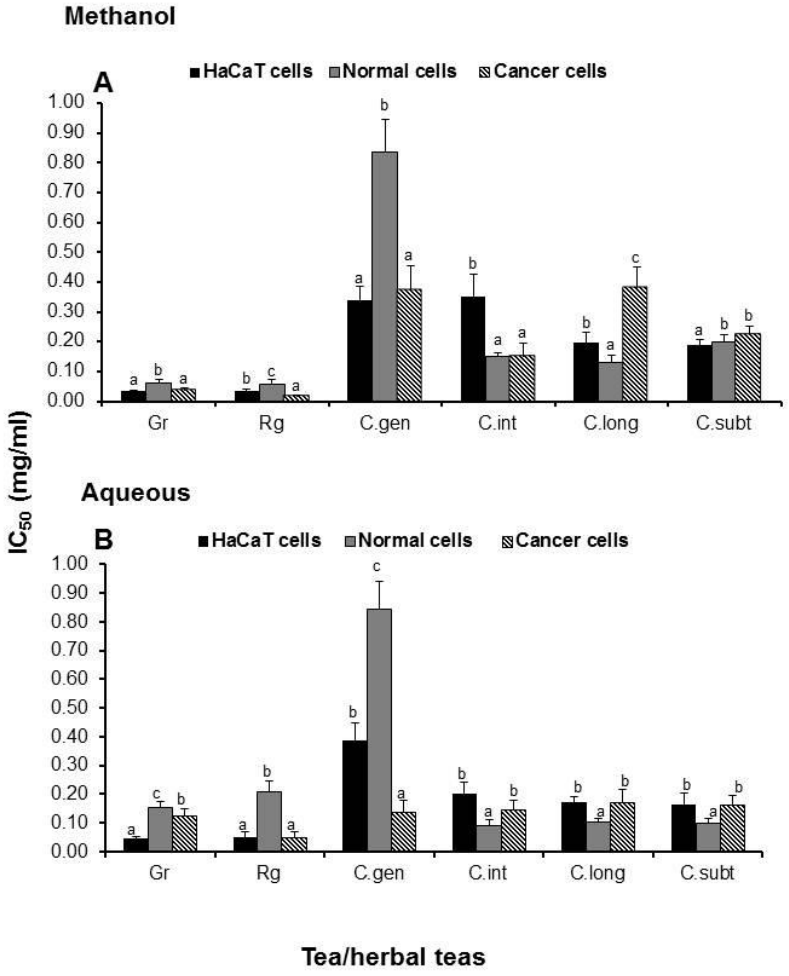

Fig. 4.1. Susceptibility of the different cell lines against the anti-proliferative properties of aqueous (Fig. 4.1A) and methanol (Fig. 4.1B) extracts of green tea, rooibos and different honeybush species. Some original variables had non-parametric distributions, but were individually transformed* to become parametric (See Statistics for details). Statistical analyses for significant group effects were assessed by either the One-way ANOVA F-test or the Welch's test, depending if homogeneity of group variances were present (Levene's test). If significant group differences were present, the post hoc Tukey's Studentized Range Test was used for between group comparisons at 5% significance level. Means followed by the same letter (lower super scrip do not differ significantly, if letters differ then $P<0.05$. Abbreviations: *C. sinensis - Camellia sinensis; A. linearis- Aspalathus linearis, C.genistoides - Cyclopia genistoides,; C.intermedia - Cyclopia intermedia; C.longifolia - Cyclopia longifolia; C.subternata - Cyclopia subternata.*

weaker when compared to green tea and rooibos extracts (Addendum: Table B1 and Table B2).

Once again, HaCaT cells were the most sensitive cell line cells whilst cancer cells were the most resistant cell line to the pro-apoptotic effects of honeybush aqueous extracts. In contrast to the aqueous extracts, the methanol extracts had no significant effect in the HaCaT and normal cell lines. However, in cancer cells *C. intermedia* methanol extract (Fig 4.4A), the significantly reduced the fold increase at the highest concentration, although it was increased at the lowest concentration. On the other hand *C. subternata* had no significant effect (Fig. 4.4B). The reduction of the fold increase by *C. intermedia* was closely associated with the reduction of ATP content (Addendum B; Table B2). Normalisation of caspase-3 activity against viable cells (ATP content), a similar effect to fold increase was noticed with HaCaT cells exhibited the highest % caspase-3 activity (Figs 4.3C and 4.3D). However, contrary to the effect on the caspase-3 fold increase, the methanol extract of *C. intermedia* had no significant effect on % caspase-3 activity per viable cancer cells (Fig 4.4C) while *C. subternata* significantly ($P<0.05$) increased the % caspase-3 activity in viable cancer cells in a dose-dependent manner (Fig 4.4D).

When considering the relationship between caspase-3 fold increase and ATP content, the aqueous extract of *C. subternata* exhibited a strong inverse relationship in HaCaT and normal cells however, this relationship was moderate in cancer cells (Table 4.2). In, the methanol extracts of *C. subternata*, a strong negative correlation was observed in HaCaT cells, a weak negative correlation in normal cells while no correlation was noticed in cancer cells. For *C. intermedia*, there was a moderate negative correlation in HaCaT cells, a positive correlation in cancer cells while normal cells lacked a correlation between ATP and caspase-3 activity.

Morphological alteration in cells associated with apoptosis: The Hoechst 33342 nuclear DNA stain provided qualitative data on the mode and degree of apoptotic cell death induced by the different tea/herbal tea extracts in normal and HaCaT cells. The extracts did not induce any morphological changes in cancer cells. In untreated control slides, cells exhibited a low intensity blue colour and reflected cell

proliferation as compact DNA fluoresced brightly, indicating pro-metaphase during mitosis (Fig. 4.5A).

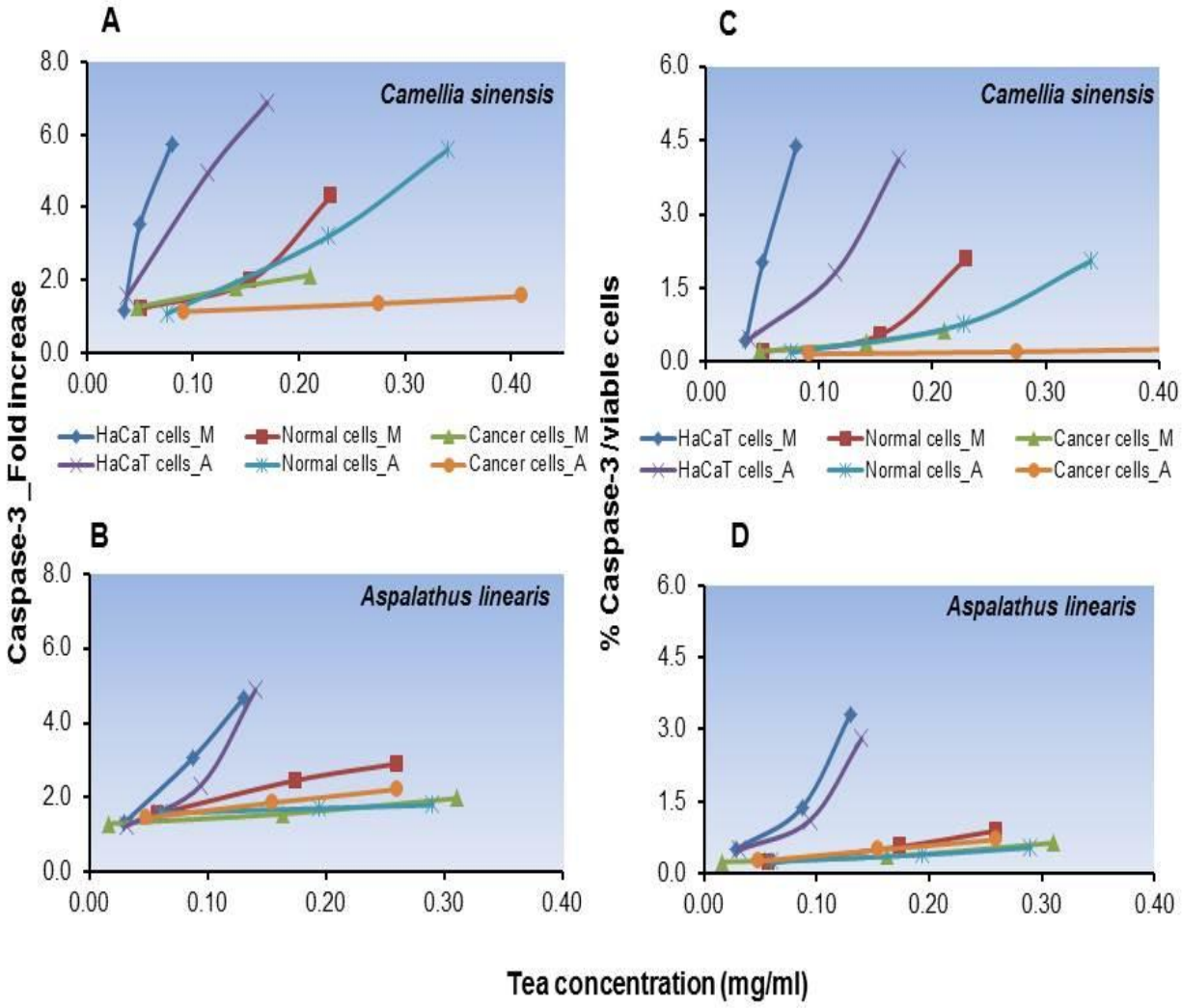

Fig. 4.2. The dose-dependent induction of caspase-3 fold increase of green tea (*Camellia sinensis*) (Fig. 4.2 A) and rooibos (*Aspalathus linearis*) (Fig. 4.2 B) in the different cell lines as well as the % caspase-3 activity expressed per viable cells (Figs 4.2 C and 4.2D). Abbreviation: M - methanol extracts; A- aqueous extracts.

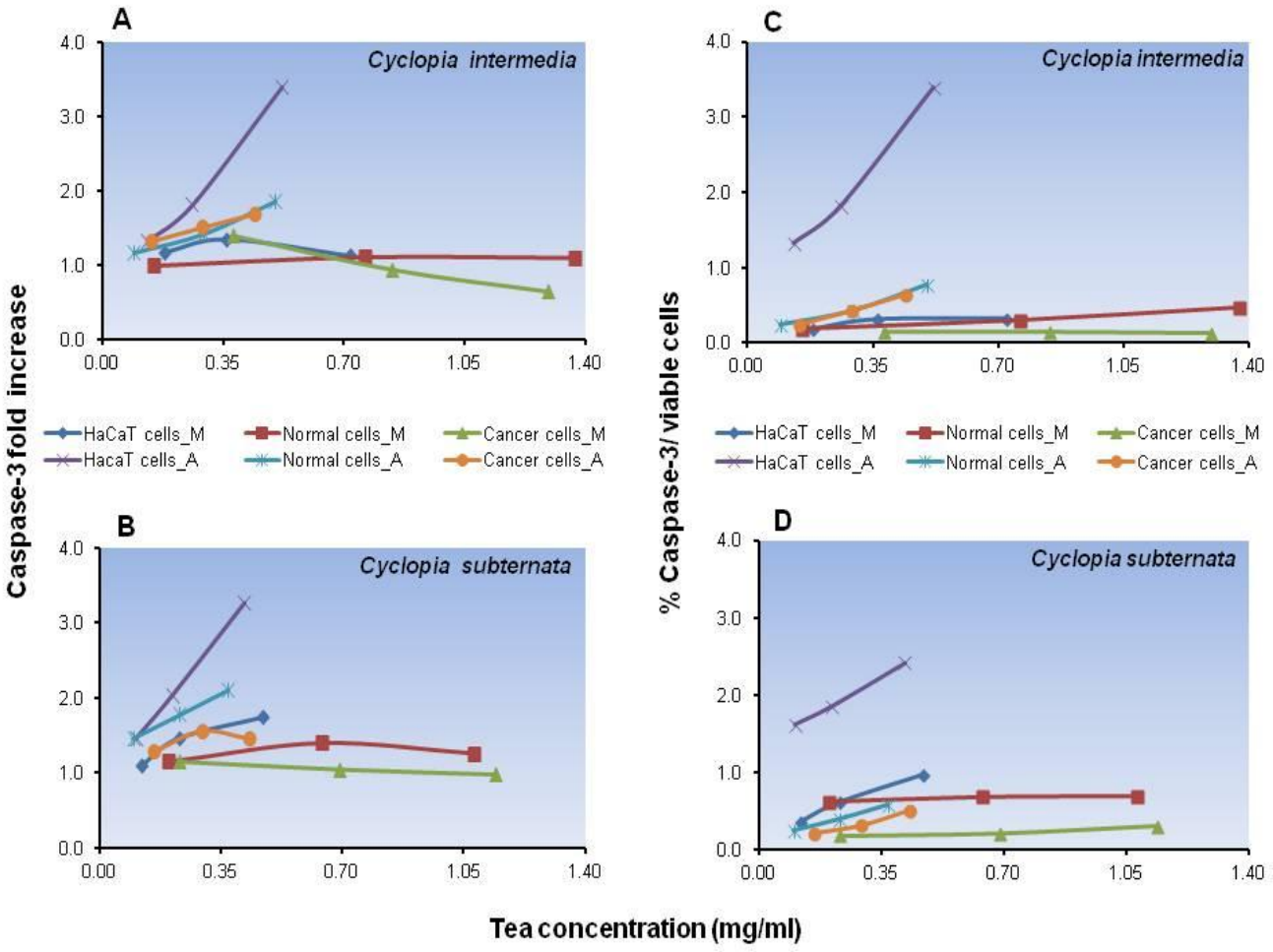

Fig. 4.3. Dose-dependent induction of caspase-3 activity by selected honeybush extracts, *Cyclopia intermedia* (Fig. 4.3 A) and *Cyclopia subternata* (Fig. 4.3 B) and the susceptibility of different skin cells lines. The dose dependent induction of caspase-3 activity, normalised against cell viability (ATP content) in Figs 4.3C and 4.3D. Abbreviation: M - methanol extracts; A- aqueous extracts.

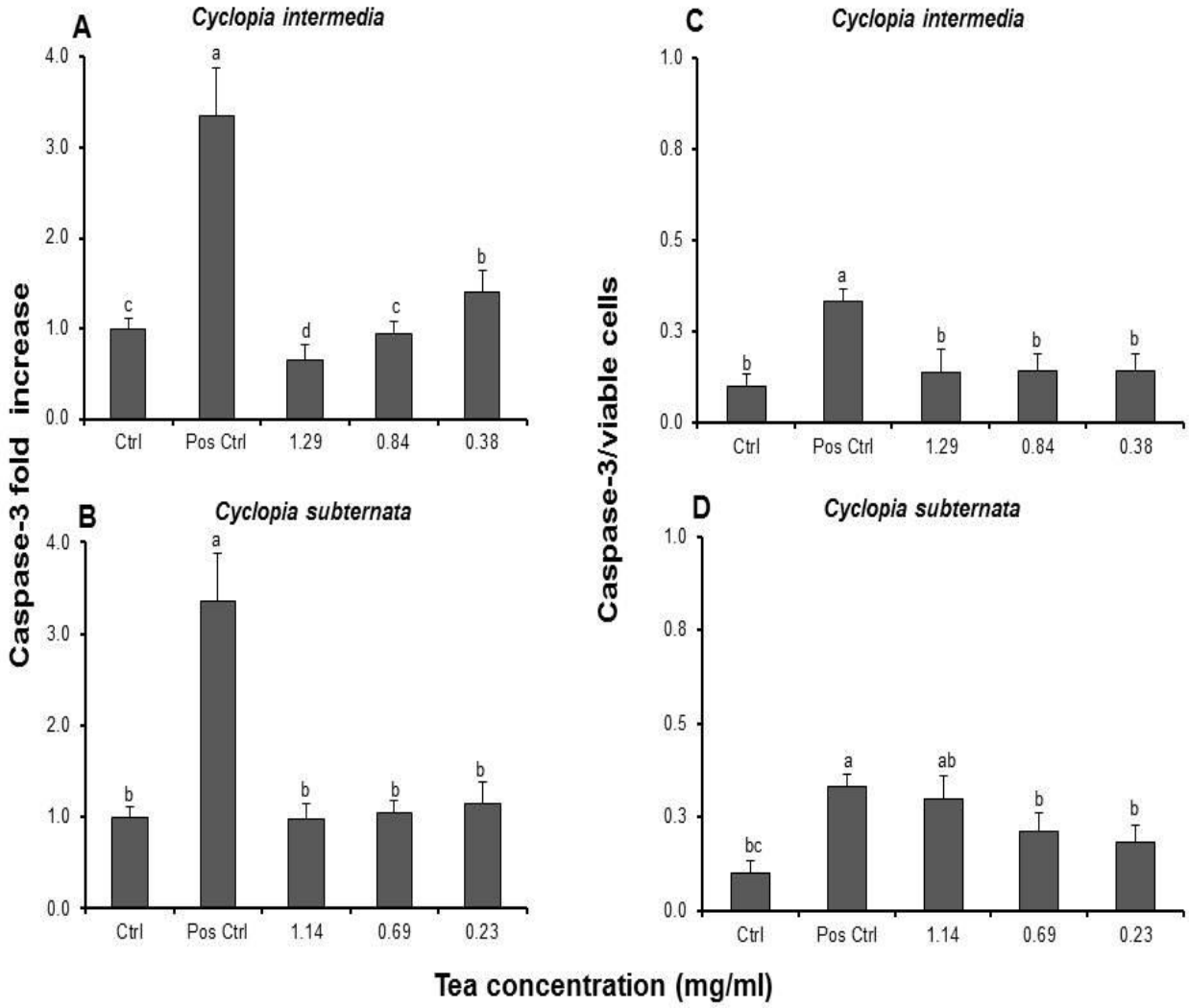

Fig 4.4 Effect of honeybush (*Cyclopia intermedia* and *Cyclopia subternata*) methanol extracts on caspase-3 activity in cancer cells. Figures 4.4A and 4.4B depict the effect of different extracts' concentrations on caspase-3 fold increase in cells. Normalisation of the effect of the extracts on caspase-3 activity against cell viability Figs 4.4C and 4.4D) Values represent means±standard deviation. Statistical analyses for significant group effects (more than 2 groups) were assessed by either the One-way ANOVA F-test or the Welch's test, depending if homogeneity of group variances were present (Levene's test). If significant group differences were present, the post hoc Tukey's Studentized Range Test was used for between group comparisons at 5% significance level. Abbreviations: M - methanol extract; A – aqueous extract; Ctrl - control; Pos. ctrl - positive control (staurosporine 200nm)

Table 4.2. Correlation between caspase-3 fold increase and ATP production from the green tea and different herbal tea extracts

Tea/herbal tea Unit of measure	Methanol extracts			Aqueous extracts		
	HaCaT cells	Normal cells	Cancer cells	HaCaT cells	Normal cells	Cancer cells
Camellia sinensis						
Casp-3_F and ATP	r -0.881 P<0.0001	r -0.888 P<0.0001	r -0.800 P<0.0001	r -0.742 P<0.0001	r -0.878 P<0.0001	r -0.672 P<0.0001
Aspalathus linearis						
Casp-3_F and ATP	r -0.881 P<0.0001	r -0.802 P<0.0001	r -0.769 P<0.0001	r -0.745 P<0.0001	r -0.547 P=0.0002	r -0.724 P<0.0001
Cyclopia intermedia						
Casp-3_F and ATP	r -0.527 P=0.0005	-	r 0.479 P=0.0015	r -0.819 P<0.0001	r -0.699 P<0.0001	r -0.599 P<0.0001
Cyclopia subternata						
Casp-3_F and ATP	r -0.841 P<0.0001	r -0.588 P=0.0004	-	r -0.783 P<0.0001	r -0.864 P<0.0001	r -0.554 P=0.0027

Spearman correlations were used to calculate correlation coefficients (r values) and P<0.05 was considered statistically significant. Abbreviations: Casp-3_F-caspase-3 fold increase; ATP - adenosine triphosphate; *Camellia sinensis* - green tea, *Aspalathus linearis* - rooibos, honeybush species (*C. intermedia, C. subternata*)

In slides treated with staurosporine, cells exhibited apoptotic features as they stained brightly, showed nuclear fragmentation and unstained nucleolar regions (Fig. 4.5B). Cells treated with tea/herbal extracts also exhibited apoptotic features which included brightly stained nuclei, cell shrinkage, membrane blebbing, globular and crescent shaped figures (Figs. 4.5C to 4.5H). In cells treated with the methanol extracts of green tea (Figs. 4.5C to 4.5D) and rooibos (Figs 4.5E to 4.5F), apoptotic features were more prominent and intensified at the highest concentration. Apoptotic features in cells treated with the aqueous extracts of *C. subternata* (Fig. 4.5G) and *C. intermedia* (Fig. 4.5H) were only exhibited by the highest concentration.

Characterisation of rooibos apoptotic activity in normal cells by flow cytometry: As the methanol extract of rooibos exhibited the highest apoptotic activity against normal cells when considering the herbal teas, further analysis was conducted utilising flow cytometry. Upon treatment with the methanol extract at the

concentration equalling the IC_{50} value for reduction of ATP content, more than 30% of the treated cells displayed caspase-3 activity when compared to the control (Figs. 4.6A and 4.6B). When monitoring the uptake of JC-1 into the mitochondria the red fluorescent JC-1 aggregates accumulated in the mitochondria of untreated cells, with only a small population of cells exhibiting a reduction in fluorescence thus indicating intact polarised mitochondrial membrane (Figs. 4.6C to 4.6D). In contrast, treatment with the rooibos methanol extract disrupted mitochondrial potential as a significant amount of JC-1 remained in the cytoplasm, indicated by an intense fluorescence in the FL-2 channel (second lower quadrant) as well as a shift to the left in the histogram (Figs. 4.6E to 4.6F). Untreated cells did not show any increase in DNA fragmentation whilst the treated cells exhibited DNA fragmentation associated with the late stages of apoptosis (Figs. 4.6G to 4.6H). Cell towards the left of the vertical line are in sub-G1 or pro-apoptotic while most of the non-apoptotic cells were in G0/G1 (200 to 400 FL2 Area).

3.3 Inhibition of cell proliferation related to phenolic composition

Differences in the phenolic compositional parameters between the methanol and aqueous extracts of green tea and the herbal teas indicated a lower recovery of phenolic constituents in the aqueous extracts (Chapter 3). Of interest is that far less of the monomeric xanthones (mangiferin and isomangiferin) and flavanones (hesperidin) polyphenols were recovered in the aqueous phase, specifically in the flavanone-rich species of *C. intermedia* and *C. subternata* (Table 4.3). In the methanol extract of green tea, strong inhibitory activity (IC_{50}) against the proliferation of HaCaT cells was associated with high levels of total polyphenols (TP), total catechins, specifically EGCG and caffeine. The specific TP to flavanol/proanthocyanidin ratios between the extracts was similar with the weaker response of the aqueous extract related to a reduction in TP, flavanol/proanthocyanidins and total catechins including EGCG. The caffeine content was also significantly reduced but not to the same extent as TP and total catechin content. Similarly, the methanol extract of rooibos, exhibiting the highest activity

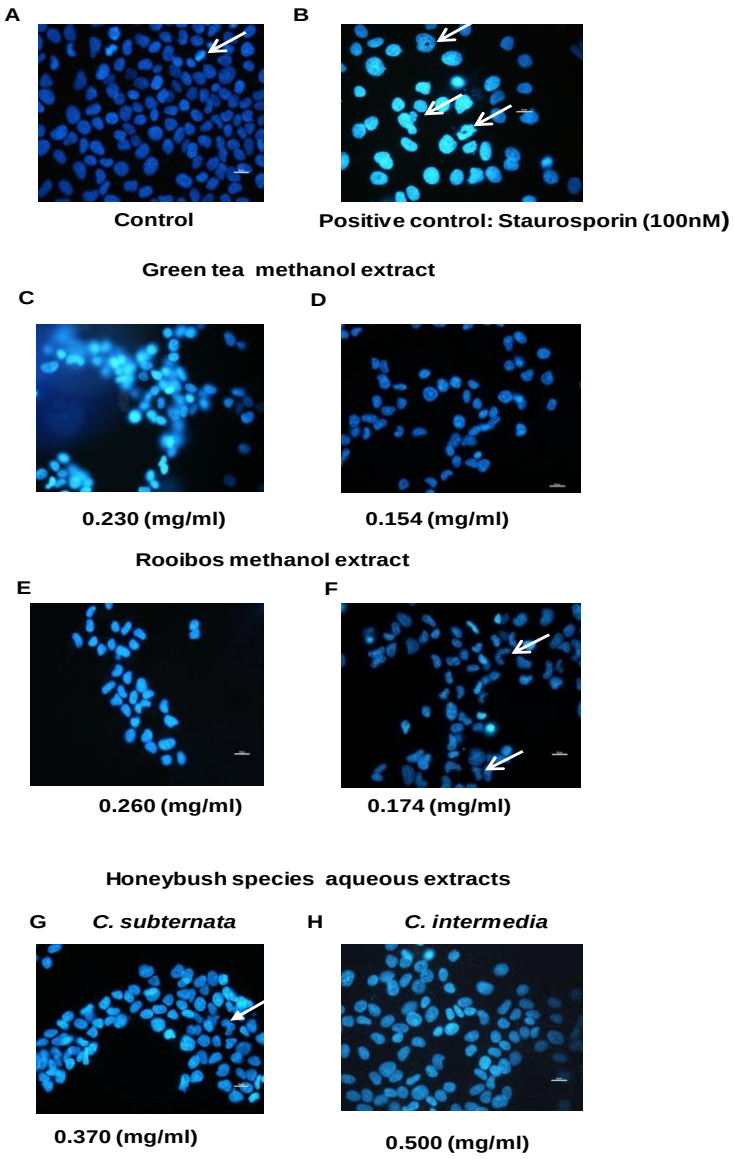

Fig. 4.5. Morphological features in cells stained with Hoechst after 24 h incubation with the various tea/herbal tea extracts. Control cells displayed dimly stained nuclei; arrow indicates pro-metaphase (Fig. 4.4 A). Positive control cells were brightly stained; arrow heads indicate nuclear fragmentation and unstained nucleolar region (Fig. 4.4B). Cells exposed to methanol extracts of green tea (Figs. 4.4 C, 4.4 D) and rooibos (Figs. 4.4 E, 4.4 F) displayed smaller nuclei, were fewer in field and some were brightly stained. Rooibos exhibited crescent-shaped figures and membrane blebbing (arrows in Fig. 4.4 F). *Cyclopia subternata* also exhibited membrane blebbing (arrow in Fig. 4.4 G) and some cells were brightly stained as also seen for *C. intermedia* (Fig. 4.4 H). Bar = 20 µM; Magnification 40 x

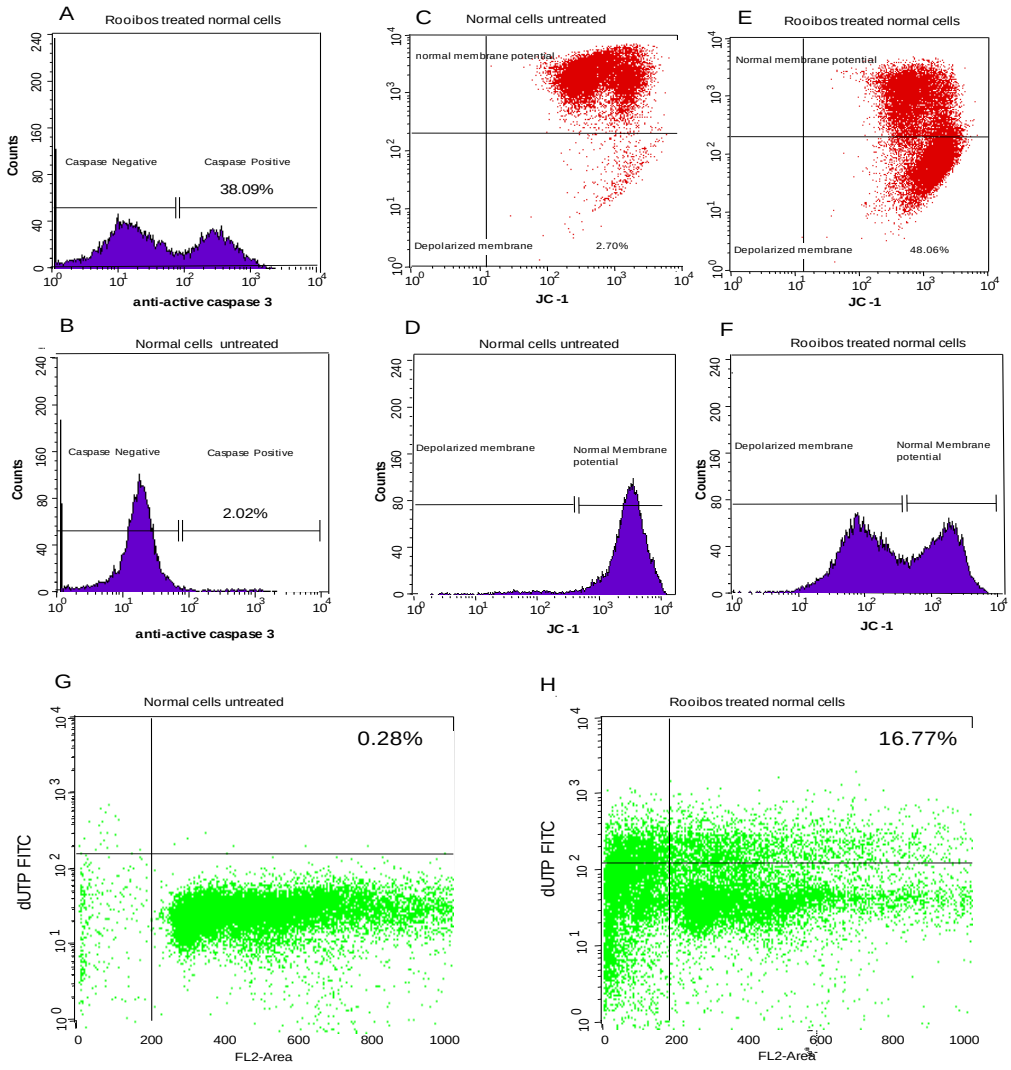

Fig. 4.6. Flow cytometric analyses of normal cells treated with the rooibos methanol extract (0.32 mg/ml). Caspase-3 activity was induced in treated cells (Fig. 4.5A) when compared to untreated control (Fig 4.5B). Control cells also exhibited an intact polarized membrane as red fluorescent JC-1 aggregates accumulated in the mitochondria of the untreated viable cells (Figs. 4.5C to 4.5D) while treatment caused membrane depolarization with JC-1 remaining in the cytoplasm (Figs. 4.5 E to 4.5 F). High concentration of non-apoptotic cells in the lower channel indicated very little DNA fragmentation in control cells (Fig. 4.5G). Treated cells exhibited DNA fragmentation as damaged cells were located in the upper panel while most of the cells were pro-apoptotic (sub G1 ; to the left of the vertical axes). The majority of non apoptotic cells are in G0/G1 - between 200 and 400 FL2. (Fig. 4.5 H).

Table 4.3. Concentration of TP, FLAVA and monomeric polyphenolic compounds of the methanol and aqueous extracts of green tea and herbal teas expressed as the TP/FLAVA ratios.

Tea/herbal extracts Subgroups	Polyphenols	Concentration (µg/mg extract)		TP/FLAVA Ratio	
		MeOH	Aq	MeOH	Aq
C. sinensis	TP	$256.5\pm36.9_A$	$161.0\pm20.8_B$	1.94	2.07
	FLAVA	$132.3\pm3.4_A$	$77.6\pm1.5_B$		
Catechins	EGCG	$111.9\pm3.0_A$	$46.1\pm1.5_B$		
	Total catechins	$200.8\pm6.2_A$	$100.0\pm2.8_B$		
Alkaloid	Caffeine	$57.5\pm1.6_A$	$40.1\pm0.3_B$		
A. linearis	TP	$350.7\pm35.0_A$	$250.5\pm16.4_B$	12.92	13.92
	FLAVA	$27.1\pm0.8_A$	$18.0\pm0.6_B$		
DHC	Asp_notho	$151.8\pm1.7_A$	$100.6\pm1.8_B$		
	Total_ mono	$209.1\pm1.8_A$	$138.1\pm3.6_B$		
Cyclopia spp.					
C. genistoides	TP	$215.1\pm24.8_A$	$193.9\pm10.9_B$	17.63	11.68
	FLAVA	$12.2\pm0.8_B$	$16.6\pm0.3_A$		
Xanthones	Mang_isomang	$196.1\pm4.7_A$	$107.6\pm0.7_B$		
Flavanone	Hesperidin	$32.9\pm1.0_A$	$4.1\pm0.1_B$		
	Total_ mono	$235.1\pm5.2_A$	$117.4\pm0.5_B$		
C. longifolia	TP	$261.0\pm27.0_A$	$239.5\pm27.8_B$	21.75	16.52
	FLAVA	$12.0\pm0.8_B$	$14.5\pm0.1_A$		
	Mang_isomang	$229.0\pm5.6_A$	$160.1\pm4.1_B$		
	Hesperidin	$24.6\pm0.7_A$	$6.7\pm0.4_B$		
	Total_ mono	$261.5\pm6.3_A$	$172.1\pm4.4_B$		
C. intermedia	TP	$172.1\pm4.1_A$	$164.5\pm11.3_B$	15.23	9.19
	FLAVA	$11.3\pm0.9_B$	$17.9\pm0.9_A$		
	Mang_isomang	$87.8\pm2.9_A$	$54.0\pm0.8_B$		
	Hesperidin	$88.8\pm11.6_A$	$7.3\pm0.6_B$		
	Total_ mono	$186.2\pm13.0_A$	$64.0\pm0.6_B$		
C. subternata	TP	$220.5\pm14.5_A$	$175.0\pm24.1_B$	16.96	7.64
	FLAVA	$13.0\pm0.6_B$	$22.9\pm0.9_A$		
	Mang_isomang	$78.5\pm1.3_A$	$30.7\pm4.4_B$		
	Hesperidin	$63.1\pm8.6_A$	$8.0\pm0.2_B$		
	Total_ mono	$174.4\pm9.0_A$	$51.3\pm4.5_B$		

Values represent means ± standard deviations. Statistical comparison between aqueous and methanolic extracts was analysed with the student t-test and significant differences ($P<0.05$) are indicated with upper case letters in subscript. Means in a row (MeOH vs Aqueous) followed by the same letter (lower and upper case) do not differ significantly, if letters differ then $P<0.05$. Abbreviations: MeOH – methanol; Aq – aqueous; TP- total polyphenol content; FLAVA - flavanol/proanthocyanidins; Total_mono – total monomeric polyphenols. *Camellia sinensis* - green tea: EGCG - epigallocatechin gallate; *Aspalathus linearis* – rooibos: DHC – dihydrochalchones; Asp_notho -aspalathin and nothofagin; *Cyclopia* spp- Honeybush species: Mang_isomang – mangiferin and iso-mangiferin (Shaded area: Data obtained from chemical characterization of extracts as described in Chapter 3).

against cell proliferation, contained high levels of TP with the dihydrochalcones (DHC) constituting the major component of the total monomeric polyphenolic compounds (ca 70%). However, as rooibos contained relatively low levels of flavanol/proanthocyanidins, the TP to flavanol/proanthocyanidin ratio was markedly higher when compared to green tea. The flavanol/proanthocyanidins, the DHCs as well as other monomeric polyphenols were significantly decreased in the aqueous extracts.

In contrast, the strong inhibitory activity of honeybush aqueous extracts against proliferation was associated with far lower levels of TP and total monomeric polyphenols with methanol extracts containing the highest levels of xanthones and flavanones. The polyphenolic constituent also significantly decreased in the aqueous extracts of the respective honeybush species. However, for rooibos, the flavanol/proanthocyanidins increased in the aqueous extracts especially when considering the flavanone-rich species (*C. intermedia and C. subternata*) resulting in a decreased TP to flavanol/proanthocyanidin ratio. A high TP to flavanol/proanthocyanidin ratio, as shown in the methanol extract is associated with decreased activity against cell proliferation and apoptosis.

4. Discussion

Mitochondrial dysfunction resulting from genetic mutations in the respiratory chain is one of the factors that contribute to cancer development (Tsuneoka et al., 2009; Chen et al., 2010; Verrax et al., 2011). Mitochondrial defects in the respiratory chain causes dysfunction in oxidative phosphorylation which results in reduced ATP synthesis. This forces cancer cells into aerobic glycolysis for energy production, leading to increased ROS production, the resulting oxidative stress alters growth responses and causes mutations that favour tumor growth (Tsuneoka et al., 2009). Since cancer cells are postulated to be more susceptible to oxidative stress, their altered energy metabolism has been identified as a primary target for preferential killing during chemoprevention. Subsequently, several *in vitro* studies have indicated the ability of natural and synthetic compounds to selectively kill cancer cells by enhancing mitochondrial dysfunction causing oxidative stress that leads to cell cycle

arrest and apoptosis. (Armstrong et al., 2001; Cariati et al., 2003; Neuzil et al., 2001, 2007; Byun et al., 2008; Chen et al., 2010a).

Unfermented extracts of rooibos and the different honeybush species have been shown to reduce cell viability by decreasing ATP content in skin cells and this implicated induction of mitochondrial dysfunction (Chapter 3). Since mitochondrial dysfunction is known to suppress the growth of cancer cells through cell cycle arrest and apoptosis (Tsuneoka et al., 2009), the anti-proliferative and pro-apoptotic effects of the herbal tea extracts in relation to ATP inhibition was of interest. As the reduction of ATP in cells can be associated with either apoptotic and/or necrotic cell death (Eguchi et al., 1997; Leist et al., 1997), clarity on the mode of cell death associated with reduction of ATP content by extracts was essential. In this regard, necrosis is considered as an inappropriate mechanism of cell death for chemoprevention studies as it elicits an inflammatory response (Ahmad et al., 1997; Elmore, 2007).

Dysregulation of machinery involved in oxidative phosphorylation and inhibition of ATP formation has been the target of various chemopreventive and chemotherapeutic agents for the suppression of tumor growth (Tsuneoka et al., 2009). However, the focus for cancer management has been on partial dysregulation of mitochondrial function in cancer cells as complete ATP inhibition has been associated with cytototoxic effects that would also impede on the growth of normal cells. Therefore, the aim of this study was to evaluate modulatory activity of herbal teas against cell proliferation and apoptosis in skin cells and to determine whether this activity is associated with selective toxicity against cancer cells.

The different tea and herbal tea extracts inhibited the proliferation of skin cells at lower concentrations than those required for ATP inhibition (IC_{50} values previously demonstrated in Chapter 3). This indicated that reduction of cell proliferation by tea/herbal tea extracts does not result from complete ATP loss but may result from other mechanisms that involve induction of cell cycle arrest. Apoptotic activity of the extracts was achieved at levels equalling ATP IC_{50} (values generated in Chapter 3), implying that reduction of ATP production by extracts results in apoptosis and not necrosis and that the apoptotic activity of the extracts is an ATP-dependent

mechanism. The highest activity against cell proliferation and apoptosis was exhibited by the extracts of green tea and rooibos.

Green tea extracts selectively inhibited the proliferation of the HaCaT and cancer cell line without adversely affecting normal cells. However, the pro-apoptotic activity of green tea extracts was not selective for the cancer cell line as it was more prominent in normal cell line. In contrast, the HaCaT cells were the most sensitive cell line to the pro-apoptotic activity of the extracts. The HaCaT cell line utilised in the present study is regarded as a premalignant cell model due to a p53 mutation and other features of premalignant and cancer cells (Adhami et al., 2003; Han et al., 2011). Thus these results showed that the green tea extracts target premalignant cancer cells and this corroborates with available literature on chemopreventive properties of this tea involving pro-apoptotic activity against damaged cells. The poor response exhibited by the cancer cell line to the pro-apoptotic effects of the green tea extracts in this study, may be due to the unique metabolic phenotype of cancer cells effecting aerobic glycolysis that is associated with a state of apoptotic resistance (Bonnet et al., 2007).

The methanol extract of green tea containing high levels of polyphenols exhibited higher activity than the aqueous extract which implicated the role of catechins in the cytototoxic effects against the proliferation of skin cells. In this regard, the anti-proliferative activity of the major polyphenolic compound, EGCG, has been demonstrated in skin cells (Ahmad et al., 1997; Nihal et al., 2005). These studies indicated that, at lower concentrations, EGCG selectively inhibits the proliferation of cancer cells by inducing cell cycle arrest at the G_1 and S-phases. At higher concentrations EGCG and other green tea polyphenols, selectively induce apoptosis in cancer cells via the mitochondrial pathway (Nihal et al., 2005; Roy et al., 2009). The proposed mechanisms involve modulation of the Bcl-2 family, release of cytochrome *c*, activation of caspase-3-cascade which leads to cleavage of PARP and subsequent DNA degradation (Roy et al., 2009). Therefore, selective cytostatic and pro-apoptotic activity of green tea extracts against cancer cells and HaCaT cells may also involve the same mechanisms and may be mediated by catechins. However, caffeine, which is one of the major constituents of green tea may also

contribute to the pro-apoptotic effects of green tea extracts as it is known to promote apoptosis of unrepaired keratinocytes (Han et al., 2011).

Rooibos extracts displayed similar effect to green tea against cell proliferation and induction of apoptosis when considering the fold increase and the % caspase-3 activity expressed per viable cells. However, extracts of this herbal tea were more effective than green tea extracts at selectively inhibiting the proliferation of cancer cells. The pro-apoptotic activity of rooibos extracts, similar to green tea extracts, was more prominent in the HaCaT cells, which suggested that this herbal tea may also preferentially target precancerous lesion during skin carcinogenesis. The strong activity exhibited by the methanol extract of rooibos implicated the role of the monomeric polyphenolic compounds in the anti-proliferative and pro-apoptotic activity of the rooibos extracts. The selective anti-proliferative and pro-apoptotic effects against different cancer cell lines have been demonstrated for some of the flavonoids found in rooibos which include luteolin, rutin and vitexin (Kawaii et al., 1999; Seelinger et al., 2008; Cai et al., 2011; Lee et al., 2012). Mechanistic studies indicated that most of these compounds induce cell cycle arrest either at G_0-G_1 phase, G_2/M phase or S-phase while apoptosis is mediated via the mitochondrial pathway (Lee et al., 2012). In the present study flow cytometric analysis indicated that the rooibos extract arrest cell cycle progression of normal cells at the G_0-G_1. Therefore, the mechanisms underlying the anti-proliferative activity of rooibos extracts in the different skin cells are likely to involve the delay of cell cycle progression at G_0-G_1 while apoptosis may be mediated by the mitochondrial pathway. The latter is evident from flow cytometric analyses in normal cells exposed to the methanol extract which showed induction of caspase-3 activity and membrane depolarisation (JC-1) associated with the release of cytochrome *c*, as well as DNA fragmentation associated with the late stages of apoptosis in treated cells (Ziegler and Groscurth, 2004; Elmore, 2007). Since membrane depolarisation and apoptosis was effected at a concentration equalling the IC_{50} concentration for ATP inhibition, this provided evidence that reduction of cell viability by rooibos results in mitochondrial dysfunction that leads to cell cycle arrest arresting cells in the G_1/G_0 phase at lower concentration whilst inducing apoptosis at higher concentrations.

Apoptotic activity was confirmed with Hoechst stain which indicated numerous morphological changes associated with early and late stages of apoptosis in cells.

In the previous study, the reduction of skin cell viability induced by rooibos was attributed to both the monomeric rooibos flavonoids and polymeric proanthocyanidins (Chapter 3). The latter was proposed to play a more important role in the lipid environment while the monomeric flavonoids are likely to intercalate in the lipid/aqueous interphase (Snijman et al., 2009). It would appear that these compounds also play an important role in the reduction of cell proliferation and apoptosis in skin cells. However, specific interactions between reactive constituents seem to play a role in the cytotoxic effects of rooibos in skin cells. This is deduced from the fact that the methanol and aqueous extract exhibited a similar ratio between TP content and flavanol/proanthocyanidin content but the methanol extract displayed a higher activity. Therefore, interesting interactive dynamics and specific rooibos flavonoid to flavanol/proanthocyanidin ratios seem to exist when utilising complex mixtures. The reactive constituents of the methanol extract appear to exhibit synergistic or additive effects and their reduction in the aqueous extract seem to alter these interactive dynamics. There is also a possibility that the water-soluble constituents may provide a protective effect.

Of the honeybush species, unlike green tea and rooibos, the aqueous extracts exhibited a higher inhibitory effect against the proliferation of the skin cells than the methanol extracts. Of the different honeybush species, only the extracts of *C. genistoides*, selectively inhibited the proliferation of cancer cells with aqueous extracts being more effective than the methanol extracts. In contrast, the aqueous extracts of *C. intermedia* and *C. subternata* induced apoptotic activity that was more prominent in HaCaT cells. This suggested that the aqueous extracts of honeybush (*C. intermedia* and *C. subternata*) may also target premalignant cells during skin carcinogenesis. On the other hand, the methanol extracts of the honeybush species exhibited a far lower apoptotic effect in the different skin cells. Although dose response effects were noticed, no effect was noticed in cancer cells when considering *C. intermedia* when expressed as a % of viable cells. As cancer cells are known to have a pro-oxidative status (Gibellini et al., 2010), it is likely that the reduced and/or lack of effect, could be related to a protective effect, possibly related

to a reduction of oxidative stress resulting from the antioxidant properties of xanthones and flavanone. In this regard, C subternata, containing lower levels of these polyphenols, exhibited a lower protective effect against the induction of apoptosis. It was previously suggested that the monomeric polyphenols of honeybush such as mangiferin and hesperidin may confer a protective activity against oxidative stress in skin cells which may be reduced in the aqueous phase due the poor extraction of these compounds (Chapter 3). This was further emphasised by the higher cytotoxic effects displayed by the aqueous extracts in this study. Since the aqueous extracts contain higher flavanol/proanthocyanidin content (Chapter 3), the anti-proliferative activity and pro-apoptotic activity of honeybush species in skin cells are likely to be mediated by flavanol-like proanthocyanidins, as previously suggested in Chapter 3. The underlying mechanisms in the anti-proliferative and pro-apoptotic activity of the honeybush species, although the less active, may involve similar mechanisms to those described for green tea and rooibos. However, contrary to green tea and rooibos, the cytotoxic effect of honeybush species seems to be dependent on a specific TP to flavanol/proanthocyanidin ratio which is reduced in the aqueous extracts, due to the flavanol/proanthocyanidin content. In this regard, proanthocyanidins from different plant origins have been shown to inhibit the proliferation of different carcinoma cells, emphasizing their possible role as chemopreventive agents (Agarwal et al., 2007; Kresty et al., 2008). On the other hand, the interactive dynamic of the reactive constituent in the methanol extracts of honeybush seem to be associated with protective effects which are then reduced in the aqueous extracts.

In summary the anti-proliferative activity of the herbal teas does not seem to result from complete ATP loss, as inhibition of cell proliferation was effected at far lower concentrations than those reported for reduction of cell viability (Chapter 3). It would appear that cell growth is modulated at lower herbal extract concentrations possibly via mechanisms that involve delay of cell cycle progression at certain checkpoints. Premalignant keratinocytes (HaCaT) are sensitised to the induction of apoptosis by the rooibos and honeybush extracts, which and may therefore target precancerous lesions during skin carcinogenesis. However, extracts of rooibos and *C. genistoides*

may also be effective in delaying the progression of cancer cells into malignant tumors as these herbal teas selectively induce cytostatic effects in cancer cells.

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Chapter 5

Interleukin-1α induction in human keratinocytes (HaCaT): an *in vitro* model for chemoprevention in skin

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Interleukin-1α induction in human keratinocytes (HaCaT): an *in vitro* model for chemoprevention in skin

ABSTRACT

Long-term exposure to UV irradiation and toxic chemicals is associated with chronic inflammation that contributes to skin cancer development with interleukin-1alpha (IL-1α), constitutively produced by keratinocytes, playing a pivotal role in skin inflammation. The aim of this study was to investigate the modulation of IL-1α production in the HaCaT keratinocyte cell line. Phorbol 12-myristate 13-acetate failed to induce IL-1α in HaCaT cells and this might be associated with the specific deficiency known to affect down-stream signalling of the MEK/ERK pathway in these cells. The calcium ionophore, ionomycin, slightly enhanced the production of intracellular (icIL-1α) but this resulted in a necrotic release at higher concentrations. UVB exposure significantly increased the production of icIL-1α in a dose dependent manner with a maximal induction exhibited at 24 h with minimal necrotic and apoptotic effects. Validation of the HaCaT cell model indicated that the non-steroidal anti-inflammatory drug (NSAID), ibuprofen, and the glucocorticoid, dexamethasone, inhibited icIL-1α production and this was associated with a slight inhibition of cell viability. The UVB induced keratinocyte cell model provides an *in vitro* system that could, apart from phorbol ester-like compounds, be utilised as a screening assay in identifying skin irritants and/or therapeutic topical agents via the modulation of IL-1α production.

1. Introduction

Inflammation is a physiological response that protects the body against various insults, such as physical injury, pathogens, exposure to toxic chemicals and UV irradiation. An acute inflammatory response has a therapeutic consequence when manifested over a short period of time, however, prolonged inflammation can lead to cancer development (Ohshima et al., 2003; Mueller, 2006; Suter et al., 2009). Various inflammatory mediators are considered to be key role players in the development of an acute and chronic response with IL-1 and TNF-α being the primary cytokines propagating this process (Feghali and Wright, 1997; Dinarello, 2006; Kundu and Surh, 2008). IL-1 and TNF-α initiate a signalling cascade that induces the gene expression and production of secondary mediators, which include cytokines and chemokines, growth factors, adhesion molecules, cyclo-oxygenase type 2 (COX-2), inducible nitric oxide synthase (iNOs) and other pro-inflammatory factors. This facilitates the chemo-attraction of reactive oxygen species (ROS)-producing immune cells which aid in the repair of damaged tissue (Galley and Webster, 1996; Williams and Kupper, 1996). Studies have linked the role of IL-1 and TNF-α to the different stages of tumorigenesis with the ROS produced by immune cells mainly functioning as chemical effectors in malignant transformation (Apte and Voronov, 2002; Apte et al., 2006). The chronic activation of the other secondary inflammatory mediators functions in promoting the proliferation and survival of malignant cells and also contributes to their progression as invasive tumor cells. The link between chronic inflammation and carcinogenesis indicates that opportunities exist where modulation of the primary cytokine production can be used in the development of new anti-inflammatory drugs that can be utilised as possible chemoprevention agents (Aggarwal et al., 2006).

Keratinocytes, the main epidermal cell, function as a major contributor to cytokine production (Grone, 2002). Of the primary cytokines, IL-1α predominates, as it is constitutively synthesised as a biologically active precursor protein (proIL-1α) while IL-1β exist in an inactive precursor form (Mizutani and Kupper, 1991). In contrast, TNF-α, which is inducible upon exposure to stimuli occurs at very low levels in cultured keratinocytes and seems to play a lesser role regarding inflammation *in vivo* when compared to IL-1α (Köck et al., 1990; Newby et al., 2000; Murphy et al., 2000).

IL-1α remains cell-associated in keratinocytes and its biological activity, which involves wound healing and leukocyte recruitment, is regulated through its cell expressed surface receptors. Type 1 receptor is responsible for transducing pro-inflammatory signalling while type 2 antagonises these effects and is often regarded as a “decoy” receptor (Sauder et al., 1990; Dinarello, 1997; Palmer et al., 2007). IL-1α production in keratinocytes is increased by various stimuli which include phorbol esters, UVB irradiation and ionomycin (Kupper et al., 1987; Lee et al., 1994a; Kavita and Mizel, 1995; Luo et al., 2004). Since preformed IL-1α is only passively released during cell injury various studies have utilised this cytokine in inflammatory and carcinogenesis models as a means to investigate the molecular mechanisms involved in the progression of inflammatory skin disease and cancer promotion (Lee et al., 1994b; Li et al., 2002; Hobbs and Watt, 2003). The modulation of IL-1α has also been identified as a useful screening tool for cell irritants and there is growing interest on the use of this cytokine in the development of anti-inflammatory drugs that can also function as chemopreventative agents (Wilmer et al., 1994; Zhang et al., 1994; Katiyar and Mukhtar, 1997; Van Och et al., 2004; Shitnikind et al., 2006).

Although the use of IL-1α in *in vivo* models is more physiologically relevant for humans, ethical issues and the laborious procedures involved in the use of animals have seen cultured keratinocytes become the prototype model for skin toxicity and chemoprevention studies (Corsini et al., 1998; Suh et al., 2001;Welss et al., 2004; Chow et al., 2008). In this regard, the use of transformed keratinocytes, particularly the immortalised HaCaT cell line, has been considered as a valid and easy to operate substitute for primary cultures (Bonifas et al., 2010). The HaCaT cell line has been widely used in various studies related to irritancy and drug development in skin (Van Och et al., 2004; Shitnikind et al., 2006; Wu et al., 2009). However, recent studies have cautioned against their appropriateness in pharmacological and/or toxicity screening assays, especially when considering phorbol ester-like compound. It has been suggested that a detailed characterization of these cells should be conducted before their application as an *in vitro* model (Ridd et al., 2010; Pastore et al., 2011).

The current study sought the suitability of different stimulants, including PMA, ionomycin, a calcium ionophore and UVB on IL-1α induction utilising the

immortalised transformed keratinocytic HaCaT cell line. The induction of IL-1α was verified by the use of known anti-inflammatory drugs in order to validate the *in vitro* cell model to be utilised as a screening assay in identifying skin irritants and/or therapeutic topical agents.

2. Materials and methods

2.1 Chemicals and reagents

Phorbol 12-myristate 13-acetate (PMA), dexamethasone, ibuprofen, dimethyl sulfoxide (DMSO), Bovine serum albumin (BSA) (Sigma-Aldrich, USA). Fetal bovine serum (FBS) (Invitrogen, USA). RPMI-1640, Dulbeco's phosphate buffered saline (DPBS), L-glutamine, heat inactivated fetal bovine serum, trypsin-versene, Hank's buffered salt solution (HBSS) were obtained from (Lonza, Belgium). Human recombinant IL-1α ELISA kit (R&D systems, USA), CytoTox 96® Non-Radioactive cytotoxicity assay, CellTiter-Glo luminescent cell viability, Caspase-3/7 assay (Promega, USA). Triton-x100 (BDH chemical Ltd, Poole England), Tween®-20 (ICN Biomedicals Inc, USA) and, ionomycin calcium salt (Synexa, South Africa).

2.2 *Keratinocyte cell culture*

Spontaneously immortalised keratinocytes (HaCaT) were a gift from the Department of Human Biology at the University of Cape Town, South Africa. The cells were cultured in RPMI-1640 supplemented with heat-inactivated fetal bovine serum (10%), L-glutamine (2 mM) in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were passaged every 3 days at a 1:3 split ratio and p70-to p80 was used in experiments

2.3 Effect of PMA on the induction IL-1α

Determination of optimal cell density and incubation time. HaCaT cells were seeded in RPMI-1640 media containing 10% FBS (100 µL) at a density of $1.5x10^4$ in a 96-well microtiter plate (Corning Costar, New York) and incubated at 37°C for 24 h to reach 70-80% confluency. Fresh RPMI-1640 medium containing 0.5% FBS and different PMA concentrations (0.31-100 ng/mL) was used. PMA was dissolved in DMSO giving a final concentration of <0.1% and plates were incubated for 6, 12 and

24 h, the supernatants decanted and the cells were washed with PBS (3x). Cells were lysed with a combination of 0.5% triton in PBS and one cycle of freeze (-80°C) thawing. Cell lysates were stored at -20°C for intracellular IL-1α determination.

2.4 Extracellular (exIL-1α) and intracellular IL-1α (icIL-α) induction by PMA and ionomycin.

Cells were seeded at a higher density ($3x10^4$) to intensify the IL-1α signal. Cells were treated either with higher concentration of PMA (6.25-100 ng/mL) or with ionomycin (0.2-10 µg/mL) and incubated for 6 h. Following incubation, supernatants were collected and stored at -20°C for exIL-1α determination. The cells were washed with PBS (3x) and lysed with 0.5% triton-x100 combined with one freeze-thawing cycle. Cell lysates were resuspended and kept at -20°C for the determination of icIL-1α production.

IL-1 α determination. Both icIL-1α and exIL-1α in cell lysates and supernatants, respectively, were determined with an IL-1α ELISA kit according to the manufacturer's instruction. Standards were prepared and assayed in duplicates while five replicates were prepared for each sample. Absorbance was measured at 450 nm with a Dynex plate reader (Dynex technologies, USA) and data were analysed using a standard curve generated from GraphPad prism (version 5 for windows) (GraphPad software Inc, La jolla, CA). Values were expressed as pg/mL of the supernatant or cell lysate.

Cytotoxicity and viability assays (LDH release and ATP production). Cytotoxicity of the tested chemicals was assessed either by monitoring LDH release or both LDH release and ATP production when determining the effect of PMA and ionomycin, respectively. For LDH release, samples were analysed utilising the colorimetric CytoTox 96® kit and absorbance was measured at 490 nm (Dynex microplate reader). LDH release was expressed as a percentage of the total LDH released (cells were lysed by one freeze thawing-cycle) based on the following calculation:

$$\% \text{ Cytotoxicity} = \text{Absorbance}_{(\text{supernatant})} / \text{Absorbance}_{(\text{total LDH activity})} \times 100$$

For determining cell viability, the CellTiter-Glo Luminescent viability kit was used to monitor ATP production in cells utilising white solid plates (Porvair Sciences, Shepperton, UK). To monitor ATP production, the luciferase reagent was added and plates rotated for 2 min and incubated at room temperature for 10 min in the dark. ATP production was monitored with the Veritas^{TM} microplate luminometer (Promega, USA). The luminescent signal was measured in relative light units (RLU) and data expressed as a percentage (%) of the control cells as follows:

$$\% \text{ ATP production} = RLU_{\text{treated cells}} / RLU_{\text{control}} \times 100$$

2.5 UVB-induced IL-1α production, cytotoxicity and apoptosis

Cells were seeded in 96-well tissue culture plate at a density of $3x10^4$ in media (100 µL) containing 10% FBS and incubated for 24 h. After removing the cultured media, cells were exposed to different doses (20, 40, 80, 160, 240 mJ/cm^2) of UVB light in DPBS (100 µL) without the plastic lid. The UVIlink UV crosslinker (UVitek limited, UK) was fitted with six 8 Watt UV tubes with a wavelength of 302 nm (Vilber Lourmat, France). Immediately after irradiation, treated cells were supplemented with fresh RPMI-1640 medium containing 0.5% FBS and incubated for different time periods (6, 12 and 24 h).

Cytotoxicity, apoptosis and IL-1α were determined after 6, 12 and 24 h. Cytotoxicity was monitored by determining LDH release as described above. For apoptosis determination, cells were lysed with a cell lysis buffer (20 µL) in combination with one freeze-thaw cycle. Cell lysates were transferred (25 µL) into a white solid plate and incubated with the caspase 3/7 reagent (25 µL) for 1 h in the dark at room temperature. Following incubation, plates were analysed in a Veritas microplate luminometer and caspase-3 activity was calculated as a fold increase compared to the control.

2.6 Inflammatory model validation utilising anti-inflammatory drugs

Cells were seeded at a density of $3x10^4$ incubated for 24 h in RPMI-1640 medium containing 10% FBS. Cells were first exposed to UVB light (80 mJ/cm^2) in DPBS (100 µL) as described above and then incubated with varying dexamethasone and

ibuprofen concentrations (0.31 to 1.25 mM) for 24 h in RPMI-1640 media containing 0.5% FBS. After the removal of the supernatants, cells were washed with DPBS and lysed with 0.5% triton combined with one cycle of freeze-thawing. Cell lysates prepared in different plates were analysed for icIL-1α and ATP production whilst the supernatant was used for LDH release as describe above.

2.7 Statistical analysis

All parameters were tested for normality using the Kolmogorov-Smirnof Test. The homogeneity of group variances for all parametric parameters were tested using Levene's Test. Group differences for these parametric parameters were then tested using One-way ANOVA's (GLM in SAS) and post-hoc Tukey Tests, which are post hoc multiple pairwise comparisons between the means of all the different groups. For those parameters with two or more fixed effects, interaction terms were also investigated to see if they improved model fit indicating that the interaction of main effects influences the outcome. For ibuprofen, data on cytotoxicity the original variables had non-parametric distributions, but were individually transformed (inverse square root) to become parametric. Least Squares Means (LS Means) were used to estimate group differences and included 95% confidence intervals for the effects and differences. The Tukey-Kramer adjustment was made automatically if the data were unbalanced. For non-parametric parameters, significant group differences were investigated using the Kruskal-Wallis test, as well as the post-hoc Tukey-type test to ascertain which groups differed from which. For parametric comparisons when only two groups were compared, the T-tests were used. Statistical analyses were performed with SAS v9.2 and statistical significance was considered at 5% ($p<0.05$).

3. Results

3.1 IL-1α induction by PMA

PMA is known to increase the *in vitro* production of intracellular IL-1α in keratinocytes at non-cytotoxic concentrations (Kupper et al., 1987) however, recent studies have reported PMA specific defects in HaCaT cells but the effect has not been demonstrated for IL-1α production (Sudbeck et al., 1999; Ridd et al., 2010).

The current study investigated the modulating effect of PMA on IL-1α induction and release, as a function of varying concentrations and time of incubation. A significant reduction in icIL-1α production was noticed as a function of time with the lowest level recorded at 24 h (Table 5.1). However, PMA did not significantly increase intracellular IL-1α (icIL-1α) production at any of the concentrations and different time points. PMA also neither induced extracellular IL-1α (exIL-1α) release nor did it exhibit any cytotoxic effects (Table 5.2).

3.2 Effect of ionomycin on IL-1α production and cytotoxicity

The ability of ionomycin to induce *de novo* synthesis and release of IL-1α at subcytotoxic concentrations has been demonstrated in normal keratinocytes (Köck et al., 1990). The calcium ionophore facilitates the processing and secretion of mature IL-1α in monocytes and macrophages (Kobayashi et al., 1990; Matsushima and Oppenheim, 1985). The current study sought to characterise its effect on the induction and release of icIL-1α as a function of cell viability in HaCaT cells. Ionomycin (0.2-10 µg/mL) treatment increased icIL-1α in a dose dependent manner with a two-fold increase at 1.25 µg/mL (Fig. 5.1A). At higher ionomycin concentrations (5 and 10µg/mL), the level of exIL-1α was significantly increased but this was associated with a significant ($P<0.05$) cytotoxic effect related to a reduction in cell viability (ATP production) and an increase in cytotoxicity (LDH release) (Fig. 5.1B).

3.3 Effects of UVB exposure in keratinocytes

Dose and time effects of UVB exposure on IL-1α production as a function of different cell viability parameters. Intracellular IL-1α production differed significantly ($P<0.05$) between the incubation periods, with highest level obtained after 6 h while it decreased after 12 and 24 h (Table 5.3). Extracellular IL-1α (exIL-1α) followed the same trend although the level was very low when compared to that of icIL-1α. When considering the different UVB treatments at 6 h, icIL-1α levels were unaffected at the lowest dose (20 mJ/cm^2), while induction was effected from 40 mJ/cm^2 onwards with the highest levels observed in cells exposed to 80 mJ/cm^2.

Table 5.1. Effect of different incubation times and PMA concentrations on the intra cellular production of IL-1α.

PMA conc (ng/ml)	Ctrl	6.25	12.5	25	50	100
	IL-1α concentration (pg/ml)					
6 h	$168.85\pm32.57^{a}{}_{A}$	$132.75\pm6.07^{a}{}_{A}$	$203.97\pm8.97^{a}{}_{A}$	$180.16\pm12.27^{a}{}_{A}$	$189.38\pm16.36^{a}{}_{A}$	$162.66\pm27.76^{a}{}_{A}$
12 h	$112.59\pm0.83^{a}{}_{B}$	$60.64\pm10.49^{b}{}_{B}$	$71.22\pm8.99^{ab}{}_{B}$	$86.85\pm11.71^{ab}{}_{B}$	$75.06\pm9.90^{ab}{}_{B}$	$73.73\pm4.07^{ab}{}_{B}$
24 h	$15.69\pm1.15^{a}{}_{B}$	$9.61\pm0.48^{a}{}_{C}$	$10.29\pm0.66^{a}{}_{C}$	$9.82\pm2.48^{a}{}_{C}$	$9.15\pm1.38^{a}{}_{C}$	$9.61\pm0.37^{a}{}_{C}$

Cells were seeded at a density of $1.5x10^{4}$. Values represent the mean ± standard deviation of four replicates. Interactions were investigated to see if they improved model fit indicating that the interaction of main effects influences the outcome. Least Squares Means (LS Means) were used to estimate group differences and included 95% confidence intervals for the effects and differences. The Tukey-Kramer adjustment was made automatically if the data were unbalanced. Different lower case letters in superscript indicate significant differences (in a row) between control and PMA concentrations. Different upper case letters in subscript indicate significant difference (in a column) between incubation periods. Values were considered significant when $P<0.05$. If the letters were similar then means did not differ statistically.

Table 5.2. Effect of PMA on the intracellular and extracellular IL-1α production and determination of cytotoxic effects

PMA conc (ng/ml)	Ctrl	6.25	12.5	25	50	100
	IL-1α concentration (pg/ml)					
Intracellular (pg/ml)	$256.30\pm26.24^{a}{}_{A}$	$266.71\pm25.06^{a}{}_{A}$	$223.29\pm82.14^{a}{}_{A}$	$200.91\pm69.97^{a}{}_{A}$	$234.73\pm31.16^{a}{}_{A}$	$233.17\pm36.38^{a}{}_{A}$
Extracellular (pg/ml)	$1.74\pm0.23^{a}{}_{B}$	$2.60\pm0.87^{a}{}_{B}$	$1.78\pm0.69^{a}{}_{B}$	$2.28\pm0.81^{a}{}_{B}$	$1.86\pm0.50^{a}{}_{B}$	$1.93\pm0.40^{a}{}_{B}$
%Toxicity	7.12 ± 0.93^{a}	7.90 ± 1.65^{a}	6.91 ± 0.68^{a}	9.48 ± 2.67^{a}	6.52 ± 0.65^{a}	7.22 ± 0.69^{a}

Cells were seeded at a density of 30 000 cells and incubated for 6hrs. PMA- phorbol 12-myristate 13-acetate; Values represent the mean±standard deviation of 6 replications. Interactions were investigated to see if they improved model fit indicating that the interaction of main effects influences the outcome. Least Squares Means (LS Means) were used to estimate group differences and included 95% confidence intervals for the effects and differences. The Tukey-Kramer adjustment was made automatically if the data were unbalanced. Different lower case letters in superscript indicate significant differences (in a row) between control and PMA concentrations. Different upper case letters in subscript indicate significant difference (in a column) between incubation periods. Values were considered significant when $P<0.05$. If the letters were similar then means did not differ statistically. % toxicity represents LDH released in the supernatant as a percentage of the total lysed cells.

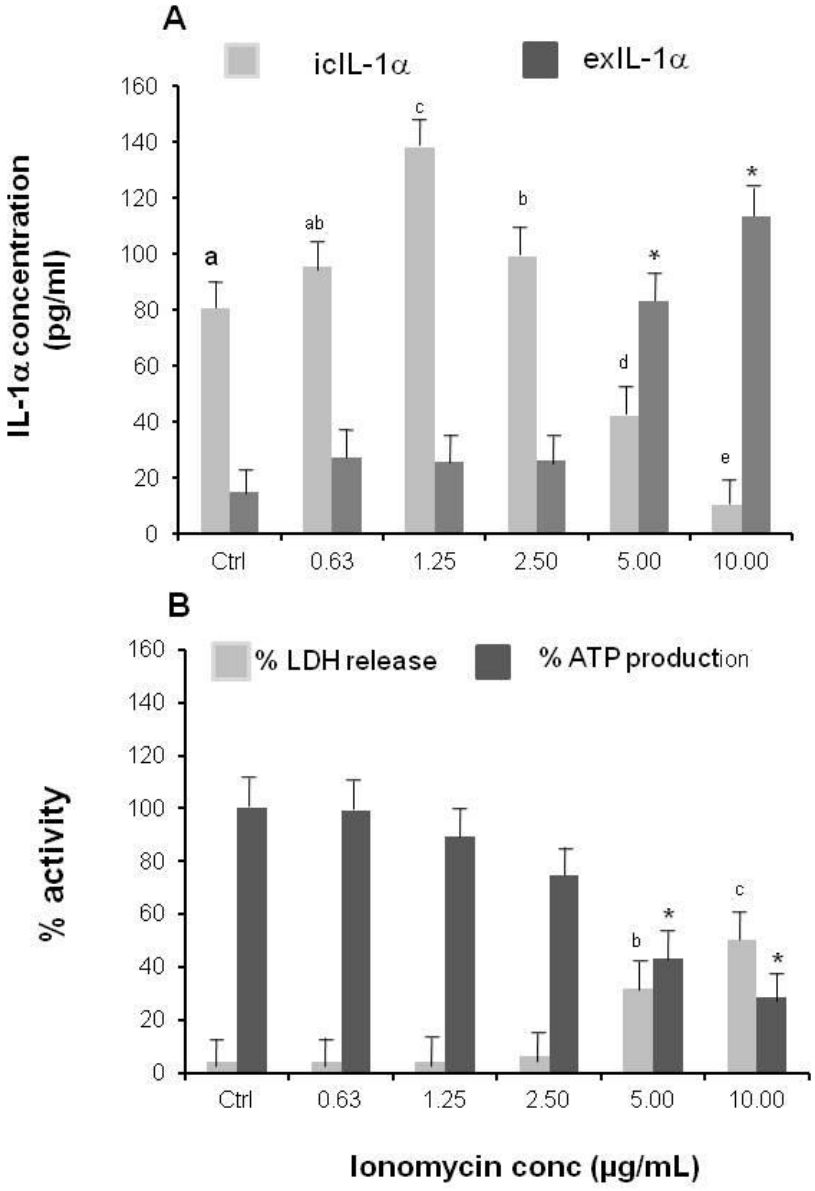

Fig 5.1. Effect of ionomycin on interleukin-1 alpha concentration in the intracellular (icIL-1α) and extracellular (exIL-1α) environment utilising HaCaT (Fig.5.1A). Effect on cytotoxicity and cell viability monitored by the % LDH release and the % ATP production, respectively (Fig. 5.1B). Values are the means ± SD. (n=4). Least Squares Means (LS Means) were used to estimate group differences and included 95% confidence intervals for the effects and differences. The Tukey-Kramer adjustment was made automatically if the data were unbalanced. Different lower case letters indicate significant differences between the concentration for icIL-1α (Fig 5.1A) and % LDH release (Fig 5.1B). *indicates significant difference between concentration and control for exIL-1α (Fig 5.1A) and % ATP production (Fig 5.1B). Abbreviations: LDH-lactate dehydrogenase, ATP- adenosine triphosphate

The icIL-1α levels decreased significantly at the two highest doses but levels were still significantly higher than the untreated control. Extracellular IL-1α levels did not exhibit response to UVB when compared to the unexposed cells.

.After 12 h, the lowest two UVB dosages had no significant effect on icIL-1α levels, while cells exposed to 80 mJ/cm^2 effected the highest icIL-1 production. However, at the two highest UVB doses the induction was significantly ($P<0.05$) reduced in a dose dependant manner reaching levels that were similar to the untreated control cells. In general the icIL-1α levels significantly decreased when compared to the 6 h levels. Regarding exIL-1α, there was a marked increase in the levels from 40 to 240 mJ/cm^2 which became significant ($P<0.05$) at the two highest doses.

After 24 h, cells exhibited a similar pattern to that observed at 12 h when considering the induction of icIL-1α. In contrast to the earlier time points, the 160 mJ/cm^2 dose yielded similar results to 80mJ/cm^2 exposure treatment. However, at 240 mJ/cm^2, icIL-1α was significantly reduced to levels that were similar to the untreated control cells. This reduction co-incided with a significant ($P<0.05$) increase in the exIL-1α levels similar to the 12 h time point. Of interest was the fold increase of icIL-1α in cells exposed to 80 mJ/cm^2 and 160 mJ/cm^2 which was markedly higher (3-fold) at 24 h when compared to the 6 and 12 h time points (2-fold).

When considering the % LDH release as a function of cytotoxicity, no effect was noticed after 6 h. However, at 12 h, a dose dependent effect became evident as a slight increase in LDH release was observed in the UVB treated cells. At 24 h, an increase in LDH release was effected at 80 mJ/cm^2 with a maximal activity observed at the two highest doses (160 mJ/cm^2 and 240 mJ/cm^2). Comparison of the cytotoxicity effects between the different incubation periods indicated no significant difference between 6 and 12 h while the highest doses displayed a significant increase in cytotoxicity at 24 h.

The UVB induced caspase-3 activity also varied when considering the different incubation periods and dose of exposure. When considering caspase-3 activity at 6 h, a 2 fold increase was observed from 80 mJ/cm^2 and above.

Table 5.3. UVB induced IL-1α production, cytotoxic and apoptotic effects

Incubation period	Unit of measure	UV dose (mJ/cm^2)					
		Ctrl	20	40	80	160	240
6 h	icIL-1α (pg/ml)	$78.52\pm8.50^{c}_{Aa}$	$65.56\pm10.45^{c}_{Aa}$	$102.16\pm18.78^{b}_{Aa}$	$159.66\pm32.91^{a}_{Aa}$	$113.40\pm28.04^{b}_{Aa}$	$110.96\pm26.28^{b}_{Aa}$
	icIL-1α fold increase	$1.00\pm0.11^{d}_{a}$	$0.83\pm0.13^{d}_{a}$	$1.30\pm0.24^{c}_{a}$	$2.03\pm0.42^{a}_{a}$	$1.44\pm0.36^{b}_{a}$	$1.29\pm0.46^{c}_{a}$
	exIL-1α (pg/ml)	$14.57\pm4.1^{a}_{B}$	$14.72\pm4.37^{a}_{B}$	$17.39\pm4.58^{a}_{B}$	$12.57\pm2.76^{a}_{B}$	$16.21\pm4.44^{a}_{B}$	$18.91\pm5.57^{a}_{B}$
	% LDH release	$8.69\pm1.64^{a}_{a}$	$8.39\pm3.10^{a}_{a}$	$11.55\pm2.79^{a}_{a}$	$11.12\pm3.69^{a}_{a}$	$11.90\pm1.58^{a}_{a}$	$11.82\pm2.25^{a}_{a}$
	Caspase-3 (fold increase)	$1.00\pm0.06^{c}_{a}$	$1.08\pm0.08^{bc}_{a}$	$1.54\pm0.34^{b}_{ab}$	$2.55\pm0.28^{a}_{a}$	$2.96\pm0.55^{a}_{a}$	$2.56\pm0.12^{a}_{a}$
12 h	icIL-1α (pg/ml)	$54.69\pm10.50^{c}_{Aa}$	$43.18\pm11.67^{c}_{Aa}$	$37.81\pm12.01^{c}_{Ab}$	$148.36\pm38.69^{a}_{Aa}$	$104.12\pm23.10^{b}_{Aa}$	$51.27\pm12.49^{c}_{Ab}$
	icIL-1α fold increase	$1.00\pm0.19^{d}_{a}$	$0.79\pm0.21^{d}_{a}$	$0.69\pm0.22^{d}_{a}$	$2.71\pm0.71^{a}_{b}$	$1.90\pm0.42^{b}_{b}$	$0.94\pm0.23^{c}_{a}$
	exIL-1α (pg/ml)	$6.30\pm1.64^{b}_{B}$	$6.97\pm1.35^{b}_{B}$	$10.32\pm4.72^{b}_{B}$	$9.85\pm2.71^{b}_{B}$	$9.04\pm1.71^{a}_{B}$	$11.14\pm1.56^{a}_{B}$
	% LDH release	$6.59\pm2.51^{b}_{a}$	$10.13\pm0.81^{a}_{a}$	$11.33\pm1.33^{a}_{a}$	$12.72\pm2.56^{a}_{a}$	$13.11\pm2.67^{a}_{a}$	$13.93\pm2.48^{a}_{a}$
	Caspase-3 (fold increase)	$1.00\pm0.05^{d}_{a}$	$1.28\pm0.24^{d}_{a}$	2.09 ± 0.68^{c}	$6.86\pm0.64^{b}_{b}$	$12.55\pm0.82^{a}_{b}$	15.32 ± 1.16^{a}
24 h	icIL-1α (pg/ml)	$17.49\pm6.65^{b}_{Ab}$	$18.45\pm3.74^{b}_{Ab}$	$22.115.95^{b}_{Ac}$	$55.01\pm11.61^{a}_{Ab}$	$55.76\pm6.15^{a}_{Ab}$	$38.15\pm9.25^{b}_{Ab}$
	icIL-1α fold increase	$1.03\pm0.39^{c}_{a}$	$1.05\pm0.21^{c}_{a}$	$1.26\pm0.34^{b}_{a}$	$3.14\pm0.66^{b}_{c}$	$3.19\pm0.35^{b}_{c}$	$2.18\pm0.53^{a}_{b}$
	exIL-1α (pg/ml)	$5.89\pm1.89^{b}_{B}$	$6.67\pm2.21^{b}_{B}$	$7.54\pm1.18^{b}_{B}$	$5.29\pm2.19^{b}_{B}$	$13.94\pm3.08^{a}_{B}$	$14.97\pm4.27^{a}_{B}$
	% LDH release	$8.24\pm1.15^{c}_{a}$	$9.27\pm1.49^{c}_{a}$	$11.05\pm1.87^{c}_{a}$	$15.43\pm3.84^{b}_{a}$	$25.71\pm6.26^{a}_{b}$	$25.53\pm4.50^{a}_{b}$
	Caspase-3 (fold increase)	$1.00\pm0.08^{c}_{a}$	$1.42\pm0.11^{b}_{a}$	$1.40\pm0.12^{b}_{a}$	$3.25\pm0.54^{a}_{a}$	$3.92\pm0.46^{a}_{a}$	$3.73\pm0.21^{a}_{b}$

Cells were seeded at a density of 3x10^4/well. Values represent mean±standard deviation of quadruplicates. Least Squares Means (LS Means) were used to estimate group differences and included 95% confidence intervals for the effects and differences. The Tukey-Kramer adjustment was made automatically if the data were unbalanced. Different lower case letters in superscript indicate difference between control and UV dose (mJ/cm^2) (in a row). Different upper case letters in subscript indicates significant difference between intracellular IL-1α (pg/ml) and extracellular IL-1α (pg/ml) (in a column); lower case letters in subscript indicate significant difference between incubation periods (hours) for icIL-1α (pg/ml) (in a column), icIL-1 fold increase, toxicity and capsase-3. Values were considered significant if $P<0.05$. icIL-1α= intracellular interleukin 1α; exIL-1α = extracellular interleukin 1α; LDH=lactate dehydrogenase.

After 12 h, a significant fold increase (2x) was noticed from 40 mJ/cm^2 with a dose response increase of up to 15 fold obtained with the highest dose of 240 mJ/cm^2. The icIL-1α to apoptosis ratio, considering the fold increase, was 1:3 at the 80 mJ/cm^2 dose of exposure. After 24 h the apoptotic fold increase was only observed from 80 mJ/cm^2 and above. The fold increase in apoptosis significantly ($P<0.05$) decreased when compared to the 12 h time point with a icIL-1α to apoptosis ratio of approximately 1:1 similar to that obtained after 6 h.

3.4 Anti-inflammatory activity (dexamethasone and ibuprofen)

To investigate the modulation of the UVB-induced icIL-1α production in HaCaT cells, the inhibitory effect of known anti-inflammatory compounds were used to validate the cell model as screening tool in identifying compounds to be utilised as chemopreventive agents. Dexamethasone inhibited UV-induced icL-1α production after 24 h in a dose-dependent manner while ibuprofen only exhibited the strongest inhibitory effect at the highest concentration used (Table 5.4).

Table 5.4. Effect of dexamethasone and ibuprofen on icIL-1α production, cell viability and cytotoxicity

Dexamethasone conc (mM)	**Ctrl**	**Positive Ctrl**	**1.25**	**0.63**	**0.31**
icIL-1α (pg/ml) (% inhibition)	19.40±2.50$^{c}_{A}$	76.04±7.20$^{a}_{A}$	48.90±9.81$^{b}_{A}$ *40.77±5.40*	67.70±10.40$^{a}_{A}$ *15.41±7.80*	67.60±9.22$^{a}_{A}$ *17.05±12.02*
% ATP production	100.00±3.25$^{a}_{A}$	85.65±6.12$^{b}_{A}$	76.39±6.09$^{b}_{A}$	78.69±4.63$^{b}_{A}$	86.59±3.37$^{b}_{A}$
% Cytotoxicity	3.48±0.83$^{d}_{A}$	8.84±0.96$^{a}_{A}$	5.51±0.41$^{b}_{A}$	5.49±0.92$^{b}_{A}$	4.45±0.71$^{c}_{A}$
Ibuprofen conc (mM)	**Ctrl**	**Positive Ctrl**	**1.25**	**0.63**	**0.31**
icIL-1α (pg/ml) (% inhibition)	19.40±2.50$^{b}_{A}$	76.04±7.20$^{a}_{A}$	20.67±3.69$^{b}_{B}$ *72.89±5.60*	86.84±11.27$^{a}_{B}$ (-)	86.45±12.77$^{a}_{B}$ (-)
% ATP production	100.00±3.80$^{a}_{A}$	90.19±9.38$^{a}_{A}$	64.76±5.89$^{b}_{B}$	84.75±11.99$^{b}_{A}$	88.98±14.01$^{a}_{A}$
% Cytotoxicity*	4.65±0.19$^{c}_{A}$	10.17±0.84$^{a}_{A}$	5.77±0.59$^{b}_{A}$	5.30±0.55$^{bc}_{A}$	4.75±0.72$^{bc}_{A}$

Values presented are the mean ±standard deviations; icIL-1α intracellular interleukin-1alpha. Statistical analyses for a significant group effect included either an ANOVA F-test or the Welch's test, depending on the presence of homogeneity of group variances (Levene's test). If significant group differences are present, the post hoc Tukey's Studentized Range Test was used for between group comparisons at 5% significance level. For ibuprofen data on cytotoxicity the original variables had non-parametric distributions, but were individually transformed* to become parametric. Different lower case letters in superscript indicate significant differences between treatment concentration, positive control and untreated control (in a row). Different upper case letters in subscript indicate significant difference between dexamathesone and ibuprofen treatment concentrations (in a column). Values in italics represent % inhibition of icIL-1α by dexamethasone and ibuprofen; (-) indicates no inhibition; % inhibition calculated from the positive control. Abbreviations: ATP – adenosinetriphosphate. icIL-1α – interleukin-1alpha. Ctrl-control represents cells that have not been exposed to UV-B light; Pos Ctrl-Positive control represents cells exposed to 80mJ/cm^2 of UV-B light.

Ibuprofen (1.25 mM) exhibited the strongest inhibition when compared to dexamethasone with icIL-1α reduced to levels similar to the control. However, the opposite was true at the lower concentrations. The inhibition of icIL-1α by dexamethasone was not associated with a significant decrease in ATP production when compared to the UVB treated cells while the LDH release was significantly lower and comparable to the untreated control keratinocytes. However, the inhibitory effect of ibuprofen was associated with a significant decrease in ATP production when compared to the treated keratinocytes. The LDH release was significantly lower than the UVB treated cells and exhibited levels similar to the untreated cells.

4. Discussion

Cytokine production by keratinocytes has been investigated *in vitro* utilising primary and a variety of keratinocyte cell lines (Ansel et al., 1988; Mizutani and Kupper, 1991; Newby et al., 2000; Grone, 2002; Van Och et al., 2005). These studies have established that IL-1α is produced as a biologically active precursor protein in both mouse and human keratinocytes and that injured human keratinocytes release preformed IL-1α which can be used to predict the effect of external stimuli on the *in vivo* inflammatory processes and skin carcinogenesis (Wilmer et al., 1994; Suh et al., 2001). *In vitro* models showed that the biological activity of IL-1α induced by phorbol esters and UVB, can be modulated by various agents including synthetic and natural compounds (Eberlein-köning and Przybilla, 1998; Shitnikind et al., 2006). Thus indicating that IL-1α, or the modulation thereof, can play a significant role in the treatment of inflammatory disease and chemoprevention in skin. However, data obtained from these *in vitro* keratinocyte models should be interpreted with care as differences exists between normal keratinocytes and transformed cell lines (Ridd et al., 2010; Pastore et al., 2011). Of particular importance is the HaCaT cell line which is widely used as a screening tool in irritancy and chemoprevention studies. This immortalised spontaneously transformed cell line has been reported to have dysregulated molecular mechanisms related to carcinogenesis/chemoprevention. As a result it has been suggested that the characterization of HaCaT cells and other transformed cell lines should be critically evaluated before application as an in *vitro* model in skin toxicity and chemoprevention studies. Thus the present study aimed to

characterize the induction of IL-1α and the resulting effect on cell integrity following exposure to PMA, ionomycin and UVB irradiation.

Although the skin cancer promoter PMA is known to induce the gene expression and IL-1α production in normal keratinocytes (Kupper et al., 1987; Lee et al., 1994a), HaCaT cells have been shown to have a PMA-specific defect that is associated with an impaired mitogen-activated protein kinases (MAPK) down-stream to the MEK/ERK signalling pathway (Matsushima and Oppenheim, 1985; Ridd et al., 2010). However, PMA signalling involved in the induction of IL-α gene expression in mouse keratinocytes has been suggested to occur via protein kinase C and not MAPK (Lee et al., 1994a). In the present study PMA did not induce IL-1α production, suggesting that dysregulated PMA-signalling in HaCaT cells is not only specific to MAPK but also involves other signalling pathways. Therefore, HaCaT cells may not be a useful model to investigate the effect of phorbol ester-like chemicals in skin toxicity and/or on cytokines induction. However, it is known that HaCaT cells differentiate normally (Boukamp et al., 1988) and are known to provide the micro environment relevant for skin sensitisation e.g., by responding to epidermal growth factor, transforming growth factor (TGF)–β1, TGF-α, IL-6, interferon gamma and TNF-α to stimulate the production of IL-1α (Phillips et al., 1995; Bonnifas et al., 2010). The HaCaT cell model was also found to be a suitable *in vitro* model to investigate phase I and phase II epidermal metabolism of chemicals and drugs.

The calcium ionophore, ionomycin, has been reported to initiate the processing of precursor IL-1α into the active cytokine in many cell types via the action of calpain, a calcium-dependent membrane-associated protease (Matsushima and Oppenheim, 1985; Kobayashi et al., 1990). Ionomycin augmented the proteolytic processing of preformed IL-1α and release of mature IL-1α in lipopolypsaccharide (LPS) - activated cell line that constituvely produces human IL-1α. In keratinocytes, however, constitutive icIL-1α is rarely secreted with the full-length pre-cursor form but sub-cytotoxic concentrations of ionomycin have been shown to increase icIL-1α levels associated with the *de novo* synthesis (Newby et al., 2000). On the other hand, the presence of IL-1α at low levels in the extracellular environment, implicates secretion of 17kDa mature protein the processing of which, is facilitated by ionomycin via the calcium dependent calpain proteases (Dinarello, 1996). In the present study,

ionomycin increased the intracellular IL-1α level at lower concentrations and this was accompanied by a slight release into the supernatant, however at higher concentrations there was a cytotoxic release. This is in agreement with other studies indicating that IL-1α is only released under certain severe pathological conditions associated with cell death (Watanabe and Kobayashi, 1994). The uncontrolled and/or necrotic release of IL-1α may not be appropriate for chemoprevention studies concerned with a "controlled" response as the former exacerbates inflammation. Unlike the processed mature IL-1α, icIL-1α protein is non-secretable but during necrosis it "leaks" out to the extracellular environment where it augments the inflammatory activity in cells through receptor signalling (Cohen et al., 2010).

UVB exposure of HaCaT cells resulted in a dose-dependent increase in the level of icIL-1α with the release into the external environment associated with a slight cytotoxic effect at higher dose levels. This is in agreement with a similar study that was conducted in primary keratinocytes where IL-1α was reported to increase intracellularly at non-lethal doses while it was progressively released at lethal doses (>100 mJ/cm^2) due to membrane damage (Cohen et al., 1991). Optimal fold induction of icIL-1α was achieved after 24 h at a UVB dose of 80 mJ/cm^2 with minimal cytotoxicity and a low level of apoptosis when compared to the 12 h time point. Induction of apoptosis by UVB light in HaCaT cells corresponds with another study which provided evidence that UV induced apoptosis proceeds via the intrinsic (mitochondrial) and extrinsic pathways (Takasawa et al., 2005). The extent of apoptosis will depend on the amount of DNA damage induced by UVB and modulation of the latter could have a primary influence on the induction of skin carcinogenesis.

Interleukin-1α is suggested to exhibit a dual function, i.e., intracellular related to the activation of transcriptional machinery with its effect on the progression of inflammation and its extracellular receptor-mediated signal transduction (Werman et al., 2004; Cohen et al., 2010). In UVB exposed keratinocytes, it was shown that IL-1α regulates not only its own production but also increase the production of other pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8 (Chung et al., 1996). Modulation of the intracellular function of IL-1α, therefore, seems to be a useful target in some inflammatory conditions. Inactivation of the biologically active IL-1α, via the

induction of apoptosis for instance could prevent inflammation since it is retained in the chromatin fraction and not released with the cytoplasmic content. This suggests that UV-induced IL-1α production could be a useful model to modulate inflammation thereby disrupting mechanisms related to inflammatory diseases via the modulation of apoptosis.

UVB irradiation is also known to induce the release of natural IL-1 inhibitors, epidermal cell contra IL-1 and the endogenous IL-1 receptor antagonist (IL-1ra) in keratinocytes (Schwarz et al., 1987; Hirao et al., 1996). The release of these molecules is implicated in the regulation of immune and proliferative responses by blocking IL-1 activity. IL-1ra has been studied extensively (Dinarello, 1997) , and its antagonist activity is attributed to a specific ratio between IL-1ra/IL-1α which can be modulated by other cytokines and various agents including UVB irradiation (Corradi et al., 1995; Lew et al., 1995; Phillips et al., 1995). Modulation of the IL-1ra/ IL-1α ratio by UVB is dependent on the dose where low doses are associated with high levels of IL-1ra while higher doses shift the balance towards IL-1α (Mühlberg et al., 2000). An imbalance towards IL-1α is associated with inflammatory disease including skin carcinogenesis (Corradi et al., 1995) as it was shown that the IL-1ra/IL-1a ratio differs between the different stages of carcinogenesis. The highest levels of IL-1ra were found in cells representing the early stages of skin carcinogenesis where it is implicated in anti-tumor activity (La et al., 1999). Modulating the ratio between IL-1ra/IL-1α offers an alternative mechanism that can be explored and incorporated into developing chemoprevention cell models *in vitro*.

The inhibitory effect by the glucocorticoid receptor (GR), dexamethasone, and the anti-inflammatory drug, ibuprofen on IL-1α production showed that the model could also be used to test anti-inflammatory effects, apart from the induction of apoptosis. Dexamethasone is known to activate cytosolic GR receptor that antagonises activity of transcription factors such as activated protein-1 (AP-1), nuclear factor- kappa beta (NF-κB), and these are required for the gene expression and production of pro-inflammatory cytokines (Almawi et al., 2003). Ibuprofen has also been reported to down-regulate the production of pro-inflammatory cytokines at high doses, apart from its effect on cyclo-oxygenase downstream (Angst et al., 2008). As the inhibition of IL-1α in the current study was associated with a reduced cell metabolic activity in the

absence of any cytotoxicity, the modulation of apoptotic cell death should be further investigated.

The current UVB/HaCaT *in vitro* model provides ample opportunities to regulate the production of IL-1α, either by the disrupting signal transduction pathways related to its induction and/or by enhancing apoptosis thereby indirectly removing cells containing excessive levels of the primary cytokine. Except for the activity of phorbol ester-like compounds, HaCaT cells behave similarly to normal skin keratinocytes, especially in their response to UVB exposure and could therefore be used to monitor the modulation of IL-1α content by potential anti-inflammatory and or pro-apoptotic compounds. However, subsequent validation utilising different *in vivo* systems in mouse and human skin should be used to verify their modulating properties prior to developing possible therapeutic products for modulating IL-1α production.

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Chapter 6

In vitro effects of herbal tea extracts (rooibos and honeybush) on UVB-induced cell growth parameters and interleukin-1α accumulation in HaCaT cells

Part of this work was presented* at:

- 12th Indigenous Plant Use Forum (IPUF) Conference held at the Olive Grove Hall, Infruitec-Nietvoorbij Campus, Stellenbosch (6-9 July) 2009.
- 4th annual MRC research open day held at MRC head office in Parow, Cape Town, 14-15 October 2010
- DTI Technology Awards function to be held at Gallagher Estate in Midrand on 6 and 7 October 2010

*Presentation titles listed in Addendum A

In vitro effects of herbal tea extracts (rooibos and honeybush) on UVB-induced cell growth parameters and interleukin-1α accumulation in HaCaT cells

ABSTRACT

In skin, UVB irradiation elicits a wide range of biological effects which may lead to cancer development after chronic exposure. These effects include induction of pro-inflammatory cytokines, cell-cycle arrest and the induction of apoptosis in cells with irreparable DNA damage. Dietary components such as tea have been shown to prevent against UVB-induced damage by modulating the inflammatory response and the proliferation of the exposed cells. Although anti-inflammatory properties of rooibos and honeybush have been demonstrated on mouse skin, their effects on the early stages of UVB-induced inflammation and cell integrity still needs to be elucidated. The aim of the current study was to determine the modulatory effects of rooibos and different honeybush methanol and aqueous extracts against IL-1α accumulation. Different growth parameter indices including cell viability, cell proliferation and apoptosis following UVB exposure of skin keratinocytes (HaCaT) were also evaluated and green tea was used as a benchmark. Green tea and rooibos extracts seem to enhance UVB effects as they exacerbated UVB-induced inhibition of ATP production, cell proliferation, and promoted apoptosis which facilitated removal of icIL-1α containing cells. The underlying mechanisms may involve the pro-oxidant potency of their respective polyphenolic constituents which result in the disruption of mitochondrial function . The methanol extracts of honeybush, containing high levels of the polyphenolic xanthones and flavanones, protected against the UVB-induced reduction of cell viability, cell proliferation and induction of apoptosis presumably via the reduction of oxidative stress leading to an increased accumulation of icIL-1α. The protective effect against oxidative stress could provide opportunities to reduce UVB-induced skin damage prior to exposure. The aqueous extracts, similar to green tea and rooibos, enhanced apoptosis and may be useful in the prevention of photo-induced inflammation resulting from UVB exposure.

Key words: anti-inflammatory properties, IL-1α, UVB-irradiation, cell viability, apoptosis

1. Introduction

Ultraviolet B (UVB) radiation (280-315 nm) is regarded as one of the major predisposing risk factors of skin cancer (de Gruijl et al., 1999, Kim and Amstrong, 2012). In skin, acute exposure to UVB irradiation causes erythrema, edema, sunburn cells, hyperplasia, inflammation and immunosuppresion, while chronic exposure leads to photo aging and cancer development (Clydesdale et al., 2001, Melnikova and Ananthaswamy, 2005). Due to their anatomical position at the skin surface, keratinocytes are the primary targets of UVB-induced damage and as a result the most commonly occurring cancers arise in the epidermal layer (Soehnge et al., 1997). In the complex process of photocarcinogenesis, two distinct pathways regulating immunosuppression and neoplastic transformation have been identified. During cell cycle arrest, DNA misrepair accumulates genetic mutations, which dysregulate cell proliferation conferring growth advantage to initiated cells upon long-term exposure, while irreparable damage activates apoptotic signalling in normal cells. In the other pathway, UVB activates the production of various pro-inflammatory mediators including the primary cytokines, TNF- α and IL-1 (α and β), as well as secondary cytokines such as IL-6, IL-8, IL-10 and IL-4 (Shwarz, 2005). These cytokines mediate the inflammatory response and suppression of T-cell mediated immunity. The early events in cutaneous inflammation are initiated by the primary cytokine, IL-1α which is constitutively produced in keratinocytes (Dinarello, 1997). The necrotic release of this cytokine from damaged cells, upon injury, plays an important role in driving the inflammatory process. Consequently IL-1α has been used as a biomarker in various *in vitro* and *in vivo* models to evaluate the progression of inflammatory process during cancer development (Lee et al., 1994a; Lee et al., 1994b; Li et al., 2002; Hobbs and Watt. 2003). However, it has been suggested that the necrotic release of IL-1α may not be suitable for chemoprevention as a results mechanisms that involve inactivation of the biological activity of this cytokine via apoptosis have been suggested (Cohen et al., 2010).

In vitro models, utilising primary or cultured keratinocytes, have been useful screening tools in mechanistic and drug development studies evaluating the inflammatory process and efficacy of compounds against cytokine production

(Cohen et al., 1991; Tebbe et al., 1997; Vazquez et al., 2004; Tomaino et al., 2006; Pastore et al., 2011). A recent study optimised an *in vitro* UVB model that could be used to evaluate the chemopreventive efficacy of novel anti-inflammatory agents against IL-1α induction in HaCaT keratinocytes (Magcwebeba et al., 2012). It showed that intracellular IL-1α (icIL-1α) induction could be modulated directly by inhibiting synthesis or indirectly by enhancing UVB-induced apoptosis. As UVB-induced reactive oxygen species play an important mechanistic role in the inflammatory process, research has focused on the anti-inflammatory activity of naturally occurring antioxidants such as polyphenolic plant constituents (Kostyuk et al., 2008). Studies have indicated that polyphenols modulate molecular mechanisms of inflammation via inhibition of pro-inflammatory markers such as the cytokines (Mantena and Katiyar, 2006; Potapovich et al., 2011; Ramachandran and Prasadan, 2012). It has been shown that tea polyphenols prevent inflammation by inhibiting the synthesis of IL-1α in mouse skin and this has been proposed to be one of the underlying mechanisms in the anti-tumor activity of green and black tea (Katiyar et al., 1995; Katiyar and Mukhtar, 1997).

Rooibos and honeybush are South African herbal teas that possess biological properties including antioxidant, anti-inflammatory, immunomodulatory, anti-proliferative and anticancer activities (Joubert et al. 2008a). The anti-carcinogenic properties of rooibos and honeybush have been demonstrated in mouse skin carcinogenesis models, as the topical application of extracts exhibited anti-tumor and photoprotective effects (Marnewick et al., 2005; Petrova et al., 2011). The anti-inflammatory properties of rooibos and honeybush against UVB and PMA- induced COX-2 expression and edema have been demonstrated and implicated in their anti-tumor activity in skin (Na et al., 2004; Marnewick et al., 2005; Petrova et al., 2011). However, the modulatory activity of the herbal teas against primary cytokines involved in the early stages of UVB-induced inflammation as well as their effect on the integrity of exposed cells still needs to be elucidated. The aim of the current study was to determine the effect of methanol and aqueous extracts of rooibos and different honeybush species against UVB-induced IL-1α production in keratinocytes in relation to various cell growth parameters.

2. Material and methods

2.1. Chemicals

Bovine serum Albumin (BSA) was purchased from (Sigma-Aldrich, USA). Heat inactivated fetal bovine serum (FBS) was obtained from (Invitrogen, USA). RPMI-1640, Dulbeco's phosphate buffered saline (DPBS), L-glutamine, trypsin-versene, Hank's buffered salt solution (HBSS) were obtained from (Lonza, Belgium). Human recombinant IL-1α ELISA kit purchased from (R&D systems, USA). The non-radioactive cytotoxicity assay, CellTiter-Glo luminescent cell viability, Cell proliferation ELISA, BrDU chemiluninescent were from (Roche, Germany) and Caspase-3/7 assay purchased from (Promega, USA). Triton-x100 BDH chemical Ltd, Poole (England), and Tween®-20 were obtained from (ICN Biomedicals Inc, USA).

2.2. *Plant material and tea preparation*

Unfermented rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.: *C. intermedia, C. subternata, C. genistoides and C. longifolia*) herbal teas were obtained from the Agriculture Research Council, Infruitec-Nietvoorbij, Stellenbosch, South Africa. Green tea (*Camellia sinensis*), imported from China, was a gift from Vital Health Foods Kuilsriver, South Africa. The aqueous extracts of the different tea/herbal teas were prepared by adding freshly boiled deionised water to the plant material (10%m/v) and filtering after 30 min through double layer cheese cloth and subsequently though Whatman No 4 and 1. Filtered extracts were freeze-dried and stored desiccated in amber vials at room temperature until used. Preparation of methanol extracts involved extraction of plant material (50 g) with chloroform (3 x 300 mL) by stirring in an Erlenmeyer for 24 h. Residual tea plant material was subsequently extracted with methanol (3 x 300 mL) for 1 h and filtered. Solvent was evaporated under reduced pressure at 40˚C and dried extracts pulverized in a mortar and pestle and stored in a desiccator in the dark at room temperature. Chemical analyses of the different tea/herbal tea extracts were conducted as described previously (Chapter 3).

2.4. UVB exposure keratinocyte cell culture model

Spontaneously immortalised keratinocytes (HaCaT) were a gift from the Department of Human Biology at the University of Cape Town, South Africa. The cells were cultured in RPMI-1640 supplemented with inactivated fetal bovine serum (10%), L-glutamine (2 mM) and incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were passaged every 3 days at 70 to 80 % confluence in a 1:3 split ratio and passages 70 to 80 were utilised in experiments. When conducting the experiments, cells were seeded (100 µL) in 96-well tissue culture microtiter plates at a density of 3 x 10^4. After removing the cultured media, cells were either exposed to UVB light (80 mJ/cm^2) in DPBS (100 µL) without the plastic lid or for the control treatment (without UVB exposure) cells were washed with DPBS (100 µL). The UVIlink UV crosslinker (UVitek limited, UK) was fitted with six 8 Watt UV tubes with a wavelength of 302 nm (Vilber Lourmat, France).

2.5. Modulation of IL-1α production and different cell growth parameters by tea/herbal tea extracts in UVB irradiated and non-irradiated cells

Irradiated or non-irradiated cells were either exposed to fresh RPMI-1640 media containing 0.5% FBS (control wells) or to 0.5% FBS RPMI-1640 containing varying concentrations of the tea extracts (experimental wells). Tea extracts were prepared in the culture media using DMSO and filtered (0.22 µm). Final concentration of DMSO in the cultured media did not exceed 0.5%. Microtiter plates were incubated for 24 h and their effect on cell viability and cell proliferation monitored as described below. The effect on IL-1α and apoptosis was also monitored, but only in UVB irradiated cells in the presence of varying concentrations of the tea extracts. Separate experiments were conducted to evaluate the interrelationship between IL-1α and cell viability as well as between apoptosis and cell viability. Experiments were conducted utilising five replicates for each tea concentration and all analyses were repeated two to three times.

2.6. Cell growth parameters

Cell viability assay. In order to determine cell viability, the CellTiter-Glo Luminescent kit was used to monitor ATP production in cells cultured using white solid plates (Porvair Sciences, UK). To monitor the effect of extracts on ATP production, microplates were first equilibrated at room temperature for 30 min, luciferase agent added to the wells and plates rotated for 2 min and incubated at room temperature for 10 min in the dark. ATP content was monitored with the Veritas^{TM} microplate luminometer (Promega, USA). The luminescent signal was measured in relative light units (RLU) and data expressed as a percentage (%) of the control cells as follows:

% ATP production =RLU $_{\text{treated cells}}$/RLU $_{\text{control}}$x100

Cell proliferation assay. Cell proliferation was determined with the BrdU chemiluminescent immunoassay kit which measures BrdU incorporation during DNA synthesis in cells plated in black solid micro plates (Porvair Sciences, UK). Briefly, after 24 h incubation with tea extracts, cells were labelled with BrdU solution (10 µL) for 2 h at 37 °C. After incubation, media was decanted, cells fixed with denaturing solution (200 µL) and incubated at room temperature for 30 min. After removal of the denaturing solution, cells were incubated with the BrdU antibody (100 µL) for 1 h 30 min. Plates were washed 3 times with saline, treated with the substrate (100 µL), covered with foil and rotated for 3 min before quantification of DNA synthesis with the Veritas^{TM} microplate luminometer (Promega, USA). The luminescent signal was measured in relative light units (RLU) and data expressed as a percentage (%) of the control cells as follows:

% cell proliferation = RLU $_{\text{treated cells}}$/RLU $_{\text{control}}$ x100

IC_{50} values for ATP and BrDU assays were calculated on the basis of the best fit for dose-response data using the 4-parameter logistic curve (Sigmoidal variable slope) in GraphPad Prism version 5.04 for Windows (GraphPad Software, USA).

Determination of apoptosis. The ability to enhance apoptosis in cells was only determined for green tea, rooibos and the two flavanone–rich honeybush species (*C. intermedia* and *C. subternata*). The honeybush species were selected based on their reactivity against cell viability and proliferation (Chapter 3). Assays were conducted in clear microplates and cells were lysed with a combination of a lysis buffer (20 µL) and one freeze-thaw cycle. Cell lysates were transferred (25 µL) into a white solid micro plates and incubated with the caspase 3/7 reagent (25 µL) in the dark at room temperature for 1 h. Analyses were conducted in a Veritas microplate luminometer and the data, generated as relative light units, was either expressed as fold increase representing the augmentation of apoptosis in cells or as % apoptotic activity per viable cells calculated as follows:

% apoptotic activity = $RLU_{caspase\text{-}3}$ / $RLU_{ATPcontent}$ x100.

IL-1α determination. The concentrations of the different extracts utilised to determine the activity against IL-1α production were selected at sub-cytotoxic levels based on the activity (IC_{50}) of the extracts against cell viability (ATP). Intracellular (icIL-1α) and extracellular interleukin-1α (exIL-1α) were determined in cell lysates and supernatants, respectively, utilising an IL-1α ELISA kit based on the manufacturer's instruction. Absorbance was measured at 450 nm with a Dynex plate reader (Dynex technologies, USA) and data was analysed using a IL-1α standard curve generated by GraphPad prism (version 5 for windows) (GraphPad software Inc, USA) and expressed as pg/mL of the supernatant or cell lysate.

2.7 Statistics.

All parameters were tested for normality using the Kolmogorov-Smirnof Test, as well as for homogeneity of group variances which were tested using Levene's Test. Group differences for the parametric parameters as well as those that could be transformed were then tested using One-way ANOVA's (GLM in SAS) and post-hoc Tukey Tests for multiple pairwise comparisons between the means of all the groups. When only two groups were compared the student T-test was used. When the original variables were not normally distributed they were individually transformed to become parametric [Table 6.3; *C. genistoides* MeOH extract (% ATP – square root;

and icIL-1α - inverse)]. The following transformations were conducted for the methanol extracts: the square root for *C. sinensis*; 1/squared root for *C. genistoides* and the inverse transformation for *C. intermedia* and *C. subternata*. For the non-parametric Kruskal-Wallis Test (Rank Sum for >2 groups) was used assess group differences using the post-hoc Tukey-type test for pairwise groups comparisons. Where only two groups were compared, the non-parametric Wilcoxon Two-sample Test was used for comparison. Spearman correlations were used to calculate all the correlation coefficients. Statistical analyses were performed with SAS v9.2 and statistical significance was considered at 5% ($p<0.05$).

3. Results

3.1 Comparative effect of different tea/herbal tea extracts tea on cell viability (ATP) and cell proliferation (BrdU) in UVB irradiated and non-irradiated cells

Cell viability: Green tea and rooibos extracts were the most active in reducing the cellular ATP content when compared to the extracts of the different honeybush species (Table 6.1). The effect of green tea and rooibos extracts was not significantly different between UVB irradiated and non-irradiated cells. The green tea methanol extract was more active than its aqueous extract whilst there was similar activity between rooibos extracts. In contrast, honeybush extracts were more active in non-irradiated cells and, except for *C. longifolia,* the aqueous extracts displayed the higher activity than the methanol extracts. The aqueous extract of *C. intermedia* exhibited the highest activity in cells whilst the effects of *C. longifolia and C. subternata* were similar. *Cyclopia genistoides* displayed the weakest response against cell viability.

Cell proliferation: Both green tea and rooibos extracts exhibited the highest activity in inhibiting cell proliferation with no significant difference between UV irradiated and non-irradiated cells (Table 6.1). There was also no significant difference when considering the activity of methanol and aqueous extracts of green tea and rooibos. For the different honeybush species, the methanol extracts were more sensitive in the non-irradiated keratinocytes with the extracts of *C. intermedia*, *C. subternata* and *C. longifolia* exhibiting a similar activity followed by *C. genistoides*. A similar order of

Table 6.1. Comparative effect of different herbal and green teas on growth parameters of UV irradiated and non-irradiated HaCaT cells.

Extract	UV exposure	*C. sinensis*	*A. linearis*	*C. genistoides*	*C. longifolia*	*C. intermedia*	*C. subternata*
ATP IC_{50} (mg/ml)							
MeOH	(-) UV-B	**$0.08\pm0.01^{d}_{A}$**	$0.15\pm0.02^{d}_{A}$	1.85 ± 0.22^{a}	0.73 ± 0.05^{c}	1.25 ± 0.21^{b}	1.85 ± 0.39^{a}
	(+) UV-B	$0.07\pm0.01^{a}_{A}$	$0.13\pm0.01^{a}_{A}$	> 2.00	>1.80	>1.46	>1.80
Aq	(-) UV-B	$0.14\pm0.01^{d}_{A}$	$0.13\pm0.01^{d}_{A}$	**$1.18\pm0.14^{a}_{A}$**	$0.76\pm0.05^{b}_{A}$	**$0.49\pm0.04^{c}_{A}$**	**0.89 ± 0.12^{b}**
	(+) UV-B	$0.10\pm0.02^{d}_{A}$	$0.10\pm0.01^{d}_{A}$	$1.33\pm0.24^{a}_{B}$	$1.01\pm0.10^{b}_{B}$	$0.60\pm0.06^{c}_{B}$	> 1.00
BrdU IC_{50} (mg/ml)							
MeOH	(-) UV-B	$0.06\pm0.02^{c}_{A}$	$0.06\pm0.01^{c}_{A}$	**0.59 ± 0.07^{a}**	0.40 ± 0.09^{b}	0.37 ± 0.08^{b}	0.27 ± 0.03^{b}
	(+) UV-B	$0.09\pm0.01^{b}_{A}$	$0.08\pm0.01^{b}_{A}$	>0.82	>0.94	>0.71	>1.80
Aq	(-) UV-B	$0.07\pm0.01^{d}_{A}$	$0.08\pm0.01^{d}_{A}$	1.06 ± 0.34^{a}	**0.25 ± 0.0^{b}**	$0.33\pm0.07^{b}_{A}$	$0.31\pm0.04^{b}_{A}$
	(+) UV-B	$0.10\pm0.02^{b}_{A}$	$0.10\pm0.01^{b}_{A}$	>1.50	>0.55	$0.55\pm0.33^{a}_{B}$	$0.53\pm0.26^{a}_{B}$

Values represent mean ± standard deviation of 4-5 replicates and at least 2 independent experiments; Statistical analyses for significant group effects (more than 2 groups) were assessed by either the One-way ANOVA F-test or the Welch's test, depending if homogeneity of group variances were present (Levene's test). If significant group differences were present, the post hoc Tukey's Studentized Range Test was used for between group comparisons at 5% significance level. Statistical comparisons for only two groups, i.e. MeOH vs Aq for each tea, the Student's T-test were used. Significant differences ($P<0.05$) between the different tea/herbal tea extracts are indicated with differing lower case letters in superscript (in a row). Significant differences between non-irradiated and UVB-irradiated are indicated with differing upper case letters in subscript (in a column). Means followed by the same letter do not differ significantly. Data in bold indicates significant differences between methanol and aqueous extracts within the non-irradiated cells (in a column), Abbreviations: IC_{50} – concentration (mg/ml) yielding 50% inhibition; ATP – adenosinetriphosphate; BrdU - 5-bromo-2'-deoxyuridine; MeOH – methanol; Aq – aqueous; (-) UVB indicates non-irradiated cells; (+) UV-B indicates cells irradiated with a dose of 80mJ/cm^2.

activity was noticed for the aqueous extracts. When the methanol and aqueous extracts were compared in the non-irradiated samples, the aqueous extract of *C. longifolia* exhibited a higher activity whilst for *C. genistoides* the methanol extract exhibited a higher activity. No significant difference was noticed between the methanol and aqueous extracts of *C. intermedia* and *C. subternata*. However, in the UVB-irradiated cells the aqueous extracts of *C. intermedia* and *C. subternata* exhibited the highest activity. *Cyclopia genistoides* again, exhibited the weakest activity when considering the inhibition of cell proliferation.

3.2 *Modulation of tea/herbal tea extracts of UVB-induced IL-1α levels in relation to cell viability*

The methanol and aqueous extracts of green tea and rooibos inhibited UVB-induced intracellular IL-1α (icIL1-α) accumulation in a dose-dependent manner exhibiting no release of extracellular IL-1α (exIL1-α) (Table 6.2). For green tea, the methanol extract was significantly more effective than the aqueous extract but for rooibos the was no significant difference between extracts however the methanol extract seemed to be slightly more effective (Fig. 6.1). The reduction of IL-1α was closely related to the decrease in the ATP content for both green tea and rooibos extracts. In this regard, slightly lower concentrations (IC_{50} values) were required for reducing the ATP content as compared to the icIL-1α accumulation. However, green tea and rooibos extracts had no significant effect on the icIL-1α accumulation when normalised against cell viability (Fig 6.2). The honeybush species displayed a different effect when compared to green tea and rooibos, as the methanol extracts increased icIL-1α accumulation at all the tested concentrations (Table 6.3). However, at lower concentrations no stimulatory effects were observed for *C. intermedia* and *C. subternata*. The increase in icIL-1α accumulation was accompanied by a far weaker dose-dependent inhibition of the ATP content as compared to green tea and rooibos. The effect of methanol extracts from *C. intermedia* (3%) and *C. subternata* (15%) on the ATP content was only slightly lower when compared to the positive control. In contrast, the aqueous extract of *C. intermedia* exhibited a dose-dependent inhibition of icIL-1α while *C. longifolia* and *C. genistoides* aqueous extracts only inhibited icIL-1α accumulation at the highest concentration.

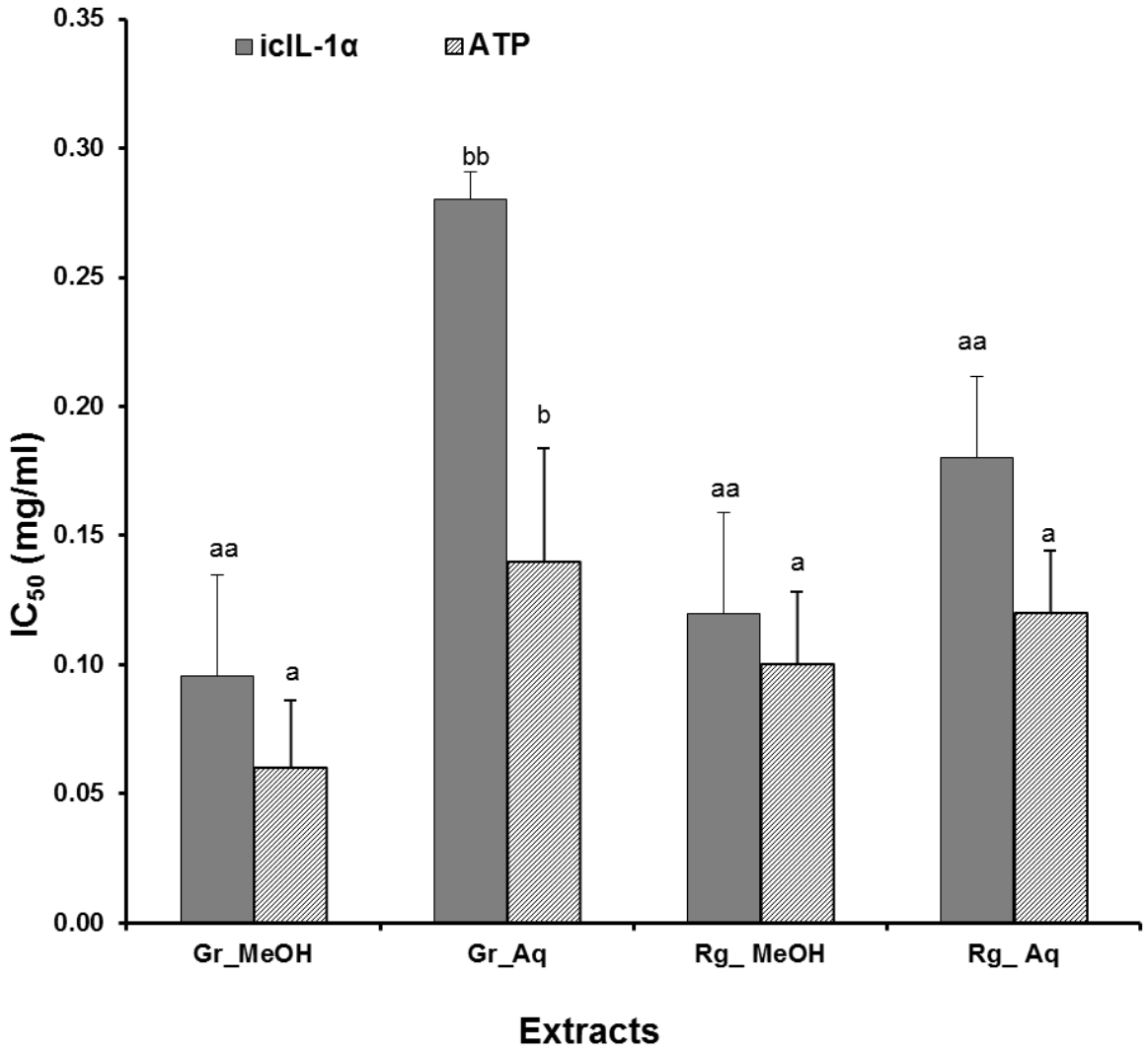

Fig 6.1 Comparison of the activity of green tea and rooibos extracts in reducing intracellular IL-1α (icIL-1α) accumulation and cell viability (ATP content). Values are the mean ± standard deviation of IC_{50} values (mg/ml) of 4 to 5 replicates of at least two experiments. Statistical comparisons between MeOH vs Aq for each tea were conducted using the Student's T-test. Statistically significant differences ($P<0.05$) for ATP are indicated with differing lower case letters while for icIL-1 α with lower case double letters. Means followed by the same letter do not differ significantly. Abbreviations: Gr-green tea, Rg- rooibos green; MeOH, methanol, Aq- aqueous.

Table 6.2. Modulation of intra and extracellular IL-1α accumulation by green tea and rooibos extracts in relation to their effect on cell viability (ATP content)

Tea/ herbal tea	Unit		Controls		Tea and herbal extract concentrations							
					Methanol extracts (mg/ml)				Aqueous extracts (mg/ml)			
			Ctrl	**Pos Ctrl**	**0.107**	**0.054**	**0.027**	**0.013**	**0.431**	**0.216**	**0.108**	**0.054**
Camellia sinensis	IL-1α conc (pg/ml)	icIL-1α	15.4±6.0^{e}	42.8±13.7^{a}	20.7±6.2^{d}	37.4±14.3^{c}	47.2±18.2^{ab}	46.5±16.9^{ab}	8.6±3.82^{f}	15.9±5.8^{de}	22.1±7.4^{c}	25.9±8.0^{b}
		exIL-1α	2.9±0.78^{d}	4.0±0.6^{a}	1.5±0.2^{e}	1.4±0.3^{e}	2.5±0.8^{c}	3.3±1.6^{b}	1.9±0.2^{b}	1.7±1.0^{b}	1.7±0.8^{b}	2.1±0.9^{c}
	% production	icIL-1α	-	100.0±10.2^{a}	42.4±8.5^{d}	75.3±13.7^{c}	91.1±17.1^{b}	92.3±11.5^{ab}	25.1±10.5^{e}	45.0±16.1^{d}	63.0±20.4^{c}	72.0±20.8^{b}
		ATP	100.0±5.6^{a}	90.4±10.3^{b}	37.0±8.4^{f}	54.8±10.8^{e}	68.6±7.2^{d}	80.3±8.6^{c}	16.±6.5^{f}	37.4±12.7^{e}	57.9±9.2^{d}	73.7±7.5^{c}
% icIL-1/viable cells			**15.4±5.9^{a}**	**47.3±15.2^{b}**	**56.1±16.9^{b}**	**68.3±26.1^{b}**	**68.9±26.6^{b}**	**58.0±21.1^{b}**	**54.3±22.4^{b}**	**41.6±15.3^{b}**	**37.5±12.7^{b}**	**38.6±7.9^{b}**
			Ctrl	**Pos Ctrl**	**0.190**	**0.095**	**0.048**	**0.024**	**0.554**	**0.277**	**0.139**	**0.069**
Aspalathus linearis	IL-1α conc (pg/ml)	icIL-1α	9.7±2.8^{e}	31.1±3.7^{a}	11.0±2.2^{d}	17.6±5.6^{c}	24.6±8.8^{b}	30.0±7.2^{a}	6.7±1.7^{f}	10.4±2.3^{d}	19.1±4.2^{c}	25.3±5.2^{b}
		exIL-1α	3.1±1.6^{a}	3.0±1.7^{a}	3.2±1.9^{a}	2.7±1.3^{b}	2.3±0.7^{b}	2.5±0.4^{b}	1.9±0.6^{b}	1.6±0.5^{b}	2.2±0.7^{c}	2.1±1.2^{c}
	% production	icIL-1α	-	100.0±10.2^{a}	35.6±8.1^{d}	57.6±20.9^{c}	80.4±30.9^{b}	96.8±22.9^{ab}	21.1±4.4^{e}	32.9±6.2^{d}	61.1±12.9^{c}	80.6±14.2^{b}
		ATP	100.0±4.6^{a}	88.4±12.6^{b}	28.2±11.2^{f}	47.2±10.7^{e}	71.4±5.3^{d}	75.7±6.3^{c}	15.0±5.13^{f}	28.9±7.27^{e}	49.4±6.16^{d}	62.8±5.04^{c}
% icIL-1/viable cells			**9.7±2.8^{a}**	**35.2±4.2^{b}**	**38.9±7.6^{b}**	**37.2±11.9^{b}**	**34.4±12.4^{b}**	**39.6±9.5^{b}**	**44.3±11.0^{b}**	**35.9±7.9^{b}**	**38.8±8.5^{b}**	**40.3±8.3^{b}**

Values represent mean ± standard deviation of 4-5 replicates and at least 2 independent experiments. The effect of the individual tea/herbal tea concentrations on both IL-1α and ATP levels were compared to the Control and Positive control using the non-parametric Wilcoxon Two-sample Test. Statistically significant differences between the control and the different tea concentrations was indicated with differing lower case letters in superscript (in a row) for each extract. Means followed by the same letter do not differ significantly. Abbreviations: Ctrl- control indicates cells that were not exposed to UVB light; Pos Ctrl-positive control indicates cells that have been exposed to 80mj/cm^2 of UVB light; IL-1α conc-IL-1α concentration in (pg/ml); icIL-1α-intracellular interleukin-1α; exIL-1α- extracellular interleukin-1α; ATP – adenosinetriphosphate; % production indicates the percentage calculated as a function of the relevant control treatment.

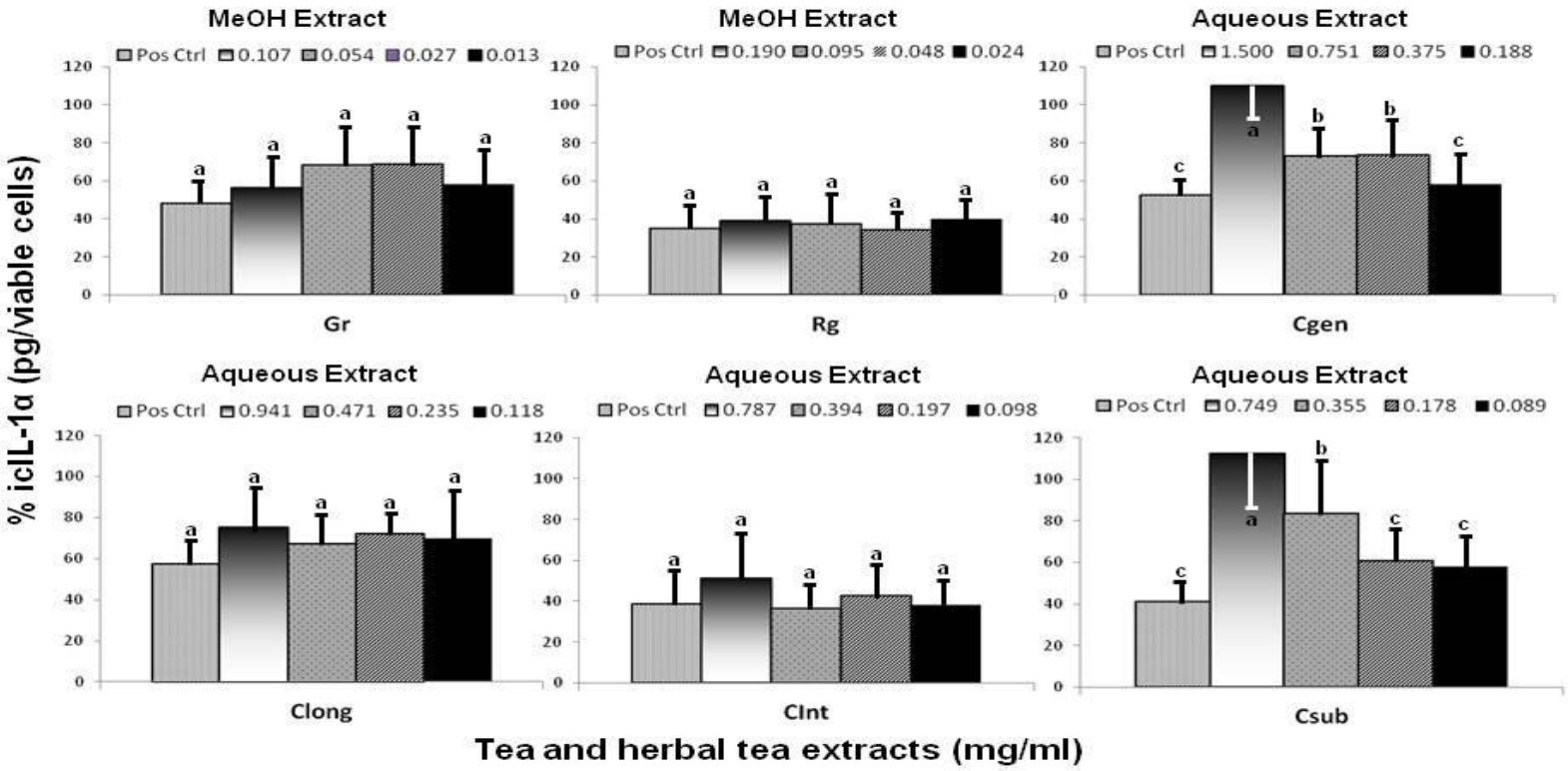

Fig 6.2. Depicts the effect of the different tea/herbal tea concentration on the amount of intracellular interleukin-1 alpha (icIL-1) in viable cells (ATP content). Activity expressed as % icIL-1 content per viable cells. Values represent mean ± standard deviations. Statistical analyses for significant group effects (more than 2 groups) were assessed by either the One-way ANOVA F-test or the Welch's test, depending if homogeneity of group variances were present (Levene's test). If significant group differences were present, the post hoc Tukey's Studentized Range Test was used for between group comparisons at 5% significance level. Differing letters in lower case indicate a significant difference ($P<0.05$), if letters are the same means do not differ significantly. Abbreviations: icIL-1α-intracellular IL-1 alpha production, Rg-rooibos green, Gr-green tea, Cgen-*Cyclopia genistoides*, Clong-*Cyclopia longifolia*, Cint-*Cyclopia intermedia*, Csub-*Cyclopia subternata*.

Table 6.3. Modulation of IL-1α accumulation by the different honeybush extracts in relation to their effect on cell viability (ATP content)

Cyclopia spp	Unit of measure		Controls		Methanol extracts (mg/ml)				Aqueous extracts (mg/ml)			
			Ctrl	**Pos Ctrl**	**0.820**	**0.410**	**0.205**	**0.103**	**1.500**	**0.750**	**0.375**	**0.188**
C. genistoides	IL-1α conc (pg/ml)	icIL-1α	9.05±2.0^{e}	39.04±6.3^{c}	48.4±12.2^{a}	46.0±6.9^{a}	45.7±11.7^{a}	48.7±12.0^{a}	25.8±2.0^{d}	39.3±6.0^{c}	49.3±11.0^{ab}	44.9±9.1^{a}
		exIL-1α	1.4±0.4^{e}	6.0±1.5^{a}	3.5±1.5^{c}	3.3±1.4^{c}	4.0±1.7^{b}	6.1±1.32^{a}	5.5±3.3^{d}	3.1±0.9^{c}	3.3±0.7^{d}	4.8±2.7^{b}
	% production	icIL-1α	-	100.0±11.1^{b}	133.4±42.7^{a}	124.7±20.1^{a}	122.8±26.7^{a}	130.3±22.8^{a}	61.9±6.4^{d}	94.3±15.1^{c}	118.6±28.8^{b}	108.2±23.5^{b}
		ATP	100.0±3.0^{a}	74.5±8.1^{b}	54.1±11.9^{e}	65.0±10.3d	71.1±10.4^{bc}	73.1±9.5^{b}	23.5±8.0	54.0±9.2	67.2±11.5	77.4±12.2
% icIL-1/viable cells			**9.0±2.0**	**52.4±8.4**	**81.6±12.7**	**70.8±10.6**	**64.2±16.4**	**66.7±16.4**	**110.0±8.7**	**72.8±11.1**	**73.4±16.4**	**58.0±11.8**
			Ctrl	**Pos Ctrl**	**0.941**	**0.471**	**0.235**	**0.118**	**0.550**	**0.275**	**0.138**	**0.069**
C. longifolia	IL-1α conc (pg/ml)	icIL-1α	12.3±5.1^{e}	49.77±12.0^{c}	62.4±12.1^{a}	65.5±20.3^{a}	71.5±28.9^{a}	69.7±29.3^{a}	41.3±6.4^{d}	45.4±8.6^{d}	53.0±8.0^{b}	56.9±11.6^{a}
		exIL-1α	1.1±0.1	6.4±1.2^{b}	9.30±1.0^{a}	4.0±0.4^{c}	3.5±0.4^{d}	4.8±1.4^{c}	4.1±1.7^{e}	3.2±0.9^{e}	4.6±1.3^{d}	5.4±1.3^{c}
	% production	icIL-1α	-	100.0±11.8^{b}	143.7±32.9^{a}	144.1±27.0^{a}	142.7±35.8^{a}	145.2±35.9^{a}	80.7±9.7^{d}	88.6±11.9^{c}	103.6±10.1^{b}	111.6±16.2^{a}
		ATP	100.0±4.8^{a}	86.5±12.9^{b}	59.4±10.3^{f}	73.8±1.3^{e}	79.0±9.6^{d}	82.0±8.1^{c}	52.1±8.2^{f}	67.3±8.9^{e}	73.2±11.3^{d}	77.2±10.8^{c}
% icIL-1/viable cells			**12.3±5.1**	**57.5±13.8**	**104.9±20.3**	**88.6±27.4**	**90.4±36.5**	**85.1±35.7**	**75.3±21.4**	**67.4±12.7**	**72.4±10.9**	**70.0±22.0**
			Ctrl	**Pos Ctrl**	**0.730**	**0.365**	**0.183**	**0.091**	**0.787**	**0.394**	**0.197**	**0.098**
C. intermedia	IL-1α conc (pg/ml)	icIL-1α	9.0±4.6^{d}	33.3±12.5^{b}	46.1±10.4^{a}	33.7±9.2^{b}	29.7±11.5^{c}	30.0±10.2^{c}	17.9±7.4^{a}	22.1±7.3^{a}	28.9±7.4^{a}	29.5±7.6^{a}
		exIL-1α	Nd	1.4±0.2^{b}	1.7±0.2^{b}	1.7±0.4^{b}	1.9±0.7^{b}	2.4±0.2^{a}	2.6±0.1^{a}	1.4±0.3^{b}	1.4±0.1^{b}	1.5±0.1^{b}
	% production	icIL-1α	-	100.0±10.8^{b}	145.7±32.2^{a}	103.0±16.6^{b}	85.1±12.9^{c}	87.5±11.2^{c}	60.1±12.5^{d}	71.2±12.0^{c}	86.8±14.3^{b}	91.2±19.0^{a}
		ATP	100.0±5.0^{a}	87.0±6.3^{b}	84.1±12.3^{b}	87.3±11.1^{b}	88.9±13.4^{b}	81.4±11.6^{b}	35.1±7.1^{f}	55.6±8.0^{e}	68.2±7.6^{d}	74.8±10.5^{c}
% icIL-1/viable cells			**9.0±4.6**	**38.3±14.3**	**54.8±12.4**	**38.6±10.5**	**33.4±12.9**	**36.8±12.5**	**51.0±21.1**	**36.1±9.8**	**42.4±10.8**	**38.1±8.9**
			Ctrl	**Pos Ctrl**	**0.710**	**0.355**	**0.178**	**0.089**	**0.749**	**0.355**	**0.178**	**0.089**
C. subternata	IL-1α conc (pg/ml)	icIL-1α	15.5±3.5^{d}	35.9±6.9^{c}	51.9±13.4^{a}	43.5±9.1^{b}	40.8±8.2^{b}	36.2±7.8^{c}	49.8±16.8^{b}	54.6±18.9^{a}	47.0±1.7^{b}	46.1±13.7^{c}
		exIL-1α	2.7±0.7^{e}	4.3±0.6^{b}	3.9±1.6^{c}	4.4±2.0^{b}	4.4±1.9^{b}	5.2±2.7^{a}	1.4±0.3^{d}	2.3±1.2^{c}	3.3±2.0^{b}	4.39±2.9^{a}
	% production	icIL-1α	-	100.0±11.5^{c}	134.9±16.5^{a}	114.8±15.8^{b}	108.5±18.2^{b}	95.8±15.2^{c}	144.1±35.7^{a}	158.5±42.9^{a}	137.4±25.9^{a}	137.2±27.7^{a}
		ATP	100.0±4.1^{a}	87.4±11.8^{b}	68.1±7.1^{f}	77.5±10.3^{e}	79.51±10.1^{d}	82.6±9.9^{c}	42.0±10.9^{f}	62.2±7.00^{e}	77.3±9.5^{d}	79.37±8.5^{c}
% icIL-1/ viable cells			**16.2±3.1**	**41.2±8.5**	**72.3±25.6**	**56.1±11.7**	**51.3±10.3**	**43.8±9.5**	**118.5±39.9**	**83.5±35.5**	**60.9±15.1**	**58.1±17.3**

Values represent mean ± standard deviation; ; Statistical analyses for significant group effects (more than 2 groups) were assessed by either the One-way ANOVA F-test or the Welch's test, depending if homogeneity of group variances were present (Levene's test). If significant group differences were present, the post hoc Tukey's Studentized Range Test was used for between group comparisons at 5% significance level. Some of original variables had non-parametric distributions, but were individually transformed to become parametric (See Statistical analyses). Means of both IL-1α and ATP levels were compared to the Control and Positive control using the non-parametric Wilcoxon Two-sample Test. Statistically significant differences was indicated with differing lower case letters in superscript for each extract. Means followed by the same letter do not differ significantly. Abbreviations: Ctrl- control indicates cells that were not exposed to ultraviolet-B light; Pos Ctrl-positive control indicates cells that have been exposed to 80mj/cm^{2} of ultraviolet- B light; IL-1α conc- IL-1α concentration in (pg/ml); icIL-1α-intracellular interleukin-1α; exIL-1α- extracellular interleukin-1α; % production-indicate the percentage production calculated from the relevant control cells;
ATP – adenosinetriphosphate

Of interest, is the *C. subternata* aqueous extract, which displayed similar activity to the methanol extract as it increased IL-1α production in cells at all the concentrations tested, independent of the reduction of cell viability. The aqueous extracts of *C. subternata* and *C. genistoides* significantly enhanced icIL-1 content in viable cells at the highest concentrations an effect that was reduced at the lower concentrations to levels which did not differ significantly from the UVB exposed cells (Table 6.4; Fig 6.2). This effect was also evident in cells exposed to the methanol extract of *C. longifolia*. However, the aqueous extract of *C. longifolia* and the methanol and aqueous extracts of *C. intermedia* exhibited no significant effect on icIL-1α accumulation in viable cells.

3.3 Modulation of UVB-induced apoptosis in relation to cell viability by tea/herbal tea extracts

The green tea and rooibos methanol extracts exhibited the highest activity in enhancing UVB-induced apoptosis in a dose-dependent manner, evidenced by caspase-3 fold increase and activity in viable cells (Table 6.4). The aqueous extracts also exhibited a similar effect although this was achieved a concentrations that were 3 to 4 fold higher than methanol extracts. Green tea extracts appeared to be more effective at inducing apoptosis than rooibos extracts when considering the highest concentrations. The apoptotic effect of this tea/herbal tea was closely related to the reduction of cell viability as caspase-3 activity was inversely related to the ATP content. Only the two honeybush species (*C. intermedia* and *C. subternata*) that exhibited the highest anti-proliferative effect against UVB irradiated HaCaT cells were selected to determine the modulatory effect on UVB-induced apoptosis. The methanol extracts of *C. intermedia* and *C. subternata* dose-dependently reduced UVB-induced caspase-3 activity in cells. This activity was either comparable to the negative control or below the positive control, respectively. The reduction of apoptotic activity in cells by the honeybush species was accompanied by a relatively weaker inhibition of cell viability when compared to that of green tea and rooibos. The aqueous extracts, similar to green tea and rooibos, dose-dependently increased UVB-induced caspase-3 activity in cells and this was accompanied by a resultant decrease in cell viability with *C. subternata* exhibiting a weaker activity.

Table 6.4. Modulation of UVB induced apoptosis and activity of ATP production by green tea and different herbal teas

Tea/ herbal tea	**Unit measurement**	**Controls**		**Methanol extracts (mg/ml)**			**Aqueous extracts (mg/ml)**		
		Ctrl	**Pos Ctrl**	**0.107**	**0.054**	**0.027**	**0.431**	**0.216**	**0.108**
Camelia sinensis	*Casp-3 fold increase*	1.00±0.19^{d}	3.89±0.68^{c}	7.86±0.93^{a}	7.82±0.76^{a}	6.10±0.21^{b}	6.51±0.77^{a}	6.20±0.87^{a}	6.09±1.11^{a}
	% ATP production	100.00±3.97^{a}	74.63±4.79^{b}	27.74±2.35^{d}	49.16±5.51^{c}	69.47±5.05^{b}	8.90±3.08^{d}	37.98±4.83^{c}	43.95±7.36^{c}
	% Casp-3/ viable cells	0.40±0.12^{e}	1.89±0.49^{d}	8.35±0.99^{a}	4.69±0.45^{b}	2.59±0.09^{c}	36.84±4.35^{a*}	8.22±1.15^{b*}	6.98±1.27^{c*}
		Ctrl	**Pos Ctrl**	**0.190**	**0.095**	**0.048**	**0.554**	**0.277**	**0.139**
A.linearis	*Casp-3 fold increase*	1.00±0.14^{e}	3.59±0.62^{d}	10.12±0.88^{a}	9.03±0.66^{b}	6.97±1.02^{c}	5.14±0.50^{a}	4.61±0.36^{b}	3.56±0.19^{d}
	% ATP inhibition	100.00±3.97^{a}	74.63±4.79^{b}	28.43±3.41^{d}	48.18±3.32^{d}	66.74±4.02^{c}	15.94±2.34^{c}	29.56±3.36^{b}	48.94±5.55^{a}
	% Casp-3/ viable cells	0.40±0.12^{e}	1.89±0.49^{d}	10.49±0.91^{a}	5.52±0.40^{b}	3.08±0.45^{c}	16.24±1.59^{a}	7.86±0.62^{b}	3.66±0.19^{c}
		Ctrl	**Pos Ctrl**	**0.730**	**0.365**	**0.183**	**0.787**	**0.394**	**0.197**
C.intermedia	*Casp-3 fold increase*	1.00±0.16^{f}	2.99±0.41^{c}	1.19±0.16^{f}	1.66±0.38^{e}	2.19±0.68^{d}	4.92±0.66^{a}	4.33±0.56^{a}	3.27±0.40^{b}
	% ATP production	100.00±3.80^{a}	88.54±8.84^{b}	63.80±6.92^{d}	79.56±9.30^{c}	81.54±7.34^{b}	39.61±6.12^{e}	59.75±9.31^{d}	75.28±7.77^{c}
	% Casp-3/ viable cells	0.45±0.21^{e}	1.58±0.74^{a}	0.60±0.34^{d}	0.73±0.57^{c}	1.03±0.80^{b}	7.11±2.04	4.20±1.13	2.44±0.35
		Ctrl	**Pos Ctrl**	**0.710**	**0.355**	**0.178**	**0.730**	**0.365**	**0.183**
C.subternata	*Casp-3 fold increase*	1.00±0.13^{e}	3.40±0.49^{c}	2.46±0.69^{d}	3.41±0.78^{c}	3.93±0.52^{b}	6.57±1.05^{a}	5.44±1.30^{b}	4.99±1.15c
	% ATP production	100.00±3.87^{a}	84.20±12.53^{b}	55.12±25.27^{d}	70.06±18.28^{c}	73.99±13.69^{c}	44.12±13.75^{e}	67.79±16.02^{d}	77.40±17.34^{c}
	% Casp-3/ viable cells	0.49±0.10^{d}	1.71±0.25^{c}	1.46±0.36^{a}	1.53±0.36^{b}	1.93±0.56^{c}	4.61±1.10^{a}	3.11±0.43^{b}	2.66±0.45^{c}

Values represent mean±standard deviation; *Statistical analyses included One-Way ANOVA using generalised linear models to test for group differences between control groups and tea concentration for each extract. If significant group differences were present, the post hoc Tukey's Studentized Range Test was used at 5% significance level Means of the remainder samples were compared to the Control and Positive control using the non-parametric Wilcoxon Two-sample Test. Statistically significant differences between the control and the different tea concentration indicated with differing lower case letters in superscript (in a row) for each extract. Means followed by the same letter do not differ significantly. Abbreviations: Ctrl- control; Pos Ctrl-positive control % casp-3/ viable cells- percentage caspase-3 activity calculated against ATP concentration reflecting viable cells; ATP – adenosinetriphosphate; % ATP production calculated against control cells Abbreviations: Ctrl- control indicates cells that were not exposed to ultraviolet-B light; Pos Ctrl-positive control indicates cells that have been exposed to UVB light.

3.4 Interrelationships between icIL-1 inhibition, cell viability and apoptosis

A strong positive correlation existed between decreased of cell viability and the concomitant reduction of icIL-1α content by the methanol and aqueous extracts of both green tea and rooibos (Table 6.5). When considering the decrease of icIL-1α content in relation to caspase-3 fold increase as well as between caspase-3 increase and cell viability, a strong negative or inverse correlation prevailed.

Table 6.5. Correlation between cell viability, caspase-3 activity (fold) and increase icIL1-α content effected by the different tea/herbal tea extracts.

Tea/herbal tea Unit of measure	**IL-1_ATP**	**Casp-3_F_ATP**	**Casp-3_F _IL-1**
C. sinensis			
MeOH	r 0.618 P<0.0001	r -0.895 P<0.0001	r -0.863 P<0.0001
Aq	r 0.754 P<0.0001	r -0.880 P<0.0001	r -0.833 P<0.0001
A. linearis			
MeOH	r 0.742 P<0.0001	r -0.878 P<0.0001	r -0.899 P<0.0001
Aq	r 0.796 P<0.0001	r -0.848 P<0.0001	r -0.876 P<0.0001
C. intermedia			
MeOH	-	-	r -0.494 P=0.0005
Aq	r 0.720 P<0.0001	r -0.820 P<0.0001	r -0.555 P<0.0001
C. subternata			
MeOH	r -0.309 P=0.0017	r -0.597 P<0.0001	-
Aq	r -0.229 P=0.023	r -0.822 P<0.0001	r 0.517 P=0.0001

Spearman correlations were used to calculate correlation coefficients. r values represent correlation coefficient. P<0.05 was considered statistically significant. Abbreviations: Casp-3_ F- caspase-3 fold increase; IL-1_ATP- correlation between intracellular IL-1α inhibition and ATP inhibition; Casp-3_F_ATP- correlation between caspase-3 fold increase and ATP inhibition; Casp-3_F_IL-1 – correlation between caspase-3 fold increase and IL-1 inhibition; caspase-3 fold increase; ATP- adenosine triphosphate; MeOH- methanol; Aq- aqueous extract *Camellia sinensis*- green tea*, Aspalathus. linearis*- rooibos, *Cyclopia* spp. (*C. intermedia, C. subternata*)..

In contrast, the methanol extract of honeybush, *C. intermedia*, exhibited no correlation between icIL-1α inhibition, cell viability or caspase-3 activity, while only a weak inverse correlation existed between icIL-1α and the increased caspase-3 activity. However, the aqueous extract exhibited similar results as compared to green tea and rooibos, exhibiting a strong positive correlation between the reduction of icIL-1α content and cell viability, an inverse correlation between caspase-3 activity and cell viability while a somewhat weaker correlation existed between with icIL-1α and caspase-3 activity. In contrast to the other tea/herbal teas, both extracts of *C. subternata* exhibited a weak inverse correlation between icIL-1α and cell viability. For the methanol extract, a weak inverse correlation was noticed between caspase-3 activity and cell viability while the aqueous extract behaved similarly to the other tea/herbal tea extracts. However, no correlation existed between the caspase-3 activity and decreased icIL-1α, while a positive correlation existed for the aqueous extract.

3.5. Chemical characterisation of the extracts in relation to their activity in cells

The methanol extract of rooibos contained higher levels of polyphenols and the major compounds present were the dihydrochalcones (DHC), aspalathin and to a lesser extent, nothofagin (Table 6.6). Other minor polyphenols included flavones, isoorientin> orientin > isovitexin > luteolin > vitexin. The flavonols decreased as follows: quercetin robionobioside (QROB) > isoquercitrin > rutin> hyperoside. These extracts also contained phenylpyruvic acid glucoside which is a non-flavonoid. Of interest is that, for rooibos, the levels of the dihydrochalcones, flavones and flavonols associated with activity (IC_{50}) against icIL-1α and cell viability were similar between the methanol and aqueous extracts.

Except for caffeine and EGC, the levels of the major polyphenol compounds in green tea extract associated with the IC_{50} values for the inhibition of cell viability and the decrease in icIL-1α accumulation was also higher in the methanol extracts exhibiting the highest activity.

When considering the TP and flavanol/proanthocyanidin content of green tea and rooibos extracts, lower levels were associated with the methanol extract which exhibited the highest activity against cell viability and the decrease in icIL-1α accumulation. However, the opposite was noticed when considering the level of EGCG and some of the catechins associated with the methanol extract of green tea. In rooibos the levels of TP and flavanols/proanthocyanidin associated with activity were similar as no significant difference was noticed between the IC_{50} levels of the methanol and aqueous extracts.

Mangiferin, isomangiferin and the flavanone, hesperidin, constituted the major polyphenols in the methanol extracts of the xanthone (*C. longifolia* and *C. genistoides*) and flavanone (*C. subternata* and *C. intermedia*) rich extracts of honeybush (Table 6.7). The recovery yield of compounds was significantly ($P<0.05$) lower in the aqueous extracts, specifically when considering hesperidin. However, the flavanol/pro-anthocyanidin content in the honeybush species was higher in the aqueous extracts. As no IC_{50} values for icIL-1α accumulation and cell viability could be obtained (Table 6.3) for the methanol and aqueous extracts after UVB irradiation, the association with specific polyphenolic constituents could not be assessed. However, a higher xanthone and flavanone content of the methanol extracts of the different honeybush species was associated with a dose-dependent increase in the level of icIL-1α.

The stimulation of icIL-1α by the methanol extracts was accompanied by relatively lower reduction of cell viability as compared to the aqueous extracts. Clear dose–dependent inhibitory responses on cell viability and the accumulation of icIL-1α were noticed with the aqueous extracts of *C. intermedia, C. longifolia* and *C. genistoides* and this was associated with a significant ($P<0.05$) reduction in TP, the xanthones, mangiferin and isomangiferin and the flavanone, hesperidin content. However, the aqueous extract *C. subternata,* containing the highest flavanol/proanthocyanidins levels, was associated with the stimulation of icIL-1α. The inhibitory activity of the aqueous extracts on icIL-1α accumulation was associated with marked reduction of cell viability.

Table 6.6. Concentration of total polyphenol, flavanol/proanthocyanidin and monomeric compounds of green tea and rooibos extracts expressed as a function of the IC_{50} values for icIL-1α and cell viability following UVB irradiation in HaCaT cells.

Tea/herbal extracts/polyphenol subgroup	Polyphenols	Concentration (µg/mg extract)		Polyphenol equivalents/IC_{50} (µg/ml)			
				icIL_1α		ATP	
		MeOH	Aq	MeOH	Aq	MeOH	Aq
C. sinensis							
	TP	$256.49\pm36.85_A$	$161.04\pm20.77_B$	23.76±4.39	28.31±18.06	17.06±5.14	22.15±7.52
	FLAVA	$132.30\pm3.44_A$	$77.57\pm1.46_B$	13.43±5.18	23.26±14.84	8.36±3.51	18.20±5.77
Catechins	EGCG	$111.93\pm3.01_A$	$46.10\pm1.49_B$	11.36±4.38	7.15±5.06	7.07±2.89	6.24±2.01
	ECG	$20.37\pm0.35_A$	$7.48\pm0.43_B$	2.08±0.82	1.08±0.79	1.29±0.53	1.01±0.33
	EGC	$42.25\pm1.79_A$	$32.00\pm3.20_B$	4.29±1.66	4.96±3.51	2.67±1.09	4.33±1.39
	EC	$14.92\pm1.06_A$	$11.28\pm1.16_B$	0.13±0.05	0.18±0.12	0.08±0.03	0.15±0.05
	Catechin	$1.32\pm0.08_A$	$1.13\pm0.01_B$	1.51±0.58	1.75±1.24	0.94±0.38	1.53±0.49
	Total	**$190.78\pm6.23_A$**	**$97.97\pm2.79_B$**	**19.38±4.44**	**12.05±2.77**	**15.11±2.93**	**13.2±2.54**
Alkaloid*	Caffeine	$57.54\pm1.59_A$	$40.10\pm0.30_B$	5.84±2.25	6.22±4.40	3.63±1.48	5.43±1.75
A. linearis							
	TP	$350.73\pm35.00_A$	$250.51\pm16.42_B$	40.94±13.58	45.69±7.86	33.74±9.89	29.93±5.95
	FLAVA	$27.10\pm0.82_A$	$18.03\pm0.60_B$	3.16±1.05	5.05±0.82	2.61±0.76	3.20±0.74
DHC	Aspalathin	$124.24\pm1.44_A$	$83.87\pm2.08_B$	14.50±4.81	15.30±2.63	11.95±3.50	10.02±1.99
	Nothofagin	$27.59\pm0.38_A$	$16.68\pm0.27_B$	3.22±1.07	3.04±0.52	2.65±0.78	1.99±0.40
	Total	**$151.82\pm1.66_A$**	**$100.55\pm1.81_B$**	**17.72±7.98**	**18.34±8.67**	**14.60±6.58**	**12.01±5.68**
Flavones	Isoorientin	$15.79\pm0.10_A$	$10.94\pm1.95_B$	1.84±0.61	1.99±0.34	1.52±0.45	1.31±0.26
	Orientin	$11.60\pm0.06_A$	$8.88\pm1.45_B$	1.35±0.45	1.62±0.28	1.12±0.33	1.06±0.21
	Vitexin	$1.60\pm0.00_A$	$1.20\pm0.00_B$	0.19±0.15	0.22±0.04	0.15±0.05	0.14±0.03
	Isovitexin	$2.62\pm0.02_A$	$1.51\pm0.16_B$	0.31±0.25	0.28±0.05	0.25±0.07	0.18±0.04
	Luteolin	$1.78\pm0.09_A$	$0.45\pm0.11_B$	0.21±0.07	0.08±0.01	0.17±0.05	0.05±0.01
	Total	**$33.39\pm0.09_A$**	**$22.98\pm3.47_B$**	**3.90±0.77**	**4.19±0.90**	**3.21±0.64**	**2.74±0.59**
Flavonols	Rutin	$4.26\pm0.01_A$	$3.60\pm0.01_B$	0.50±0.41	0.66±0.11	0.41±0.12	0.43±0.09
	Hyperoside	$3.53\pm0.07_A$	$1.48\pm0.77_B$	0.41±0.34	0.27±0.05	0.34±0.10	0.18±0.04
	Iso-Quercitrin	$4.51\pm0.03_A$	$2.00\pm0.89_B$	0.53±0.43	0.37±0.06	0.43±0.13	0.24±0.05
	QROB	$11.6\pm0.00_A$	$7.50\pm0.00_B$	1.35±1.12	2.12±0.36	1.11±0.33	1.39±0.28
	Total	**$23.90\pm0.10_A$**	**$14.57\pm1.66_B$**	**2.79±0.44**	**3.42±0.86**	**2.29±0.75**	**2.24±0.61**
Alpha-hydroxy acid	PPAG	$0.39\pm0.02_B$	$0.42\pm0.01_A$	0.45±0.37	0.77±0.13	0.37±0.11	0.50±0.10

Values represent means±standard deviations. Statistical significance between the methanol and aqueous extracts was analysed with the student t-test and significant differences ($P<0.05$) are indicated with upper case letters in subscript. Means followed by the same letter do not differ significantly. Abbreviations: TP – total polyphenols; FLAVA – flavanols/proanthocyanidins; IC_{50} – concentration yielding 50% inhibition; MeOH – methanol; Aq – aqueous; SS – soluble solids; icIL-1α- intracellular inteleukin-1 alpha; ATP- adenosine triphosphate; *C. sinensis* - green tea; Alkaloid* - non-polyphenolic compound. *A. linearis* – rooibos; EGCG - epigallocatechin gallate, EGC - epigallocatechin, ECG - epicatechin gallate, EC - epicatechin, DHC – dihydrochalchones, QROB - quercetin-3-robinobioside, PPAG - phenylpyruvic acid glycoside. (Shaded area: Data obtained from HPLC characterization of extracts as described in Chapter 3).

Table 6.7. Concentration of total polyphenols, flavanol/proanthocyanidin and monomeric compounds in methanol and aqueous extracts prepared from different honeybush species

Polyphenolic subgroups		**Concentration (µg/ mg instead)**			
***Cyclopia* spp. (xanthone-rich)**		**MeOH**	**Aq**	**MeOH**	**Aq**
		C. genistoides		***C. longifolia***	
TP		251.1±24.8$_A$	193.9±10.9$_B$	261.0±27.0$_A$	239.5±27.8$_B$
Flavanols		12.2±0.8$_A$	16.6±0.3$_B$	12.0±0.8$_A$	14.5±0.10$_B$
Xanthones	Mangiferin	156.1±4.3$_A$	77.5±0.3$_B$	180.0±6.1$_A$	126.7±3.4$_B$
	Isomangiferin	40.0±0.9$_A$	30.1±0.8$_B$	49.0±1.9$_A$	33.1±0.8$_B$
	Total	**196.1±5.0$_A$**	**107.6±0.1$_B$**	**229.0±5.6$_A$**	**160.1±4.1$_B$**
Flavanone	Hesperidin	32.9±1.0$_A$	0.41±0.0$_B$	24.6±0.7$_A$	6.7±0.4$_B$
***Cyclopia* spp. (flavanone-rich)**		***C. intermedia***		***C. subternata***	
TP		172.1±4.1$_A$	164.5±11.3$_A$	220.5±14.5$_A$	175.0±24.1$_B$
Flavanols		11.3±0.9$_B$	17.9±0.9$_A$	13.0±0.6$_B$	22.9±0.9$_A$
Xanthones	Mangiferin	67.7±2.2$_A$	40.0±0.4$_B$	62.6±1.5$_A$	22.0±3.0$_B$
	Isomangiferin	20.0±0.7$_A$	14.3±0.4$_B$	16.0±0.3$_A$	8.7±1.5$_B$
	Total	**87.8±2.9$_A$**	**54.0±0.7$_B$**	**78.5±1.3$_A$**	**30.7±4.4$_B$**
Flavanone	Hesperidin	88.8±11.6$_A$	7.3±0.6$_B$	63.3±8.6	8.0 **±** 0.2

Values represent means ± standard deviations of triplicate determinations. Statistical significance between the methanol and aqueous extracts was analysed with the student t-test and significant differences ($P<0.05$) are indicated with upper case letters in subscript. Data from the table derived chemical characterization of extracts in Chapter 3 (Table 3.2).

4. Discussion

One of the adverse biological effects in skin, following UVB radiation, is the increased production and release of intracellular IL-1α (icIL-1α) from keratinocytes (Kupper et al., 1987). The release of this primary cytokine leads to the activation of other epidermal and dermal cells which results in the induction of an inflammatory response and facilitation of cell proliferation (Dinarello, 1997; Freedberg et al., 2001). Chronic release, as mediated by different external factors such as exposure to UVB or cancer tumor promoters, is associated with epidermal hyperplasia and skin

carcinogenesis (Cohen et al., 1991; Lee et al., 1994b). Ultraviolet B radiation is also known to increase the level of reactive oxygen species (ROS) by disrupting mitochondrial dysfunction resulting in oxidative DNA damage (Soehnge et al., 1997; Gniadecki et al., 2000). The protective mechanism against DNA damage involves the induction of cell cycle arrest that allows for DNA repair while irreparable damage triggers apoptosis in a p53 dependent manner (Melnikova and Ananthaswamy, 2005). Unrepaired DNA lesions can generate mutations that confer a selective growth advantage to pre-neoplastic cell populations as they become more resistant to apoptotic cell death eventually resulting in the development of cancer. A key step that has been identified for chemoprevention of UVB-induced cell damage is the elimination of transformed cells by apoptotic cell death (Fresco et al., 2010).

Naturally occurring plants polyphenols have been targeted for use in cutaneous photo protection as they can modulate UVB-induced inflammatory and apoptotic signalling pathways associated with oxidative stress and DNA damage in skin (Mantena and Katiyar, 2006; Fresco et al., 2010; Ramachandran et al., 2012). Rooibos and honeybush also possess antioxidant and anti-inflammatory properties which have been implicated in their photoprotective and anti-carcinogenic mechanisms in skin (Na et al., 2004; Marnewick et al., 2005; Petrova et al., 2011). The anti-inflammatory activity against cytokine production by quercetin, rutin and luteolin, also present in rooibos, has been shown to inhibit the synthesis of pro-inflammatory cytokines by suppressing the activity of transcription factors and MAP kinases (Potapovich et al., 2011; Vicentini et al., 2011). Mangiferin, the major polyphenolic constituent of honeybush, was shown to inhibit the inflammatory response by suppresing cytokines as well as other pro-inflammatory mediators such as adhesion molecules and prostaglandins (Leiro et al., 2004; Garrido et al., 2004; Yeh et al., 2007). However, the activity of these herbal teas against the adverse effects of UVB irradiation on cell integrity and the induction of primary cytokines still needs to be elucidated. The current study investigated the modulatory activity of methanol and aqueous extracts of rooibos and selected honeybush species against UVB-induced effects on different cell growth parameters as well as the induction of IL-1α production in HaCaT skin keratinocytes. The activity of the herbal teas was

benchmarked against the protective properties of green tea that has been studied extensively in skin (OyetakinWhite et al., 2012).

Green tea and rooibos extracts, exhibited the highest activity against cell viability and cell proliferation although there was no significant differences between UVB irradiated and non-irradiated cells. Since UVB is known to induce oxidative stress in cells (Soehnge et al., 1997), it would appear that these extracts do not provide protection against ROS-induced cellular damage. This implies synergistic interactions between UVB and extracts of green tea and rooibos in enhancing oxidative stress in the keratinocytes, possibly due to the pro-oxidant effects of their polyphenolic constituents. It was previously hypothesised that the pro-oxidant effects of the herbal teas may be due to polyphenol/iron interactions that involve autoxidation and modulation of the mitochondrial respiratory chain complexes related to iron depletion (Chapter 3).

In vitro studies indicated that oxidative stress is associated with a decline in ATP levels and cell cycle arrest and this primarily results from the inhibition of complex II in the mitochondrial electron transport chain (Yoon et al., 2003; Byun et al., 2008). Complex II defects have also been shown to mediate UVA-induced ROS production in HaCaT epidermal keratinocytes (Gniadecki et al., 2000). Therefore, it is likely that reduction of cell viability and cell proliferation exhibited by the green tea and rooibos extracts further enhances oxidative stress either through pro-oxidant effects that involve autoxidation and/or by inducing complex II defects. Oxidative stress resulting from complex II inhibition and autoxidation of green tea polyphenols has been associated with induction of apoptosis in different cell lines (Yang et al., 2000; Elbing et al., 2005; Lemarie et al., 2011). In the present study, rooibos and green tea extracts displayed similar activity by enhancing the UVB-induced reduction in cell viability and resultant induction of apoptosis in the keratinocytes. As pre-neoplastic or initiated cells are more prone to undergo apoptosis due to DNA damage(Schulte-Herman et al., 1993), it is likely that the pro-oxidant properties of these extracts and the resultant oxidative stress further accelerates the UVB damaged keratinocytes to undergo apoptosis.

The pro-apoptotic activity of the green tea extracts may involve the major compounds, EGCG and the alkaloid, caffeine. The compounds have been shown to prevent tumor formation by enhancing apoptosis in UVB irradiated mouse skin *in vivo* (Lu et al., 2002, 2004). In addition, the underlying mechanisms involved in the pro-apoptotic activity of caffeine have been demonstrated in HaCaT cell premalignant model known to have UVB typical p53 mutations (Adhami et al., 2003; Han et al., 2011). It was shown that caffeine specifically targets keratinocytes with unrepaired DNA damage by inhibiting AKT/protein kinase B involved in UVB-induced anti-apoptotic mechanisms. Since the pro-apoptotic activity of rooibos and green tea extracts was also demonstrated in the HaCaT cell line in the present study, this suggests that they also targeted cells with unrepaired DNA damage. This in agreement with previous studies where the HaCaT cell line was also the most sensitive cell line with respect to the reduction in cell viability and the induction of apoptosis by extracts of rooibos and green tea when compared to normal and cancer skin cells (Chapters 3 and 4). The pro-apoptotic activity of green tea extracts in the UVB-exposed keratinocytes may mainly be attributed to the major catechin, EGCG since the methanol extract, containing the highest levels, exhibited a higher activity than the aqueous extract. Although caffeine was significantly higher in the methanol extract, no appreciable difference in the alkaloid equivalents associated with the IC_{50} level against cell viability was noticed between the methanol and aqueous extracts (Table 6.6). Therefore, even though caffeine is likely to play a role in the pro-apoptotic activity of green tea it may not be the main active constituent. In rooibos, the polyphenolic equivalents associated with IC_{50} values against cell viability was also similar between the methanol and aqueous extracts. However, when considering apoptosis, the methanol extract was more active further emphasising the role of pro-oxidant effects by the monomeric and/or the flavanol/proanthocyanidins polyphenolic compounds in the promotion of UVB-induced apoptosis associated with oxidative stress.

The honeybush species displayed different effects as compared to green tea and rooibos which could be related to the activity of the major polyphenolic constituents, the xanthones and flavanones. UVB-irradiated cells exhibited resistance to reduction of cell viability and inhibition of cell proliferation when exposed to the honeybush

aqueous and methanol extracts. This suggests that the extracts exhibited a protective effect against UVB-induced oxidative damage which was also noticed when considering the reduction of UVB-induced inhibition of cell proliferation and the induction of apoptosis, specifically exhibited by the methanol extracts of *C. intermedia* and *C. subternata* (Tables 6.1 and 6.4). The protective effect exhibited by the methanol extracts implicated a cytoprotective role of the major polyphenolic constituents the xanthones, mangiferin and isomangiferin, and the flavanone, hesperidin as suggested previously (Chapters 3 and 4). Mangiferin has been shown to protect human skin keratinocytes against oxidative cell death (Chae et al., 2011) while hesperidin has been found to exhibit protective effects against radiation-induced DNA damage and cell proliferation in bone marrow cells (Hosseinmehr and Nemati, 2006). Other minor constituents of honeybush, such as the flavanone, eriocitrin and flavone, scolymoside, have been reported to be more effective at protecting lipid membrane against oxidative stress and scavenging radicals(Kim et al., 2000; Joubert et al., 2008b). Of interest is that *C. subternata*, containing high level of scolymoside (Chapter 3) exhibited an enhanced protection against apoptosis. Therefore, it is possible that the xanthones may be acting additively and/or synergistically with hesperidin as well as other polyphenolic compounds such as eriocitrin and scolymoside to protect against UVB-induced oxidative stress. The protective effect of the methanol extracts against the UVB-induced apoptosis was reduced when considering the lower recovery of the xanthones and flavanones in the aqueous extract. This implies that with the reduction of the protective activity of the aqueous extracts, pro-oxidant conditions prevail with a resultant increase in cytotoxic effects. In this regard the pro-oxidant activity of the polymeric flavanols, presumably the proanthocyanidins that occur in higher levels in the aqueous extracts, could be of importance (Chapter 3). However, the role of these polymeric compounds in the pro-oxidant and pro-apoptotic activity of the honeybush still needs to be elucidated

Regarding the modulatory role on pro-inflammatory cytokine production, green tea and rooibos extracts inhibited UVB-induced icIL-1α accumulation without increasing the levels of extracellular IL-1α (Table 6.2). The effect on icIL-1α was strongly correlated with reduction in cell viability while the modulation of cell viability was achieved at lower concentrations (Fig. 6.1). This indicated that the inhibitory effect on

icIL-1α production resulted from a reduction in cell viability and not direct modulation of its transcription machinery as previously demonstrated for dexamethasone and ibuprofen (Magcwebeba et al., 2012). Since the extracts did not induce the release of icIL-1α, reduction of cell viability did not result in necrotic cell death. Green tea and rooibos extracts did not affect icIL-1 accumulation in viable cells, suggesting that the mechanism underlying anti-inflammatory activity does not involve modulation of cytokine synthesis. However, there strong inverse correlation between icIL-1α inhibition and enhanced UVB-induced apoptotic activity indicated that the reduction of the pro-inflammatory cytokine is facilitated by the removal of cells with increased levels of icIL-1α via apoptosis. It is known that, in cells undergoing apoptosis, icIL-1α is retained in the nuclear chromatin fraction and this prevents its release into the extracellular environment (Cohen et al., 2010). A similar mechanism is proposed whereby rooibos and green tea extracts counters the accumulation of UVB-induced icIL-1α in the keratinocytes by effecting apoptosis.

Honeybush tea displayed different effects when considering the modulation of the UVB-induced icIL-1α accumulation. Except for *C. subternata*, the aqueous extracts inhibited icIL-1α accumulation in a dose dependent manner, although not to the same extent when compared the extracts of green tea and rooibos. The inhibitory effect of *C. intermedia* on icIL-1α was dose-dependent and correlated strongly with the induction of apoptosis (Table 6.5), indicating that the decrease in icIL-1α accumulation followed a similar mechanism as described for green tea and rooibos. However, as the aqueous extracts of *C. genistoides*, and *C. subternata* and methanol extract of *C. longifolia* enhanced icIL-1 accumulation in viable cells, the possible mechanisms underlying this pro-inflammatory activity from the honeybush species may involve stimulation of IL-1 synthesis. In addition, these extracts exhibited pro-inflammatory activity in UVB-induced HaCaT cells despite the significant reduction of cell viability and promotion of apoptosis, the mechanisms of which needs further characterisation.

Of interest is that, *C. subternata* exhibited the highest content of scolymoside in the aqueous extract while the aqueous extract of *C. longifolia*, also contained high levels of scolymoside exhibiting lower inhibitory activity against icIL-1α. The possible stimulatory role this flavanone on icIL-1 production needs to be investigated in the

future. It would appear that the aqueous extract of *C. subternata* protect against oxidative stress effected by UVB radiation resulting in a lower level of apoptosis when compared to *C. intermedia* (Table 6.4). This became apparent as this species was far weaker in inducing apoptosis although it inhibited ATP content to a similar extent. A threshold level of apoptosis seems to exist for the effective removal of UVB-induced icIL-1α keratinocytes, therefore, a stimulatory effect induced by the extract of *C. subternata* was noticed (Table 6.3). Alternatively, it could be argued that the aqueous extract of *C. subternata* stimulate the formation of icIL-1α, as suggested for scolymoside, thereby exhibiting a pro-inflammatory response. A similar effect is noticed for the methanol extract of *C. subternata* where icIL-1α production increased in a dose dependent manner of while the inhibition of cell viability was affected at a far lesser extent as compared to the aqueous extracts (Table 6.4). This suggested that the reduction in cell viability is likely to also exhibit a threshold as discussed for apoptosis but it is not an overt marker in the modulation of icIL-1α. Both methanol extracts of *C. intermedia* and *C. subternata* exhibited the lowest inhibitory activity of cell viability whilst effectively reducing UVB-induced apoptosis (Table 6.4). For *C. intermedia*, the apoptotic activity was reduced to levels that were comparable to that of the negative control. This provided further evidence that the removal of icIL-1α from of UVB damaged cells by the different teas occurred via the stimulation of apoptosis although threshold effects are likely to exist.

In summary, the current study suggested that the polyphenolic compounds of green tea and rooibos extracts induced mitochondrial dysfunction presumably via iron-related mechanisms, enhancing UVB-induced oxidative stress and induced cell cycle arrest that specifically earmarked unrepaired cells to undergo apoptosis. Thus the inhibitory effects on icIL-1α by these tea/herbal extracts did not result from the direct modulation of its transcription as associated with dexamethasone and ibuprofen (Magcwebeba et al., 2012). Therefore, rooibos compounds, similar to green tea, may prevent against photo carcinogenesis by causing oxidative damage that accelerates the removal of unrepaired UVB-irradiated cells via apoptosis. On the other hand, the honeybush major polyphenolic compounds protected against UVB-induced oxidative stress and apoptotic cell death, which seems to potentiate inflammation as cells containing increased icIL-1α levels, are retained. The

application of methanol extracts from honeybush therefore, may not be suitable for preventing tumorigenesis after UVB-exposure as they seem to protect against the removal of harmful effects resulting in oxidative stress. These extracts should rather be considered for application in a pre-exposure model for protection against UVB induced oxidative stress in normal cells, as the major polyphenol, mangiferin has also been shown to possess anti-genotoxic activity (Satish Rao et al., 2009). However, the aqueous extracts of honeybush seem to be promising for utilisation to reduce the risk of cancer development following after UVB. Except for *C. subternata* these extracts exhibited pro-apoptotic activity associated with the removal of damage cells similar to green tea and rooibos. A previous study suggested a role for proanthocyanidins in the activity of the aqueous extracts of honeybush (Chapter 3) but this still needs to be clarified as well as how these high molecular weight compounds are transported across cellular membranes. Future studies for rooibos and honeybush aqueous extracts should further investigate the role on iron-related pro-oxidant mechanisms effecting mitochondrial impairment which results in oxidative stress and apoptosis in the protection against UVB-induced inflammation.

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Chapter 7

General discussion and conclusion

General discussion and conclusions

Anecdotal evidence of rooibos and honeybush alleviating certain skin problems and knowledge of their phenolic composition providing a scientific basis, led to the development of skin care products containing extracts of these herbal teas (Joubert et al., 2008a; Joubert and De Beer, 2011; Joubert et al., 2011). Topical use of extracts of these herbal teas has been substantiated in several studies which have also indicated their potential as chemopreventive agents against skin carcinogenesis (Na et al., 2004; Marnewick et al., 2005; Petrova, 2009). The anti-tumor and anti-inflammatory activity exhibited by rooibos and honeybush (*C. intermedia)* extracts when utilised in a mouse skin two-stage carcinogenesis model provided evidence of anti-cancer properties against tumor promotion. Although the biological properties of the polyphenolic compounds were implicated against tumor promotion, the underlying mechanisms involved are still not clear.

In order to further elucidate the possible mechanisms involved in the anti-carcinogenic activity of the herbal teas, the present study investigated the *in vitro* chemopreventive properties of aqueous and methanol extracts of the unfermented plant material of rooibos and several honeybush species in different skin cells, utilising green tea as benchmark. The first tier determined the biological activity of the extracts against the growth parameters in skin cell lines including premalignant skin keratinocytes (HaCaT), as well as malignant (CRL 7762) and non-malignant (CRL 7761) skin cells. The role of chemical constituents and antioxidant properties as predictive tools for assessing the anti-proliferative effects of the different extracts in skin cells *in vitro* was evaluated. The second tier focused on the development and validation of a post UVB exposure *in vitro* chemopreventive model. This model was used to monitor the efficacy of the extracts as chemopreventive agents against the early stages of inflammation using the primary cytokine interleukin-1α (IL-1α) as biomarker.

Effect of rooibos extracts on the growth of skin cells and the possible role of its polyphenolic constituents

Of the different herbal teas tested, rooibos extracts were the most active against the proliferation of skin cells, displaying a similar effect as green tea. These extracts inhibited the growth of skin cells by inducing mitochondrial dysfunction via membrane depolarisation. This was associated with reduction of cell viability, inhibition of cell proliferation at lower concentrations and induction of apoptosis at higher concentrations of the rooibos extracts. Induction of mitochondrial dysfunction by rooibos extracts possibly resulted from oxidative stress caused by the pro-oxidant activity of its polyphenolic constituents. In the present study, this was deduced from the strong antioxidant properties of the extracts that co-incided with the reduction in cell viability, particularly by the methanol extract which displayed the highest antioxidant activity and levels of monomeric polyphenols in Chapter 3. Previous *in vitro* studies have indicated that the strong antioxidant properties of rooibos extract (Joubert et al., 2004) can also result in iron-related pro-oxidant effects that are mainly mediated by high levels of the dihydrochalcones, aspalathin and nothofagin present in unfermented rooibos (Joubert et al., 2005). Pro-oxidant effects involving specific polyphenol-iron interactions have been implicated in the cytotoxic effects of rooibos aqueous extracts displayed against the development of altered preneoplastic foci in rat liver *in vivo* (Marnewick et al., 2009). Interaction of the rooibos polyphenolic constituents with iron was further substantiated as there was a significant reduction of the total serum iron levels in rats consuming this herbal tea. Therefore, it is likely that the *in vitro* cytotoxic effects exhibited by rooibos extracts in the present study also emanates from pro-oxidant effects resulting from interaction of aspalathin and other monomeric flavonoids with iron.

However, as the methanol extract also contained higher flavanol/pro-anthocyanidin content than the aqueous extract with the flavanol/proanthocyanidin fraction largely consisting of proanthocyanidins, in a particular procyanidin type heteropolymer (Joubert et al., 2008a), additive and/or synergistic interactions may exist between the monomeric flavonoids and the polymers. The monomeric rooibos flavonoids are likely to exert their effect at the aqueous/lipid interphase (Snijman et

al., 2009) while the polymeric compounds are known to be more effective in the lipid environment (Joubert et al., 2005). Although the iron related pro-oxidant activity of crude polymeric fractions has been demonstrated in the deoxyribose assay *in vitro*, the activity was weaker when compared to ethyl acetate fractions containing higher levels of monomeric polyphenols (Joubert et al., 2005). Thus the pro-oxidant activity of polymeric proanthocyanidins, present as minor compounds in the rooibos extracts may contribute only to a lesser extent to the cytotoxic effects when compared to the monomeric flavonoids. In addition, it has been shown that the pro-oxidative effects of rooibos monomeric and polymeric fractions decreases with fermentation while their antioxidant activity improves as fermented extracts display more protective effects in the lipid environment (Joubert et al., 2004; 2005). This could be due to enhanced levels of specific compounds in the extract from fermented rooibos. It would appear that a specific monomeric to polymeric polyphenol ratio in the rooibos extracts is required to determine whether antioxidant or pro-oxidant conditions will prevail.

Possible mechanisms involved cytotoxic effects of rooibos extracts in skin cells

The comparable activity displayed by rooibos and green tea extracts in cells *in vitro* as well as the similar ABTS radical cation scavenging potency of the major polyphenolic compounds aspalathin and EGCG (Snijman et al., 2009), suggests that the mechanisms underlying their pro-oxidative polyphenol/iron interactions are related. The two possible mechanisms that may be involved in the induction of oxidative stress leading to the reduction of cell viability by green tea and rooibos flavonoids include autoxidation and/or complex II defects in the mitochondrial respiratory chain resulting from iron depletion. Due to their low redox potential green tea catechins are known to generate free radicals in the presence of oxygen and transition metals (Korkina et al., 2008; Lambert and Elias, 2010). This interaction has been associated with mitochondrial dysfunction, cell cycle arrest and apoptosis. Therefore, it is likely that rooibos flavonoids, such as aspalathin may also cause oxidative stress resulting in cytotoxic effects by generating reactive oxygen species, phenoxyl radicals and the highly reactive Fe^{2+} in a reaction involving oxygen and an iron catalyst, as has been demonstrated for EGCG (Fig. 7.1) (Lambert and Elias, 2010).

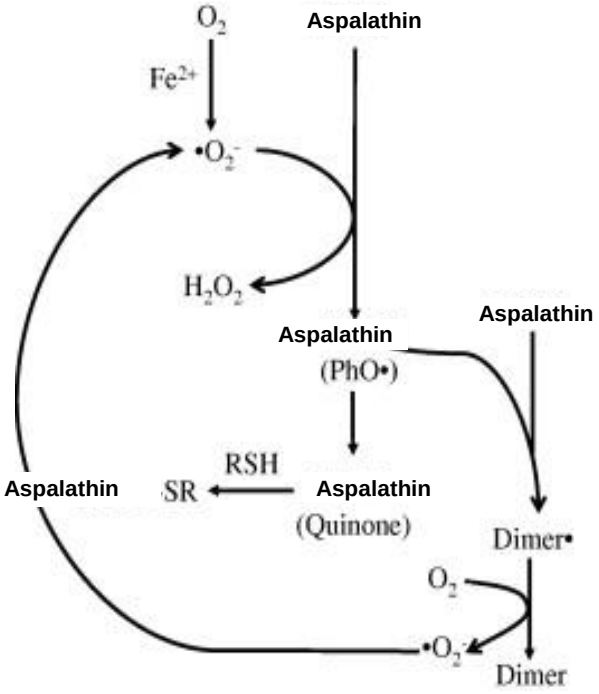

Fig. 7.1. Proposed polyphenol/ iron interaction pro-oxidative mechanism for rooibos extracts involving autoxidation of aspalathin and generation of free radicals (Adapted from Lambert and Elias, 2010). Abbreviations: RSH-sulfur containing fragment, PhO•-semi-quinone radical; SH-sulfur hydryl groups; SR-cysteine-conjugates

In the present study, the methanol extract of rooibos reduced ATP content which was accompanied by inhibition of cell proliferation at lower concentration while higher concentrations equalling ATP IC_{50} induced membrane depolarisation and caspase-3 activity associated with apoptosis. The effects of this herbal teas on cellular intergrity correlated with strong activity in the iron-related assays, this suggested that second possible mechanism (Fig. 7.2) in the cytotoxic effects of rooibos may involve the iron-chelating properties of its flavonoids which have been demonstrated *in vitro* (Snijman et al., 2009). The underlying mechanisms may involve reduction of iron levels in cells which then s cause defects in the electron transport chain that primarily target iron sulphur subunits in complex II, as previously

described for a known iron chelator, desferrioxamine (DFO) (Yoon et al., 2003). This may lead to oxidative stress as complex II inhibition has been found to increase the levels of reactive oxygen species in cells (Byun et al., 2008). Complex II defects are associated with reduction of intracellular ATP content, cell cycle arrest and membrane depolarisation involved in the initiation of apoptosis (Yoon et al., 2003). Pro-oxidant effects resulting from autoxidation and complex II defects have been associated with the preferential killing of cancer cells, as they have pre-existing mitochondrial abnormalities and a pro-oxidative status that sensitises them to further oxidative damage and cell death (Chen et al., 2010a; Lambert and Elias, 2010).

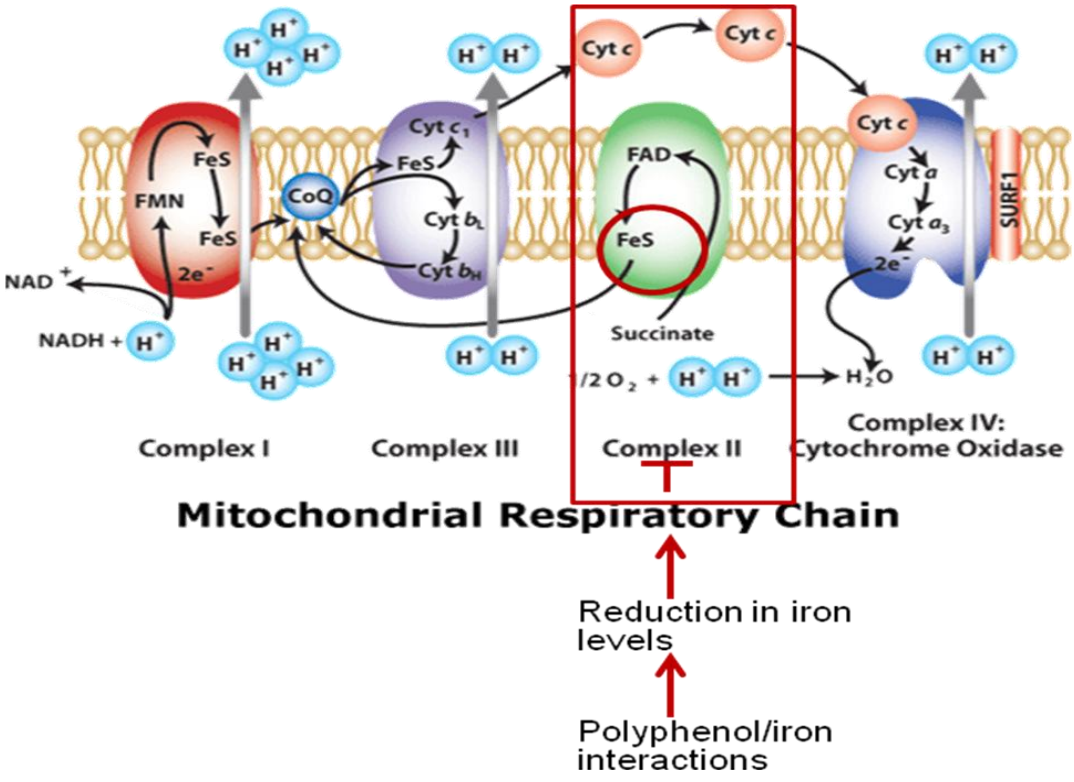

Fig 7.2. Possible mechanism in the pro-oxidant activity of rooibos extract involving iron depletion and complex II defect in respiration chain (Adapted from Baylor College of Medicine. http://www.bcm.edu/pediatrics/cardiology/surf.)

Selective toxicity of rooibos extracts against skin cells and implications for prevention of skin carcinogenesis

In the present study, a lower concentration (0.016 to 0.048 mg/mL) of the rooibos extracts inhibited the proliferation of malignant cancer cells without adversely affecting non-malignant cells. However, at higher extract concentrations of 0.130 to 0.140 mg/mL (Table B.1: Addendum B), induction of apoptosis was not selective for the malignant cancer cells. This implies that, when utilising low concentrations of the extract, rooibos may effectively retard the development of malignant tumors, however, higher concentrations present a risk of toxicity towards non-malignant healthy cells. Although malignant cells can still be removed through the induction of apoptosis, the extracts may not be effective against the late stages of carcinogenesis due to the metabolic phenotype of cancer cells, characterised by aerobic glycolysis, which confers to them apoptotic resistance (Bonnet et al., 2007). On the other hand, the premalignant cells (HaCaT) were the most responsive cell line with respect to the reduction of cell viability, inhibition of cell proliferation and induction of apoptosis when compared to the non-malignant cells. This suggests that the lower concentrations of rooibos extract will effectively delay the evolvement of early precancerous lesions during cancer promotion and target them for removal through apoptosis at a level that exhibit minimal toxicity towards non-malignant cells.

A study utilising a cancer initiation and promotion liver model in rats indicated that rooibos effectively reduces the total number of diethylnitroasamine-induced pre-neoplastic foci during cancer promotion utilising the mycotoxin fumonisin B_1 as promoter, (Marnewick et al., 2009). A similar finding was obtained in the induction of oesophageal carcinogenesis during which an aqueous extract of unfermented rooibos significantly reduced the development of papillomas induced by methylbenzylnitrosamine (Sissing et al., 2011). It was proposed that the rooibos extract could delay the altered growth pattern of these early pre-neoplastic lesions via the induction of apoptosis through mechanisms related to oxidative stress caused by the pro-oxidant activity of rooibos flavonoids. Of interest is that, with respect to the oesophageal carcinogenesis model, the epithelial tissue type in the rat is similar to that of the skin thereby closely mimicking mouse skin (Marnewick et al., 2005) as well as the skin cells utilised in the present study. This is of relevance as, in the

current study, the rooibos extracts also exhibited anti-initiation properties in the *in vitro* UVB/HaCaT model by sensitising cells to toxic effects of UVB which led to inhibition of cell proliferation and promotion of apoptosis. UVB irradiation is known to cause DNA damage which triggers apoptosis to eliminate cells with irreparable DNA damage as a way of protecting against cancer initiation (Soehnge et al., 1997). However, the genotoxic damage in surviving cells activates DNA repair mechanisms which may, due to inadequate removal of photolesions, result in p53 mutations essential for the initiation of carcinogenesis in skin. As HaCaT cells are known to contain p53 mutations that are characteristic of induction by UVB irradiation (Lehman et al., 1993), the pro-apoptotic activity exhibited by rooibos extracts indicated that this herbal tea potentiates the hosts' anti-initiation mechanisms specifically by removing keratinocytes with unrepaired DNA damage. A similar mechanism was demonstrated *in vitro* and *in vivo* for major green tea compounds, EGCG and caffeine, indicating prevention of tumorigenic transformation by stimulating UVB-induced apoptosis (Lu et al., 2002; Han et al., 2011). Therefore, one alternative for chemoprevention in the skin following UVB irradiation is to use rooibos extracts to enhance the removal of the initiated cell populations.

Anti-inflammatory activity of rooibos extracts against icIL-1α in the UVB/HaCaT model and the implication for chemoprevention

One mechanism by which the growth of precancerous cells in skin is promoted, is through the chronic induction of pathophysiological inflammation with cytokines IL-(1α/β) playing a pivotal role (Apte et al., 2002; Apte et al., 2006). Interleukin-1α (IL-1α), a primary cytokine that is constitutively produced in keratinocytes, and one of the key role players in initiating the early inflammatory events in skin and epidermal hyperplasia during skin carcinogenesis (Lee et al., 1994; Li et al., 2002). The production of IL-1α in the *in vitro* UVB/HaCaT model was optimised in the current study (Magcwebeba et al., 2012) and used a biomarker to evaluate the chemopreventive efficacy of the herbal tea extracts against the onset of inflammation. In the current study UVB irradiation was found to be the most effective pro-inflammatory agent relevant for chemoprevention studies and optimally enhanced intracellular IL-1α (icIL-1α) production with minimal cytotoxic release associated with an exacerbated inflammatory response. Validation of the

UVB/HaCaT model with known anti-inflammatory compounds, dexamethasone and ibuprofen, indicated that icIL-1α could be directly inhibited, presumably through the modulation of its transcription. The UVB-induced apoptosis in the HaCaT keratinocyte model offered an indirect anti-inflammatory mechanism, whereby icIL-1α could be biologically inactivated via retention in the nuclear chromatin network and removal through apoptosis (Cohen et al., 2010). Application of rooibos extracts, post-UVB exposure in the HaCaT keratinocyte model, indicated the role of an indirect anti-inflammatory mechanism, as the reduction of icIL-1α co-incided with its ability to promote UVB-induced apoptosis. This suggested that rooibos may prevent the onset of inflammation following UVB exposure presumably by enhancing oxidative stress through the pro-oxidant activity of its polyphenolic compounds. This would then earmark icIL-1α containing keratinocytes with DNA damage for removal by apoptosis. It would appear that rooibos extracts are likely to prevent carcinogenesis in the skin through pro-oxidative and pro-apoptotic mechanisms. These extracts may block cancer initiation and promotion by facilitating apoptotic removal of premalignant cells and preventing the onset of inflammation (Fig. 7.3). The polyphenolic compounds seem to play an important role in mediating the anti-carcinogenic effects of rooibos but their activity is likely to be dependent on concentration, polyphenol/iron interactions and a specific ratio between monomeric flavonoids and proanthocyanidin type compounds as discussed above. A summary of the major findings regarding the possible mechanisms involved in (Table 7.1)

Effect of honeybush extracts on growth of skin cells and the possible role of the polyphenolic constituents

Extracts of the different honeybush species, characterised by qualitative and quantitative differences in phenolic composition, exhibited weaker effects against the measured growth parameters of skin cells when compared to green tea and rooibos extracts. Honeybush species also inhibited the growth of skin cells presumably by causing mitochondrial dysfunction associated with reduction of cell viability and cell cycle arrest. However, the varying anti- and pro-apoptotic effects displayed by the species suggested different cytoprotective and cytotoxic roles for their polyphenolic constituents. The methanol extracts, containing higher levels of monomeric polyphenols than aqueous extracts, displayed weaker activity against the reduction

of cell viability and the inhibition of cell proliferation compared to the aqueous extracts. In addition, the methanol extracts of the relatively flavanone-rich species, *C. intermedia* and *C. subternata,* as opposed to the xanthone-rich species. *C. genistoides* and *C. longifolia*, significantly protected against apoptosis in cancer cells and UVB-exposed HaCaTs. The weaker inhibitory effects against cell viability, proliferation as well as the anti-apoptotic activity of the methanol extracts implicated the role of monomeric polyphenols in the cytoprotective effects of the honeybush extracts.

Although additive and/or synergistic interactions may exist between the monomeric polyphenolic compounds of honeybush, the xanthone, mangiferin and flavanone, hesperidin appears to be the key role players in the cytoprotective activity of the honeybush extracts. For instance, the pure compounds, mangiferin and hesperidin, did not alter the viability of treated cells even when relatively high concentrations were used. In addition, a significant reduction in the mangiferin and specifically hesperidin content in the aqueous extracts is associated with a decrease in the protective effect. The protective effect of the extracts may be dependent on a specific xanthone to flavanone ratio as the weakest activity was exhibited by the xanthone-rich species (*C. longifolia* and *C. genistoides*) containing high levels of mangiferin and isomangiferin. However, when comparing these two species, mangiferin content does not appear to be the only contributing factor as the weakest effects associated with protection were exhibited by *C. genistoides* which contained slightly lower mangiferin but higher hesperidin content than *C. longifolia*. A shift in the specific xanthone to flavanone ratio, under certain conditions, appears to favour cytotoxic effects. As indicated above, aqueous extracts with lower levels of mangiferin and hesperidin exhibited a higher activity against cell viability, inhibition of cell proliferation and exhibited pro-apoptotic activity in the different skin cells as well as in UVB exposed keratinocytes. However, other compounds may also contribute to the cytotoxicity of the honeybush extracts. The higher flavanol/proanthocyanidin content of the aqueous extracts suggested a role for polymeric proanthocyanidins in the cytotoxic effects of honeybush extracts.

Table 7.1: A summary of the findings, possible mechanisms and implications for skin carcinogenesis for rooibos extracts

Chemical and biological properties	Findings	Possible mechanisms	Implication for skin carcinogenesis
Chemical characteristics	Higher TPC and FLAVA content in MeOH extract	Polyphenol/iron interactions may enhance oxidative stress via autoxidation and complex II defects resulting from iron depletion These are likely to result from polyphenol/iron interactions involving monomeric flavonoids and polymeric proanthocyanidins Strong antioxidant properties and promotion of UVB effects implicated pro-oxidative mechanisms	Rooibos extracts may effectively block initiation by removing premalignant cell via apoptosis Extracts may also block the promotion stage by preventing onset of inflammation and inhibiting the growth of premalignant cancer cells Extracts may also effectively delay progression by inhibiting the proliferation of malignant cancer cells
	Higher levels of monomeric flavonoids in MeOH extract-		
	Aspalathin and nothofagin major monomeric flavonoids		
Antioxidant properties	MeOH extract exhibited strong activity in ORAC, ABTS, FRAP and LPO antioxidant assays		
Cell viability parameters	MeOH extract most active in reducing cell viability (ATP), proliferation (BrDU) and inducing apoptosis (Caspase-3)		
	Reduction of cell viability (IC_{50}) by MeOH extract was associated with caspase-3 activity, membrane depolarisation and DNA fragmentation		
	Selective inhibition of cancer cell proliferation at lower concentrations (0.016 to 0.048 mg/mL).		
	Selective induction of apoptosis in premalignant cells at higher concentrations (0.130 to 0.140 mg/mL)		
In vitro UVB/HaCaT model	Extracts enhanced UVB-induced reduction of cell viability, proliferation and promoted apoptosis		
	Inhibition of icIL-1α associated with apoptosis		
Predictive potential of TPC, FLAVA antioxidant properties	Strong relationship between TPC, FLAVA antioxidant properties and reduction of cell viability	Useful indicators to predict biological activity of extracts *in vitro*	May serve as quality control parameters to ensure efficacy of extracts *in vitro*

Abbreviations: ABTS - 2,2'-azinobis 3-ethyl-benzothizoline-6-sulfonic acid; ORAC- Oxygen radical absorbance capacity; FRAP- Ferric reducing antioxidant power ; LPO - Lipid peroxidation; ATP - adenosine triphosphate; BrDU; icIL-1α – intracellular interleukin 1-alpha; UVB; IC_{50} – concentration yielding 50% inhibition; TPC-total polyphenolic content; FLAVA - flavanol/proanthocyanidin content; MeOH - methanol extract.

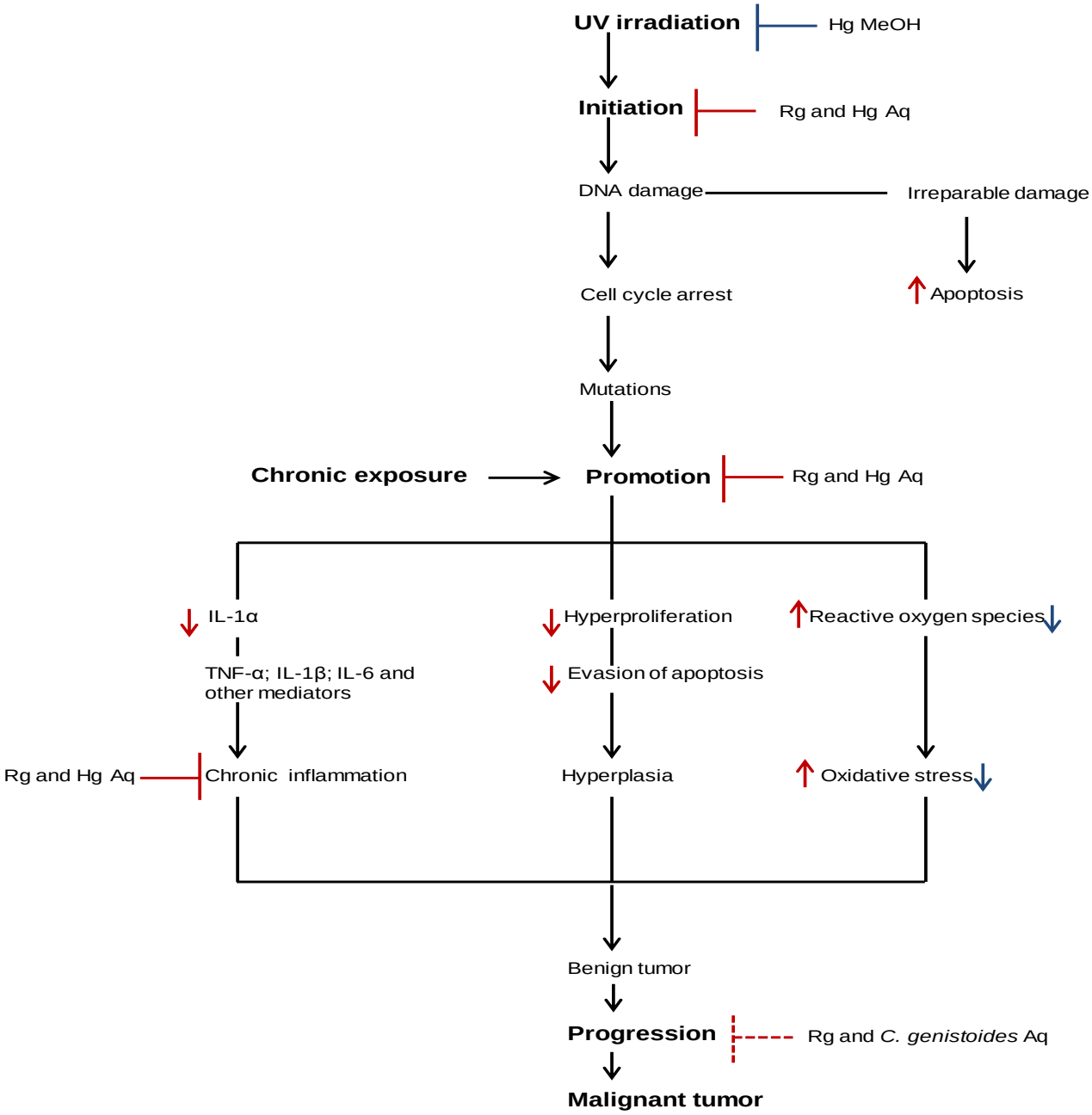

Fig. 7.3. Proposed chemopreventive targets of herbal tea extracts during skin carcinogenesis. Rooibos and aqueous extracts of honeybush may block cancer initiation and promotion by facilitating the apoptotic removal of damaged and icIL-1α containing cells. Underlying mechanisms may involve exacerbation of oxidative stress. Rooibos extracts and aqueous extracts of *C. genistoides* may also delay progression by inhibiting the proliferation of malignant cancer cells. The methanol extracts of honeybush may protect the skin against UVB-induced oxidative stress. Abbreviations: Rg - rooibos 'green' unfermented extracts; Hg - honeybush 'green' unfermented extracts; Aq - aqueous extracts; MeOH - methanol extracts. Red lines and arrows indicate target areas and activity of rooibos and aqueous extracts of honeybush. Full lines indicate blocking of the stage and broken lines indicate delay. Blue lines and arrows indicate target area and activity of honeybush methanol extracts.

Therefore specific ratios of xanthone to flavanone to flavanol/proanthocyanidins, amongst others, seem to exist in the cytoprotective and cytotoxic effect of honeybush extracts and should be further investigated.

Proposed mechanisms in the cytoprotective effects of honeybush methanol extracts

The activity of honeybush extracts, similarly to green tea and rooibos, also seems to involve modulation of cellular oxidative status by its polyphenolic compounds. The anti-apoptotic activity of the methanol extracts against cancer cells and UVB exposed HaCaT cells suggested that these extracts target cells with a pro-oxidative status and protects them against cell death by reducing oxidative stress. The underlying mechanisms involved in the cytoprotective effects of the methanol extracts may result from the antioxidant activity of the monomeric polyphenols. As the xanthone-rich species displayed high activity in the iron-related assays antioxidant assays (FRAP, LPO), a specific role for the iron chelating properties of mangiferin in the cytoprotective activity of honeybush extracts is proposed. However, unlike rooibos flavonoids and green tea catechins, polyphenol-iron interactions of mangiferin may not be associated with pro-oxidant activity. Studies have shown that complex formation of mangiferin with iron improves its cytoprotective activity and prevents its autoxidation by increasing its redox potential (Pardeu-Andreu et al., 2006a; Pardeu-Andreu et al., 2006b). The cytoprotective effects of mangiferin against metal-induced apoptosis in HepG2 liver cancer cells have been associated with reduction of oxidative stress attributed to the radical scavenging activity of this xanthone (Satish Rao et al., 2009). Hesperidin may also play a role as its cytoprotective effects have been demonstrated in primary hepatocytes and were associated with reduction of oxidative stress via radical scavenging, inhibition of lipid peroxidation and stimulation of endogenous antioxidant defense systems (Chen et al., 2010b). Although mangiferin and hesperidin may be key role players in the antioxidant effects of honeybush a role for minor polyphenols such as eriocitrin and scolymoside is indicated. High levels of these compounds were found in extract that exhibited the strongest activity against lipid peroxidation. Eriocitrin has been suggested to be as effective as mangiferin in the protection of lipid membranes

against peroxidation (Joubert et al., 2008b) whilst the radical scavenging activity of scolymoside has been reported (Kim et al., 2000). Its aglycone, luteolin, is a well-known radical scavenger (Horváthová et al., 2004; Joubert et al., 2008b). Therefore the underlying antioxidant mechanisms in the cytoprotective effects of the methanol extracts of honeybush may involve polyphenol-iron interactions, radical scavenging and stimulation of antioxidant defense system via its monomeric polyphenolic compounds.

Proposed mechanisms in the cytotoxic activity of honeybush aqueous extracts

The pro-apoptotic activity of aqueous extracts that was more prominent in the premalignant cell line and the promotion of apoptosis in the UVB/HaCaT cell model suggested that their cytotoxic activity also results from induction of oxidative stress, presumably due to the proanthocyanidin fraction. *In vitro* studies have indicated that induction of ROS-dependent cytotoxicity involving apoptosis by proanthocyanidins is a concentration dependent mechanism with higher concentrations being more pro-oxidative whilst lower concentrations are associated with cytoprotective antioxidant activity (Shao et al., 2003; Chung et al., 2009). The weak or lack of relationship between the iron related assays and the flavanol/proanthocyanidins content of the extracts as well as the reduction of cell viability in the present study, suggested that polyphenol/iron interactions may not be involved in the pro-oxidant activity of honeybush extracts. However, pro-oxidative effects involving specific polyphenol iron interactions have been implicated in the hepatotoxicity of honeybush aqueous extract in the liver carcinogenesis model (Marnewick et al., 2009). Mangiferin was considered to be involved as key role player, although it would appear that in the present study it is more associated with cytoprotection. The possible pro-oxidant effects of honeybush extracts may involve polyphenol/iron interactions that are likely to result from proanthocyanidins which has been reported previously (Wu et al., 2010). Autoxidation of catechins, monomeric flavanols known to be the building blocks of proanthocyanidins, has been ascribed to their electron donating properties (Aron and Kennedy, 2008; Korkina et al., 2008). In the present study, a moderate relationship was established between the flavanol/proanthocyanidin content, reduction of cell viability and electron donating properties of honeybush extracts as

assessed with the ABTS assay. This further suggested that the role of electron donating properties, involving the proanthocyanidins in the pro-oxidative polyphenol iron interaction, underlies the cytotoxic effects of honeybush aqueous extracts. The other mechanism proposed for the rooibos extracts was induction of respiratory defects resulting from iron depletion resulting in oxidative stress. However, this may not be applicable for honeybush, as interaction of an honeybush aqueous extract (*C. intermedia*) with iron *in vivo* was not associated with a significant reduction of total serum iron levels as was demonstrated for rooibos extracts (Marnewick et al., 2009).

Selective toxicity of honeybush extracts in skin cells and implications for prevention of skin carcinogenesis

Selective toxicity of honeybush against the survival of skin cells seems to be dependent on the species, extract concentration and cell type. For instance, at lower concentrations (0.098 to 0.385 mg/mL), selective inhibitory activity against the proliferation of malignant cancer cells was only exhibited by *C. genistoides* with the aqueous extract being more effective than methanol extract. The extracts of *C. genistoides* also displayed the weakest effect against non-malignant cells. This implies that *C. genistoides* may be the most effective of the honeybush species to delay the progression of cancer cells into malignant tumors with minimal effects on normal tissue.

However, higher concentrations of 0.209 to 0.520 mg/mL (Table 8.2; Addendum B) of the aqueous extracts of *C. subternata* and *C. intermedia* selectively induced apoptosis in premalignant cells at levels that did not adversely affect non-malignant cells. Pro-apoptotic activity associated with the pro-oxidant effects of polyphenolic constituents of honeybush extracts (*C. intermedia*) has been implicated in the altered growth kinetics of early pre-neoplastic lesions in the rat liver model and thought to play a preventive role during cancer initiation and promotion (Marnewick et al., 2009). In addition, the similar anti-tumor activity of *C. intermedia* extracts, displayed in the mouse skin and rat eosophogeal cancer models, has been suggested to interfere with cancer promotion during carcinogenesis (Marnewick et al., 2005; Sissing et al., 2011). Therefore, flavanone-rich species are more likely to target

cancer initiation and promotion during carcinogenesis, and results of the current study indicated that the aqueous extracts of these species may effectively inhibit tumor development by selectively removing premalignant cells via apoptosis (Fig 7.3).

Modulation of icIL-1α by honeybush extracts in the UVB/HaCaT model and implications for inflammation and chemoprevention

Modulation of icIL-1α content in the UVB/HaCaT model by the honeybush extracts indicated different roles for their polyphenolic constituents during inflammation and cancer promotion. The protective role of monomeric polyphenols of honeybush seems to be via potentiation ofinflammation as the methanol extracts enhanced icIL-1α by preventing UVB-stimulated cells from undergoing apoptosis. In contrast, aqueous extracts, similarly to rooibos extracts, exhibited indirect anti-inflammatory effects against icIL-1α as they removed cells containing high levels by promoting UVB-induced apoptosis. The indirect anti-inflammatory effects exhibited by the aqueous honeybush extracts are likely to result from proanthocyanidins as these compounds were also implicated in the antitumor activity against cancer promotion induced by the known pro-inflammatory 12-O-tetradecanoylphorbol-13-acetate (TPA) in the two-stage mouse skin model (Marnewick et al., 2005). Similar to rooibos extracts, the mechanisms underlying the indirect anti-inflammatory effects of honeybush extracts may involve the pro-oxidant activity of the pro-anthocyanidins which targets damaged cells with increased levels of icIL-1α to undergo apoptosis. Therefore, during cancer promotion aqueous extracts containing high levels of proanthocyanidins may prevent the onset of inflammation through the pro-oxidant and pro-apoptotic properties, thereby delaying the process of skin carcinogenesis (Fig. 7.3). In contrast, the methanol extracts containing higher levels of xanthones and flavanones may not be a viable option, as the cytoprotective activity of its monomeric compounds may promote carcinogenesis by protecting damaged cells from undergoing apoptosis. The methanol extracts may, therefore be more useful in protecting normal cells to undergoing UVB-induced damage due to their protecting effects against oxidative stress (Fig. 7.3).

Table 7.2: A summary of the findings, possible mechanisms and implications for skin carcinogenesis for honeybush extracts

Chemical and biological properties	Findings	Possible mechanisms	Implication for skin carcinogenesis
Chemical characteristics	MeOH extracts exhibited higher TPC and monomeric polyphenols Distribution of major monomeric polyphenols categorised extracts into xanthone-rich and flavanone-rich species Aqueous extracts exhibited higher FLAVA content	Cytotoxic effects of aqueous extracts are also likely to involve pro-oxidative polyphenol/iron interaction resulting from the flavanol-like proanthocyanidins Cytoprotective effects of MeOH may result from reduction of oxidative stress via the antioxidant properties of mangiferin and hesperidin involving iron chelation	Honeybush Aq extracts may also target initiation stage and promote apoptotic removal of premalignant cells Honeybush Aq extracts may also block the promotion stage by preventing onset of inflammation and inhibiting the growth of premalignant cancer cells
Antioxidant properties	MeOH extracts of xanthone-rich species were more active in ORAC, FRAP and LPO assays Aqeous extracts were highly active in ABTS assay with *C. subternata* displaying highest activity		
Cell viability parameters	Aqueous extracts exhibited stronger cytotoxic effects involving reduction of cell viability, proliferation and pro-apoptotic activity in skin cells *C. genistoides* Aqueous extracts were more effective at selectively inhibiting proliferation of malignant cancer cells at lower concentrations Higher concentrations of the flavanone-rich species selectively induced apoptosis in premalignant cells		
In vitro UVB/HaCaT model	Aq extracts enhanced UVB-induced reduction of cell viability, proliferation and promoted apoptosis Inhibition of icIL-1α associated with apoptosis MeOH extract increased resistance of cells against UVB-induced cytotoxic effects MeOH enhanced icIL-1α production by protecting UVB exposed cells from undergoing apoptosis		
Predictive potential of polyphenolic content and antioxidant properties	Strong activity of Aqueous extracts in cells correlated with FLAVA and ABTS. MeOH extracts activity correlated with ORAC	FLAVA and ABTS may be useful indicators for cytotoxic activity of extracts ORAC may be a useful predictive tool for cytoprotection	May serve as quality control parameters to ensure efficacy of extracts *in vitro*

Abbreviations: ABTS - 2,2'-azinobis 3-ethyl-benzothizoline-6-sulfonic acid; ORAC- Oxygen radical absorbance capacity; FRAP- Ferric reducing antioxidant power ; LPO - Lipid peroxidation; ATP - adenosine triphosphate; BrDU; DNA - Deoxyribonucleic acid ; icIL-1α – intracellular interleukin 1- alpha; UVB -. Ultraviolet-B; IC_{50} – concentration yielding 50% inhibition; TPC-total polyphenolic content; FLAVA - flavanol/proanthocyanidins: MeOH- methanol extract.

Thus, methanol extracts containing high levels of xanthones and flavanone may be useful additives in skin care products such as sunscreens and anti-ageing cream formulated for prevention of UV induced skin damage, as suggested previously (Petrova, 2009)

Relevance of UVB/HaCaT model and different cell lines as preclinical screening models to determine chemopreventive efficacy of compounds

The National Cancer Institute indicated that a battery of *in vitro* assays need to be applied when testing the chemopreventive efficacy of novel compounds (Steele et al., 1998). The development of relevant new *in vitro* models for rapid and efficient screening of chemopreventive agents was further suggested as those commonly used may not be inclusive of all the relevant mechanisms involved. The criteria recommended for consideration in the development of *in vitro* model include the use of cell lines with epithelial origin relevance to human cancers, organ specificity and possession of a normal genome. In the present study an *in vitro* UVB/HaCaT model was developed to determine the specific biological mechanisms involved in the chemopreventive properties of the herbal teas. The HaCaT cell line is known to have p53 mutations that are characteristic of UVB damage (Lehman et al., 1993). Consequently, studies have utilised it as an *in vitro* premalignant model (Lei et al., 2010; Han et al., 2011) that represents the stable populations of cancer prone p53 mutated keratinocyte cells that frequently occur in sun-exposed areas (Jonason et al., 1996).

In the present study, it was further developed as model that could be used to determine the modulatory effects of chemopreventive compounds against the onset of inflammation in skin after UVB exposure using icIL-1α as a biomarker. Optimisation of icIL-1α accumulation indicated that the production and release following UVB exposure was similar to the reported response of primary human keratinocytes with a normal genome (Cohen et al., 1991). Modulation of icIL-1α was validated with known anti-inflammatory compounds, dexamethasone and ibuprofen, indicating that the model could be used to test the efficacy of novel compounds against the onset of inflammation. The pro-apoptotic activity demonstrated for green tea extract in the current model corroborated the pro-apoptotic effects of green tea

compounds in the UVB/mouse carcinogenesis model *in vivo* (Lu et al., 2002). In addition, the protective activity of the honeybush extracts in the *in vitro* UVB/HaCaT model was in line with the protective properties of honeybush extracts, prepared from *Cyclopia intermedia*, previously reported for the UVB-induced skin carcinogenesis model *in vivo* (Petrova, 2009). Thus, when considering all the above mentioned properties, the UVB/HaCaT may be a useful chemoprevention *in vitro* model to screen for the anti/pro-inflammatory activity of herbal tea extracts/compounds and other agents as well as to predict their mechanisms *in vivo* and in humans. However, caution must be exercised when the inflammatory properties of phorbol-ester like compounds are evaluatedas the HaCaT keratinocytes contain phorbol ester specific defects that affect down-stream cell signalling responses (Sudbeck et al., 1999; Ridd et al., 2010).

The premalignant (HaCaT) and malignant skin cell lines verified the possible mechanisms suggested for anti-cancer properties of herbal tea extracts *in vivo* (Marnewick et al., 2004, Marnewick et al., 2009; Petrova, 2009; Sissing et al., 2011). The non-malignant cell line offered an opportunity to assess the resultant effect of the herbal teas on normal tissue. The different skin cell lines, as well as the UVB/HaCaT model, may be useful pre-screening *in vitro* models to predict the efficacy of extracts and other agents against the different stages of skin carcinogenesis *in vivo* and in human skin.

Polyphenolic content and antioxidant properties as potential predictive tools and quality control parameters for assessing efficacy of extracts

Evaluation of the relationship between the phenolic composition and the biological properties of the herbal teas in skin cells indicated that TP content, flavanol/proanthocyanidin content and different antioxidant assays (FRAP, ABTS and ORAC) can be used as indicators to predict the cytotoxic effects of rooibos extracts *in vitro*. The methanol extract, containing the highest polyphenolic and flavanol/proanthocyanidin content and antioxidant activities, displayed the highest activity against the different cell growth parameters in the different skin cells. In contrast, the lack of correlation between TP content of honeybush extracts and biological activity in skin cells implied that the polyphenolic content may not be useful

indicators to predict the biological activity of herbal teas prepared from *Cyclopia* species. This may be attributed to the presumed dual role of the monomeric and polymeric compounds regarding the cytoprotective and cytotoxic activity of the extracts. However, the strong relationship between flavanol/proanthocyanidin content, ABTS radical cation scavenging activity and reduction of cell viability suggest that these parameters may be good predictors of cytotoxic activity. On the other hand, ORAC levels and to a certain extent, inhibition of lipid peroxidation may be used as indicators of cytoprotection effected by the honeybush extracts.

Conclusions

South Africa, due to its latitudinal position, is amongst the countries with the highest incidence rate of UVB-induced skin cancer in the world and prevention strategies involving behavioural changes are not effective due to non-compliance. The focus on cancer management in skin has been on locally devised intervention strategies that can help reduce the incidence of this preventable disease (Mqoqi et al., 2004). The chemopreventive properties exhibited by the herbal teas investigated in the present study, as well as from preceding studies, suggests that rooibos and honeybush extracts might assist in curbing the morbidity of skin cancer by being incorporated as additives into skin care products such as ointment and sunscreens as previously suggested (Petrova, 2009; Joubert and De Beer, 2011; Joubert et al., 2011). Application of rooibos extracts containing high levels monomeric flavonoids and polymeric proanthocyanidins following UVB exposure might prevent cancer development in the skin by facilitating the removal of damaged cells with pro-inflammatory activity via stimulation of UVB induced apoptosis, as indicated in the UVB/HaCaT cell model. For the first time, this study has indicated that the polyphenolic constituents of honeybush extracts may have different cytoprotective and cytotoxic roles when compared to rooibos. The honeybush aqueous extracts containing higher levels of flavanol/proanthocyanidins may also exhibit the same pro-apoptotic and indirect anti-inflammatory effects as rooibos extracts following UVB exposure. However, honeybush methanol extracts containing higher levels of the xanthones, mangiferin and iso-mangiferin, and flavanone, hesperidin, may be useful

in protecting the skin against UVB-induced oxidative stress due to their cytoprotective effects implying their use in sunscreens prior to exposure.

As a cancer cell takes a long period to develop into a malignant tumor, herbal tea extracts may be useful in controlling skin cancer by (i) targeting initiation to remove the DNA damaged cell via apoptosis and (ii) delaying the promotion stage by preventing the onset of inflammation via the induction of apoptosis and selectively inhibiting their growth. However, the levels of the active polyphenolic compounds in extracts as well as their biological properties should be standardised through a set of *in vitro* antioxidant and cell-based assays which together with the chemical characterisation could be used as quality control parameters to ensure the chemopreventive efficacy of the extracts in skin.

Future studies

Monomeric and the polymeric proanthocyanidins fractions need to be prepared from the extracts of the herbal teas and characterised chemically and standardised using different biological screening assays. The focus of the research on the rooibos monomeric and polymeric fractions as well as the polymeric proanthocyanidins of honeybush should be on cytotoxicity. Conversely, for the monomeric xanthone and flavanones of honeybush the focus should be on cytoprotection. Future studies should also investigate the effects of the extracts and polyphenolic fractions on the oxidative status of the cells utilising known markers of oxidative stress, as well as endogenous antioxidant enzymes such as catalase and superoxide dismutase and glutathione peroxidase. Special emphasis should be placed on the different roles of the polyphenolic fractions i.e. pro-oxidant activity of the monomeric polyphenols and polymeric proanthocyanidins of rooibos and polymeric proanthocyanidins fraction of honeybush as well as the antioxidant effects of the xanthone and flavanone fractions of honeybush. The relationship between their modulatory activity on the oxidative status of the cells and their cytoprotective/cytotoxic effects needs to be established. The UVB/HaCaT cell model needs to be further characterised for the production of other pro-inflammatory mediators as well as including normal skin keratinocytes. Finally, biological and molecular markers of oxidative stress, inflammation and different cell growth parameters developed *in vitro*, need to be validated in animal

models and in human skin biopsies prior to developing chemopreventive products against skin carcinogenesis.

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Addendum A

Presentations, awards and manuscripts indicated in Chapter 2, 3,4,5 and 6

Presentations, awards and manuscripts indicated in Chapter 2, 3, 4, 5 and 6

*Presentations are listed in the order indicated in the research chapters 2, 3, 4 and 6

Chapter 2

Magcwebeba, T., Riedel, S., Joubert, E., De Beer, D., and Gelderblom, W.C.A., Investigating the chemopreventive activity of selected South African herbal teas rooibos (*Aspalathus linearis*)and honeybush spp in human skin cells. International conference on African indigenous research and development initiatives, Goodnews Convention Centre Muldersdrift, Johannesburg in South Africa. Oral presentation

Manuscript published

Magcwebeba, T., Riedel, S., Joubert, E., De Beer, B., Swart, P., and Gelderblom, W., 2012. The Chemopreventive properties of selected South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp). IRCAB Journal of Natural and Applied Sciences 2, 83-95.

Chapter 3 and 4

Magcwebeba T., Gamieldien, K., Joubert, E., Swart P. and Gelderblom W.C.A. Investigating the chemopreventive activity of selected South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush *spp.* in normal and cancer human skin cells. MRC Research Open Day Conference in at the MRC Convention Center, Tygerberg, 16 - 17 October 2008. Oral presentation

Magcwebeba T., Gamieldien, K., Joubert, E., Swart P. and Gelderblom W.C.A. Investigating the chemopreventive activity of selected South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush *spp.* in normal and cancer human skin cells. PAEMS International conference, 2008, Cape Town International Convention Centre, 3-5 November 2008. Poster Presentation

Magcwebeba T., Gamieldien, K., Joubert, E., Swart P. and Gelderblom W.C.A. Investigating the anti-proliferative and pro-apoptotic activity of selected South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush *spp.* in human skin cells. PAEMS International conference, 2008, Cape Town International Convention Centre, 3-5 November 2008. Oral Presentation (Awarded highly commended presentation)

Chapter 5 and 6

Magcwebeba T., Riedel, S., Joubert, E., Swart P., and Gelderblom W.C.A., Investigating the modulatory effects of selected South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush *spp.* against primary cytokines (IL-1α and TNF-α) in skin cells. 12th Indigenous Plant Use Forum (IPUF) Conference held at the Olive Grove Hall, Infruitec-Nietvoorbij Campus, Stellenbosch (6-9 July) 2009.

Magcwebeba T., Riedel, S., Joubert, E., Swart P., and Gelderblom W.C.A.The *in vitro* modulation of IL-1alpha in keratinocytes by *Aspalathus linearis* and different *Cyclopia* spp. as a biomarker for early chemoprevention 4th annual MRC research open day held at MRC head office in Parow, Cape Town, 14-15 October 2010. Poster presentation (Awarded 3rd prize in the PhD category).

Magcwebeba, T., and Gelderblom, WCA. attended the DTI Technology Awards function to be held at Gallagher Estate in Midrand on 6 and 7 October 2010. An exhibition of the research was conducted for school children from Tembisa and the general public. Title of poster presentation: Herbal teas food for skin. The research was a runner-up in the section SMME development for the project “Biological properties - Rooibos and Honeybush tea”.

Manuscripts published

Magcwebeba, T., Riedel, S., Swanevelder, S., Bouic, P., Swart, P., and Gelderblom, W., 2012. Interleukin-1α induction in human keratinocytes (HaCaT): an *in vitro* model for chemoprevention in skin. J. Skin Cancer 2012, doi:10.1155/-2012/393681

Addendum B

Dose response effects of green tea and herbal tea extracts on the pro-apoptotic activity and the viability (ATP production) of different skin cells.

Table B1 Dose response effects of methanol and aqueous extracts of green tea and rooibos on the pro-apoptotic activity and the viability (ATP production) of different skin cells.

Teas	Skin cells	Units	Methanol extracts			Aqueous extracts		
C. sinensis	HaCaT cells	**Tea conc (mg/ml)**	**0.080**	**0.050**	**0.035**	**0.170**	**0.114**	**0.038**
		Caspase-3_F	5.75±1.26^{a}	3.54±1.00^{a}	1.14±0.22^{c*}	6.88±1.65^{a}	4.94±2.43^{ab}	1.57±0.76^{c*}
		% ATP production	36.12±6.90^{c}	49.08±12.55^{a}	79.19±7.79^{a}	47.49±5.88^{c}	67.63±3.81^{b}	83.67±8.18^{a}
		% Caspase-3/viable cells	4.40±0.67^{a}	2.01±0.40^{b}	0.42±0.18^{c}	4.11±1.45^{a}	1.81±0.19^{b}	0.47±0.06^{c}
	Normal cells	**Tea conc (mg/ml)**	**0.230**	**0.154**	**0.051**	**0.340**	**0.228**	**0.075**
		Caspase-3_F	4.33±0.68^{a}	1.98±0.36^{b}	1.22±0.18^{c*}	5.58±2.01^{a}	3.20±1.47^{b}	1.07±0.15^{c*}
		% ATP production	28.58±4.22^{c}	50.11±1.50^{b}	82.37±5.04^{a}	42.69±14.23^{c}	60.64±15.10^{b}	95.57±26.22^{a}
		% Caspase-3/viable cells	2.10±0.28^{a}	0.55±0.12^{b}	0.21±0.06^{c}	2.05±0.29^{a}	0.77±0.09^{b}	0.19±0.06^{c}
	Cancer cells	**Tea conc (mg/ml)**	**0.210**	**0.141**	**0.047**	**0.410**	**0.275**	**0.091**
		Caspase-3_F	2.13±0.25^{a}	1.79±0.20^{a}	1.26±0.37^{b*}	1.55±0.22^{a}	1.35±0.12^{a}	1.13±0.17^{b*}
		% ATP production	62.27±8.70^{c}	75.41±7.10^{b}	96.70±8.59^{a}	58.92±4.58^{c}	73.75±6.09^{b}	88.35±7.24^{a}
		% Caspase-3/viable cells	0.99±0.17^{a}	0.60±0.05^{a}	0.33±0.05^{c}	0.73±0.06^{a}	0.43±0.07^{b}	0.29±0.01^{c}
A.linearis	HaCaT cells	**Tea conc (mg/ml)**	**0.130**	**0.087**	**0.029**	**0.140**	**0.094**	**0.031**
		Caspase-3_F	4.67±0.63^{a**}	3.06±0.28^{a}	1.32±0.40^{b*}	4.89±0.78^{a}	2.33±0.59^{b}	1.24±0.25^{c*}
		% ATP production	41.74±5.09^{c}	68.12±3.27^{b}	83.04±4.51^{a}	50.41±5.35^{b}	69.76±7.86^{b}	76.36±6.61^{a}
		% Caspase-3/viable cells	4.48±1.03^{a}	1.94±0.32^{b}	0.88±0.05^{c}	3.62±0.35^{a}	1.73±0.21^{b}	0.65±0.18^{c}
	Normal cells	**Tea conc (mg/ml)**	**0.260**	**0.174**	**0.058**	**0.290**	**0.194**	**0.060**
		Caspase-3_F	2.91±0.29^{a}	2.46±0.30^{a}	1.57±0.21^{b*}	1.80±0.22^{a}	1.71±0.33^{a}	1.58±0.44^{a*}
		% ATP production	38.68±5.91	54.29±9.58	89.76±10.73	41.09±6.74^{b}	54.11±6.55^{b}	78.65±15.47^{a}
		% Caspase-3/viable cells	1.08±0.24^{a}	0.71±0.12^{b}	0.27±0.03^{c}	0.63±0.08^{a}	0.46±0.12^{a}	0.26±0.06^{b}
	Cancer cells	**Tea conc (mg/ml)**	**0.310**	**0.163**	**0.016**	**0.260**	**0.154**	**0.048**
		Caspase-3_F	1.98±0.17^{a}	1.56±0.19^{b}	1.30±0.24^{b*}	2.22±0.25^{a}	1.86±0.26^{ab}	1.46±0.39^{b*}
		% ATP production	54.53±6.51^{c}	72.00±5.23^{b}	90.60±15.84^{a}	51.72±2.39^{c}	63.19±5.56^{b}	87.05±6.65^{a}
		% Caspase-3/viable cells	0.94±0.16^{a}	0.47±0.03^{b}	0.31±0.09^{c}	0.98±0.11^{a}	0.68±0.09^{b}	0.31±0.04^{c}

Values represent means ± standard deviations of five replicates of at least two experiments. Statistical analyses for significant group effects (more than 2 groups) were assessed by either by the One-way ANOVA F-test or the Welch's test, depending if homogeneity of group variances were present (Levene's test). If significant group differences were present, the post hoc Tukey's Studentized Range Test was used for between group comparisons at 5% significance level. Statistically significant differences($P<0.05$)between tea/herbal tea concentrations (in a row) are indicated with differing lower case letters in superscript. If letters are the same, means do not differ significantly. *indicates no significant difference between tea concentration and control. Abbreviations: Caspase-3_F – caspase fold increase; Ctrl - negative control; Pos Ctrl - positive control (staurosporine); ATP – adenosine triphosphate; MeOH – methanol; Aq – aqueous. *C sinensis,* - green tea*; A. linearis,* - rooibos. Negative control: caspase-3 fold increase-1.00±0.14, % ATP production-100.00±4.88. Positive control - Staurosporine (concentration), [caspase-3 activity & cell viability]: HaCaT cells- (75nM), [4.73±0.89, % ATP production-51.83±7.95]; Normal cells- (100nM), [3.50±0.23, % ATP production-58.16±9.50]; Cancer cells- (100nM), [3.35±0.53, % ATP production-69.18±2.71]. % Caspase-3/viable cells: HaCaT cells [Ctrl-0.16±0.04; Pos Ctrl-2.08±0.55]; Normal cells [Ctrl-0.16±0.06; Pos Ctrl-1.95±0.28]; Cancer cells [Ctrl-0.14±0.04; Pos Ctrl-0.38±0.10].

Table B2 Dose response effects of methanol and aqueous extracts of honeybush species on the pro-apoptotic activity and cell viability (ATP production) in different skin cells.

Herbal teas	Skin cells	Units	Methanol extracts			Aqueous extracts		
C. intermedia	HaCaT cells	**Tea conc (mg/ml)**	**0.720**	**0.360**	**0.180**	**0.520**	**0.260**	**0.130**
		Caspase-3 fold increase	1.12±0.34^{a}*	1.34±0.29^{b}*	1.17±0.25^{a}*	3.40±0.95^{a}	1.81±0.26^{b}	1.32±0.18^{c}*
		% ATP production	60.05±12.31^{b}	72.21±11.72^{b}	94.66±4.73a	39.08±2.50^{c}	60.23±8.09^{b}	78.90±10.09^{a}
		% Caspase-3/viable cells	0.46±0.16^{a}	0.44±0.11^{a}	0.20±0.05^{b}	1.54±0.27^{a}	0.40±0.10^{b}	0.27±0.07^{b}
	Normal cells	**Tea conc (mg/ml)**	**1.370**	**0.760**	**0.150**	**0.500**	**0.295**	**0.091**
		Caspase-3 fold increase	1.09±0.11^{a}*	1.10±0.18^{a}*	0.98±0.26^{a}*	1.86±0.61^{a}	1.42±0.31^{ab}	1.16±0.26^{b}
		% ATP production	39.33±4.46^{c}	61.95±11.40^{b}	89.50±20.60^{a}	38.13±12.42^{b}	54.03±11.80^{b}	80.35±20.17^{a}
		% Caspase-3/viable cells	0.68±0.13^{a}	0.43±0.04^{b}	0.26±0.03^{c}	0.77±0.10^{a}	0.43±0.13^{b}	0.24±0.07^{c}
	Cancer cells	**Tea conc (mg/ml)**	**1.290**	**0.84**	**0.38**	**0.440**	**0.291**	**0.143**
		Caspase-3 fold increase	0.65±0.17^{c}	0.94±0.14^{b}*	1.40±0.23^{a}	1.69±0.32^{a}	1.51±0.13^{a}	1.32±0.20^{b*}
		% ATP production	49.57±5.00^{c}	66.6±07.97^{b}	95.48±9.11^{a}	48.65±8.56^{b}	63.32±8.60^{b}	92.17±8.98^{a}
		% Caspase-3/viable cells	0.14±0.06$^{a•}$	0.14±0.05$^{a•}$	0.14±0.05$^{a•}$	0.91±0.17^{a}	0.59±0.13^{b}	0.32±0.07^{c}
C. subternata	HaCaT cells	**Tea conc (mg/ml)**	**0.468**	**0.234**	**0.117**	**0.417**	**0.209**	**0.104**
		Caspase-3 fold increase	1.73±0.20^{a}	1.46±0.12^{a}	1.10±0.25^{b}*	3.27±1.00^{a}	2.03±0.44^{b}	1.45±0.22^{c}*
		% ATP production	52.72±10.24^{c}	71.09±7.65^{b}	87.17±9.40^{a}	50.33±7.21^{b}	61.50±8.53^{b}	75.80±6.51^{a}
		% Caspase-3/viable cells	1.08±0.31^{a}	0.87±0.15^{b}	0.31±0.09^{c}	2.94±0.77^{a}	1.63±0.31^{b}	1.61±0.13^{b}
	Normal cells	**Tea conc (mg/ml)**	**1.080**	**0.640**	**0.200**	**0.370**	**0.230**	**0.098**
		Caspase-3 fold increase	1.25±0.27^{a}*	1.39±0.37^{a}*	1.14±0.16^{a}*	2.11±0.61^{a}	1.77±0.37^{ab}	1.45±0.27^{a}*
		% ATP production	40.17±6.89^{c}	64.71±5.99^{b}	84.98±8.28^{a}	49.96±6.09^{c}	63.28±7.45^{b}	80.42±9.88^{a}
		% Caspase-3/viable cells	0.37±0.11^{a}	0.25±0.06^{a}	0.15±0.03^{b}	0.58±0.15^{a}	0.40±0.14^{b}	0.26±0.07^{b}
	Cancer cells	**Tea conc (mg/ml)**	**1.140**	**0.680**	**0.223**	**0.430**	**0.294**	**0.158**
		Caspase-3 fold increase	0.97±0.26^{a}*	1.04±0.12^{a}*	1.15±0.06^{a}*	1.45±0.33^{a}*	1.55±0.35^{a}*	1.28±0.23^{a}*
		% ATP production	36.09±1.31^{c}	60.68±3.46^{b}	76.79±0.70^{a}	39.68±2.28^{c}	66.38±1.77^{b}	82.00±8.73^{a}
		% Caspase-3/viable cells	0.30±0.08^{a}	0.21±0.02^{b}	0.18±0.01^{b}	0.51±0.10^{a}	0.31±0.09^{b}	0.21±0.03^{b}

Values represent means ± standard deviations of five replicates of at least two experiments. Statistical analyses for significant group effects (more than 2 groups) were assessed either by the One-way ANOVA F-test or the Welch's test, depending if homogeneity of group variances were present (Levene's test). If significant group differences were present, the post hoc Tukey's Studentized Range Test was used for between group comparisons at 5% significance level. Statistically significant differences(P<0.05)between tea/herbal tea concentrations (in a row) are indicated with differing lower case letters in superscript. If letters are the same means do not differ significantly. Statistically significant differences(P<0.05)between tea/herbal tea concentrations (in a row) are indicated with differing lower case letters in superscript. If letters are the same, means do not differ significantly. *indicates no significant difference between tea concentration and control. Abbreviations: ATP – adenosine triphosphate. Negative control: caspase-3 fold increase-1.00±0.14, % ATP production-100.00±4.88. Positive control - Staurosporine (concentration), [caspase-3 fold increase & cell viability]: HaCaT cells- (75nM), [4.73±0.89, % ATP production-51.83±7.95]; Normal cells- (100nM), [3.50±0.23, % ATP production-58.16±9.50]; Cancer cells- (100nM), [3.35±0.53, % ATP production-69.18±2.71]. %Caspase-3/viable cells: HaCaT cells [Ctrl-0.16±0.04; Pos Ctrl-2.08±0.55]; Normal cells [Ctrl-0.16±0.06; Pos Ctrl-1.95±0.28]; Cancer cells [Ctrl-0.14±0.04; Pos Ctrl-0.38±0.10].

Addendum C
Certificates

MRC Research Day - 2009

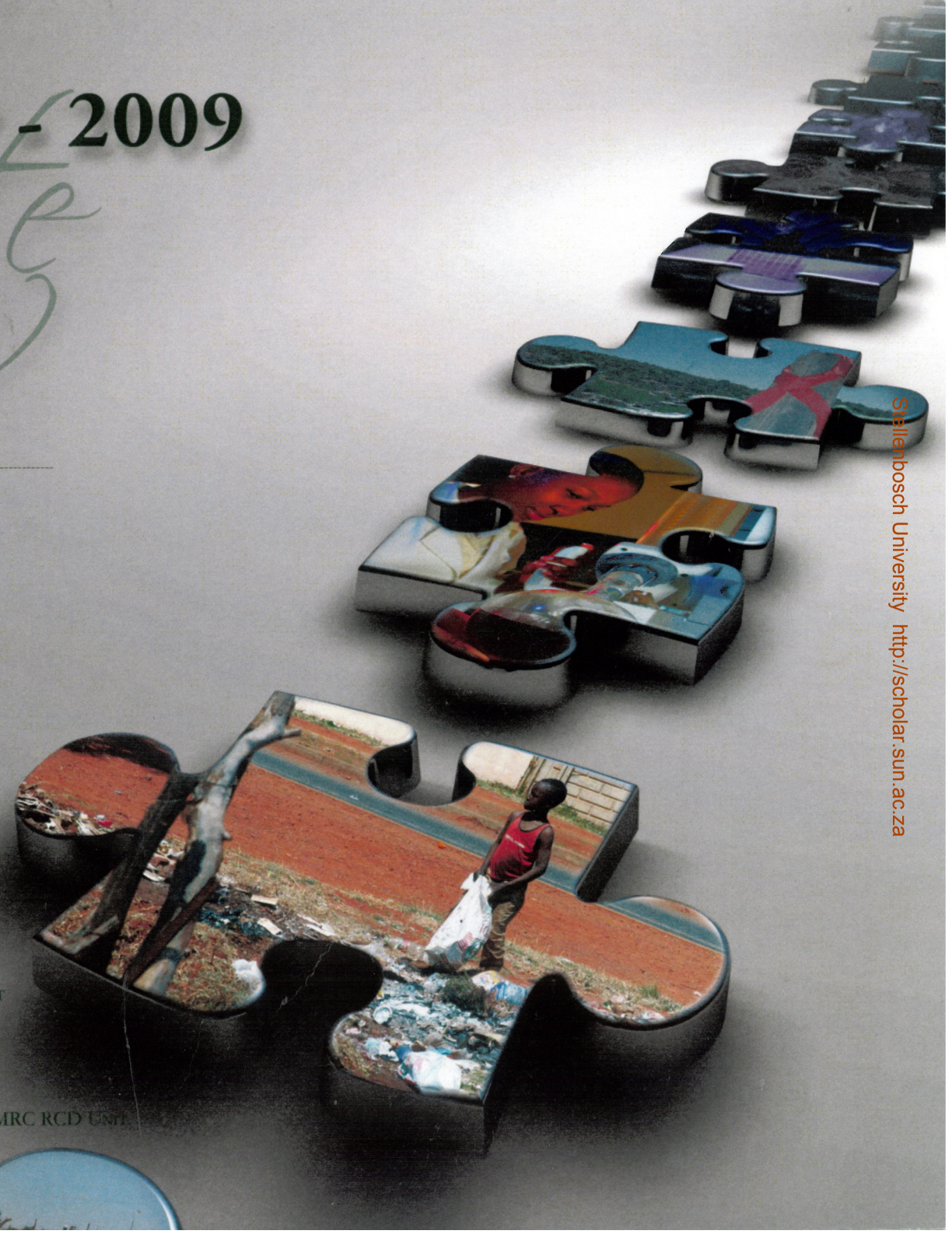
Certificate

This is to certify that

Ms T. U. Magcwabeba

has been awarded highly commended
in the Disease and Disease Mechanism category

at the MRC Annual Research Day, 2009



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4th MRC Research Day

2010

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has been awarded the 3rd prize
In the PhD category

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This is to certify that

Prof. W.C.A. Gelderblom

is the Runner-up in the THRIP SMME
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7 October 2010
Date

Mrs Bongi Maria Ntuli (MP)
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