MODULATION OF ULTRAVIOLET LIGHT-INDUCED SKIN CARCINOGENESIS BY EXTRACTS OF ROOIBOS AND HONEYBUSH USING A MOUSE MODEL: ELUCIDATING POSSIBLE PROTECTIVE MECHANISMS

By

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P R E FA C E

This thesis is submitted to fulfil the requirements for the degree Master of Technology in the discipline of Biomedical Technology. In chapter 1, a brief introduction is given to the study and the aims and approach are stated. A literature review in chapter 2 discusses concepts, which are key in understanding why the study was done and to help interpret the results obtained. Two articles, which will be submitted for publication, form chapter 3 and 4, and investigate the two main aims of the project. These two chapters contain separate introductions, results and discussions. A general summary concludes the thesis in chapter 5 and is followed by addendums, which contain additional material and results relevant to the study. As the thesis is written in article format, each chapter has separate numbering systems and references listed according to the respective journals and university requirements.

D E C L A R A T I O N

I, Antoinette Petrova, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed …… Date ……

A B S T R A C T

This thesis provides the first scientific evidence of the photoprotective properties of rooibos and honeybush herbal tea extracts and to some extent, two major honeybush polyphenols, hesperidin and mangiferin. These properties were demonstrated using *in vivo* models by:

- Providing evidence for the inhibition of tumour promotion by ultraviolet B (UVB) radiation in a two-stage skin carcinogenesis mouse model. Topical application of polyphenol-rich extracts of rooibos and honeybush prior to UVB tumour promotion of 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated mouse skin, inhibited the formation of tumours. The rooibos and honeybush extracts decreased the incidence and volume of the tumours. Topical application of hesperidin and mangiferin were less effective than the honeybush extracts as only the tumour volume was decreased, but not the incidence.

- Providing evidence for the inhibition of photodamage of the skin by UVB exposure in a mouse model. Topical application of polyphenolic rich extracts of honeybush prior to UVB irradiation of mouse skin reduced erythema, peeling, oedema and hyperplasia. The depletion of antioxidant enzymes catalase and superoxide dismutase (SOD) was prevented. The extracts protected the skin from oxidative and direct DNA damage, and reduced lipid peroxidation. The induction of cyclooxygenase-2 (COX-2) and ornithine decarboxylase (ODC) was also reduced. Topical application of the polyphenols hesperidin and mangiferin showed reduced protective effects compared to the extracts.

- Suggesting the possible mechanisms by which honeybush and the polyphenols protect against photocarcinogenesis such as reducing tumour promotion, inflammation and oxidative stress.

- Suggesting the benefits of including honeybush and rooibos as cosmeceuticals in skin care products and sunscreens as part of the strategy for preventing skin cancer.

- Discussing the recommendations for further study such as investigating more specific chemopreventive activities of these two South African herbal teas and their polyphenols, dose response studies and clinical evaluations.

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G L O S S A R Y

Actinic keratosis	:	A thick, rough or scaly spot on the skin, which occurs due to sun exposure.
Angiogenesis	:	The formation of new blood vessels.
Antioxidant	:	A molecule that reduces or prevents oxidation of other molecules by being oxidized.
Apoptosis	:	A controlled process of cell suicide that results in the degradation of a cell that is cleared by neighbouring cells without causing any spillage of the cellular contents.
Benign tumour	:	A tumour that does not grow aggressively, invade the surrounding tissue or metastasize.
Biomarker	:	A measurable biochemical indicator of a biological state, such as the progress of disease.
Carcinogenesis	:	The molecular process in which normal cells are transformed into cancer cells.
Carcinoma	:	A malignant tumour derived from epithelial tissue.
Chemoprevention	:	The use of naturally occurring or synthetic substances to prevent or slow the development of cancer.
Chromophore	:	The part of a molecule that absorbs ultraviolet radiation.
Cytokines	:	Signalling molecules made by cells, which modulate the immune response.
Cytoplasmic	:	Relating to the cytoplasm of a cell and not the nucleus or membranes.
Cytotoxicity	:	The degree to which an agent is toxic to cells.
Dermis	:	The lower of the two layers of the skin, which contains nerves, blood and lymph vessels, hair follicles and glands.
Differentiation	:	The process in which a cell progressively changes to become more specialized.
Dismutation	:	A chemical reaction in which two identical molecules are converted to two different molecules.
Endogenous	:	Originating from within an organism.
Epidermis	:	The upper of the two layers of the skin, which consists of epithelial tissue.

Epigenetic	:	Modification of gene expression without altering DNA sequence.
Epithelial tissue	:	Cells covering internal and external surfaces of organs and cavities. These cells protect the surfaces they cover against the harmful environment surrounding them.
Erythema	:	Redness of the skin due to dilatation of capillaries.
Ex vivo	:	Experimentation done on living tissue in an artificial environment.
Exogenous	:	Originating from outside of an organism.
Fenton reaction	:	The nonenzymatic reaction of hydrogen peroxide with ferrous iron (Fe_2^+) to produce hydroxyl radicals and ferric iron (Fe_3^+).
Flavonoid	:	A subgroup of polyphenols.
Free radical	:	A molecule that contains one or more unpaired electrons.
Genotoxic chemicals	:	Chemicals capable of causing damage to DNA that leads to mutations.
Hyperplasia	:	The enlargement of tissue due to an increase in proliferation of cells.
IC_{50}	:	The concentration of a substance that is required to inhibit 50% of its target biological process.
Initiation	:	The introduction of a permanent and heritable change in a cell's DNA due to exposure to a carcinogen.
In vitro	:	Experimentation done outside of a living organism, e.g. test tube.
In vivo	:	Experimentation done inside a living organism.
Inflammation	:	The process that occurs in response to injury, characterized by redness, swelling, warmth and pain that is due to an increase in blood flow, white blood cells and inflammatory chemicals.
Keratinocyte	:	The most common skin cell residing in the epidermis that produces keratin.
Malignant	:	Having the ability to invade and destroy local tissue and metastasize to other parts of the body.
Melanocyte	:	The melanin-producing cell in the epidermis of the skin.
Metastasize	:	To spread from one part of the body to another part.
Minimal erythema dose	:	The dose of ultraviolet radiation, which causes a slight redness to the skin after 24 h.

Mitogenic	:	Having the ability to induce mitosis.
Mutagenesis	:	The process of causing a genetic mutation.
Mutagenic	:	Capable of causing genetic mutations.
Oedema	:	An accumulation of fluid in intercellular tissue causing swelling.
Oncogene	:	A mutated form of a normal gene, which now contributes to the transformation of a normal cell to a tumour cell by promoting mitogenic processes in a cell.
Oxidation	:	The loss of electrons from a molecule.
Oxidative stress	:	Damage of cellular structures due to an unbalanced state between reactive species and antioxidants.
Papilloma	:	A benign tumour of derived from non-glandular or non-secretory epithelial tissue.
Perinuclear	:	Relating to the area around the nucleus.
Photochemoprevention	:	Using naturally occurring or synthetic substances to prevent or slow the development of ultraviolet light induced cancer.
Polyphenol	:	A group of compounds containing one or more phenol rings.
Progression	:	The process in which tumour cells acquire abilities such as invasiveness, metastasis and angiogenesis.
Proliferation	:	The increase in cell numbers by division.
Promotion	:	The stimulation of an initiated cell to become a tumour due to exposure to a promoter.
Proto-oncogene	:	A normal gene that when mutated, contributes to the development of cancer.
Reactive oxygen species	:	Molecules produced by the incomplete reduction of oxygen.
Reduction	:	The gain of electrons from a molecule.
Sunburn	:	The visible reaction of the skin to ultraviolet radiation exposure, which includes redness, swelling, peeling, blistering and pain.
Sunburn cell	:	Keratinocytes undergoing apoptosis after exposure to ultraviolet radiation.
Tumour suppressor gene	:	A gene that functions to protect a cell from transformation by regulating the cell cycle and apoptosis.
Ultraviolet light	:	Electromagnetic radiation with a wavelength shorter than the wavelength of violet light but longer than the wavelength of x-rays.

A B B R E V I A T I O N S

2-AAF	:	2-Acetylaminofluorene
8-OxoG	:	8-Oxo-7,8-dihydro-2'-deoxyguanosine
A	:	Adenine
AAPH	:	2,2'-Azo-bis(2-methylpropionamidine)dihydrochloride
ABTS	:	2,2-Azino-bis(3-ethylbenzothiozoline-6-sulphonic acid)
AFB_1	:	Aflatoxin B_1
ATP	:	Adenosine triphosphate
BCA	:	Bicinchoninic acid
BCC	:	Basal cell carcinoma
BHT	:	Butylated hydroxytoluene
BPO	:	Benzoyl peroxide
BSA	:	Bovine serum albumin
C	:	Cytosine
CAT	:	Catalase
CD	:	Conjugated diene
CPD	:	Cyclobutane pyrimidine dimer
COX	:	Cyclooxygenase
DAB	:	Diaminobenzidine
DETAPAC	:	Diethylenetriaminepentaacetic acid
DMACA	:	4-(Dimethylamino)-cinnamaldehyde
DMBA	:	7,12-Dimethylbenz[*a*]anthracene
DNA	:	Deoxyribonucleic acid
DNPH	:	2,4-Dinitrophenylhydrazine
DTNB	:	5,5'-Dithiobis(2-nitrobenzoic acid)

EDTA	:	Ethylenediaminetetraacetic acid
FBS	:	Foetal bovine serum
Fe(II)	:	Ferrous
FRAP	:	Ferric reducing antioxidant power
G	:	Guanine
GADD45α	:	Growth arrest and DNA damage inducible protein 45 α
GRed	:	Glutathione reductase
GPx	:	Glutathione peroxidase
GSH	:	Reduced glutathione
GSSG	:	Oxidized glutathione
HCL	:	Hydrochloric acid
H_2O	:	Water
H_2O_2	:	Hydrogen peroxide
HNE	:	4-Hydroxynonenal
HOCl	:	Hypochlorous acid
HPLC	:	High performance liquid chromatography
J	:	Joule
L•	:	Lipid radical
LH	:	Fatty acid
LOO•	:	Peroxyl radical
LOOH	:	Hydroperoxide
LPO	:	Lipid peroxide
M2VP	:	1-Methyl-2-vinylpyridinium triflate
MAPK	:	Mitogen activated protein kinase
MDA	:	Malondialdehyde
MED	:	Minimal erythema dose
MIF	:	Migration inhibitory factor
NADPH	:	β-Nicotinamide adenine dinucleotide phosphate reduced

NMSC	:	Non-melanoma skin cancer
NO•	:	Nitric oxide radical
NO^+	:	Nitrosonium cation
NO^-	:	Nitroxyl anion
NO^-_2	:	Nitrite
NO_2•	:	Nitrous oxide radical
NO_3^-	:	Nitrate
NOS	:	Nitric oxide synthase
O_2	:	Oxygen
O_2•$^-$	:	Superoxide radical
$^1O_2$•	:	Singlet oxygen radical
OH•	:	Hydroxyl radical
ODC	:	Ornithine decarboxylase
$ONOO^-$	:	Peroxynitrite
ORAC	:	Oxygen radical absorbance capacity
PBS	:	Phosphate buffered saline
PG	:	Prostaglandin
PMSF	:	Phenylmethanesulfonyl fluoride
RO_2•	:	Peroxyl radical
RNA	:	Ribonucleic acid
RNS	:	Reactive nitrogen species
ROS	:	Reactive oxygen species
RS•	:	Thiyl radical
SCC	:	Squamous cell carcinoma
SOD	:	Superoxide dismutase
T	:	Thymine
TBA	:	2-Thiobarbituric acid
TBARS	:	Thiobarbituric acid reacting substances
TEAC	:	Trolox equivalent antioxidant capacity

TPA	:	12-*O*-tetradecanoylphorbol-13-acetate
TPTZ	:	2,4,6-Tris(2-pyridyl)-s-triazine
USA	:	United States of America
UV	:	Ultraviolet

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 4: Photoprotection by honeybush extracts, hesperidin and mangiferin against UVB-induced skin damage in SKH-1 mice

ADDENDUMS

LIST OF EQUATIONS

CHAPTER 2: Literature review

ADDENDUMS

LIST OF ADDENDUMS

C H A P T E R 1

Introduction

Introduction

The skin acts as a protective barrier between the body and the external environment, and is therefore constantly exposed to damaging insults such as radiation, pathogens and chemicals. The skin is divided into two main layers, the outer epidermis and the inner dermis (Young & Heath, 2000). Ultraviolet (UV) radiation from the sun, which forms a component of natural sunlight, penetrates these two layers, with detrimental effects including inflammation, oxidative stress and damage of important cellular macromolecules such as deoxyribonucleic acid (DNA), proteins and lipids (Clydesdale *et al*., 2001; Tedesco *et al*., 1997). Chronic exposure to UV radiation can ultimately result in the development of skin cancer, which is the most prevalent cancer worldwide (Stewart & Kleinhues, 2003). Squamous cell carcinoma, a type of skin cancer, develops from keratinocytes, cells located in the epidermis of the skin, due to overexposure to UVB radiation (Markey, 1995). Squamous cell carcinoma is also the most common type of skin cancer after basal cell carcinoma. The latter develops from basal cells in the epidermis (Boi *et al*., 2003; Brooke, 2005). Melanoma, which is the third most common skin cancer, develops from melanocytes, the pigment producing cells located in the basal layer of epidermis. Melanoma can metastasize to other organs of the body, increasing the risk of mortality (Kalkman & Baxter, 2004). South Africa has one of the highest incidence rates of skin cancer in the world, and therefore, locally developed strategies to reduce the incidence of skin cancer are of interest (Mqoqi *et al.,* 2003). In order to develop these strategies, it is important to understand how skin cancer develops. Skin carcinogenesis consists of three stages; initiation, promotion and progression (Pitot & Dragan, 1991). Initiation is the introduction of a mutation in the genome of a cell and the process is generally considered an unavoidable and irreversible event. Promotion is a longer process in which initiated cells are exposed to promoting agents, which induce a favourable environment for an initiated cell to proliferate. Initiated cells respond differently to these conditions than normal cells due to the mutations gained, giving it a growth advantage over the surrounding cells. These conditions include inflammation, proliferation and changes in cell signalling (Pitot & Dragan, 1991). Promotion is considered to be a process that is reversible and therefore has become a target of cancer prevention. Progression is an accelerated process in which the cancer cells acquire abilities such as invasiveness, metastasis and angiogenesis (Hanahan & Weinberg, 2000).

Polyphenolic compounds, that are secondary plant metabolites found naturally in plants, are being investigated for potential use in inhibiting the promotion and progression stages of skin carcinogenesis (Afaq *et al*., 2002). The findings of many of these compounds have been so compelling, that they have already been included in skin care products such as anti-aging creams and sunscreens (Allemann & Baumann, 2008). Very well known examples of such

polyphenolic compounds are the catechins found abundantly in green tea produced from the plant *Camellia sinensis* (Katiyar *et al*., 2007). In South Africa, two plants that only grow locally are being investigated for their health promoting properties. *Aspalathus linearis* is harvested to produce the herbal tea, rooibos, and *Cyclopia* spp*.* is harvested to produce the herbal tea, honeybush (Joubert *et al*., 2008). The anecdotal health benefits of drinking these two herbal teas has been known for many years by local inhabitants, with growing scientific evidence to support and substantiate these beliefs (Cape Honeybush Tea, 2003; Joubert *et al*., 2008; Van Niekerk & Viljoen, 2008). Recent studies showed that rooibos and honeybush do indeed have biological properties that benefit health, such as being rich in antioxidants and having anti-inflammatory and antimutagenic activity (Joubert *et al*., 2004; Marnewick *et al*., 2000; Na *et al*., 2004; Rotelli *et al*., 2003). These properties make honeybush and rooibos good candidates for the treatment or prevention of skin carcinogenesis. Indeed, a study published in 2005 by Marnewick and coworkers demonstrated that extracts of rooibos and honeybush plant material significantly inhibited the promotion of skin carcinogenesis in mice induced by the promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA). This suggests that rooibos and honeybush may very likely prevent UV radiation-induced skin carcinogenesis as well. The health-promoting properties of rooibos have been studied for longer than honeybush, with much more literature available. Therefore it would be of interest to investigate the health-promoting properties of honeybush in more detail.

Aims and approach of this study

The aim of this study was to determine if honeybush could prevent UVB-induced skin carcinogenesis. Specific properties of honeybush that may be responsible for inhibiting UVB-induced skin carcinogenesis were investigated as possible mechanisms of protection. These properties included DNA protective, anti-inflammatory, antiproliferative and antioxidant activities. Rooibos was used as a reference in this study, as there is considerably more literature published on its health-promoting properties. The two most abundantly occurring polyphenolic compounds found in honeybush, the flavanone hesperidin and the xanthone mangiferin, were also investigated in this study to determine if these two compounds were mainly responsible for the protective properties of honeybush. Hairless SKH-1 mice were used as a model, with ethical approval for the study obtained from the Research Ethics Committee of the University of Cape Town, South Africa[1]. The following methods (summarized in **Figure 1**) were followed:

- Ethanol soluble extracts of honeybush and rooibos plant material were prepared and analysed for antioxidant capacity and flavonoid content. As the levels of specific polyphenols in plants can vary in different subspecies and geographic location, as well as by harvesting and extract preparation conditions, the content of two main polyphenolic compounds, hesperidin and mangiferin, was determined in the honeybush extracts.

- A two-stage skin carcinogenesis study investigated the possible protective effects of honeybush extracts, hesperidin and mangiferin on 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated, UVB-promoted skin carcinogenesis in female SKH-1 mice. The development of tumours was monitored over 22 weeks. Rooibos extracts were included to serve as a reference.

- A ten-day study addressed the activities of honeybush extracts, hesperidin and mangiferin on the short-term exposure of UVB radiation to the skin of female SKH-1 mice. The study monitored the sunburn response of the skin after daily exposure to UVB radiation with application of the honeybush extracts and polyphenolic compounds before exposure. Mice skin samples were also analyzed for biochemical markers of oxidative stress, inflammation and DNA damage.

[1] See Addendum 1: Ethics approval obtained

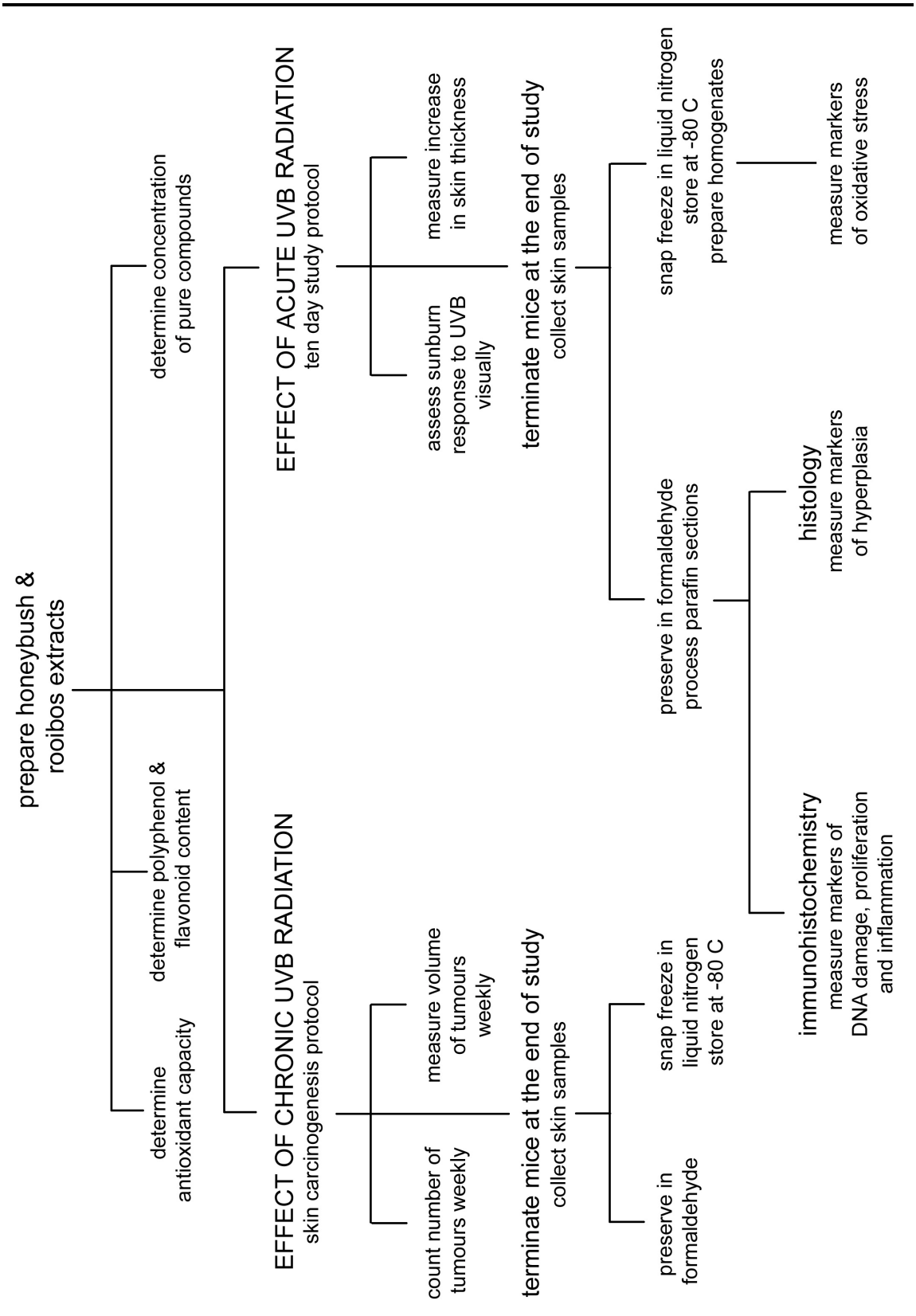

Figure 1. Scheme of the approach followed in this study

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C H A P T E R 2

Literature review

1. The skin

The primary role of the skin is to act as a protective barrier between the body and the external environment. This barrier functions to prevent physical, mechanical, biological, and chemical damage, such as harmful radiation, abrasions and wounds, microbial infection and dehydration. Skin cells damaged by the environment are removed and replaced to maintain the barrier structure (Young & Heath, 2000). Microorganisms that evade the barrier are detected by the residing skin cells, which secrete signals such as cytokines to recruit immune cells to the site of infection (Nickoloff & Turka, 1993). The most outer layer of the skin prevents dehydration by controlling the permeability of fluids (Bazzoni & Dejana, 2002). The skin is also involved in metabolic and sensory functions. Adipose tissue situated at the lowest level of the skin is a source of energy (Kuzawa, 1999), and essential vitamin D is produced in the epidermis (Holick, 2004). Cutaneous nerves are abundant in the skin, with different types of receptors to detect sensations such as touch, temperature and pain (Oaklander & Siegel, 2005). Nerves also interact with other structures in the skin to regulate internal body temperature. Nerve receptors signal sweat glands to produce sweat to cool off the surface of the skin, to arrector pili muscles of the hair shafts to insulate the body and to blood vessels to constrict or dilate to control the loss of heat through blood flow (Oaklander & Siegel, 2005; Sawasaki *et al.*, 2001).

Human skin, shown in **Figure 1**, consists of two main layers, the outer epidermis and inner dermis, separated by a basement membrane. A deeper layer to the dermis is called the hypodermis and consists of extracellular matrix and fatty connective tissue. The epidermis consists of five layers or strata: the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and the stratum basale (**Figure 2**). The most abundant cell type of the epidermis is the keratinocyte. The skin is constantly in contact with the environment, therefore a continuous process of renewal occurs, in which old and damaged keratinocyte cells, are sloughed or shed from the surface and replaced by new cells from the stratum basale (Young & Heath, 2000).

A single layer of cuboidal cells forms the stratum basale. Epidermal stem cells residing in this layer are the source of transiently amplifying daughter cells, which frequently divide to produce cells that detach from the basement membrane and move up through the epidermal layers (Alonso & Fuchs, 2003; Baba *et al.*, 2005). During this migration, the keratinocytes gradually differentiate into corneocytes. In the stratum spinosum, the keratinocytes become large polyhedral shaped with a “prickle”-like appearance due to cytoplasmic projections, which attach to neighbouring cells. Keratinocytes in this layer are actively synthesizing the protein keratin. In the stratum granulosum, the cells appear granular, due to a high density of

maturing keratin granules in the cytoplasm. In the upper parts of the stratum granulosum, cell death begins, which extends into the stratum lucidum. In the stratum corneum, organelles and the nucleus are completely lost and the resulting terminally differentiated dead cells are referred to as corneocytes. The corneocytes are filled with matured keratin filaments and are cemented together with lipids, which form a barrier to the environment and protects against water loss (Fuchs, 2007; Kanitakis, 2002; Powell & Soon, 2002).

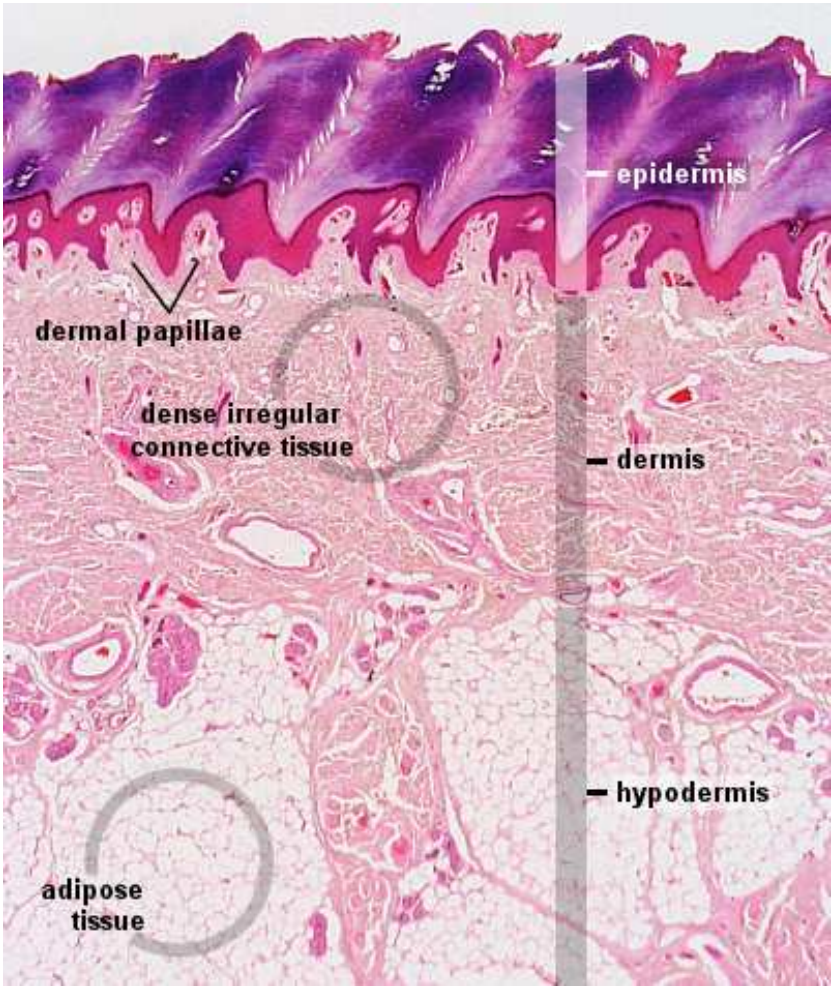

Figure 1. The structure of human skin (from University of Western Australia. 1 October 2006. *Blue histology – integumentary system.* http://www.lab.anhb.uwa.edu.au/mb140/CorePages/ Integumentary/ Integum.htm#labepidermis [11 February 2009])

Other cells in the epidermis are scattered sparsely between the keratinocytes. Melanocytes are situated in the stratum basale and are distinguishable from the basal cuboidal keratinocytes by its round shape with cellular processes, which extend between the other cells. Melanocytes produce a brown pigment called melanin, which is distributed to

surrounding keratinocytes to protect the deoxyribonucleic acid (DNA) from harmful ultraviolet (UV) radiation (Hirobe, 2004). Langerhans cells are situated in all the layers of the epidermis, as well as around blood vessels in the dermis. Langerhans cells resemble melanocytes, and are involved in immunological functions (Liu, 2001). Merkel cells are found in the stratum basale and may function as mechanoreceptors or may have neuroendocrine functions (Haeberle & Lumpkin, 2008; Kanitakis, 2002).

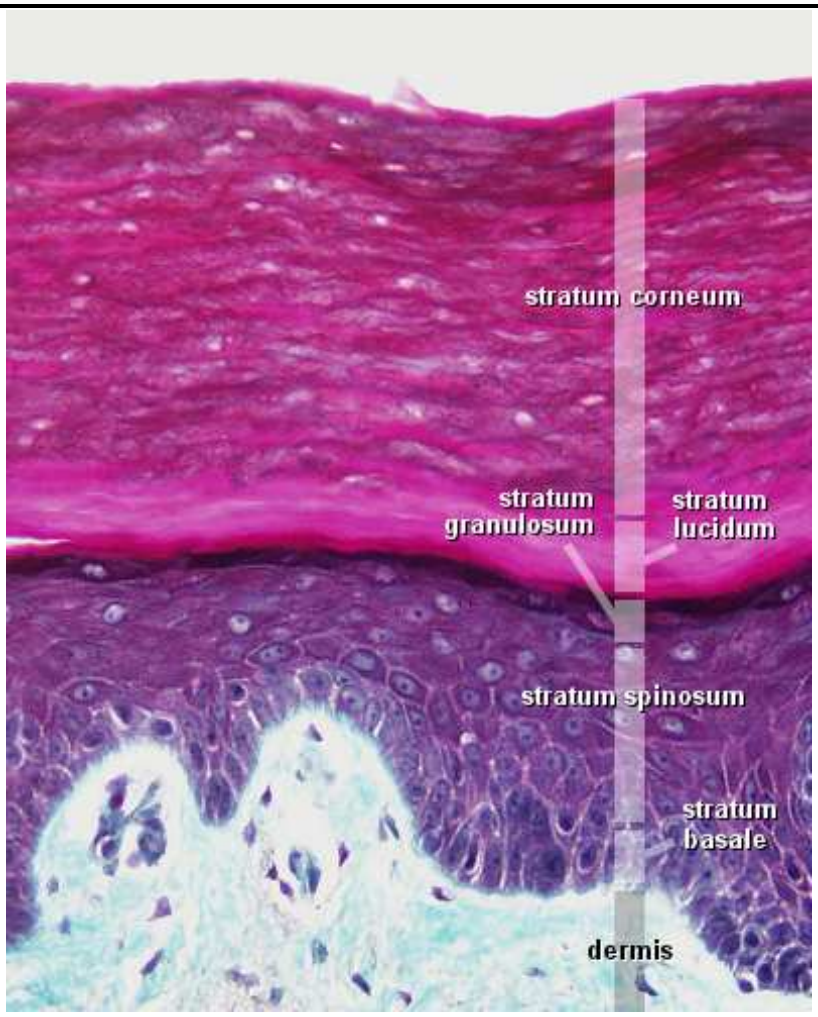

Figure 2. The epidermis (from University of Western Australia. 1 October 2006. *Blue histology – integumentary system.* http://www.lab.anhb.uwa.edu.au /mb140/CorePages/ Integumentary/ Integum. htm#labepidermis [11 February 2009])

The dermis is attached to the epidermal stratum basale by a basement membrane (lamina lucida) and consists mainly of an extracellular matrix of collagen and elastin fibres. The border between the dermis and epidermis is irregular, with dips and ridges forming the dermal papillae (**Figure 1**). Cells such as fibroblasts, mast cells, lymphocytes and

macrophages are situated mainly in this region of the dermis, which is also known as the papillary layer. Fibroblasts produce collagen, which gives the dermis its strength, elasticity, and acts as a shock absorber. Mast cells, lymphocytes and macrophages are involved in the immune response. Nerves, glands, blood vessels and lymph vessels are situated in the lower dermis region, known as the reticular layer. The hair follicle, which is based in the hypodermis, passes through the dermis and epidermis to the surface. The hypodermis situated below the dermis consists of adipose tissue and contains adipocyte cells, which produce fatty acids and triglycerides. The hypodermis is an important part of thermoregulation, energy store and a buffer against mechanical injury (Kanitakis, 2002; Young & Heath, 2000).

Disturbances in the regulation of proliferation and differentiation of the epidermis can result in skin diseases, disorders and cancers. Numerous publications have reported the use of various mouse skin models to study the pathology of skin diseases and cancer development in humans. The mouse is used to study skin carcinogenesis by inducing tumours through the repeated application of specific chemicals or UV radiation and investigating possible preventative treatments (Afaq *et al*., 2003; Zhao *et al*., 1999). Also, grafting of human skin to mice is used for the investigation of carcinogenesis in human skin directly (Beermann, 2006). Many genetically modified mouse models have been developed to understand skin diseases in humans by altering, removing or increasing the expression of specific genes involved in skin development and homeostasis (Beermann, 2006; Chen & Roop, 2008). Hairless mice are particularly popular for skin research as they are more similar to fine haired human skin than haired mice. Also, it is not necessary to use hair removal techniques, which can be time consuming, cause inflammation and interfere with hair regrowth. Hairless mice are used to study immunobiology, wound healing and the acute and chronic effects of UV radiation such as sunburn, skin cancer and aging. Hairless mice possess a mutated allele of the *Hr* gene, which causes hairlessness in mammalian skin. A number of strains of hairless mice are available with the albino SKH-1 mouse supplied by Charles River Laboratories (Wilmington, Massachusetts) one of the most commonly used (Benavides *et al*., 2009).

There are some differences in the structure of the mouse skin when compared to the human skin, which are important to consider when investigating human skin conditions using mouse models. Human skin has the irregular dermal papillae border between the epidermis and dermis called rete ridges, while the border in mouse skin is regular (Lowes *et al*., 2007). Both the epidermis and dermis of human skin is much thicker than mouse skin. The mouse skin has fewer cell layers in the epidermis and the differentiation process from the basal layer to the corneus takes 10 to 14 days to complete a cycle, whereas in the human the differentiation process takes approximately 28 days (Menon, 2002).

2. Skin Cancer

Skin cancer is the most commonly occurring cancer type in the world (Stewart & Kleinhues, 2003). Most cases of skin cancer are manageable and seldom result in mortality, as they are easily identified, diagnosed and treated. Melanoma, however, is aggressive and metastasizes to other sites of the body, resulting in secondary tumours, which require conventional cancer therapy and causes increased mortality rates. Skin cancer places a huge burden on health care services due to the high incidence and cost of treatment. Skin cancer is a public health problem in many countries, as considerable amounts of money and resources are wasted on a disease, which is preventable by employing simple personal sun protection strategies. For example, it was estimated in 2002 that in England, the total costs contributed by skin cancer was over £190 million and approximately a third of a dermatologist's and a plastic surgeon's workload is accounted for by just skin cancer care alone (Hiom, 2006). In the United States of America (USA), non-melanoma skin cancer is reported to be a substantial burden on the health care system and it is estimated that melanoma treatment will cost approximately $5 billion annually by 2010 (Housman *et al.,* 2003; Rigel *et al*., 1996). Skin cancer is Australia's most costly cancer and results in over $300 million in expenses for the healthcare system every year (International Union Against Cancer, 2006). A study in 2004 estimated that skin cancer cost the health care system of Germany €406 million annually and it is speculated that these amounts were severely underestimated, as it only reflects direct costs recorded at hospitals, and not costs from private practices and indirect costs incurred due to morbidity and mortality (Stang *et al*., 2008).

2.1. Types of skin cancer

The most common skin cancers (**Figure 3**) are malignant melanoma and the non-melanoma carcinomas. The non-melanoma carcinomas include basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Rare skin cancers include sarcoma, Merkel cell carcinoma, Paget's disease and adnexal carcinoma (Riou-Gotta *et al*., 2009).

Accounting for more than 70% of all non-melanoma skin cancers, BCC (**Figure 3a**) is the most common skin cancer worldwide (Boi *et al*., 2003). This skin cancer develops from the basal cells of the epidermis in the follicular bulges and therefore occurs mostly on hair bearing skin. As BCC is locally invasive, slow growing and rarely metastasizes, it is seldom life threatening (Brooke, 2005).

The second most common skin cancer worldwide, accounting for 22% of all non-melanoma skin cancers is SCC (**Figure 3b)** (Boi *et al*., 2003). This type of skin cancer develops from keratinocytes in the epidermis and usually occurs on the head, neck and hands. It also develops from existing actinic keratosis and on damaged skin such as ulcers and burns (Markey, 1995; Onuigbo, 2006). It is invasive and can sometimes metastasize to other organs and result in mortality (Efird *et al*., 2002).

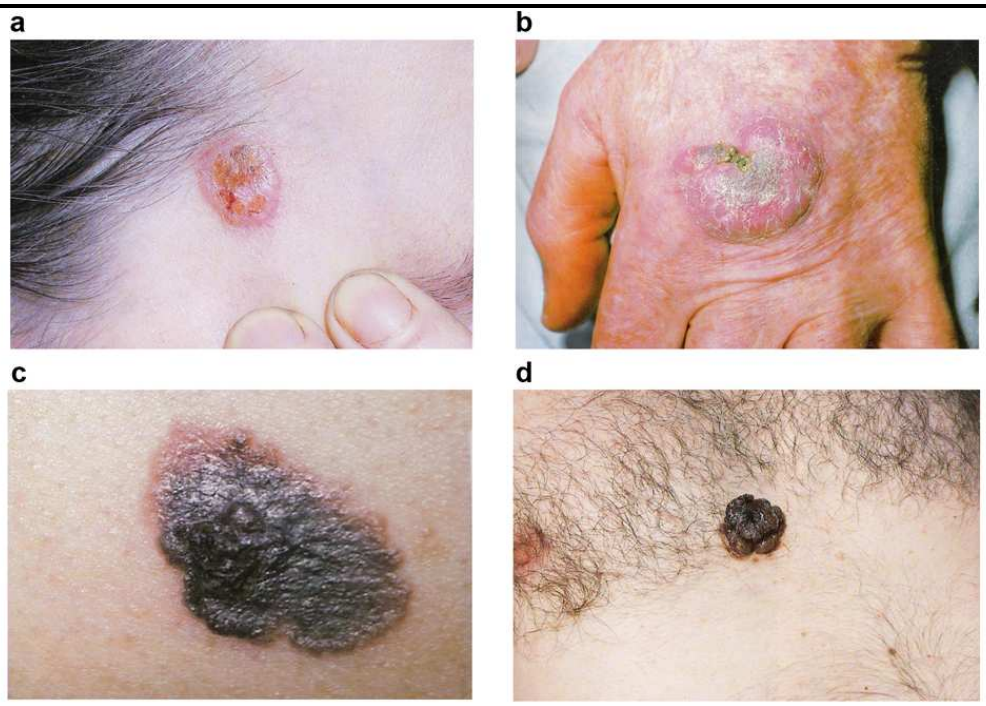

Figure 3. Common skin cancer types: (**a**) basal cell carcinoma (**b**) squamous cell carcinoma (**c**) superficial melanoma (**d**) nodular melanoma (adapted from MacKie, 1996)

Cutaneous malignant melanoma (**Figure 3 c** and **d**) accounts for approximately 9% of all skin cancers (Boi *et al.,* 2003) and is the third most common cancer worldwide (Mqoqi *et al.,* 2003). Melanoma arises from melanocytes that invade the dermis, and can develop from precursor lesions such as lentigines (freckles) or nevi (moles) or directly from the skin. Melanoma is an aggressive cancer that metastasizes quickly to organs such as the brain, lung, liver, intestine and other skin sites with increased mortality risk (Kalkman & Baxter, 2004). Tumours often occur on sun-exposed areas such as the head and neck, but also on less exposed areas like the trunk and legs (Stang *et al*., 2003).

2.2. The incidence of skin cancer

The incidence rates for skin cancer increases in countries located closer to the equator, therefore countries in Europe have lower rates than countries like Zimbabwe, South Africa and Australia. Even though some studies have reported a plateau in the incidence and mortality rates, it is estimated that the global annual rate of increase in the incidence of skin cancer is 10%, and is not declining (Bulliard & Cox, 2000; Cohn-Cedermark *et al*., 2000; Crocetti & Carli, 2003; Czarnecki & Meehan, 2000). In the USA, it is estimated that more than 1 million new cases of skin cancer occur each year and one in six Americans will acquire skin cancer in their lifetime (Housman *et al*., 2003, Jemal *et al*., 2008). There have even been increases in skin cancer incidence in European countries, which are not normally considered areas of high risk. In the Netherlands, where skin cancer occurrence is low (accounting for only 17% of all cancers), a study concluded that from 1973-2000 there was a 100% increase in cases of BCC reported (De Vries & Coebergh, 2004). In a report from Italy, the incidence of melanoma increases at a rate of 5.5% per year (Crocetti & Carli, 2003).

In the Southern hemisphere, New Zealand, Australia, Zimbabwe and South Africa have the highest incidence rates of melanoma amongst the Caucasian population groups, in the world (Giblin & Thomas, 2007; Jones *et al*., 1999; Mqoqi *et al.,* 2003). According to the South African National Cancer Registry for 1998 to 1999 (Mqoqi *et al.,* 2004), one in four South African males and one in five South African females will develop cancer in their lifetime. Basal cell carcinoma is the most common cancer in South African men and the 3rd most common in South African women, while SCC is the 3rd most common in South African men and 4th most common in South African woman. Melanoma is the ninth most common in women and the tenth most common in men (**Figure 4**).

Cancer registries show skin cancer that affects mainly the elderly, is now increasing in younger age groups as well (Czarnecki & Meehan, 2000). Since sun exposure is known to be the major cause of skin cancer, this increase in incidence is assumed to be the result of changing trends in lifestyle and depletion of the stratospheric ozone that protects the earth from damaging radiation, causing an increase in UV radiation levels (de Gruijl *et al*., 2003; United Nations Environmental Programme & Environmental Effects Assessment Panel, 2006). In the early 1900's, clothing styles were more modest. Larger areas of the body were covered with the use of hats and clothing, covering the extremities of the body. Today, clothing styles tend to allow more sun exposure of the skin. Also, travelling to sunny resorts for annual holidays has increased in popularity over the decades, resulting in more increased duration of exposure (Kojo *et al.*, 2006).

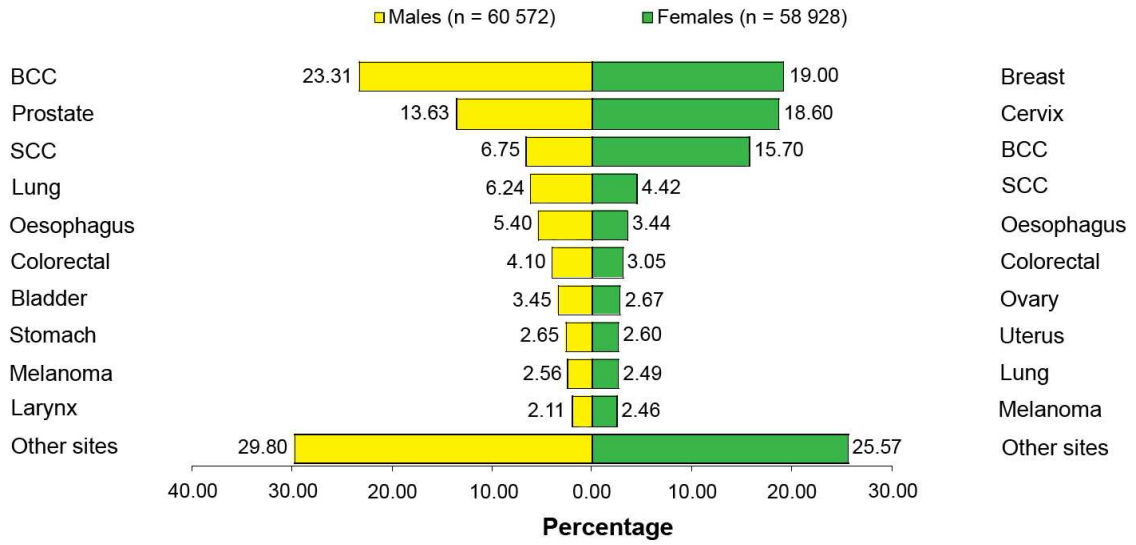

Figure 4. Cancer incidence in South Africa (adapted from Mqoqi *et al*., 2004)

Also, time spent sun tanning and sunbathing has increased, as obtaining a tan became desirable and a sign of good health. As a consequence, the use of tanning beds as an alternative to sun tanning has become popular as well. These social attitudes and beliefs have been difficult to change, even with significant increases in skin cancer education campaigns and the development of better sunscreens (Albert & Ostheimer, 2003).

2.3. Ultraviolet radiation is the major risk factor for skin cancer

The electromagnetic spectrum consists of various radiation types ranging from high frequency gamma radiation to low frequency radio waves, and also UV radiation (Diffey, 2002). The major source of UV light originates from the sun, but artificial sources such as salon sun beds and skin treatment lamps are also now available. Solar UV radiation is divided into three categories according to wavelength:

UVA (320-400 nm)
UVB (280-320 nm)
UVC (100-280 nm)

The stratospheric ozone absorbs most of the dangerous solar UV radiation, particularly UVC and up to 90% of UVB. The average UV radiation reaching the ground consists of approximately 90 - 99% UVA and 1-10% UVB, but the level of UV radiation exposure is affected by a number of conditions, such as the season and time of day, the latitude and

altitude, cloud cover and air pollution (Boyle & Levin, 2008; McKenzie *et al*., 2003). The quantity of UV radiation is measured in the radiometric unit called the joule (J) and the dose is expressed as joules per square meter (J/m^2) (Diffey, 2002).

Although the exact causes of skin cancer are not fully understood, UV radiation unquestionably plays a major role, and numerous population-based studies support this theory. For example, skin cancer occurs mainly on sun-exposed areas such as the head and neck (Lovatt *et al*., 2004; Boi *et al.*, 2003; Chuang *et al*., 1995). Skin cancer also occurs on the trunk and limbs, areas that are typically exposed during outdoor vacation and sunbathing activities (Lovatt *et al*., 2004; Berwick *et al*., 2005*)*. Furthermore, melanoma risk was found to increase with increasing hours spent outdoors (Fears *et al.*, 2002). Chronically exposed outdoor workers have an increased risk of developing skin cancer compared to indoor workers who spend less time in the sun (Pichon *et al.,* 2005). Moreover, skin cancer incidence increases with increasing ambient solar radiation levels across the latitudes. In Australia, a high ambient radiation area, studies show that people born locally have a greater risk than people who emigrated from lower ambient radiation countries (Armstrong & Kricker, 2001; Fears *et al*., 2002). In addition, a history of severe sunburns, especially during childhood, correlates with skin cancer occurrence (Lovatt *et al.*, 2004*)*. The use of UV emitting sun beds in salons has been identified as another possible risk for the development of non-melanoma skin cancer *(*Boyd *et al.*, 2002; Karagas *et al.,* 2002*).* Also, in the majority of cases, skin cancer occurs amongst the fair skinned population groups who have less UV protecting melanin than darker skinned populations. The fair skinned phenotype includes a fair complexion, blue or green eyes, red hair, freckling and the inability to tan (Cho *et al*., 2005). For example, a study in New Mexico compared the rates of incidence between darker skinned Hispanic and fair skinned non-Hispanic people. The non-Hispanic fair skinned people displayed a 5-10 times higher incidence of skin cancer than the darker skinned Hispanics (Armstrong & Kricker, 2001). For melanoma, the number of moles (nevi) is an indication of risk. Nevus counts are related to the level of sunlight exposure and a history of sunburn (Bittencourt *et al*., 2000; Cho *et al*., 2005; Dulon *et al.,* 2002). Therapy relying on the use of UV lamps to treat the skin disease psoriasis is associated with an increased risk for the development of non-melanoma skin cancers at the site of treatment (Lim & Stern, 2005).

It is estimated that UVB causes approximately 80-90% of skin cancers in humans, while UVA contributes 10-20% (Kelfkens *et al*., 1990). Animal studies indicate that both UVA and UVB cause SCC. There are however, no animal models for BCC to study experimentally, and animal models for melanoma are scarce and often questionable (International Agency for Research on Cancer, 2005). Melanoma is inducible with UVA irradiation in opossum models, and by UVB irradiation in *xiphophorus* fish, opossum and transgenic mouse models (De

Fabo *et al*., 2004; Ley, 1997; Wood, *et al*., 2006; Yamazaki *et al*., 2005). Ultraviolet B was found to be significantly more effective than UVA at inducing melanoma (Ley, 1997). Studies confirm that UVB irradiation produces squamous cell papillomas and SCC. Ultraviolet B irradiation twice a week for approximately 30 weeks at a low dose of approximately 200 mJ/cm^2 produces 100% tumour incidence among hairless mice (Katiyar *et al*., 1997). Ultraviolet B irradiation daily for approximately 15 weeks at a low dose of 900 J/m^2, produces over 90% tumour incidence in hairless mice (van Kranen *et al*., 2005). Ultraviolet A irradiation also produces papillomas and SCC in hairless mice but UVA induced skin cancer requires much longer latency periods (over 200 days) and higher doses (220 – 240 kJ/m^2) of daily irradiation for tumours to develop compared to UVB (De Laat *et al.,* 1997; Sterenborg & van der Leun, 1990). Therefore, UVA is estimated to be up to 10 000 times less effective than UVB per J/m^2, and thus more experimental models for skin carcinogenesis studies are based on UVB-induced skin cancer (International Agency for Research on Cancer, 1992).

3. Skin carcinogenesis

Squamous cell skin carcinogenesis has been studied for many years as a model for understanding cancers of other organs. Compared to internal organs of the body, the skin is easily accessible and the experimental environment easily controlled. Carcinogenesis of the skin appears to develop in a sequential manner according to a process called multistage carcinogenesis (Yuspa, 2000).

3.1. Multistage carcinogenesis

Multistage carcinogenesis is a widely accepted hypothesis in the scientific community for the development of skin cancer. Skin carcinogenesis is divided into three stages, which occur due to disturbances in growth regulating pathways, namely: initiation, promotion and progression (Pitot & Dragan, 1991).

Initiation (**Figure 5**) is a single irreversible genetic event within an individual cell, in which a genotoxic agent called the initiator, binds directly to DNA causing damage, resulting in DNA mutations in critical genes of the genome. These critical genes are usually tumour suppressors or proto-oncogenes. Transformation inhibiting tumour suppressors are deactivated, and transformation-encouraging oncogenes are activated by these mutations (Bowden *et al*., 1994). Mutated genes commonly found in skin cancers include the p53 tumour suppressor gene and Ha-*ras* oncogene (Bardeesy *et al*., 2001; Giglia-Mari & Sarasin, 2003; Pierceall *et al*., 1992). Initiated cells are the precursors for the formation of tumours as the mutation is passed on to daughter cells during clonal expansion. Initiated cells have

acquired abilities such as evading apoptosis and growth self-sufficiency, but remain phenotypically normal as they are not distinguishable from surrounding cells and tissues until exposed to further stimuli which encourage these properties (Brown *et al.*, 1990; Hanahan & Weinberg, 2000; Yuspa, 2000).

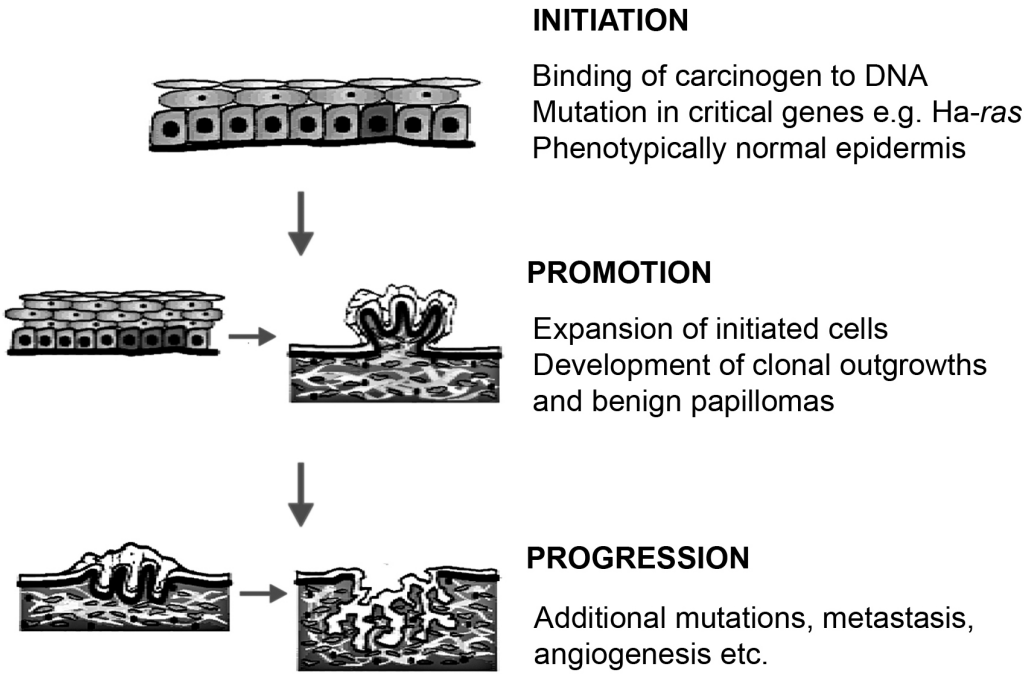

Figure 5. Multistage skin carcinogenesis (adapted from Hursting *et al*., 1999)

In the skin (**Figure 6**) initiated keratinocytes will evade terminal differentiation signals and apoptosis and continue to divide, forming benign lesions such as actinic keratosis. The initiated cell may never change further in its lifetime if not exposed to a promoter. The polycylic aromatic hydrocarbon, 7,12-dimethylbenz[*a*]anthracene (DMBA) is an example of a skin cancer initiator, which reacts with purine bases in DNA forming depurinated DNA adducts that, when erroneously repaired by DNA repair enzymes, result in mutation of the Ha-*ras* oncogene (Brown *et al*., 1990; Chakravarti *et al.,* 2008). Ultraviolet B radiation is also an initiator as it has been shown to cause genetic mutations in critical genes such as p53 and Ha-*ras* (Giglia-Mari & Sarasin, 2003; Pierceall *et al*., 1992).

Promotion (**Figure 5**) is a long-term event in which there must be continuous exposure to a promoter or promoters. During promotion, proliferation and clonal expansion occurs among initiated cells due to selective pressure from a promoter, forming a premalignant tumour (Yuspa, 2000). A promoter is thought to have properties, which confer a growth advantage to

the mutated cells. These properties promote oxidative stress and inflammation, an environment, which is not favourable for normal cells. Promoters can also increase proliferation and the synthesis of DNA, ribonucleic acid (RNA) and proteins in cells, decrease epidermal differentiation, alter cell morphology and have epigenetic effects that encourage carcinogenesis by altering gene expression. Promotion is reversible in the early stages, as seen when tumours regress after removal of the promoter, but later becomes irreversible (Slaga *et al*., 1980). Promotion in the skin (**Figure 6**) leads to the formation of benign tumours called papillomas. Cutaneous cancer promoters include chemical agents such as phorbol esters and physical agents such as chronic wounding. Benzoyl peroxide (BPO), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and UVB are commonly used promoters in skin carcinogenesis studies, which cause proliferation or hyperplasia of keratinocytes, inflammation and oxidative stress in the skin (Afaq *et al*., 2003; Jang & Pezzuto, 1998; Zhao *et al*., 2000).

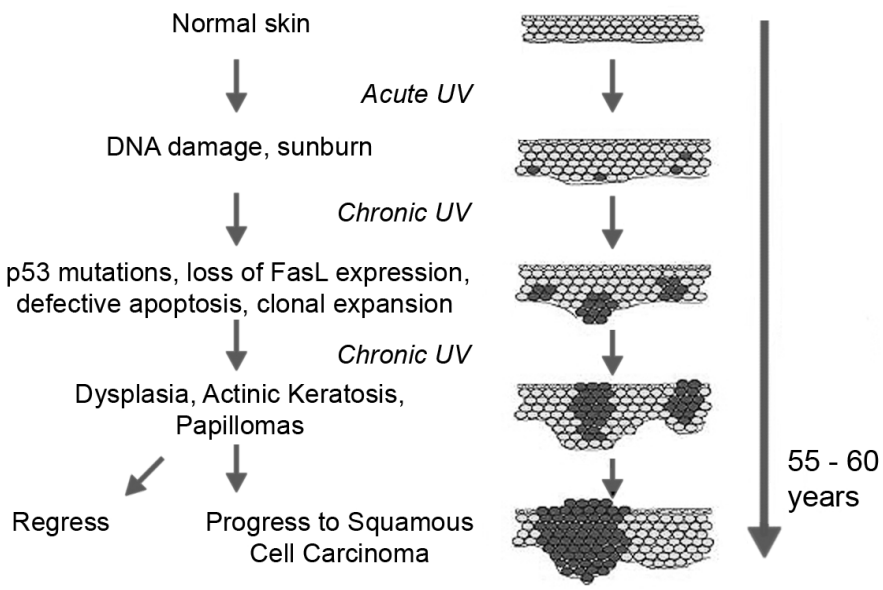

Figure 6. The development of ultraviolet radiation-induced human squamous cell carcinoma (adapted from Melnikova & Ananthaswamy, 2005)

Progression (**Figure 5**) is an accelerated process in which the tumour exhibits characteristic phenotypes such as invasiveness, metastasis and angiogenesis (Hanahan & Weinberg, 2000). During progression, there is further exposure to mutagens, which produce mutations in genes such as tumour suppressors and oncogenes (Bowden *et al*., 1994). In the skin (**Figure 6**), benign papillomas may progress to malignant SCC.

3.2. Animal models for squamous cell carcinoma

As it is difficult to obtain human tissue to study tumourigenesis, animals such as mice, which are readily available, have been useful in elucidating the process of carcinogenesis (Odashiro *et al*., 2005; Wu & Pandolfi 2001). The benefits of using the mouse include a short life span and high metabolic rate, characteristics which allow for the faster development of cancers than in humans, thus reducing experimental time. The process of skin carcinogenesis in the mouse is also very similar to humans. For example, mice develop tumours in the same tissues and in a similar histopathological course as seen in humans (Balmain & Harris, 2000). In addition, the types of mutations found in murine skin cancers are similar to those found in human skin cancers, therefore identifying genes involved in human carcinogenesis can be done using transgenic mice where specific genes are knocked out or over-expressed (Dumaz *et al.*, 1997; Wu & Pandolfi, 2001). Signal transduction pathways, activated after UVB irradiation and epigenetic changes such as aberrant DNA methylation patterns in human skin cancers are comparable with the mouse skin carcinogenesis model. For example, the activation of the mitogen-activated protein kinase (MAPK) pathways results in changes in protein phosphorylation and gene transcription (Einspahr *et al*., 2008). The gene *E-cadherin,* which is involved in cell to cell adhesion in the skin, is silenced through increased methylation in both human and murine cancers resulting in increased invasiveness (Fraga *et al*., 2004). All these features contribute in justifying the use of the mouse for skin carcinogenesis studies.

Mouse skin cancer models for UV radiation-induced melanoma are limited and there are no models available for UV radiation-induced basal cell carcinoma (Ha *et al*., 2005). Two-stage mouse models for squamous cell carcinoma are however, well established. Initiation occurs with a single topical application of the carcinogen DMBA to the skin, and promotion occurs with multiple exposures to UVB (Kyriazi *et al*., 2006; Tanaka *et al*., 2004; Wang *et al*., 1994; Zhaorigetu *et al*, 2003). Application of a promoter leads initiated cells to the development of benign squamous papillomas, which sometimes progress to SCC. Potential cancer preventing agents are commonly tested at the promotion stage, as promotion is reversible while initiation is irreversible and less avoidable (Zhao *et al*., 1999). The potential agent is tested before, after, or concurrently with promoter exposure and the delay in the development of tumours can be monitored.

Skin tumours are also initiated in human skin grafted to immunodeficient mice using a single topical application of DMBA and promoted by UVB radiation or a combination of UVB and UVA radiation (Berking *et al*., 2002; Soballe *et al*., 1996). In a study by Berking *et al*. (2002), tumours developed more rapidly in the mouse skin next to the grafted human skin, possibly

because the mouse skin is much thinner and more susceptible to transformation than human skin. The xenografted human skin studies have shown that human skin carcinogenesis also develops in a multistage manner, though slower than mice skin.

4. UVB effects that contribute towards skin carcinogenesis

The biological effects of UVB radiation can be detrimental to the skin by initiating and promoting carcinogenesis. Cellular macromolecules such as DNA, RNA, lipids and proteins in the skin absorb UVB, causing direct structural damage. Damaged DNA that is not repaired, can result in mutations that produce initiated cells. Inflammation, hyperplasia and the generation of free radicals are also important damaging effects of excessive exposure to UVB radiation, promoting carcinogenesis. Cells that are damaged by UVB are removed by apoptosis, evident by an increase in sunburn cells in the epidermis (Katiyar *et al*., 1997).

4.1. Oxidative stress

Free radicals are molecules that have one or more unpaired electrons occupying atomic or molecular orbits. As an electron is stable when paired with another electron within the same atomic orbit, these molecules are highly reactive with other molecules. Free radicals are created by a loss or gain of electrons, and molecules will therefore lose or gain electrons to become stable. Non-radical molecules are targeted for their electrons, and as a consequence are oxidized, resulting in the generation of new free radicals (Halliwell & Gutteridge, 2007). In this way, if the non-radical molecules involved are part of important cellular structures or systems, considerable cellular destruction can occur. Free radicals include superoxide ($O_2\bullet^-$), hydroxyl radical ($OH\bullet$), singlet oxygen ($^1O_2\bullet$), peroxyl radical ($RO_2\bullet$) and thiyl ($RS\bullet$). Oxidative stress is defined as an imbalance between highly reactive molecules and the body's defence system against radicals favouring the reactive molecules, which can result in oxidative damage to cellular macromolecules (Sies, 1997). Oxidative stress has been linked to aging, carcinogenesis and diseases such as rheumatoid arthritis, Alzheimer disease, cataracts and cancer (Gracy *et al*., 1999; Leiria *et al*., 1995; Mazzetti *et al*., 2001; Smith *et al*., 1991; Spector *et al*., 1993).

4.1.1. Reactive oxygen and nitrogen species

The generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) occurs in tissues by endogenous sources such as metabolic, respiratory, and inflammatory processes (Liu *et al*., 2002; Schrader & Fahimi, 2004), and due to exogenous sources such

as carcinogens, pollutants and radiation (Pouget & Mather, 2001; Romieu *et al*., 2008; Sinkó *et al*., 2005).

Reactive oxygen species are oxygen centred reactive molecules derived from the incomplete reduction of oxygen (O_2) to water (H_2O). The stepwise reduction of oxygen by the addition of one electron at a time produces superoxide, hydrogen peroxide (H_2O_2) and the hydroxyl radical (**Equation 1**), respectively. Some ROS are free radicals, while others are non-radicals and include hypochlorous acid (HOCl) and hydrogen peroxide (Dröge, 2002; Sies, 1997). Superoxide radicals are dismutated to hydrogen peroxide (**Equation 2**), which is converted to the highly reactive hydroxyl radical by ferrous Fe (II) in the Fenton reaction (**Equation 3**) (Mori & Iwahashi, 2007; Stadtman & Berlett, 1991).

During respiration, the mitochondria produce the energy molecule adenosine triphosphate (ATP) in the respiratory electron transport chain through the four-step electron reduction of oxygen to water (**Equation 1**). In this process electrons are leaked out of the chain at Complexes I and III and react with oxygen to produce the superoxide radical (Liu *et al*., 2002; St-Pierre *et al*., 2002). An organelle called the peroxisome is involved in metabolic processes such as β-oxidation of fatty acids, biosynthesis of cholesterol and oxidation of amino acids, and produces hydrogen peroxide enzymatically by various oxidases (Schrader & Fahimi, 2004). Inflammatory cells such as neutrophils and macrophages produce ROS, such as the superoxide radical and hydrogen peroxide during the oxidative respiratory burst by the enzyme nicotine adenine dinucleotide phosphate reduced (NADPH) oxidase in order to destroy invading organisms and foreign particles (Bablor, 1984; Forman & Torres, 2002). Exogenous sources such as ionizing radiation produce ROS such as hydroxyl radicals by the ionization of water molecules (Pollycove & Feinendegen, 2003). Viruses such as hepatitis C and D induce oxidative stress by expressing proteins, which depolarize the mitochondria and inhibit and down-regulate the complexes in the electron transport chain causing an increase in ROS production (Gong *et al*., 2001; Korenaga *et al*., 2005; Lee *et al*., 2004). Viruses also activate the respiratory burst in inflammatory cells during the host defence, which causes an increase in ROS levels (Peterhans, 1997).

Equation 1: $O_2 \xrightarrow{e^-} O_2\bullet^- \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} OH\bullet \xrightarrow{e^-} H_2O$

Equation 2: $O_2\bullet^- + O_2\bullet^- \rightarrow H_2O_2 + O_2$

Equation 3: $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH\bullet + OH^-$

Reactive nitrogen species are nitrogen centred reactive molecules, which include radicals such as nitric oxide radical (NO•), nitrosonium cation (NO^+), peroxynitrite ($ONOO^-$), nitrous oxide radical (NO_2•) and nitroxyl anion (NO^-). The nitric oxide radical is the precursor of all other RNS and is produced by the enzyme nitric oxide synthase (NOS) through the oxidation of the amino acid arginine to citrulline (**Equation 4**) (Farrell & Blake, 1996; Marletta, 1993). Peroxynitrite is the product of the reaction of nitric oxide with superoxide radical (**Equation 5**) and is formed during the respiratory burst by inflammatory cells (Carreras *et al*., 1994). Nitroxyl anion can react with oxygen to produce peroxynitrite (**Equation 6**) (Kirsch & de Groot, 2002). Nitrite (NO_2^-) is formed by the reaction of nitric oxide radical with oxygen (**Equation 7**), which is then converted to nitrous oxide radical (**Equation 8**) by enzymes such as myeloperoxidase and lactoperoxidase or by ROS such as hypochlorous acid (Ignarro *et al*., 1993; Van der Vliet *et al*., 1997). Nitric oxide also reacts with oxyhemoglobin in erythrocytes to form methemoglobin and nitrate (NO_3^-) (**Equation 9**) (Liu *et al*., 1998). Nitrosonium cations and nitroxyl anions are formed through the reaction of nitric oxide ions such as iron, or by the reaction of peroxynitrite with superoxide radical (Pryor & Squadrito, 1995; Stojanovic *et al*., 2004).

Equation 4: L-arginine + O_2 $\xrightarrow{\text{NOS}}$ citrulline + NO•

Equation 5: NO• + O_2•$^-$ $\rightarrow$ $ONOO^-$

Equation 6: NO^- + O_2 $\rightarrow$ $ONOO^-$

Equation 7: NO + O_2 $\rightarrow$ NO^-_2

Equation 8: NO^-_2 + H_2O_2 $\xrightarrow{\text{peroxidase}}$ NO•$_2$

Equation 9: $Fe(II)O_2$ + NO• $\rightarrow$ Fe(III) + NO_3^-

At lower concentrations, ROS and RNS play important physiological roles within cells, such as regulation of gene expression, redox-sensitive signalling and cell growth regulation (Dröge, 2002). For example, it is well established that nitric oxide plays a critical role in regulating blood pressure, neurotransmission and inflammation (Clough, 1999; Farrell & Blake, 1996). However, at increased levels, ROS and RNS can cause considerable damage to DNA, lipids and proteins, leading to disease.

4.1.2. Oxidative DNA damage

Reactive oxygen species such as the hydroxyl radical attack the 2'-deoxyribose backbone, the nucleoside bases guanine (G), cytosine (C), adenine (A) and thymine (T), as well as the sugar moiety structures of nuclear and mitochondrial DNA. These reactions produce many different types of DNA adducts (**Figure 7**) that can lead to mutations if left unrepaired, as well as inter- and intra-strand DNA cross-links, single and double strand breaks and DNA-protein cross-links (Dizdaroglu *et al*., 2002; Yakes & Van Houten, 1997). For example, oxidative damage of nucleoside bases produce DNA adducts such as 7,8-dihydro-8-oxo-2'deoxyadenosine from A and 5-hydroxy-2-'deoxycytidine from C (Wagner *et al*., 1992; Wang *et al*., 1998).

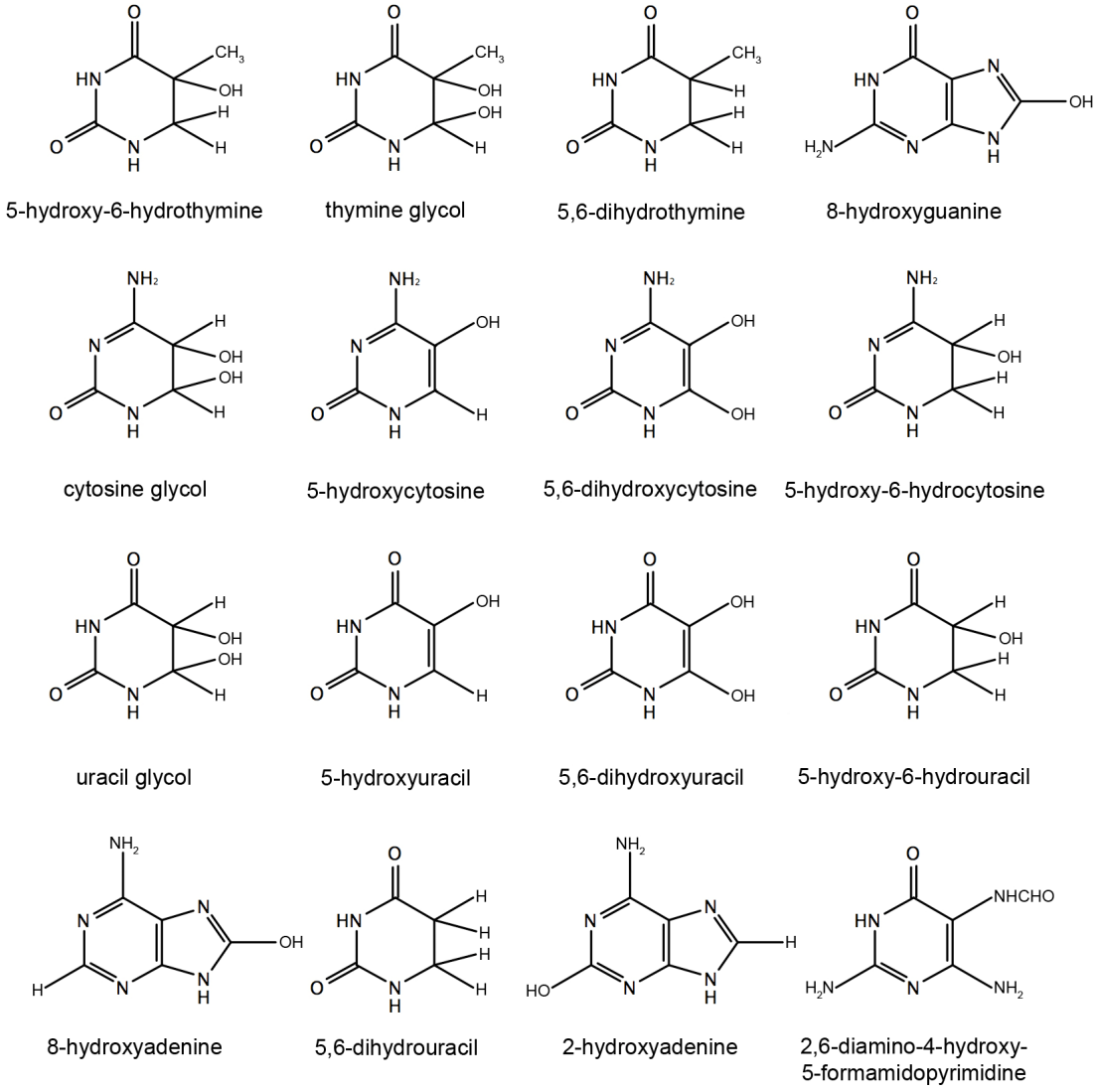

Figure 7. Structures of DNA bases modified by oxidation (adapted from Cooke *et al*., 2003)

Oxidation of G forms the common DNA adduct 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxoG) (Kasai *et al*., 1986). This adduct can cause mutations by pairing with A instead of C, causing a G to T transversion and plays an important role in mutagenesis and tumourigenesis (Cheng *et al*., 1992; Tsuzuki *et al*., 2001). Of all the DNA adducts, 8-oxoG is one of the most commonly measured as a biomarker for oxidative DNA damage. Nitrative DNA adducts such as 8-nitroguanine and 8-nitrotyrosine are formed when RNS such as peroxynitrite and nitric oxide react with DNA (Akuta *et al*., 2006; Ohshima *et al*., 2006).

4.1.3. Oxidative protein damage

The amino acid residues of proteins such as cysteine and methionine are oxidized by ROS to produce protein carbonyls, protein fragmentation and cross-linked proteins. Reactive nitrogen species such as nitrous oxide radicals can cause damage to proteins by the nitration of tyrosine residues in proteins. Lipid peroxidation products react with proteins to form adducts and cross-linking by reacting with lysine, histidine and cysteine residues, as well as amino-terminal amino acids (Requena *et al*., 1996). Protease enzymes degrade oxidized proteins, but the system is not perfect. Some oxidized proteins are resistant to degradation and can inhibit proteases from degrading other proteins. Accumulation of oxidized proteins occurs over time and causes changes in cellular function as oxidation can reduce or abolish enzyme activities and the integrity of cellular structures. Accumulation of oxidized proteins is associated with aging and many diseases such as Alzheimer's disease, cataractogenesis, arthritis, atherosclerosis and diabetes (Berlett & Stadtman, 1997; Stadtman & Levine, 2006).

4.1.4. Oxidative lipid damage

Fatty acids in membrane lipids can be damaged by ROS through oxidation reactions to form lipid peroxidation (LPO) products (**Figure 8**). These LPO products are radicals, which react with neighbouring fatty acids in the membrane, causing a chain reaction. The fatty acid (LH), which contains double bonded carbon chains, is oxidised by a free radical to produce a lipid radical (L•). This lipid radical stabilizes itself by reacting with oxygen to produce a new radical called peroxyl radical (LOO•) that then oxidizes a neighbouring fatty acid to become a stable hydroperoxide (LOOH). The oxidized fatty acid neighbour becomes a lipid radical, which propagates the oxidation process across the lipid membrane. The hydroperoxides formed are then converted to conjugated dienes or decomposed to various other compounds such as aldehydes, alcohols, enals, hydrocarbons (Laguerre *et al*., 2007). These products are measured in tissue as biomarkers of lipid peroxidation, with the most commonly

measured products been malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Requena *et al*., 1996). Lipid radicals oxidize proteins and react with DNA to form DNA adducts such as ethano- and propano- DNA adducts and MDA deoxyguanine adducts (Negre-Salvayre *et al*., 2008; Wang *et al*., 1996; Williams *et al*., 2006). Malondialdehyde is a mutagen and also a carcinogen (Niedernhofer *et al*., 2003). Malondialdehyde reacts with DNA nucleoside bases to form MDA-DNA adducts (Marnett, 1999). These adducts can be mutagenic by causing G to T, A to G and C to T transversions (Benamira *et al*., 1995).

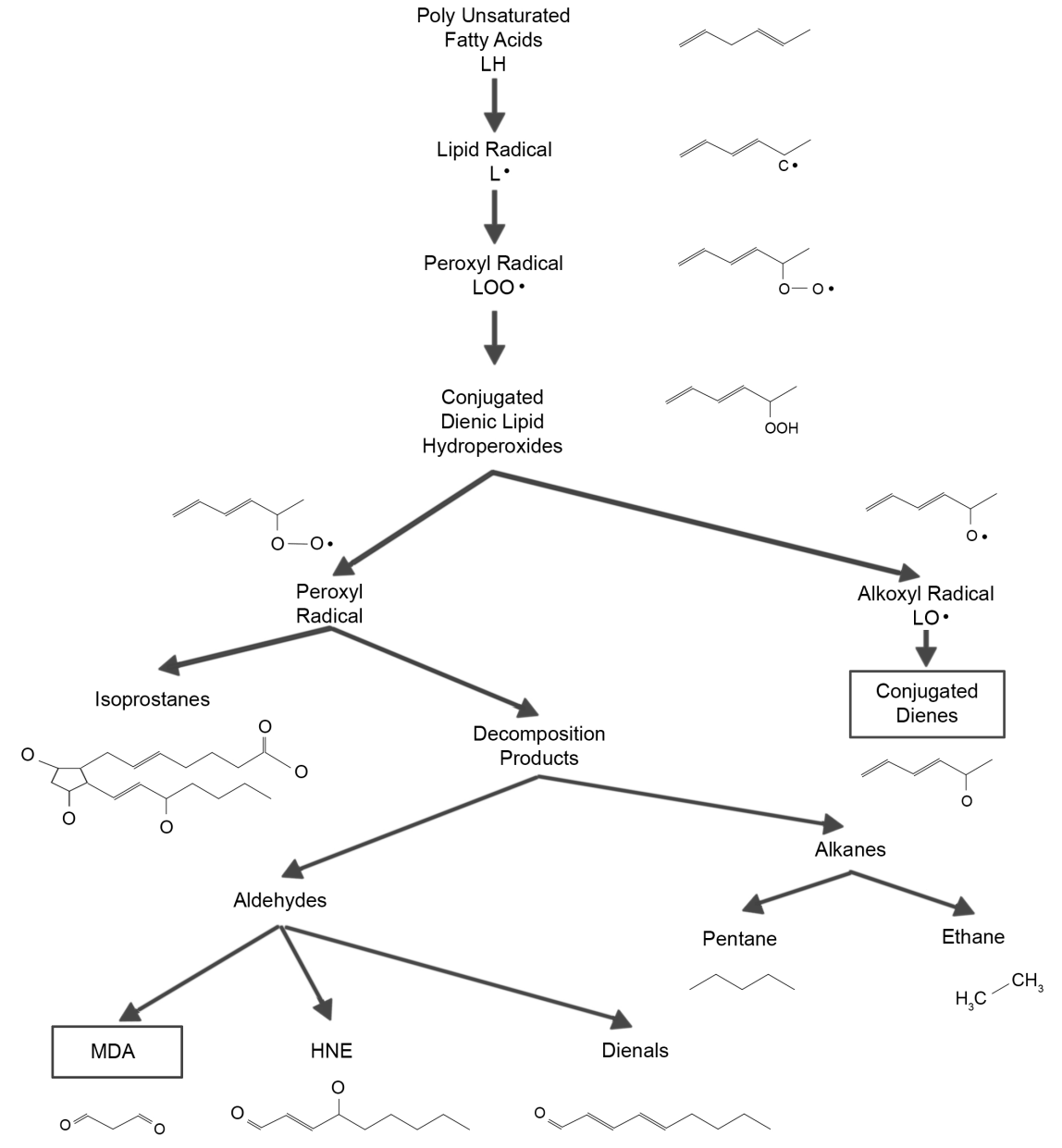

Figure 8. Lipid peroxidation pathway (adapted from Dotan *et al*., 2004)

4.1.5. The antioxidant defence system

The skin contains an antioxidant defence system to protect itself from oxidative stress. The defence system consists of a group of antioxidant enzymes and non-enzymatic antioxidants that work together to prevent oxidative stress-induced damage by neutralizing free radicals, ROS and RNS. These include enzymes such as catalase, glutathione peroxidase (GPx), glutathione reductase (GRed), and superoxide dismutase (SOD). The non-enzyme antioxidants include glutathione and vitamins such as ascorbic acid (vitamin C) and tocopherol (vitamin E), as well as other antioxidants such as albumin, uric acid and bilirubin (Prior & Cao, 1999). The antioxidants work together to convert free radicals and ROS into harmless products such as water (**Figure 9**). Ascorbic acid, a cofactor for some enzymes and a reducing agent, is important in quenching free radicals. It scavenges superoxide, singlet oxygen, thiyl radicals, hypochlorous acid and hydroxyl radicals and also recycles oxidized tocopherol (Niki, 1991). Beta-carotene and α-tocopherol are lipophilic vitamins protecting membranes from free radicals by scavenging peroxyl and hydroxyl radicals. When these vitamins quench radicals, they in turn become radicals, which are recycled by reduced glutathione (GSH) and ascorbic acid (Liebler *et al*., 1986; Machlin & Bendich, 1987).

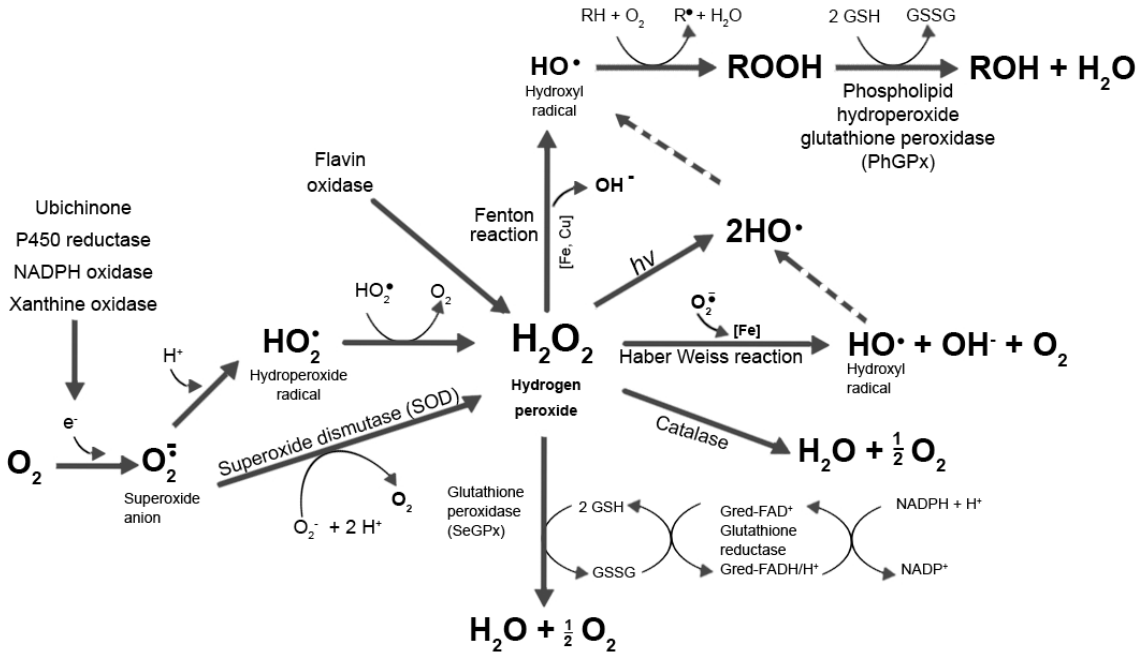

Figure 9. Endogenous antioxidant defence system (from Steiling *et al*., 1999)

Superoxide dismutase is responsible for the dismutation or reduction of superoxide radicals to hydrogen peroxide (**Equation 10**). It is hypothesized that the superoxide radical is a major factor in oxygen toxicity, and SOD is essential in removing it (Dreher & Junod, 1996). Catalase converts hydrogen peroxide to water and oxygen (**Equation 11**), while GPx can also reduce hydrogen peroxide to water (**Equation 12**) through the oxidation of GSH to oxidized glutathione (GSSG). Additionally, GPx neutralizes lipid peroxides using GSH and GSSG generated from these reactions are recycled back to GSH by GRed (**Equation 13**) (Afaq & Mukhtar, 2001).

Equation 10: $O_2^- + O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$

Equation 11: $2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$

Equation 12: $H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + 2H_2O$

Equation 13: $GSSG + NADPH + H^+ \xrightarrow{GRed} 2GSH + NADP^+$

Glutathione reductase is essential in maintaining the balance between GSSG and GSH. The ratio between GSH and GSSG levels is a commonly used marker for oxidative stress. A high ratio of GSH/GSSG indicates a healthy balanced state, but a low ratio is indicative of oxidative stress (Rossi *et al*., 2006). Glutathione can also work independently of GPx by directly scavenging radicals such as hydroxyl radicals and singlet oxygen (Afaq & Mukhtar, 2001).

4.1.6. Oxidative stress and cancer

Free radicals and reactive species can exhibit anti-cancer effects on cells such as senescence, apoptosis, and the inhibition of angiogenesis. However, these molecules can also exhibit pro-cancer effects such as proliferation, invasiveness, angiogenesis, metastasis and the suppression of apoptosis (Halliwell, 2007). Oxidative stress therefore, plays an important role in carcinogenesis. The introduction of mutations in DNA is an essential initiating step in the process of carcinogenesis and reactive species-induced DNA damage is a major cause of mutations (Cooke *et al*, 2003). Cancerous tissues contain increased levels of free radical induced mutations such as G to T transversions (Jaruga *et al*., 1994; Olinski *et al*., 2002). Also, it was shown that mice deficient in *MTH1*, a gene that codes for an enzyme that degrades the common oxidation induced DNA adduct 8-oxoG, are prone to tumourigenesis (Tsuzuki *et al*., 2001). Exogenous sources of ROS such as chronic

inflammatory conditions, tobacco smoking and exposure to ionizing radiation are known to cause cancer (Dreher & Junod, 1996).

Oxidation of DNA bases not only causes mutations, but also epigenetic changes to DNA such as methylation and histone modifications, which affect the expression of genes, and are involved in carcinogenesis (Fraga *et al*., 2004; Franco *et al*., 2008; Valinluck & Sowers, 2007). Oxidative damage to other cellular molecules such as lipids and proteins also play a role in the promotion of carcinogenesis. For example, ROS can alter signal transduction pathways important in maintaining normal cell cycle regulation. Many cancers have antioxidant enzyme imbalances, as well as increased oxidative stress-induced lipid peroxidation (Oberley, 2002). Mice lacking the radical scavenging enzymes SOD and GPx have an increased risk of developing cancer (Elchuri *et al*., 2005; Van Remmen *et al*., 2003; Chu *et al*., 2004).

4.1.7. UVB-induced oxidative stress

Exposure to UVB causes the generation of free radicals such as hydrogen peroxide, superoxide, nitric oxide and peroxynitrite endogenously within keratinocytes, by the induction of enzymes such as NADPH oxidase, nitric oxide synthase, xanthine oxidase, catalase and cyclooxygenase (COX) (Beak *et al*., 2004; Deliconstantinos *et al*., 1996a; Deliconstantinos *et al*., 1996b; Heck *et al*., 2003; Tedesco *et al*., 1997). Antioxidant enzymes such as SOD, catalase and GPx as well as the non-enzymatic GSH, α-tocopherol and ascorbic acid levels are depleted in the skin after UVB irradiation (Katiyar *et al*., 1997; Larsson *et al*., 2005; McArdle *et al*., 2002; Podda *et al*., 1998; Vayalil *et al.*, 2003). There is an increase in GSSG and lipid peroxidation products such as MDA and HNE, as well as an increase in oxidation of proteins, producing carbonyl groups (Afaq *et al*., 2003; Podda *et al*., 1998; Vayalil *et al*., 2003). Ultraviolet B induced ROS such as hydrogen peroxide and hydroxyl radical oxidises DNA with 8-oxoG a common adduct causing cytosine-adenine transition mutations (Ichihashi *et al.,* 2003; Kunisada, *et al*., 2005; Pelle *et al*., 2003; Zhang *et al*., 1997).

4.1.8. UVB-induced DNA damage

Genomic DNA is a chromophore, which absorbs UVB resulting in conformational changes that cause DNA damage (Clydesdale *et al*., 2001). Specific mutations are produced in the DNA of skin cells called “signature” mutations, which are distinct from mutations induced by other genotoxic compounds. Adducts such as the cyclobutane pyrimidine dimers (CPD) and 6-4 pyrimidine-pyrimidones are prevalent and cause predominantly cytosine-thymine transition mutations at dipyrimidine sites in the DNA structure (de Gruijl *et al*., 2001). When

these mutations occur at sites, which when translated to amino acids, are involved in the function of proteins, then this can be detrimental. For example, UVB targets the dipyrimidine site in the p53 gene that translates to arginine at codon 248. This arginine is essential in the DNA binding ability of p53, so a mutation at this site causes a disruption of p53 function, and hence the cell loses the ability to regulate the cell cycle and apoptosis (Giglia-Mari & Sarasin, 2003).

4.2. Inflammation in cancer

Reactive oxygen species generated during the inflammatory process damage DNA and causes mutations, which contribute towards the development of cancer (Bartsch & Nair, 2006). Inflammation may also contribute towards carcinogenesis by other epigenetic events such as altered DNA methylation and histone modifications (Chan *et al*., 2003; Valinluck & Sowers, 2007). Also, mediators of inflammation such as cytokines and prostaglandins (PGs) are associated with carcinogenesis by stimulating cell proliferation, angiogenesis and inhibiting apoptosis (Leahy *et al*., 2002; Li *et al*., 2003; Lin & Karin, 2007; Wang & DuBois, 2006). Epidemiological studies indicate that chronic inflammatory conditions increase the risk of cancer incidence. For example, chronic inflammatory conditions of the skin such as non-healing wounds and burn scars are associated with the development and progression of skin cancer (Mueller, 2006). Besides skin cancer, many other cancer types are linked to chronic inflammation. Examples include bladder, stomach, cervical, liver, prostate, pancreas, and colorectal cancers (Castle *et al*., 2001; Farrow & Evers, 2002; Lowenfels *et al*., 1993; Michaud, 2007; Narayanan *et al*., 2008; Rutter *et al*., 2004; Yoshimura *et al*., 2000). Cyclooxygenase-2 plays an essential role in the development of skin tumours (Fischer *et al*., 2007). Tumours of the skin have increased levels of PGs and an increased expression of the PG producing enzyme COX-2 (Buckman *et al*., 1998). Administration of COX-2 inhibitors such as celecoxib reduces the number of skin tumours in mouse skin (Pentland *et al*., 1999).

4.2.1. UVB-induced inflammation

Shortly after UVB exposure, reddening of the skin (erythema) and damage such as tissue swelling (oedema) and peeling occurs (Afaq *et al*., 2003; Malcotti *et al*., 2001). Ultraviolet B irradiation of the skin causes the induction of NOS and xanthine oxidase enzymes to produce nitric oxide, superoxide and peroxynitrite (Deliconstantinos *et al*., 1996a). These RNS play a role in the vasodilatation of blood vessels in the skin, causing an increase in blood flow and vascular permeability (Deliconstantinos *et al*., 1996b; Tedesco *et al*., 1997). Vasodilatation allows inflammatory cells such as macrophages and neutrophils to migrate into the skin and phagocytose damaged tissue (Afaq *et al*., 2003; Wilgus *et al*., 2000). Inflammatory cells

protect the skin against invading microorganisms with the “respiratory burst” (Mueller, 2006). Therefore, inflammatory cells recruited after UVB exposure, contribute to cellular damage, by inadvertently damaging the healthy skin tissue via ROS production (Schäfer & Werner, 2008). Infiltrating macrophages to the dermis and epidermis secrete the cytokine interleukin-10 and exert immunosuppressive properties to help resolve the inflammatory response after UVB irradiation (Kang *et al*., 1994; Kang *et al*., 1998).

An increase in proliferation in the basal layer of the skin then occurs in response to cytokines and PG secretion. Stem cells located in the rete ridges of the epidermis in human skin have been shown to undergo emergency proliferation after UVB irradiation as it would during wound healing to replace damaged tissue. Ultraviolet B radiation causes an increase in transiently dividing cells and hence an increase in cell numbers. Non-proliferating cells are forced upwards towards the surface by proliferating cells in the basal layer. This results in a thickening of the epidermis, which is referred to as hyperplasia (Baba *et al*., 2005; Mueller, 2006; Tripp *et al*., 2003). The increase in PGs is a common biomarker for inflammation, as PGs not only affect cell proliferation, but also angiogenesis, immune surveillance and apoptosis (Chun & Surh, 2004). The major PG produced in the skin after UVB irradiation is PGE_2, which is involved in the regulation of proliferation and differentiation of normal keratinocytes (Tripp *et al*., 2003). Other PGs produced include $PGF_{2\alpha}$ and PGD_2 (Afaq *et al*., 2003; Katiyar *et al*., 1997). Prostaglandins are produced from the precursor arachidonic acid by the COX enzymes, COX-1 and -2 (Afaq *et al*., 2003).

Polyamines are also involved in UVB-induced inflammation and hyperplasia. Putrescine, spermine and spermidine are involved in growth and differentiation by binding to DNA, regulating DNA replication, transcription and translation. Polyamines are synthesized from the amino acid L-arginine, which is converted to L-ornithine by the enzyme arginase (**Figure 10**). Ornithine is then converted to putrescine by the enzyme ornithine decarboxylase (ODC) that is further converted to spermine and spermidine by spermine and spermidine synthase, respectively (Gilmour, 2007). Ornithine decarboxylase activity and protein expression is increased in the skin after UVB irradiation (Afaq *et al*., 2003).

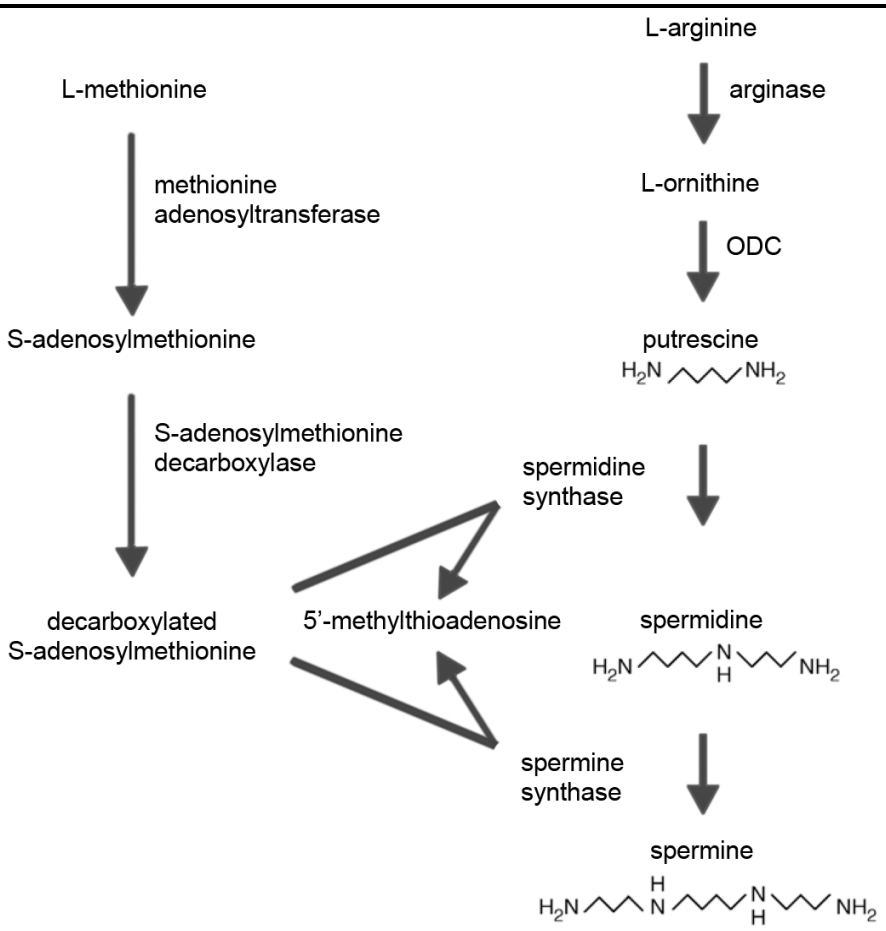

Figure 10. Polyamine synthesis pathway (adapted from Gilmour, 2007)

5. Chemoprevention

It is estimated that approximately a third of cancers can be prevented by correct diets, and a healthy lifestyle with regular exercise and weight management (Williams & Hord, 2005; Willet, 2000). It is well known that populations that are on the Mediterranean diet, which is rich in fruits and vegetables, and low in red meats and refined carbohydrates, have reduced risk for cancers (La Vecchia, 2004). Many other epidemiological studies also show that there is a positive association between a diet rich in fruits and vegetables and a reduced incidence of cancer (Van't Veer *et al*., 2000).

Besides relying on a healthy diet and lifestyle as a source for preventing cancer, specific compounds isolated from foods, have recently been investigated for use in cancer prevention. This relatively new concept is referred to as chemoprevention, which is defined by Zhao *et al*. (1999) as, "… a means of cancer control where the induction of disease can be totally prevented or the rate of development slowed or reversed partially or substantially

by the administration of one or more naturally occurring or synthetic chemical agents". Furthermore, the term "photochemoprevention" is used to refer to chemoprevention of UV radiation induced carcinogenesis (Afaq *et al*., 2002). Photochemoprevention may target the initiation, promotion or progression stages of photocarcinogenesis. Intervention at the initiation stage e.g. use of sunscreens, is not as practical as intervention at the promotion or progression stages. Intervention at the promotion stage using anti-tumour promoting agents is the most practical approach to follow in humans, as there is a long latency period between initiation and promotion of cancer before the promotion stage becomes irreversible. Targets for UV radiation-induced carcinogenesis include DNA damage, inflammation, immunosupression, oxidative stress and changes in signalling pathways and cell cycle regulation (Afaq *et al*., 2005).

Numerous animal and *in vitro* studies have demonstrated the photochemopreventive effects of naturally occurring botanical phenolic compounds by ingestion or applied to the skin, many occurring in foodstuffs, e.g. resveratrol from grapes (Afaq *et al*., 2003; Aziz *et al*., 2005), delphinidin from pomegranates (Afaq *et al*., 2007), (-)-epigallocatechin-3-gallate from green tea (Mittal *et al*., 2003), genistein from soybeans (Wei *et al*., 2003) as well as complex extracts from plants e.g. extracts of beetroot (Kapadia *et al*., 2003), green tea (Mnich *et al*., 2009), pine bark (Kyriazi *et al*., 2006), and grape seeds (Mantena & Katiyar, 2006). Clinical trials have begun, in which some compounds are being investigated in humans for the prevention of various cancer types, such as genistein for breast cancer and pancreatic cancer, resveratrol for colon cancer, curcumin for pancreatic cancer and colorectal cancer, and (-)-epigallocatechin-3-gallate for cervical cancer (Russo, 2007). From these studies, it is clear that there is potential for these phenolic compounds to be used as additives in products such as supplements, sunscreens and skin care products. Plant-derived polyphenolic compounds are already used in skin care formulations for sunscreen and anti-aging, such as green tea catechins, silymarin from the milk thistle plant, resveratrol from grapes, pomegranate extracts, and genistein from soybeans (Afaq *et al*., 2002; Allemann & Baumann, 2008). Other products are also been supplemented with health promoting botanical antioxidants, such as toothpastes, shampoos, depilatory creams, scented sprays and shower gels (Afaq *et al*., 2002). The incidence of skin cancer may possibly be reduced by a combination of a healthy lifestyle, a diet rich in naturally occurring chemopreventive compounds and the use of sunscreens and specially formulated health care products containing botanical antioxidant compounds (Afaq et al., 2002; Katiyar, 2002; Velasco *et al*., 2008).

There is a lack of studies and data from Africa on the chemopreventive properties of polyphenolic compounds, which are a common part of the African diet. Studies like these

would be pertinent for improving the health of the African population, by increasing awareness of the importance of living a healthy lifestyle and the use of natural chemopreventive compounds to reduce the risk of disease.

6. Polyphenols and flavonoids

Flavonoids are a group of polyphenolic compounds found in plants that are consumed as part of the human diet. Flavonoids play key functions in the development and growth of plants and in plant-insect interactions (Simmonds, 2001; Taylor & Grotewold, 2005). Flavonoids also play an important role in protecting plants from the harmful effects of radiation by absorbing UVB and assisting the antioxidant defence system by scavenging ROS produced by UVB irradiation (Xu *et al*., 2008). They also display health promoting biological effects for humans that consume the plants, such as prevention of oxidative stress, lipid peroxidation, inflammation, cardiovascular disease, cancer, as well as antiviral, antibacterial and immune modulating activities (Bonina *et al*., 1996; Huang *et al*., 2007; Lahiri-Chatterjee *et al*., 1999; Lee & Jang, 2004; Rotelli *et al*., 2003). Flavonoids are well known for their antioxidant ability as scavengers of free radicals such as hydroxyl, peroxyl radicals and superoxide anions (Bonina *et al*., 1996; Saija *et al*., 1995). Flavonoids have also been shown to protect membranes by interacting with lipids in the membranes, forming phospholipid complexes or by incorporating into the membrane itself (Saija *et al*., 1995). Some polyphenols show activity that inhibits carcinogenesis, such as affecting signal transduction pathways, reducing angiogenesis and cell growth, and encouraging apoptosis (Lambert *et al*., 2005).

6.1. Types and Structure

Polyphenols possess one or more aromatic rings bearing one or more hydroxyl groups, and may include functional substituents such as esters, methyl esters and glycosides. Polyphenols also occur unconjugated or conjugated with sugars, organic acids, lipids and amino acids (Aherne & O'Brien, 2002). Polyphenols are divided into groups based on the number of aromatic rings and substituents. These groups are the phenolic acids, phenolic alcohols, lignans, stilbenes and flavonoids (**Figure 11**) (D'Archivio *et al*., 2007; Manach *et al*., 2004). Phenolic acids consist of the benzoic and cinnamic acid derivatives. Caffeic acid (derived from cinnamic acid) is the most common phenolic acid, and is abundant in coffee (Nardini *et al*., 2002). Ferulic acid (derived from cinnamic acid) and gallic acid (derived from benzoic acid) are other well-known phenolic acids found in cereal and tea, respectively (Renger & Steinhart, 2000; Shahrzad, *et al*., 2001). Tyrosol is a phenolic alcohol found in olive oil, whereas resveratrol is a stilbene that occurs mainly in grapes, and linseed is the

main source of lignans in the diet (Fernández-Bolaños *et al*., 1998; Gürbüz *et al*., 2007; Zimmermann *et al*., 2006).

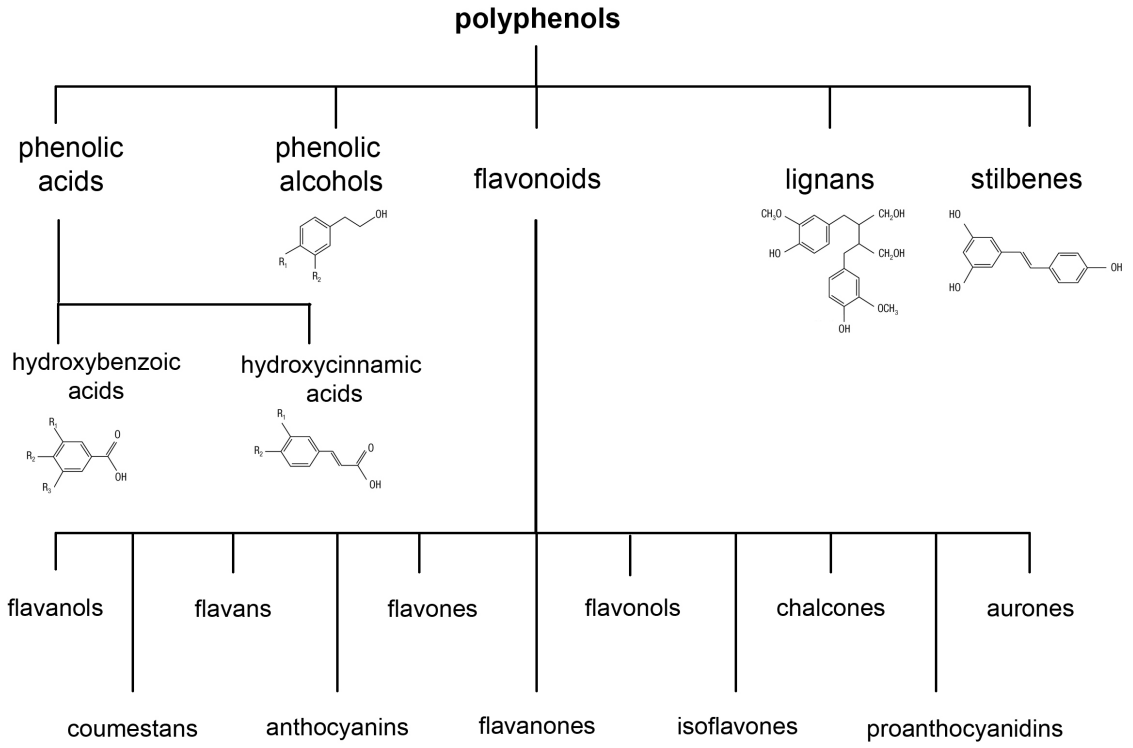

Figure 11. Classification of polyphenols (adapted from D'Archivio *et al*., 2007)

The flavonoids are characterized by a C6-C3-C6 backbone structure. This structure is called the flavan nucleus (**Figure 12**), which consists of two benzene rings (ring A and ring B) joined by a three-carbon atom chain that forms a pyran ring (ring C) (D'Archivio *et al*., 2007).

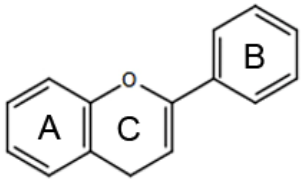

Figure 12. Flavan nucleus (adapted from Aherne & O'Brien, 2002)

The flavan nucleus is altered by various substitutions and functional groups in different positions to produce different subclasses of flavonoids. The main subclasses of flavonoids consist of flavanols (catechins), anthocyanins, flavones, flavonols, isoflavones and flavanones (**Figure 13**). Other classes include the coumestans, flavans, proanthocyanidins, chalcones and aurones (Aherne & O'Brien, 2002).

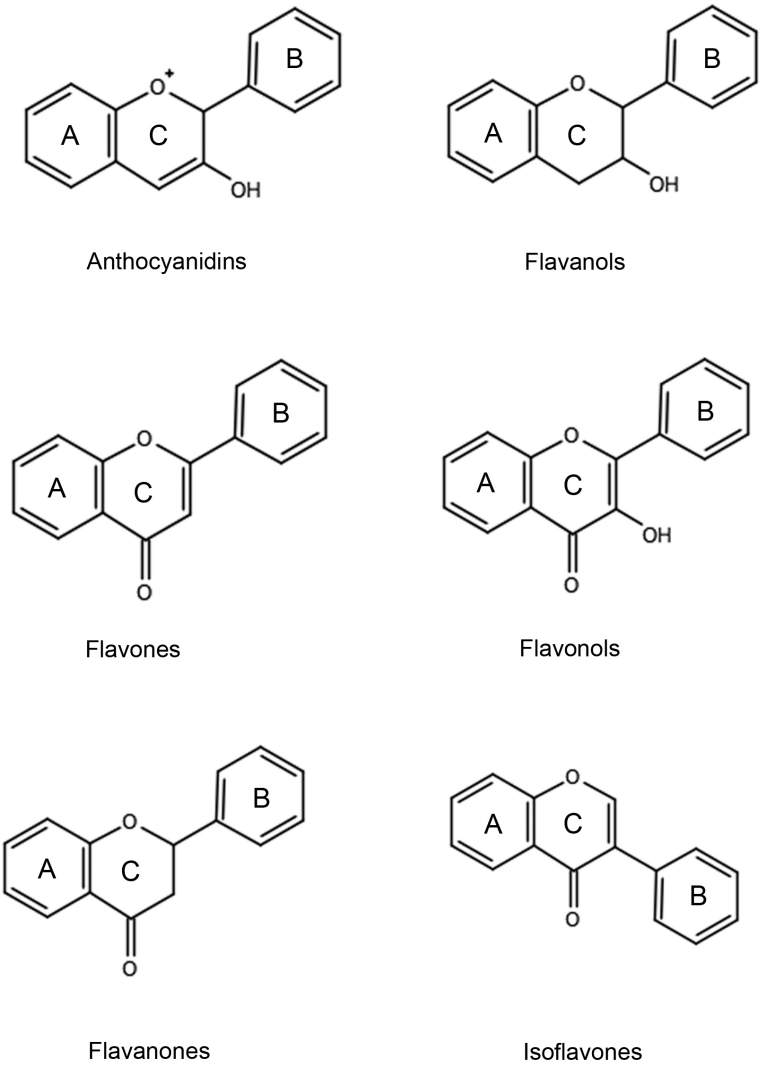

Figure 13. Main flavonoid classes (adapted from Aherne & O'Brien, 2002)

6.2. Dietary sources of flavonoids

Flavonoids are present mainly as glycosides in fruits, vegetables, herbs, spices, confectionaries and chocolate, as well as in beverages such as wine and tea. Flavonoids occur in the skin and flesh of fruit, as well as in the leaves and flowers of the plant (Andersen

& Markham, 2006). The flavonoid subclasses and examples from each are listed in **Table 1**. Flavonols are the most commonly occurring flavonoids in many vegetables, cereal, fruits and beverages. Flavones are less common, occurring mainly in parsley, celery and some spices. Citrus fruits are particularly rich in flavanones with isoflavones occurring mainly in leguminous foods such as soybeans and tofu. Anthocyanins are found mainly in the skin of fruits and vegetables that are purple, red and blue in colour, like berries and vegetables such as cabbage and radish. Flavanols or catechins, although found in many fruits, are most abundant in tea and chocolate (D'Archivio *et al*., 2007).

Table 1. Flavonoid classes and food source (adapted from Andersen & Markham, 2006)

Class	Compounds	Food source
Flavonol		
	Kaempferol	Apples, berries, broccoli, onions, tea
	Myricetin	Berries, beans
	Quercetin	Apples, berries, grapes, olives, raisins, chilli pepper, lettuce, onions, tomatoes, coriander, tea
Flavone		
	Apigenin	Olives, celery, parsley, marjoram, sage
	Luteolin	Olives, lemon, chilli pepper, oregano, sage, thyme
Flavanol		
	Catechin	Apples, apricots, berries, grapes, beans, chocolate, tea
	Epigallocatechingallate	Beans, tea
Flavanone		
	Hesperetin	Lemon, lime, orange
	Naringenin	Grapefruit, orange
Anthocyanidin		
	Cyanidin	Apples, berries, cocoa, asparagus
	Delphinidin	Pomegranate
	Malvidin	Grape, blueberry
Isoflavone		
	Genistein	Soybeans
	Daidzein	Soybeans

7. South African herbal tisanes

Teas infused from herbs and other plants, called tisanes or infusions, are gaining popularity due to their low tannin and caffeine content, and other health promoting properties (McKay & Blumberg, 2006a; McKay & Blumberg, 2006b). Two tisanes produced from plants that are grown locally and that are particularly popular in South Africa, are rooibos and honeybush (**Figure 14**).

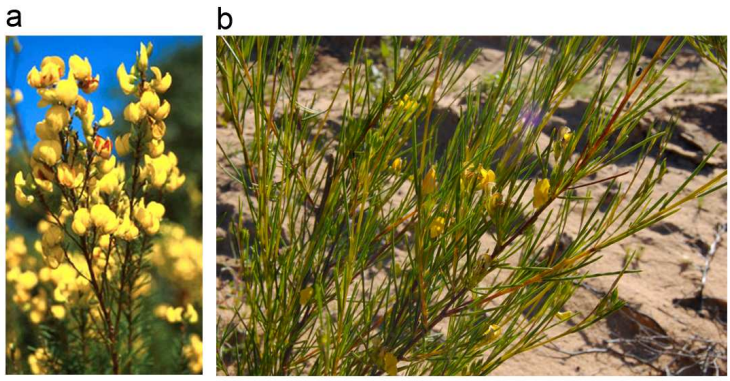

Figure 14. The (a) honeybush plant *Cyclopia* spp. and the (b) rooibos plant *Aspalathus linearis* ((a) from Institute for Traditional Medicine, 2004; (b) photograph taken on Elandsberg farm, Clanwilliam South Africa)

7.1. Honeybush

The *Cyclopia* genus, which is a member of the Fabaceae plant family, consists of over 20 species of legume shrubs that grow indigenously in the Cape fynbos biome (**Figure 15**) of the Western and Eastern Cape of South Africa (Ferreira *et al*., 1998; Kokotkiewicz & Luczkiewicz, 2009). *Cyclopia* spp. plants (**Figure 14a**) have sweetly scented yellow flowers, long needle-like trifoliate leaves and woody stems (Le Roux *et al*., 2008). Honeybush is a sweet honey flavoured tea made from the leaves and stems of the most common species of the *Cyclopia* genus; *C. intermedia, C. genistoides*, *C. sessiliflora* and *C. subternata* (Ferreira *et al*., 1998; Kokotkiewicz & Luczkiewicz, 2009).

The first documented use of *Cyclopia* spp. as a herbal tea was in 1705, which described the local inhabitants harvesting the leaves of the plants growing naturally in the mountains. Honeybush was commercialized in the 1960s and wild honeybush was nearly marketed to extinction (Cape Honeybush Tea, 2003c). A programme was therefore initiated in 1992 by

the National Botanical Institute of South Africa, in which honeybush was cultivated on farms and its export controlled (Joubert *et al*., 2008a).

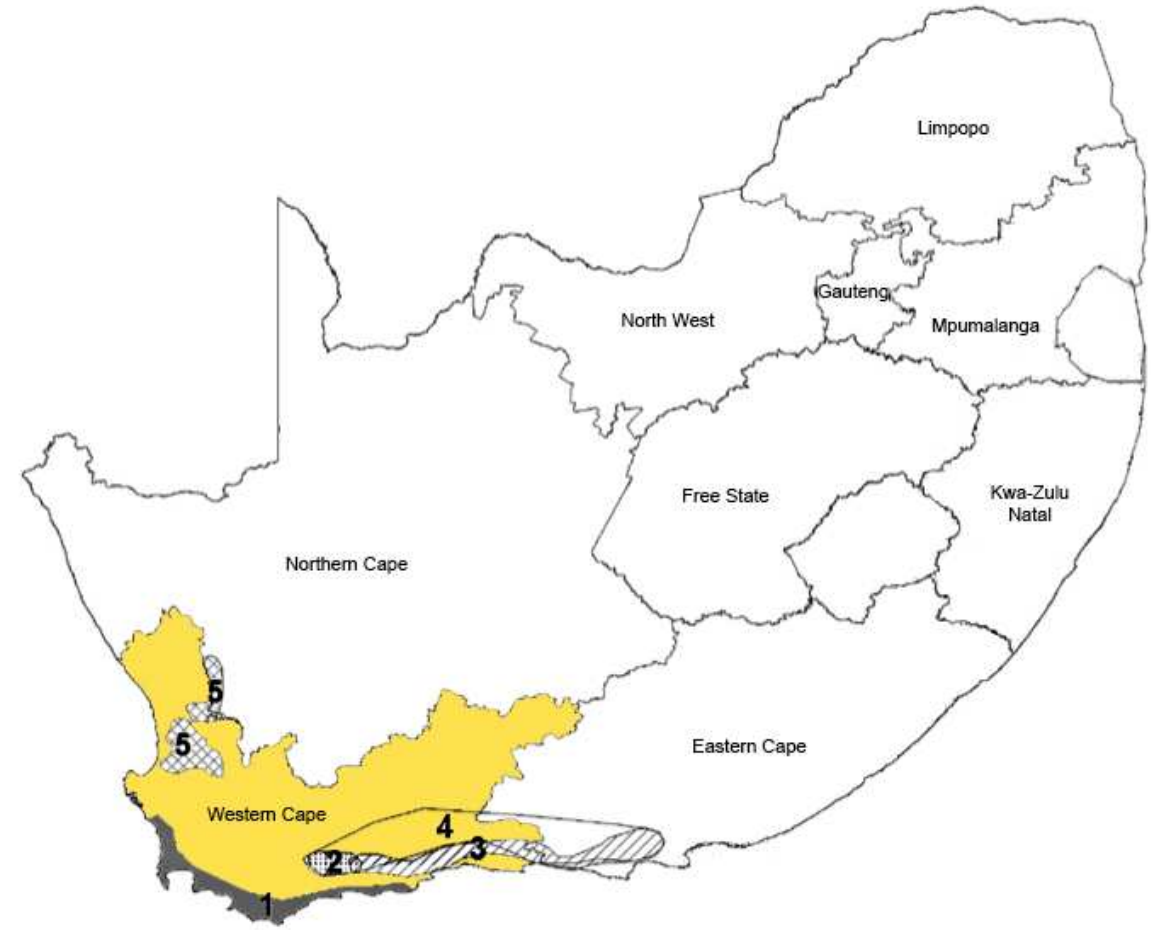

Figure 15. Natural distribution areas of the most common *Cyclopia* species and production areas of *Aspalathus linearis* in South Africa. Key: (1) *Cyclopia genistoides*; (2) *Cyclopia sessiliflora*; (3) *Cyclopia subternata*; (4) *Cyclopia intermedia*; (5) *Aspalathus linearis* (adapted from Joubert *et al*., 2008a)

The export of honeybush has since increased from 50 tons in 1999 to 300 tons in 2005 with the demand for honeybush in countries such as Japan, Germany and Switzerland where health-conscious consumers are discovering the health-promoting properties of this herbal tea (Cape Honeybush Tea, 2003a). Presently, commercial growers contribute about 30% of the production, while 70% is still harvested from natural uncultivated areas (Agricultural Research Council, 2007).

7.1.1. Processing

Cultivated honeybush is planted from *Cyclopia subternata* seedlings or *Cyclopia genistoides* cuttings, while *Cyclopia intermedia* are only harvested from uncultivated areas (Joubert *et al*., 2008). Seeds are treated by scarification to encourage germination, and planted in soil inoculated with rhizobium and michorriza (National African Farmers Union of South Africa, nd). As shown in **Figure 16**, *Cyclopia* spp*.* (**a**) is harvested (**b**) every one to three years after planting. After harvesting, the leaves and stems are shredded to disrupt cellular integrity (**c**), fermented at 70-85°C at controlled humidity for 18-60 h in an oven (**d**) thereafter it is sun-dried on trays for 1-2 days (**e**). The plant material develops a red to dark brown colour during fermentation (Du Toit & Joubert, 1998). The dried fermented tea is then sieved into size categories and graded for quality. It is then packaged (**f**) and sold as herbal tea or processed further to produce extracts for food, beverage and cosmetics (Agricultural Research Council, 2007). The traditional method for processing honeybush is to use a curing heap for fermentation instead of an oven. The plant material is allowed to oxidise in a heap outdoors for three to four days. During this time, heat from the sun raises the temperature within the heap up to 60°C, which is covered with a canvas or hessian bag. After fermentation, the plant material is spread out on a canvas or tray to dry (Du Toit *et al*., 1999). This method is no longer used as it was difficult to obtain a consistent quality necessary for large-scale production, and the plant material was prone to microbial contaminations (Du Toit *et al*., 1999; Joubert *et al*., 2008a).

Figure 16. Honeybush production process: (**a**) *Cyclopia* spp. (**b**) harvesting (**c**) shredding (**d**) fermentation (**e**) drying (**f**) packaging (images kindly donated by Melmont Honeybush Tea CC, South Africa)

7.1.2. Composition

Polyphenols such as flavonoids and xanthones are the main compounds found in the *Cyclopia* shrub. De Nysschen *et al*. (1996) screened methanol extracts of 22 species of *Cyclopia* and found that the three major flavonoids are the xanthone, mangiferin (**Figure 17a)** and the flavanones hesperidin (the glucoside of hesperitin) (**Figure 17b**), and didymin (the glycoside of isosakuranetin). Further studies done by Ferreira *et al*. (1998) and Kamara *et al.* (2004) using methanol extracts of fermented *Cyclopia intermedia* and unfermented *Cyclopia subternata* respectively, showed that the following phenolic compounds are present

in honeybush: the isoflavones formononetin, afrormosin, calycosin, psuedobaptigen and fujikinetin and orobol; the flavanones naringenin, eriodictyol, hesperitin and hesperidin; the coumestans medicagol, flemichapparin and sophoracoumestan; the xanthones mangiferin and isomangiferin; the flavones luteolin and scolymoside (rutinosyl); the flavanol epigallocatechin gallate; and the flavonol glycosylkaempferol. Other polyphenols found in *Cyclopia* include (+)-pinitol, (+)-shikimic acid, 4-hydroxycinnamic acid, p-coumaric acid (4-hydroxy-*trans*-cinnamic acid), 4-glycosyltyrosol and very low amounts of tannins. Joubert *et al*. (2003) determined that the quantity of each polyphenol varies between species of *Cyclopia*, as well as by location and harvest date. Also, fermentation of *Cyclopia* plant material was shown to decrease the quantity of polyphenols (Joubert *et al*., 2008b).

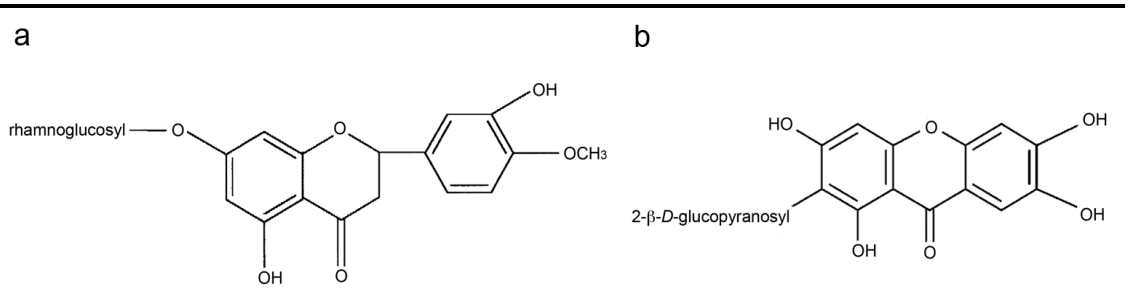

Figure 17. Chemical structures of (a) hesperidin and (b) mangiferin (adapted from Joubert *et al*., 2006)

Essential elements in honeybush include calcium, copper, iron, potassium, magnesium, manganese, phosphorous and zinc (Malik *et al*., 2008). Other compounds present in unfermented honeybush include alcohols, aldehydes and ketones producing characteristic grassy odours of green tea. In fermented honeybush, terpenoids are abundant and are responsible for the sweet floral odours (Le Roux *et al*., 2008).

7.1.3. Anecdotal properties

It is said that the Khoi tribe of South Africa discovered the healing properties of honeybush centuries ago, and that the knowledge of the medicinal beverage was shared with people who colonized the country (Cape Honeybush Tea, 2003a). Honeybush is now used as a folk medicine to treat a variety of symptoms and as a general health tonic to reduce the degenerative effects of lifestyle diseases. Honeybush is used to boost the immune system and to treat colds and influenza and their symptoms, as well as hay fever and asthma. Women use honeybush to treat symptoms of menopause and to protect them from

cardiovascular disease and osteoporosis. The nervous system is said to be soothed of headaches, tension, insomnia and depression with honeybush. The tisane is also used to relieve digestive complaints such as constipation, stomach ulcers, nausea, heartburn and colic in babies. It is also claimed that honeybush used topically can relieve skin irritations such as eczema and nappy rash (Cape Honeybush Tea. 2003b. *Health benefits / focus on organic production*; Kamara *et al.,* 2004; Verhoog *et al*., 2007).

7.1.4. Biological properties

Extensive literature is available on the biological properties of green and black tea (*Camellia sinensis*) and the major polyphenols found in green and black tea, the catechins, thearubigins and theaflavins (Hsu, 2005; Katiyar *et al*., 2007; Yang *et al*., 2009). However, there is very little scientific literature available on the health promoting properties of honeybush. The literature available show the effects of honeybush or the major polyphenols hesperidin and mangiferin, in *in vitro* studies using cells grown in culture and *in vivo* studies using rodents. From these studies, honeybush is shown to have antioxidant, antimutagenic and antitumourigenic properties.

7.1.4.a. **Anti-inflammatory activities**

There are currently no studies available on possible anti-inflammatory activities of honeybush. Animal studies by Guardia *et al*. (2001) and Rotelli *et al*. (2003) showed that hesperidin reduced inflammation in rats by inhibiting arthritis in carrageenan-induced arthritis, xylene-induced ear oedema and cotton pellet-induced granuloma. An *in vitro* study showed that hesperidin was able to prevent lipopolysaccharide-induced inflammation in macrophage cells by inhibiting COX, inducible NOS expression, and the production of PGE_2 (Sakata *et al*., 2003).

7.1.4.b. **Antioxidant activities**

Honeybush has been shown to protect the antioxidant defence system and prevent lipid peroxidation in a small number of studies. Marnewick *et al*. (2003) showed that honeybush protected against oxidative stress in the liver of Fischer 344 rats given aqueous extracts of fermented or unfermented honeybush (*Cyclopia intermedia*) as drinking fluid. Oxidised glutathione levels decreased and GSH levels increased in the liver. Methanol extracts of fermented and unfermented honeybush were also shown to protect against lipid peroxidation in microsomal liver fractions from Fischer 344 rats (Marnewick *et al*., 2005). Aqueous extracts of fermented and unfermented honeybush fed to Fischer 344 rats, reduced

fumonisin B_1-induced lipid peroxidation and catalase depletion in the liver (Marnewick *et al*., 2009).

A number of studies demonstrated the protective effect of the flavanone hesperidin and the xanthone mangiferin against oxidative stress by protecting the antioxidant defence system, scavenging radicals and preventing ROS induced damage. Hesperidin prevented the formation of malonaldehyde *in vitro* through the oxidation of ethyl arachidonate by hydroxyl radicals, indicative of radical scavenging activity (Lee *et al*., 2003). Yi *et al*. (2008) confirmed that hesperidin scavenged organic free radicals, hydroxyl radicals, superoxide anions and hydrogen peroxides *in vitro*. Hesperidin reduced DNA damage and lipid peroxidation in γ-irradiated cultured lymphocytes through antioxidant radical scavenging activity (Hosseinimehr *et al*., 2009; Kalpana *et al*., 2009). Kamaraj *et al*. (2009) also demonstrated that hesperidin protected against benzo(a)pyrene-induced oxidative stress in Swiss albino mice lungs, by reducing lipid peroxides, depletion of antioxidant enzymes and GSH. Hesperidin also reduced dextran sulphate sodium-induced lipid peroxidation and myeloperoxidase activity in the colon of mice (Xu *et al*., 2009). Furthermore, oral administration of hesperidin to Swiss albino rats resulted in a significant reduction of acrylonitrile-induced lipid peroxidation and decrease in SOD, catalase and glutathione peroxidase activity in the brain (El-Sayed *et al*., 2008). Mangiferin orally administered to Wistar rats, reduced iron-dextran induced oxidative stress by decreasing lipid peroxidation and increasing GSH in the blood and liver (Pardo-Andreu *et al*., 2008). Mangiferin also reduced the production of ROS in stimulated macrophages of Wistar rats (Garcia *et al*., 2002). Mangiferin injected intraperitoneally into Wistar rats, reduced cyclophosphamide-induced lipid peroxidation and catalase and SOD depletion in lymphocytes and macrophages (Muruganandan *et al*., 2005). Mangiferin was also found to reduce oxidative stress induced by TPA in mice. Mangiferin reduced the production of ROS in macrophages, prevented SOD depletion in blood, lipid peroxidation in serum and liver, and DNA damage in the liver and brain (Sanchez *et al*., 2000). Mangiferin also protected the antioxidant defence system by preventing GSH depletion in 1-methyl-4-phenyl-pyridine ion-induced oxidative stress in a mouse neuroblastoma cell line (Amazzal *et al*., 2007). Mangiferin decreased the formation of ROS in cultured neuron cells and increased the cell survival of lymphocytes in culture exposed to hydrogen peroxide (Campos-Esparza *et al*., 2009; Muruganandan *et al*., 2005). Furthermore, mangiferin showed antioxidant activity *in vitro* by scavenging superoxide radicals, inhibiting the formation of ROS induced by cadmium chloride in hepatoma cells and reducing lipid peroxidation in erythrocytes treated with hydrogen peroxide (Leiro *et al*., 2003; Rao, 2009; Rodriquez *et al.,* 2006).

7.1.4.c. Antimutagenic activities

Marnewick *et al.* (2000) and Van der Merwe *et al*. (2006) showed that aqueous extracts of fermented and unfermented honeybush have antimutagenic activity against the occupational mutagen 2-acetylaminofluorene (2-AAF) and the mycotoxin aflatoxin B_1 (AFB_1). Van der Merwe *et al*. (2006) also showed that the polyphenols hesperidin and mangiferin have antimutagenic activity against 2-AAF and AFB_1. Cytosolic liver fractions from rats that were fed aqueous extracts of fermented and unfermented honeybush were found to render protection against mutagenesis by 2-AAF and AFB_1 in the *Salmonella* mutagenicity assay (Marnewick *et al*., 2004).

7.1.4.d. Antitumourigenic activities

Honeybush extracts and the polyphenols hesperidin and mangiferin show ability to prevent carcinogenesis by reducing tumourigenesis in a number of carcinogenesis models. Marnewick *et al*. (2005) showed that methanol extracts of fermented and unfermented honeybush significantly suppressed skin tumourigenesis in ICR mice initiated with DMBA and promoted with TPA. Tumour volume and number of tumours per mouse decreased, as well as a delay in the time of tumour appearance. Aqueous extracts of fermented and unfermented honeybush fed to Fischer rats, protected against diethylnitrosamine-initiated, fumonisin B_1-induced liver carcinogenesis (Marnewick *et al*., 2009).

Hesperidin inhibited the development of oesophageal cancer in Wistar rats initiated by the carcinogen N-methyl-N-amylnitrosamine (Tanaka *et al*., 1997). Hesperidin inhibited DMBA-initiated, TPA-promoted skin tumourigenesis in mice (Berkada *et al*., 1998) and benzo(a)pyrene induced lung tumourigenesis in Swiss albino mice (Kamaraj *et al*., 2009). Yoshimi *et al.* (2001) showed that mangiferin protects against the carcinogen azoxymethane induced aberrant crypt foci in rats, which is a measure of tumourigenesis in the intestine. Hesperidin showed anti-metastatic effects and antiproliferative effects in hepatoma cells in culture showing potential as an anti-cancer drug for hepatocellular carcinoma (Bellocco *et al*., 2009; Yeh *et al*., 2009). Park *et al*. (2008) also showed that hesperidin possesses anti-cancer properties as the polyphenol induced apoptosis in human colon cancer cells.

7.1.4.e. Other activities

Hesperidin and mangiferin are known to also have other biological activities, such as the broad-spectrum antimicrobial activity of hesperidin and the cardioprotective, antidiabetic and hypolipidemic properties of mangiferin (Nair & Devi, 2006; Prabhu *et al*., 2008; Sellamuthu *et al*., 2009; Yi *et al*., 2008).

7.2. Rooibos

Rooibos herbal tea is made from the leaves and stems of the plant *Aspalathus linearis* spp. (**Figure 14 b**), which is a legume of the family Fabaceae that grows indigenously in the Cederberg region of South Africa (**Figure 15**). The plant is easily recognizable by its thin needle-like green leaves, woody red stem and small yellow sweet pea-like flowers. Rooibos is consumed as an herbal health tea as it is caffeine free, low in tannin and rich in antioxidants. Extracts are also used in the food industry and is becoming popular in cosmetics (Manley *et al*., 2006). The plant was harvested from the wild and used by local inhabitants long before it was cultivated for commercial use. Trading first began on a small scale in the early 1900s, initiated by the Russian immigrant Benjamin Ginsberg. In 1954, the Rooibos Tea Control Board was formed to control the quality of tea produced (Rooibos Limited, nd, b). Export since increased from 524 tons in 1955 to over 7200 tons in 2007, with major markets in Germany, Japan, Netherlands, United Kingdom and USA (Joubert *et al*., 2008a).

7.2.1. Processing

As shown in **Figure 18**, farmers plant rooibos seeds collected from adult plants in nursery seed beds (**a**). When the seedling reaches 10-20 cm in height, it is replanted to the fields. The plant is harvested once it has grown 1.5 meters in height (**b**). To produce fermented rooibos, the harvest (**c**) is shredded and bruised to encourage fermentation (**d**). The rooibos is then fermented in curing heaps for 12 – 14 hours at 38-42°C or in ovens where the oxidation of green rooibos develops the sweet flavour and characteristic red colour. After fermentation, the plant material is spread out and sun-dried outdoors (**e**), steam pasteurized and then sieved, sorted into grades and packaged (**f**) (Rooibos Limited, nd. a). Green rooibos is produced by a method that excludes the fermentation step. Plant material is steamed or pre-dried in the sun to inactivate enzymes and then shredded (Joubert *et al*., 2008a).

Figure 18. Rooibos production process: (**a**) *Aspalathus linearis* seedlings. (**b**) and (**c**) harvesting (**d**) shredding and fermentation (**e**) drying (**f**) packaging (images kindly provided by Rooibos Ltd, Clanwilliam South Africa)

7.2.2. Composition

Aspalathus linearis contains a unique flavonoid called aspalathin, which is the major polyphenolic compound found in rooibos (**Figure 19**). Other compounds have also been found in aqueous extracts of green rooibos. These include the flavonols quercetin, isoquercetin and rutin, the aglycons luteolin and chrysoeriol, the dihydrochalcones aspalathin and nothofagin, and the flavones orientin, iso-orientin, vitexin and iso-vitexin (Bramati *et al*., 2002; Joubert, 1996). Phenolic acids caffeic acid, ferulic acid, p-coumaric acid, p-hydroxybenzoic acid, vanillic acid, protocatechuic acid and syringic acid are also found in rooibos (Joubert, 1996).

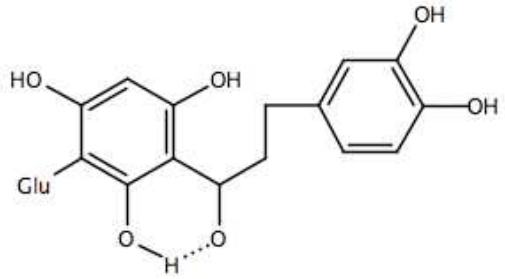

Figure 19. Chemical structure of aspalathin (from Huang *et al*, 2008)

The fermentation of rooibos reduces the content of polyphenols. Joubert (1996) found that fermented rooibos contained only 7% of the dihydrochalcones found in unfermented rooibos. The total flavonoids detected in fermented rooibos are only 9% of that found in unfermented rooibos, with the levels of aspalathin up to 50 times lower (Bramati *et al*., 2003). Essential elements in rooibos include calcium, copper, iron, potassium, magnesium, manganese, nickel, phosphorous and zinc (Malik *et al*., 2008). Volatile compounds identified in aqueous fermented rooibos extracts include mainly ketones, aldehydes, alcohols and esters and few hydrocarbons, phenols, acids and ethers (Habu *et al*., 1985).

7.2.3. Anecdotal properties

The indigenous Khoi tribe that lived in the Cedarberg region, knew the medicinal properties of rooibos, and shared their knowledge with the settlers. Rooibos was marketed in those days as a beverage capable of calming the nervous and digestive system and to improve sleep (Van Niekerk & Viljoen, 2008). In 1968, the South African, Annique Theron, noticed that rooibos was particularly good at relieving colic in babies. This led her to investigate the medicinal properties of rooibos further, which she published (Annique, 2003). Today, rooibos is consumed as a herbal tea to soothe the nervous system of irritability, depression, insomnia, tension and headaches. Digestive complaints such as nausea, vomiting, diarrhoea, heartburn, stomach cramps, constipation, and colic in babies, are also treated with rooibos. Allergies like hay fever and asthma are said to be relieved, and rooibos is used topically on the skin for conditions such as eczema, nappy rash and acne (Gilani *et al*., 2006; Rooibos Limited. nd. c.). Rooibos has already been included in cosmetic skin products to help combat aging and the harmful effects of UV radiation. Also, rooibos has been included in hair care products to improve hair growth and reduce hair loss (Tiedtke & Marks, 2002). Rooibos is included in baby products due to the anti-inflammatory and soothing effects (Annique, 2009).

7.2.4. Biological properties

Both fermented and green rooibos have been shown in various studies to have antioxidant, antimutagenic and antitumourigenic activity. Other properties have also been found, such as protecting the immune system.

7.2.4.a. **Antioxidant activities**

Rooibos showed antioxidant activity both *in vivo* and *in vitro*. Aqueous preparations of fermented and unfermented rooibos, and rooibos flavonoids showed *in vitro* antioxidant activity by scavenging free radicals (Joubert *et al*., 2004; Joubert *et al*., 2005; Lee & Jang, 2004; Standley *et al*., 2001; Von Gadow *et al*., 1997). In addition, aqueous extracts of fermented rooibos fed to rodents reduced lipid peroxidation in the blood of diabetic rats, in the brain of aged rats, in the liver of TPA treated mice and in the blood of γ-ray irradiated mice (Inanami *et al*., 1995; Marnewick *et al*., 2005; Shimoi *et al*., 1996; Ulicná *et al*., 2006). Also, levels of GSSG were reduced and GSH levels increased in the livers of rats fed with aqueous extracts of fermented and unfermented rooibos (Marnewick *et al*., 2003). Furthermore, water and 75% ethanol extracts of rooibos prevented ROS induced DNA damage (Lee & Jang, 2004). Aqueous extracts of rooibos tea prevented carbon tetrachloride-induced liver damage in rats, by inhibiting lipid peroxidation, and oxidation of coenzyme Q_9 and α-tocopherol (Kucharská *et al*., 2004). Rooibos tea reduced dextran sodium sulfate induced oxidative stress in Wister rats by decreasing 8-oxoG levels in urine and prevented SOD depletion in blood serum (Baba *et al*., 2009).

7.2.4.b. **Antimutagenic activities**

Aqueous extracts of fermented and unfermented rooibos, as well as liver fractions from rats that were fed these extracts, protected against 2-AAF and AFB_1 induced mutagenesis (Marnewick *et al*., 2000; Marnewick *et al*., 2004; Standley *et al*., 2001; Van der Merwe *et al*., 2006). The flavonoids aspalathin, nothofagin, luteolin and chrysoeriol from rooibos also show antimutagenic properties using the same test (Snijman *et al*., 2007). Rooibos protected against 2-AAF and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine induced genotoxicity in Chinese hamster lung fibroblasts (Edenharder *et al*., 2002).

7.2.4.c. **Antitumourigenic activities**

Methanolic extracts of fermented and unfermented rooibos suppressed skin tumourigenesis in DMBA-initiated TPA-promoted ICR mice (Marnewick *et al*., 2005). Unfermented rooibos aqueous extracts reduced the number of foci in the liver of Fischer rats initiated with diethylnitrosamine and promoted with fumonisin B_1 (Marnewick *et al*., 2009).

7.2.4.d. **Other activities**

Rooibos has also been shown to reduce inflammation, stimulating antibody production and suppressing human immunodeficiency virus-induced cytotoxicity (Ichiyama *et al*., 2007; Na *et al*., 2004; Nakano, 1997). Another study demonstrated bronchodilatory, antispasmodic and blood pressure lowering effects of rooibos (Khan & Gilani, 2006).

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C H A P T E R 3

Inhibition of UVB-promoted skin tumours by extracts of rooibos and honeybush in SKH-1 mice

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Inhibition of UVB-promoted skin tumours by extracts of rooibos and honeybush in SKH-1 mice

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Abstract

The inhibition of 7,12-dimethylbenz[a]anthracene (DMBA)-initiated and ultraviolet B (UVB)-promoted skin tumourigenesis by polyphenolic extracts of honeybush (*Cyclopia intermedia*) and the main polyphenols in honeybush, hesperidin and mangiferin was investigated in a two-stage skin carcinogenesis mouse model.

Hesperidin, mangiferin and ethanol extracts of fermented and "green" (unfermented) honeybush were dissolved in ethanol: acetone (1:1, v/v). Skin tumourigenesis was initiated in female hairless SKH-1 mice with topical application of DMBA and afterwards, irradiation with UVB twice weekly for 22 weeks. The extracts were applied topically to the skin after initiation but before irradiation while tumour incidence was monitored.

"Green" honeybush extract was more effective in reducing the number of tumours per mouse (85.92%, $P < 0.05$), than fermented honeybush (63.61%). Hesperidin (33.14%) and mangiferin (20.23%) were less effective individually when compared to the extracts. All the extracts and polyphenols also reduced the tumour volume. Fermented honeybush extract was the most effective in reducing the tumour volume (95.12%, $P < 0.05$), followed by "green" honeybush (91.12%, $P < 0.05$), hesperidin (85.39%) and mangiferin (74.33%). "Green" and fermented rooibos extracts used as reference, reduced the number of tumours per mouse by 75.37% and 91.39% ($P < 0.05$), respectively, and the tumour volume by 90.74% and 97.28% ($P < 0.05$), respectively.

Polyphenolic extracts of honeybush have anti-tumourigenic properties and show photoprotective properties for the skin. The antioxidant activity, but also other biological activities of polyphenols may play a role in the protective activity of the extracts. Xanthones, flavonols and flavones were identified as a subgroup of polyphenols, which most likely

contributed significantly towards these properties.

Keywords: honeybush, rooibos, hesperidin, mangiferin, UVB, SKH-1, chemoprevention, DMBA, tumourigenesis

1. Introduction

The incidence of skin cancer worldwide is by far higher than for any other cancer type even though this cancer can be prevented by conventional sun protection methods such as using sunscreen daily, wearing protective clothing and improving education about the harmful effects of UV irradiation (1). New strategies are needed to reduce the incidence of skin cancer, as treatment places an unnecessary financial burden on health care services (2; 3; 4; 5). Various epidemiological studies indicate that exposure to UV radiation is the major risk factor in the development of non-melanoma and melanoma skin cancers (6; 7). In experimental animal studies, chronic exposure to UVB has resulted in the development of tumours (8; 9; 10). Skin carcinogenesis is a complex process occurring in three stages; initiation, promotion and progression. The two-stage skin carcinogenesis mouse model with DMBA as the initiator and UVB as the promoter is widely used to study carcinogenesis and preventative strategies (8; 9; 11). Besides causing mutations by direct DNA damage, UVB irradiation also produces reactive oxygen species (ROS) in excess, which can result in oxidative stress if the pro-oxidant/antioxidant equilibrium is disturbed, hence promoting skin carcinogenesis (12). Therefore, the use of photochemopreventive compounds that can target ROS may play an important role in preventing or modulating skin carcinogenesis.

Photochemoprevention is a strategy in which the occurrence of skin cancer is reduced by using naturally occurring compounds e.g. phytochemicals, or synthetic compounds (13). Polyphenols are a group of compounds found ubiquitously in plants and play important functions in plant development and plant-insect interactions (14; 15). They are known to possess antioxidant, anti-inflammatory and antimicrobial as well as antitumourigenic properties (9; 16; 17). Some have been shown to reduce UVB-induced skin tumourigenesis, such as catechins in green tea, silymarin in milk thistle plants, apigenin found in many fruits and vegetables, and resveratrol in grapes and wine (8; 11; 18; 19; 20).

South Africa produces two herbal teas made from the leaves and stems of legume plants that grow indigenously, called honeybush (*Cyclopia* spp.) and rooibos or “red bush” (*Aspalathus linearis*). The most abundant polyphenol in rooibos is the dihydrochalcone aspalathin, and in honeybush the xanthone, mangiferin and the flavanone, hesperidin (21;

22; 23). Just as green tea from *Camellia sinensis* is oxidised to produce black tea during processing, the unprocessed/ "green" herbal teas are also oxidised to produce the fermented herbal teas, which have reduced polyphenol content (24; 25). Polyphenol rich extracts from these plants have antioxidant activity and previous studies have shown that honeybush and rooibos have chemopreventive properties, such as antimutagenic activity and the reduction of oxidative stress (26; 27; 28; 29). Both herbal teas inhibited DMBA-initiated, 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-promoted skin tumourigenesis in mice (30). In the latter study, the honeybush extracts were found to be more effective than the rooibos extracts in reducing tumour growth, even though the rooibos extracts showed a higher antioxidant capacity and polyphenol content than the honeybush extracts. Ethanol: acetone (1:1, v/v) soluble fractions prepared from methanol extracts of these two herbal teas were used. Since the lower antioxidant activity of the methanolic honeybush extracts did not relate to its more effective anti-tumourigenic property, specific polyphenols in the honeybush extracts might be exerting specific biological effects. Hesperidin is an antioxidant and an anti-inflammatory and was previously shown to also possess antitumourigenic activity by inhibiting TPA-induced skin carcinogenesis, benzo(a)pyrene-induced lung carcinogenesis and *N*-methyl-*N*-amylnitrosamine-induced oesophageal carcinogenesis (31; 32; 33; 34; 35). Mangiferin is an antioxidant and previously showed antitumourigenic activity by inhibiting azoxymethane-induced colon carcinogenesis (36; 37). There are however, no data available on the possible modulating effects of these polyphenols and herbal teas on UVB-induced skin carcinogenesis.

In this study, the protective effect of hesperidin, mangiferin and polyphenolic rich ethanol extracts of "green" and fermented honeybush and rooibos was investigated on DMBA-initiated, UVB-promoted skin tumourigenesis using female SKH-1 mice.

2. Materials and Methods

2.1. Chemicals

The chemicals L-ascorbic acid, 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2-azino-di-3-ethylbenzthiazoline sulphonate (ABTS), (+)-catechin hydrate, 7,12-dimethylbenz[a]anthracene (DMBA), fluorescein sodium salt, gallic acid, quercetin dihydrate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), hesperidin and mangiferin were purchased from Sigma-Aldrich (Steinheim, Germany). The chemicals 4-dimethylaminocinnamaldehyde (DMACA) and Folin Ciocalteu's phenol reagent were purchased from Merck (Hohenbrunn, Germany). "Green" and fermented honeybush (*Cyclopia intermedia*) and rooibos (*Aspalathus linearis*) plant material from the

Clanwilliam area was kindly donated by Mr Arend Redelinghuys (Rooibos Limited, Clanwilliam, South Africa). Clear and black flat bottom 96-well microplates were obtained from Greiner Bio-one (Germany).

2.2. Preparation of extracts

Ethanol soluble polyphenolic rich extracts of both fermented and "green" honeybush and rooibos were prepared as previously described (30). Plant material 10% (m/v) was extracted three times with chloroform for 12 h each time on a stirrer and filtered through Whatmann no. 1 paper to remove chlorophyll and lipophilic compounds. The retained plant material was further extracted three times with absolute ethanol for 12 h each time on a stirrer. The plant material was filtered through Whatmann no. 1 paper and the filtrate retained. The filtrate was then evaporated at 45°C and 150 rpm under reduced pressure in a rotary evaporator (Laborota 4000, Heidolph Instruments, Germany) and the remaining solids weighed and stored in the dark at room temperature in a desiccator. The dried extracts were prepared as a 1% (w/v) solution in ethanol: acetone (1:1, v/v) for the determination of total polyphenol, flavonol and flavanol content and antioxidant capacity. The solid content of the honeybush and rooibos extracts and pure compounds dissolved in ethanol: acetone (1:1, v/v) was determined gravimetrically after drying aliquots at 50°C for 48 h. The analysis of each extract was done in triplicate and each assay was repeated at least three times.

Ethanol extracts were prepared for the animal study at a concentration of 30 mg/ml in ethanol: acetone (1:1, v/v) as described by Marnewick *et al*. (30). Pure compounds, hesperidin and mangiferin were prepared at a concentration of 3 mg/ml (3.93 mM) and 4 mg/ml (9.47 mM) in the same solvent, respectively. The doses of pure compounds were chosen according to the ratio (3:4, hesperidin:mangiferin) and concentration (less than 5 mg pure compound per 30 mg of extract) occurring in the "green" honeybush tea extract determined by HPLC analysis (see Table 2).

2.3. Total polyphenol and flavonoid content of the extracts

The total polyphenol content of the ethanol extracts was determined using the Folin Ciocalteu method described by Singleton *et al*. (38). A volume of 25 µl of the various extracts was incubated in a clear 96-well flat bottom plate for 5 min with 125 µl freshly prepared 0.2 N Folin Ciocalteu's phenol reagent where after 100 µl 7.5% sodium carbonate was added and incubated for 2 h. The absorbance at 765 nm was measured on a plate spectrometer (Multiskan, Thermo Electron Corporation) and total polyphenols calculated using the

standard gallic acid in 10% ethanol. Results were expressed as mg gallic acid equivalents per gram extract.

The flavanol/proanthocyanidin and flavonol/flavone/xanthone content of the ethanol extracts was determined colourimetrically at 640 nm and spectrophotometrically at 360 nm, respectively (39; 40; 41). For the flavanol assay, a freshly prepared 0.05% DMACA solution was made by dissolving DMACA in 8% HCl prepared in methanol. The extracts (50 µl) were incubated in a clear 96-well flat bottom plate with 250 µl DMACA solution for 30 min. For the flavonol assay, a solution of 0.1% HCl prepared in 95% ethanol was made. The extracts (12.5 µl) were incubated in a clear 96-well flat bottom plate with 12.5 µl 0.1% HCl-ethanol solution and 225 µl 2% HCl for 30 min. The absorbance was measured on a plate spectrometer (Multiskan, Thermo Electron Corporation) and flavanols and flavonols calculated using the standards catechin in methanol and quercetin in 95% ethanol, respectively. Results were expressed as mg standard equivalents per gram herbal tea extract.

2.4. Total antioxidant capacity of the extracts and pure compounds

The oxygen radical absorbance capacity (ORAC) was determined according to the fluorometric method described by Ou *et al*. (42). A 36 ng/ml fluorescein and a 2.5% AAPH radical solution were freshly prepared in 75 mM phosphate buffer (pH 7.4). The reaction was initiated by incubating 12 µl of herbal tea extract in a black 96-well flat bottom plate with 138 µl fluorescein solution and 50 µl AAPH solution. The decrease in fluorescence was then measured every 5 min for 2 h on a plate fluorometer (Fluoroskan, Thermo Electron Corporation). The ORAC values were determined by comparing the sample curve to the standard curve obtained for trolox. Results were expressed as µmol trolox equivalents per gram extract.

The ferric reducing antioxidant power (FRAP) and the trolox equivalent antioxidant capacity (TEAC) was determined according to the spectrophotometric methods described by Benzie & Strain (43) and Pellegrini *et al*. (44), respectively. For the FRAP assay, the ethanol extracts (10 µl) were incubated in a clear 96-well flat bottom plate in 300 µl FRAP solution (250 mM sodium acetate in acetic acid, pH 3.6; 0.83 mM TPTZ in 40 mM hydrochloric acid; 1.67 mM Iron (III) chloride hexahydrate) at 37°C for 30 min. The reaction was then measured at 593 nm on a plate spectrometer (Multiskan, Thermo Electron Corporation) and the FRAP value determined by comparing to the standard ascorbic acid. Results were expressed as µmol ascorbic acid equivalents per gram extract. For the TEAC assay, an ABTS radical solution

was prepared by incubating 7 mM ABTS with 2.42 mM potassium peroxodisulfate in the dark overnight. The solution was diluted with ethanol to an absorbance of 2. The ethanol extracts (25 µl) were incubated in a clear 96-well flat bottom plate in 300 µl ABTS radical solution for 30 min. The TEAC value was then determined by measuring the reaction at 734 nm on a plate spectrometer (Multiskan, Thermo Electron Corporation) and compared to the standard trolox in ethanol. Results were expressed as µmol trolox equivalents per gram extract.

2.5. HPLC quantification of major polyphenols in the honeybush extracts

High performance liquid chromatography (HPLC) was used to determine the concentrations of hesperidin and mangiferin in the honeybush ethanol extracts according to an adapted method described by Bramati *et al*. (45). HPLC analysis was done on a Finnigan Spectra SCM1000 system, which consisted of an AS3000 autosampler, P2000 pump and UV1000 UV detector. A YMC-Pac Pro C18 (5 µm, 150x4.6 mm I.D.) column was used at a flow rate of 0.8 ml per min, with a 20 µl sample injection volume, and a 95% - 5% linear gradient of 1% acetic acid – acetonitrile over 30 min. Detection was at 280 nm and data was interpreted using the ChromQuest 4.2 system manager. Mangiferin and hesperidin standards (50 µg/ml) were injected in triplicate, and repeated at least three times, to determine calibration curves[2].

2.6. Animals and UV source

Breeding pairs of SKH-1 mice were purchased from Charles River Laboratories (Kent, United Kingdom) and quarantined at the Animal Unit (Medical School, University of Cape Town, South Africa). Thereafter, mice were bred and 6-week-old female offspring were selected for the study. The animals were housed in filter top cages with corncobs as bedding material, which was changed once weekly. They had free access to standard rodent diet pellets (protein 160 g/kg, moisture 120 g/kg, lipid 25 g/kg, fibre 60 g/kg, phosphorous 7 g/kg, calcium 18 g/kg) and drinking water and were kept in a ventilated room at approximately 21°C on a 12 h photoperiod. The selected mice were acclimatized for one week prior to experimental procedures. An UVIlink crosslinker (UVItec Limited, UK) was used as the UVB source. The unit was fitted with an ultraviolet source of six 8 Watt T-8M UVB tubes (Vilber Lourmat, France) emitting a wavelength of 312 nm at a distance of 18 cm from the base. Ultraviolet radiation energy output was monitored by the built-in radiometer, which is controlled by a microprocessor, allowing precise and consistent dosage. Ethical approval

[2] See Addendum 2: HPLC quantification of hesperidin and mangiferin
[3] See Addendum 1: Ethics approval obtained

was obtained from the University of Cape Town Research Animal Ethics Committee before commencement of the study (REC REF: 005/025)[3].

2.7. Skin carcinogenesis protocol

The skin carcinogenesis study[4] was performed according to the protocol[5] described by Zhaorigetu *et al*. (10). Female SKH-1 mice were randomly divided into nine groups of ten mice each with five mice per cage. Initiation was introduced with a single topical application of DMBA [200 nmol, 100 µl ethanol: acetone (1:1, v/v)]. One week after initiation, skin cancer was promoted by exposing the mice to 180 mJ/cm^2 UVB, twice a week for 22 weeks. To test the protective effect of the honeybush herbal tea extracts and pure compounds, mice were treated with 100 µl of extract (30 mg/ml), hesperidin (3 mg/ml) or mangiferin (4 mg/ml) 30 min before UVB irradiation. Rooibos extracts were included as a reference. The following experimental control groups were also included in the study: (a) positive control group (initiated with DMBA) receiving a topical application of 100 µl solvent 30 min before UVB irradiation, (b) a solvent control group (not initiated with DMBA) receiving a topical application of 100 µl solvent but without UVB irradiation and (c) a negative control group (initiated with DMBA) receiving a topical application of 100 µl solvent without UVB irradiation. The weight of the mice, the number of skin tumours and the volume of tumours were recorded weekly, for each animal. The inhibition of tumour development by the extracts and pure compounds was calculated as a percentage of the positive control. After 22 weeks the mice were terminated by carbon dioxide asphyxiation and tumour size and numbers were noted. Samples of tumours were collected and preserved in 37% formaldehyde for future analysis.

2.8. Statistical analysis

The honeybush and rooibos extracts and pure compounds data was analysed using ANOVA one-way analysis of variance with MedCalc v 9.4.2.0 software. The tumour incidence data was analysed using the Kruskal-Wallis one-way ANOVA on ranks hypotheses with NCSS v 07.1.14 software. To determine statistically significant differences between each mouse group, the Tukey-Kramer Multiple-comparison test was used. Data not normally distributed was log transformed. A *p* value of 0.05 was considered statistically significant. Correlations were calculated using Microsoft Excel 2008 for Mac v 12.0 software. A *r* value of 0.8 was considered a good correlation.

[4] See Addendum 3: Animal study conditions
[5] See Addendum 4: Skin carcinogenesis protocol

3. Results

3.1. Total polyphenol and flavonoid content of the extracts

The total polyphenol content (**Table 1**)[6] of the ethanol: acetone (1:1, v/v) soluble fraction of "green" honeybush extract (179.618 mg/g) was significantly ($P < 0.05$) higher than the corresponding fermented honeybush extract (69.916 mg/g). Similarly, the total polyphenol content of the reference "green" rooibos extract (435.613 mg/g) was significantly ($P < 0.05$) higher than the corresponding fermented rooibos extract (166.502 mg/g), with the "green" rooibos extract exhibiting a much higher ($P < 0.05$) total polyphenol content than any of the other extracts. The flavanol/proanthocyanidin content of the "green" honeybush extract (15.691 mg/g) was significantly ($P < 0.05$) higher than the corresponding fermented honeybush extract (1.566 mg/g), and the reference "green" rooibos extract (33.421 mg/g) significantly ($P < 0.05$) higher than the fermented rooibos extract (1.693 mg/g), with "green" rooibos having the highest ($P < 0.05$) content of flavanols/proanthocyanidins compared to the other extracts. "Green" honeybush had a significantly ($P < 0.05$) higher content of flavonols/flavones/xanthones (85.147 mg/g) than fermented honeybush (51.862 mg/g), while the reference "green" rooibos extract (103.845 mg/g) a lower ($P < 0.05$) content of flavonols/flavones/xanthones than fermented rooibos (136.113 mg/g).

3.2. Hesperidin and mangiferin content in the honeybush extracts

The concentrations of hesperidin and mangiferin were significantly ($P < 0.05$) higher in the "green" honeybush extract than in the fermented honeybush extract (**Table 2**). In the "green" honeybush extract, the concentration of mangiferin (62.721 mg/g) was significantly ($P < 0.05$) higher than hesperidin (40.742 mg/g), while in the fermented honeybush extract the concentration of mangiferin (2.559 mg/g) was significantly ($P < 0.05$) lower than hesperidin (24.260 mg/g).

[6] See Addendum 5: Contribution of flavonoids to the total polyphenol content

Table 1. Total polyphenol and flavonoid content of the honeybush and rooibos extracts

Extract	Total polyphenol content		Flavanol/proanthocyanidin content		Flavonol/flavone/xanthone content	
(Ethanol: acetone)	(mg GAE/g)	(mg GAE/100 µl)	(mg CE/g)	(mg CE/100 µl)	(mg QE/g)	(mg QE/100 µl)
"Green" Honeybush	$179.618 \pm 5.018^{Hf,Rg,Rf}$	0.539 ± 0.015	$15.691 \pm 1.597^{Hf,Rg,Rf}$	0.047 ± 0.005	$85.147 \pm 7.238^{Hf,Rg,Rf}$	0.255 ± 0.022
Fermented Honeybush	$69.916 \pm 2.708^{Hg,Rg,Rf}$	0.210 ± 0.008	$1.566 \pm 2.144^{Hg,Rg}$	0.005 ± 0.006	$51.862 \pm 3.874^{Hg,Rg,Rf}$	0.156 ± 0.012
"Green" Rooibos	$435.613 \pm 5.757^{Hg,Hf,Rf}$	1.307 ± 0.017	$33.421 \pm 3.322^{Hg,Hf,Rf}$	0.100 ± 0.010	$103.845 \pm 9.493^{Hg,Hf,Rf}$	0.312 ± 0.028
Fermented Rooibos	$166.502 \pm 6.699^{Hg,Hf,Rg}$	0.500 ± 0.020	$1.693 \pm 1.061^{Hg,Rg}$	0.005 ± 0.003	$136.113 \pm 3.786^{Hg,Hf,Rg}$	0.408 ± 0.011

Values in columns are means ± SD of three determinations (n = 3). Superscripts indicate significant differences $P < 0.05$. Abbreviations: GAE, gallic acid equivalents; CE, catechin equivalents; QE, quercetin equivalents; Hg, "green" honeybush; Hf, fermented honeybush; Rg, "green" rooibos; Rf, fermented rooibos.

Table 2. Concentration of hesperidin and mangiferin in the honeybush extracts

Extract (Ethanol: acetone)	Hesperidin (mg/g)	(mg/100 µl)	Mangiferin (mg/g)	(mg/100 µl)
"Green" honeybush	$40.742 \pm 5.268^{HgM,HfH,HfM}$	0.122 ± 0.016	$62.721 \pm 3.253^{HgH,HfH,HfM}$	0.188 ± 0.010
Fermented honeybush	$24.260 \pm 2.156^{HgH,HgM,HfM}$	0.073 ± 0.006	$2.559 \pm 1.019^{HgH,HfH,HfM}$	0.008 ± 0.003

Values in columns are means ± SD of three determinations (n = 3). Superscripts indicate significant differences (P < 0.05). Abbreviations: HgH, "green" honeybush hesperidin; HgM, "green" honeybush mangiferin; HfH, fermented honeybush hesperidin; HfM, fermented honeybush mangiferin.

3.3. Total antioxidant capacity of the extracts and pure compounds

Three different assays were used to determine the antioxidant capacity of the extracts and pure compounds at the concentration applied to the skin. Results from the FRAP and TEAC assays correlated well with each other (r = 0.998), and results from the ORAC assay showed correlations with the FRAP (r = 0.938) and TEAC (r = 0.916) assays. The "green" extracts showed significantly (P < 0.05) higher antioxidant capacities than their corresponding fermented extracts, in all three assays (**Table 3**). "Green" honeybush had a slightly higher antioxidant capacity (8.14 µmol, 3.01 µmol, 14.40 µmol) than fermented rooibos (6.45 µmol, 2.23 µmol, 14.12 µmol). Both hesperidin and mangiferin had significantly (P < 0.05) lower antioxidant capacities when compared to the extracts. Hesperidin (0.02 µmol, 0.02 µmol, 0.28 µmol) had a lower antioxidant activity than mangiferin (0.13 µmol, 0.05 µmol, 0.24 µmol) in the FRAP and TEAC assays, but not the ORAC assay. The antioxidant capacity of "green" rooibos extract was the highest (22.81 µmol, 9.48 µmol, 24.89 µmol), followed by "green" honeybush extract (8.14 µmol, 3.01 µmol, 14.40 µmol), fermented rooibos extract (6.45 µmol, 2.32 µmol, 14.12 µmol), fermented honeybush extract (2.47 µmol, 0.75 µmol, 8.46 µmol), mangiferin (0.13 µmol, 0.05 µmol, 0.24 µmol) and hesperidin (0.02 µmol, 0.02 µmol, 0.28 µmol). The antioxidant capacity of the extracts correlated well with the total polyphenol content (FRAP r = 0.996, TEAC r = 0.994, ORAC r = 0.991), indicating that an increase in antioxidant capacity may be directly related to the total polyphenol content.

Table 3. Antioxidant capacity of the extracts and pure compounds

Extract / pure compound	Soluble solids	FRAP		TEAC		ORAC	
Ethanol: acetone	(mg/100 µl)	(µmol AAE/g)	(µmol AAE/ 100 µl)	(µmol TE/g)	(µmol TE/100 µl)	(µmol TE/g)	(µmol TE/100 µl)
"Green" honeybush	$2.67 \pm 0.03^{Hf,H,M}$	2714.31 ± 239.79	$8.14 \pm 0.72^{Hf,H,M,Rg,Rf}$	1003.13 ± 284.03	$3.01 \pm 0.85^{Hf,H,M,Rg}$	4798.55 ± 315.16	$14.40 \pm 0.95^{Hf,H,M,Rg}$
Fermented honeybush	$1.97 \pm 0.13^{Hg,Rg,H,M}$	823.54 ± 52.91	$2.47 \pm 0.16^{Hg,H,M,Rg,Rf}$	248.27 ± 12.88	$0.75 \pm 0.04^{Hg,H,M,Rg,Rf}$	2818.38 ± 79.07	$8.46 \pm 0.24^{Hg,H,M,Rg,Rf}$
Hesperidin	$0.04 \pm 0.01^{Hg,Hf,Rg,Rf}$	61.01 ± 3.42	$0.02 \pm 0.001^{Hg,Hf,M,Rg,Rf}$	51.77 ± 0.80	$0.02 \pm 0.0002^{Hg,Hf,M,Rg,Rf}$	923.46 ± 1.77	$0.28 \pm 0.001^{Hg,Hf,Rg,Rf}$
Mangiferin	$0.03 \pm 0.01^{Hg,Hf,Rg,Rf}$	320.67 ± 5.99	$0.13 \pm 0.002^{Hg,Hf,H,Rg,Rf}$	133.07 ± 3.36	$0.05 \pm 0.001^{Hg,Hf,H,Rg,Rf}$	603.72 ± 18.53	$0.24 \pm 0.01^{Hg,Hf,Rg,Rf}$
"Green" rooibos	$2.53 \pm 0.03^{Hf,H,M}$	7602.32 ± 194.15	$22.81 \pm 0.58^{Hg,Hf,H,M,Rf}$	3160.10 ± 682.81	$9.48 \pm 2.05^{Hg,Hf,H,M,Rf}$	8296.43 ± 57.11	$24.89 \pm 0.17^{Hg,Hf,H,M,Rf}$
Fermented rooibos	$2.32 \pm 0.09^{H,M}$	2150.11 ± 170.75	$6.45 \pm 0.51^{Hg,Hf,H,M,Rg}$	743.40 ± 161.40	$2.23 \pm 0.48^{Hf,H,M,Rg}$	4705.28 ± 64.77	$14.12 \pm 0.19^{Hf,H,M,Rg}$

Values in columns are means ± SD of three determinations (n = 3). Superscripts indicate significant differences $P < 0.05$. Abbreviations: ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant power; TEAC, trolox equivalent antioxidant capacity; AAE, ascorbic acid equivalents; TE, trolox equivalents; Hg, "green" honeybush; Hf, fermented honeybush; Rg, "green" rooibos; Rf, fermented rooibos; H, hesperidin; M, mangiferin.

3.4. Modulation of UVB-promoted skin tumours

There were no deleterious effects on the mice as a result of the protocol followed, as indicated by the similar weight profile (data not shown)[7], nor were there any other noticeable signs of illness. The positive control mice (DMBA/solvent/UVB) developed an average of 7.75 tumours per mouse, with 100% of mice developing tumours by the end of the study (**Table 4**)[8]. The first tumours developed in the eleventh week of promotion and the rate (number of tumours per unit of time) of tumour incidence increased throughout the experiment (**Figure 1a**). The solvent control group (solvent only) did not develop any tumours, nor did the negative control group (DMBA/solvent). Application of the honeybush extracts inhibited the development of tumours, but there was no real delay in the onset of tumours. The "green" honeybush extract showed the highest significant ($P < 0.05$) protection with only 1.09 tumours per mouse (85.92% inhibition). "Green" honeybush was effective in delaying the onset of the first tumour by two weeks (thirteenth week) and also maintained a low rate of tumour incidence compared to the positive control. Fermented honeybush extract showed high protection against tumour development with 2.82 tumours per mouse (63.61% inhibition). Fermented honeybush did not delay the onset of the first tumours (tenth week), but did maintain a lower rate of tumour incidence compared to the positive control. Hesperidin and mangiferin showed a lower protection against the development of tumours compared to the honeybush extracts, with hesperidin-treated mice developing 5.18 tumours per mouse (33.14% inhibition) and mangiferin-treated mice developing 6.18 tumours per mouse (20.23% inhibition). Hesperidin delayed the onset of the first tumours by one week (twelfth week), while mangiferin did not delay the onset of the first tumours (ninth week). The rate of tumour incidence in the hesperidin group and the mangiferin group were high compared to the honeybush groups. The rooibos extracts used as a reference also inhibited the development of tumours. Fermented rooibos showed the highest significant ($P < 0.05$) protection with only 0.67 tumours per mouse (91.39% inhibition) and "green" rooibos with 1.91 tumours per mouse (75.37% inhibition). The rooibos extracts did not delay the onset of first tumours (tenth and ninth week), but the rate of incidence was very low compared to the positive control.

The average volume of tumours per mouse for the positive control group was 490.87 mm^3 (**Table 4**) and the average rate of increase in volume was higher in the positive control than the other groups (**Figure 1b**). The volume of tumours per mouse was significantly ($P < 0.05$) reduced by topical application of the honeybush extracts. Fermented honeybush-treated

[7] See Addendum 6: Weight profile of mice
[8] See Addendum 7: Tumours on the dorsal skin of SKH-1 mice

mice had a mean tumour volume per mouse of 23.95 mm^3, thus showing a 95.12% inhibition when compared to the positive control group. “Green” honeybush-treated mice had a tumour volume per mouse of 43.61 mm^3, showing a 91.12% inhibition when compared to the positive control. The honeybush extract-treated mice maintained a lower average tumour volume per mouse until the final week of the study, when an increase in size was noted. Hesperidin and mangiferin resulted in the development of larger tumours when compared with the honeybush extract-treated groups, but were still smaller than the mean tumour volume of the positive control. The average tumour volume per mouse for hesperidin and mangiferin was 71.73 mm^3 (85.39% inhibition) and 126 mm^3 (74.33% inhibition), respectively.

Table 4. The mean number of tumours and tumour volume per mouse in each group at the end of the skin cancer study

Experimental group	*n*	Number of tumours (% mice)	% Inhibition	Tumour volume (mm^3)	% Inhibition
Positive control	12	7.75 ± 4.78 (100)$^{N, Hg, Rf}$	-	490.87 ± 726.92$^{N, Hg, Hf, Rf}$	-
Negative control	10	nd	-	nd	-
Solvent control	10	nd	-	nd	-
"Green" honeybush	11	1.09 ± 1.14 (54.5)$^{P, N, H, M}$	85.92	43.61 ± 88.62$^{P, N, M}$	91.12
Fermented honeybush	11	2.82 ± 1.66 (90)$^{N, Rf}$	63.61	23.95 ± 47.06$^{P, N}$	95.12
Hesperidin	11	5.18 ± 3.03 (90.9)$^{N, Hg, Rf}$	33.14	71.73 ± 115.26$^{N, Rf}$	85.39
Mangiferin	11	6.18 ± 3.25 (100)$^{N, Hg, Rf}$	20.23	126.00 ± 126.94$^{N, Hg, Rf}$	74.33
"Green" rooibos	11	1.91 ± 1.08 (90.9)$^{N, Rf}$	75.37	45.46 ± 83.93^{N}	90.74
Fermented rooibos	12	0.67 ± 0.78 (50)$^{P, N, Hf, H, M, Rg}$	91.39	13.35 ± 37.01$^{P, H, M}$	97.28

Values in columns are means ± SD. Values in brackets indicate % mice with tumours. Superscripts indicate significant differences $p < 0.05$. Abbreviations: N, negative; P, positive control; Hg, "green" honeybush; Hf, fermented honeybush; Rg, "green" rooibos; Rf, fermented rooibos; H, hesperidin; M, mangiferin; nd, none detected.

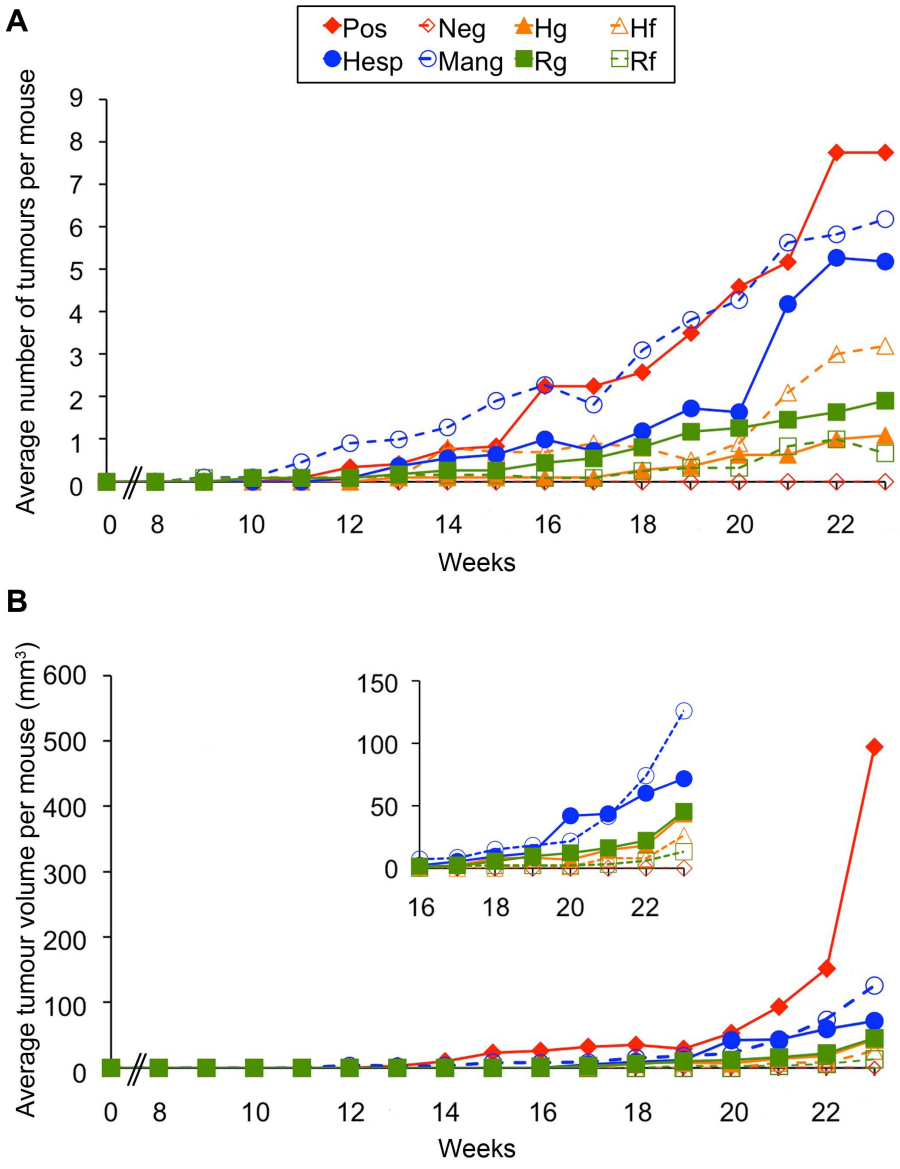

Figure 1. (**A) Mean number of tumours and (B) mean tumour volume per mouse over each week** (Abbreviations: Pos, positive control; Neg, negative control; Hg, "green" honeybush; Hf, fermented honeybush; Hesp, hesperidin; Mang, mangiferin; Rg, "green" rooibos; Rf, fermented rooibos)

Hesperidin and mangiferin-treated mice maintained small tumour sizes for the first few weeks of tumour development, but then increased during the last five weeks of the study. The rooibos extracts used as a reference also reduced the volume of tumours per mouse. Fermented rooibos developed significantly ($P < 0.05$) smaller tumours with a volume per mouse of 13.35 mm^3, showing a 97.28% inhibition when compared to the positive control. Fermented rooibos-treated mice maintained a low average tumour volume per mouse throughout the study. "Green" rooibos-treated mice developed small tumours with an

average tumour volume per mouse of 45.46 mm^3, showing a 90.74% inhibition when compared to the positive control. “Green” rooibos-treated mice maintained a lower average tumour volume per mouse until the final week of the study.

4. Discussion

Basal cell carcinoma and squamous cell carcinoma, the non-melanoma skin cancers, are the most commonly occurring cancers worldwide, accounting for approximately 70% and 21% of skin cancers, respectively (46). Melanoma skin cancer is less common, accounting for approximately 9% of all skin cancers, but is aggressive and causes increased mortality due to metastasis to other organs of the body (46; 47). Ultraviolet B (280-320 nm) is the major etiological factor in the development of skin cancer, which occurs mainly on sun-exposed areas of the body (48). Although sun protection strategies are encouraged, skin cancer incidence remains a worldwide burden. An alternative to conventional sun protection methods is chemoprevention, which is defined as “a means of cancer control in which the occurrence of the disease can be entirely prevented, slowed or reversed by topical or oral administration of naturally occurring or synthetic compounds or their mixtures” (49).

Excessive exposure to UVB produces ROS in the skin by reacting with photosensitive molecules and through the induction of the inflammatory response, and causes ROS related photodamage to the skin (50). Many antioxidant polyphenolic compounds and polyphenol rich extracts, which scavenge ROS, have been shown to possess anticarcinogenic activity. For example, green tea polyphenols, which are potent antioxidants, prevent UVB-induced skin carcinogenesis when applied topically to the skin of mice or administered orally (51; 11). Also, the polyphenol silymarin from the milk thistle plant is an antioxidant and has been shown to prevent UVB-induced skin tumourigenesis when applied topically to the skin of mice (8). A polyphenolic rich bark extract from the French Maritime Pine, *Pinus maritima*, which has strong antioxidant activity, prevented UVB-induced skin tumourigenesis when administered orally to mice (9). Polyphenols function in plants not only as protective UV filters, but also in many other biological effects such as plant development and insect interaction (15; 14; 52). In humans, polyphenols show numerous biological activities such as antimicrobial, anti-inflammatory and immunomodulatory activities. It has been hypothesized that the scavenging of ROS may be an important mechanism of chemoprevention by these compounds, but the many other biological properties may also play a role. Polyphenols have therefore been identified as a group of compounds that show greatest potential as chemopreventives for skin disease such as melanoma and non-melanoma skin cancer (53).

In a previous study, polyphenolic methanol extracts of honeybush and rooibos significantly protected against DMBA-initiated, TPA-promoted mouse skin tumourigenesis (30). The present study confirms that polyphenolic ethanol extracts of honeybush and rooibos also inhibits the formation of mouse skin tumours promoted by UVB. A significant decrease in the average tumour incidence per mouse was shown for both fermented and "green" extracts. The reference extract, fermented rooibos, which was the most effective in reducing the incidence of tumours, was also most effective in reducing the average tumour volume per mouse. Fermented honeybush, "green" honeybush and the reference extract "green" rooibos were also highly effective at reducing the tumour incidence and volume. In a previous study by Marnewick *et al*. (30), the fermented honeybush extract was most effective in reducing the number of skin tumours per mouse, followed by "green" honeybush extract, fermented rooibos extract and "green" rooibos extract. The trend found in the present study (Rf>Hg>Rg>Hf) does not match the trend found in the above-mentioned study (Hf>Hg>Rf>Rg). The differences in the efficacy of the various extracts from each study could be attributed to a number of factors, such as different harvest and production location of plant material used, extract preparation methods where ethanol was used instead of methanol, and skin cancer model differences such as the use of SKH-1 mice instead of ICR mice, and promotion with UVB and not TPA.

In order to determine if the phenolic content of the various extracts played an important role in the antitumourigenic effects observed, the total polyphenol content of each extract was determined. Both "green" herbal extracts had higher contents of polyphenols than their respective fermented extracts, confirming that during fermentation, the total phenolic content as well as antioxidant capacity is reduced. The total polyphenol content of each extract correlated well with the FRAP, TEAC, and ORAC data, suggesting that the antioxidant capacity is related to an additive effect of various polyphenols in the extract. In the previous study by Marnewick *et al*. (30), the total polyphenol content in methanol extracts showed the same trend with very similar total polyphenol contents. In this study, the antioxidant capacity as determined by FRAP, TEAC and ORAC did not directly correlate with the degree of protection against tumourigenesis (FRAP r = 0.566, TEAC r = 0.518, ORAC r = 0.810) and the total polyphenol content did not directly correlate with the degree of protection against tumourigenesis either (r = 0.102). "Green" rooibos extract, which had the highest antioxidant capacity and total polyphenol content, and fermented honeybush extract, which had the lowest antioxidant capacity and total polyphenol content, were both the least effective of the extracts in protecting against tumourigenesis. Fermented rooibos and "green" honeybush, which shared similar antioxidant capacities and total polyphenol contents, were the most effective in protecting against tumourigenesis. This could indicate that an optimum antioxidant capacity and polyphenol content may be needed to prevent tumourigenesis

through the reduction of ROS levels, as it is known that polyphenols show pro-oxidant activity at high concentrations and insufficient antioxidant activity at lower polyphenol concentrations (54).

It was previously suggested by Marnewick *et al.* (30), that there may be specific polyphenols or polyphenol subgroups, which are active against tumour promotion, as the total polyphenol content that correlated with the antioxidant capacity, did not correlate with the degree of tumour inhibition observed. In the present study, the polyphenols hesperidin and mangiferin, the main honeybush polyphenols, were tested as possible active compounds in honeybush. Hesperidin, which had a lower antioxidant capacity according to FRAP and TEAC data compared to mangiferin, was more effective in protecting against tumour incidence than mangiferin, and also reduced the volume of tumours more effectively than mangiferin. The ORAC data indicates that hesperidin and mangiferin have similar antioxidant capacities. If an optimum antioxidant capacity was necessary for a high degree of protection against tumourigenesis, then mangiferin with a higher antioxidant capacity should have shown a greater protective effect than hesperidin, but this was not the case. Also, the very low antioxidant capacities of hesperidin and mangiferin at the concentrations tested, compared to the much higher antioxidant capacities of the extracts do not explain how hesperidin and mangiferin were able to protect against tumour development by a modest 33.14% and 20.23%, respectively. This suggests that hesperidin and mangiferin may be active against tumourigenesis in a dose dependent manner or by mechanisms other than scavenging ROS. This is plausible, as both hesperidin and mangiferin have been shown to exhibit other biological properties such as anti-inflammatory and antimutagenic effects (31; 32; 33; 34; 35; 36; 37). As hesperidin and mangiferin were not as effective as “green” and fermented honeybush extracts in protecting against tumourigenesis, other compounds in fermented and “green” honeybush, may be contributing to the protective effect of the extracts by working synergistically. However, hesperidin and mangiferin were able to significantly reduce the tumour volume, and are therefore included as possible contributing active compounds in honeybush herbal tea.

The flavanol/proanthocyanidin and flavonol/flavone/xanthone content of the extracts were analyzed to determine if other groups of polyphenols from the flavonoid subgroup were prevalent. In this study, the “green” extracts had higher flavanol/proanthocyanidin contents than their respective fermented extracts. “Green” rooibos had the highest flavanol/proanthocyanidin content, followed by “green” honeybush, fermented rooibos and fermented honeybush. Fermented rooibos and “green” rooibos had the highest content of flavonols/flavones/xanthones, followed by “green” honeybush and fermented honeybush. The flavanol/proanthocyanidin content did not correlate with the degree of protection of each

extract (r = -0.034), however the flavonol/flavone/xanthone content did show a good correlation (r = 0.829). Fermented rooibos, the most effective in preventing tumourigenesis, had the highest flavonol/flavone/xanthone content, while fermented honeybush, the least effective in preventing tumourigenesis, had the lowest flavonol/flavone/xanthone content. This suggests that other polyphenolic compounds in the herbal tea extracts, specifically xanthone, flavonol and flavone compounds, may contribute towards the inhibition of tumourigenesis. Results from this study corroborate our earlier data to further demonstrate that honeybush and rooibos extracts are not only effective against chemical-induced skin carcinogenesis, but also against UVB radiation-induced skin tumourigenesis (30).

In conclusion, ethanol extracts of rooibos and honeybush are both effective in modulating UVB-induced skin carcinogenesis, and have potential use as a cosmeceutical for sun protection and as a strategy for the prevention of melanoma and non-melanoma skin cancers in humans. Hesperidin, mangiferin, other polyphenols and combinations of polyphenols found in honeybush and rooibos should be further investigated i.e. dose response studies, to determine specific chemopreventive activities and possible synergistic effects, to fully maximize the use of this property. Hereafter, clinical evaluations are needed to confirm the photoprotective value of these two unique herbal teas. Also, mechanisms that may be responsible for the modulation of UVB-promoted skin tumours by these two herbal teas should be investigated. Possible mechanisms may include the reduction of UVB penetration into the skin and the inhibition of UVB-induced DNA damage, inflammation, induction of ornithine decarboxylase and immune suppression.

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CHAPTER 4

Photoprotection by honeybush extracts, hesperidin and mangiferin against UVB-induced skin damage in SKH-1 mice

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Photoprotection by honeybush extracts, hesperidin and mangiferin against UVB-induced skin damage in SKH-1 mice

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Abstract

The mechanism of possible photoprotection by polyphenolic extracts of honeybush and the two most abundant polyphenols found in honeybush, hesperidin and mangiferin were determined using a mouse model. Ethanol: acetone soluble extracts and pure compounds were applied topically to the skin of SKH-1 mice before daily exposures to ultraviolet B (UVB) (180 mJ/cm^2) for 10 days.

The honeybush extracts reduced signs of sunburn, such as erythema, peeling and hardening of the skin. Oedema was significantly ($P < 0.05$) inhibited by "green" (61.04 %) and fermented (40.26 %) honeybush extracts as well as mangiferin (42.86 %). Epidermal hyperplasia was significantly ($P < 0.05$) inhibited by extracts of "green" (30.17 % - 40.11 %) and fermented honeybush (33.26 % - 42.44 %) extracts, and by hesperidin (32.23 % - 40.29 %). Depletion of the antioxidant enzymes catalase and superoxide dismutase (SOD) was significantly ($P < 0.05$) inhibited by the fermented honeybush extract, while mangiferin also inhibited the depletion of SOD. The fermented honeybush extract treated mice showed a significant reduction in UVB-induced lipid peroxidation. Both the "green" and fermented honeybush extracts significantly ($P < 0.05$) prevented the induction of cyclooxygenase-2 (78.06 % and 77.63 %) and ornithine decarboxylase (63.52 % and 79.26 %) respectively, while mangiferin prevented the induction of ornithine decarboxylase (65.61 %). All antioxidant treated mice showed a significant ($P < 0.05$) reduction in GADD45 expression, suggesting that UVB-induced DNA damage was reduced. The "green" honeybush extract showed a 90.38% protection, fermented honeybush extract a 92.98 % protection, hesperidin an 84.91 % protection and mangiferin a 37.16 % protection against DNA damage. A significant ($P < 0.05$) reduction in the expression of OGG1 was also shown, suggesting that oxidative DNA damage was reduced by the "green" (99.62 %) and fermented (76.72 %)

honeybush extracts and hesperidin (75.31 %).

These results show that extracts of honeybush and to some extent, hesperidin and mangiferin, renders protection against UVB-induced skin damage. The mechanisms investigated suggest that honeybush extracts protected the skin via modulation of oxidative stress-induced damage. Other specific biological properties such as anti-inflammatory and modulation of signalling pathways could also be involved.

Keywords: Honeybush, UVB, SKH-1, oxidative stress, inflammation, DNA damage

1. Introduction

Acute exposure of the skin to UVB irradiation not only causes tanning due to increased melanogenesis, but also sunburn [1]. The skin reddens due to an inflammatory response called “erythema”, which occurs immediately after exposure, and peaks within minutes to hours afterwards [2]. Histological changes such as oedema and hyperplasia occur after irradiation and peak within 24-48 hours. Cutaneous oedema is the intercellular and perivascular swelling of the dermis due to the dilatation of blood vessels resulting in the infiltration of inflammatory cells, while hyperplasia is epidermal and dermal thickening due to increased cell proliferation [3]. Ultraviolet B irradiation also generates reactive oxygen species (ROS) within the skin by reacting with photosensitive molecules, and also via the inflammatory response. Oxidative stress occurs due to the excessive increase in ROS levels, resulting in an imbalance between ROS and the skin’s antioxidant defence system [4]. Ultraviolet B radiation causes indirect damage through ROS production and direct damage to important molecular structures in the cells of the skin, such as DNA, proteins and lipids. When epidermal cells are irreversibly damaged, apoptosis occurs, resulting in the production of sunburn cells, which is visible as peeling of the skin. Chronic or repeated exposure to UVB irradiation therefore causes skin photoaging and both the initiation and promotion of photocarcinogenesis [3].

Polyphenols, such as resveratrol from grapes and silymarin from the milk thistle plant, that scavenge UVB-generated ROS in the skin have also been shown to prevent other damaging effects of UVB such as oedema, hyperplasia, inflammation, lipid peroxidation and carcinogenesis [5; 6]. Honeybush (*Cyclopia* spp.*)* is a fynbos plant species that only grows in South Africa. The leaves and stems of honeybush are traditionally used to make a herbal tea. The unprocessed or “green” plant material is oxidised to produce a fermented honeybush herbal tea. Honeybush is rich in antioxidant polyphenols and has previously been

shown to have chemopreventive properties such as reducing oxidative stress, inhibiting mutagenesis and inhibiting tumourigenesis [7; 8; 9]. In a study by Marnewick and coworkers [9], extracts of honeybush significantly reduced the promotion of tumours by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in 7,12 dimethylbenz[*a*]anthracene (DMBA)-initiated mice skin. Honeybush may also protect the skin against UVB, which could contribute towards reducing the damaging effects of acute UVB irradiation, and ultimately prevent carcinogenesis and aging due to chronic exposure. The mechanisms by which honeybush may show photoprotective effects, will therefore be of interest. The two most abundant polyphenols in honeybush are the flavanone hesperidin and the xanthone mangiferin [10]. Both hesperidin and mangiferin have been shown to exhibit antioxidant activity, anti-inflammatory activity and antitumourigenic activity [11; 12; 13; 14; 15]. Due to the known properties of these two polyphenols, it may be that they are active compounds responsible for the previously shown chemoprotective effects of honeybush.

Therefore, the aim of this study was to investigate the effects of “green” and fermented honeybush extracts, and the polyphenols hesperidin and mangiferin on the damaging effects of acute UVB exposure to mice skin.

2. Materials and methods

2.1. Chemicals

The chemicals L-ascorbic acid, 2,2’-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,2-azino-di-3-ethylbenzthiazoline sulphonate (ABTS), 2,6-di-tert-butyl-4-methylphenol (BHT), (+)-catechin hydrate, 5,5’dithiobis-2-nitrobenzoic acid (DTNB), fluorescein sodium salt, formaldehyde, Folin Ciocalteu’s phenol reagent, gallic acid, glutathione reductase, hesperidin, histological grade formaldehyde, 6-hydroxydopamine, mangiferin, 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP), β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), quercetin dihydrate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2-thiobarbituric acid (TBA) and 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ) was obtained from Sigma-Aldrich (Steinheim, Germany). Diethylenetriaminepentaacetic acid (DETAPAC), 4-(dimethylamino)-cinnamaldehyde (DMACA) and malondialdehyde bis (diethyl acetal) (MDA) were purchased from Merck (Hohenbrunn, Germany). The primary antibodies cyclooxygenase-2 (COX-2) (M-19 goat polyclonal), ornithine decarboxylase (ODC) (D-15 goat polyclonal), 8-oxoguanine DNA gylcosylase (OGG1/2) (L-19 goat polyclonal) and Growth arrest and DNA damage inducible protein 45 (GADD45α) (C-4 mouse monoclonal) were purchased from Santa Cruz

Biotechnology (CA, USA), the histobond microscope slides from Marienfeld Laboratory Glassware (Lauda-Königshofen, Germany) and the diaminobenzidine (DAB+) liquid substrate chromogen system was purchased from Dako (South Africa). “Green” and fermented honeybush (*Cyclopia intermedia*) from the Clanwilliam area was kindly donated by Mr Arend Redelinghys (Rooibos Limited, Clanwilliam, South Africa).

2.2. Preparation of honeybush extracts and pure compounds

Ethanol soluble polyphenolic-rich extracts of fermented and “green” honeybush were prepared as described by Marnewick *et al.* [9]. Plant material 10% (m/v) was extracted three times with chloroform for 12 h each time on a stirrer and filtered through Whatmann no. 1 paper to remove chlorophyll and lipophilic compounds. The retained plant material was further extracted three times with absolute ethanol for 12 h each time on a stirrer. The plant material was filtered through Whatmann no. 1 paper and the filtrate retained. The filtrate was then evaporated at 45°C and 150 rpm under reduced pressure in a rotary evaporator (Laborota 4000, Heidolph Instruments Germany) and the remaining solids weighed and stored in the dark at room temperature in a desiccator. The dried extracts were prepared as a 1% (w/v) solution in ethanol: acetone (1:1, v/v) for the determination of total polyphenol, flavonol and flavanol content and antioxidant capacity. The solid content of the honeybush extracts and pure compounds dissolved in ethanol: acetone (1:1, v/v) was determined gravimetrically after drying aliquots at 50°C for 48 hours. The analysis of each extract was done in triplicate and each assay was repeated at least three times for reproducibility.

2.3. Determination of total polyphenol and flavonoid content

The total polyphenol content of the ethanol extracts was determined using the Folin Ciocalteu’s phenol reagent according to the method described by Singleton *et al*. [16]. The total polyphenols were expressed as mg gallic acid standard equivalents per gram extract. The flavanol/proanthocyanidin content of the ethanol extracts was determined colourimetrically at 640 nm using the aldehyde DMACA and expressed as mg catechin standard equivalents per gram extract [17; 18]. The flavonol/flavone/xanthone content of the ethanol extracts was determined spectrophotometrically at 360 nm and expressed as mg quercetin standard equivalents per gram extract [19].

2.4. HPLC quantification of hesperidin and mangiferin

The concentration of hesperidin and mangiferin in the honeybush extracts[9] was determined by HPLC analysis according to an adapted method described by Bramati *et al*. [20], where the elution gradient was adjusted. The HPLC analysis was done on a Finnigan Spectra SCM1000 system, which consisted of an AS3000 autosampler, P2000 pump and UV1000 UV detector. A YMC-Pac Pro C18 (5 μm, 150x4.6 mm I.D.) column was used at a flow rate of 0.8 ml per min, with a 20 μl sample volume, and a 95% - 5% linear gradient of 1% acetic acid – acetonitrile over 30 min. Detection was at 280 nm and data was interpreted using the ChromQuest 4.2 system manager.

2.5. Determination of antioxidant capacity

The oxygen radical absorbance capacity (ORAC) of the ethanol extracts was determined according to the fluorometric method described by Ou *et al*. [21] using the fluorophore fluorescein and the radical AAPH. The ORAC value was expressed as μmol trolox standard equivalents per gram extract. The ferric reducing antioxidant power (FRAP) was determined according to the spectrophotometric method described by Benzie & Strain [22] using the reaction of TPTZ with Fe (II) generated by the donation of hydrogen to iron (III) chloride hexahydrate by antioxidants. The trolox equivalent antioxidant capacity (TEAC) was determined according to the spectrophotometric method described by Pellegrini *et al*. [23] using the ABTS radical generated by potassium peroxodisulfate. The FRAP value was expressed as μmol ascorbic acid standard equivalents per gram extract while the TEAC value was expressed as μmol trolox standard equivalents per gram extract.

2.6. Animals and UV source

Breeding pairs of SKH-1 mice were obtained from Charles River Laboratories (Kent, United Kingdom). The mice were quarantined according to the standard procedures of the Animal Unit at the University of Cape Town (Cape Town, South Africa) and bred. Four to six week old female offspring[10] were selected for the study. The mice had free access to rodent diet cubes (protein 160 g/kg, moisture 120 g/kg, lipid 25 g/kg, fibre 60 g/kg, phosphorous 7 g/kg, calcium 18 g/kg) and water and were kept at 20-23°C on a 12 h dark-light cycle in a room that was ventilated with approximately 16-20 total air changes per h.

[9] See Addendum 2: HPLC quantification of hesperidin and mangiferin
[10] See Addendum 8: Age of the mice

The UVIlink UV crosslinker (UVItec Limited, UK) was fitted with six 8-Watt T-8M 312 nm tubes (Vilber Lourmat, France) at a distance of 18 cm from the base with the UV energy output monitored by the built-in radiometer. Ethical approval for the study was obtained from the University of Cape Town Research Ethics Committee before commencement of the study (REC REF: 005/025)[11].

2.7. Animal study protocol

The short term UVB exposure study was performed according to the protocol described by Vayalil *et al*. [24] (**Figure 1**)[12]. The SKH-1 mice were randomly divided into seven groups of ten mice each; positive, negative and vehicle control groups, "green" and fermented honeybush treated groups, and hesperidin and mangiferin treated groups. The honeybush extracts were prepared in ethanol: acetone (1:1, v/v) at a concentration of 30 mg/ml according to the method of Marnewick *et al*. [9], while hesperidin was prepared at a concentration of 3 mg/ml and mangiferin at 4 mg/ml in ethanol: acetone (1:1, v/v) according to representative concentrations in the "green" honeybush extract as determined by HPLC analysis.

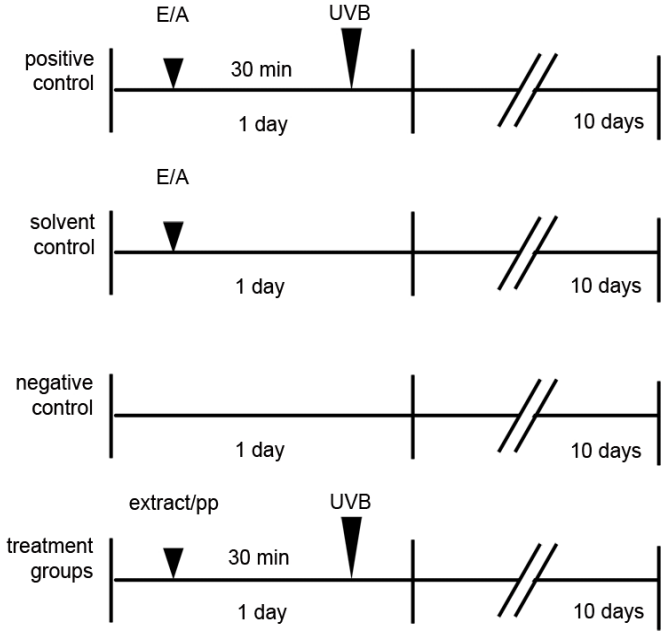

Figure 1. The short term UVB exposure protocol. Mice in the treatment groups were treated with a topical application of herbal tea extract (100 µl) or pure compound (100 µl), and the solvent and positive control groups with solvent (100 µl). Treatment and positive control groups were irradiated 30 min later with 180 mJ/cm^2 UVB. This was done once a day for 10 days. (Abbreviations: E/A, ethanol: acetone; pp, pure polyphenol compound)

[11] See Addendum 1: Ethics approval obtained
[12] See Addendum 3: Animal study conditions

On the first day, the mice in the solvent control group and the positive control group were treated topically on the dorsal skin with 100 µl of ethanol: acetone (1:1, v/v), while the negative control group was left untreated. The "green" and fermented honeybush groups were treated topically on the dorsal skin with 100 µl of the respective honeybush extracts. The hesperidin and mangiferin groups received a 100 µl application of the respective polyphenol on the dorsal skin. Thirty minutes after this treatment, all groups except the negative control and solvent control groups were irradiated with 180 mJ/cm^2 UVB. The duration of the exposure was approximately 45 sec. This treatment was done daily, for ten consecutive days. Mice were monitored daily for any possible harmful effects. The mice were then terminated by carbon dioxide asphyxiation 24 h after the last treatment and the dorsal skin harvested. The weight of the mice was noted before and after the study to serve as a health indicator. Harvested skin tissue samples were fixed for sectioning or snap frozen in liquid nitrogen and then homogenized with a polytron homogenizer in phosphate buffered saline (with or without M2VP). The homogenate was centrifuged at 10 000 g at 4°C for 15 min and the skin preparation collected and stored at -80°C until further analysis.

The sunburn response of the skin, which indicates damage, was monitored every day and recorded. Skin erythema, thickening and peeling was estimated visually and recorded on a scale of degree of damage in a similar manner as described by Zhaorigetu *et al*. [25]. The term "erythema" was used to describe the reddening of the skin. The term "thickening" was used to describe scaling and plaque-like lesions on the skin. "Peeling" referred to the flaking of skin from the surface. The increase in bifold skin thickness, which is an indicator of oedema and hyperplasia, was measured using a Digi-Max slide calliper (Sigma, USA) before the first treatment on day one and before termination. Measurements were taken on three random areas of the dorsal skin and the average thickness determined [26].

2.8. Histology and immunohistochemistry

Skin samples harvested from the mice were immediately fixed in 37% histological grade formaldehyde where after it was processed and embedded in Histotec™ paraffin wax (Merck)[13]. The samples were then sectioned (5 µm) and mounted onto microscope slides and stained with haematoxylin and eosin according to standard histology procedure in the histology laboratory. The average number of epidermal cell layers and the thickness of the epidermis from the stratum basale to the stratum corneum was determined from approximately 10 random fields of three representative samples from each group at 200x magnification using a Zeiss Axioskop 2 light microscope (Carl Zeiss, Germany) and

[13] See Addendum 9: Histology and immunohistochemistry protocol

Photoshop CS3 v10.0 software. A graticule was used to determine the scale. Paraffin embedded tissue was sectioned (5 μm) and collected onto silane treated microscope slides for immunohistochemical staining according to standard procedure in the laboratory. The paraffin embedded sections were dewaxed with xylene and rehydrated through graded alcohols, permeabilized using 0.2% Triton X100 in PBS and boiled in 10 mM sodium citrate, pH 6.0 for 10 min. The sections were then treated with 3% hydrogen peroxide in methanol for 10 min to quench endogenous peroxidases and blocked for 1 hr with 1% BSA in PBS. Primary antibodies for COX-2, ODC, OGG1/2 and GADD45α were incubated on separate sections at 4°C overnight and then with the appropriate secondary horseradish peroxidase-conjugated antibodies for 1 hr at room temperature. The slides were stained with diaminobenzidine (DAB) and the nuclei counterstained with haematoxylin. Slides were viewed using a Zeiss Axioskop 2 light microscope (Carl Zeiss, Germany). Stained cells were counted using Photoshop CS3 v10.0 software in approximately ten random fields of three representative samples from each group at 400x magnification, and expressed as the number of cells per 10 μm in that field. A graticule was used to determine the scale.

2.9. Catalase and superoxide dismutase activity

Catalase activity in the skin preparations were determined using the method described by Aebi [27]. In a quartz cuvette, 170 μl 50 mM potassium phosphate, pH 7.0 and 75 μl 0.1% hydrogen peroxide in 50 mM potassium phosphate, pH 7.0 was added, followed by 5 μl skin preparation. The reaction was measured at 240 nm with a spectrophotometer (Nicolet Evolution 300, Thermo Electron Corporation, Finland) and the decrease in hydrogen peroxide recorded for 2 min in 15 sec intervals. Catalase activity (μmole/min/mg) was determined using the millimolar extinction coefficient of 0.00394. The activity of superoxide dismutase (SOD) was determined spectrophotometrically [28]. In a 96 well plate, 170 μl 0.1 mM DETAPAC in 50 mM sodium phosphate buffer, pH 7.4 and 15 μl crude extract was added followed by 15 μl 1.6 mM 6-hydroxydopamine. The reaction was measured at 490 nm for 4 min at 30 sec intervals and SOD activity expressed as U/mg of protein. Protein concentration was determined using the BCA protein assay kit supplied by Pierce (Illinois, USA).

2.10. Determination of glutathione content

The ratio of reduced (GSH) and oxidized (GSSG) glutathione was determined according to the method described by Asensi *et al*. [29]. In a clear 96-well flat bottom plate, 1U glutathione reductase, 50 μl 0.3 mM DTNB and 50 μl skin preparation for GSH or 50 μl skin preparation

treated with M2VP for GSSG was incubated at 25°C for 5 min. The reaction was then initiated with 50 µl 1 mM NADPH and measured at 412 nm for 5 min at 30 sec intervals and the concentration of total glutathione, GSH and GSSG determined with GSH as standard.

2.11. Determination of TBARS

Thiobarbituric acid reacting substances (TBARS) content in the skin preparations were assessed as an indicator of lipid peroxidation. For the determination of TBARS, a volume of 300 µl sample was mixed with 6.25 mM BHT on ice and then centrifuged at 2000 rpm, 4°C for 15 min. A volume of 350 µl supernatant was added to 0.25% TBA and incubated at 90°C for 20 min. The sample was then transferred to a clear 96-well flat bottom plate and the absorbance measured at 532 nm. The concentration of TBARS per sample was determined using the standard malondialdehyde (MDA) and expressed as nmole per mg protein.

2.12. Statistical analysis

The herbal tea extracts and pure compounds data was analysed using ANOVA one-way analysis of variance with MedCalc v 9.4.2.0 software. All other data was analysed using the Kruskal-Wallis one-way ANOVA on ranks hypotheses with NCSS v 07.1.14 software. To determine statistically significant differences, the Tukey-Kramer Multiple-comparison test was used. Data not normally distributed was log transformed. A *p* value of 0.05 was considered statistically significant. Correlations were calculated using Microsoft Excel 2008 for Mac v 12.0 software. A *r* value of 0.8 was considered a good correlation.

3. Results

3.1. Total polyphenol and flavonoid content

The fermented honeybush extract (69.916 mg/g) contained significantly ($P < 0.05$) less total polyphenols (61.08 %) than the “green” honeybush extract (179.618 mg/g) (**Table 1**). Flavonols/flavones/xanthones were most abundant and contributed 47.40 % (85.147 mg/g) and 74.18 % (51.862 mg/g) of the total polyphenols in the “green” and fermented honeybush extracts, respectively, while the flavanol/proanthocyanidin content of the “green” honeybush extract (15.691 mg/g) comprised only 8.74 % and 2.24 % for the fermented counterpart (1.566 mg/g). The “green” honeybush extract contained significantly ($P < 0.05$) more flavanols/proanthocyanidins and flavonols/flavones/xanthones than the fermented honeybush extract.

Table 1. Total polyphenol and flavonoid content of honeybush extracts topically applied to the skin of SKH-1 mice

Extract	Total polyphenol content		Flavanol/proanthocyanidin content		Flavonol/flavone/xanthone content	
(Ethanol: acetone)	(mg GAE/g)	(mg GAE/100 µl)	(mg CE/g)	(mg CE/100 µl)	(mg QE/g)	(mg QE/100 µl)
"Green" honeybush*	179.618 ± 5.018 ^{Hf}	0.539 ± 0.015	15.691 ± 1.597 ^{Hf}	0.047 ± 0.005	85.147 ± 7.238^{Hf}	0.255 ± 0.022
Fermented honeybush	69.916 ± 2.708 ^{Hg}	0.210 ± 0.008	1.566 ± 2.144 ^{Hg}	0.005 ± 0.006	51.862 ± 3.874 ^{Hg}	0.156 ± 0.012

Values in columns are means ± SD of three determinations (n = 3). Superscripts indicate significant differences ($P < 0.05$). Abbreviations: GAE, gallic acid equivalents; CE, catechin equivalents; QE, quercetin equivalents; Hg, "green" honeybush; Hf, fermented honeybush. *Similar batches not stated by producer.

3.2. Hesperidin and mangiferin content

The concentrations of both hesperidin and mangiferin were significantly ($P < 0.05$) higher in the "green" honeybush extract (40.742 mg/g and 62.721 mg/g) than in the fermented honeybush extract (24.260 mg/g and 2.559 mg/g) respectively (**Table 2**). The concentration of mangiferin in "green" honeybush extract was also significantly ($P < 0.05$) higher than the hesperidin content, while in the fermented honeybush extract, the concentration of mangiferin was significantly ($P < 0.05$) lower than the hesperidin content.

Table 2. Concentration of hesperidin and mangiferin in honeybush extracts

Extract (Ethanol: acetone)	Hesperidin (mg/g)	(mg/100 μl)	Mangiferin (mg/g)	(mg/100 μl)
"Green" honeybush	$40.742 \pm 5.268^{HgM,HfH,HfM}$	0.122 ± 0.016	$62.721 \pm 3.253^{HgH,HfH,HfM}$	0.188 ± 0.010
Fermented honeybush	$24.260 \pm 2.156^{HgH,HgM,HfM}$	0.073 ± 0.006	$2.559 \pm 1.019^{HgH,HfH,HgM}$	0.008 ± 0.003

Values in columns are means ± SD of three determinations (n = 3). Superscripts indicate significant differences ($P < 0.05$). Abbreviations: HgH, green honeybush hesperidin; HgM, green honeybush mangiferin; HfH, fermented honeybush hesperidin; HfM, fermented honeybush mangiferin.

3.3. Antioxidant capacity of honeybush extracts and pure compounds

Using the FRAP, TEAC and ORAC methods for determining antioxidant capacity, the "green" honeybush extract (8.14 μmol/100 μl, 3.01 μmol/100 μl; 14.40 μmol/100 μl) exhibited a significantly ($P < 0.05$) higher antioxidant capacity when compared to the fermented honeybush extract (2.47 μmol/100 μl, 0.75 μmol/ 100μl; 8.46 μmol/100 μl) (**Table 3**). The antioxidant capacity of hesperidin and mangiferin used at the concentrations applied, were greatly reduced compared to the honeybush extracts, with mangiferin (0.13 μmol/100 μl; 0.05 μmol/100 μl; 0.24 μmol/100 μl) exhibiting a higher capacity than hesperidin (0.02 μmol/100 μl; 0.02 μmol/100 μl; 0.28 μmol/100 μl) using the FRAP and TEAC methods, but not with the ORAC method. All three methods for determining antioxidant capacity correlated well with each other (FRAP/TEAC r = 0.998, FRAP/ORAC r = 0.938, TEAC/ORAC r = 0.916).

Table 3. Antioxidant capacity of extracts and pure compounds

Extract / pure compound	Soluble solids	FRAP		TEAC		ORAC	
Ethanol: acetone	(mg/100 µl)	(µmol AAE/g)	(µmol AAE/100 µl)	(µmol TE/g)	(µmol TE/100 µl)	(µmol TE/g)	(µmol TE/100 µl)
"Green" honeybush	$2.67 \pm 0.03^{Hf,H,M}$	2714.31 ± 239.79	$8.14 \pm 0.72^{Hf,H,M}$	1003.13 ± 284.03	$3.01 \pm 0.85^{Hf,H,M}$	4798.55 ± 315.16	$14.40 \pm 0.95^{Hf,H,M}$
Fermented honeybush	$1.97 \pm 0.13^{Hg,H,M}$	823.54 ± 52.91	$2.47 \pm 0.16^{Hg,H,M}$	248.27 ± 12.88	$0.75 \pm 0.04^{Hg,H,M}$	2818.38 ± 79.07	$8.46 \pm 0.24^{Hg,H,M}$
Hesperidin	$0.04 \pm 0.01^{Hg,Hf}$	61.01 ± 3.42	$0.02 \pm 0.001^{Hg,Hf,M}$	51.77 ± 0.80	$0.02 \pm 0.0002^{Hg,Hf,M}$	923.46 ± 1.77	$0.28 \pm 0.001^{Hg,Hf}$
Mangiferin	$0.03 \pm 0.01^{Hg,Hf}$	320.67 ± 5.99	$0.13 \pm 0.002^{Hg,Hf,H}$	133.07 ± 3.36	$0.05 \pm 0.001^{Hg,Hf,H}$	603.72 ± 18.53	$0.24 \pm 0.01^{Hg,Hf}$

Values in columns are means ± SD of three determinations (n = 3). Superscripts indicate significant differences ($P < 0.05$). Abbreviations: ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing antioxidant power; TEAC, trolox equivalent antioxidant capacity; AAE, ascorbic acid equivalents; TE, trolox equivalents; Hg, "green" honeybush; Hf, fermented honeybush; H, hesperidin; M, mangiferin.

3.4. UVB-induced sunburn of mice skin

Daily UVB irradiation of the dorsal skin of SKH-1 mice resulted in a sunburn response, which included erythema, peeling, thickening of the skin and oedema. Mice in the positive control group responded with strong erythema, hardening and peeling of the skin at days four and five, which gradually subsided towards the end of the study (day 10) (**Table 4**)[14]. Neither the negative control, nor the solvent control group mice showed any changes in dorsal skin appearances. The application of "green" and fermented honeybush extracts markedly reduced the sunburn effects of UVB irradiation as mice from these groups showed only mild hardening and erythema from day five, while no peeling occurred. The pure compounds hesperidin and mangiferin did not reduce the sunburn effects of UVB irradiation and showed a similar sunburn response as the positive control group.

Oedema was determined by measuring the increase in bifold skin thickness compared to the normal increase in skin thickness due to growth of the mice. UVB irradiation of mice skin resulted in a significant ($P < 0.05$) increase in bifold skin thickness in the positive control group (0.77 mm) compared to the increase observed in the unirradiated negative control (0.32 mm) and the solvent control group (0.14 mm) (**Table 5**)[15]. Topical application of the "green" honeybush (0.30 mm) and fermented honeybush (0.46 mm) extracts significantly ($P < 0.05$) inhibited the UVB-induced increase in bifold skin thickness, by 61.04% and 40.26%, respectively. Topical application of mangiferin (0.44 mm) also significantly ($P < 0.05$) inhibited the increase in bifold skin thickness by 42.86% while hesperidin only moderately inhibited the increase in bifold skin thickness (0.56 mm) by 27.27%. There was no significant weight loss observed (data not shown)[16]. The "green" honeybush extract was the most effective in inhibiting the increase in bifold skin thickness, followed by the fermented honeybush extract, mangiferin and hesperidin.

[14] See Addendum 10: UVB-induced sunburn
[15] See Addendum 11: Increase in bifold skin thickness
[16] See Addendum 12: Monitored weight of mice

Table 4. Sunburn response of mice skin to UVB irradiation

	Hardening										
Experimental group	**Day**										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**T**
Positive control	-	-	-	-	XXX	XX	X	X	-	-	-
Negative control	-	-	-	-	-	-	-	-	-	-	-
Solvent control	-	-	-	-	-	-	-	-	-	-	-
"Green" honeybush	-	-	-	-	XX	X	-	-	-	-	-
Fermented honeybush	-	-	-	-	XX	X	-	-	-	-	-
Hesperidin	-	-	-	-	XXX	XX	X	X	-	-	-
Mangiferin	-	-	-	-	XXX	XX	X	X	-	-	-
	Peeling										
Experimental group	**Day**										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**T**
Positive control	-	-	-	XXX	XXX	-	-	-	-	-	-
Negative control	-	-	-	-	-	-	-	-	-	-	-
Solvent control	-	-	-	-	-	-	-	-	-	-	-
"Green" honeybush	-	-	-	-	-	-	-	-	-	-	-
Fermented honeybush	-	-	-	-	-	-	-	-	-	-	-
Hesperidin	-	-	-	XXX	XX	X	X	X	-	-	-
Mangiferin	-	-	-	XXX	XX	XX	X	X	-	-	-
	Erythema										
Experimental group	**Day**										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**T**
Positive control	-	-	-	-	X	XXX	XXX	XX	XX	X	X
Negative control	-	-	-	-	-	-	-	-	-	-	-
Solvent control	-	-	-	-	-	-	-	-	-	-	-
"Green" honeybush	-	-	-	-	X	-	-	-	-	-	-
Fermented honeybush	-	-	-	-	X	X	X	X	X	-	-
Hesperidin	-	-	-	-	X	XXX	XXX	XX	X	X	X
Mangiferin	-	-	-	-	X	XXX	XXX	XX	X	-	-

Abbreviations: XXX, severe damage; XX, moderate damage; X, mild damage; -, no damage; T, termination.
n = 10

3.5. UVB-induced epidermal hyperplasia

Daily UVB irradiation of mice skin in the positive control group resulted in epidermal hyperplasia and showed a significant ($P < 0.05$) 2.5-3 fold increase in the thickness of the epidermis (6.21 μm) and the number of cell layers (5.15) when compared to the negative control (2.49 μm, 1.96 cell layers) and the solvent control (1.98 μm, 1.74 cell layers) groups

(**Table 5**)[17] that did not show any hyperplasia. Topical application of "green" honeybush extract to the mice skin inhibited UVB-induced hyperplasia in the epidermis, evident by the lower epidermal thickness (4.34 μm) resulting in a 30.17% inhibition, and the significantly ($P < 0.05$) lower number of cell layers (3.08), resulting in a 40.11% inhibition compared to the positive control group. Topical application of fermented honeybush extract to mice skin also resulted in a significantly ($P < 0.05$) lower epidermal thickness (4.15 μm), resulting in a 33.26% inhibition and a significantly ($P < 0.05$) lower number of cell layers (2.96), resulting in a 42.44% inhibition compared to the positive control group. While application of hesperidin did not significantly reduce the epidermal thickness (4.21 μm), hesperidin did significantly ($P < 0.05$) reduce the number of cell layers (3.07) by 40.29%. Topical application of mangiferin did not have any effect in reducing the epidermal thickness (7.24 μm) nor the number of cell layers (5.19). The fermented honeybush extract was most effective in inhibiting the increase in hyperplasia, followed by hesperidin and green honeybush extract.

[17] See Addendum 13: H&E stained sections of skin

Table 5. Average increase in bi-fold skin thickness and epidermal hyperplasia in response to UVB irradiation

Group	Bi-fold Thickness		Epidermal hyperplasia			
	Increase in bi-fold thickness (mm)	% Inhibition	Thickness (μm)	% Inhibition	Number of cell layers	% Inhibition
Positive control	$0.77 \pm 0.28^{N,S,Hg,Hf,M}$	-	$6.21 \pm 1.54^{N,S,Hf}$	-	$5.15 \pm 1.35^{N,S,Hg,Hf,H}$	-
Negative control	0.32 ± 0.11^{P}	-	$2.49 \pm 0.58^{P,M}$	-	$1.96 \pm 0.59^{P,M}$	-
Solvent control	$0.14 \pm 0.17^{P,Hf,H,M}$	-	$1.98 \pm 0.86^{P,Hg,Hf,H,M}$	-	$1.74 \pm 0.66^{P,M}$	-
"Green" honeybush	$0.30 \pm 0.14^{P,H}$	61.04	$4.34 \pm 1.30^{S,M}$	30.17	$3.08 \pm 0.93^{P,M}$	40.11
Fermented honeybush	$0.46 \pm 0.24^{P,S}$	40.26	$4.15 \pm 1.49^{P,S,M}$	33.26	$2.96 \pm 0.94^{P,M}$	42.44
Hesperidin	$0.56 \pm 0.26^{S,Hg}$	27.27	$4.21 \pm 1.34^{S,M}$	32.23	$3.07 \pm 0.73^{P,M}$	40.29
Mangiferin	$0.44 \pm 0.29^{P,S}$	42.86	$7.24 \pm 1.97^{N,S,Hg,Hf,H}$	-16.55	$5.19 \pm 1.15^{N,S,Hg,Hf,H}$	-0.72

Values in columns are means ± SD of three determinations. Superscripts indicate significant differences ($P < 0.05$). Abbreviations: P, positive control; N, negative control; S, solvent control; Hg, "green" honeybush; Hf, fermented honeybush; H, hesperidin; M, mangiferin. n = 10 (except negative control, n = 6)

3.6. UVB-induced modulation of catalase and superoxide dismutase activity

Ultraviolet B irradiation of the mice skin resulted in a significant ($P < 0.05$) decrease in catalase (1.99 μmole/min/mg) and SOD (2.32 U/mg) activity in the positive control group when compared to the negative (2.34 μmole/min/mg, 3.43 U/mg) and solvent control (2.51 μmole/min/mg, 3.15 U/mg) groups, indicating depletion in enzyme activity (**Table 6**). Topical application of the "green" honeybush extract resulted in a marked although not significant protection against the decrease in catalase (2.36 μmole/min/mg) and SOD (3.01 U/mg) activity when compared to the positive control group. However, topical application of the fermented honeybush extract resulted in a significant ($P < 0.05$) protection against the decrease in catalase (2.39 μmole/min/mg) and SOD (3.92 U/mg) activity when compared to the positive control group. Hesperidin showed a moderate but not significant protection against the decrease in catalase (2.21 μmole/min/mg) and SOD (2.82 U/mg) activity, though not as effectively as the honeybush extracts. Mangiferin did not show any protection against the decrease in catalase (2.05 μmole/min/mg) activity but did significantly ($P < 0.05$) increase SOD (3.92 U/mg) activity. Oxidised glutathione (GSSG) could not be detected in the skin samples because of the detection limit of the assay therefore total glutathione (GSHt) levels were determined. Ultraviolet B irradiation of the skin did not have any effect on the levels of GSHt in the skin. The application of hesperidin did however cause a significant ($P < 0.05$) decrease in the levels of GSHt in the skin, when compared to all the other groups.

3.7. UVB-induced lipid peroxidation

Malondialdehyde was assessed as an indicator of lipid peroxidation[18]. Daily UVB irradiation of the mice skin for ten days resulted in a significant ($P < 0.05$) increase in lipid peroxidation (**Table 6**) in the positive control group (8.254 nmole/mg) when compared with the negative control group (3.857 nmole/mg) and solvent control group (5.445 nmole/mg). Topical application of the "green" honeybush extract resulted in a marked although not significant protection against UVB-induced increase in lipid peroxidation (6.405 nmole/mg) when compared to the positive control group.

[18] See Addendum 14: Levels of conjugated dienes in the skin

Table 6. Total glutathione levels, catalase and superoxide dismutase activity, and malondialdehyde content in the skin of mice irradiated with UVB

Group	CAT activity (μmole/min/mg)	SOD activity (U/mg)	GSHt (μM/mg)	TBARS (nmole/mg)
Positive control	$1.987 \pm 0.470^{S,Hf}$	$2.317 \pm 0.935^{N,Hf,M}$	1.712 ± 0.216^{H}	$8.254 \pm 2.492^{N,S,Hf}$
Negative control	2.340 ± 0.502	3.428 ± 0.246^{P}	1.867 ± 0.317^{H}	$3.857 \pm 0.566^{P,Hg,H,M}$
Solvent control	$2.514 \pm 0.468^{P,M}$	3.148 ± 0.676	1.783 ± 0.274^{H}	5.445 ± 1.068^{P}
“Green” honeybush	2.362 ± 0.500	$3.006 \pm 1.030^{Hf,M}$	1.681 ± 0.360^{H}	6.405 ± 0.605^{N}
Fermented honeybush	2.387 ± 0.425^{P}	$3.918 \pm 1.606^{P,Hg,H}$	1.804 ± 0.271^{H}	5.200 ± 0.931^{P}
Hesperidin	2.214 ± 0.470	$2.818 \pm 1.305^{Hf,M}$	$1.080 \pm 0.241^{P,N,S,Hg,Hf,M}$	6.881 ± 2.054^{N}
Mangiferin	2.048 ± 0.534^{S}	$3.921 \pm 0.841^{P,Hg,H}$	1.732 ± 0.442^{H}	6.542 ± 1.596^{N}

Values in columns are means ± SD of three determinations. Superscripts indicate significant differences ($P < 0.05$). Abbreviations: CAT, catalase; SOD, superoxide dismutase; GSHt, total glutathione; MDA, malondialdehyde; P, positive control; N, negative control; S, solvent control; Hg, “green” honeybush; Hf, fermented honeybush; H, hesperidin; M, mangiferin. n = 10 (except negative control, n = 6)

However, topical application of the fermented honeybush extract resulted in a significant ($P < 0.05$) decrease in lipid peroxidation (5.200 nmole/mg) when compared to the positive control group. Topical application of hesperidin (6.881 nmole/mg) and mangiferin (6.542 nmole/mg) resulted in a marked although not significant protection against UVB-induced increase in lipid peroxidation.

3.8. UVB-induced inflammation

Irradiation of mice skin daily for ten days with UVB resulted in a significant ($P < 0.05$) increase in the expression of COX-2 in the epidermis of the positive control (15.92 cells/10 μm) group skin when compared to the negative control (1.01 cells/10 μm) and solvent control (1.44 cells/10 μm) groups (**Table 7**)[19]. Topical application of the "green" honeybush extract (3.49 cells/10 μm) and the fermented honeybush extract (3.56 cells/10 μm) resulted in significant ($P < 0.05$) protection against the increase in COX-2 expression in the skin when compared to the positive control group, resulting in a 78.06% and 77.63% inhibition, respectively. Topical application of hesperidin (12.26 cells/10 μm) and mangiferin (15.84 cells/10 μm) did not protect against the increase in COX-2 expression.

3.9. UVB-induced induction of ornithine decarboxylase

There was a significant ($P < 0.05$) increase in the expression of ODC, a marker of cell proliferation, in the positive control (13.09 cells/10 μm) group skin when compared to the negative (0.54 cells/10 μm) and solvent (0.85 cells/10 μm) control groups (**Table 7**)[20]. Topical application of "green" honeybush (4.78 cells/10 μm) and fermented honeybush (2.72 cells/10 μm) extracts significantly ($P < 0.05$) protected against the increase in expression of ODC in the skin, with a 63.52% and 79.26% inhibition, respectively. Topical application of hesperidin (7.60 cells/10 μm) was only moderately but not significantly effective in protecting against the increase in ODC expression in the epidermis, while mangiferin (4.50 cells/10 μm) was as effective as the "green" honeybush.

[19] See Addendum 15: COX-2 expression in the epidermis
[20] See Addendum 16: ODC expression in the epidermis

Table 7. Expression of COX2, ODC, GADD45 and OGG1/2 proteins in mouse skin sections.

Experimental group	Inflammation marker		Proliferation marker		DNA damage marker		Oxidative DNA damage marker	
	COX-2 positive cells		ODC positive cells		GADD45 positive cells		OGG1/2 positive cells	
	(cells/10 μm)	% Inhibition	(cells/10 μm)	% Inhibition	(cells/10 μm)	% Inhibition	(cells/10 μm)	% Inhibition
Positive control	$15.92 \pm 4.43^{N,S,Hg,Hf}$	-	$13.09 \pm 3.66^{N,S,Hg,Hf,M}$	-	$15.89 \pm 5.00^{N,S,Hg,Hf,H,M}$	-	$13.71 \pm 2.51^{N,S,Hg,Hf,H}$	-
Negative control	$1.01 \pm 0.75^{P,H,M}$	-	$0.54 \pm 0.56^{P,H}$	-	$0.48 \pm 0.40^{P,M}$	-	$0.38 \pm 0.37^{P,M}$	-
Solvent control	$1.44 \pm 1.07^{P,H,M}$	-	$0.85 \pm 1.05^{P,H}$	-	$0.20 \pm 0.30^{P,M}$	-	$0.17 \pm 0.41^{P,M}$	-
"Green" honeybush	$3.49 \pm 2.57^{P,H,M}$	78.06	4.78 ± 2.27^{P}	63.52	$1.53 \pm 1.52^{P,M}$	90.38	$0.05 \pm 0.11^{P,M}$	99.62
Fermented honeybush	$3.56 \pm 2.46^{P,H,M}$	77.63	2.72 ± 2.85^{P}	79.26	$1.12 \pm 1.14^{P,M}$	92.98	$3.19 \pm 2.98^{P,M}$	76.72
Hesperidin	$12.26 \pm 4.52^{N,S,Hg,Hf}$	23.02	$7.60 \pm 3.51^{N,S}$	41.91	$2.40 \pm 0.98^{P,M}$	84.91	$3.39 \pm 2.77^{P,M}$	75.31
Mangiferin	$15.84 \pm 5.06^{N,S,Hg,Hf}$	0.25	4.50 ± 1.62^{P}	65.61	$9.98 \pm 3.09^{P,N,S,Hg,Hf,H}$	37.16	$17.32 \pm 4.41^{N,S,Hg,Hf,H}$	-26.27

Values in columns are means ± SD of three determinations. Groups found to be significantly different ($P < 0.05$) are indicated in superscript. The number of cells expressing each protein was expressed as the average number of positive cells per 10 μm length and as the percentage of the positive control. Abbreviations: P, positive control; N, negative control; S, solvent control; Hg, "green" honeybush; Hf, fermented honeybush; H, hesperidin; M, mangiferin. n = 10 (except negative control, n = 6)

3.10. UVB-induced DNA damage

A significant ($P < 0.05$) increase in the expression of GADD45 (15.89 cells/10 μm), a marker of DNA damage, and OGG1/2 (13.71 cells/10 μm), a marker of oxidative DNA damage, was noted in the positive control group skin when compared to the negative (0.48 cells/10 μm and 0.38 cells/10 μm) and solvent (0.20 cells/10 μm and 0.17 cells/10 μm) control groups (**Table 7**)[21,22]. Application of the "green" honeybush extract significantly ($P < 0.05$) protected against the increase in expression of GADD45 (1.53 cells/10 μm) and OGG1/2 (0.05 cells/10 μm) when compared to the positive control group, with an inhibition of 90.38% and 99.62%, respectively. Topical application of the fermented honeybush extract significantly ($P < 0.05$) protected against the increase in expression of GADD45 (1.12 cells/10μm) and OGG1/2 (3.19 cells/10 μm) when compared to the positive control group with an inhibition of 92.98% and 76.72%, respectively. Topical application of hesperidin was effective in significantly ($P < 0.05$) protecting against the increase in expression of OGG1/2 (3.39 cells/10 μm) but not GADD45 (7.60 cells/10 μm) when compared to the positive control groups, with a 75.31% inhibition in OGG1/2 expression. Topical application of mangiferin showed a significant ($P < 0.05$) protection against the increase in expression of GADD45 (9.98 cells/10 μm) but no effect on OGG1/2 (17.32 cells/10 μm) expression.

4. Discussion

The development of skin cancer occurs in three stages; initiation, promotion and progression. During initiation, DNA mutations occur in genes, which result in epidermal cells that respond abnormally to stimuli compared to uninitiated cells. Promotion drives clonal expansion of initiated cells due to altered signal transduction pathways, producing malignant and pre-malignant tumours. These tumours then progress to metastatic and invasive malignant tumours [30]. Processes that are involved in skin carcinogenesis include; DNA damage, proliferation, inflammation, immune suppression, dysregulation of the cell cycle and signal transduction pathways, depletion of the antioxidant defence system, induction of cyclooxygenase and increased prostaglandin production, and induction of ornithine decarboxylase [31]. It is well established that chronic exposure to UVB (280-320 nm) can cause skin cancer and that short-term exposure damages the skin by all these processes.

Ultraviolet B is absorbed by DNA, and results in cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone (6-4 PP) adducts, which contribute towards mutagenesis [32].

[21] See Addendum 17: GADD45 expression in the epidermis
[22] See Addendum 18: OGG1/2 expression in the epidermis

Increased proliferation occurs in the epidermis of the skin in response to UVB irradiation, resulting in epidermal hyperplasia [33]. Ultraviolet B also stimulates the inflammatory response, causing erythema, oedema and an influx of inflammatory cells such as neutrophils and lymphocytes [34]. Cyclooxygenase-2 is induced, causing an increase in the production of prostaglandins, which are involved in proliferation, inflammation and promotion of carcinogenesis [35; 36]. Ornithine decarboxylase is induced to generate polyamines and encourages proliferation in the epidermis [36]. Ultraviolet B also produces ROS, directly and via the inflammatory response, causing the depletion of the cellular antioxidant defence system and an increase in oxidative damage [37; 38]. Ultraviolet B also activates signalling pathways that resist apoptosis, such as proliferation and differentiation, thus driving clonal expansion of tumour cells [39].

Because chronic exposure to UV irradiation can cause skin cancer, the International Agency of Research on Cancer [40] recommend that to protect against skin damage, one must wear clothing that adequately covers the body, a hat that adequately covers the head, seek shade, use sunscreens and avoid going outdoors during peak sun hours. These recommendations imply that preventing the early cancer initiating and promoting effects of UV irradiation is important for the prevention of skin cancer. However, the use of these strategies is insufficient in preventing skin cancer due to non-compliance. Also, these strategies do not protect against the later stages of carcinogenesis, such as metastasis [3]. Photochemoprevention is the “means of (skin) cancer control in which the occurrence of photodamage can be entirely prevented, slowed or reversed by topical or oral administration of naturally occurring or synthetic compounds” [30]. Numerous publications have shown that botanical polyphenolic antioxidants are effective in reducing skin carcinogenesis and also the damaging effects of short-term exposure to UVB. For example, green tea catechins applied to the skin of mice showed photoprotective mechanisms against UVB irradiation such as preventing the depletion of antioxidant defence system enzymes glutathione peroxidase, catalase and glutathione, oxidative damage such as lipid peroxidation and protein oxidation and changes in MAPK signal transduction pathways [24]. Resveratrol, a stilbene found in grapes, prevented UVB irradiation-induced oedema and hyperplasia, induction of COX-2 and ODC, and lipid peroxidation when applied topically to the skin of mice [5]. Lycopene reduced DNA damage and the induction of ODC and inflammation [42]. Also, the soybean genistein reduced sunburn erythema, hyperplasia and DNA damage [43]. Polyphenols in combinations, can show synergism and be more active than individual compounds [44]. For example, the flavonols and flavones identified in propolis are more active in combination than individually [45].

In this study, polyphenol-rich extracts of honeybush (*Cyclopia intermedia*) plant material and the most abundant polyphenols found in honeybush, hesperidin and mangiferin, were investigated to determine their possible modulation of the harmful effects of UVB. It was noted that processing of 'green" honeybush to produce fermented honeybush caused a decrease in the total polyphenol, flavanol and flavonol content, which is in accordance with previously published studies [9; 46; 47]. The antioxidant capacity of fermented honeybush was also decreased. High performance liquid chromatography quantification of the polyphenols mangiferin and hesperidin showed that processing of honeybush reduced hesperidin and mangiferin levels, with mangiferin significantly more reduced than hesperidin. Pure hesperidin and mangiferin also had much lower antioxidant capacities than the honeybush extracts.

Daily UVB irradiation of SKH-1 hairless mice resulted in sunburn, which included erythema, peeling and hardening or scaling of the skin. Oedema and hyperplasia was also evident. Topical application of both "green" and fermented honeybush extracts reduced sunburn, oedema and hyperplasia of the skin. The lack of peeling of the skin in the extract-treated mice shows that the extracts prevented the development of sunburn cells undergoing apoptosis. The "green" honeybush extract was more effective than the fermented honeybush extract. Topical application of hesperidin and mangiferin also showed some protective effects against hyperplasia and oedema, though not as effective as the honeybush extracts, but were ineffective in preventing sunburn. These results suggest that the extracts and pure compounds either prevented the penetration of UVB into the skin by acting as a sunscreen, and/or modulated the inflammatory and proliferative response to UVB. The expression of COX-2 in the epidermis was also determined as a marker of inflammation and proliferation. Cyclooxygenase-2 is the enzyme responsible for production of prostaglandins during the inflammatory response and is expressed after acute irradiation of skin with UVB, as well as chronic UVB irradiation [35; 48]. As prostaglandins also regulate proliferation and differentiation in keratinocytes, an increase in COX-2 expression and the resultant increase in prostaglandins cause an increase in proliferation in the epidermis and probably contribute towards the promotion of carcinogenesis by UVB [35]. Fischer *et al*. [36] demonstrated with transgenic and knockout mice that COX-2 plays a crucial role in UVB-induced skin carcinogenesis. In this study, the "green" and fermented honeybush extracts significantly reduced UVB-induced COX-2 expression in the epidermis, showing anti-inflammatory activity and probably anti-proliferative activity. Hesperidin only showed a weak inhibition in the increase in COX-2 expression, while mangiferin had no effect.

Ornithine decarboxylase is the rate-limiting enzyme responsible for polyamine synthesis and is involved in normal cell proliferation and differentiation of the skin [49]. Some studies have

found that expression of ODC is an early marker of carcinogenesis, as ODC is highly expressed in early papillomas [50]. Polyphenols such as green tea catechins and silymarin from the milk thistle plant have previously been shown to concurrently inhibit ODC activity and the development of tumours in mice, indicating the role of proliferation in carcinogenesis [6; 51]. Ornithine decarboxylase expression in the skin increases with UVB irradiation dose, suggesting that this enzyme may also play a role in UVB-induced skin carcinogenesis. In this study, there was a significant increase in ODC expression in the skin after UVB irradiation, but was reduced when the “green” and fermented honeybush extracts and mangiferin were topically applied. Hesperidin also reduced the levels of ODC, but not as effectively as the extracts.

Ultraviolet B irradiation of the skin results in the depletion of the antioxidant defence system enzymes such as catalase, glutathione peroxidase, SOD and the ratio of oxidised and reduced glutathione due to quenching the increased levels of ROS that are produced [6; 24]. In this study, both catalase and SOD activities decreased after daily UVB irradiation to mice skin showing that there was a depletion of enzyme levels, but no changes in total glutathione was observed. Both the “green” and fermented honeybush extracts protected against the decrease in activities of catalase and SOD when applied topically with hesperidin also protecting against the decrease in activities of catalase and SOD, though not as effectively as the honeybush extracts. Mangiferin did not protect against UVB-induced decrease in the activity of catalase, but did significantly increase SOD activity. These results suggest that the honeybush extracts and to a certain extent, the pure compounds, either assisted the antioxidant defence system by quenching ROS, or preventing the production of ROS, or increased the expression of antioxidant enzymes to prevent depletion. Further studies are needed to ascertain the exact mechanisms involved.

Ultraviolet B-induced ROS production damages lipids, producing lipid peroxides that are converted to products such as MDA and conjugated dienes [52; 53]. In this study, irradiation with UVB resulted in an increase in MDA levels. “Green” and fermented honeybush extracts, as well as hesperidin and mangiferin protected against UVB-induced increase in MDA levels in the skin.

The most common DNA lesion produced by UVB-induced ROS is the 8-hydroxydeoxyguanosine (8-oxoG) adduct, formed by the oxidation of the guanine base [38; 54]. This DNA adduct may play an important role in UVB-induced skin carcinogenesis by producing GC to TA transversions in critical genes such as p53 [55; 56]. The protein, 8-oxoguanine DNA gylcosylase (OGG1), is a base excision repair enzyme, which excises this lesion from DNA [57]. Studies have demonstrated the important role of 8-oxoG in skin

carcinogenesis [55; 56]. In this study, the expression of OGG1/2 was significantly increased after UVB irradiation, indicating that there was an increase in ROS production and ROS-induced 8-oxoG DNA adducts. However, topical application of the “green” and fermented honeybush extracts significantly reduced the expression of OGG1/2, suggesting that the honeybush extracts prevented the production of ROS or quenched ROS and ROS-induced 8-oxoG DNA adducts, thus not requiring OGG1/2 expression to be increased. Hesperidin was also effective in reducing OGG1/2 expression while mangiferin had no effect.

Growth arrest and DNA-damage-inducible protein (GADD45) is upregulated in cells exposed to UVB [58]. Cells damaged by UVB are induced by GADD45 to undergo apoptosis (producing sunburn cells) or cell cycle arrest for DNA repair. This occurs by activation of p38 and c-JNK MAPK pathways. Transgenic mice lacking GADD45 are more susceptible to the development of tumours than normal mice, indicating the important role of this protein in protecting against skin carcinogenesis [59]. In this study, UVB irradiation of mice skin resulted in a significant increase in the expression of GADD45 in the epidermis, indicating that extensive DNA damage occurred. The “green” and fermented honeybush extracts significantly reduced the expression of GADD45 in the epidermis, suggesting that DNA damage was prevented and GADD45 expression was not needed. Hesperidin was also effective in reducing GADD45 expression, while mangiferin was only moderately effective.

All of these protective effects may be as a direct result of the scavenging of ROS by the antioxidant polyphenols present in the extracts. The processing of “green” honeybush to produce fermented honeybush reduces the antioxidant capacity and total polyphenol content, suggesting that the fermented honeybush extract should be less effective in protecting against UVB-induced damage. This did, however, not always correlate with the protective effects of the fermented honeybush extract compared to the “green” honeybush extract. This observation was also made when comparing the antioxidant capacity of hesperidin and mangiferin with the various protective effects. Mangiferin, which had a greater antioxidant capacity than hesperidin, did not protect against UVB-induced damage as well as hesperidin did. Also, the significantly lower antioxidant capacity of hesperidin compared to the antioxidant capacity of the honeybush extracts does not explain the protective effect of this flavonoid. This suggests that specific polyphenols present in both green and fermented honeybush extracts, including hesperidin and mangiferin, may be responsible for specific protective properties, such as the anti-inflammatory effects and modulation of signalling pathways and can explain why the plant extracts, containing a plethora of polyphenolic compounds, performed best. A synergism amongst the various phenolic compounds could exist to exert the protective effect, and that is why a single compound does not perform at a similar level.

In conclusion, this short-term study provides the first evidence that phenolic-rich honeybush extracts, and to a certain extent the purified honeybush compounds, hesperidin and mangiferin can modulate UVB-induced damage. The photoprotective effects may be mediated by mechanisms such as i) preventing the penetration of UVB into the skin ii) preventing erythema and the inflammatory response of the skin, iii) reducing proliferation, iv) preventing DNA damage, v) reducing oxidative stress by modulating the antioxidant defence system and scavenging ROS, vi) reducing the expression of ODC and vii) reducing the expression of COX-2. The photoprotective effect of the honeybush extracts may be suggestive of including the extracts into skin care products such as sunscreens and anti-aging products for the prevention of UV-induced skin damage. Clinical trials will further validate these claims.

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C H A P T E R 5

Summary and conclusions

Summary

Skin cancer, especially non-melanoma skin cancer is the most common cancer type world wide with over a million new cases of non-melanoma cancer reported every year in the United States of America alone. Since skin cancer places an enormous financial burden on health care services worldwide, new approaches of prevention other than sun protection strategies need to be implemented. Skin carcinogenesis occurs in three stages, namely: initiation, promotion and progression. Exposure to ultraviolet B (UVB) radiation causes skin damage that contributes towards the development of skin cancer. Irradiation causes direct DNA damage and oxidative stress through the generation of reactive oxygen species (ROS). Inflammation, immune suppression and modulation of cell signaling are also important effects of UVB irradiation of the skin, which contribute towards carcinogenesis. Photochemoprevention is the prevention or reduction of skin cancer by the use of naturally occurring or synthetic compounds. Some mechanisms by which these compounds may act include the prevention of UVB penetration into the skin, UVB-induced inflammation, immunosuppression, modulation of cell cycle and signalling pathways, induction of cyclooxygenase-2 (COX-2) and ornithine decarboxylase (ODC) and generation of ROS. The use of botanical polyphenolic compounds for their health promoting properties has recently been investigated. The chemopreventive properties of green tea catechins for example, are now well known.

The legumes rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.), indigenous to South Africa, are used locally to prepare a herbal tea. Local inhabitants have known the medicinal properties of these herbal teas for many years. A limited number of scientific studies have demonstrated some biological properties of rooibos and even fewer studies haven been done on honeybush, therefore honeybush was investigated in more detail in this study. Properties that have been identified using polyphenol-rich extracts of honeybush and rooibos include antioxidant, antimutagenic and antitumourigenic effects. A previous study showed that both rooibos and honeybush protected against fumonisin B_1-induced liver carcinogenesis and a two-stage skin carcinogenesis model showed that both rooibos and honeybush protected against dimethylbenz[*a*]anthracene (DMBA)-initiated, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-promoted tumourigenesis in mice. Research suggests that the polyphenol content is responsible for the biological properties, as these compounds possess chemopreventive activities, but other contributors cannot be excluded. Processing of “green” honeybush and rooibos to produce fermented honeybush and rooibos results in a decrease in the total polyphenol content and a resultant decrease in antioxidant capacity. The degree of protection against tumourigenesis by these two herbal teas in these studies compared to their antioxidant capacities do not always correlate, suggesting that specific biological activities and not the antioxidant activity of the polyphenols are important.

This study investigated the possible photoprotective properties of honeybush and the two most abundant polyphenols, hesperidin and mangiferin using mouse models and rooibos extracts as a reference.

The first study investigated the protective properties of polyphenol-rich ethanol extracts of “green” and fermented honeybush and the pure compounds hesperidin and mangiferin against UVB-induced tumourigenesis in a two-stage skin carcinogenesis model. Skin tumourigenesis was initiated with DMBA and promoted with UVB radiation in SKH-1 mice. Mice treated topically with the honeybush extracts before irradiation exhibited a reduction in the incidence and volume of tumours, while the pure compounds were less effective. The “green” honeybush showed a greater protection against tumourigenesis than the fermented honeybush. Even though the fermented honeybush extract had a reduced antioxidant capacity and polyphenol content, the protection against tumourigenesis was high. This suggests that specific biological activities of the polyphenols and not the antioxidant activity is responsible for protection observed. Hesperidin and mangiferin were not excluded as possible polyphenolic compounds, which contribute towards the antitumourigenic effect of honeybush. When determining the flavonoid content of the extracts, the flavonol/flavone/xanthone content correlated well with the protective effect of the extracts, suggesting that this flavonoid subgroup plays an important role in the photoprotective properties of honeybush. The “green” and fermented rooibos extracts used as a reference were both effective in protecting against tumourigenesis, with fermented rooibos showing the greatest protection.

The second study investigated the possible photoprotective mechanisms of the polyphenol-rich ethanol extracts of “green” and fermented honeybush and the pure compounds hesperidin and mangiferin, in SKH-1 mice against short-term exposure of the skin to UVB radiation. The extracts and pure compounds were applied topically to the skin of mice before irradiation with UVB. Both honeybush extracts protected against UVB-induced sunburn, oedema and hyperplasia, while the pure compounds had little effect. The honeybush extracts protected against UVB-induced lipid peroxidation, oxidative DNA damage and probably also direct DNA damage. Depletion of the antioxidant enzymes catalase and superoxide dismutase (SOD), as well as the induction of COX-2 and ODC was also prevented. As with the first study, the reduced polyphenol content and antioxidant capacity of the fermented honeybush extract did not correlate with the degree of photoprotection against short-term exposure to UVB irradiation. The pure compounds showed some protective effects but were less pronounced compared to the extracts. Hesperidin and mangiferin were not excluded as possible polyphenolic compounds, which contribute towards the photoprotective effect of honeybush. Hesperidin, mangiferin and other polyphenols in honeybush probably exert

different biological properties and work synergistically in the extract.

The results obtained suggest that the honeybush extracts protected the skin from UVB-induced damage and ultimately against tumourigenesis by mechanisms such as i) preventing the penetration of UVB into the skin, ii) preventing erythema and the inflammatory response of the skin, iii) reducing proliferation, iv) protecting DNA from direct damage by UVB radiation, v) preventing oxidative stress such as ROS-induced damage to macromolecules and depletion of the antioxidant defence system, by scavenging ROS, vi) preventing the induction of ODC and vii) preventing the induction of COX-2.

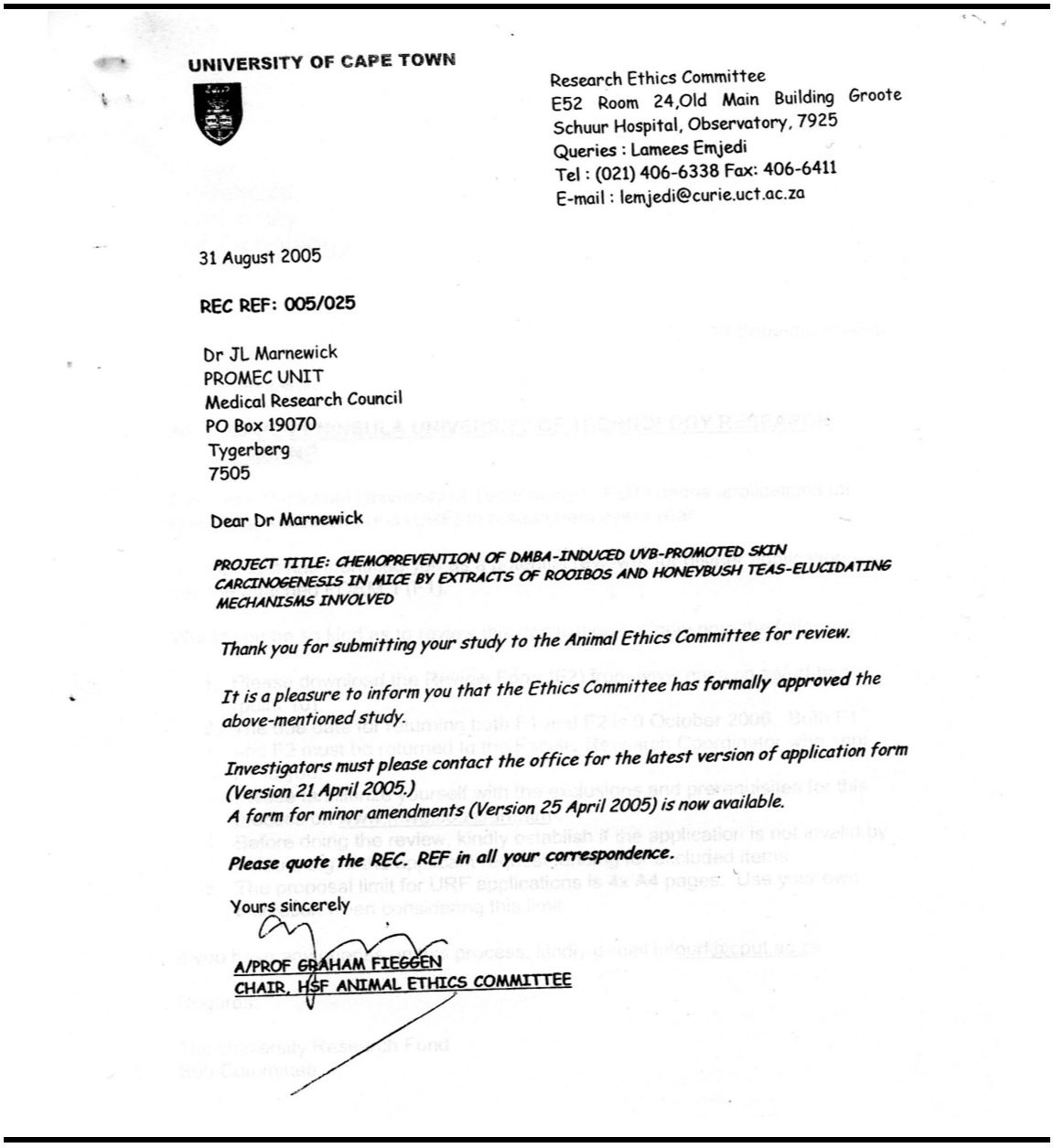
Conclusions

Results from these two studies generated new knowledge, which will be a valuable addition to the literature available on the health promoting properties of honeybush, rooibos, hesperidin and mangiferin. Also, these two studies substantiate the anecdotal claims for the two herbal teas, which are recommended for the treatment of skin conditions. Results indicate that both “green” and fermented honeybush may be valuable additions to skin care products such as sunscreens and anti-aging products for the prevention of UVB-induced skin damage and carcinogenesis.

Further studies may be useful to fully maximize the use of the skin protecting properties. These future studies may include the investigation of the photoprotective properties of other compounds in honeybush and the dose response the compounds and herbal extracts. The elucidation of the possible photoprotective effects of honeybush and the pure compounds against UVB-initiated, UVB-promoted instead of DMBA-initiated, UVB-promoted skin carcinogenesis. UVA-induced photodamage may also be a beneficial investigation, as UVA also plays a role in carcinogenesis and aging. From this study, it was not clear what role the extracts and pure compounds played in preventing the penetration of UVB into the skin of the mice. Therefore, applying the extract immediately after UVB irradiation instead of before irradiation, as well as the administration of aqueous extracts orally instead of topical application, may help to elucidate this effect. If the prevention of UVB penetration is not the main mechanism of protection, other specific biological mechanisms of photoprotection identified in this study may also be investigated in more detail to determine which properties of honeybush are most beneficial. These mechanisms may be better elucidated using *in vitro* keratinocyte cell cultures in which cellular responses can be carefully monitored. Other models for carcinogenesis may also be investigated, such as melanoma as well as other cancer types. Clinical trials will also further validate the biological properties proposed.

A D D E N D U M S

ADDENDUM 1: Ethics approval obtained for the use of SKH-1 mice in experimentation



UNIVERSITY OF CAPE TOWN

Research Ethics Committee
E52 Room 24,Old Main Building Groote Schuur Hospital, Observatory, 7925
Queries : Lamees Emjedi
Tel : (021) 406-6338 Fax: 406-6411
E-mail : lemjedi@curie.uct.ac.za

31 August 2005

REC REF: 005/025

Dr JL Marnewick
PROMEC UNIT
Medical Research Council
PO Box 19070
Tygerberg
7505

Dear Dr Marnewick

PROJECT TITLE: CHEMOPREVENTION OF DMBA-INDUCED UVB-PROMOTED SKIN CARCINOGENESIS IN MICE BY EXTRACTS OF ROOIBOS AND HONEYBUSH TEAS-ELUCIDATING MECHANISMS INVOLVED

Thank you for submitting your study to the Animal Ethics Committee for review.

It is a pleasure to inform you that the Ethics Committee has formally approved the above-mentioned study.

Investigators must please contact the office for the latest version of application form (Version 21 April 2005.)
A form for minor amendments (Version 25 April 2005) is now available.

Please quote the REC. REF in all your correspondence.

Yours sincerely

A/PROF GRAHAM FIEGGEN
CHAIR, HSF ANIMAL ETHICS COMMITTEE

ADDENDUM 2: HPLC quantification of hesperidin and mangiferin in the honeybush extracts

The concentration of hesperidin and mangiferin in the ethanol extracts of honeybush was determined by HPLC. The samples and standard concentrations of hesperidin and mangiferin were passed through the column separately. The retention times for the hesperidin and mangiferin standards were determined and the areas of the peaks measured. The area of the peaks obtained at the same retention times for the "green" and fermented honeybush extracts were also measured. To determine the concentration of hesperidin and mangiferin in the extracts, the area of the extract peak was divided by the area of the standard peak and then multiplied by the concentration of the standard and by the dilution factor.

Equation 1: $[\text{polyphenol}]_{\text{extract}} = [(\text{area of peak})_{\text{extract}}/(\text{area of peak})_{\text{standard}}] \times [\text{standard}] \times \text{dilution factor}$

A concentration of 50 μg/ml standard was injected into the HPLC column and diluted samples of the "green" and fermented honeybush extracts. In **Figure 1**, the arrows indicate that hesperidin eluted from the column at approximately 10.5 min and mangiferin, **Figure 2**, eluted at approximately 8 min. The arrows in **Figure 3** and **Figure 4** show the corresponding peaks for hesperidin and mangiferin in the "green" and fermented honeybush extract samples, respectively. The results are representative of samples injected in triplicate, and repeated in triplicate for reproducibility.

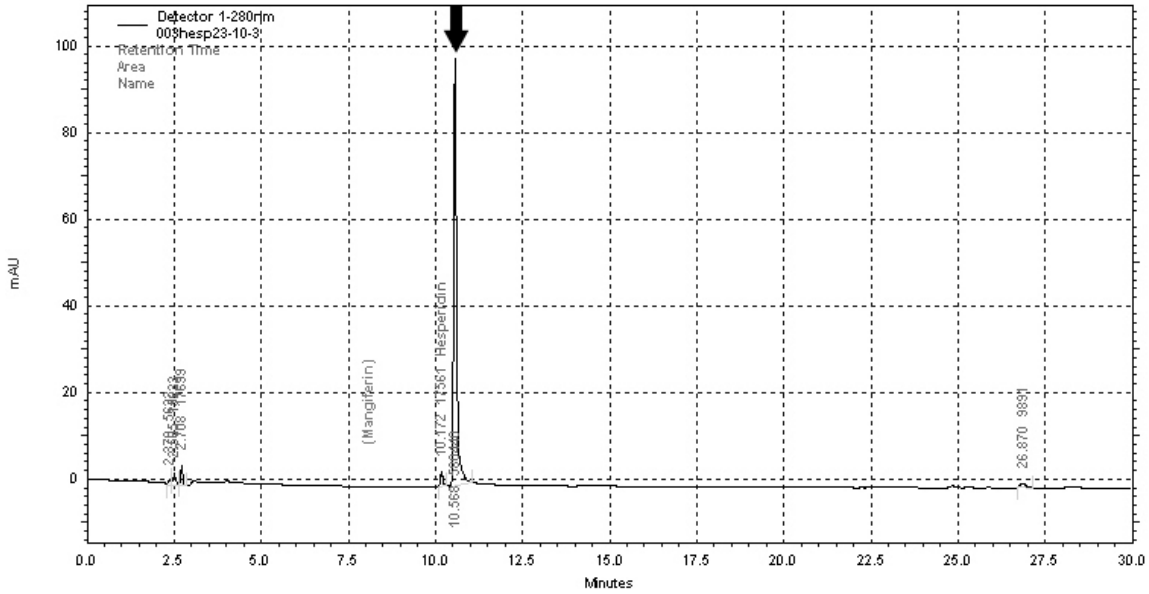


Figure 1. HPLC analysis of hesperidin (The arrow indicates when hesperidin eluted from the column)

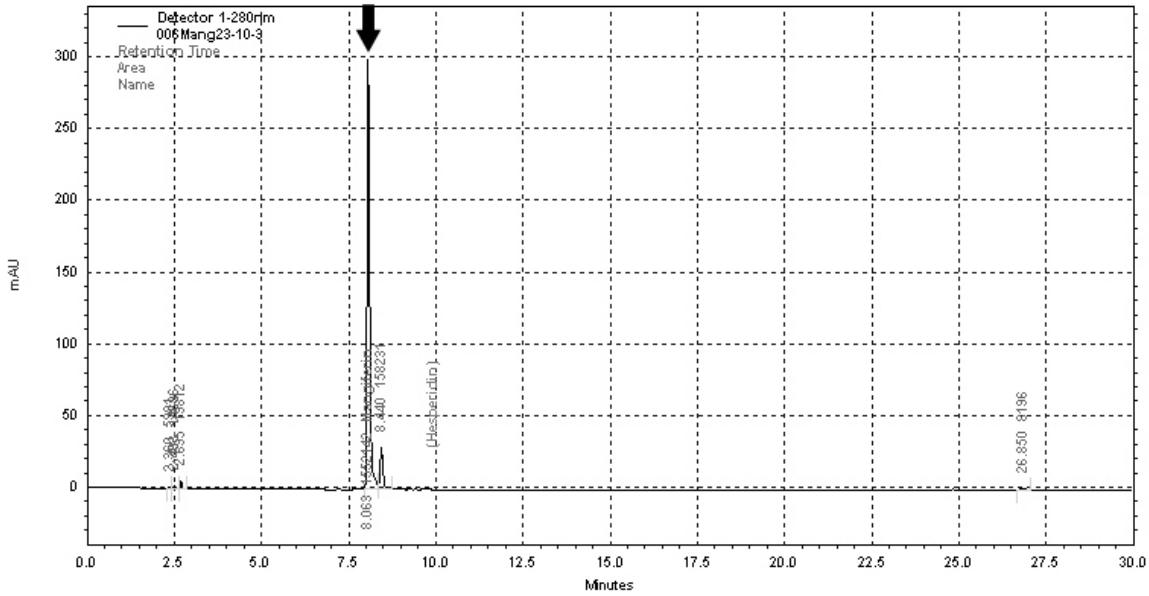


Figure 2. HPLC analysis of mangiferin (The arrow indicates when mangiferin eluted from the column)

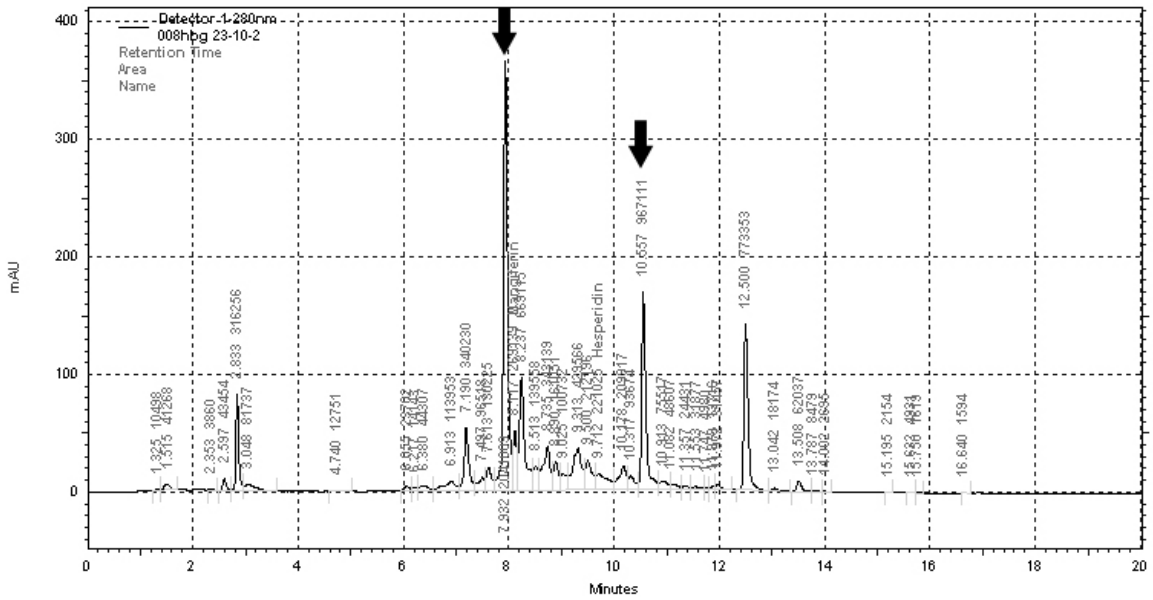


Figure 3. HPLC analysis of the "green" honeybush extract (The arrow on the left indicates when mangiferin eluted and the arrow on the right indicates when hesperidin eluted from the column)

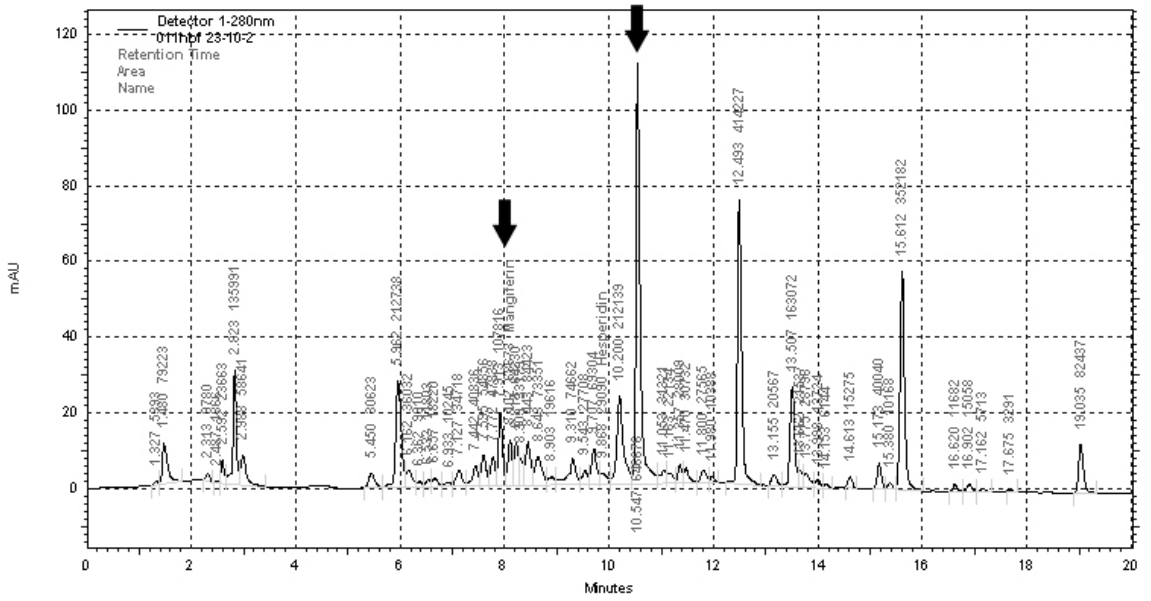


Figure 4. HPLC analysis of the fermented honeybush extract (The arrow on the left indicates when mangiferin eluted and the arrow on the right indicates when hesperidin eluted from the column)

ADDENDUM 3: Animal study conditions

As shown in **Figure 5**, for both studies, the female SKH-1 mice were acclimatized in their new cages for one week before the study began (**a**). Extracts and pure compounds or solvent were applied to the skin by using a micropipette (**b**). The solution was spread gently onto the skin from the base of the neck to the beginning of the tail using the flat surface of the pipette tip to avoid any injury to the mice. This was followed 30 min later by 180 mJ/cm^2 UVB irradiation in a UV crosslinker (**c**). Mice were placed in a cardboard box layered with paper towelling to prevent soiling (**d**). The mice were able to move freely within the box without any stress and remained for a maximum of 1 min in the machine during irradiation.

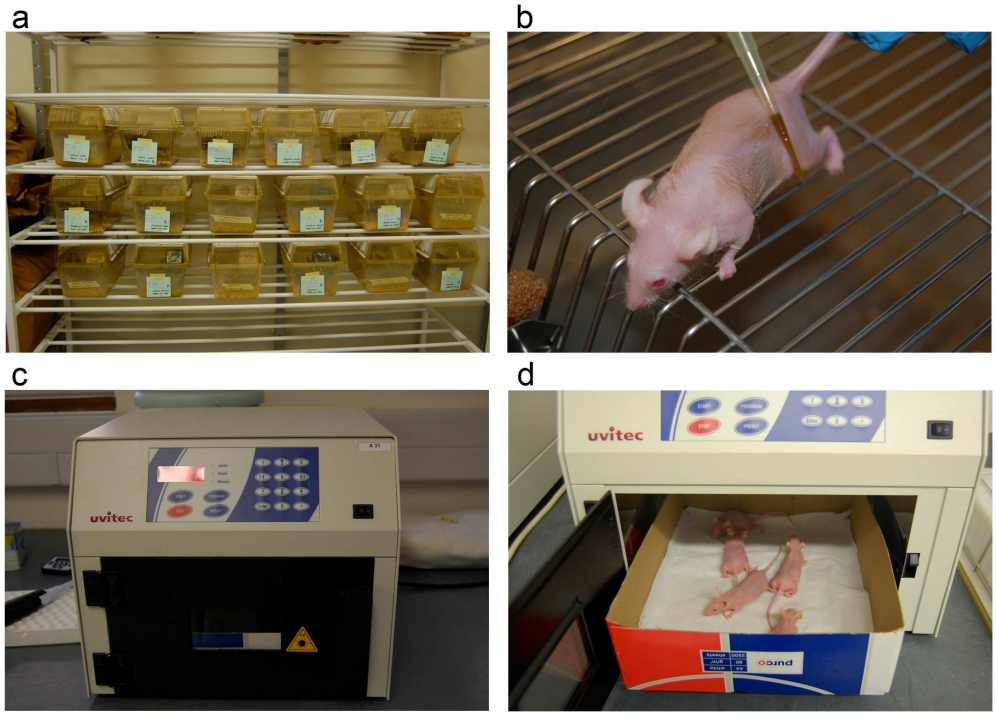


Figure 5. Animal study conditions (**a**) Cages, (**b**) application of extract, pure compound or solvent, (**c**) UVllink UV crosslinker, (**d**) irradiation of mice**.**

ADDENDUM 4: Skin carcinogenesis protocol

As shown in **Figure 6**, initiation was introduced with a single topical application of DMBA dissolved in ethanol: acetone (1:1, v/v). One week after initiation, skin cancer was promoted by exposing the mice to UVB radiation, twice a week for 22 weeks. To test the protective effect of the honeybush herbal tea extracts and pure compounds, mice were treated with extract or pure compound 30 min before UVB irradiation. Rooibos extracts were included as a reference. The following experimental control groups were also included in the study: (a) positive control group (initiated with DMBA) receiving a topical application of solvent 30 min before UVB irradiation, (b) a solvent control group (not initiated with DMBA) receiving a topical application of solvent but without UVB irradiation and (c) a negative control group (initiated with DMBA) receiving a topical application of solvent without UVB irradiation.

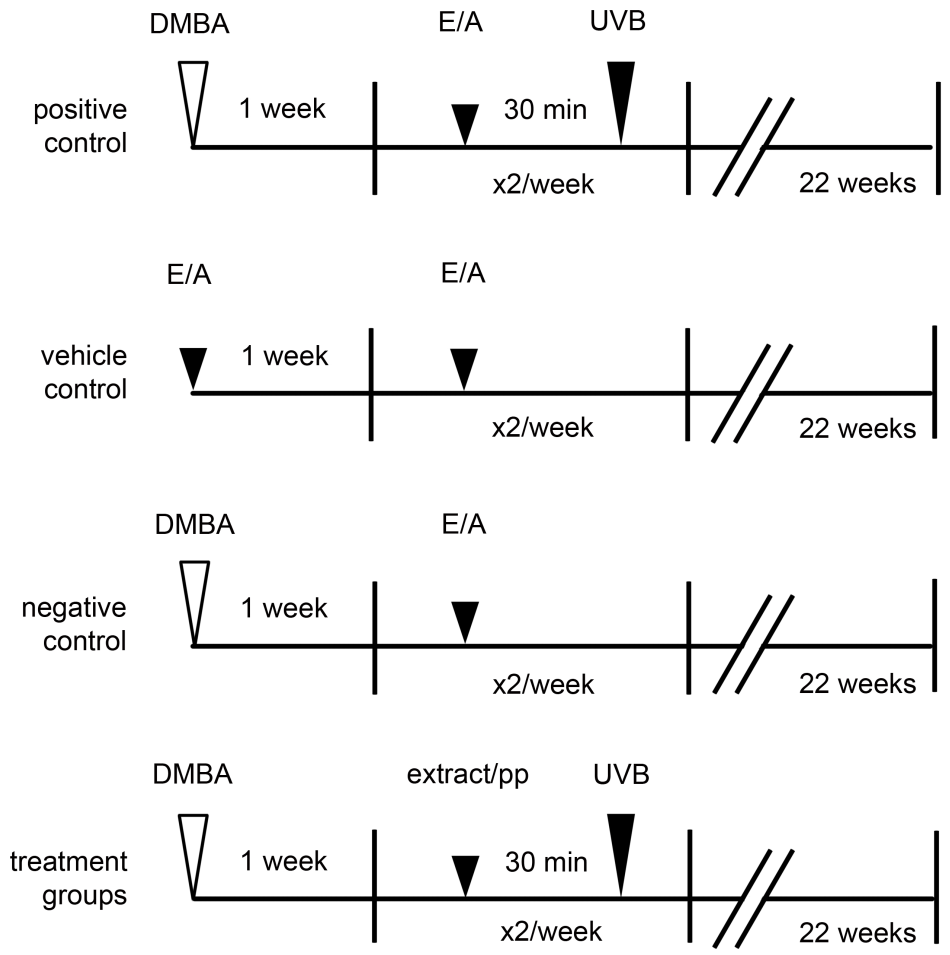


Figure 6. Scheme of the skin carcinogenesis protocol (Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; E/A, ethanol: acetone; pp, polyphenol; UVB, ultraviolet B)

ADDENDUM 5: Contribution of flavonoids to the total polyphenol content of the herbal tea extracts

The total polyphenol and flavonoid content of the herbal tea extracts dissolved in ethanol: acetone (1:1, v/v) were determined and represented in **Figure 7**. The flavanol/proanthocyanidin and flavonol/flavone/xanthone contribution towards the total polyphenol content is shown. Flavonols/flavones/xanthones contribute a large percentage and flavanols/proanthocyanidins a small percentage of the total polyphenols in the “green” honeybush, fermented honeybush and fermented rooibos extracts. Other polyphenols contribute the majority of the total polyphenol content of the “green” rooibos extract.

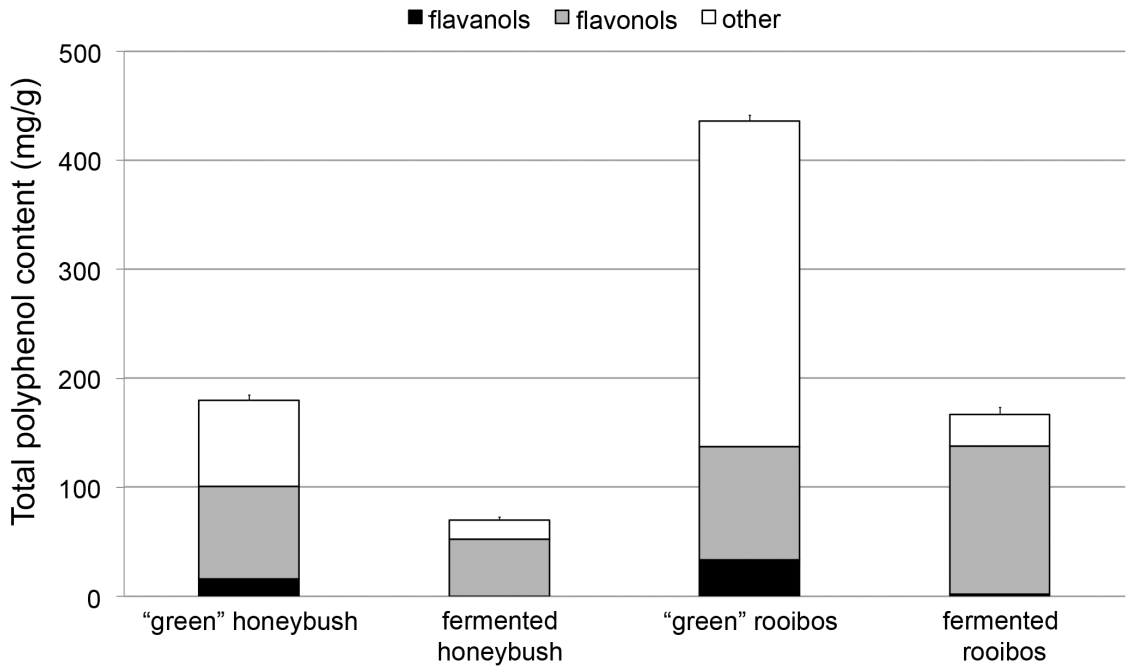


Figure 7. Contribution of flavonoids to the total polyphenol content of honeybush and rooibos extracts (Total polyphenols were expressed as mg gallic acid equivalents, flavanols/proanthocyanidins as mg catechin and flavonols/flavones/xanthones as mg quercetin equivalents per gram extract)

ADDENDUM 6: Weight profile of mice during the skin carcinogenesis study

The weight of each mouse used in the skin carcinogenesis study was monitored every week until termination (**Figure 8**). No weight loss was observed in any of the groups, indicating that the experiment did not adversely affect the health of the mice other than producing skin tumours. There was also no significant difference in weight gain observed between each group of mice during the skin carcinogenesis study.

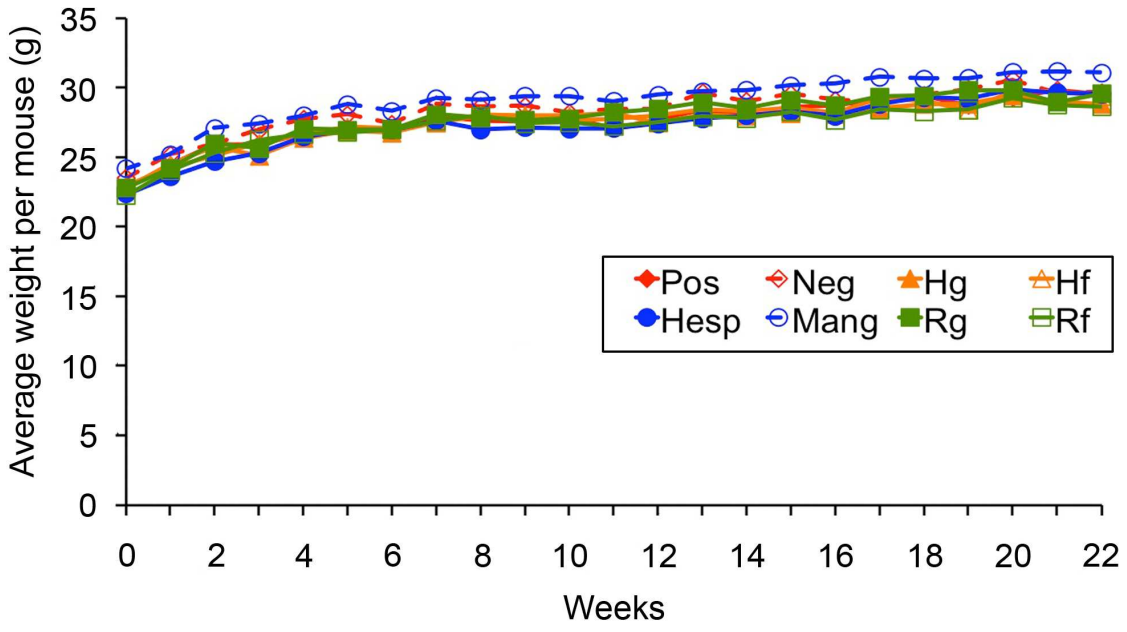


Figure 8. The average increase in weight per mouse in each group during each week of the skin carcinogenesis study (Abbreviations: Pos, positive control; Neg, negative control; Hg, "green" honeybush extract; Hf, fermented honeybush extract; Hesp, hesperidin; Mang, mangiferin; Rg, "green" rooibos extract; Rf, fermented rooibos extract. Solvent control group excluded.)

ADDENDUM 7: Tumours on the dorsal skin of SKH-1 mice initiated with DMBA and promoted with UVB

Figure 9 and **Figure 10** show the dorsal skin of each mouse used in the skin carcinogenesis study after termination. Mice in the positive control group **Figure 9** (**a**) developed the largest and most tumours per mouse, while no tumours developed in the negative control **Figure 9** (**b**) or solvent control. Application of the “green” and fermented extracts of rooibos **Figure 9** (**c**) and (**d**) and honeybush **Figure 10** (**e**) and (**f**) reduced the number of tumours, which developed, as well as the size of the tumours. Mangiferin and hesperidin **Figure 10** (**g**) and (**h**) were not effective in reducing the number of tumours, which developed on the skin, but were able to reduce the average size of tumours. Two mice did however develop benign lipomas in the fermented honeybush extract treated group. These two growths were excluded from statistical analysis, as the aim of the study was to obtain tumours, which develop from the epidermis and not from adipocytes. Also, lipomas are not considered skin tumours, but rather subcutaneous soft tissue tumours.

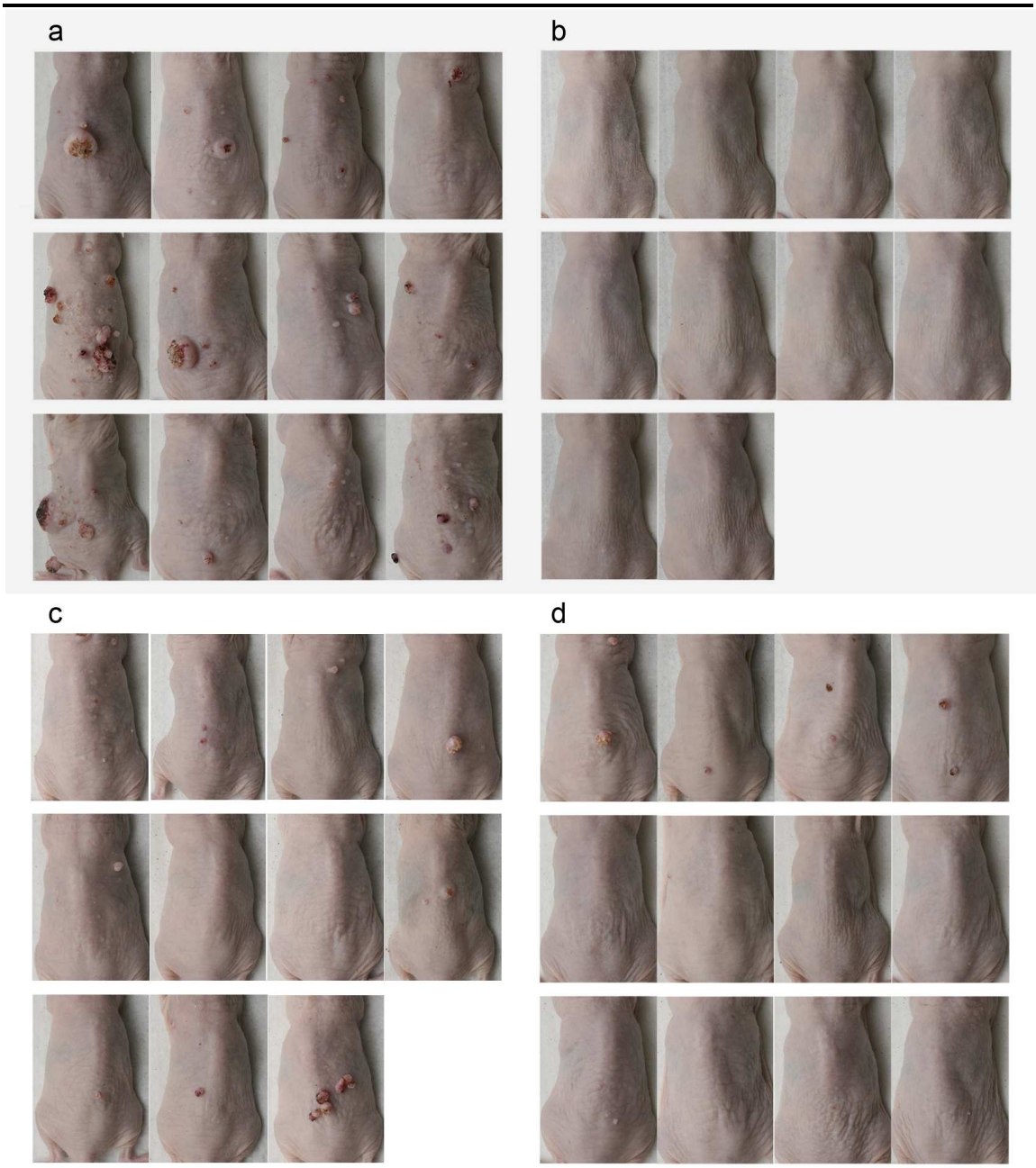


Figure 9. Tumours that developed by the end of the skin carcinogenesis study (**a**) positive control group, (**b**) negative control group, (**c**) green rooibos extract group, (**d**) fermented rooibos extract group

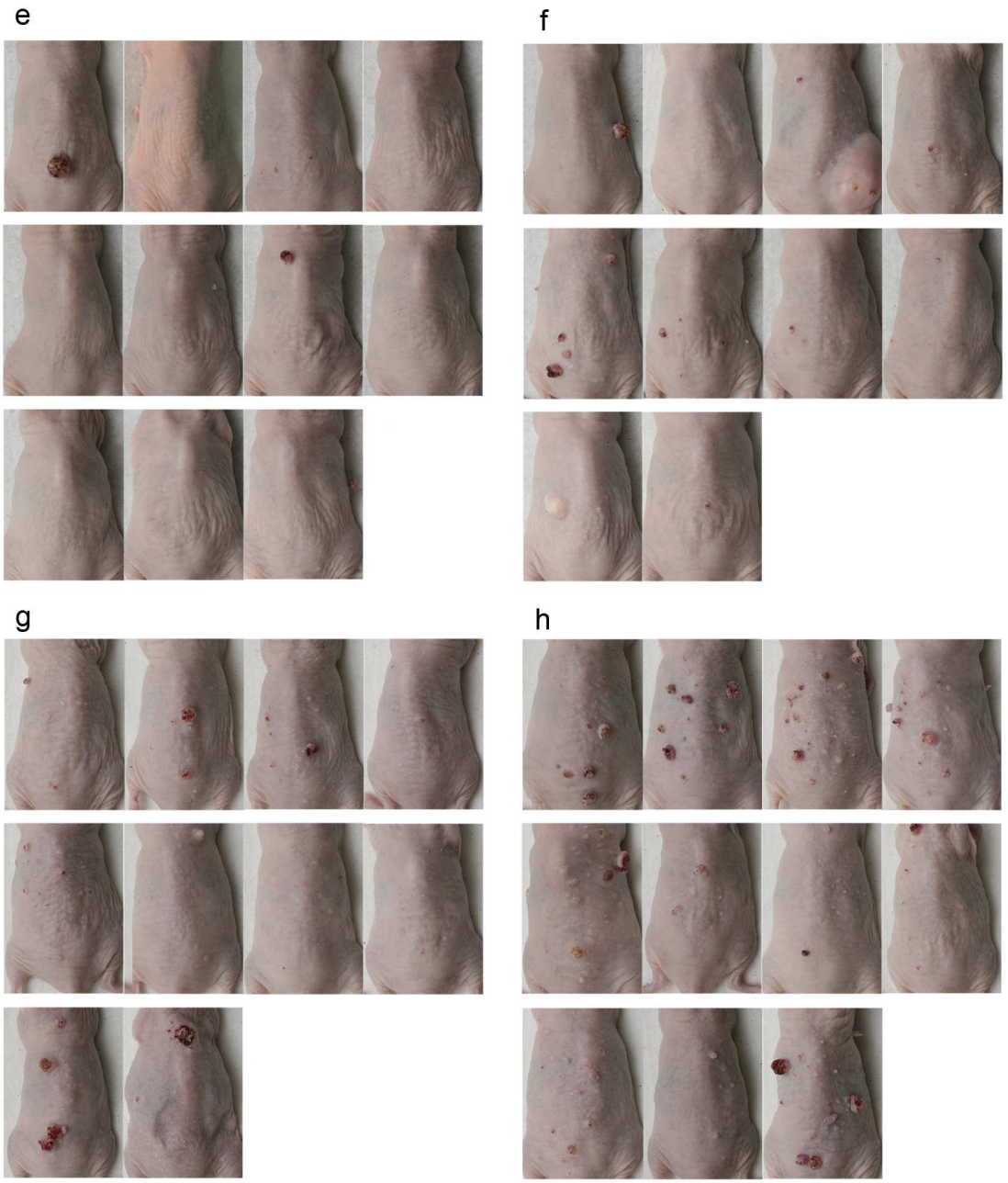


Figure 10. Tumours that developed by the end of the skin carcinogenesis study (**e**) green honeybush extract group, (**f**) fermented honeybush extract group (**g**) hesperidin group, (**h**) mangiferin group (solvent control group excluded)

ADDENDUM 8: Age of the mice used for the short term UVB exposure study.

The mice used in the short term UVB exposure study were obtained from litters from various birth dates; therefore the ages are given in **Table 1**.

Table 1. Age of female SKH-1 mice used for the short term UVB exposure study

Group	Number of mice	Date born	Date issued	Date on 1st day of study	Age (Days)	Age (Days)
Positive control	5	02-10-2007	12-11-2007	19-11-2007	41	
	5	25-09-2007	12-11-2007	19-11-2007	49	45
Negative control	5	25-09-2007	12-11-2007	19-11-2007	49	
	1	02-10-2007	12-11-2007	19-11-2007	41	45
Solvent control	5	16-10-2007	12-11-2007	19-11-2007	28	
	5	25-09-2007	12-11-2007	19-11-2007	49	38
“Green” honeybush	1	16-10-2007	12-11-2007	19-11-2007	28	
	4	18-09-2007	12-11-2007	19-11-2007	56	
	5	02-10-2007	12-11-2007	19-11-2007	41	41
Fermented honeybush	5	25-09-2007	12-11-2007	19-11-2007	49	
	5	16-10-2007	12-11-2007	19-11-2007	28	38
Hesperidin	5	02-10-2007	12-11-2007	19-11-2007	41	
	5	25-09-2007	12-11-2007	19-11-2007	49	45
Mangiferin	5	30-10-2007	10-12-2007	17-12-2007	41	
	5	23-10-2007	10-12-2007	17-12-2007	49	45

ADDENDUM 9: Histology and immunohistochemistry protocol

Preparation of slides

Fix skin tissue samples in 37 % formaldehyde at 4°C overnight.

Wash the tissue in phosphate buffered saline (PBS), 100 % ethanol and 80 % ethanol on a shaker for 5 min each, and then store in 70 % ethanol until further processing.

Place the tissue in a cassette in 90 % alcohol for 1 h and again for 2 h in fresh 90 % alcohol. Then place the tissue in absolute alcohol for 2 h, and repeat twice in fresh absolute alcohol. Place the tissue in xylol for 2 h, and again in fresh xylol.

Place the tissue in molten paraffin wax (Histotec™ wax pastilles, Merck) for 6 h and then embed the tissue by allowing the wax to cool.

Prepare 5 μm sections on microscope slides using a rotary microtome (Leica, RM2125 RT).

Incubate the slides for 1 h or longer at 56°C to adhere the sections to the slides and to melt the wax. Place the slides into xylol for 10 min and repeat using fresh xylol, and then again briefly. Place the slides in absolute alcohol 1 min and then repeat twice with fresh alcohol. Place the slides in 90 % alcohol for 1 min and then in 70 % alcohol for 1 min. Rinse in running tap water.

Haematoxylin and Eosin staining

After rinsing in running tap water, stain the slides in Haematoxylin for 5 min and then rinse in running tap water. Differentiate in 1 % acid alcohol and rinse in tap water. Place the slides in Scott's tap water for 1 min and rinse in running tap water. Stain the slides in Eosin/phloxine for 2 min and rinse in running tap water.

Dehydrate the slides briefly in 96 % alcohol and repeat twice and then in absolute alcohol twice. Clear the slides with xylol and again in fresh xylol.

Mount the slides with coverslips using Entellan® for permanent preparations.

View slides with light microscope.

Immunohistochemical staining

After rinsing in running tap water, permeabilize the lipid membranes with 0.2 % Triton X-100 in PBS for 10 min and then rinse in PBS three times for 5 min.

Boil the slides in 10 mM sodium citrate, pH 6.0 for 10 min to unmask the antigens. Cool the slides for 10 min and then rinse in PBS three times for 5 min.

Inactivate endogenous peroxidases, which could interfere with the horseradish peroxidase (HRP)-conjugated secondary antibody by incubating the slides in 3 % hydrogen peroxide in methanol for 10 min. Rinse the slides in PBS three times for 5 min.

Reduce background staining of the primary antibody by incubating the slides for 1 hour in 1 % bovine serum albumin (BSA) in PBS. Incubate the primary antibody in 1 % BSA at a predetermined optimal dilution overnight at 4°C on a shaker. Rinse the slides in PBS three times for 5 min.

Incubate the slides with HRP-conjugated secondary antibody in PBS for 1 hr at room temperature and then rinse three times with PBS for 5 min.

To detect the staining, incubate the slides with the chromogen diaminobenzidine (DAB) (which oxidises the chromogenic substrate HRP to form a brown colour) at a predetermined concentration for 1-2 min. Rinse the slides in running tap water.

Counterstain the slides lightly with haematoxylin. Dehydrate and mount the slides according to the haematoxylin and eosin staining method.

View slides with light microscope.

ADDENDUM 10: UVB-induced sunburn

The sunburn response of the SKH-1 mice skin in the short term UVB exposure study is demonstrated in **Figure 11**. These images are representative of the response observed in the positive control, mangiferin and hesperidin groups. The fermented and "green" honeybush groups showed considerably reduced sunburn. The skin remained normal in appearance on day one, day two and day three. On day four and day five, the skin began to peel, followed by a strong erythema response on day six, which slowly subsided by day ten. The skin appeared to be normal again on the last day when the mice were terminated.

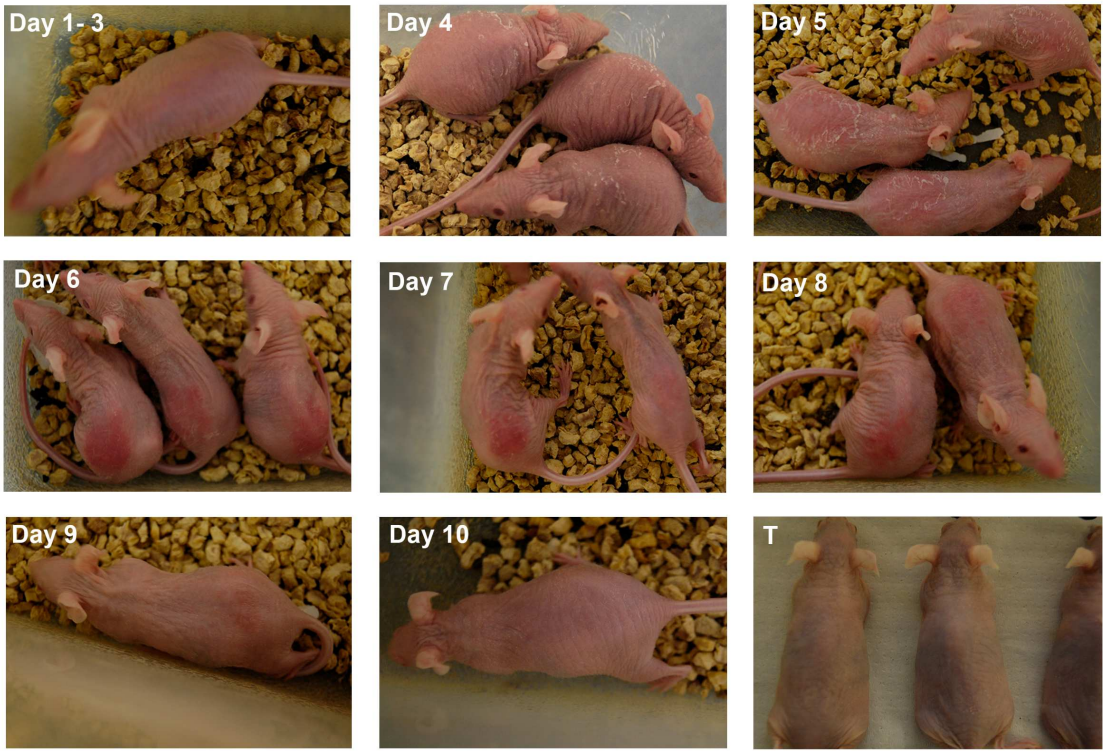


Figure 11. Sunburn response of mice to UVB irradiation (Abbreviation: T, termination)

ADDENDUM 11: Increase in bifold skin thickness in the mice skin

Table 2 shows the UVB-induced average bifold skin thickness of each group before and after the study, with the average increase expressed in mm increase and percentage increase.

Table 2. The UVB-induced increase in bifold skin thickness of mice in the short term UVB exposure study

Group	Bifold thickness before (mm)	Bifold thickness after (mm)	Increase (mm)	% increase
Positive control	0.40 ± 0.00	1.17 ± 0.28	0.77 ± 0.28	192.50
Negative control	0.45 ± 0.05	0.77 ± 0.09	0.32 ± 0.11	71.11
Solvent control	0.49 ± 0.07	0.63 ± 0.15	0.14 ± 0.17	28.57
“Green” honeybush	0.46 ± 0.05	0.76 ± 0.15	0.30 ± 0.14	65.22
Fermented honeybush	0.45 ± 0.07	0.91 ± 0.22	0.46 ± 0.24	102.22
Hesperidin	0.39 ± 0.03	0.95 ± 0.27	0.56 ± 0.26	143.59
Mangiferin	0.57 ± 0.05	1.01 ± 0.28	0.44 ± 0.29	77.19

ADDENDUM 12: Monitored weight of mice during the short term UVB exposure study

The weight of each mouse was measured before the study began and before termination to determine if the experiment had any adverse effects on health resulting in weight loss (**Table 3**). There was no significant difference in the weight gain or loss measured.

Table 3. Weight gain observed during the short term UVB exposure study

Experimental group	Weight before (g)	Weight after (g)	Gain in weight (g)
Positive control	22.64 ± 1.44	23.87 ± 1.84	1.23
Negative control	20.67 ± 2.38	24.08 ± 1.11	3.42
Solvent control	23.01 ± 1.12	23.31 ± 1.70	0.30
“Green” honeybush	22.51 ± 1.96	22.13 ± 1.65	-0.38
Fermented honeybush	21.39 ± 3.23	23.62 ± 1.82	2.23
Hesperidin	22.22 ± 1.59	21.89 ± 1.93	-0.33
Mangiferin	21.83 ± 2.23	24.64 ± 2.22	2.81

ADDENDUM 13: H&E stained sections of mice skin

UVB irradiation of the skin resulted in hyperplasia in the epidermis, shown in **Figure 12,** evident by the increase in thickness and number of cell layers in the positive control (**a**) compared to the negative (**b**) and solvent control (**c**) The application of the “green” (**d**) and fermented honeybush (**e**) extracts and hesperidin (**f**) reduced hyperplasia indicative of the decreased epidermal thickness, while mangiferin (**g**) had no effect. Micrographs are representative of 10 random sections photographed from 3 samples stained form each group. The structure of the skin is indicated on the positive control section (**a**), showing the lower dermis (D) separated from the upper epidermis (E) by a basement membrane (BM) and the keratinized (K) protective layer above the epidermis.

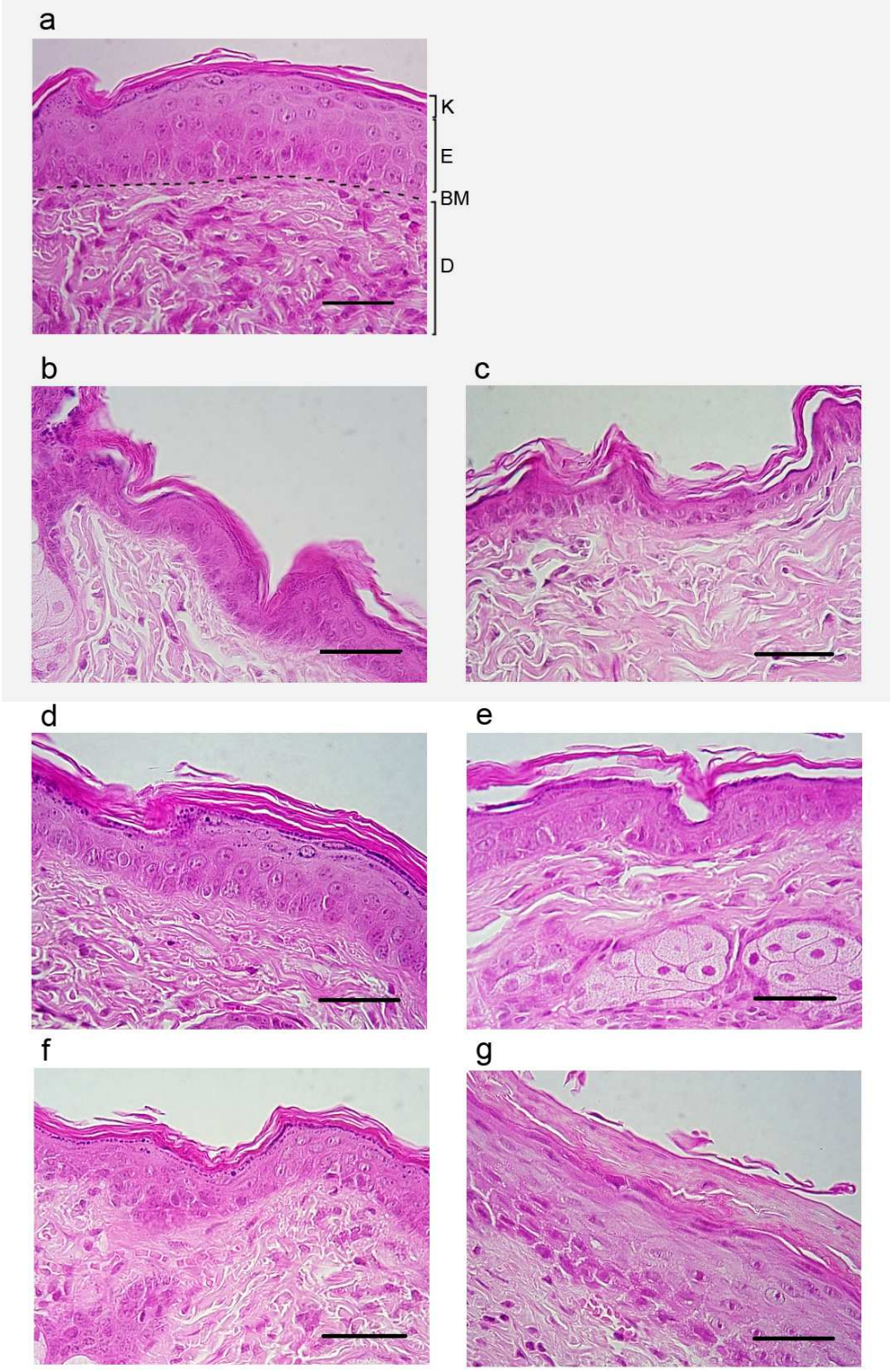


Figure 12. Haematoxylin and eosin stained mouse skin sections (**a**) Positive control, (**b**) negative control, (**c**) solvent control, (**d**) "green" honeybush, (**e**) fermented honeybush, (**f**) hesperidin, (**g**) mangiferin. Scale bar 5 µm, magnification 400x (Abbreviations: K, keratinized top layer; E, epidermis; BM, basement membrane; D, dermis)

ADDENDUM 14: Levels of conjugated dienes in the skin

Conjugated dienes (CDs) content in the skin preparations were assessed as an indicator of lipid peroxidation. For CDs, a volume of 200 µl sample was mixed with 400 µl chloroform/methanol (2/1, v/v) and then centrifuged at 4°C for 15 min at 8000 rpm. The lipid soluble layer was dried with N_2 and dissolved in 350 µl cyclohexane. The sample was then transferred to a 96 well plate and the absorbance at 234 nm was measured. The concentration of CDs was calculated by using the extinction coefficient 2.95 x 10^4 and expressed as nmole per mg protein.

The results (**Table 4**) were excluded from the second article due to the unusually high conjugated dienes value obtained for the negative control group. As this group consisted of only six mice, compared to ten to twelve mice in the other groups, outlier values could not be excluded from the data obtained in order to reduce the standard deviation.

Table 4. Conjugated dienes levels in skin samples of mice irradiated with UVB

Experimental group	Conjugated dienes (nmole/mg)
Positive control	$26.116 \pm 3.323^{N,M}$
Negative control	$34.930 \pm 9.700^{P,S,Hg,Hf,H,M}$
Solvent control	$23.434 \pm 3.645^{N,M}$
"Green" honeybush	$25.553 \pm 4.899^{N,M}$
Fermented honeybush	$27.356 \pm 4.836^{N,M}$
Hesperidin	$26.860 \pm 4.688^{N,M}$
Mangiferin	$17.265 \pm 8.821^{P,S,Hg,Hf,H}$

Superscripts indicate significant values $P < 0.05$. Abbreviations: P, positive control; N, negative control; S, solvent control; Hg, "green" honeybush; Hf, fermented honeybush; H, hesperidin; M, mangiferin

ADDENDUM 15: COX-2 expression in the epidermis

The expression of COX-2 in the epidermis of UVB irradiated mice skin was determined by immunohistochemistry as a measure of inflammation. The number of cells expressing COX-2 in the epidermis was counted in 10 random fields using a light microscope and expressed as the number of cells per 10 μm length (**Figure 13**). Three samples were analyzed for each group. Micrograph images were taken with a camera attached to the microscope to show the distribution of ODC in the epidermis (**Figure 14**). Irradiation of mice skin daily for 10 days with UVB resulted in a significant increase in the expression of COX-2, with intense cytoplasmic staining in the keratinocytes in the upper layers of the epidermis and weaker staining in the basal layer (**a**). Unirradiated mice skin showed very low levels of COX-2 expression, with some perinuclear stained cells (**b**) and (**c**). Topical application of "green" honeybush (**d**) and fermented honeybush (**e**) extracts resulted in a significant decrease in the expression of COX-2 compared to the positive control. Staining occurred weakly and predominantly in the perinuclear and less in cytoplasmic regions of keratinocytes of the upper layers of the epidermis. Far fewer cells of the basal layer were stained. Topical application of hesperidin (**f**) and mangiferin (**g**) did not reduce the expression of COX-2. Strong cytoplasmic staining occurred in keratinocytes in the upper layers of the epidermis with weak staining in the basal layer cells.

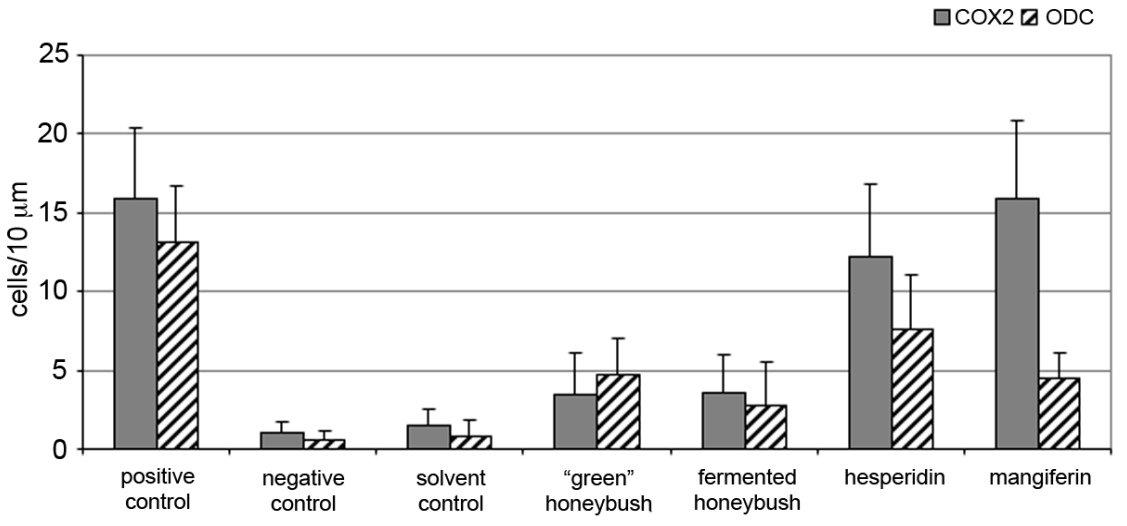


Figure 13. The number of positive COX-2 and ODC expressing cells in the epidermis of mouse skin sections

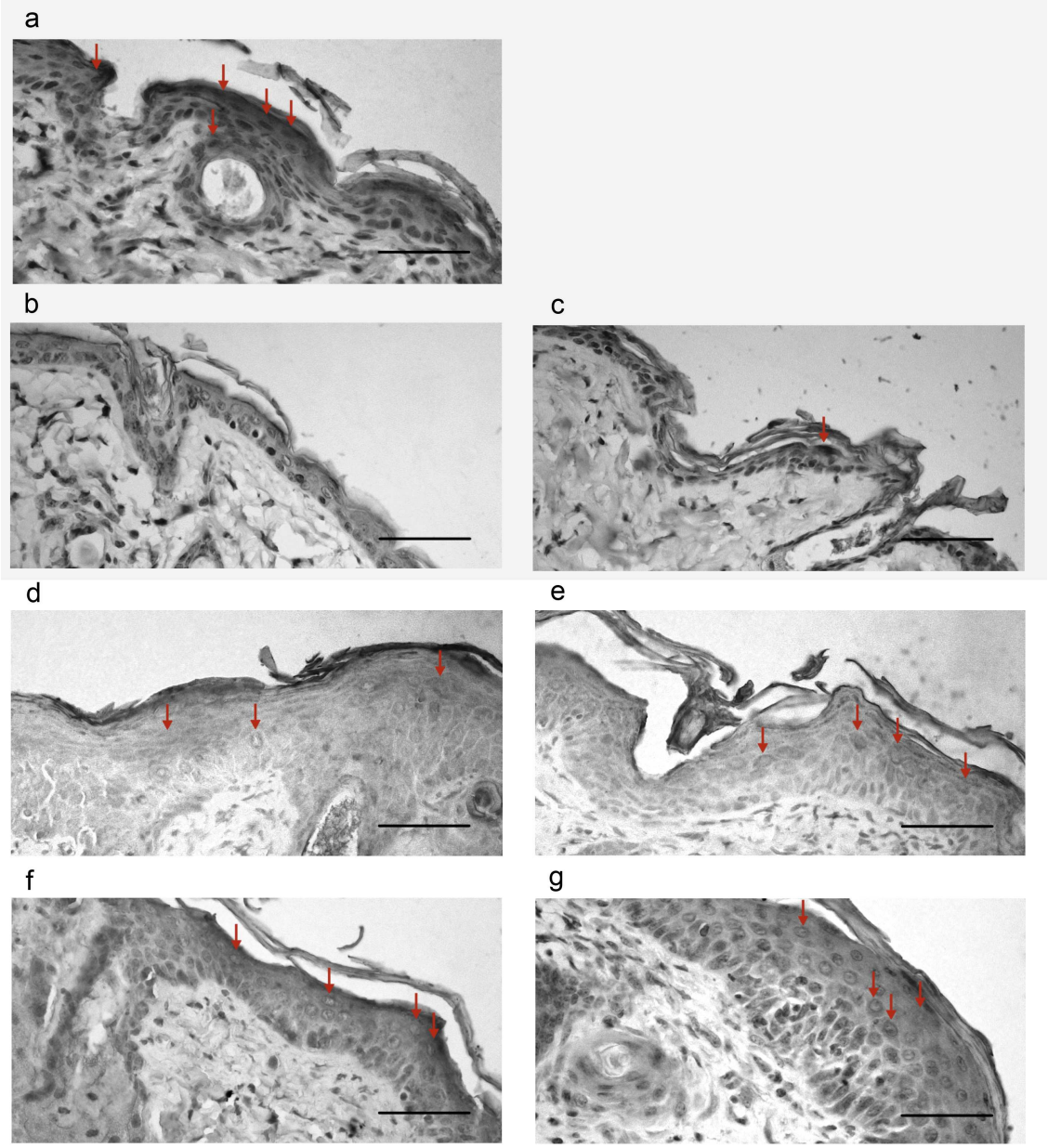


Figure 14. COX-2 expression in the epidermis of mouse skin sections Red arrows indicate COX-2 positively stained cells (**a**) positive control, (**b**) negative control, (**c**) solvent control, (**d**) "green" honeybush, (**e**) fermented honeybush, (**f**) hesperidin, (**g**) mangiferin. Scale bar 10 µm, magnification 400x.

ADDENDUM 16: ODC expression in the epidermis

The expression of ODC in the epidermis of UVB irradiated mice skin was determined by immunohistochemistry as a measure of increased proliferation. The number of cells expressing ODC in the epidermis was counted in 10 random fields using a light microscope and expressed as the number of cells per 10 μm length (**Figure 13**). Three samples were analyzed for each group. Micrograph images were taken with a camera attached to the microscope to show the distribution of ODC in the epidermis (**Figure 15**). UVB-irradiation resulted in a significant increase in the expression of ornithine decarboxylase (ODC) in the positive control group (**a**). Strong cytoplasmic and perinuclear staining occurred in the upper layers of the epidermis, with weaker perniculear staining occurring in lower layers of the epidermis. Unirradiated mice skin showed very low levels of ODC staining, with very few cells of the upper epidermis showing weak cytoplasmic staining (**b**) and (**c**). Topical application of the “green” honeybush (**d**) and fermented honeybush (**e**) extracts significantly reduced the expression of ODC, with fermented honeybush extract more effective than the “green” honeybush extract. Weak staining occurred diffusely across the epidermis of both “green” and fermented honeybush treated skin. “Green” honeybush treated skin showed occasional stronger cytoplasmic and perinuclear stained cells in the upper layer of the epidermis. Topical application of hesperidin (**f**) was only moderately effective in reducing ODC expression in the epidermis, while mangiferin (**g**) was more effective in reducing ODC expression. Strong cytoplasmic staining occurred in the upper layers of the epidermis and weak staining in the lower layers of the epidermis.

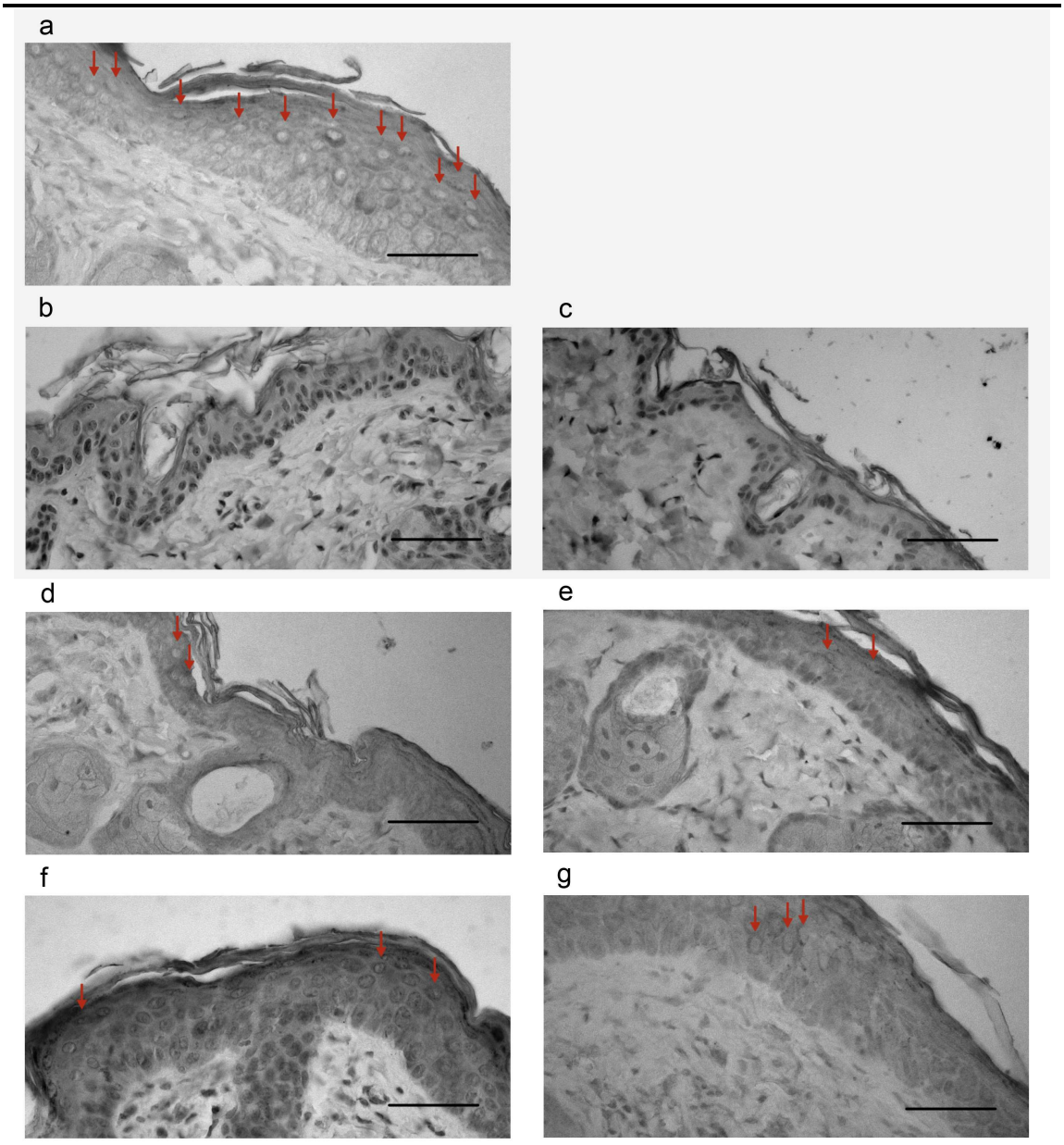


Figure 15. ODC expression in the epidermis of mouse skin sections Red arrows indicate ODC positively stained cells (**a**) positive control, (**b**) negative control, (**c**) solvent control, (**d**) “green” honeybush, (**e**) fermented honeybush, (**f**) hesperidin, (**g**) mangiferin. Scale bar 10μm, magnification 400x.

ADDENDUM 17: GADD45 expression in the epidermis

The expression of GADD45 in the epidermis of UVB irradiated mice skin was determined by immunohistochemistry as a measure of UVB-induced DNA damage. The number of cells expressing GADD45 in the epidermis was counted in 10 random fields using a light microscope and expressed as the number of cells per 10 μm length (**Figure 16**). Micrograph images were taken with a camera attached to the microscope to show the distribution of GADD45 in the epidermis (**Figure 17**). Irradiation of mice skin daily for 10 days with UVB resulted in a significant increase in the expression of GADD45, with intense cytoplasmic and perinuclear staining in the keratinocytes in the upper layers of the epidermis and weaker cytoplasmic staining in the basal layer (**a**). Unirradiated mice skin showed very low levels of GADD45 expression, with few weakly stained cells (**b** and **c**). Topical application of "green" honeybush (**d**) and fermented honeybush (**e**) extracts resulted in a significant decrease in the expression of GADD45 compared to the positive control. Staining occurred weakly and predominantly in the cytoplasmic regions of keratinocytes of the upper layers of the epidermis with no cells of the basal layer stained. Topical application of hesperidin (**f**) also significantly reduced the expression of GADD45 in the skin. Weak staining occurred in the upper layers of the epidermis, predominantly in the cytoplasm. Topical application of mangiferin (**g**) did not reduce the expression of GADD45 as effectively as hesperidin or the extracts. Cytoplasmic staining occurred in keratinocytes in the upper layers of the epidermis with some perinuclear staining as well. Staining did not occur in the basal layer.

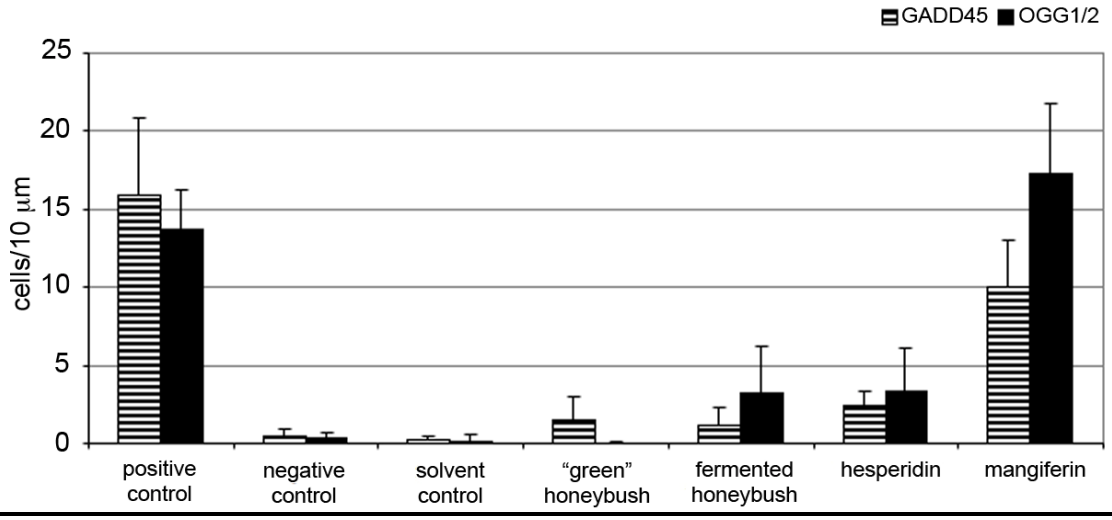


Figure 16. The number of positive GADD45 and OGG1/2 expressing cells in the epidermis of mouse skin sections

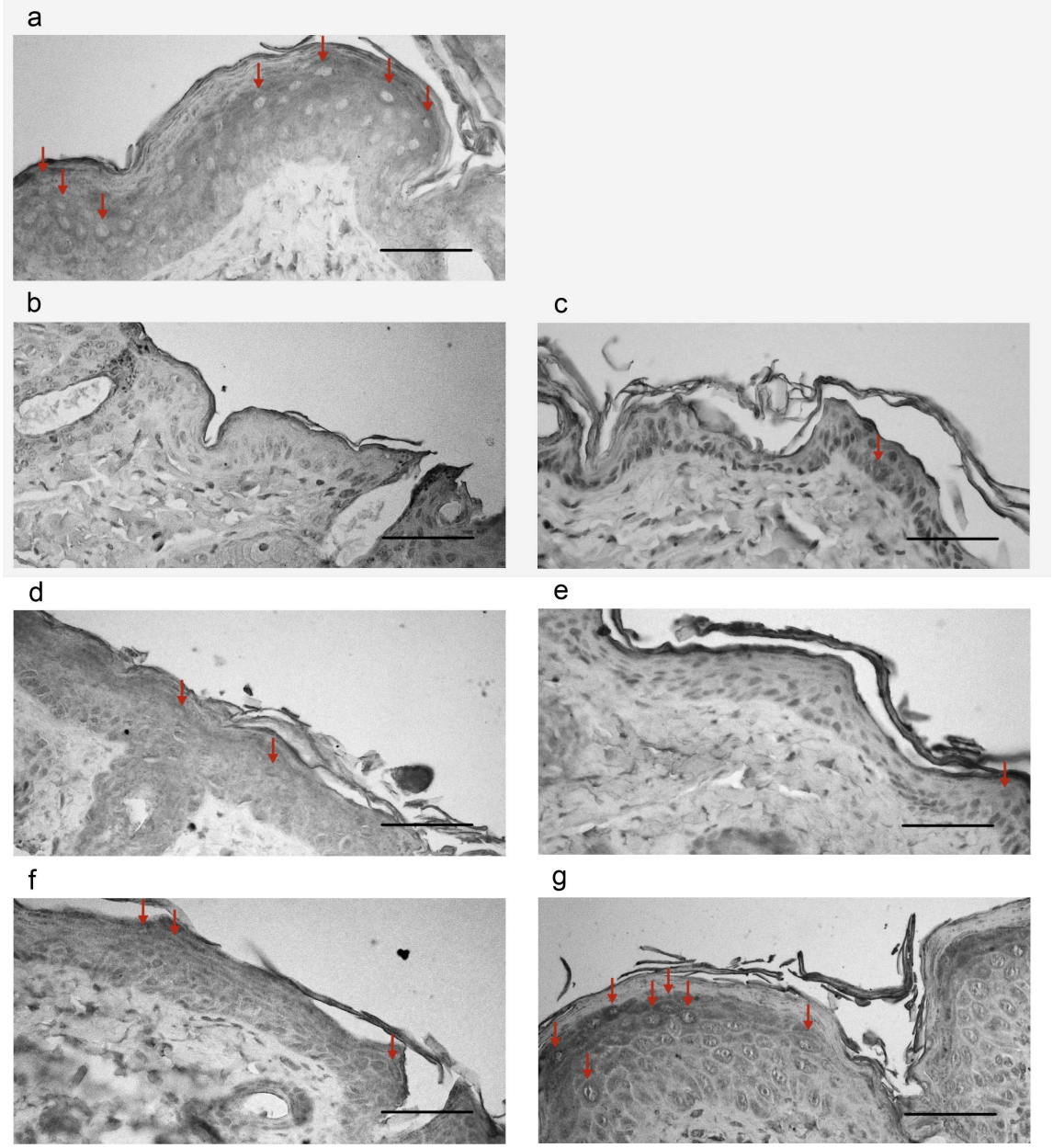


Figure 17. GADD45 expression in the epidermis of mouse skin sections Red arrows indicate GADD45 positively stained cells (**a**) positive control, (**b**) negative control, (**c**) solvent control, (**d**) "green" honeybush, (**e**) fermented honeybush, (**f**) hesperidin, (**g**) mangiferin. Scale bar 10 µm, magnification 400x.

ADDENDUM 18: OGG1/2 expression in the epidermis

The expression of OGG1/2 in the epidermis of UVB irradiated mice skin was determined by immunohistochemistry as a measure of ROS produced 8-oxoG DNA adducts. The number of cells expressing OGG1/2 in the epidermis was counted in 10 random fields using a light microscope and expressed as the number of cells per 10 μm length (**Figure 16**). Three samples were analyzed for each group. Micrograph images were taken with a camera attached to the microscope to show the distribution of OGG1/2 in the epidermis (**Figure 18**). Irradiation of mice skin daily for 10 days with UVB resulted in a significant increase in the expression of OGG1/2, with intense cytoplasmic staining in the keratinocytes in all the layers of the epidermis (**a**). Unirradiated mice skin did not express OGG1/2 (**b** and **c**). Topical application of “green” honeybush (**d**) resulted in a significant decrease in OGG1/2 expression with very weak cytoplasmic staining in some cells in the epidermis. Fermented honeybush (**e**) extract was not as effective as “green” honeybush extract but significantly decreased the expression of OGG1/2 compared to the positive control. Staining occurred weakly in the cytoplasmic regions of keratinocytes of the upper layers of the epidermis with no cells of the basal layer stained. Topical application of hesperidin (**f**) also significantly reduced the expression of OGG1/2 in the skin. Weak staining occurred in the upper layers of the epidermis, predominantly in the cytoplasm. Topical application of mangiferin (**g**) did not reduce the expression of OGG1/2. Strong cytoplasmic staining occurred in keratinocytes in the upper layers of the epidermis with some staining in the basal layer.

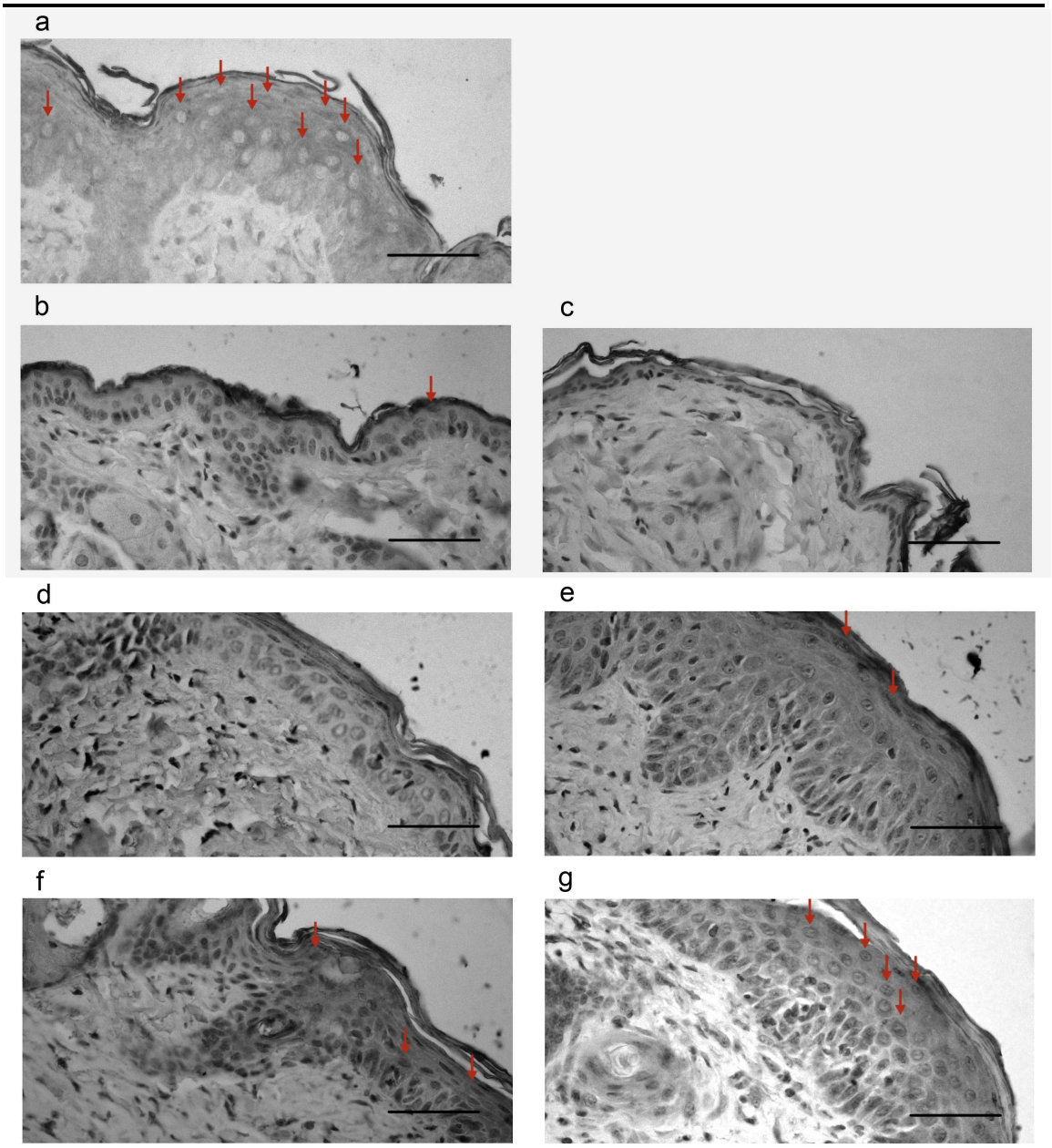


Figure 18. OGG1/2 expression in the epidermis of mouse skin sections Red arrows indicate OGG1/2 positively stained cells (**a**) positive control, (**b**) negative control, (**c**) solvent control, (**d**) "green" honeybush, (**e**) fermented honeybush, (**f**) hesperidin, (**g**) mangiferin. Scale bar 10 μm, magnification 400x.