Determination of Cancer Induction and Cell-Killing Potential of Beauty Products and Personal Care Products Using Human Skin Cells

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THESIS SUMMARY

The *in vitro* toxicity and genotoxicity of four cosmetic products were determined using a human keratinocyte (HaCaT) and human fibroblast CCD-1064SK cells lines. The products were: Nivea Visage Q10plus Anti-Wrinkle cream including synthetic chemicals with TiO_2 (NVAW+ TiO_2); Nivea Visage Q10plus Anti-Wrinkle cream including synthetic chemicals without TiO_2 (NVAW); Facial Moisturizer: Camellia & Geranium Blossom (FMCGB); with glycerol as the negative control. Two human skin cell lines (human keratinocytes HaCaT and human fibroblast CCD1064SK) were employed. Four popular products for head lice treatment were also examined for both types of toxicity: Lice breaker (Permethrin 1% w/w); KP24 Medicated lotion (Maldison0.5% w/w); Organix Pyrethrum treatment (4g/L Pyrethrins, 16g/LPiperonyl Butoxide); Tea Tree Oil (100% pure); and Lavender oil (100% pure) on a human skin cell line (HaCaT). Toxicity was measured by Crystal Violet assay and Methyl tetrazolium (MTT) assay and the proportion of apoptosis or necrosis was monitored by Flow Cytometry. Genotoxicity was detected via the cytokinesis block micronucleus (CBMN) assay. NVAW+ TiO_2 induced the highest toxicity and genotoxicity levels of all the tested beauty products. No toxicity or genotoxicity observed with FMCGB, but there was a significant necrosis. Glycerol did not induce any toxicity or genotoxicity. Populations of cells treated with diluted (NVAW+ TiO_2) and (NVAW) products showed increased proportions of apoptosis and necrosis. The decrease in NDI by NVAW+ TiO_2 and NVAW (1.4 ($P < 0.05$)) was observed at the 0.3% w/v dose and by FMCGB at the 0.05% w/v dose. (NVAW+ TiO_2) and (NVAW) products showed increased frequency of micronuclei (MNi). (NVAW+ TiO_2) product proved to induce significantly more micronuclei (MNi) than the product without TiO_2. (NVAW+ TiO_2) product induced genetic damage manifested as chromosomal damage determined by CBMN assay at a frequency significantly higher (43 MNi/1000 binucleated cells, n=3) than the background frequency (media alone control; MNi range= 9 MNi /1000 binucleated cells, n=3). Head lice treatments Tea Tree Oil (TTO), Pure Lavender oil and Pyrethrum did induce significant cytotoxicity. Also, they enhanced both early apoptosis and late apoptosis or necrosis. However, two head lice treatments, Permethrin (Lice Breaker) and Maldison (Malathion) (KP24) did not induce cytotoxicity. Early apoptosis and necrosis were observed in Permethrin treatment, and late apoptosis and early necrosis were measured in Maldison (Malathion) (KP24). Moreover, Permethrin (Lice Breaker) and Maldison (Malathion) (KP24) induced micronuclei (MNi) at a frequency significantly higher (range= 15-25 MNi/1000 binucleated cells, n=3) than the background frequency (media alone control; MNi

range= 6 MNi /1000 binucleated cells, n=3). Moreover, Calendula officinalis extracts A (C5), B (C6), D and C were examined for their ability to protect against hydrogen peroxide (H_2O_2) induce cell killing and chromosomal damage to HaCaT skin cells. Using the MTT cytotoxicity assay and CBMN genotoxicity assay, it was observed that extracts of Calendula officinalis gave time-dependent and concentration-dependent protection against H_2O_2-induced oxidative stress *in vitro* using human skin cells. Pre-incubation with the Calendula extracts for 24 and 48 hours increased survival relative to the population without extract by 20% and 40% respectively following oxidative challenge. The frequency of MNi in the presence of extracts was reduced by an average increment of 20 MNi/1000 BN cells to 2-9 MNi/1000 BN cells at all doses tested. Finally, Calendula extract was examined for its protective effects against the cytotoxicity of selected beauty products and head lice treatments and titanium dioxide (TiO_2) on HaCaT cells. Pre-incubation with the Calendula extract for 48 hours significantly increased survival relative to the population without the extract by 30% and 50%, following treatment with personal care products.

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LIST OF ABBREVIATIONS

6-TG	6- thioguanine
7-AAD	7-amino-actinomycin D
ANOVA	analysis of variance
BN	binucleated
BP	British Pharmacopoeia
Cyt-B	cytochalasin B
CBMN	cytokinesis-block micronucleus
CE	cloning efficiency
CO_2	carbon dioxide
CV	crystal violet
Cyt-B	Cytochalasin B
DDT	dichloro diphenyl trichloroethane
DEA	diethanolamine
DMSO	dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picryl-hydrazyl
EDTA	ethylenediamine tetra acetic acid
FBS	foetal bovine serum
FDA	Food and Drug Administration (US)
FMCGB	Facial Moisturizer—Camellia and Geranium Blossom
FITC	fluorescein isothiocyanate
GAE	gallic acid equivalent
H_2O_2	hydrogen peroxide

HSD	honestly significant difference (in Tukey's HSD post hoc test)
IARC	International Agency for Research on Cancer
IC20	20% inhibitory concentration
IMDM	Iscove's Modified Dulbecco's Medium
MF	mutant frequency
ml	millilitre
µM	micromolar
MNi	micronuclei
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NBUD	nuclear bud
NDI	nuclear division index
NICNAS	National Industrial Chemical Notification and Assessment Scheme
NPB	nucleoplasmic bridge
NVAW+TiO_2	Nivea Visage Q10plus Anti-Wrinkle with TiO_2
NVAW	Nivea Visage Q10plus Anti-Wrinkle without TiO_2
OD	optical density
OH	hydroxyl radical
PAHs	polycyclic aromatic hydrocarbons
PBS	phosphate-buffered saline
PCBs	polychlorinated biophenyls
PI	propidium iodide
PS	phospholipid phosphatidylserine
RNS	reactive nitrogen species

ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	room temperature
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TB	trypan blue
TiO_2	titanium dioxide
TTO	tea tree oil
US	United States of America
UV	ultraviolet
UVA	ultraviolet A
UVB	ultraviolet B
v/v	volume/volume percentage concentration
w/v	weight/volume percentage concentration

DECLARATION

I certify that this thesis does not incorporate, without acknowledgement, any material submitted for a degree or diploma in any university; and that, to the best of my knowledge and belief, it does not contain any material previously published or written by another person except where due reference is made in the text.

Abdullah Alnuqaydan

August 2016

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Doing a PhD in science is a long process and represents a new stage of life. A PhD in medical research means living your life in a laboratory for four years or more, arriving early in the morning and leaving late at night. It requires critical thinking and reading most of the recent publications and references in your field of study. This long process could not have been brought to a successful conclusion without the support of many different people.

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PUBLICATIONS AND PRESENTATIONS

Research articles

1. Alnuqaydan, AM & Sanderson, BJS 2016, 'Toxicity and genotoxicity of beauty products on human skin cells in vitro', *Journal of Clinical Toxicology.* In Press, Accepted Manuscript.
My contribution: AMA carried out all of the experiments, analysed the data and drafted the manuscript. BJSS designed the study and reviewed the work.

2. Alnuqaydan, AM & Sanderson, BJS 2016, 'Genetic damage and cell killing induction by five head lice treatments on HaCaT human skin cells', *Journal of Carcinogenisis and Mutagenesis*, vol. 7, no. 2, pp. 1–6.
My contribution: AMA carried out all of the experiments, analysed the data and drafted the manuscript. BJSS designed the study and reviewed the work.

3. Alnuqaydan, AM, Lenehan, CE, Hughes, RR & Sanderson, BJS 2015, 'Extracts from calendula officinalis offer in vitro protection against H2O2 induced oxidative stress cell killing of human skin cells', *Phytotherapy Research*, vol. 29, pp. 120–124.
My contribution: AMA carried out the majority of the experiments, analysed the data and drafted the manuscript. CEL and RRH prepared the extracts and carried out the Folin-Ciocalteu and DPPH assays. BJSS designed the study, edited the manuscript and reviewed the work.

4. Alnuqaydan, AM, Lenehan, CE, Hughes, RR & Sanderson, BJS 2015, 'Calendula officinalis extracts protect against H_2O_2 induced chromosome damage on HaCaT human skin cells', *Journal of Carcinogenisis and Mutagenesis*, vol. 6, no. 6.
My contribution: AMA carried out the majority of the experiments, analysed the data and drafted the manuscript. CEL and RRH prepared the extracts and carried out the Folin-Ciocalteu and DPPH assays. BJSS designed the study and reviewed the work.

Research articles in submission

5. Alnuqaydan, AM & Sanderson, BJS 2016, 'Calendula officinalis protection against cytotoxicity effects of personal care products on HaCaT human skin cells', *Journal of Clinical Toxicology*.
My contribution: AMA carried out all of the experiments, analysed data and drafted the manuscript. BJSS designed the study and reviewed the work

Conference presentations

1. Alnuqaydan, AM, Lenehan, CE, Hughes, RR & Sanderson, BJS 2013, 'The potential of naturally derived plant compounds to act as antioxidants to protect skin cells in culture', poster presented at the *BioProcessing Network (BPN) Annual Conference*, 22–24 October 2013, Gold Coast, Queensland.

2. Alnuqaydan, AM & Sanderson, BJS 2012, 'Toxicity and genotoxicity of facial moisturisers detected via human cell culture', presentation at the *Australian College of Toxicology and Risk Assessment (ACTRA) 5th Annual Scientific Meeting*, October 2012, Adelaide, South Australia.

Professional membership

1. American Society of Toxicology (SOT)

CHAPTER 1: INTRODUCTION

1.1 Toxicology principles for products involving application to humans

1.1.1 Definition and history of toxicology

Toxicology is now formally described as the study of adverse effects of chemical, physical or biological agents on the ecosystem and living organisms, how they could harm us and what mechanisms can prevent that harm (ToxLearn 2015). The definition of toxicology can be expanded to other areas such as pharmacology, which studies the side effects of new medicinal drugs (Gilbert 2004). The adverse effects under study can range from obvious ones such as cancer or death, to the unexpected outcomes of high doses of caffeine that result from drinking too much coffee every day.

Most toxicology studies examine a single agent, and there has been limited research on agents that are chemical mixtures (Mumtaz, Suk and Yang 2010; Zeliger 2008a; Yang, 2016). Commercial products, such as beauty products, skin care and head lice treatments, are an important area of study because these products are applied to people. Cosmetics are somewhat different from other products, in that they are formed from a combination of chemical ingredients or nanoparticles that can cause a variety of harm to consumers (Basketter & Lea 2009). The application of an appropriate toxicological review of product ingredients and of the final formulation is key to meeting the requirement that such products will not harm the consumer. Failure to undertake toxicological examinations on commercial products containing chemical mixtures to ensure that they meet an adequate standard increases the risk of adverse effects. Chemical toxicology covers a wide range of topics as well as most chemicals and synthetics, and has produced a long list of chemical classifications and their effects (Epstein & Fitzgerald 2009; Thomas 2008; NICNAS 2014). In the case of products containing chemical mixtures, these mixtures can be toxic and cause genetic damage due to the number of active chemicals that are carcinogenic (Zeliger 2008a). However, some chemical mixtures become toxic only when the components interact with each other, making the mixture the causative agent of the harm, and not a single component (Zeliger 2008a). Therefore, mixtures can have different effects than their individual ingredients. For example, in the American city of Woburn, in Middlesex County, Massachusetts, a group of children suffered from leukaemia from 1969 to 1979. This particular group drank water contaminated with different chemicals, none of which are known to cause leukaemia individually (Zeliger 2008a; Zeliger 2004). Various studies have examined the effect of pesticides and their active ingredients on human health (Alavanja et al. 2003; Bernard et al. 2008; Carozza et al. 2009). In

contrast, few analyses have considered commercial or personal products. Therefore, further examination needed for all beauty products and personal care products before releasing them onto the market.

There are government regulations for commercial products. However, it focuses on single chemicals rather than mixtures. The Australian federal government's National Industrial Chemical Notification and Assessment Scheme (NICNAS) maintains a classification list of industrial chemicals used as cosmetic ingredients, while the United States Food and Drug Administration (US FDA) has classified many single chemical ingredients used in cosmetics as carcinogens or hormone-disrupting (NICNAS, 2014; U.S.FDA, 2006). Cosmetics companies conduct short-term tests for their products before releasing them onto the market for sale to consumers. The products are also checked by a dermatologist or doctor who examines the test results before a product can be approved (Meridjanian 2015). This type of fast track test assesses the sensitivity of human skin to a product, such as the potential of a moisturiser to cause an allergic reaction, or the quality of under-arm products. However, commercial products such as beauty or skin care products could carry some risks resulting from long-term exposure to the variety of different mixtures used. Many commercial products contain mixtures. Therefore, toxicology studies need to be done on the whole commercial product. This project looked at selected four beauty products containing a mixture of chemical ingredients or a mixture of natural ingredients. Moreover, five popular use head lice treatments were examined for both types of toxicity. Finally, four *Calendula officinalis* extracts used in cosmetic were tested for their antioxidants activity on human skin cells.

1.1.2 Toxicology's relevance to daily life focussing on consumer products

Most people use beauty and skin care products every day to look after themselves and their children. We are exposed to various commercial products by applying them directly to our skin or by using them in the household. We go through this routine every day, without thinking about the cellular consequences and never imagining that we may be harming ourselves. Therefore, it is essential to carry out toxicology research to evaluate modern lifestyles and identify hidden toxins that can cause damage. Toxicology research ensures that the benefits of therapeutic or environmental agents are not outweighed by any problems or adverse impacts. The use of chemicals in commercial products has increased in the last 100 years. Up to 100,000 chemicals are currently used in commercial/ products, and approximately 1,000–3,000 new ones are introduced every year (Agency for Toxic Substances and

Disease Registry (ATSDR) 2009; Zeliger 2008a). Basic toxicity information is missing for about 72% of the top 3,000 chemicals in production, and nothing is known about the mixtures of these chemicals (Zeliger 2008a). Each day, humans are exposed to a huge number of chemicals at home, at work and in their general environment. Toxicology knowledge is improving, with more specialised branches that seek to control commercial products and protect us from contaminated food and drinking water and exposure to heavy metals. Thus, toxicology helps to mitigate danger in our daily lives, especially through the following subclasses: toxigenomics, nano-toxicology, food toxicology and environmental toxicology.

It is possible to be exposed to vast quantities of chemical agents in a single day (figure 1.1). In taking a shower early in the morning one may use a shampoo containing at least 15 chemicals. This is followed by breakfast or lunch, which is likely contaminated with a variety of natural, synthetic or artificial substitutes or and may be accidently contaminated with polychlorinated biophenyls (PCBs), methyl mercury found in fish, Escherichia coli or fungal toxins. The use of cosmetic products entails an especially high risk of exposure to various amounts of chemicals. Every day, on average, women use up to seven cosmetic and approximately 13 personal care products (Epstein & Fitzgerald 2009). Preparing one's self for a special occasion could mean exposing oneself to up to 475 chemicals hidden inside cosmetic products such as eye shadow, lipstick, perfume, nail varnish, fake tan, foundation, deodorant, blusher and hair sprays. Other toxins are hidden in candles and air fresheners that people use every day (Environmental Working Group (EWG) 2015).

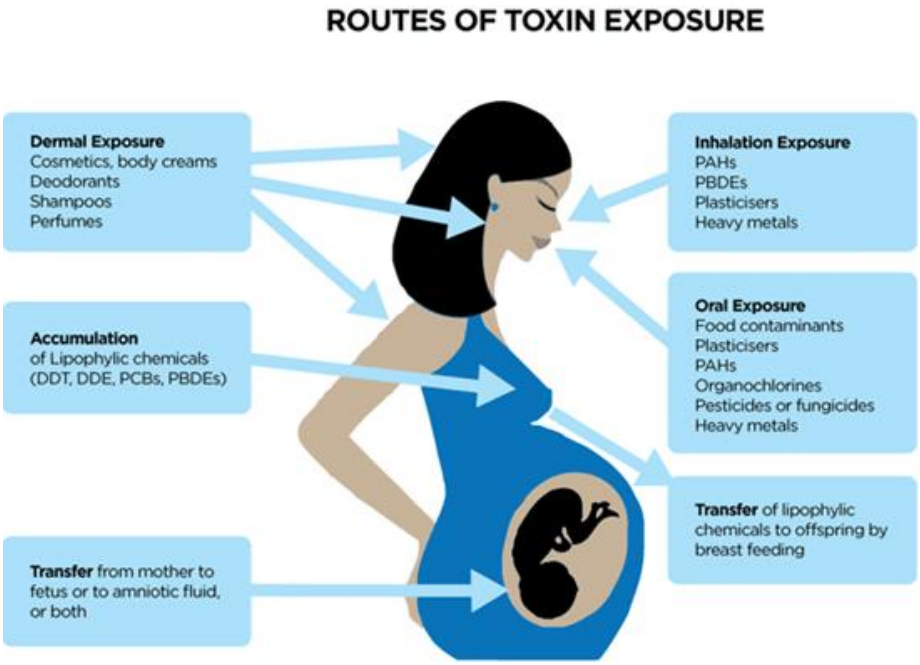

Figure 1.1: Everyday routes of toxin exposure in consumer products. Source: CosmeticsInfo (2015).

1.1.3 Routes of exposure

The route of exposure is that when a substance comes into contact with the body that determines how much of the substance enters and which organs are initially exposed to the highest concentration of the substance (Williams et al. 2015). This project used human cells to study toxicity. The main routes of exposure are ingestion (digestive tract), inhalation (respiratory tract) and dermal or skin contact which uses in this project (Gilbert 2004; Staskal, 2005). The consequences of a given dose are not determined by the dose response and route of exposure alone, but are strongly influenced by the following factors:

- Dose amount: A quantitative measure of the dose.
- Dose frequency: How often the exposure occurs (e.g., daily, weekly, biweekly or monthly).
- Dose duration: The length of the total period of dose exposure (e.g., 24 hours or one week).
- Individual characteristics: Natural traits such as age, sex, state of health and genetic background.
- Route exposure: How the person is exposed (e.g., through ingestion, inhalation or dermal contact) (see Figure 1.3).

1.1.3.1.1 Types of exposure

As shown in Figure 1.3, there are several different types of exposure. Acute exposure occurs when the subject is exposed to chemicals for 24 hours or less; subacute exposure occurs over a period of 1 month or less; chronic exposure occurs over a period of 3 months or less and subchronic exposure lasts for more than 3 months (Williams et al. 2015).

1.1.4 Absorption

1.1.4.1 Dermal exposure (skin contact)

This project uses human skin cells and therefore focuses on dermal exposure. The skin is the most common route for exposure to toxic substances from pharmaceuticals or consumer products that are intentionally applied (ATSDR 2009). As illustrated in Figure 1.4, the skin's structure is composed of three layers:

A) Epidermis: The rate of absorption can be determined by the stratum corneum, or the outermost layer of the epidermis. For example, malathion penetrates the stratum corneum, moves easily through the layers of the skin and is absorbed into the bloodstream at a high rate (ATSDR 2009). Conversely, the toxic pesticide dichlorodiphenyltrichloroethane (DDT) hardly penetrates the stratum corneum and has a slower rate of absorption (ATSDR 2009; Blickenstaff et al. 2014).

B) Dermis: This is the inner layer of the skin containing the hair follicles, sweat glands and oil glands. This layer plays a limited role in the absorption of toxic substances through the skin. See table 1.1 for an example of allergic contact dermatitis.

C) Subcutaneous tissue: This is the lowest layer of the integumentary system (Anderson 1994).

Dermal absorption of toxic substances can be affected by a variety of factors:

- Skin condition: A healthy stratum corneum (epidermis) protects against the absorption of some toxins. However, any physical damage to this layer, such as a cut, can easily shift toxic substances to the dermis and then to the bloodstream (ATSDR 2009). Healthy skin can also prevent the absorption of organic chemicals such as lead and mercury. As skin is impermeable to water, organic chemicals dissolved in water do not move easily through the skin. However, organic solvents such as gasoline are easily absorbed through the epidermis.

- Increasing the period of exposure to or concentration of the substance can increase the amount of absorption. Dermal toxicity can manifest as irritant dermatitis, allergic contact dermatitis, systemic contact dermatitis, photosensitivity, contact urticaria syndrome, toxic epidermal necrosis, acneiform dermatoses, pigment disturbances, ulcers and skin cancer (Williams et al. 2015). *In vitro* methods are important as a first step to calculate skin permeation and the potential of compounds or chemical mixtures to be directly toxic, such as to the skin, or systemically toxic. Two human skin cells models are used in this study: the human skin keratinocyte cell line (HaCaT), and the human normal fibroblast cell line (CCD-1064Sk).

1.1.4.1.1 HaCaT human skin cells

The HaCaT cell line grows as a monolayer and works as an adherent human epithelial skin cell line derived from histologically normal skin that maintains full epidermal differentiation capacity. It is in fact the outermost layer of the skin (Krejčí et al. 2014; Hughes & Edwards 2010; Schoop et al. 1999). The HaCaT used in this study was donated by a 62 year-old Caucasian male (CLS 2011). It is resistant to transformation *in vitro* (immortal > 140 passages), is similar to normal keratinocytes and is the first preeminent epithelial cell line from the skin cell that displays normal differentiation for examining the regulation of keratinization in normal human cells (Boukamp et al. 1988). Karyotyping revealed that this line is aneuploid, with unique chromosomes as stable markers indicating that it is monoclonal. The HaCaT cell can undergo spontaneous transformation of human adult keratinocytes after stress *in vitro.* This process is also associated with karyotype alternations (Boukamp et al. 1988).

1.1.4.1.2 Human fibroblast skin cells

Human dermal fibroblast cells CCD-1046 (CRL-2076, ATCC, USA) within the dermis layer of skin are responsible for generating connective tissue (Krejčí et al. 2014; Kopanska et al. 2013).

Table 1.1: Examples of contact allergens (Williams et al. 2015)

Source	Allergen(s)	Examples
Plants and trees	Rhus	Poison oak and ivy
Metals	Nickel and chromium	Earrings, coins and watches
Glues and bonding agents	Bisphenol A, formaldehyde and acrylic monomers	Glues, building materials and paints
Hygiene products and topical medications	Bacitracin, neomycin, benzalkonium chloride, lanolin, benzocaine and propylene glycol	Creams, shampoos and topical medications
Antiseptics	Chloramine, glutaraldehyde, thimerosal and mercurials	Betadine
Leather	Formaldehyde and glutaraldehyde	
Rubber products	Hydroquinone, diphenylguanidine and p-phenylenediamine	Rubber gloves and boots

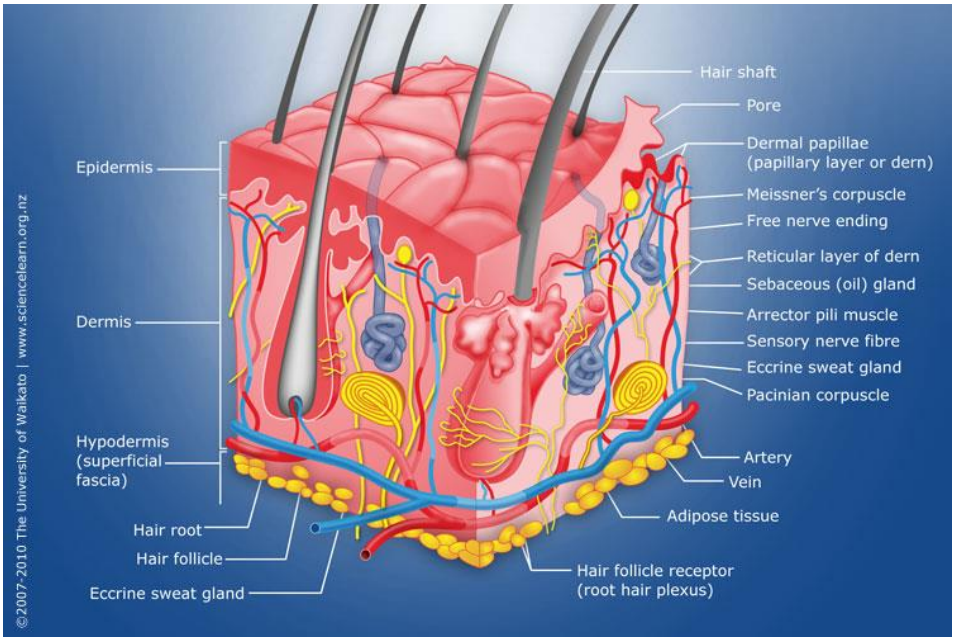

Figure 1.4: Human skin structure. Source: sciencelearn.org (2011).

1.1.4.2 Inhalation exposure (the respiratory tract)

Inhalation is the absorption of toxic agents through the respiratory tract after exposure to these agents (ATSDR 2009). The lining of the respiratory tract does not prevent toxic substances from entering the body (ATSDR 2009). The respiratory tract is composed of the upper airway, including the nasal passages, paranasal sinuses,

pharynx and larynx; and the lower airway, comprising the portion of the larynx below the vocal cords, trachea and lungs (Figure 1.5). The inhalation of toxic substances involves many factors, such as the concentration of toxic substances in the air, respiratory rate, solubility of substances in the blood and tissue, period and length of exposure, state of health of the respiratory tract and the size of toxic particles (ATSDR 2009; Zeliger 2008a).

1.1.4.3 Ingestion exposure (gastrointestinal tract)

The ingestion route of exposure can happen accidently when we eat. The digestive tract consists of the mouth, esophagus, stomach, and large and small intestines (Sherwood 2015). The absorption rate is affected by how long the food containing the toxic substance remains in the body. Factors that can affect the dose of exposure are as follows: the concentration of the chemical in the target organs, its chemical and physical form, its condition after absorption, and the period of time it remains in the tissue or organ. It is also important to know what happens to the chemical after it is absorbed into the bloodstream; for example, it can move from one organ to another (a process known as translocation), or change into a new compound form (known as biotransformation) (ATSDR 2009).

1.1.5 Distribution

After absorption, toxic substances are carried through the body by the blood into various organs (Williams et al. 2015). Barriers in the body, such as the blood brain barrier and placenta, can affect this movement. The chemicals bind to blood proteins in forms that depend on the form of chemical compound. However, the distribution of chemical mixtures results in one species that is then transported to one organ (Zeliger 2008a). The metabolism is another important factor in the transportation of toxic chemicals through the body; for example, xenobiotics can be transported to another organ when metabolised, as shown in Figure 1.6 (Zeliger 2008a).

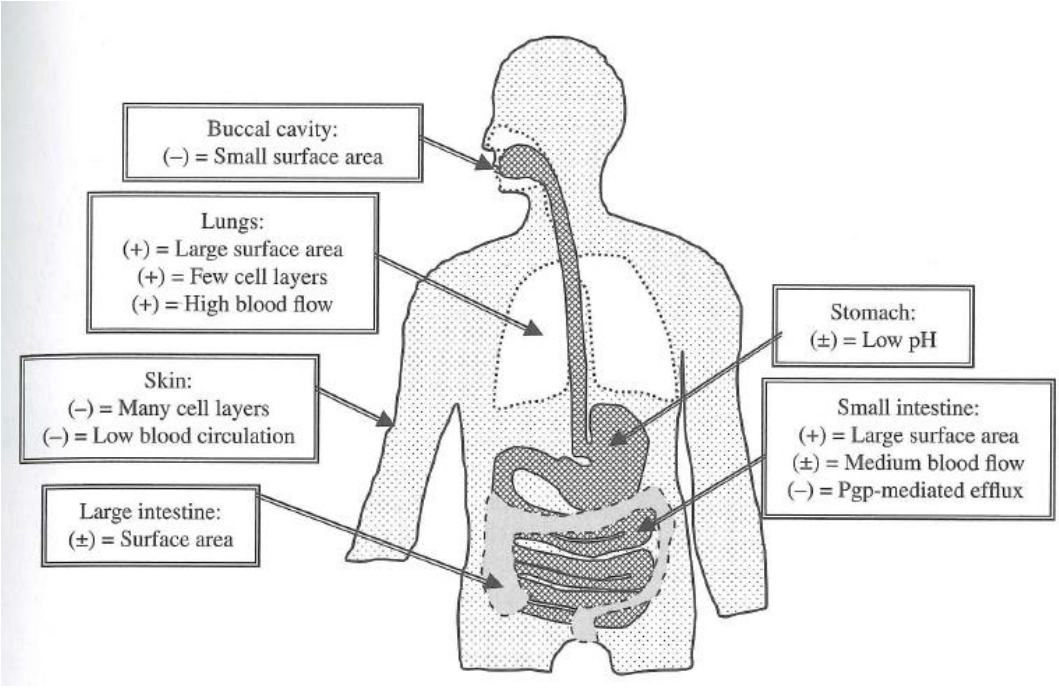

Figure 1.6: The main sites of xenobiotic absorption. Source: Williams et al. (2015).

1.1.6 Eliminating toxins from the body

Chemicals are eliminated from the human body through the urine, kidneys, lungs and liver. The kidneys eliminate more toxins than any other organ in the human body, the lungs eliminate gases such as carbon dioxide and the liver removes metals such as lead, or pesticide components such as DDT, by excreting them into the bile duct and moving them to the small intestine. Sweat glands, skin, hair, breast milk and the gastrointestinal tract are not important means of excreting toxins from the body (ATSDR 2009).

1.2 Classification of commercial products applied to human skin

1.2.1 Children's products

Children are exposed to chemicals before and after birth, both in the household and outdoors. They are more sensitive than adults to the impact of toxins because they have a lower phonological ability to detoxify carcinogens, and because their cells divide more than those of adults making them more likely to experience mutation (Epstein & Fitzgerald 2009; Zeliger 2008a). The most common chemicals applied to children's skin include lead, head lice treatments and skin care and beauty products, in addition to possible exposure to pesticides or herbicides in household agents, which often include various toxic ingredients also found in personal care products, as shown in Table 1.2 (Zeliger 2008a; Epstein & Fitzgerald 2009; Roberts et al. 2012). Children

should not be exposed to products containing titanium dioxide (TiO_2) because it is classified as a carcinogen, as documented in several animal studies noting its toxic effects in rats (Skocaj et al. 2011).

Table 1.2: Toxic ingredients in children's products (Epstein & Fitzgerald 2009; Winter 2009)

Ingredients	Toxic Effects
Benzyl alcohol	Allergen
Ceteareths	Contaminated with the carcinogens ethylene oxide and dioxane
Diazolidinyl urea	Precursor of the carcinogen formaldehyde
1,3-Dimethylol-5,5-dimethylhydantoin	Precursor of the carcinogen formaldehyde
Ethylenediaminetetraacetic acid	Hormone disrupter and penetration enhancer
Food dye and colouring red 40	Carcinogen
Lanolin	Allergen
Laureths	Contaminated with the carcinogens ethylene oxide and dioxane
Parabens	Hormone disrupters
Phthalates	Toxic group used in children's toys and products
Polyethylene	Contaminated with carcinogens
Quaternium-15	Precursor of the carcinogen formaldehyde
Sodium lauryl sulphate	Penetration enhancer
Talc (talcum powder)	Carcinogen and lung irritant
Triethanolamine	Precursor of the carcinogen nitrosamine (TEA)

1.2.2 Women's products

Women currently use cosmetics in vast quantities, often without considering the cellular consequences to their bodies, and as a result absorb the chemical ingredients found in such products through their skin. Approximately 90% of 14 year-old girls use make up regularly (Epstein & Fitzgerald 2009). The most common products used daily by 18 year-old women are toner, moisturiser, foundation, blush, eyeliner, eyeshadow, mascara, lipstick, lip gloss, perfume, hairspray, nail polish and personal care products, including deodorant, mouthwash, fragrances and soap (Thomas 2008). These are used daily and contain hundreds of chemical ingredients. These chemicals can be absorbed transferred to a fetus *in utero*. Ten blood samples from newborn American babies were analysed for 413 consumer products, and 287 chemicals were discovered in babies' blood samples including pollutants such as mercury, pesticides,

polycyclic aromatic hydrocarbons (PAHs) and PCBs (Environmental Working Group 2004).

1.2.3 Men's products

The most common consumer products used by men are deodorants and antiperspirants, shampoos and conditioners, shaving products and fragrances. These products contain many of the same chemicals as women's products.

1.2.4 Types of carcinogenic ingredients

The toxic effects of carcinogenic consumer products are classified into two types. The first type is frank carcinogens, which comprise up to 40 substances (such as acrylamide, acesulfame, aspartame, auramine, butadiene and butyl benzyl phthalate); these substances are labelled as carcinogens on product containers (Epstein & Fitzgerald 2009; Zeliger 2008a). The second type is that of hidden carcinogens; these include approximately 30 substances that are unlabelled in consumed products. They are not innately carcinogenic but can become so when they interact with other substances (see Tables 1.3, 1.4 and 1.5) (Epstein & Fitzgerald 2009). Hidden carcinogens have been classified into the following three groups (Epstein & Fitzgerald 2009):

A) Contaminant: non-carcinogens hidden in known carcinogens;
B) Formaldehyde: non-carcinogenic ingredients that release formaldehyde when they break down in products or the skin; and
C) Nitrosamines precursors: non-carcinogens react with nitrites in products to release nitrosamine.

Table 1.3: Summary of hidden carcinogenic ingredients (Epstein & Fitzgerald 2009)

Contaminated ingredients	Contaminated with
Acrylate and methacrylate polymers	Ethylhexyl acrylate
Amorphous silicate	Crystalline silica
Alcohol ethoxylates Laureths Oleths Polyethylene glycol (PEG) Polysorbates	Ethylene oxide and 1,4-dioxane
Butane	Butadiene
Coal tar dyes	Arsenic and lead
Glyoxal and polyoxymethylene urea	Formaldehyde
Lanolin	Organochlorine pesticides, PCBs and ceteareth
Petroleum	PAHs
Phenol ethoxylates Nonoxynols Octoxynols	Ethylene oxide and 1,4-dioxane
Polyacrylamide and polyquaternium	Acrylamide
Formaldehyde releasers	
Diazolidinyl urea DMDM-hydantoin Imidazolidinyl urea Metheneamine polyoxymethylene quaterniums Sodium hydroxymethylglycinate	
Nitrosamine precursors	
Bronopol (nitrite donor) cocamidopropyl betaine DEA and fatty acid condensates DEA sodium lauryl sulphate diethanolamine (DEA) Morpholine Padimate-O Quaterniums Sarcosine Triethanolamine (TEA)	

Table 1.4: Other toxic ingredients (Becker et al. 2010; Epstein & Fitzgerald 2009; Thomas 2008; Trouiller et al. 2009)

Ingredient	Toxic effects
TiO_2	Carcinogen
Tea tree oil (TTO)	Hormone disrupter
Formaldehyde	Carcinogen and allergen
Henna	Allergen
Lactic acid	Penetration enhancer
Lavender oil	Hormone disrupter and allergen
Lead	Carcinogen and hormone disrupter
Lead acetate	Carcinogen

Table 1.5: Common allergen products (Epstein & Fitzgerald 2009; Thomas 2008)

Product	Ingredients
Shampoo	Formaldehyde, fragrances, lanolin, solvents and surfactants
Hair dye	p-Phenylenediamine and p-toluenediamine
Waving solution	Ammonium thioglycolate and glyceryl thioglylcolate
Artificial nails	Methyl methacrylate
Nail base coat	Phenol formaldehyde resin
Nail varnish	Resins (aryl sulfonamide, formaldehyde and methyl methacrylate)
Nail hardener	Formaldehyde
Lipstick	Castor oil, colophony and pigments (e.g., eosin, azo dyes, carmine), perfumes, preservatives and propyl gallate)
Eyebrow pencil	Pigments
Eye shadow	Colophony, preservatives (e.g., parabens and triclosan) and pigments
Mascara	Colophony, preservatives (e.g., triclosan and parabens) and pigments
Deodorant	Fragrances (e.g., cinnamic salicylate, jasmine, methyl anisate and balsam of Peru)
Shaving products	Propylene glycol
Depilators	Thioglycolate
Toner	Arnica, coumarin, lanolin and oak moss
Face cream	Benzyl alcohol, lanolin, cetyl alcohol, parabens, propylene glycol and stearic acid

Sunscreen	Benzophenone-3 (oxybenzone), benzy l salicylate, coumarin and para-aminobenzoic acid

1.3 Types of hazards posed by commercial products

1.3.1 Sources of hazards

A free radical is any atom or molecule species with a single unpaired electron in an atomic orbital (Young & Woodside 2001b). Radicals are paramagnetic and highly reactive in nature. Therefore, they can donate or extract an electron from other molecules. As a result of this reactivity, the life expectancy of the majority of free radicals is about 10^{-10} seconds or less. Oxygen derivatives, particularly superoxide and hydroxyl radicals, are the most important radicals in many diseases. These free radicals occur in the body through various mechanisms involving both endogenous and exogenous sources such as pesticides or environmental factors. Superoxide (O_2^-) is produced via the addition of a single electron to oxygen (O_2) (Figure 1.7)

Various mechanisms can produce superoxide *in vivo*. A number of molecules, such as adrenaline, glucose, flavine nucleotides and thiol compounds, can be oxidized by oxygen producing superoxide. These reactions occur due to the phenomenon of transition metals, such as copper or iron.

The electron transport chain into the mitochondrial membrane reduces the amount of oxygen in water. As a result of these processes, free radicals are generated and some electrons also leak into the mitochondrial matrix due to the activity of various enzymes involved in the synthesis of adrenal hormones, such as cytochrome p450 oxidase in the liver.

Hydrogen peroxide (H_20_2) can be produced from any biological system such as superoxide dismutase and hydroxyl radical pathways that generates superoxide as a result of the reaction of a spontaneous dismutation. Further, glycolate oxidase and D-amino acid oxidase can be catalysed to produce H_2O_2 directly. H_2O_2 cannot be classified as a free radical by itself, but is usually included with a reactive oxygen species (ROS).

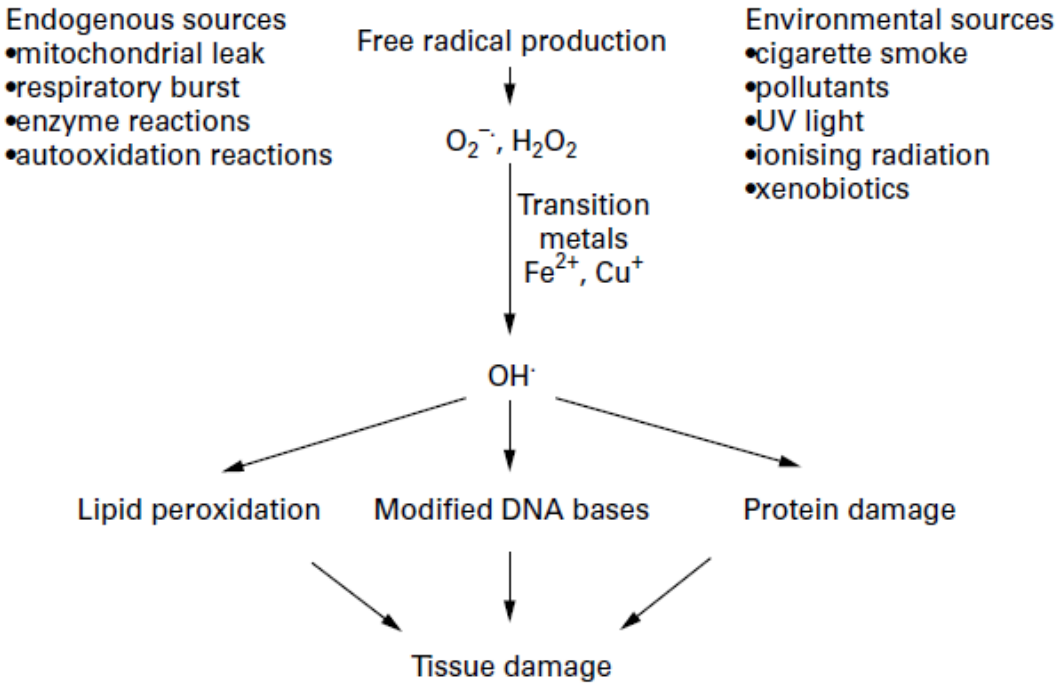

Figure 1.7: Main sources of free radicals in the body and the results of damage caused by free radicals. Source: Young and Woodside (2001a).

A weak oxidising agent may directly damage proteins and enzymes. H_2O_2 acts as a conduit that transmits free radicals, which in turn induce cell damage. H_2O_2 also works to generate a single oxygen and myeloperoxidase to kill bacteria with phagocyte. The hydroxyl radical (OH) is the final mediator that could cause tissue damage. However, a high rate of H_2O_2 formation is central to the pathological effects of the ROS. It is found at a high constant rate in living cells such as sugars, lipids, amino acids, lipids and nucleotides.

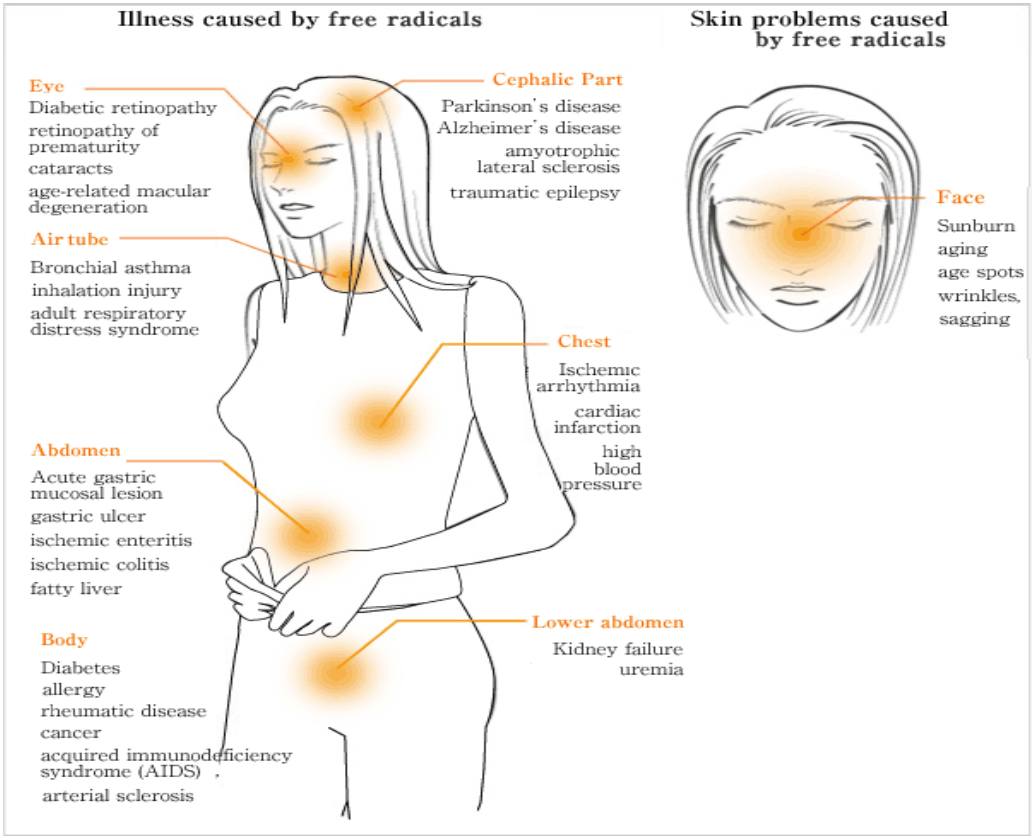

Figure 1.8: Summary of the illness caused by free radicals. Source: http://thedetoxdiva.com/what-are-free-radicals-and-why-should-you-care (2012).

As described in Figure 1.8, free radicals are formed as a consequence of endogenous reactions. Exogenous environmental factors can also promote the formation of free radicals that play an important role in the function of a normal cell. Ultraviolet (UV) light can lead to the formation of single oxygen (O) and other ROS in the skin. Other atmospheric pollutants, such as ozone and nitrogen peroxide, lead to radical formation and may cause respiratory diseases. Cigarette smoke also contains free radicals, as shown in Figure 1.9.

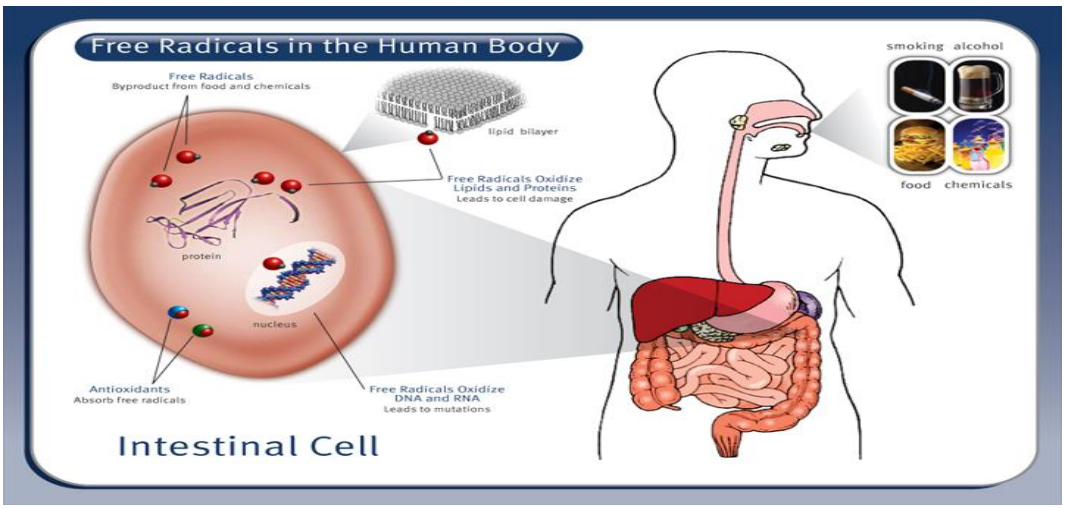

Figure 1.9: Free radical sources and the role of antioxidant defences system in protecting the human body. Source: http://humanphysiology2011.wikispaces.com/14.+Metabolism (2011).

1.3.2 Cell death

Exogenous agents induce a number of cellular effects, including cytotoxicity, necrosis, cell cycle arrest, apoptosis and the stimulation or inhibition of proliferation. Figure 1.10 depicts some of the processes that lead to toxicity, for example, altered metabolism, binding to plasma transport proteins, binding to a hormone receptor agonist or antagonist or the alteration of biosynthesis or metabolism. Exposure to environmental agents causes toxicity, leading to inflammation and cell death. Cell death is defined as being caused by three processes: apoptosis, necrosis and autophagy. Apoptosis is a normal program of cell death that protects cells from damage or infection and is a response to cell stress (Edinger & Thompson 2004; D.L 2002). For cell death to be classified as apoptotic, nuclear fragmentation and cleavage of chromosomal DNA into fragments of inter-nucleosomal into the apoptotic bodies must occur. It should also observed that there is no plasma membrane breakdown in apoptotic cells (Edinger & Thompson 2004).

In contrast, necrosis is a type of cell death that occurs accidentally when cells are exposed to noxious chemicals, biological agents or physical situations (Edinger & Thompson 2004). For cell death to be classified as necrosis there must be a breakdown of the plasma membrane, changes in the morphology of nuclear and an inflammatory reaction (White et al. 2015). Many morphological differences differentiate apoptosis from necrosis. For example, the two factors that will alter the process of an ongoing apoptotic process into a necrotic one are declines in the availability of caspases and of intracellular ATP' (Elmore 2007). In addition, whether cells are killed by necrosis or apoptosis depends in part on the nature of the cell death signal, the tissue type, the stage of development of the tissue and the physiological

background (Elmore 2007). Conversely, cell death classified as autophagic involves cells digesting themselves. This form of death differs from apoptosis and necrosis (Edinger & Thompson 2004). The characterization of autophagic is characterised by the sequestration of cytoplasm and organelles in the vesicles of double or multi-membrane and their delivery to the cells' own lysosomes for subsequent degradation (Elmore 2007). Figure 1.10 depicts the morphological differences between the classes of cell death.

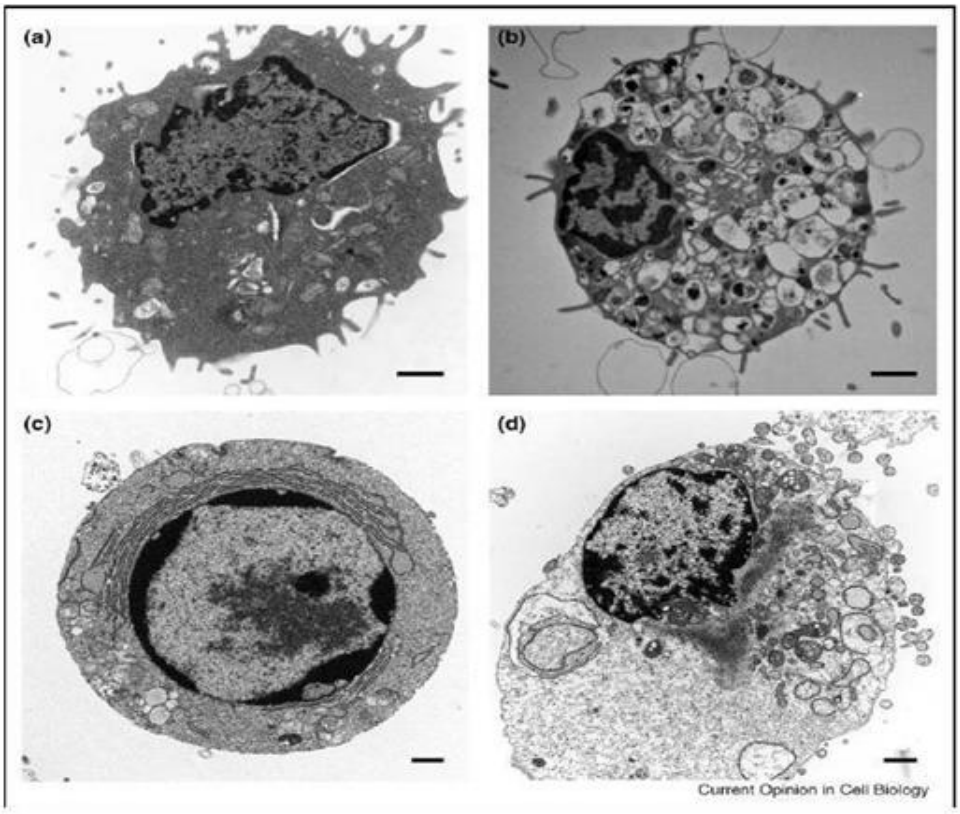

Figure 1.10: The morphological features of cell death classes: (a) normal, (b) autophagic, (c) apoptotic (d) and necrotic cells. The scale bar represents 1µm. Source: Edinger and Thompson (2004).

1.3.3 Genetic damage

1.3.3.1 Genetic damage to carcinogenesis and mutagenesis

DNA is genetic material endowed with distinct nucleotide sequences that carry hereditary information (Aidoo et al. 1997). Any alteration in these sequences resulting in deletions, base-pair substitution, insertions or frame shifts may lead to mutation (Aidoo et al. 1997). Mutations may arise from three significant sources: endogenous DNA damage, DNA replication and any exogenous agents (White et al. 2015). Mutation is important in the etiology of cancer, the aging process and the induction of human disease (Casciano et al. 1999).

Genotoxicity is defined as toxicity to DNA when exposed to chemicals or types of radiation that cause genetic changes and damage a person's DNA. Genetic changes may affect cells through carcinogenic or mutagenic changes. These changes can be classified as gene mutations, chromosome arrangements and numerical chromosome changes or gene amplification (Barrett 1993). Gene mutations, including point, missense and nonsense mutations, occur during DNA replication preceding meiosis (Lewis 1997). While cancer can represent heritable changes in genes, it can also result from exogenous agents, inducing somatic mutations. All other genetic diseases are caused by germ-line mutations. Individual cancer can occur as a result of a single mutation (Vogelstein & Kinzler 1993). Exposure to environmental agents leads to the formation of DNA adducts (Perera et al. 1992). There is a significant correlation between aromatic adducts on DNA and chromosomal mutations in that they confirm the molecular link between exposure to environmental agents and genetic conversion into cancer (Perera et al. 1992). Approximately 90% to 95% of human cancer occurs from exposure to carcinogen chemical agents (Figure 1.11). (Williams et al. 2015).

A carcinogen is an agent (chemicals, viruses, hormones or ionizing radiation) that causes neoplasia in multicellular organisms (Klaunig 2014). Carcinogenic agents can be genotoxic or non-genotoxic in terms of the damage that they cause. Genotoxic damage can result in DNA reactive or may affect gene expression, leading to an alteration (mutation) in genomic DNA (Klaunig 2014). Non-genotoxic damage can compromise the functioning of cell growth pathways. The two processes of mutation and carcinogenesis are linked to cancer, which results in DNA alteration (mutation) (Klaunig 2014) (Figure 1.12).

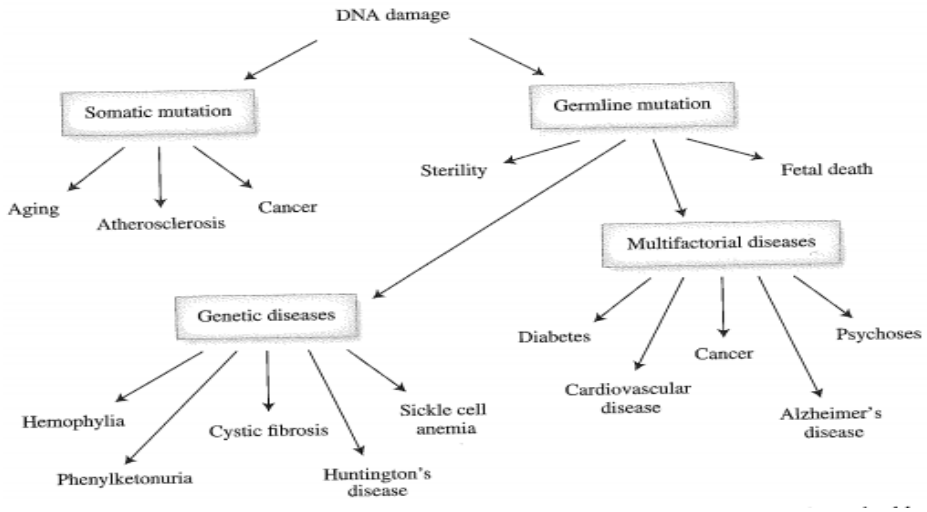

Figure 1.11: Mutation process and human health outcomes. Source: Williams et al. (2015).

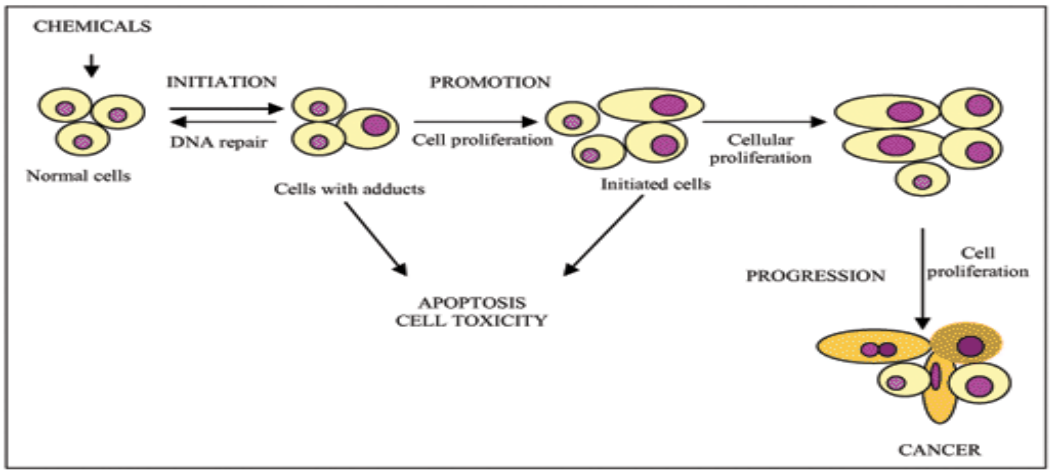

Figure 1.12 Multistage model of carcinogenesis. Source: Williams et al. (2015).

1.3.4 Other types of hazards posed by commercial products

Some commercial products have chemical ingredients that cause systemic effects (Thomas 2008). These chemicals are classified as follows:

- **Reproductive toxins:** Chemicals that affect fertility or cause impotence such as diethylene glycol dimethyl ether or alkylphenols (Thomas 2008; TOXNET 2015).
- **Teratogens:** This substance interferes with the development of an embryo when a pregnant women is exposed to this particular substance (Thomas 2008).
- **Neurotoxins:** These substances affect the nervous system, such as clostridium botulinum neurotoxin, aluminium or A-Terpineol (Thomas 2008; TOXNET 2015). Some of the diseases caused are associated with effects on the nervous system, such as Alzheimer's disease and Parkinson's disease (Thomas 2008).

1.4 Diseases associated with commercial products applied to the skin

1.4.1 Oxidative damage, stress and disease

Oxidative stress arises from an imbalance between free radicals and antioxidant defences, and is related to damage to a broad range of molecular species, including lipids, proteins and nucleic acids (Young & Woodside 2001b). Oxidative damage to proteins or nucleic acids could increase the variety of specific damage caused by products due to modifications in amino acids and nucleotides (de Zwart et al. 1999). It could also contribute to cellular dysfunction leading to the pathophysiology of a wide assortment of diseases. The role of oxidative stress can be observed in many

conditions, such as the aging process, cancers, atherosclerosis, inflammatory conditions, viral infections, trauma, cigarette smoke or environmental pollution (Ashok & Ali 1999; Frankel 2007; Hecht 1999; Rosenfeld 1998). However, oxidative stress occurs mainly due to environmental interactions and normal physiological processes where antioxidant defence systems work to protect the body against oxidative damage (Young & Woodside 2001b).

Physiological consequences of human skin aging

During the aging process there is little change in the number of layers in the structure of human skin, namely the stratum corneum, epidermis, dermis, subcutaneous tissue, eccrine sweat, apocrine sweat, sebaceous glands and hair (Figure 1.15). Changes do occur in the moisture content of the skin of elderly people compared to that of younger adults, specifically in the stratum corneum layer (Balin & Pratt 1989; Potts et al. 1984). As a result, aged skin has a brittle stratum corneum, and even the corneocytes are less cohesive. The epidermis of older people takes more time to be renewed than does that of young adults due to the turnover of the stratum corneum (Balin & Pratt 1989). In addition, aged skin observed with changes in the stratum corneum include larger cell size, increased turnover time, a decline in cellular cohesion and reduction in moisture content as shown in Figure 1.16 (Balin & Pratt 1989). Some studies have shown that the average replacement time for volar skin is approximately 20 days for women aged 83–91 years old and around 13 days in women aged 21–40 (Baker & Blair 1968; Baker & Kligman 1967). As a consequence, medications take a long time to shed, and this increases the treatment time for fungal infections due to the slower rate at which the stratum corneum is renewed (Balin & Pratt 1989).

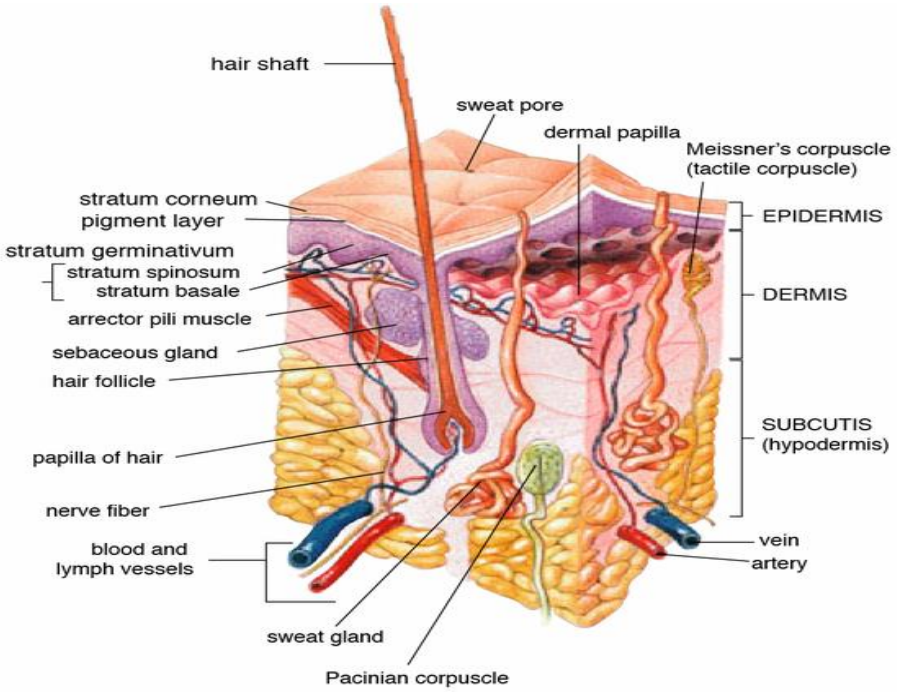

Figure 1.15: The basic structure of human skin. Source: http://thecaribbeancurrent.com/ maintaining-my-beautiful-skin (2012).

Epidermal changes with age include a decrease in the contact area between the dermis and epidermis, a decrease in Langerhans cells and a decline in sun-damaged skin and melanocytes (Balin & Pratt 1989). One study shows that sun-protected skin increases the number of cells in young adults by approximately 10 epidermal Langerhans cells/1µm, but these decreased in older adults to 5.8 cells/1µm. The decline in Langerhans cells contributes to the skin's reduced ability to sensitise with contact allergens (Balin & Pratt 1989). Otherwise, no change is observed in the rate of epidermal renewal, as shown in Table 1.6.

Table 1.6: The relationship between chronological age and epidermal turnover time (Balin & Pratt 1989a)

Age (Years)	Stratum Corneum Transit Time (Days)	Replacement Rate (Layers/Day)	Replacement Rate (Cells/mm^2/Day)
20–25	19 ± 3 (10)	0.89 ± 0.11 (10)	952 ± 154 (10)
40–45	18 ± 2 (10)	0.90 ± 0.12 (10)	939 ± 227 (10)
60–65	28 ± 7 (10)	0.62 ± 0.12 (10)	560 ± 177 (10)

Aging causes anatomical changes in the dermis, such as a decreased amount of collagen and glycosaminoglycans; alteration and loss of elastic tissue; decrease in fibroblasts, macrophages and mast cells; a decrease in the contact area between the dermis and epidermis and a loss of vasculature, which becomes thinner. Also the skin also becomes more easily damaged, responds less effectively to inflammation and is

unable to protect against UV light. Wrinkled skin and decreased sensitivity to pain become evident (Balin & Pratt 1989; Black 1969). One *in vivo* study demonstrated a decline in the thickness of forearm skin in men from 1.3mm at age 30 to 0.9mm by age 80 (Balin & Pratt 1989; Black 1969). Collagen decreases year by year and the remaining amount of collagen becomes less soluble and has less capacity and resistance to collagenase (Shuster et al.1975). Studies indicated that men have a thicker dermis than women with men's forearm skin measuring 1.3 mm on average while in women this measurement is 1.1 mm (Balin & Pratt 1989). This explains the controversy with regard to why women's skin deteriorates more with aging than men's skin, as thinner skin is more easily damaged by exposure or trauma. Table 1.7 illustrates the changes that occur to elastic fibres with intrinsic aging, including the loss of subepidermal oxytalan fibres, which leads to superficial laxity and wrinkled skin.

Table 1.7: Changes in elastic fibres with intrinsic aging (Balin & Pratt 1989)

Age	**Changes in elastic fibres with intrinsically aged skin**
30–50 years old	Loss of subepidermal oxytalan fibres Abnormal elaunin and elastic fibres
50–70 years old	Papillary dermis loses oxytalan fibres Abnormal elaunin and elastic fibres Collagen bundles have less density Beginning of cystic spaces within the elastic matrix
70 years old	Larger cystic spaces within the elastic matrix tan with lacunae separating microfibrils Thinner elastin core

Balin and Pratt (1989) demonstrated the changes of photo-aging, including an increase in the amount of elastic fibres. These changes are classified as either early or later changes and are documented in Table 1.8.

Table 1.8: Photo-aging and elastic fibre changes with aging (Balin & Pratt 1989)

Age	Early changes	Later changes
During the aging process	The fibre becomes separated from the epidermis by a narrow band of normal dermis.	The fibres become thicker and tangled. The size of microfiberillar masses also changes.
	The fibre becomes thicker or thinner and coiled.	Absence of inflammation and quiescent fibroblasts
	Hyperplasia and degeneration of fibres occurs in the reticular.	The number of diffuse clumps of amorphous and granular elastotic material increases.

The number of mast cells in the dermis also decreases with age, and the vessels decline in size, particularly the cutaneous appendages (Balin & Pratt 1989). Studies in cutaneous aging illustrate that vessels in intrinsically aged sun-protected skin become thinner with age, and the number of surrounding veil cells declines (Braverman et al. 1986; Braverman & Fonferko 1982). Age-related changes in vasculature could lead to a lower inflammatory response, a decline in clearance, absorption, sweating and urticarial reactions. Further, delays in wound healing, ecchymosis and delayed resolution and decreased thermal regulation become evident as a person ages (Balin & Pratt 1989; Marín 1995).

Moreover, as a result of aging the number of sweat glands such as eccrine and apocrine sweat glands decreases. Specifically, a decrease in apocrine appears as a sequencing of age-associated decrease in testosterone levels, which in turn is related to less body odour. This is why the need for antiperspirants decreases in the elderly (Balin & Pratt 1989). Sebaceous glands increase in size in the elderly to approximately 0.40 mm^2 from.22 mm^2 in young adults because these are endogen dependent (Plewig & Kligman 1978). Transmission time increases in an elderly person to within six days longer than that in a younger person (Balin & Pratt 1989).

Hair is also affected by age. There is a decline in the rate of growth of scalp hair as a person gets older. For example, women older than 65 have more hair on the lip and chin, but experience a decline in head hair. On the other hand, men often lose their scalp and beard hair, but experience an increased rate of growth of the hair on their ears and eyebrows (Balin & Pratt 1989). Even nail growth is influenced by age, which could explain the prolonged treatment for fungal disease in the elderly. Nails become brittle and lustreless, and are characterised by longitudinal striations (Balin & Pratt 1989; Saxon et al. 2009). Finally, the aging process involves a decrease in cutaneous

physiological functions such as pain perception, vitamin D synthesis, photo protection and alteration and immune surveillance and antigen presentation (Balin & Pratt 1989; Saxon et al. 2009).

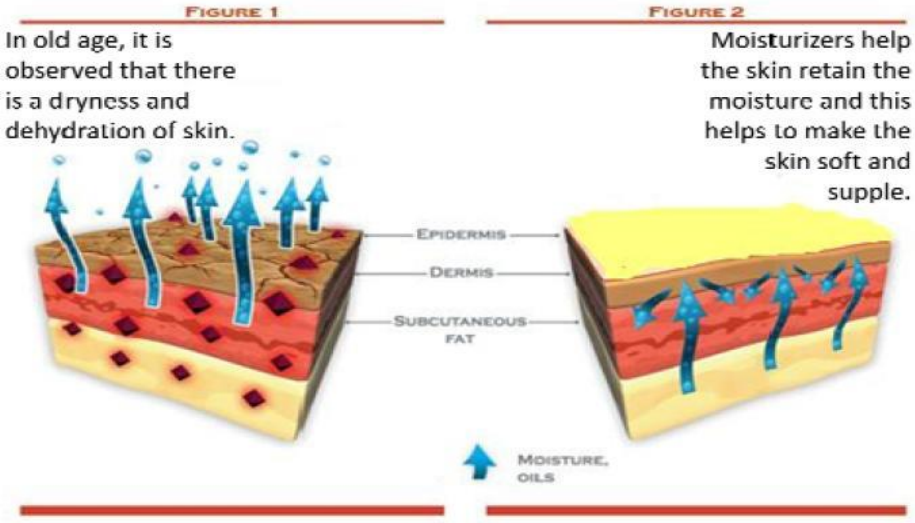

Figure 1.16: How moisturizers prevent water loss from the skin's surface. Source: Malvi (2011).

1.4.2 Skin diseases associated with the use of cosmetic products

A clinical and laboratory study indicated the consequences of long-term use of beauty lightening products on human skin (Mahé et al. 2003). Table 1.9 describes 425 female consumers' lightening products used in this study with their active ingredients, and summarise the results of using these products, which include a variety of skin diseases. As described in Tables 1.9 and 1.10, the application of one of these beauty products to the skin resulted in a dermatophyte infection in 75% of the women, as shown in Figure 1.17; 58% of woman were diagnosed with scabies as shown in Figure 1.18 and three of four woman developed tinea versicolor on the inner thighs, as seen in Figure 1.19. When first using the beauty products listed in Table 1.9, approximately half of consumers were observed to have acne; 29% of consumers were later diagnosed with inflammatory acne

Table 1.9: List of lightening products used by 425 women involved in this study (Mahé et al. 2003)

Active substance	No. of users (%)	Branded products (type of product)
Hydroquinone	378 (89%)	Skin Light (M, C, S), Niuma (M), Top-tone (C), Sivoclair (M, C), Fair White (M, C), Peau Claire (M, C), MGC (M), Akagni (C, M), Immediat Clair (M, C), Clairliss (M), Black Star (M), HT 26a (M), CBLa (M), Body Clear (M, C), Emos (C)
Glucocorticoids	297 (70%)	Tenovateb (C, G), Neoprosonec,d (G), Niuma Extra Cream (C), Movateb (C), PCb (C, G), Lumie`reb (C), Neomatb,d (C, G), Topgeld (G), Dermovateb (C, G), Clovateb (C), Maximc (G), Prosonec (G), Dianab (C), Civicb (C, G), Fashion Fairb (C)
Mercury salts	43 (10%)	Niuma (S), Rico (S), Sukisa Bango (S), Idole (S), Jaribu (S), Movate (S)
Caustic agents	72 (17%)	Liquid soaps, hydrogen peroxide, salicylic preparations (10–30% in Vaseline and Sivoderm)
Unknown	55 (13%)	Si Claire (M, C), Shirley (C), Asepso (S), Ide´al (C)

Table 1.10: Types of skin diseases observed in consumers after using lightening products (Mahé et al. 2003)

Skin disease	Skin diseases motivating visits	Dermatoses/non-motivating visits/ found by systematic examination	Total % of all users
Mycoses	105	23	
Dermatophyte infection	17	6	128 (30%)
Candidiasis and nonspecific intertrigo			23
Tinea versicolor	4		4
Scabies	69		69
Bacterial skin infection	14	8	22
Other infectious diseases	11		11
Acne	42	81	123 (29%)
Perioral dermatitis	2		2
Eczema	41	2	43
Irritant dermatitis	14	-	14
Xerosis⁄ichthyosis	6	16	22
Isolated itching	11	4	15
Dyschromia			
Hyperchromic	8	146	154 (36%)
Hypochromic	4	14	18
Exogenous ochronosis	14	12	26

Skin disease	Skin diseases motivating visits	Dermatoses/non-motivating visits/ found by systematic examination	Total % of all users
Pigmenting keratosis pilaris	3	3	6
Striae	2	167	169 (40%)
Poikilodermac	1	23	24
Blue ear	-	25	25
Facial hypertrichosis	-	43	43
Others	80	12	92

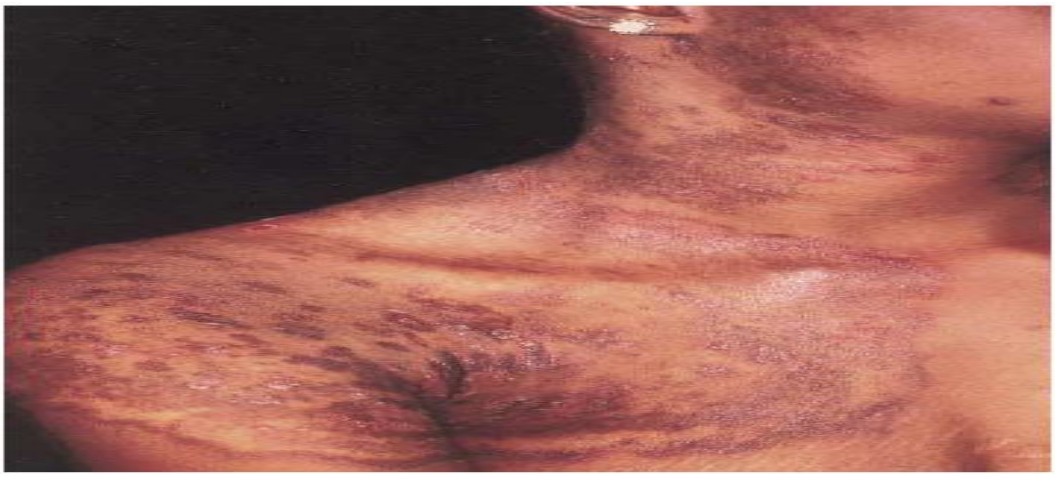

Figure 1.17: A patient diagnosed with Inflamed widespread tinea corporis. Source: Mahé et al. (2003).

Figure 1.18: A patient examined with scabies. Source: Mahé et al. (2003).

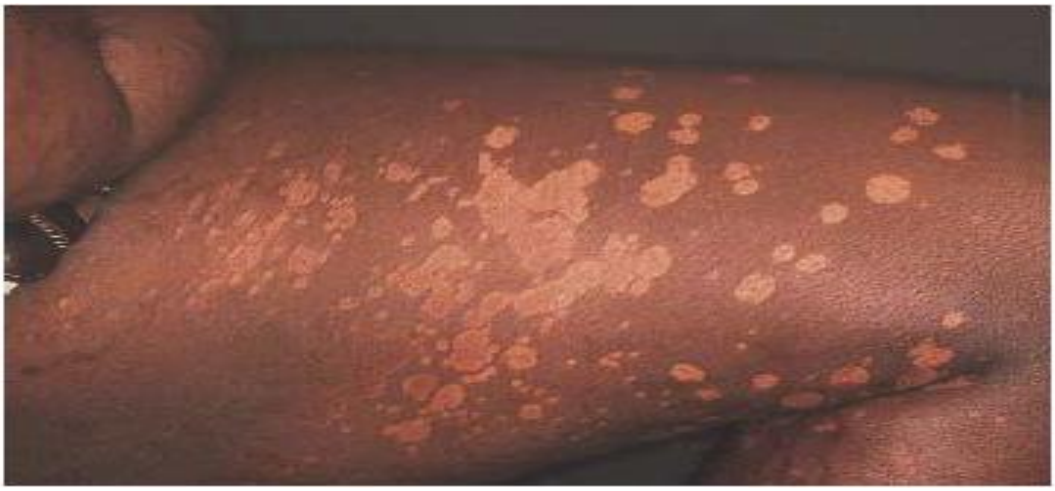

Figure 1.19: A Patient with achromic tinea versicolor. Source: Mahé et al. (2003).

1.4.3 Cancer

Cancer is defined as a group of diseases in which some of the cells in the body become abnormal and out of control, with a mass formed called a neoplasm. It then spreads through the body via the bloodstream or lymphatic system, reaches other organs and damages the tissues around them, as shown in Figure 1.24. When the spread of these cells is uncontrolled, this can lead to death. However, not all tumours result in death as there are benign tumours which do not spread to other organs in the body and rarely lead to death. As cancer cells can develop from different types of cells, tissues and organs, they are classified depending on the part of the body that they develop in.

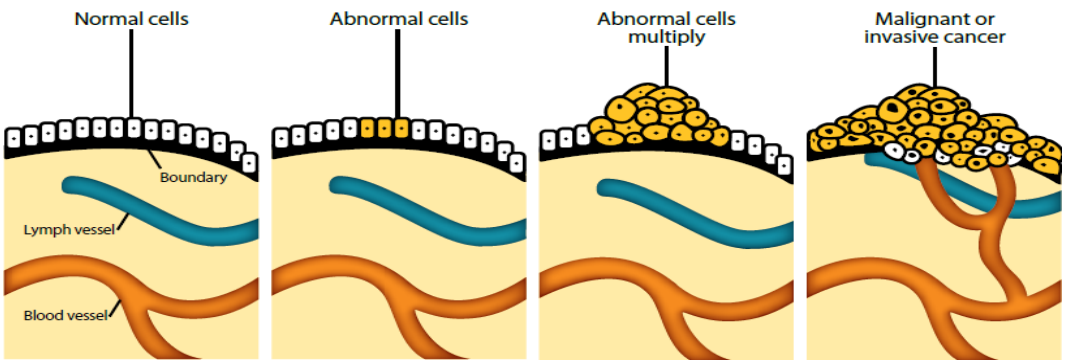

Figure 1.24: The beginning of cancer. Source: Cancer in Australia in brief (2010).

The causes of cancers are not fully clear. However, some recognised causes are biomedical, lifestyle and environmental factors, as shown in Table 1.11. In Australia more than 108,368 cases of cancer were diagnosed in 2007. Up to 60% of these cases are diagnosed in males. The average age at diagnosis before the age of 70 is 67 for men and 64 for women. The risk of being diagnosed with cancer in the age of 75 -85 years is 1in 3 in males and 1 in 4 in females (Australian Institute of Health and Welfare 2010). Cancer rates differ with age and between the sexes. The 2007 report about cancer in Australia, which indicates age-specific incidence rates for all cancers, is summarised in Figures 1.25 and 1.26.

Table 1.11: The main risk factors of cancer (Australian Institute of Health and Welfare 2010)

Biomedical factors	Lifestyle factors	Environmental factors
Genetic susceptibility, (e.g., breast cancer)	Smoking (e.g., cancer of the lungs, leukaemia, liver, pancreas and cervix)	Sunlight (e.g., melanoma and non-melanoma skin cancer)
Hormonal factors in females (e.g., breast and endometrial cancer	Alcohol consumption (e.g., cancer of the oral cavity, pharynx, larynx, oesophagus, liver, bowel and breast [in females])	Radiation (e.g., leukaemia and breast and thyroid cancer)
	Physical inactivity and obesity (e.g., cancer of the kidney, oesophagus, colon [males only], and breast and endometrium [females only])	Occupational exposure, e.g., mesothelioma and nasal cavity cancer
	Chronic infections (e.g., cancers of the liver and cervix)	Pollution [e.g., cancer of the skin, lungs and bladder]
	Diet (e.g., bowel and prostate)	

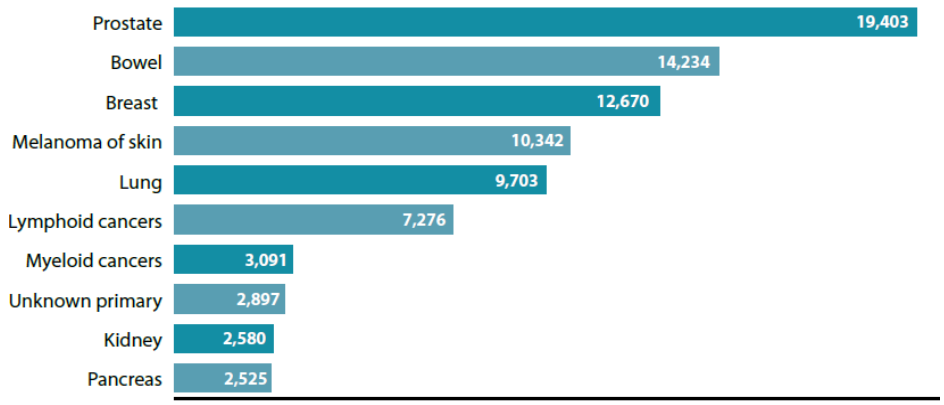

Figure 1.25: The most 10 common cancers incident in Australia in 2007. Source: Australian Institute of Health and Welfare (2010).

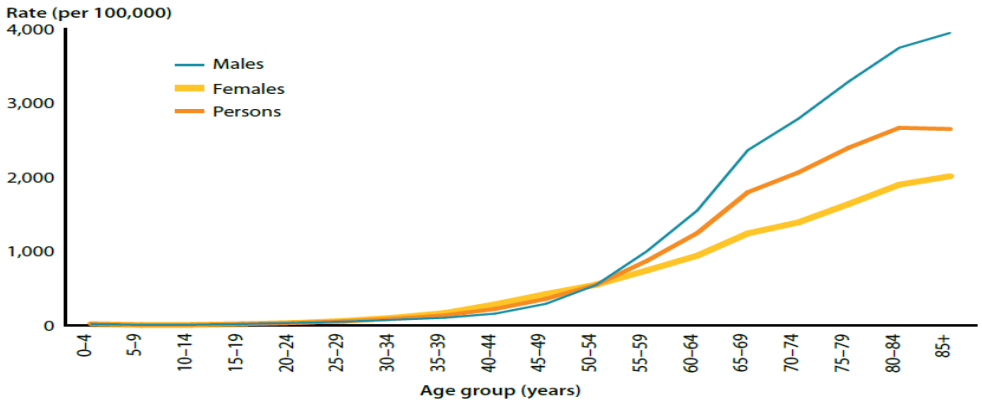

Figure 1.26: The rate of cancer according to age and between the sexes in Australia in 2007. Source: Australian Institute of Health and Welfare (2010).

Which form of cancer led to the most deaths in 2007 is currently under discussion. The 2007 report illustrating the most common causes of cancer deaths is summarised in Figure 1.27.

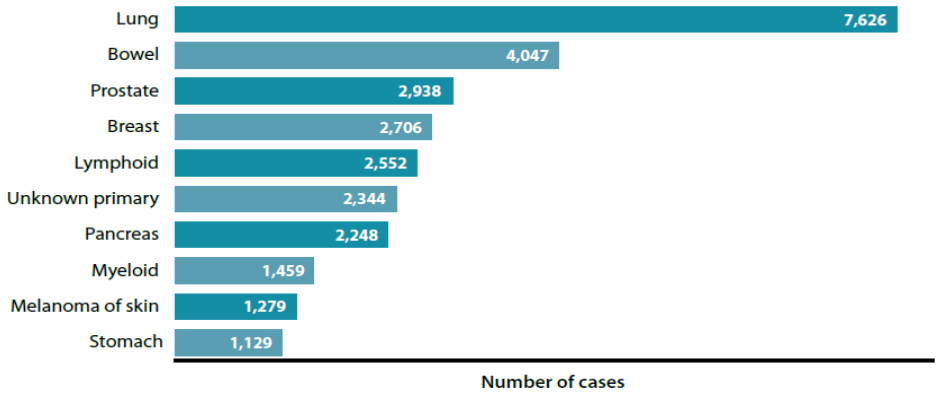

Figure 1.27: The most common causes cancer of deaths in Australia in 2007. Source: Australian Institute of Health and Welfare (2010).

Recent research has considered cancer a genetic disease (Go et al. 2003), because exposure to environmental agents can result in tumour cells classified as multiple genetic defects (Go et al. 2003). Oxidative damage to cells also leads to cancer; therefore, people who over-generate ROS have a high risk for cancer (Salganik 2001). Antioxidant defences can protect cell structures in our body from oxidative damage and thus discourage the growth of cancer. Indeed, a relationship exists between apoptosis and cancer. First of all, mitochondria play an important role in apoptosis. Anti-cancer cells and radiation kill cancer cells by inducing apoptosis (Labriola & Livingston 1999; Salganik 2001). However cancer cells may be resistant to apoptosis due to mutations in the p53 gene (Blackstone & Green 1999). A number of studies have revealed antioxidants can interfere with the therapeutic activity of anti-cancer drugs, and this may be because the pivotal role of ROS is triggering apoptosis, and antioxidants have ability to inhibit the protective mechanism of killing cancer cells

through apoptosis by depleting ROS (Labriola & Livingston 1999; Salganik 2001). The main causes of cancer involve genetic changes that are exacerbated by exposure to exogenous agents. Most cancers may occur due to exposure to environmental agents (Venitt 1994). Cancer essentially is a genetic disease occurring when environmental agents change the genes that regulate the mechanism of how cells divide. Cigarette smoking is responsible for more than 30% of all cancer deaths and an estimated 80% of lung cancer deaths (Fauci et a. 2008).

1.4.3.1 Skin cancer

Skin cancer is the most common cancer diagnosed in Western countries, with half a million new cases reported every year in the United States alone (Diepgen et al. 2012; Williams et al. 2015). UV light is the main cause of skin cancer, but chemical agents may also be to blame as they can cause skin cancer by altering the DNA of epidermal cells (Williams et al. 2015). These alterations can lead to interaction with genetic material and cause genetic damage (genotoxicity), chronic toxicity and/or cell death (cytotoxicity), ultimately leading to a cancerous lesion (Williams et al. 2015; Wurgler & Kramers 1992). One example of chemicals that induce cancer in or on the skin is PAHs, which are found in high concentrations in coal tar, creosote, pitch and soot (Siddens et al. 2012). The PAHs mechanism that causes DNA damage is bioactivation within the skin, often to a reactive epoxide, by cytochrome P450 metabolism (Siddens et al. 2012; Williams et al. 2015). Several chemicals can damage DNA, but to cause skin cancer the mechanism of genetic damage for these chemicals must be DNA damage to a lesion that in turn leads to a cancerous lesion.

1.4.3.2 Epidemiology and clinical studies

Little research has been done on the toxicity and genotoxicity of consumer products, such as commercially available mixtures. The current study investigated possible hazardous cellular side effects resulting from the use of personal care products. A study indicated a relationship between beauty product-related exposures and childhood brain tumours in seven different countries (Efird et al. 2005). Data were collected from 1218 cases of childhood brain tumours that were diagnosed between 1976 and 1994 in Milan, Italy; Valencia, Spain; Paris, France; Sydney, Australia; Winnipeg, Canada and Los Angeles, Seattle and San Francisco in the United States. 2223 matched the control of the analysis of exposure to beauty products and the workplace. The risk of a child developing a childhood brain tumour was 50% higher among children with mothers who used or were exposed to beauty products five years before giving birth compared to children of mothers never exposed to beauty products

(Efird et al. 2005). This study concluded that exposure to beauty products increased the risk of childhood brain tumours.

Another study found mercury toxicity associated with a beauty lotion in Mexico. 'Crema de Belleza' is a beauty lotion purchasable from pharmacies in Mexico (Balluz et al. 1997). Mercury is listed as one of its key ingredients. Data were collected from 25 participants (five women who were current users and 20 non-users) who contributed urine samples that were then analysed for mercury and creatinine (Balluz et al. 1997). The results of the urine samples ranged from 7.9 µg/L to 32.8 µg/L, with a median of 18.2 µg/L for current users, versus 0.70 µg/L to 23.20 µg/L with a median of 4.3 µg/L for non-users. The median urine mercury count for current users was four times higher than that of non-users (95% CI for difference in median is 3.6 to 26.6). Eight percent of current users showed symptoms of mercury toxicity, as shown in Table 1.12.

Table 1.12: The toxicity symptoms of mercury exposure from the use of 'Crema de Belleza' beauty lotion (Balluz et al. 1997)

Symptoms	Frequency in Users	%	Frequency in Non-Users	%	P Value
Fatigue	4	80	1	5	0.005
Nervousness	2	40	3	15	0.004
Memory loss	2	40	0	0	0.004
Weakness	2	40	2	10	0.004
Vision changes	2	40	2	10	0.004
Sore gums	2	40	1	5	0.004

In 1999, many consumers suffered from neurological and respiratory symptoms after using a perfume called Eternity sold by Calvin Klein. An environmental and health network run by California's government analysed the ingredients of this product and found 41 different toxic ingredients with various concentrations of 0.1%–12%, including known chemicals caused carcinogen, allergen and other harmful effects to humans. Many other different chemicals that were analysed were of unknown toxicity or have not yet investigated (Epstein & Fitzgerald 2009).

In 2007, an environmental working group investigated 400 cosmetics products containing unsafe ingredients being sold in the United States (EWG 2007). A North American dermatitis contact group survey found that the percentage of cosmetic allergies from 1994 to 1996 increased from 11% to 14% (EWG 2007). In addition, a 2007 consumer report that tested eight randomly selected perfumes found in each

one hormone-disruptive phthalates di-n-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) (Epstein & Fitzgerald 2009). Previously, in 1986, in the United States, the Congress nation academy reported the ingredients of several fragrances as neurotoxins (Epstein & Fitzgerald 2009). In 1992, a study indicated that the use of talcum powder correlated with ovarian cancer in women who applied talcum powder to their genital area (Harlow et al. 1992).

A recent epidemiology study indicated the possible sources of infant phthalate exposure from baby body care products (Sathyanarayana et al. 2008). This study measured nine phthalate metabolites in 163 infants born in 2000–2005. Most of the infants examined (up to 80%) tested positive for more than seven phthalate metabolites that were above the limit. The following baby care products were used in this study; baby lotion, baby shampoo, baby powder, destin/diaper cream and baby wipes. This study also found a relationship between the concentration of phthalates and the number of products used.

1.4.3.3 Previous in vitro and animal studies

Few clinical trials have been published on exposure to commercial products. Also, limited *in vitro* studies have investigated exposure to chemical mixtures based on human cells. Some studies have looked at the toxicity and genotoxicity of individual ingredients such as the nanoparticle TiO_2 used in the NVAW product examined in this study. TiO_2 can cause cytotoxicity and genotoxicity in human cells (Wang et al. 2007), and has been reclassified as a carcinogen by the IARC as it can damage DNA (Trouiller et al. 2009). It is also classified as a carcinogen ingredient used in cosmetics (Epstein & Fitzgerald 2009). EWG's Skin Deep (2011) also classified TiO_2 as a toxic substance, harmful and a medium human health priority. Animal studies indicate that it causes significant irritation to skin, has damaged and irradiated pig skin and caused lung tumours in rats after long-term exposure (Shi et al. 2013). As animal testing for cosmetic ingredients is not allowed by EU cosmetic regulations (Steiling 2016), *in vitro* studies are the first step to investigate the toxicity of the new ingredients, but they have limitations. Therefore, cosmetic toxicologist requested to contribute to data sources for the best safety assessment as illustrate in Figure 1.28.

EWG's Skin Deep (2011) reviewed a cosmetics database and showed that glycerol is a safe product, finding only limited evidence of its renal toxicity. One animal study investigated the effects of consumer product ingredients such as nanoparticles or permethrin in rats and cats (Dymond & Swift 2008; Shi et al. 2013). The FDA and the Act subject cosmetics to FDA premarket approval but do not specifically require the

use of animals in testing cosmetics for safety. The FDA requires cosmetic manufacturers to use appropriate and effective testing to assure the safety of their products (FDA 2006). The manufacturer is responsible for the safety of both the ingredients used and the final formulated cosmetic products in the mixture (FDA 2006).

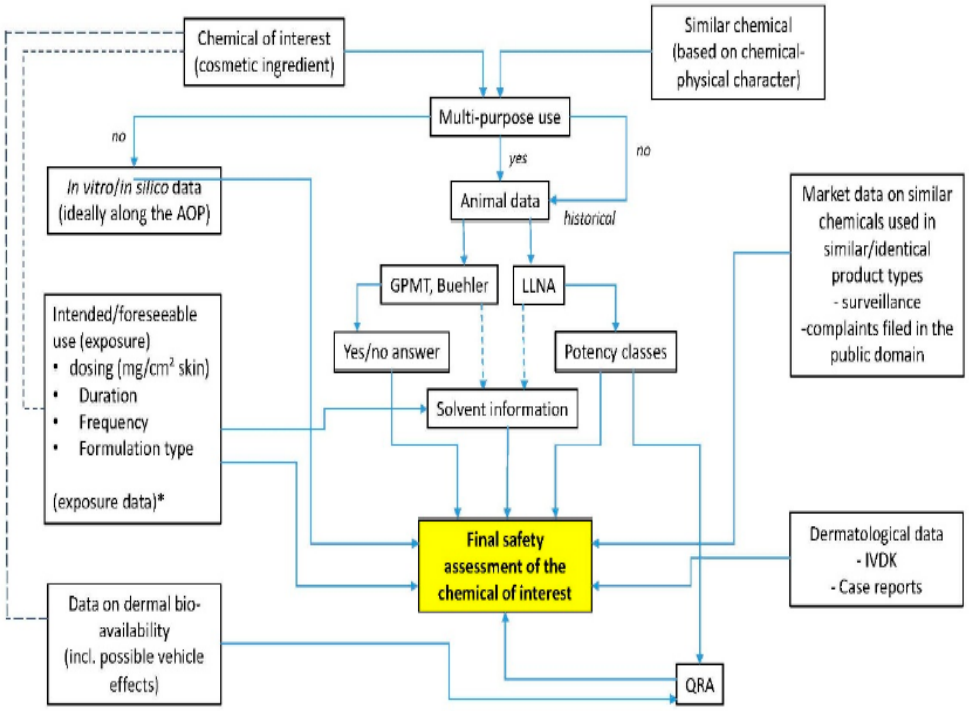

Figure 1.28: Data sourcing for a best safety assessment. Source: Steiling (2016).

1.5 Protection against cellular hazards

1.5.1 Antioxidants

An antioxidant is a substance that is able to inhibit or delay the oxidation of an oxidisable substrate, even at low concentrations (Young & Woodside 2001a). Free radicals are controlled naturally by several beneficial compounds known as antioxidants, which are very important for maintaining optimal cellular and systemic health (Percival 1998). In addition, the production of free radicals occurs constantly in all normal cells as a part of cellular function (Young & Woodside 2001b). Antioxidants work to prevent free radical-induced cellular damage by preventing the formation of radicals or scavenging them (Figure 1.29).

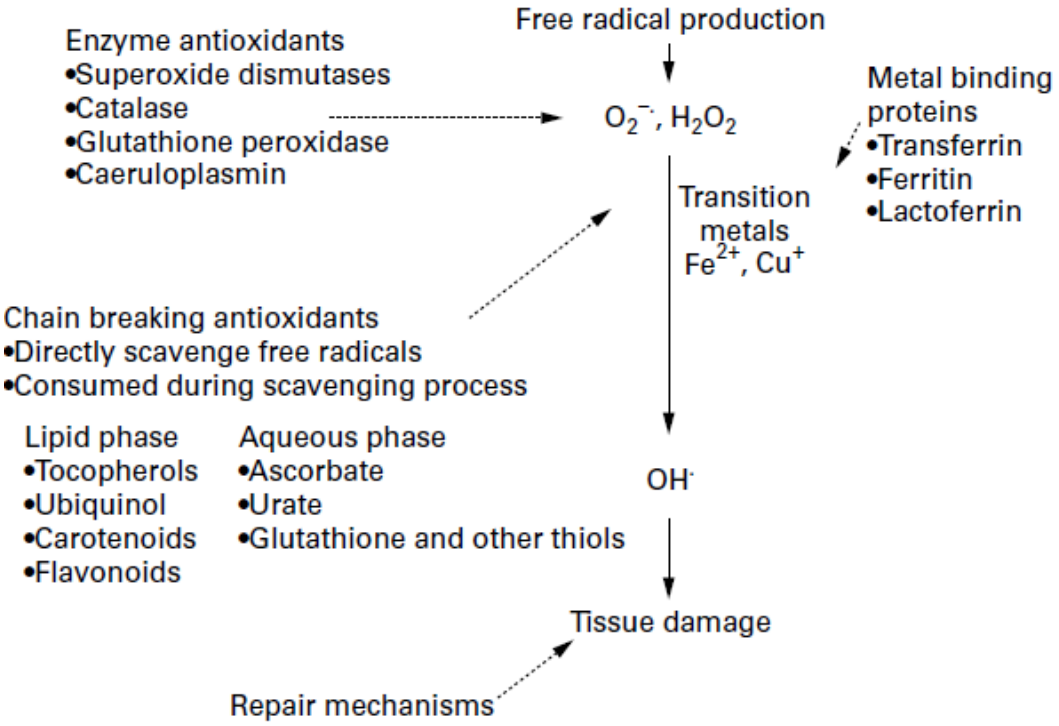

Figure 1.29: Antioxidants' defences against attack by free radicals. Source: Young and Woodside (2001b).

Many studies have examined the chemical nature of fruits, vegetables, herbs and grains for their natural antioxidant activities, and they indicate that the majority of antioxidants isolated from higher plants are polyphenols, indicating biological activity that is anti-inflammatory, anti-carcinogenic, anti-bacterial and anti-viral (Larson 1988; Shahidi 2000). The antioxidant activity of phenolics is due to their redox properties, which permits them to function as reducing agents and provide hydrogen and singlet oxygen quenchers. Many studies have been conducted to assess the presence and activity of antioxidants and the phenolic content of herbs and different types of tea (Sinha et al., 2010).

Radicals can damage any cellular component because of their ability to react indiscriminately. A wide range of antioxidant defences is present to protect cells' components from the damage that can be caused by free radicals. These antioxidant defences can be classified into three major groups: antioxidant enzymes, chain-breaking antioxidants, and transition metal-binding proteins (Young & Woodside 2001b).

1.5.1.1 The antioxidant enzymes

A) Catalase

Catalase plays a role in catalysing the two stages of H_2O_2 to water and oxygen:

$$\text{catalase–Fe(III)} + H_2O_2 \rightarrow \text{compound I}$$
$$\text{compound I} + H_2O_2 \rightarrow \text{catalase–Fe(III)} + 2H_2O + O_2$$

Catalase can also perform this function when not bound to Fe (III). Catalase works to protect cells from H_2O_2 when it diffuses freely in the membrane (Frankel 2007). However, H_2O_2 is degraded by glutathione peroxidases (Frankel 2007). The greatest activity of catalase occurs in the liver and erythrocytes, although some activity is observed in all tissues.

B) Glutathione peroxidases and glutathione reductase

The enzyme glutathione peroxidases catalyses the oxidation of glutathione at the expense of a hydro-peroxidase and may be hydrogen peroxidase (Takahashi & Cohen 1986; Frankel 2007):

$$ROOH + 2GSH \rightarrow GSSG + H_2O + ROH$$

Other substrates for these enzymes include lipid hydroperoxide, which may play a role in repairing damage resulting from lipid peroxidation. Glutathione peroxidase is a selenoprotein, meaning that it requires selenium at the active site (Young & Woodside 2001b). Glutathione peroxidase enzymes are encoded by discrete genes and their plasma forms as a synthesis mainly in the kidneys. It is distributed in approximately all tissues but the highest concentration is observed in the liver. Glutathione peroxidase works by scavenging H_2O_2 in the predominant subcellular distribution.

C) Superoxide dismutase

The superoxide dismutases catalyses the dismutation of superoxide to H_2O_2:

$$O_2^- + O_2^- + 2H+ \rightarrow H_2O_2 + O_2$$

The three different forms of superoxide dismutases in mammalian tissues differ in terms of the location of specific subcellular and tissue distribution. These are as follows: copper zinc superoxide dismutase (CuZn-SOD), which is found in the cytoplasm and organelles of virtually all mammalian cells (Liou et al. 1993); and manganese superoxide dismutase (MnSOD), which is found in the mitochondria of almost all cells.

1.5.1.2 The chain-breaking antioxidants

If free radicals react with another molecule, secondary free radicals will be generated that can react with another target to generate a more radical species. A lipid peroxidation is a simple example of a chain reaction that will continue to increase until either free radicals combine as a stable product or a chain-breaking antioxidant neutralises them (de Zwart et al. 1999). Chain-breaking antioxidants are small molecules that can accept an electron from or donate an electron to a radical, and this process leads to the formation of stable by-products (Halliwell 1990). Chain-breaking antioxidants can be divided into two phases: aqueous phase and lipid phase (Young & Woodside 2001b).

1. Lipid-phase chain-breaking antioxidants

These antioxidants scavenge radicals in membrane and lipoprotein particles and prevent lipid peroxidation. Vitamin E is the most important lipid-phase antioxidant and it occurs naturally, having probably eight forms whose biological activities differ (Horwitt 1991). The tocopherols (alpha, beta, gamma and delta) have a chromanol ring and phytyl tail, and they differ in number and position from the mythyl group. The tocotrienol (alpha, beta, gamma and delta) have almost similar structures but unsaturated tails (Young & Woodside 2001b). Moreover, vitamin E plays an important role in stabilising the membrane (Urano et al. 1992). Vitamin E deficiencies are not common in humans, although it might cause haemolysis and could lead to a peripheral neuropathy in the abetalipoproteinaemia (Sokol 1988; Swann & Kendra 1998). The antioxidant role of vitamin E is to trap peroxyl radicals and to break the chain reaction that characterises lipid peroxidation. It also works to minimise the formation of secondary carbon-centred radicals in a lipid-rich environment (Burton & Ingold 1986; Kayden & Traber 1993). α- tocopherol is the most abundant antioxidant in humans and the most potent in the tocopherol antioxidant group (Young &Woodside 2001b). Furthermore, α- tocopherol reacts with a peroxyl radical to create a relatively stable tocopheroxyl radical.

These radicals could react in several ways; α-tocopherol might react with ascorbate at the aqueous phase or a different aqueous-phase chain-breaking antioxidant such as reducing the level of glutathione or urate (Kagan & Tyurina 1998; May et al. 1998). Furthermore, the stabilised radical might react by combining two α-tocopheroxyl radicals to form a stable dimer or by oxidising a radical to form tocopherol quinone (Young & Woodside 2001b). The carotenoids are a part of the lipid-soluble antioxidants group. A β carotene is the most important antioxidant of this group. The

carotenoids play a role *in vivo* lipid peroxidation and are precursors of vitamin A (retinol), which has an antioxidant role (Chaudière & Ferrari-Iliou 1999; Keys & Zimmerman 1999).

The large group of polyphenolic antioxidants consists of flavonoids found in many fruits, vegetables and beverages such as coffee, tea and wine (Frankel 2007; Rice-Evans et al. 1996). Approximately 4000 flavonoids have been identified and are classified into groups depending on their chemical structure. These groups include flavonols (quercetin and kaempherol), flavanols (the catechins), flavones (apigenin), and isoflavones (genistein) (Young & Woodside 2001b). Some studies indicated an inverse relationship between the intake of flavonoids and the risk of coronary heart disease (Hertog et al. 1993; Hertog Ml et al. 1995; Rimm et al. 1996). However, other studies also show that the bioavailability of some flavonoids is poor, and the value of plasma low. Further, increasing the intake of flavonoids may ameliorate the biochemical indices and thus trigger oxidative damage (Hollman et al. 1995; McAnlis et al. 1999; Serafini & Ferro-Luzzini 1996; Stein et al. 1999). Other dietary compounds could affect the capacity of all antioxidants (Day et al. 1997).

Ubiquinol-10 is an effective lipid-soluble chain-breaking antioxidant. Ubiquinol-10 is the reduced form of Q10 coenzyme. It can scavenge lipid peroxidal radicals very effectively even when present in a smaller concentration than α-tocopherol (Lass & Sohal 1998).

2. Aqueous-phase chain-breaking antioxidants

Ascorbate (Vitamin C) is an example of this type of antioxidant in humans. It acts as an essential cofactor for various enzymes that catalyse the reaction of hydroxylation (Levine M 1999; Young & Woodside 2001b). It is also a key element in aqueous-phase chain-breaking antioxidants and scavenges superoxide), H_2O_2, the hydroxyl radical, hypochlorous acid, aqueous peroxyl radicals and singlet oxygen (Jialal et al. 1990;Young & Woodside 2001b). Uric acid scavenges radicals and converts them into allantoin. Urate may provide some protection against oxidising agents (Cross et al. 1992). Albumin-bound bilirubin plays a role in protecting newborn babies from oxidative damage (Gopinathan et al. 1994).

1.5.2 Anti-aging activity and the effects of antioxidants on aging

It has been argued that analyses of the free radical theory on aging indicate that ROS can affect lifespan via the accumulation of damage caused by oxidation (Frankel 2007). This damage could result from either an increase in oxidant generation or a

decrease in antioxidant defences. The final outcome of oxidative stress is a function of populating of oxidants, antioxidants defences and having to repair damage caused by oxidative processes. Moreover, nearly 300 theories have been developed to illustrate the aging process but not all are accepted by gerontologists. Free radicals are causally related to the basic process of aging (Fusco 2007; Harman 1956), and it is increasingly accepted that chemical reactions are the basis of aging (M 2002; Fusco 2007). The hypothesis of free radical theory of aging is that it is a natural process; modifiable by genetic factors and environment agents, and oxygen (O_2) derived free radicals are responsible for the age-related damage. The accumulation of endogenous radicals such as oxygen (O_2) generated in cells resulted in the oxidative modification of some biological molecules such as lipids, proteins and nucleic acid that are responsible for the aging and death of all living beings (Finkel T 2000).

Free radicals were known as early as 1972 when mitochondria were identified as being responsible for the first initiation of most free radicals reactions incidents in human cells (Fusco 2007). Mitochondrion DNA is a key target for the free radicals attached to live cells. Cells which use oxygen (O_2) and consequently ROS produced a complex antioxidant system evolved to neutralise ROS and prevent free radicals causing damage. Oxidative stress is a consequence of the imbalance between the production of free radicals and the system of antioxidant defences (Fusco 2007). One study described an ideal ‘golden triangle’ of oxidative balance in which three elements - oxidants, antioxidants and biomolecules – are situated at the apex (Carmeli et al. 2002). However, in a normal situation the over-production of free radicals may overwhelm natural cellular antioxidant defences and lead to oxidation and impairment in cellular function (Meydani et al. 1993; Fusco 2007; Balin & Pratt 1989).

1.5.3 Quercetin (a model compound)

Quercetin (3,3’,4’,5,7-pentahydroxyflavone) is one of the polyphenolic flavonoid compounds and it is ubiquitous in plants and other food resources (Abarikwu et al., 2012; Lamson and Brignall 2000). Quercetin occurs in the form of glycosides (sugar derivative) such as rutin in which the hydrogen of R-4 hydroxyl group is replaced by a disaccharide as shown in Figure 1.30. In the human diet quercetin is the main bioflavonoid and 25 mg is the average daily dietary intake of quercetin as estimated by researchers in the United States (Lamson & Brignall 2000). Recently, quercetin research has confirmed its potential as a carcinogenic and its role as an anti-cancer agent (Lamson & Brignall 2000). Clinical trials with animal and human of oral dosage of quercetin show absorption at around 20-25% (Lamson & Brignall 2000; Murray et al. 1954). A number of studies indicated that the concentration of serum quercetin

required for the activity of anti-cancer is approximately 10 µM. However, human studies show less concentration of serum quercetin than those required for anti-cancer activity, but a 1500 mg daily dose could attain a 10 µM level (Hollman et al. 1997). Finally, Morand et al. (1998) demonstrated that the required concentrations of serum quercetin are attainable with oral doses. An intravenous single dose of 100mg in humans is equal to 12 µM of a serum quercetin concentration (Gugler et al. 1975).

Figure 1.30: Quercetin structure shows R1, R2 and R3 are all OH: rutinose (β-1-l-rhamnosido-6-o-glucose) that rutin occurs in which the hydrogen of R-4 hydroxyl group is replaced by a disaccharide. Sources: Lamson and Brignall (2000); Rahman et al. (1989).

Some studies have queried the safety of quercetin, and there are no side effects in humans of a single oral dose up to 4g quercetin and an intravenous single dose 100mg in human does not show toxicity. However, intravenous 1400 mg/ m^2 once a week for long 3 weeks did show toxicity in two out of 10 participants (Gugler et al. 1975; Lamson & Brignall 2000). Ames, cell culture and human DNA tests demonstrated that quercetin is the most mutagenic of the flavonoids (Bjeldanes & Chang 1977; Duthie et al. 1997; Nakayasu et al. 1986). However, an *in vivo* study indicated that quercetin does not carcinogenic activity (Lamson & Brignall 2000).

Quercetin proved to be significant in oncological therapeutics. A study indicated that quercetin reduced skin damage especially in the head and neck cancer during radiotherapy (Rozenfel'd et al. 1990). Moreover, quercetin has been found to increase the therapeutic efficacy of cisplatin in both *in vivo* and *in vitro* studies. For example, one study used a combination of quercetin and cisplatin to treat mice from tumour and showed a reduce tumour growth (Hofmann et al. 1990). Ultimately, quercetin is found in various foods such as fruits, vegetables, leaves and grains as shown in Figure 1.31. It also might be used as an ingredient in supplements, beverages or foods.

Figure 1.31: Various foods enriched with quercetin. Source: http://www.menshealth.com/mhlists/best-supplements-for-men/quercetin.php (2012)

1.5.4 Anti-cancer properties

The specific role of free radicals in carcinogenesis is still being investigated. However, some clinical trials show that antioxidants are associated with fewer incidences of different types of cancer. For example, vitamin E has been shown to reduce the incidence of breast, lung, colon cancer and prostate cancer (Fusco 2007). A study confirmed that the supplementation of α-tocopherol reduced the incidence of prostate cancer and mortality (Albanes et al. 1995). A trial study determined the efficacy of a combination of antioxidants, vitamins and minerals as nutritional doses to control the incidence of cancer in the general population. After 7.5 years' follow-up to this study, the supplementation antioxidants did reduce the incidence of cancer in men (Hercberg et al. 2004; Fusco 2007). The cancer prevention study II cohort shows that there is a mild association between multivitamins and a low risk incidence of colorectal cancer (Jacobs et al. 2003). However, there was no sufficient evidence for antioxidant supplements protecting people against gastrointestinal cancers (Bjelakovic et al. 2004). A Chinese cancer prevention study found that there is a lower rate of gastric and oesophageal cancer and decrease in mortality among people whose daily diets are supplemented with combinations of β-carotene, vitamin E and selenium for approximately 5 years (Blot et al. 1993).

Another study confirmed that a high intake of antioxidants can prevent lung cancer in non-smokers, while β-carotene and vitamin E indicate a decrease in the occurrence of gastric cancer (Byers & Perry 1992; Hwang et al. 1994). A high intake of vitamin E reduced the risk of colon cancer and reduced the incidence of prostate cancer in male smokers (Bostick et al. 1993; Heinonen et al. 1998). Diets rich in vegetables and fruit

containing a variety of antioxidants can clearly protect humans from oxidative cellular damage and risk of cancer (Das 1994; Johnson et al. 1994; Kohlmeier et al. 1995; Thompson et al. 1999). Some antioxidants have pro-apoptotic anti-cancer properties such as α-tocopherol succinate analog of α-tocopherol (Jha et al. 1999; NEUZIL et al. 2001). Moreover, α-tocopherol inhibited the activity of pro-apoptotic of α-tocopherol succinate.

There are some contradictory findings for the effects of antioxidants. Firstly, as mentioned above antioxidants can protect healthy people but they could harm smokers due to exposure to exogenous chemicals that are in tobacco and the lung tissues of smokers may not resist mutagenized precancerous cells (Salganik 2001). Secondly, antioxidants scavenge ROS but do not completely remove chemicals that appear in smokers' lungs and this is could explain why antioxidants can protect non-smokers against lung cancer (Byers & Perry 1992; Salganik 2001). Thirdly, the ability of antioxidants to defend the human body against cancer depends on the ROS rate level in cells. This means antioxidants might be effective with a high level of ROS but could be undermined due to a low rate of ROS. This is explained by the inhibition of ROS-dependent cancer protective apoptosis and phagocytosis (Salganik 2001). *In vivo* and *in vitro* studies indicated that Q10 acts as a phenolic antioxidant and a chain-breaking antioxidant (Dallner & Stocker 2005). Q10 which is effective in keratinocytes against ultraviolet A (UVA) light-induced oxidative stress and in the collagenase in human dermal fibroblasts following UVA light irradiation (Hoppe et al. 1999).

1.6 Medicinal plants used in therapy and cosmetics

Plants have a long history of use in therapy and cosmetics. Flowers and herbal infusions have been studied for their ability to protect people against oxidative and phenolic problems (Atoui et al. 2005; Day et al. 1997). Also, different active principles have been used in the treatment of cancer such as *Angelica Gigas, Catharanthus roseus, Podophyllum peltatum, Podophyllum emodii, Taxus brevifolia, Ocrosia elliptica,* and *Campototheca acuminate* (Jiménez-medina et al. 2006). Flavones, flavanols and isoflavones are phytochemicals which have responded to tumour treatment. Treatment for cancer by numerous drugs used in chemotherapy can cause cellular damage by inducing cytotoxic and genotoxic changes. Therefore, medicinal plants constitute an important line of research for alternative treatments that are effective and non-toxic (Jiménez-medina et al. 2006).

Calendula officinalis is one example of a flower that is used in cancer therapy and different extracts of this flower have shown anti-tumoral, anti-inflammatory, anti-viral

activity, anti-HIV properties, etc. It also assists in healing wounds and overcoming cutaneous and internal inflammatory diseases and has antioxidant properties (Fonseca et al. 2010; Katalinic et al. 2006). Clinical studies indicate that calendula is highly efficacious in the prevention of acute dermatitis in cancer patients (Pommier et al. 2004). *Calendula officinalis* in time can remedy sun burn and inflammatory disease (Fonseca et al. 2010). It is an herb that is native to the Mediterranean region. In Europe and the United States is cultivated for different reasons, for example ornamental or medicinal (Fonseca et al. 2010).

1.7 Scope and Aims

The overall aim of the project was to investigate the potentially harmful effects of beauty products and personal care products on human skin cells *in vitro*. To determine the mechanism of cell killing of head lice treatments using the FITC Annexin V apoptosis detection assay on adherent keratinocytes human skin cells (HaCaT) there was a need for the optimisation of apoptosis detection assay using adherent human keratinocytes (Chapter 2). The resulting of Chapter 2 indicated that the FITC Annexin V apoptosis detection kit I followed by flow cytometry can be used for adherent cell lines, such as keratinocytes HaCaT cells. The initial study set out to determine the cytotoxicity and genotoxicity of four beauty products (Nivea Visage Q10 plus Anti-Wrinkle which contains synthetic chemicals with TiO_2 (NVAW+TiO_2), Nivea Visage Q10 plus Anti-Wrinkle which includes synthetic chemicals without TiO_2 (NVAW), Facial Moisturizer - Camellia & Geranium Blossom (FMCGB) and glycerol on two different human skin cell lines as an *in vitro* models; human keratinocytes HaCaT and normal fibroblast skin cell line CCD 1064SK (see Chapter 3). The study also aimed to investigate the cytotoxicity and genotoxicity of five popular head lice treatments (Lice breaker (Permethrin 1% w/w), KP24 Medicated lotion (Maldison0.5% w/w), Organix Pyrethrum treatment (4g/L Pyrethrins, 16g/LPiperonyl Butoxide), Tea Tree Oil (100% pure) and Lavender oil (100% pure) on HaCaT human skin cells (see Chapter 4). Four Calendula officinalis extracts were examined to determine their antioxidant ability using a bioassay to protect HaCaT skin cells against cell death and genetic damage induced by an oxidative challenge, hydrogen peroxide (H_2O_2) (see chapters 5 and 6). Moreover, Calendula officinalis extract was examined to detect its ability to protect human skin cells (HaCaT) against the cytotoxicity effects of personal care products; NVAW + TiO_2 and NVAW (without TiO_2) Lice Breaker treatment, which has 1% w/w of permethrin, and pyrethrum treatment (4g/l pyrethrin plus 16g/L piperonyl butoxide) and TiO_2 on HaCaT skin cells (see Chapter 7).

Finally, given that much of this doctoral work has already been published, some of the Chapters are in the form of the text of the journal article in accordance with Flinders University guidelines (Flinders University, 2015). Where this is the case, it is clearly stated at the beginning of the Chapter. Where contributions were made by others, their specific contribution is noted in (contribution to publications).

CHAPTER 2: OPTIMISATION OF APOPTOSIS DETECTION ASSAY USING ADHERENT HUMAN KERATINOCYTES

2.1 Introduction

Necrosis can be distinguished from apoptosis as an alternative to apoptotic cell death, which is considered to be accidental cell death or cell damage due to toxic substances (Kamiński 1993). Apoptosis is a normal physiological process that occurs during normal cell turnover or proper development (Elmore 2007). The earliest feature of programmed cell death is a loss of plasma asymmetry (Elmore 2007; Pharmingen™ 2008). During this stage of apoptosis, two processes occur: cell shrinkage and cell pyknosis (Pharmingen™ 2008; van Engeland et al. 1996). Cell shrinkage makes the cytoplasm dense with tightly packed organelles (Pharmingen™ 2008; van Engeland et al. 1996), and pyknosis, the most studied feature of apoptosis, is the result of chromatin condensation (Elmore 2007). In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) moves from the inside of the plasma membrane to the outside, and is consequently exposed to the external cellular environment. FITC Annexin V is a calcium (Ca^{+2})-dependent and phospholipid-binding protein that binds to cells with exposed PS (van Engeland et al. 1996). Propidium iodide (PI) dye is used to investigate the loss of membrane integrity. Viable cells with intact membranes prevent permeable PI. PI is negative and FITC Annexin V is positive in the early stages of apoptosis (Pharmingen™ 2008). However, the membranes of damaged or dead cells are permeable to PI (Pharmingen™ 2008). When cells at the end stage of apoptosis undergo necrosis, both PI and FITC Annexin V are positive. Cells are alive when both PI and FITC Annexin V are negative (Pharmingen™ 2008). All three cell stages can be detected using the FITC Annexin V apoptosis detection kit I with flow cytometry. The assay technical sheet indicates that the assay was tested routinely for suspension cells. However, this is not recommended for adherent cell lines due to membrane damage, possibly occurring during the harvesting phase (Pharmingen™ 2008). As an adherent HaCaT cell line was used in this study, the FITC Annexin V apoptosis detection assay was modified because dead cells lose their adherence properties, and it is necessary to know if these cells (which are in the supernatant) need to be included. Trypan blue dye exclusion staining was also used to determine relative cytotoxicity after the cells were harvested using TrypleEx before adding the supernatant and counting cells after spinning and resuspending them (checking for blue cells and evidence of debris).

2.2 Materials and methods

2.2.1 Materials

RPMI-1640 medium and FBS were purchased from Gibco® Cell Culture MediumLife Technologies (Australia). Phosphate-buffered saline (PBS) and a FITC Annexin V

apoptosis detection kit I were purchased from BD Biosciences TM. 0.1% sodium azide in PBS. All of the reagents used in this chapter were purchased from Sigma-Aldrich, unless otherwise stated.

2.2.2 Cell culture and treatment

A human non-cancer keratinocytes cell line (HaCaT) was donated by the Department of Haematology and Genetic Pathology at Flinders Medical Centre, School of Medicine at Flinders University, South Australia. The cell line was maintained in the RPMI-1640 medium (RPMI-1640 powder reconstituted medium supplemented with L-glutamine (2 mM), penicillin (100U/mL), streptomycin (100 µg/mL) and 10% FBS). Cells were seeded in tissue culture flasks at 5×10^5 cells/ml and incubated in a fully humidified atmosphere at 37°C in 5% CO_2. The cells were subcultured twice a week when they reached 60–80% confluence or more. The cell concentration and viable cell count of the HaCaT cell culture to be assayed was determined with a trypan blue assay. In brief, 3×10^5 / were treated for apoptosis assay in a six wells plate. There was a zero control for each experiment (untreated = medium plus cells). They were incubated at 37°C in 5% CO_2 for 19 hours to allow the cells to adhere. The medium was then removed, the cells were washed twice with 1 ml of PBS and treated with 2 ml of treatment/well for 1 hour and the plates were then incubated at 37°C in 5% CO_2.

2.2.3 Apoptosis assay via flow cytometry

The FITC Annexin V apoptosis detection kit I (BD Biosciences, US) was employed and the results measured using flow cytometry. After treatment, the cells were washed twice with 1×PBS. Detached cells with TrypLE express enzyme (1X) for five minutes and collected with the supernatant. Three tubes were prepared for each treatment, as illustrated in Figure 2.1. Tube A represents the method of testing without collecting the supernatant. Tube B represents the method of testing using the supernatant alone. Tube C represents the method of testing using the supernatant after spinning and resuspending. The cells were washed twice in 0.1% sodium azide in PBS and then centrifuged at 78g (1200 rpm) for five minutes'. The cells were then rinsed with the medium and harvested, and the pellets were resuspended in 50µl of the binding buffer. Next, the cells were double-stained with 25 µl of annexin V-FITC and 25µl of PI. After 15 minutes of incubation in the dark at room temperature, 200 µl of the binding buffer was added to the mixture. The intensity of annexin V-FITC and PI was recorded using BD Accuri™ flow cytometry and analysed with BD CFlow® Plus Software.

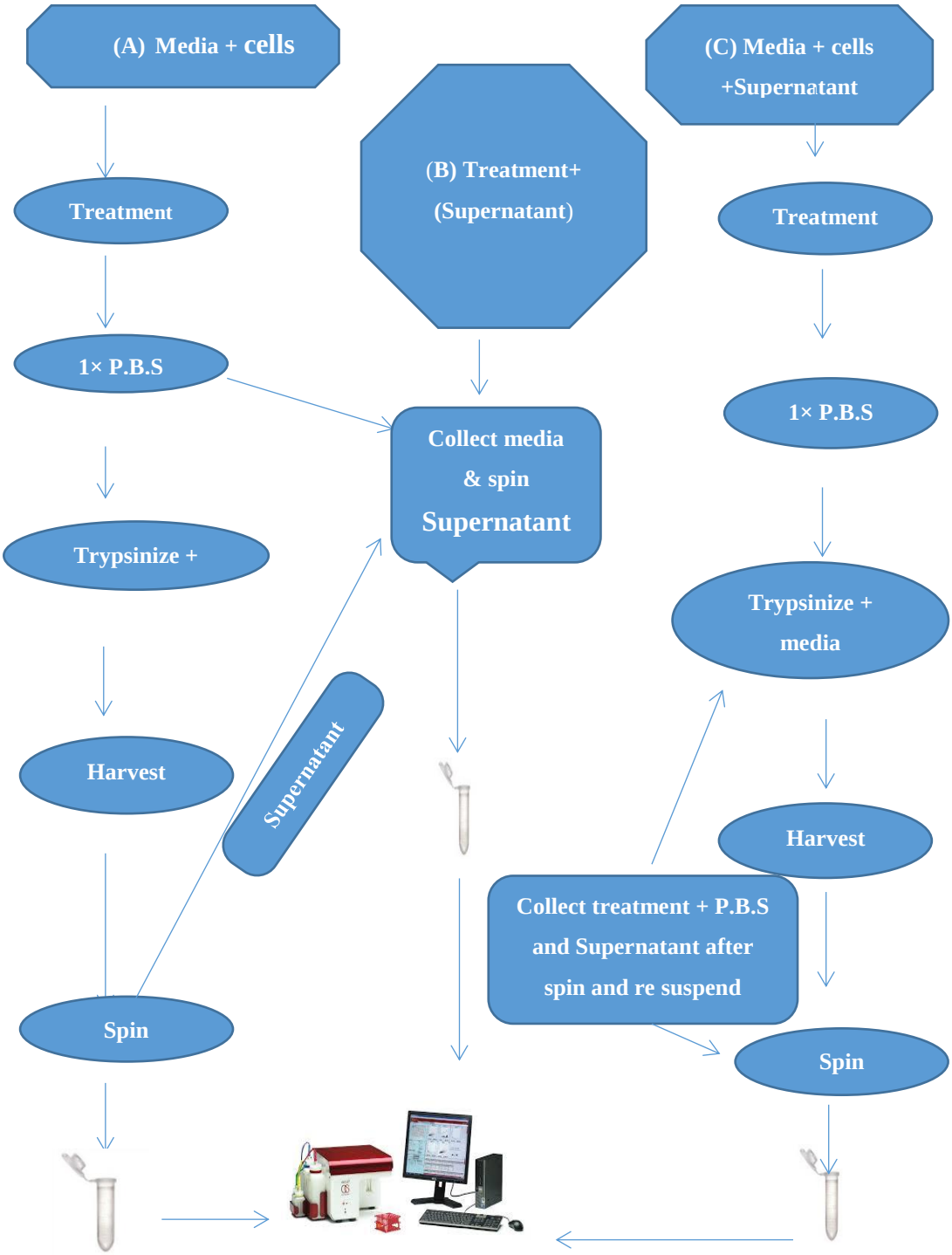

Figure 2.1 Brief outline of the methodology for improving apoptosis assay for adherent cell line by FITC Annexin V-FITC apoptosis detection kit I followed by flow cytometry analysis. (A) Tube A contains the cells after the supernatant is removed (B) Tube B contains the supernatant. (C) Tube C contains cell and supernatant after a spin and resuspend.

2.2.4 Trypan blue assay

The trypan blue assay is the most common assay used to assess cell viability. Trypan blue is a vital stain that stains nonviable cells (dead cells with a damaged membrane), which take up the dye to give the cells a blue colour, observed under a microscope, while viable cells appear unstained (due to intact cell membranes) (Louis & Siegel

2011). Fifty µl was taken from the suspension and diluted 1:1 by adding 50µl of trypan blue dye (0.2%); the mixture was then mixed gently and 10 µl of it was transferred into each of the two chambers of a hemocytometer slide. Cells were counted in the four large squares. Cell concentration per ml was calculated from the average number of cells in one large square x dilution factor x 10^4. The dilution factor was usually two (1:1 dilution with trypan blue), and 10^4 was the conversion factor to convert 10^{-4}ml (volume of one square) to 1ml.

2.2.5 Data analysis

The experiment results were replicated with at least three separate experiments. Data are presented as the mean ± SEM. Statistical analysis was carried out using ANOVA including Tukey's HSD post hoc test (version 22). The significance was determined compared to the untreated control (0 doses) when the *P* value < 0.05.

2.3 Results

This study aimed to modify the FITC Annexin V apoptosis detection assay for adherent cell lines because dead cells in the supernatant lose their adherence properties and could affect the detection of apoptosis using flow cytometry. The study also aimed to determine the relative cytotoxicity after the adherent HaCaT cells were harvested (using TrypLE Express Enzyme (1X) for five minutes) before adding the supernatant and counting cells after spinning, resuspending and checking for blue cells and evidence of debris. H_2O_2 at a dose of 300µM was used as a late apoptosis (necrosis) positive control (Qian et al. 2012). Camptothecin (1mM in DMSO) with a final concentration of 25µM was used as a positive control for early apoptosis and was used in this study as it is a recommended control used in the technical sheet (Pharmingen™ 2008). All treatments were compared to the medium untreated control. All treatments were measured in triplicate with the supernatant collected after spinning and resuspended after harvesting, or without the supernatant to determine the difference in the apoptosis results using the adherent HaCaT cell line. No significant difference was observed in the results regarding the collected supernatant or that without supernatant in any of the treatments, including the untreated control performed by the FITC Annexin V apoptosis detection assay followed by flow cytometry, as shown in Figure 2.2. H_2O_2 at a dose of 300µM (late apoptosis or positive necrosis control) revealed significant early and late apoptosis (or necrosis) induced in HaCaT cells. The supernatant alone was also measured by flow cytometry for all treatments (see Appendix VII).

The current study also used trypan blue dye exclusion staining to determine the relative cytotoxicity of all treatments after the cells were harvested using TrypleEx before adding the supernatant and counting the cells after spinning and resuspending (checking for blue cells and evidence of debris) (Louis & Siegel 2011). No significant cell killing was observed after the cells were harvested in the untreated control, as shown in Figure 2.3. In addition, the result for camptothecin demonstrated no significant cell death. After supernatant was added, there was a 17% decrease in survival (this figure did not reach significance), but that was probably due to the toxicity of the treatment on the cells.

However, H_2O_2 yielded significant cell killing, which is consistent with the results described in Chapter 5 (Alnuqaydan et al. 2015b). No membrane debris was observed, and no increase in cell killing was observed after the supernatant was added.

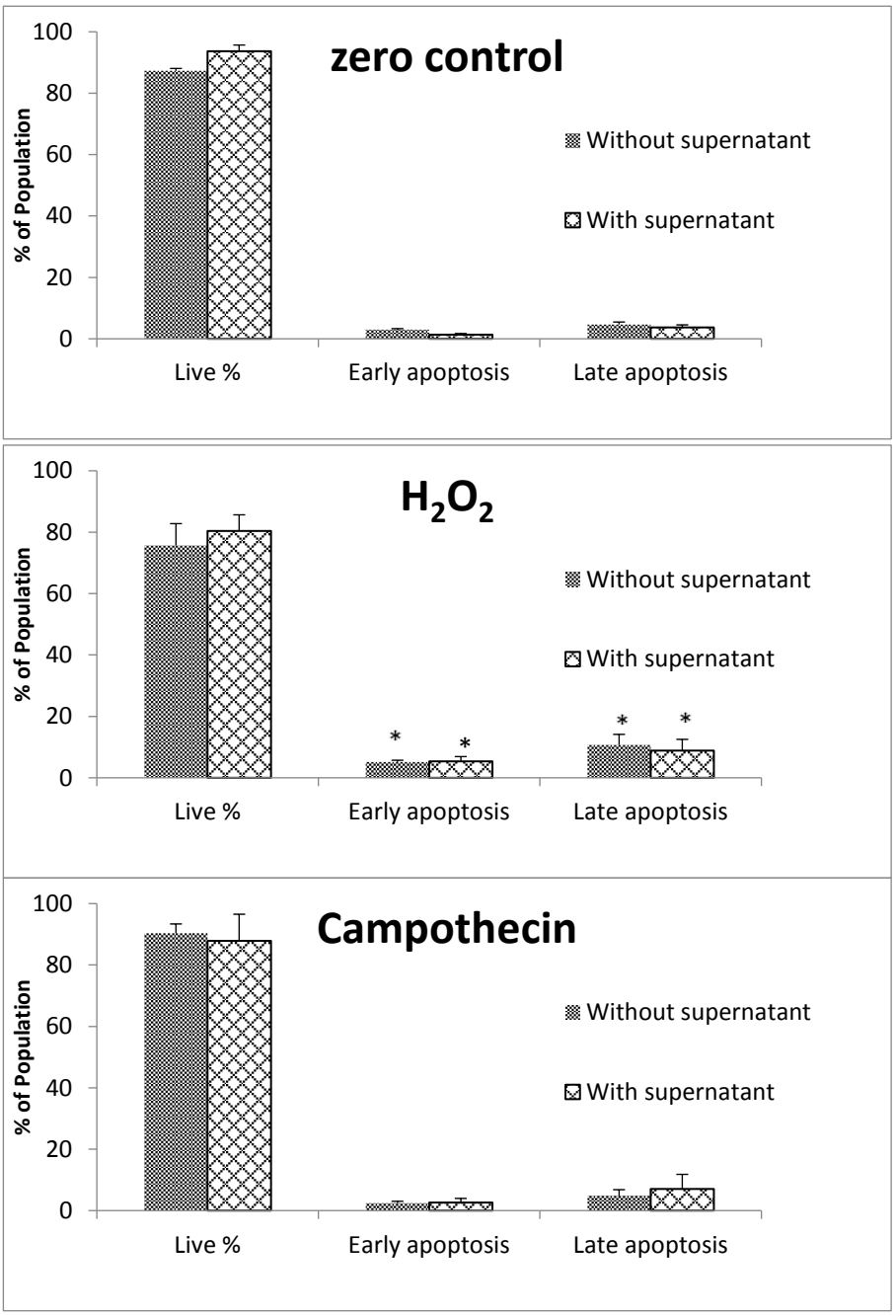

Figure 2.2: HaCaT adherent cells were used to measure apoptosis induction via FITC annexin v apoptosis detection kit I followed by flow cytometry as described in sub-section 2.2.4. HaCaT cells were treated with 300 µM Hydrogen peroxide (H_2O_2) and Camptothecin (1mM in DMSO) for 1h. Data were obtained from 20,000 events and early apoptotic cells (Annexin positive; PI negative), and late apoptotic cells (necrotic cells) (annexin positive/PI positive) are presented as a percentage of total cells analysed. The values are shown as means ± SEM. Treatments significantly differing from the untreated control at $p < 0.05$ are presented as *.

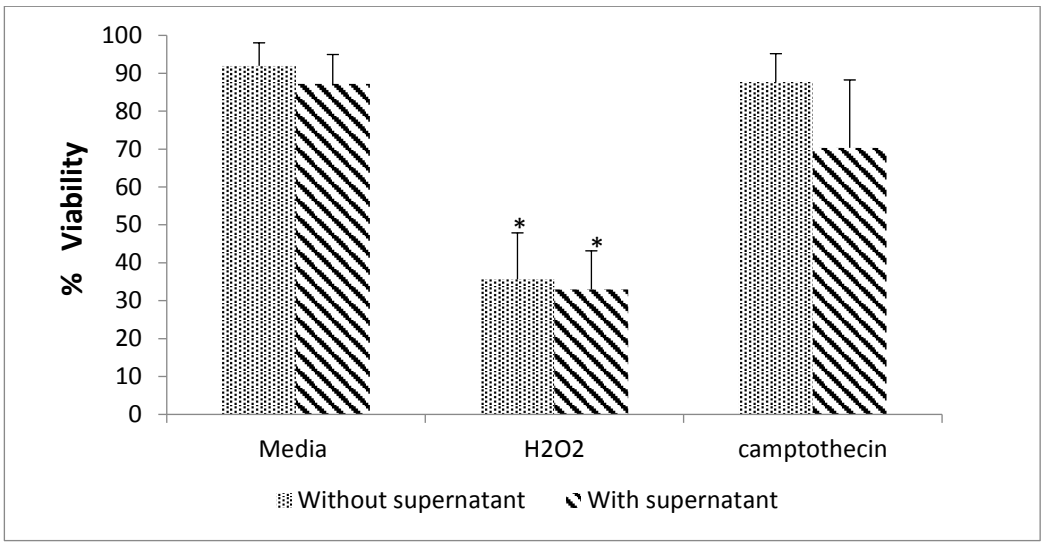

Figure 2.3: Relative cells per ml for the adherent HaCaT cell line in flasks following treatment for analysis in the apoptosis assay after the harvesting stage using the TrypLE Express Enzyme (1X). This was estimated by using the Trypan blue exclusion assay concerning for all treatments with or without supernatant. HaCaT cells were treated with 300 µM Hydrogen peroxide (H_2O_2) and camptothecin (1mM in DMSO) for 1 hour. Data are shown as a percentage of relative surviving cell numbers compared to the untreated control, and are presented as the mean ± SEM of three separate experiments. Treatments significantly differing from the untreated control at $p < 0.05$ are presented as *.

2.4 Discussion

Many researchers are using the FITC Annexin V apoptosis detection kit I followed by flow cytometry for adherent cell lines such as the human adherent lung cancer A-549 cell line, non-cancer human lung cell line MRC5, human adherent breast cancer cells T47D, MCF7 (adenocarcinoma, a p53 wild-type cell line) and human normal breast cell line 184B5 (Ramezanpour et al. 2014a; Ramezanpour et al. 2014b). However, BD Pharmingen ™ noted in its technical sheet that FITC Annexin V flow cytometric analysis on adherent cell lines is not routinely tested, as a specific membrane could be damaged during the harvesting stage, leading to a false positive result. Also, as mentioned earlier in this chapter, dead cells lose their adherence properties; therefore, it is necessary to know if these cells (which are in the supernatant) need to be included, as dead cells in the supernatant could affect the detection of apoptosis using flow cytometry.

The trypan blue exclusion dye staining assay was used to determine the relative number of live and dead cells. Trypan blue dye stains nonviable cells with a blue colour observable under a light microscope (Louis & Siegel 2011). The viability of adherent HaCaT cells was measured after harvesting to determine debris as evidence of membrane damaged. After harvesting, approximately 87% of cells survived with intact membranes with the supernatant, and 92% survived without it. The result of the

trypan blue assay confirmed that harvesting did not damage the membrane of adherent cells and did not affect the result of apoptosis detection by flow cytometry. Figure 2.3 shows the results of the untreated control and treatments with H_2O_2 and camptothecin. H_2O_2 was used as a positive control for late apoptosis (necrosis) (Saito et al. 2006). The significantly toxic effect of H_2O_2 on adherent HaCaT cells as measured by trypan blue is consistent with recently published work (Alnuqaydan et al. 2015b). Camptothecin (1mM in DMSO) with a final concentration of 25µM was used as a recommended positive control for early apoptosis (Pharmingen™, 2008). No significant toxicity observed after HaCaT cells were treated with camptothecin.

The FITC Annexin V assay was used to detect the loss of plasma membrane asymmetry when PS was transferred from the inside of the plasma membrane to the outside (Pharmingen™ 2008; van Engeland et al. 1996). Different methods were carried out to determine whether the dead cells (supernatant alone or combined with the treatment) could affect the detection of apoptosis followed by flow cytometry. There was no significant difference between the results with and without supernatant, including the untreated control as shown in Figure 2.2.

The other issue with harvesting cells is that trypsin may affect the proteolysis and chelation of Ca^{+2} from the cells' surface used to bind the annexin. To avoid this phenomenon, other harvesting options, such as mechanical scraping, can be used (van Engeland et al. 1996). However, enzymatic harvest is more efficient than mechanical scraping due to the percentage of viable cells measured by trypan blue and fluorescein diacetate uptake. Approximately 10% of scraped cells were viable, compared to 90% of enzymatic harvested cells (Dilley & Herring 1984).

In conclusion, based on the current findings, the FITC Annexin V apoptosis detection kit I followed by flow cytometry can be used for adherent cell lines, such as keratinocytes HaCaT cells. Enzymatic harvest did not affect the detection of apoptosis confirmed by the results of the trypan blue assay. Further, dead cells in the supernatant did not affect the results of apoptosis measured by flow cytometry, as shown in the result of untreated control (Figure 2.2). Therefore, combining the supernatant with the samples did not affect the detection of apoptosis in any way.

CHAPTER 3: TOXICITY AND GENOTOXICITY OF BEAUTY PRODUCTS ON HUMAN SKIN CELLS *IN VITRO*

This chapter is a reproduction of the manuscript has been accepted with minor changes for publication as Alnuqaydan, AM & Sanderson, BJS 2016, 'Toxicity and genotoxicity of beauty products on human skin cells in vitro', Journal of Clinical Toxicology. In Press, Accepted Manuscript.

3.1 Introduction

We use large quantities of beauty products every day and, in the process, are exposed to a wide variety of chemicals used in these products. These chemicals are a particularly insidious form of body pollution because they enter the human body through multiple routes. It is easy to swallow them, inhale them and absorb them through the mucous membrane of the eyes, mouth or nose. Our skin absorbs approximately 60% of the chemical ingredients and sends them into the bloodstream, from whence they can reach every organ in the body seconds after absorption (Thomas 2008). Women using many cosmetics are thought to absorb up to 2 kg of chemical cosmetic ingredients through their skin each year (Thomas 2008). Government reports in the US and EU indicate that about 90% of the cosmetic ingredients are not safe for long-term use people (Thomas 2008). Most beauty products contain a mixture of chemicals that only make the problem worse (Zeliger 2008a). Unfortunately, the companies that make the products are self-regulating, and government agencies do not press manufacturers to prove that their products are safe (Thomas 2008; Zeliger 2008a). In the US, the FDA does not regulate cosmetic and personal care products (FDA 2006; Lazarus & Baumann 2001). However, the FDA does require drugs to be extensively tested and approved (Lazarus & Baumann 2001). Studies using biochemical methods to screen blood samples from over all the world have indicated that most people are carrying a huge amount of chemicals in their bodies (Cone 2005; Thomas 2008). Another study investigating chemical exposure has demonstrated that most American children and adults carry nearly 100 chemicals substances, including pesticides and toxic compounds (Cone 2005). Many of these cause cancer, damage the immune system and affect human behaviour and the central nervous system. The sources of these chemicals include household exposure to pesticides and detergents, cosmetics, toiletries, paints and fabric treatments (Menegaux et al. 2006; Thomas 2008; Zeliger 2008a). They can have long-term effects on the body, accumulate in different organs and the bloodstream and then pass through urine, semen and breastmilk. After a while, the body becomes overloaded is and at risk of total breakdown (Thomas 2008). Some cosmetics contain mercury, which is used to lighten the skin, and people who use products containing mercury are at a high risk of mercury poisoning (Balluz et al. 1997). In the US, mercury compounds are used as preservatives in small concentrations for eye area products, and FDA regulations in the US restrict cosmetics products that contain mercury (Balluz et al. 1997). Some moisturisers contain mineral oil, which can slow down cell renewal and promote early skin aging (Thomas 2008). A study that tested 88 brands of eye shadow found that approximately 75% of these products contained at least one of the following five elements: lead, nickel, chromium, arsenic or cobalt (Sainio et al.

2000). Lead can damage any part of the human body and the nervous system in particular (Järup 2003). Even in the small doses found in these products, these elements may cause hormone disruption (Rubin 2011). Some sunblocks and moisturisers with sunblock contain TiO_2, which is a potential hazard and carcinogen (Falck et al. 2009; Tucci et al. 2013). Finally, most shampoos and other toiletries or liquid formulae contain nitrosamines, which can cause cancer (King 2011). Even products labelled as hypoallergenic probably still contain potentially carcinogenic substances (Millikan 2001). This study examined four different facial beauty products to assess their effects on two human skin cell lines (human keratinocytes HaCaT skin cells and human fibroblast CCD-1064SK cells). The products were NVAW face moisturiser, which includes synthetic chemicals and TiO_2; NVAW face moisturiser (improved formula, without TiO_2), which includes synthetic chemicals and FMCGB, which includes a mixture of natural ingredients and Glycerol British Pharmacopoeia (BP).

3.2 Materials and methods

3.2.1 Materials

RPMI-1640 media and FBS were purchased from Gibco® Cell Culture Media—Life Technologies (Australia). Cytochalasin B (Cyt-B) solution, Sodium dodecyl sulphate (SDS, approximately 99%), PBS and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (US). A spectrophotometer plate reader was purchased from BIO-TEK Instruments Inc. (US); Diff-Quik stains were purchased from Lab Aids (Australia); a cytospin centrifuge was purchased from Shandon, England and TrypLE™ was purchased from Life Technologies (Australia). All other reagents were obtained from Sigma, unless otherwise stated.

3.2.2 Products to be examined

3.2.2.1 Nivea Visage Q10 Plus Anti-Wrinkle face moisturiser with titanium dioxide

NVAW day moisturiser cream plus extra UVA protection (Sun Protection Factor 15) is produced by Nivea, a global company. The NVAW cream was purchased from a local pharmacy in Adelaide, South Australia. It suits all skin types. The product aims to increase the natural level of co-enzyme Q10 and prevent wrinkles. It also protects against UVA and UVB rays. The ingredients of NVAW are a mixture of chemicals (Table 3.1). This formula contains TiO_2, a nanoparticle used widely in pigments, cosmetics and skin care products because it protects the skin from UV light, particularly in nanoparticles less than 100 nm (Trouiller et al. 2009). TiO_2 has been

classified as carcinogen (Zhao et al. 2009). Some studies have shown that TiO_2 can damage DNA directly or indirectly via inflammatory response or oxidative stress (Trouiller et al. 2009).

Table 3.1: The ingredients and toxic effects of NVAW + TiO_2. The toxic effects of the ingredients were classified by TOXNET (2015) and EWG (2015).

Ingredient	Toxic effects
Octocrylene	Skin allergen. Restricted for use in cosmetics in Japan. Produces excess ROS that can interfere with cellular signalling, cause mutations, lead to cell death and may be implicated in cardiovascular disease. Measured to accumulate in people.
Ethylhexyl Salicylate	Low allergies and immunotoxicity, ecotoxicology
Methylpropanediol	Not expected to be potentially toxic or harmful
Glyceryl Stearate	Suspected to be an environmental toxin
Butyl Methoxydibenzoylmethane	Toxin in mice (Butt & Christensen 2000)
C12-15 Alkyl Benzoate	Suspected to be an environmental toxin
Tocopheryl Acetate	Human skin toxin or allergen—strong evidence. Has caused tumours in animals.
Chondrus Crispus	Organ system toxin (non-reproductive)
Dimethicone	Organ system toxin (non-reproductive)
Trisodium EDTA	Penetration enhancer
Caprylic/Capric Triglyceride	Ecotoxin
Limonene and Parfum	Irritant. Possible human immune system toxin or allergen. Restricted in cosmetics
Methylparaben	Human endocrine disruptor—strong evidence
Phenoxyethanol	Irritant (skin, eyes or lungs), occupational hazard, organ system toxin (non-reproductive)
Cera Microcristallina	Organ system toxin (non-reproductive)
Paraffinum Liquidum	Human immune and respiratory toxin or allergen—strong evidence
Benzyl Alcohol	Occupational hazard, organ system toxin (non-reproductive)
TiO_2	Carcinogen
Thylhexylglycerin	Irritant (skin, eyes or lungs); organ system toxin (non-reproductive)
Carbomer	No carcinogenicity data available, but it is found to be irritating to the respiratory tract.
Sodium Phenylbenzimidazole Sulfonat	May cause skin irritation, if swallowed will cause vomiting.
Trimethoxycaprylylsilane	Not expected to be potentially toxic or harmful

3.2.2.2 Nivea Visage Q10 Plus Anti-Wrinkle face moisturiser (improved formula, without titanium dioxide)

This product is an improved formula of NVAW day moisturiser. It was released into the market after the original product containing TiO_2 was removed. It has almost the same ingredients as the original one other than TiO_2. The package is labelled 'skin compatibility dermatologically approved'. This product aims to protect against UVA, and is suitable for sensitive skin.

3.2.2.3 Grown Facial Moisturizer—Camellia and Geranium Blossom.

FMCGB is a natural moisturiser made from bioactive ingredients. It is made from camellia and rose hip seed oil extracts, which contain vital phytosterols that rehydrate and nourish the skin. Cane sugar is also present, and it releases biosaccharides that soothe the skin and combats the effects of UV light and pollution. Mayblossom releases flavonoids, which normalize sebum production and reduce pore size. The product was purchased from a local chemist.

3.2.2.4 Glycerol British Pharmacopoeia

Pharmaceuticals Pty Ltd produces Glycerol BP. It was purchased from a local pharmacy. It is 90–100% glycerol (glycerine). It can be prescribed to be taken internally to act as a mild laxative or externally to soften and moisturise the skin. Glycerol may reduce food intake in diabetic rats (Brief & Davis 1982). Therefore, there is a label on the package warning diabetic patients. As glycerol is a common basic ingredient in many moisturisers, it was used as a negative control for the beauty product experiments.

3.2.3 Cell lines and cell culture

A human non-cancer keratinocytes cell line HaCaT was a gift from the Department of Haematology and Genetic Pathology, Flinders Medical Centre, School of Medicine at Flinders University, Adelaide. The skin fibroblast cell line CCD-1064Sk (normal human skin cells) was obtained from ATCC, US (ATCC® CRL-2076™). The HaCaT line was maintained in the RPMI-1640 medium with 10% FBS and 1% penicillin/streptomycin (Thermo Scientific, Australia). The CCD-1064Sk cell line was maintained in Iscove's Modified Dulbecco's medium (IMDM medium) with 10% FBS and 1% penicillin/streptomycin. The cells were seeded in tissue culture flasks and incubated in a fully humidified incubator at 37°C in 5% CO_2. The HaCaT cells were subcultured when they reached 60–80% confluence.

3.2.4 Cell treatment

The 96-well flat-bottom microplate was seeded with 10^4 cells/well and incubated for 19 hours at 37°C in 5% CO_2 to allow the cells to adhere. The media were aspirated, replaced with 100 µL of the treatment solution per well and treated for one hour prior to the bioassays or genetic assays. The negative or untreated control (0 dose) was the media.

3.2.5 Crystal violet assay

Crystal violet (CV) stains the DNA of the live cells that adhere to the plate after the dead cells are washed away (Berry et al. 1996). The relative number of viable cells was determined using the CV assay as described in Ramezanpour et al. (2012). In brief, 50 µL of CV stain (0.5% of CV in 50% methanol) was added to each well and incubated for 10 minutes at ambient temperature. The plate was then gently washed with distilled water and air-dried, and 50 µL of 33% acetic acid was added to de-stain the cells. The absorbance (ODs) was measured on a spectrophotometric plate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm.

3.2.6 Methyl tetrazolium cytotoxicity assay

The tetrazolium salt MTT assay is based on a colorimetric assay for mammalian cell growth and survival, and depends on the ability of viable cells to metabolise the yellow, water-soluble tetrazolium salt (Mosmann 1983). Cells were seeded at 10^4 in a volume of 100 µl in each well of a 96-well flat-bottom plate. The MTT solution with a final concentration of 0.5 mg/ml was added and then incubated for four hours at 37°C. After incubation, 80 µl of 20% SDS in 0.02 M HCl was added. The plates were incubated overnight in the dark at room temperature. The absorbance (ODs) was measured on a spectrophotometric plate reader using a reference wavelength of 630 nm and a test wavelength of 570 nm.

3.2.7 Cytokinesis-block micronucleus assay

The mechanism of cell killing and genotoxicity of beauty products was investigated using the CBMN assay as described by Fenech (2007) and Wang et al. (2007). In brief, after the treatment Cyt-B (4.5µg/ml) was added to the media and the cultures were incubated at 37ºC for 23 hours, the cells were trypsinised (TrypLE™ Express Enzyme (1X)) and collected onto slides by a cytospin centrifuging for five minutes at 47 × g (@6000 rpm). The slides were air-dried, fixed by DiffQuick Fixative for 10 minutes and then double-stained with Stain 1 (red DiffQuick Stain) and then Stain 2 (blue DiffQuick Stain). The slides were scored as described in Fenech (2000). The chromosomal damage induced by treatment and total number of MN_i in binucleated (BN) cells totalled 1000. The slides were scored at a magnification of 250X or 40X.

The criteria used for scoring MNi, NPBs or NBUDs were those described in Fenech (2007). Cytotoxicity induced by treatment was determined, and the percentages of apoptosis and necrosis were evaluated in 500 cells and calculated according to published formulae (Fenech 2000; Kirsch-Volders et al. 2003).

3.2.8 Statistical analysis

Data were presented as the mean ± S.E.M. of the standard error. The experiments were replicated at least three independent times. Statistical analysis of the data was carried out using ANOVA, followed by Tukey's HSD post hoc test. These tests were performed using SPSS software (Version 22). Differences were considered significant when the p value was less than 0.05. Responses to treatment were compared to the untreated control (0 doses), which is represented as 100% survival.

3.3 Results

3.3.1 Cytotoxicity effects of beauty products on human skin cells

The toxicity of four beauty products on keratinocytes human skin cells (HaCaT) and normal human skin fibroblasts (CCD-1064Sk) *in vitro* was determined by incubating cells with the treatments for 1 hour. Two cytotoxicity assays were carried out to determine the toxicity of the beauty products. The MTT cell viability assay was used to determine the relative survival of cells, as yellow MTT is reduced to purple formazan in the mitochondria of living cells. The CV assay was used to determine the relative cell number, as CV stains the DNA of the live cells that adhere to the plate after the dead cells are washed away. There was significant toxicity at doses of 3% w/v and 0.3% w/v for NVAW face moisturiser (with TiO_2) after HaCaT cells were treated for 1 hour (Figure 3.1 (C)). Significant toxicity was also induced by the highest dose (3% w/v) of the NVAW face moisturiser (improved formula, without TiO_2) on human fibroblast CCD-1064SK cells, as determined by MTT and CV assays (Figure 3.22 (C)). However, no significant toxicity emerged in the HaCaT human skin cells or human fibroblast CCD-1064SK cells when using treatments of Glycerol BP or FMCGB.

The nuclear division index (NDI) is a method employed to measure the proliferative status of viable cells that can be used to assess general toxicity (Fenech 2007; Ionescu et al. 2011). Table 3.2 shows the value of NDI for all of the beauty products examined, and reveals those that had a significantly lower NDI value in the highest dose (0.3% w/v; 1.4 ($P < 0.05$) of NVAW with or without TiO_2, and 5% w/v; 1.4 ($P < 0.05$) of FMCGB) on HaCaT cells.

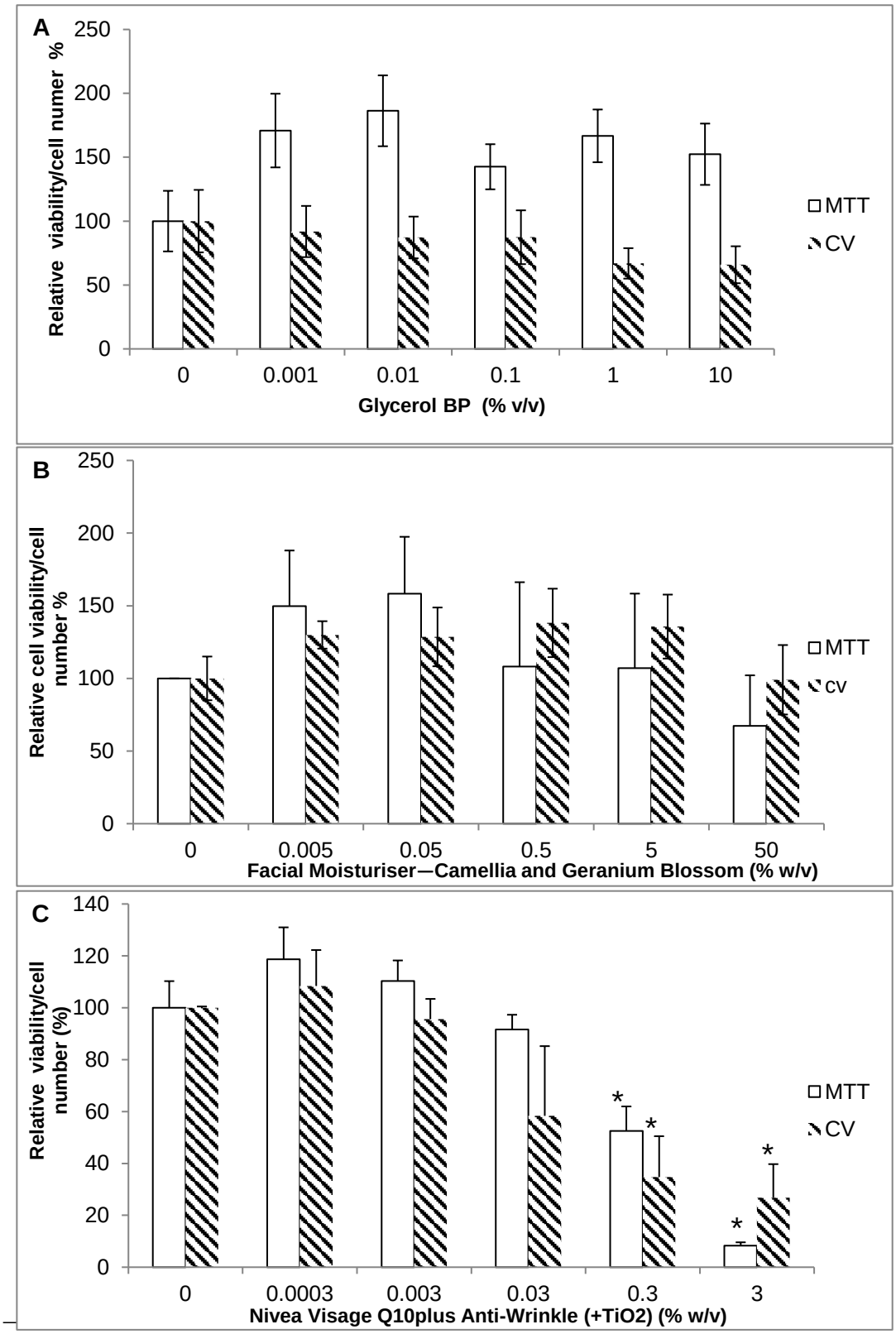

Figure 3.1: Relative cell viability and cell number (%) after treatment of HaCaT human skin cells with (A) Glycerol B.P, (B) FMCGB and (C) NVAW + TiO_2 for one hour. Relative survival was measured by the MTT assay. Relative cell number was measured by the crystal violetCV assay. Data are shown as a percentage compared to the untreated control and are the mean of three replicates ± standard error of the mean (S.EM). Treatments that differed significantly different from the untreated control at P < 0.05 are presented as '*'. This result referred into my master degree (Alnuqaydan 2011).

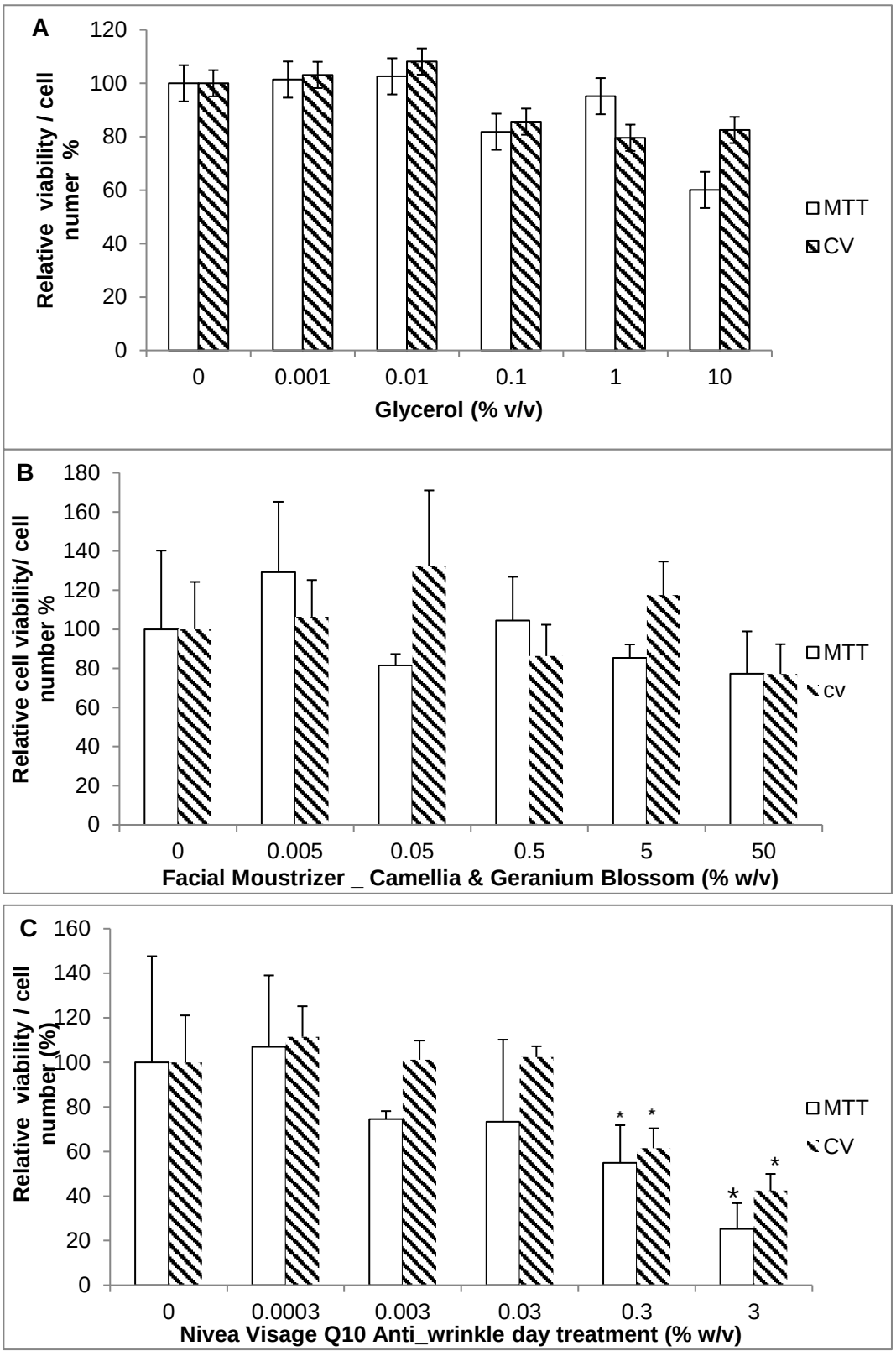

Figure 3.2: Relative cell viability and cell number (%) after treatment of CCD-1064Sk normal fibroblast human skin cells with (A) Glycerol B.P, (B) Facial Moisturizer - Camellia & Geranium Blossom and (C) Nivea Visage Q10plus Anti-Wrinkle face moisturizer (Improved formula, without TiO_2) for 1 hour. Relative survival was measured by the MTT assay. Relative cell number was measured by the crystal violet assay. Data are shown as a percentage compared to untreated control and are mean of three replicates ± standard error of the mean (S.E.M). Treatments significantly different from untreated control at P < 0.05 are presented as '*'.

Table 3.2: The NDI comparison between the untreated control (0 doses) and the beauty products. The cells were plated and described in the materials and methods section and exposed to beauty treatments for one hour. The NDI was determined by the CBMN assay. Data are shown as percentages compared to the untreated control and are the mean of three replicates ± SEM. There was a significant difference from the untreated control at *P < 0.05.

	NDI value	
Treatments	**HaCaT cell line**	**CCD_1064SK cell line**
Media control	1.8	1.6
NVAW + TiO_2 dose 0.3% w/v	*1.4	-
NVAW + TiO_2 dose 0.03% w/v	1.7	-
NVAW without TiO_2 dose 0.3% w/v	*1.4	1.5
NVAW without TiO2 dose 0.03% w/v	1.6	1.5
***FMCGB treatment* dose 5% w/v**	*1.4	1.5
***FMCGB treatment* dose 0.5% w/v**	1.6	1.6
Glycerol dose 10% v/v	1.7	1.6
Glycerol dose 1% v/v	1.8	1.6

3.3.2 Mechanism of cell killing

The CBMN results (Figure 3.3 (B)) detected a significant increase in late apoptosis and early necrosis induced by the highest dose of NVAW with TiO_2 0.3% w/v and significantly induced by the 0.3% w/v and 0.03% w/v doses of NVAW without TiO_2 after the HaCaT cells were treated for one hour. Conversely, no significant apoptosis or necrosis induction was observed in HaCaT cells treated with a natural facial treatment (FMCGB) and Glycerol BP, as shown in Figure 3.3 (A).

NVAW without TiO_2 induced significant late apoptosis and early necrosis on fibroblast cells (CCD-1064SK) at a dose of 0.03% w/v and significant early necrosis was induced by FMCGB at a dose of 5% w/v on CCD 1064SK, as shown in Figure 3.4 (see appendix VIII for photomicrograph of the cells scored in the CBMN assay). However, no significant induction of apoptosis or necrosis was observed in CCD-1064SK cells treated with Glycerol BP. This result was consistent with the previous result of necrosis induced by NVAW at dose 3% w/v, as detected by the apoptosis assay followed flow cytometry (Alnuqaydan 2011).

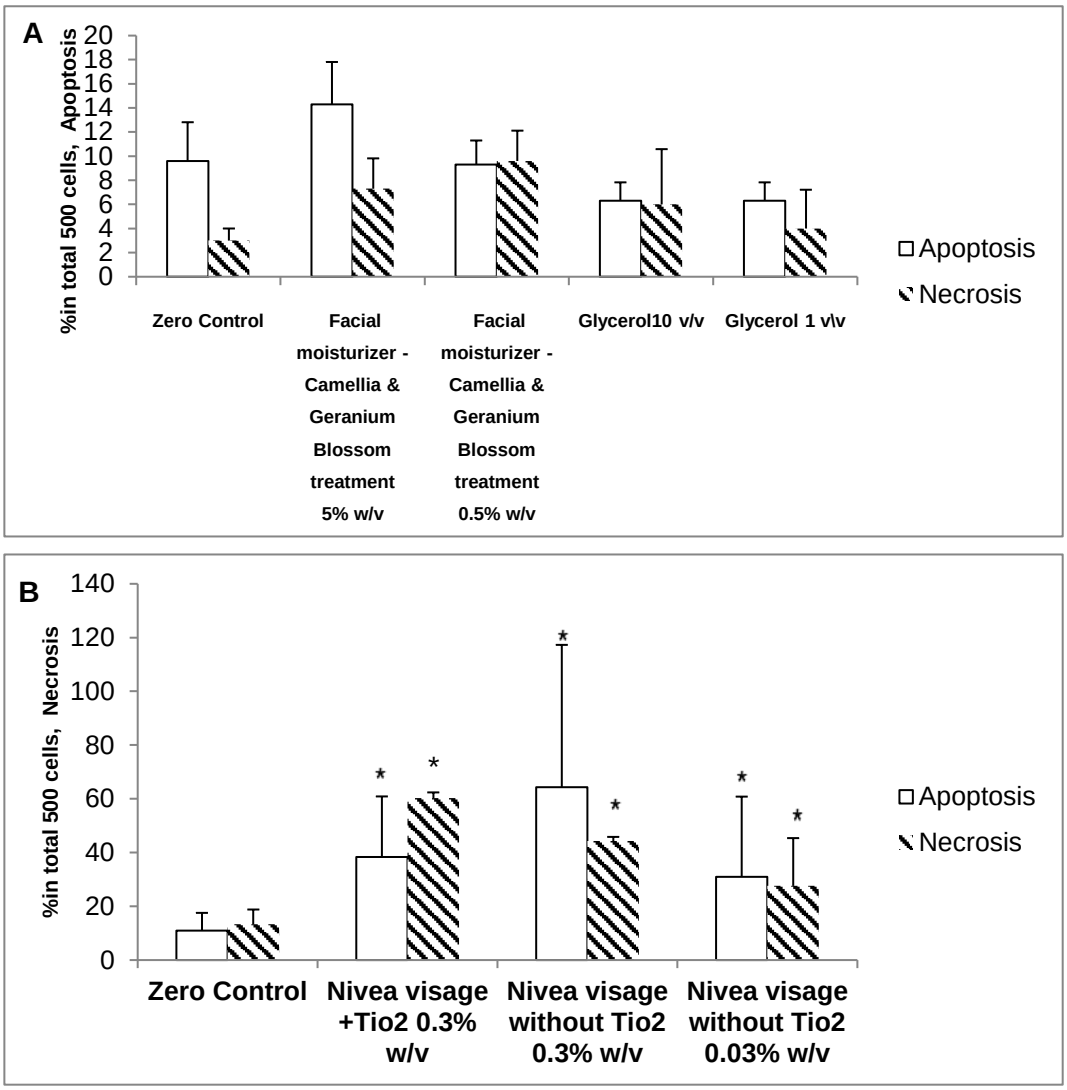

Figure 3.3: Apoptosis and necrosis induction detected by the CBMN assay for HaCaT cells followed by one hour of treatment using (A) Facial natural treatment (FMCGB) and Glycerol BP and (B) using Nivea Visage Q10plus Anti-Wrinkle face moisturizer with TiO_2 and Nivea Visage Q10plus Anti-Wrinkle face moisturizer (Improved formula, free of TiO_2). Treatment data are shown as the mean of three observations ± SEM. Treatments significantly different from untreated control at $P < 0.05$ are presented as '*'.

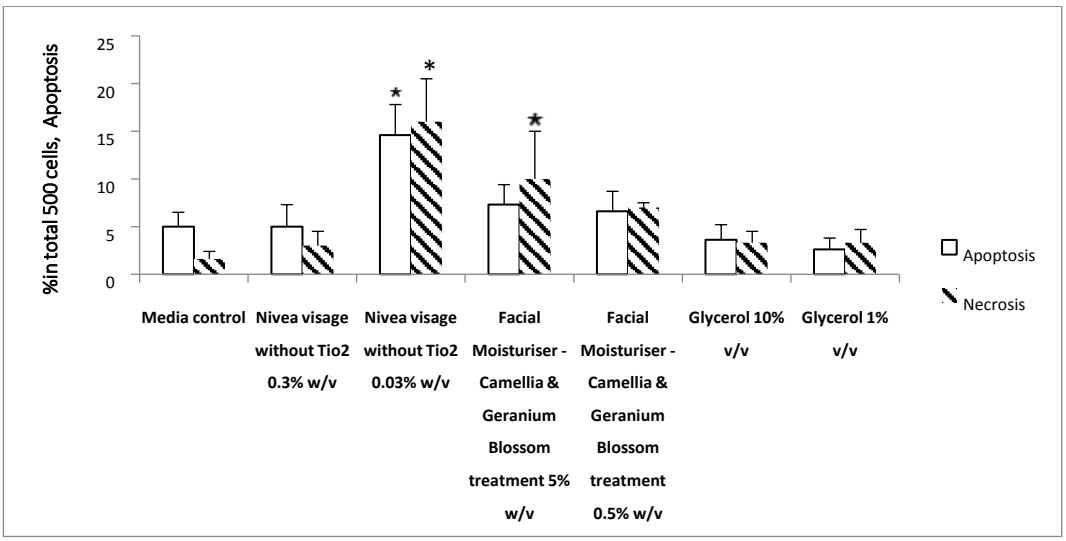

Figure 3.4: Apoptosis and necrosis induction detected by the CBMN assay for CCD_1064SK cells followed by 1h treatment using NVAW (improved formula, without TiO_2 0.3% w/v and 0.03% w/v and FMCGB 5% w/v and 0.5% w/v and Glycerol BP 10% v/v and 1% v/v. Data are shown as the mean of three observations ± SEM. Treatments significantly different from untreated control at P < 0.05 are presented as '*'

3.3.3 Genotoxicity of beauty products on human skin cells

The genotoxicity of beauty products on HaCaT human skin cells and CCD-1064SK cells was assessed using a CBMN assay. The following measures of genotoxicity were used: chromosome breakage and chromosome loss (MNi), chromosome rearrangement (NPB) and gene amplification (NBUDS) (Fenech 2008). The frequency of the induced MNi indicates the extent of chromosomal changes induced by these beauty products. The result of the CBMN assay showed the genotoxic effects of these beauty products on HaCaT cells as shown in Figure 3.5. No significant increase in MNi was observed in the results of the HaCaT cells treated with Glycerol BP and FMCGB (a natural product) for one hour (Figure 3.5 (A)). However, there was significant increase in the number of MNi at a 0.3% w/v dose of NVAW + TiO_2. Figure 3.5 (B) also showed the result of HaCaT cells treated with two different formulae of the NVAW product (with and without TiO_2). There was a significant difference between the effects of the two Nivea products on HaCaT cells. The effects of NVAW with TiO_2 differed significantly from that of NVAW without TiO_2 and from the untreated control. This means that NVAW + TiO_2 is significantly genotoxic to HaCaT cells, while the product without TiO_2 was less so. NPB and NBUDs were also observed in NVAW + TiO_2 and FMCGB (a natural product), but these did not reach a significant level. A significant increase in MNi in CCD-1065SK

cells was observed at doses of 0.3% w/v and 0.03% w/v of NVAW (improved formula, without TiO_2) after treatment (Figure 3.6). Other treatments demonstrated an increase in the number of MNi, but these did not reach a significant level (Figure 3.6). NPB and NBUDs were not observed in all of the CCD-1065SK cells treated with the products.

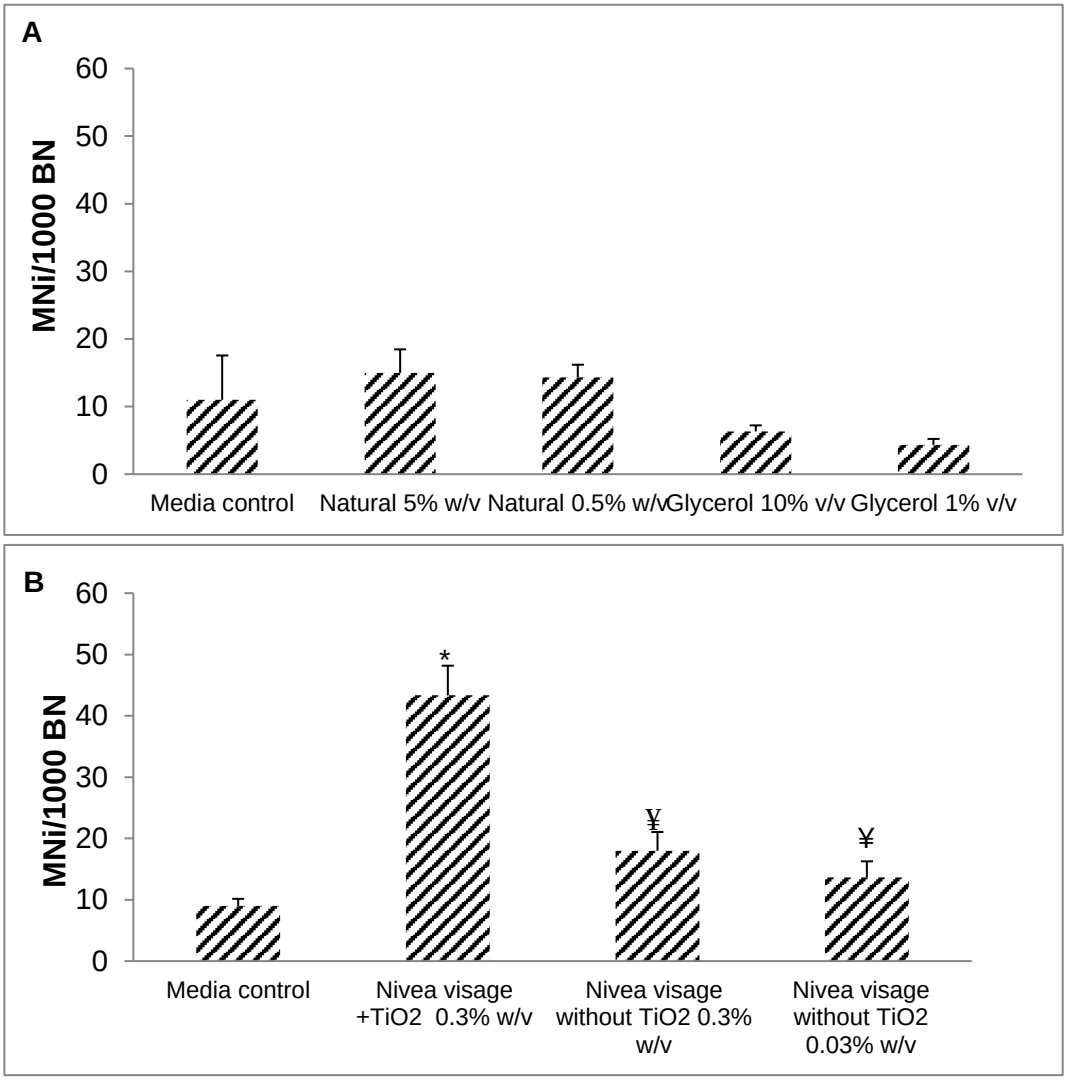

Figure 3.5: Frequency of MNi BN cells as measured by the CBMN assay following exposure of HaCaT cells to (A) Glycerol BP and FMCGB (natural product) and (B) NVAW + TiO_2 and NVAW (improved formula, without TiO_2) treatment. The data are the mean ± SEM. from three separate experiments. Treatments significantly different from the untreated control at P < 0.05 are presented as '*' or '¥'. Treatments differed significantly from NVAW + TiO_2.

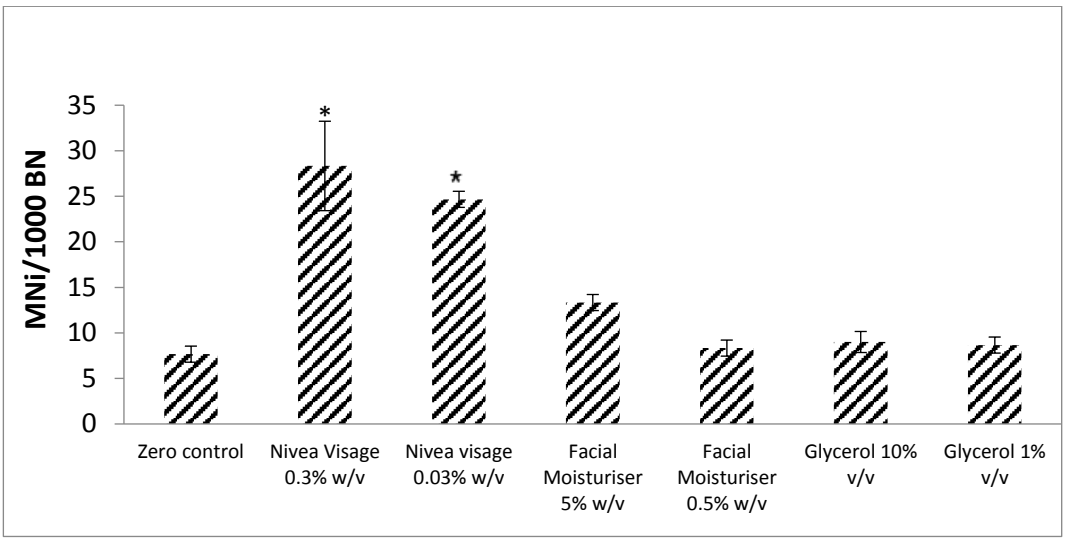

Figure 3.6: The frequency of MNi per 1000 BN cells was determined using the CBMN assay following exposure of CCD-1065SK cells NVAW (improved formula, without TiO_2) treatment, Glycerol B P, FMCGB (natural product). The data are the mean ± SEM. from three separate experiments. Treatments significantly different from untreated control at P < 0.05 are presented as '*'.

3.4 Discussion

In this study, two normal human skin cell lines were used to examine the toxicity and genotoxic effects of beauty products *in vitro*. The human keratinocyte cell line (HaCaT), which is derived from full epidermal differentiation capacity, functions as the outermost layer of the skin (Hughes & Edwards 2010; Krejčí et al. 2014; Schoop et al. 1999). Human dermal fibroblast cells CCD-1046 within the dermis are responsible for generating connective tissue (Krejčí et al. 2014; Kopanska et al. 2013).

Glycerol BP served as a negative control in this study because it is a common basic ingredient in many moisturisers. There was no cytotoxic effect on HaCaT or CCD 1064SK cells after being exposed for one hour to Glycerol BP. The NDI obtained from the CBMN assay provides a measure of cell division (Sugisawa & Umegaki 2002). There was no significant change in the NDI value, reflecting the fact that Glycerol BP did not affect the cell cycling in either cell line**.** Further, the evaluation of apoptosis and necrosis of Glycerol BP on human skin cells detected by the CBMN assay showed no significant difference from the untreated control. In addition, the no micronucleated binucleate (MNi), NPBs and NBUDs were observed per 1000 BN cells, indicating that no genetic damage occurred after treatment with glycerol. Therefore, glycerol, which is used in several cosmetic products, is a safe ingredient use.

The FMCGB treatment used in this study contains a mixture of natural ingredients, listed on the product label. It showed no significant toxicity on CCD-1064SK cells

compared to the untreated control. Similarly, no cytotoxicity was observed on HaCaT cells treated with FMCGB for one hour (Figure 3.1). Based on the result of the CBMN assay, the NDI revealed a significant decrease at a dose of 5% w/v, with a lower NDI value of 1.4 ($P < 0.05$) in HaCaT cells. This means that a change occurred in the rate of cell cycling—they either took longer to divide, or the viable cells failed to divide during the cytokinesis-block (Fenech 2007). No apoptosis or necrosis induction was observed after the HaCaT cells were treated with FMCGB for one hour at both doses. However, a small but statistically significant amount of necrosis was observed in the CCD-1064Sk cells at the higher (5% w/v) dose of the FMCGB after being treated for 1 hour.

The CBMN result indicated that no chromosomal damage occurred in human skin cells after being treated for one hour with FMCGB.

Although FMCGB did not demonstrate significant toxicity or genotoxicity on human skin cell lines, this does not mean it is a safe product to use. The change in NDI value indicates a decrease in the cell cycle of 1.4 ($P < 0.05$) on the HaCaT cell line. Significant necrosis was also observed after fibroblast cell lines were treated with FMCGB for one hour at a dose of 5% w/v.

Green or botanical products are not well-regulated by government agencies. It is advised to avoid products that use essential oils such as lavender oil or TTO, which are classified as hormone disruptors (see chapter 4) (Alnuqaydan & Sanderson 2016; Epstein & Fitzgerald 2009)

TiO_2 is a nanoparticle classified as a carcinogen (Trouiller et al. 2009). TiO_2 plays a role in the induction of apoptosis, as well as oxidative stress. Moreover, studies have shown that TiO_2 causes genetic damage linked to DNA-adduct formation in human lung cells (Bhattacharya et al. 2009; Park et al. 2008; Reeves et al. 2008). The metabolic effects of TiO_2 on keratinocytes HaCaT cells are also being investigated. One study indicated that TiO_2 affects the mitochondria (Tucci et al. 2013). Another study using transmission electron microscopy and flow cytometry demonstrated a significant uptake of TiO_2 in keratinocytes human skin cells (HaCaT) (Shukla et al. 2011).

The current study compared NVAW + TiO_2 to an identical product without TiO_2, allowing for an evaluation of the effect of the TiO_2. The result of the MTT and CV showed significant toxicity, with the two doses of NVAW+TiO_2 having killed up to 76% and 92% of cells after 1 hour of exposure to doses of 0.3% and 3% (w/v), respectively (Figure 3.1).

The mechanism of cell death was elucidated using the CBMN assay. A dose of 0.3% w/v induced significant levels of late apoptosis and early necrosis on HaCaT human skin cells (Figure 3.3 (B)). A significantly low NDI value (1.4 ($P < 0.05$)) was also observed at the 0.3% w/v dose (Table 3.2). This means that the cells took a longer time to divide. NVAW + TiO_2-induced genetic damage manifested as chromosomal damage was determined by a CBMN assay at a frequency significantly higher (43 MNi/1000 binucleated cells, n=3) than the background frequency (media alone control; MNi range = 9 MNi/1000 binucleated cells, n = 3). NPBs and NBUDs were also observed. These outcomes illustrate some of the mechanisms of chromosomal damage when using the NVAW product**.** The frequency of chromosomal rearrangement is indicated by the NPB and NBUDs. An NPB may arise from di-centric chromosomes and NBUDs from gene amplification (Fenech 2007). A di-centric chromosome and an a-centric chromosome fragment are formed as a result, and they manifest as an NPB and MNi (Fenech 2007). The formation of NPBs could lead to the misrepair of DNA strand breaks, which could also lead to a di-centric chromosome and concatenated ring chromosome. One di-centric chromosome mechanism could result in telomere-end fusion, which is caused by the shortening or loss of the telomere capping protein (Fenech 2007). This study is consistent with that conducted by (Dunford et al. 1997), which demonstrated that sunscreens containing TiO_2 can catalyse oxidative damage to DNA *in vitro* and in human cell cultures.

NVAW (improved formula, without TiO_2) showed significant toxicity on CCD-1064SK at doses of 3% and 0.3% w/v as measured by the MTT and CV assays. The mechanism of cell death scored by the CBMN assay (Figure 3.4) showed there was significant induction of apoptosis and necrosis at a dose of 0.03% w/v on CCD-1064 cells.

Genetic damage detected by the CBMN assay included a significant increase in MNi (28.3 MNi/1000 binucleated cells, n = 3) ($P < 0.05$) in CCD_1064SK cell lines. However, there was no significant increase in MNi in the HaCaT cells (Figure 3.5 (B)). A significantly low NDI value (1.4 ($P < 0.05$)) was only observed in HaCaT cells at a 0.3% w/v dose (Table 3.2). This means that the HaCaT cells took a longer time to divide after being treated with NVAW (improved formula, without TiO_2) for one hour. NPBs and NBUDs were not observed in the CCD-1064SK cells.

This product contains the same mixture of chemical ingredients as reported in Table 3.1, except TiO_2, which was removed from the improved formula. Therefore, although none of these chemicals are known to be carcinogenic individually, it is clear that the mixture has shown carcinogenicity (Zeliger 2008a). It is hypothesised that chemicals

in mixtures could interact with each other and become carcinogenic. A brain cancer cluster study concluded that different chemical mixtures can induce the same cancer types despite using different mechanisms, even if none of the chemicals are known to cause brain cancer individually (Zeliger 2008a).

Interestingly, NVAW + TiO_2 proved to induce significantly more MNi than the product without TiO_2. As mentioned earlier, this difference is compatible with findings that TiO_2 enters the nucleus and cytoplasm of keratinocytes, causing oxidative stress damage to DNA (Shukla et al. 2011).

Consumers using the NVAW product are in fact exposed to an undiluted product with the potential to cause long-term damage. Carcinogens in cosmetics and personal care products are potentially greater cancer risks than food contaminated with industrial carcinogens or pesticides because chemicals ingested through the mouth are absorbed by the intestines and pass into the venous blood. These chemicals are then transported to the liver which exists to detoxify these substances to varying degrees with enzymes before they can reach the rest of the body (Epstein & Fitzgerald 2009). However, carcinogens absorbed by the skin can bypass the liver and circulate through the bloodstream, thus reaching every organ in the body (Epstein & Fitzgerald 2009; Thomas 2008).

In conclusion, the current study has shown the possible harmful effects of several beauty products on normal human skin cells *in vitro*. In particular, the anti-aging face moisturiser, which has a synthetic chemical product (NVAW + TiO_2), induced the highest toxicity and genotoxicity of all the beauty products tested. Further, NVAW without TiO_2 also induced significant toxicity and genotoxicity on human fibroblast CCD-1064Sk cells. Conversely, the face moisturiser containing natural ingredients (FMCGB) was a relatively less toxic product than other beauty products, and Glycerol BP (the negative control) showed no toxic effect in either human cell line. Further investigation could be done to study specific chromosomal damage occurring due to NVAW using fluorescence in situ hybridisation. More work could also be done to understand the underlying mechanism of the effects of FMCGB on the NDI.

CHAPTER 4: GENETIC DAMAGE AND CELL-KILLING INDUCTION BY FIVE HEAD LICE TREATMENTS ON HACAT HUMAN SKIN CELLS

This chapter is a reproduction of the article published as Alnuqaydan, AM & Sanderson, BJS 2016, 'Genetic damage and cell killing induction by five head lice treatments on HaCaT human skin cells', *Journal of Carcinogenisis and Mutagenesis*, vol. 7, no. 2, pp. 1–6.

4.1 Introduction

Head lice infections in children, particularly those of primary school age, are an important public health issue. Reports and surveys relating to head lice infestations present them as existing in epidemic proportions. They are common among children aged between three and 12 years old in many countries. In Australia, for example, a survey of 25000 school children indicated that 48% (44/92) of long day care centres in Western Sydney reported head lice outbreaks (Jorm & Capon 1994), compred to 13% in Victoria and only 2% in Tasmania (Counahan et al. 2004). The prevalence of head lice ranges from 30% to 45% in Brazil, and is 14% in Chinese schools (Fan et al. 2004; Heukelbach et al. 2005). In the United Kingdom, only 2% of school age children are infected (Harris et al. 2003). In the US, infestation with head lice is most common in younger children in primary and elementary schools, and there are up to 12 million infestations of head lice every year (Frankowski et al. 2010b).

Parents prefer chemical treatments to treat their children due to their rapid removal of head lice. Head lice treatment based on pesticides can be absorbed through the human skin. Early exposure of children to pesticides from neonate to 7 years old can affect their neurodevelopment system (Counahan et al. 2004; Landrigan & Goldman 2011; LICE 2008; Menegaux et al. 2006; Roberts et al. 2012). Children are more sensitive to being affected and absorb a greater proportion of a given dose of a chemical than an adult (Gilbert 2004; Landrigan & Goldman 2011). Even a small dose can affect children's health and behaviour and influence their future development (Gilbert 2004). Pesticide exposure, including insecticides, has been correlated with children's leukaemia (Menegaux et al. 2006). Head lice treatments restricted due to their side effects include malathion, which is not recommended and is restricted in Canada, and Lindane which is banned in California (Frankowski et al. 2010b; LICE 2008). DDT has been banned because it is toxic to people and persists in the environment (Downs et al. 1999; Gilbert 2004). Permethrin can cause eye and skin irritation despite being not very toxic to humans (Diel et al. 1998). Malathion is available as a head lice treatment only by prescription, as it has been taken off the market twice in the US due to its flammability and odour (Devore et al. 2015). Pyrethrum (pyrethrins plus piperonyl butoxide) is neurotoxic to lice but it can be absorbed through the gastrointestinal tract and the pulmonary route (Devore et al. 2015).

Other head lice treatment products contain approximately 10% TTO (melaleuca oil) and 1% lavender oil. TTO acts as a human poison if ingested and human poisoning cases were registered in 2003 by the American Association of Poison Control Centres

(Hammer et al. 2006). A high concentration of TTO and lavender oil could result in skin irritations or burns (Bozhanov et al. 2007; Hammer et al. 2006). Pyrethrins are neurotoxic to lice, but have a reportedly low toxicity to mammalian cells. However, people who are sensitive to ragweed or chrysanthemums are allergic to pyrethrins (Frankowski et al. 2010b). This study examines the cytotoxicity and genotoxicity of five currently used chemical-based head lice treatments and monitored their effects using an *in vitro* human skin cell model.

4.2 Material and methods

4.2.1 Materials

RPMI-1640 media and FBS were purchased from Gibco® Cell Culture Media—Life Technologies (Australia). Cytochalasin B (Cyt-B) solution, SDS (approximately 99%), PBS and MTT were purchased from Sigma-Aldrich (US). A 96-plate reader was purchased from BIO-TEK Instruments Inc., US; Diff-Quik stains were purchased from Lab Aids (Australia); a cytospin centrifuge was purchased from Shandon, England; an Annexin V-FITC Apoptosis Detection Kit was purchased from BD Biosciences (Australia) and TrypLE™ was purchased from Life Technologies (Australia). All other reagents were obtained from Sigma, unless otherwise stated.

4.2.2 Head lice treatments

Three different groups of products are currently used to treat head lice: synthetic pyrethroids such as permethrin; the pyrethrins group, such as pyrethrum; organophosphates, such as maldison and herbal and essential oils such as TTO and lavender oil. Any head lice treatment product released onto the Australian market must be registered with the Australian Register of Therapeutic Goods. The head lice treatments for which a prescription is not required were obtained from a local chemist. This study examined the following treatments: Lice Breaker (permethrin 1% w/w), KP24 (maldison 0.5 w/w), TTO (100% pure), lavender oil (100% pure) and pyrethrum (4g/l pyrethrins plus 16g/L piperonyl butoxide).

4.2.3 Cell treatment

The 96-well flat bottom microplate was seeded with 10^4 cells/well and incubated for 19 hours to allow the cells to adhere at 37°C in 5% CO_2. The media were aspirated and replaced with 100 µl of the treatment solution per well and were treated for one hour prior to the bioassays. The treatment solutions included serial dilutions of different head lice treatments. The negative or untreated control (0 dose) served as the media. The two doses of head lice treatments used in the CBMN assay (Table 4.1) were diluted from the commercial product to be at least two orders of magnitude

lower in concentration. Therefore if the product is used as instructed (i.e., undiluted) to treat head lice, it will be at concentrations at the same order of magnitude or higher at which it is genotoxic and toxic *in vitro*. The choice of one hour as the duration of the cell treatment is because the recommended treatment time is 20 minutes to one hour. If any head lice are found alive in the next morning a different head lice treatment should be used for a more few hours to prevent the head lice from developing a resistance.

4.2.4 Cell line and cell culture

A human non-cancer keratinocytes cell line, HaCaT, was obtained from ATCC Cell Lines (US). It was maintained in RPMI-1640 medium with 10% FBS and 1% penicillin/streptomycin (Thermo Scientific, Australia). Cells were seeded in tissue culture flasks and incubated in a fully humidified incubator at 37°C in 5% CO_2. The HaCaT cells were subcultured when they reached 60–80% confluence.

4.2.5 Crystal violet assay

The cytotoxicity (relative cell number) of head lice treatments was monitored by a CV assay (subsection 3.2.5).

4.2.6 Methyl tetrazolium cytotoxicity assay

The cytotoxicity of head lice treatments was monitored (relative cell viability) by an MTT assay (subsection 3.2.6).

4.2.7 Apoptosis assay via flow cytometry

Annexin V-FITC apoptosis detection kit I (BD Biosciences, US) was carried out and flow cytometry was used for measuring. After the treatment, the cells were washed twice with 0.1% sodium azide in PBS and then centrifuged at 78g (1200 rpm) for five minutes. The pellets were resuspended in 50µl binding buffer. Cells were double-stained with 2.5 µl of annexin V-FITC and 2.5 µl of PI. After 15 minutes of incubation in the dark at room temperature, 200 µl of a binding buffer was added to the mixture. The intensity of annexin V-FITC and PI was recorded by BD Accuri™ flow cytometry and analysed with BD CFlow® Plus Software.

4.2.8 Cytokinesis-block micronucleus assay

The genotoxicity of head lice treatments was monitored by a CBMN assay (subsection 3.2.7).

4.2.9 Data Analysis

Data are presented as the mean ± SEM. The results of the experiments were replicated in at least three separate experiments Statistical analysis was carried out using ANOVA including Tukey's HSD post hoc test (version 22). The significance was determined at a *P* value of < 0.05. The cell responses to treatment were compared to the untreated control (0 doses), which is represented as 100% survival.

4.3 Results

4.3.1 Cytotoxic effects of head lice treatments on HaCaT human skin cells

The toxicity of head lice treatments on HaCaT human skin cells *in vitro* was determined by incubating cells with the treatments for one hour. Two cytotoxicity assays were carried out to indicate the toxicity of the head lice treatments. The relative viability of cells was determined using the MTT cell viability assay. Relative cell number was measured using the CV assay. Permethrin (Lice Breaker) and maldison (KP24) showed no significant toxicity, as shown in Figures 4.1 (A and B). However, significant toxicity was induced by the two highest doses (100 and 1000 $\times 10^{-4}$ v/v) of the TTO and pyrethrum treatments (see Figures 4.2 (A) and 4.3). Lavender oil also induced a significant level of toxicity, but only at the highest dose (1000 $\times$ 10^{-4} v/v, Figure 4.2 (B). The NDI is a method used to measure the proliferative status of viable cells to assess general toxicity (Fenech 2007; Ionescu et al. 2011). Figure 4.4 shows the results of the NDI of head lice treatments as compared to the untreated control. The NDI values were: untreated control (0 dose) 1.66, permethrin (100 $\times$ 10^{-4} (v/v)) 1.46 ($P < 0.05$) and (10 $\times$ 10^{-4} (v/v)) 1.37 ($P < 0.01$), maldison (50 $\times$ 10^{-4} (v/v)) 1.52 and (5 $\times$ 10^{-4} (v/v)) 1.52, TTO (5 $\times$ 10^{-4} (v/v)) 1.46 and (1 $\times$ 10^{-4} (v/v)) 1.49, lavender oil (10 $\times 10^{-4}$ (v/v)) 1.54 and (5 $\times$ 10^{-4} (v/v)) 1.54 ($P < 0.05$) and pyrethrum (pyrethrins plus piperonyl butoxide) (50 $\times$ 10^{-4} (v/v)) 1.53 and (10 $\times$ 10^{-4} (v/v)) 1.57.

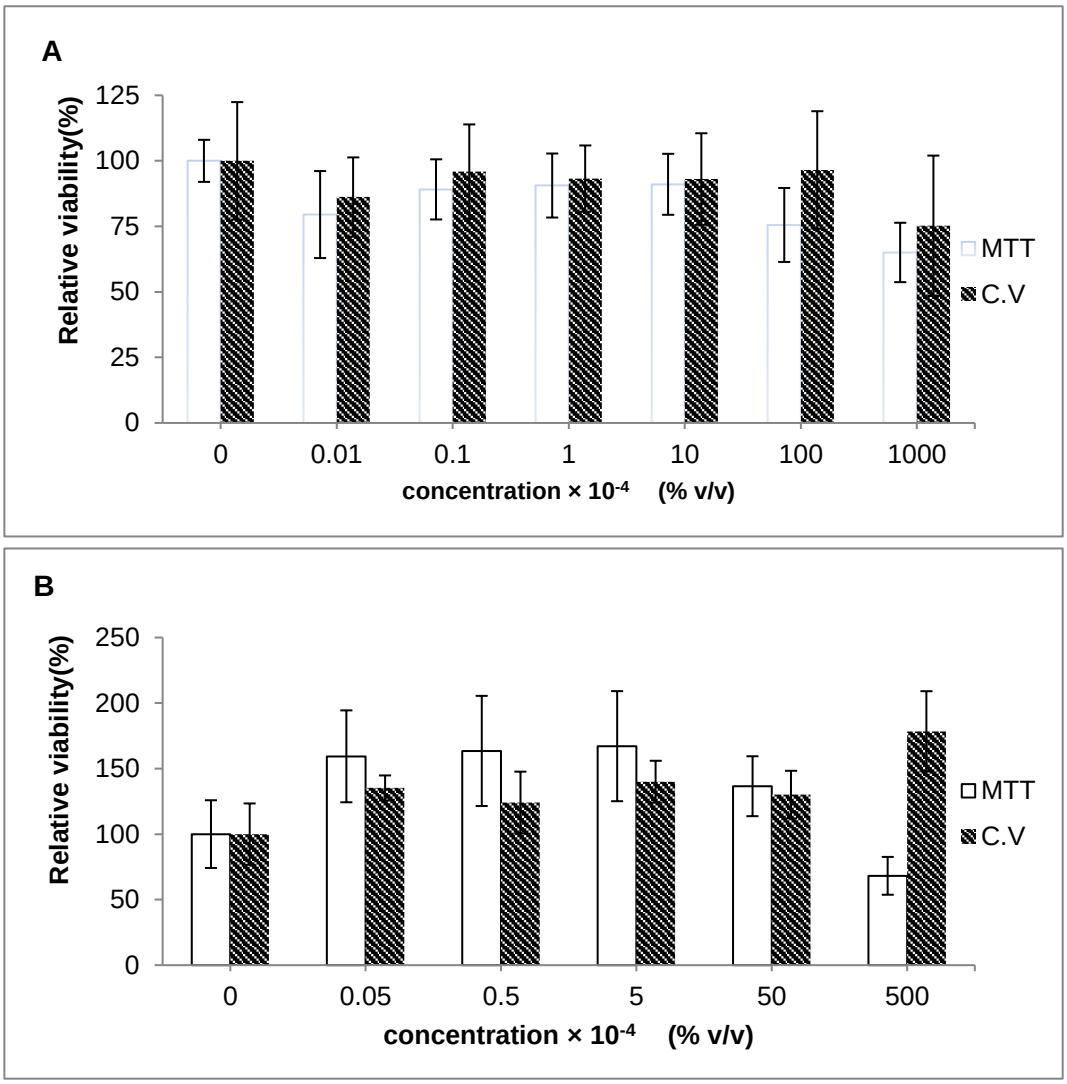

Figure 4.1: Survival relative to the untreated control, determined by the MTT assay. Relative cell number determined by Crystal Violet assay. Cells were plated in microplates at 10,000 cells/well then treated with (A) Permethrin 1% v/v and (B) Maldison 0.5 w/w for 1h. Data are shown as percentage relative to untreated control and are mean of four replicates ± S.E.M. Three replicates referred to my master's degree (Alnuqaydan 2011).

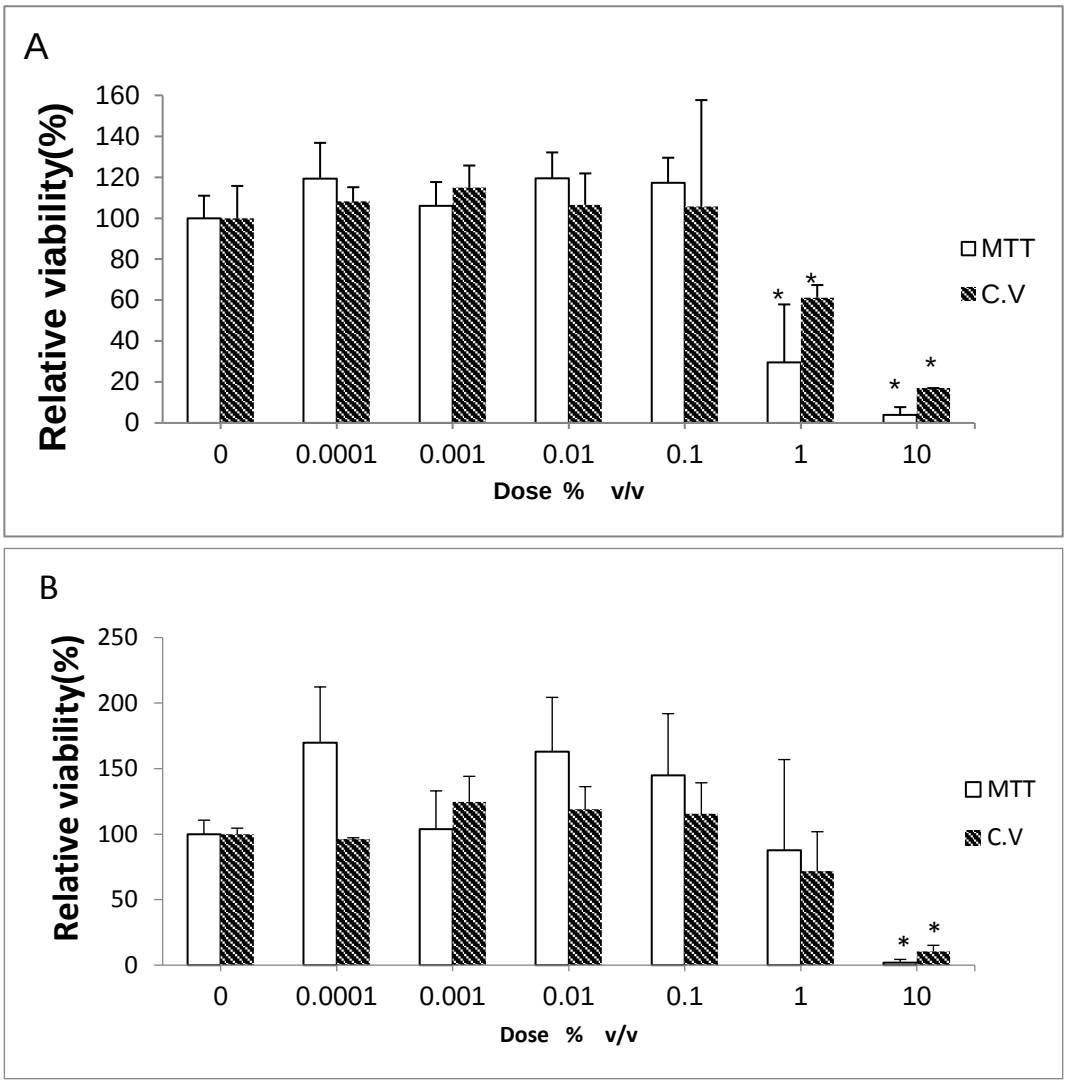

Figure 4.2: Survival relative to the untreated control, determined by the MTT assay. Relative cell number determined by crystal violet assay. The cells were plated in microplates at 10,000 cells/well then treated with (A) tea tree oil (TTO) (100%) and (B) lavender oil (100%) for one hour. *Significant difference at P < 0.05. Data are shown as percentage relative to untreated control and are mean of four replicates ± SEM. The three replicates refer to my master's degree (Alnuqaydan 2011).

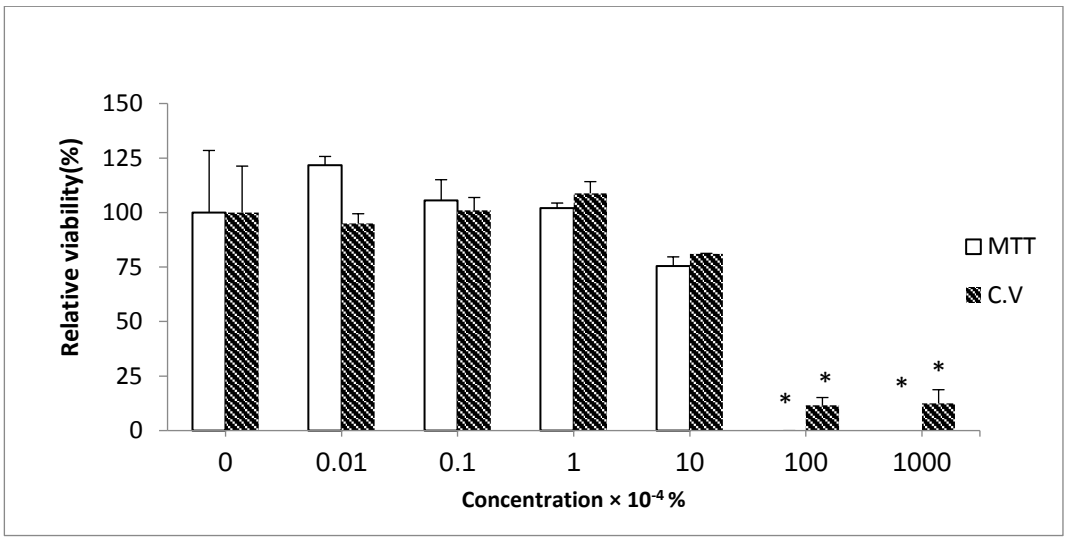

Figure 4.3: Survival relative to the untreated control, determined by the MTT assay. Relative cell number determined by the CV assay. The cells were plated in microplates at 10,000 cells/well then treated with pyrethrum (pyrethrins plus piperonyl butoxide) for one hour. Data are shown as percentages relative to the untreated control and are the mean of four replicates ± SEM.

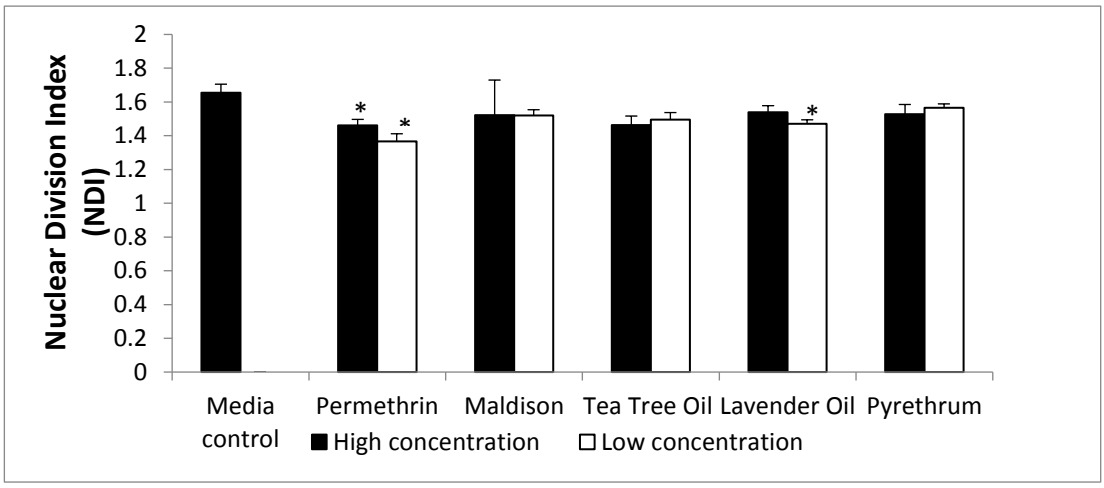

Figure 4.4: NDI comparisons between the untreated control (0 dose) and head lice treatments. The HaCaT cells were plated as described in materials and methods section. The cells received head lice treatments for one hour. NDI was determined by the CBMN assay. Data are shown as percentages compared to the untreated control and are the mean of three replicates ± SEM. Significant difference at *P < 0.05, **P < 0.01.

4.3.2 Mechanism of cell killing

The mechanism of cell death induced by head lice treatments for HaCaT cells for one hour is shown in Table 4.2. Induced apoptosis and necrosis were determined by CBMN assay and flow cytometry assay. A flow cytometric assay was used to differentiate the live cells population from early apoptotic and late apoptotic/necrotic cell populations (Jia & Misra 2007). The CBMN assay identified late apoptotic and early necrotic cells (Fenech 2007)., and also detected the induction of a significant increase in late apoptosis induced only by the highest dose of maldison (0.5 w/w). However, the flow cytometry results observed significant early apoptosis induced by

high doses of permethrin, TTO, pyrethrum (pyrethrins plus piperonyl butoxide) and both tested doses of lavender oil. Significant necrosis was detected by the CBMN assay with a high dose of permethrin and low dose of maldison 0.5 w/w. Flow cytometry indicated that significant necrosis was induced by both doses of permethrin and high doses of maldison 0.5 w/w, lavender oil and pyrethrum (pyrethrins plus piperonyl butoxide).

Table 4.1: Doses of head lice treatments use in the flow cytometry and CBMN assay

Product	Dose × 10^{-4} (V/V)	Code for Table 2 and Figures 4.4 and 4.5
Control		0 (zero)
Permethrin	100	Low
	10	High
Maldison	50	Low
	5	High
TTO	5	Low
	1	High
Lavender	10	Low
	5	High

Table 4.2: Cell-killing mechanism of head lice treatments determined by the CBMN and flow cytometry-based apoptosis/necrosis assays. The dose code is taken from Table 4.1.

		Apoptosis		Necrosis	
Product	**Concentration**	**CBMN**	**Flow cytometry**	**CBMN**	**Flow cytometry**
Control	0	6.3 ± 3.4	1.2 ± 0.3	5.3 ± 1.3	1.7 ± 0.8
Permethrin	Low	25.3 ± 3.4	1.0 ± 0.0	40.6 ± 14.31	*3.7 ± 0.4
	High	24.7 ± 7.4	*3.6 ± 0.0	*57.3 ± 4.91	*5.4 ± 0.6
Maldison	Low	7.0 ± 1.2	1.1 ± 0.4	*61.3 ± 11.46	0.9 ± 0.3
	High	*27.6 ± 3.2	1.1 ± 0.4	32.6 ± 4.25	*1.3 ± 0.2
TTO	Low	22 ± 12.9	1.3 ± 0.2	14.3 ± 6.36	1.4 ± 0.1
	High	23.6 ± 9.6	*6.7 ± 0.2	15.3 ± 2.96	13.3 ± 7.0
Lavender Oil	Low	6.6 ± 1.20	*5.6 ± 1.2	7.6 ± 4.17	6.7 ± 1.7
	High	6.0 ± 3.21	*4.6 ± 1.2	4.3 ± 1.76	*16.3 ± 5.0
Pyrethrum	Low	9.3 ± 5.23	1.4 ± 0.6	18 ± 6.66	2.3 ± 0.3
	High	9.6 ± 5.24	*6.5 ± 0.6	11 ± 1.52	*4.4 ± 0.4

Significant difference at *P < 0.05. Data are shown as the mean of three observations ± SEM. For flow cytometry, 3 × 10^5 cells were scored for each treatment population analysed. In the CBMN assay, an average of 400 cells were scored.

4.3.3 Genotoxicity of head lice treatments on HaCaT human cells

The values of genotoxicity are given in Figure 4.5. The dose code is from Table 4.1. A significant increase in MNi was observed in the high dose of permethrin and both doses of maldison. The other treatments showed an increase in the number of MNi, but this did not reach significance. NPB and NBUDs were not observed after the HaCaT cells were exposed to head lice treatments for one hour. NPB can arise from the misrepair of strand breaks in DNA that may lead to di-centric chromosomes, and NBUDs can be due to gene amplification (Fenech 2007).

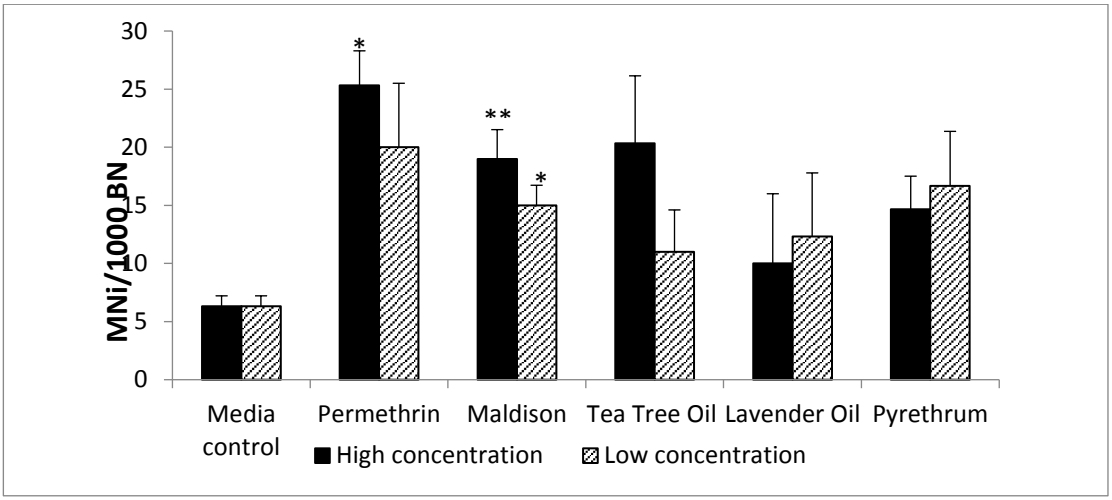

Figure 4.5: HaCaT cells were plated as described in the materials and methods section and exposed to head lice treatments for one hour. The frequency of MNi per 1000 binucleated cells (BN = BN cells) was determined using the CBMN assay as described in the methods section. Data are shown as the mean ± SEM; n = 3.

4.4 Discussion

Children are the most sensitive sub-group in the population to the effects of chemical exposure, even the smallest doses (Gilbert 2004; Landrigan & Goldman 2011). The head lice treatments examined in this study are common treatments used after the identification of head lice. Few studies have analysed the active ingredients of head lice treatments.

Permethrin (commercial concentration 1% w/w) is reported to be the least toxic to humans; however, it still shows adverse effects, such as itching and superficial reddening of the skin (Frankowski et al. 2010a). Animal studies have demonstrated the toxicity of permethrin in cats, with clinical signs that appear within hours of exposure (Dymond & Swift 2008). The current study found significant levels of HaCaT

cells in early apoptosis and late apoptosis or necrosis following treatment with permethrin. The increase in these endpoints implies that permethrin induced some level of cell death *in vitro*. Necrotic cell death *in vivo* can be associated with inflammation, which would explain the skin redness observed in some cases of the use of this product. Conversely, although the two bioassays showed decreases in viability as the doses increased (Figure 4.1 (A)), these did not reach significance. One indicator of general toxicity is NDI, a method for measuring the proliferative status of viable cells. Permethrin showed a significantly lower NDI value for 100×10^{-4} (v/v) dose; 1.46 ($P < 0.05$) and 10×10^{-4} (v/v) dose; 1.37 ($P < 0.01$) than the control (cells grown/treated with media). 1.0 is the lowest NDI value possible; this can occur if all of the viable cells have failed to divide during the period of the cytokinesis-block period of the assay (Fenech 2007). Therefore, it is clear that permethrin has a broadly toxic effect, and the amount of division of the remaining viable cells decreased dose-dependently after treatment. Permethrin also had a genotoxic effect, as it induced a significant increase in MNi (Figure 4.5). MNi can arise from either complete chromosome loss or a-centric chromosome fragments (Fenech 2007). This type of genetic damage has been linked to carcinogenesis (Fenech 2007; Ionescu et al. 2011).

Maldison (malathion) (commercial concentration 0.5% w/w), which is an organophosphate, was restricted in the US market in 1986 due to inflammation-related issues (Frankowski et al. 2010a). In addition, as it is a cholinesterase inhibitor, there is a high risk of it causing respiratory depression in young children (Frankowski et al. 2010a). Although these problems have been identified, maldison (malathion) is still available for use. The current study indicated that maldison (malathion) induced significant early apoptosis and significant late apoptosis/necrosis, depending on the concentration and method used (Table 4.2). The induction of cell death, particularly necrosis, in treated populations is consistent with the inflammation-related issues observed. Maldison also induced genetic damage and significant increases in MNi compared to the untreated control, and these levels increased with the concentration of the product. This means that, as with permethrin, exposing skin cells to the product *in vitro* resulted in a significant risk of chromosomal damage. The changes in relative viability for maldison did not follow any pattern related to concentration. At some concentrations the population expanded (showed increases in relative viability %), while at others they were non-significantly killed (showed decreases in relative viability %). It is difficult to interpret this phenomena; it may be due to the interaction of maldison with different aspects of the metabolism at different concentrations. This is consistent with the *in vivo* study, which found that malathion is genotoxic in mice

(Giri et al. 2002). In future studies it would be interesting to observe the cells microscopically for phenotypical changes and utilise a live/dead assay.

A previous study showed that TTO reduces the growth of human cells by 50% (IC50) compared to the control after one day at a range of 20–2700 µg/ml, and that TTO is not mutagenic based on the outcome of the bacterial reverse mutation assay (Hammer et al. 2006). In the present study, TTO showed significant toxicity, as measured by the MTT and CV assays (Figure 4.2 (A)). This is not unexpected, as the concentrations used included both lower dilutions in the natural products, and some higher concentrations used in other products. TTO induced significant early apoptosis in HaCaT cells, and lavender oil induced an increase in MNi as shown in Figure 4.5, although this failed to reach significance. In the future, it would be interesting to study the action of TTO further using additional genotoxic endpoints and extra replicates for the CBMN assay.

Pure lavender oil (100%) induced significant toxicity when measured by MTT and CV assays, as shown in Figure 4.2 (B). However, toxicity was significant only at the highest concentration, and at other concentrations the response of the treated populations varied, as occurred with maldison (see discussion above). Lavender oil also induced significant levels of early apoptosis and necrosis (Table 4.2). However, no MNi were observed after treatment TTO, which means that TTO does not pose a genotoxic risk. Together, these findings mean that lavender oil is toxic and acts via necrosis and apoptosis.

Pyrethrum is used as a lice shampoo to treat children suffering from head lice. The National Pesticide Information Centre reported that children who used pyrethrin-based lice shampoo experienced irritation, burns and scratches to the eyes with blurred vision and tearing (National Pesticide Information Centre 2014). Other symptoms observed were asthma, wheezing, coughing and difficulty breathing (National Pesticide Information Centre 2014). This study examined pyrethrum (pyrethrins plus piperonyl butoxide) using MTT and CV assays, and found significant toxicity (Figure 4.3). When the mechanism of this cell death was investigated using CBMN and flow cytometry assays, it was found that pyrethrum induced significant early and late apoptosis in HaCaT cells. The induction of two different mechanisms could mean that the results of this treatment include not only programmed cell death, but also necrosis, with the implications as discussed above. No significant MNi were observed in HaCaT cells after exposure to pyrethrum for one hour. Thus, the *in vitro* model used in the current study indicates that pyrethrum is not a genetic hazard, but is a toxic hazard.

In summary, pyrethrum (pyrethrins plus piperonyl butoxide) induced the highest significant toxicity level measured by MTT assay, with 100% of cells killed. TTO at a concentration of 10×10^{-4} (v/v) induced significant levels of toxicity, as measured by the MTT assay, and led to 70% of cells being killed. Permethrin (Lice Breaker) caused survival to decline, with 35% of cells killed, and maldison (KP24) killed 32% of the cells. Lavender oil was only toxic at the highest dose, but showed no toxicity on human cells at the concentration found in the head lice treatment product (1×10^{4} (v/v)). Permethrin (Lice Breaker) and maldison (KP24) induced significant levels of chromosomal damage in HaCaT cells at lower concentrations than that in the commercial concentration. As a result, exposure to the commercial mixtures of these treatments induced both early apoptosis and late apoptosis/necrosis; and also induced chromosomal damage in human skin cells. The endpoints used in this study are relevant to real-life health problems. Cancers diagnosed with a high level of necrosis most often have a poor prognosis (White et al. 2015). Necrosis also damages tissue through inflammation, which plays an important role in the expansion of oncogenesis and tumours (White et al. 2015). Cancers develop as a result of genetic instability, and many cancers can cause chromosomal breakage or complete chromosome loss (Lengauer et al. 1998). The products examined in this study were found to pose hazards related to these phenomena.

CHAPTER 5: EXTRACTS FROM *CALENDULA OFFICINALIS* OFFER *IN VITRO* PROTECTION AGAINST H2O2-INDUCED OXIDATIVE STRESS CELL KILLING IN HUMAN SKIN CELLS

This chapter is a reproduction of the article published as Alnuqaydan, AM, Lenehan, CE, Hughes, RR & Sanderson, BJS 2015, 'Extracts from calendula officinalis offer in vitro protection against H2O2 induced oxidative stress cell killing of human skin cells', *Phytotherapy Research*, vol. 29, pp. 120–124.

5.1 Introduction

Flowers and herbal infusions long used in folk medicine have been recently studied for their ability to protect against oxidative stress (Atoui et al. 2005; Day et al. 1997). *Calendula officinalis* (marigold or maravilla) is an herb that is native to the Mediterranean region. The dried flower heads of *Calendula officinalis* have been used in folk medicine, used as a spice and cultivated for ornamental purposes and medicinal properties in several countries (Fonseca et al. 2010; Ozkol et al. 2012; Re et al. 2009). Calendula extracts have been shown to exhibit antioxidant, anti-tumoural, anti-inflammatory, anti-viral and anti-HIV properties (Jiménez-Medina et al. 2006; Kalvatchev et al. 1997b). The chemical compounds identified in methanol extracts of calendula include polar compounds, phenolic acids (e.g., vanillic, protocatechuic, syringic, p-coumaric, caffeic and chlorogenic acid) and flavonoid glycosides (eg. rutin, narcissin, 3-glucoside of isohamnetin, isoquercitrin) (Matysik et al. 2005). Other constituents include sterols, steriods, terpenoids and free and esterified triterpenic alcohols (Fonseca et al. 2010; Re et al. 2009a). Thus, it is clear that calendula contains a number of compounds with antioxidant and free radical scavenging potential. Biological systems generate hydroxyl radicals ($^{\bullet}OH$) from H_2O_2 by the Fenton reaction (Chen & Schopfer 1999). For example, when free radical processing in melanin synthesis is inadequate, H_2O_2 is created, leading to the production of $^{\bullet}OH$ and other ROSs (Wang et al. 2006). Although $^{\bullet}OH$ and other ROSs are typically short-lived, they do interact with, and damage, cellular components, including DNA, membrane lipids and proteins (Chen & Schopfer 1999). Cells require antioxidants to stop these potentially destructive reactions. Plant-derived antioxidants have the potential to perform this function. However, measuring the levels of plant antioxidants in a cell system only partly correlates with the measured levels of antioxidants in the cells (Slatnar et al. 2012). Therefore non-cell-based assays and cell-based bioassays are both necessary to determine the efficacy of plant-derived extracts and individual compounds. The current study examined the antioxidant potential of two calendula extracts using an *in vitro* human skin cell model with H_2O_2 as an oxidative challenge. This was complemented by the use of the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) chemical antioxidant assay.

5.2 Materials and methods

5.2.1 Materials

The materials used were RPMI-1640 medium (Gibco, Invitrogen, Barcelona, Spain), FBS (Gibco, Invitrogen, Barcelona, Spain), penicillin/streptomycin (Gibco, Invitrogen, Barcelona, Spain), PBS (Sigma™), H_2O_2 (Sigma3-MTT (Sigma™), quercetin

(Sigma™), and DPPH (Sigma™). All other chemicals were reagent from Sigma™. The medium for growth and treatment was RPMI with 10% FBS.

5.2.2 Plant extracts

An industry partner supplied dried calendula flower heads from biodynamically grown plants, and two extracts (C5 and C6) were prepared independently using a proprietary process involving distillation and percolation with water/ethanol-based solvents (Jurlique International, Mount Barker, Australia), which were used as supplied. When appropriate, the extracts were diluted in media to prepare the required concentration of the treatment solution. The plant extracts were characterised by DPPH assay (see 5.2.7), total phenolics (GAEs, see Folin-Ciocalteu assay), dry weight and density determination. Density determination was carried out at 20°C by averaging 10 independent measurements. The data are reported (Table 5.1) as mean ± SD, with errors calculated using the propagation of uncertainty equation when division utilised (since density = mass/volume). The total dry weights (%) of the C5 and C6 extracts were determined after samples were freeze-dried at -56°C at 3.4 Pa for 24 hours. Once all volatiles were removed from the samples, the total dry solids remaining were weighed.

5.2.3 Cell cultures

The cells were cultured as described in subsection 4.2.4

5.2.4 Cell treatment

The cells were treated to allow exposure to toxicity prior to the protection assay (see 5.2.5). Then, 96 flat-bottom wells were seeded with 10^4 cells/well and incubated for 19 hours at 37°C in 5% CO_2 to allow the cells to adhere. The media were then aspirated and replaced with 200 µl of the treatment solution per well, and were treated for 4, 24 and 48 hours prior to the MTT assay analysis. The treatment solutions included the C5 or C6 extracts at doses of 0, 0.125, 0.5, 1, 2 and 5% (v/v). Quercetin (16µg/ml final concentration) was used as a positive control compound with known antioxidant capacity. The negative or zero control was the media.

5.2.5 Induction of oxidative stress to determine protection activity

After the treatment (see 5.2.4), the cells were exposed to 300 µM H_2O_2 for one hour to induce oxidative stress (Fenech et al. 1999). Then MTT assay was performed

5.2.6 MTT cell viability assay

The cytotoxicity (relative cell viability) of head lice treatments was monitored by MTT assay (subsection 3.2.5).

5.2.7 DPPH assay

The antioxidant activities of calendula extracts were measured in terms of radical scavenging ability using the stable radical DPPH. A methanolic stock solution of quercetin (1.6 mg/mL) was prepared as the positive control, and further dilutions of the stock in methanol (5, 4, 3, 2 and 1% (v/v)) were used to determine the linear range of the DPPH assay. Calendula extracts C5 and C6 were diluted in methanol (5, 4, 3, 2 and 1% (v/v)) before analysis. The sample (50 µl) was put into a cuvette with 2 ml of a methanolic DPPH solution (6×10^{-5} mol/l) and analysed immediately following the method reported by Politeo et al. (2006). After 15 minutes, the decrease in absorbance at 517 nm was determined for all samples with a Thermo Scientific Evolution Array UV-Visible spectrophotometer. Methanol was used to zero the spectrophotometer. The percent inhibition of the DPPH radical by the samples was calculated according to the following formula:

$$\% \text{ Inhibition} = ((AA(0) - AA(t) / AA(0)) \times 100$$

Where AA(0) is the absorbance of the antioxidant solution at t = 0 min and AA(t) is the absorbance of the antioxidant solution at t = 15 min.

5.2.8 The Folin-Ciocalteu assay

The Folin-Ciocalteu assay was based on the method of Atanassova and Georgieva (2010). The C5 and C6 extracts were prepared by diluting 200 µL of extract with up to 10mL of water (a 50-fold dilution). Gallic acid aqueous standards were prepared by adding 5.8, 12.8, 16.4, 22.8, 27.8 mg to 250 mL of water. For the Folin-Ciocalteu assay, 0.4 mL of diluted extract or gallic acid standard was added to 3.6 mL of water and 0.4 mL of the Folin-Ciocalteu reagent. The mixture was shaken and left for five minutes at room temperature, after which 4 mL of a 7% sodium carbonate solution and 1.6 mL water was added. The mixture was left for two hours at room temperature. The absorbance of each solution at 765 nm and 750 nm was measured using a UV-Visible spectrophotometer. A calibration curve of gallic acid concentration versus absorbance was constructed, from which the GAEs of the C5 and C6 extracts was determined. Each gallic acid standard and calendula extract was prepared and analysed in triplicate.

5.2.9 Statistical analysis

The experiment was performed in triplicate and data are presented as the mean ± SEM. A one-way ANOVA with Turkey's post hoc test was carried out using SPSS software (version 19.0). Differences were considered statistically significant when the P value was less than 0.05. The survival in each experiment following pre-treatment

with quercetin, C5 or C6 was calculated relative to the population of cells not treated with H_2O_2 (zero dose of extract and zero dose of H_2O_2), which are represented as 100% survival.

5.3 Results and discussion

5.3.1 Characterisations of calendula extracts

Multiple methods were used to gain an appreciation of the physico-chemical characteristics of the C5 and C6 extracts. Their average densities were 1.003 g/mL and 1.002 g/mL, and their total dry weights were 8.8% and 8.4%, respectively (Table 5.1). The Folin-Ciocalteu assay was carried out to estimate polyphenol composition, which was expressed using per dry weight determinations and gallic acid equivalents (GAE) (Table 5.1). The values for C5 and C6, 28 mg GAE/g dry weight and 26 mg GAE/g dry weight, respectively, were two orders of magnitude higher than those published by the authors who outlined the assay method (Atanassova & Georgieva 2010). They found values of 0.27–0.49 mg GAE/g dry weight for sage (*Salvia officianalis*), mint and lemon balm. Sage is often used as a reference plant in such circumstances because its antioxidant activity is well recorded. The calendula extracts C5 and C6 clearly contain more compounds with phenolic activity than do the extracts prepared from sage and other plant sources. The C5 and C6 extracts also had higher GAE activity than did a calendula extract from another source, which had only 6.6 mg GAE/g extract (Miliauskas et al. 2004). This would presumably be even lower if expressed per dry weight of extract, as is the case for our data.

Table 5.1: The characterisation of the calendula extracts C5 and C6. Shown are the results for density determination (see section 5.2.2); the measurement of total dry weight after freeze-drying (see section 5.2.2) and the GAE results from the Folin-Ciocalteu assay (see section 5.2.8).

Characteristic	Extract C5 (A)	Extract C6 (B)
Density at 20°C (Mean ± SD)	1.003g/ml ± 0.019	1.002g/ml ± 0.019
Total Dry Weight	8.8%	8.4%
GAE (mg GAE/g dry weight)	28	26
***The densities of C5 and C6 were 1.003 g/ml and 1.002 g/ml, respectively.**		

5.3.2 The toxicity of calendula plant extracts on HaCaT human skin cells

The toxicity and protective effects of the C5 and C6 extracts on HaCaT cells *in vitro* was explored by incubating cells with the extracts for 4, 24 and 48 hours. Re et al. (2009) state the need for toxicity studies to appreciate the safety of plant extracts. The current study found that two extracts of calendula (C5 and C6) showed only a limited toxicity, and this was at high doses. At the lowest doses (0.125, 0.5 and 1.0% (v/v)),

the C5 and C6 extracts were not toxic on HaCaT skin cells for any of the treatment periods. However, the highest dose (5%; C5 = 4.4 mg dry weight/mL and C6 = 4.2 mg dry weight/mL) of extracts was non-toxic for the four hour treatment only (Table 5.2). Conversely, at the highest doses (2% and 5% (v/v)), the C5 and C6 extracts showed significant toxicity only in the 48 hour treatment (Table 5.2). Doses of the two extracts less than 1% (v/v) (for C5 = 0.88 mg dry weight/mL; C6 = 0.84 mg dry weight/mL) were not significantly toxic at 24 hours. These results suggest that C5 and C6 extracts could be toxic at low concentrations, and have low diffusivity through the cell membrane. Thus, only high concentrations of the extracts, which exceed those currently used in commercial products, would have a sufficient quantity of toxic components to have an effect. This effect could involve a period of contact between these components and the cells, such that these toxic components would be taken up by cells, thus reducing cellular viability. In contrast, lower concentrations of these extracts would non-toxic if incorporated into products at the safe doses identified by current findings.

The toxicity observed at higher doses could be due to the concentration of some bioactives becoming toxic above a given dose and time of exposure. Alternately, the reduction in survival at higher doses could be due to pro-oxidant activity acting as a source of free radicals. Diffusion through the cell membrane over long treatment periods may be such that high, but not low, doses have a pro-oxidant effect. Consistent with this, calendula extracts, infusions and decoctions exhibit *in vitro* cytotoxic effects and growth inhibition on human cancer cells in a dose-dependent fashion (Jiménez-medina et al. 2006; Matic et al. 2013).

After treatment with low doses of the C5 and C6 extracts, cell numbers increase, as reflected in relative survival greater than 100%. Similar observations have been made with mouse (Fonesca et al. 2010) and human fibroblasts (Matysik et al. 2005) treated with calendula.

Table 5.2: Relative survival at each time point (4, 24 and 48 hours) for the C5 and C6 extracts. HaCaT cells were treated for the period of time indicated then assessed by an MTT assay (see

section 5.2). *Significant difference at P < 0.05; n=3. Data are shown as the mean survival relative to the untreated control ± SEM; n = 3.

Extract	Time	Dose					
		0% (v/v)	0.125% (v/v)	0.5% (v/v)	1% (v/v)	2% (v/v)	5% (v/v)
C5	4h	100 ± 28.0	70.2 ± 15.4	85.3 ± 0.9	85.8 ± 1.6	90.11 ±0.9	*62.1 ± 5.3
	24h	100 ± 0.4	114.6 ± 7.3	101.0 ± 6.0	*72.3 ± 2.7	*29.86 ± 2.6	*7.1 ± 3.6
	48h	100 ± 0.1	108.6 ± 1.9	107.5 ± 3.1	97.1 ± 4.7	*60.90 ± 4.7	*4.2 ± 2.1
C6	4h	100 ± 30.5	91.6 ± 0.5	94.0 ± 1.2	92.6 ± 5.9	103.0 ± 5.2	*74.8 ± 1.0
	24h	100 ± 0.1	88.9 ± 4.7	96.2 ± 5.5	83.7 ± 10.4	*52.86 ± 5.4	*10.9 ± 3.7
	48h	100 ± 0.1	107.9 ± 2.6	114.1 ± 3.7	108.1 ± 7.2	*49.97 ± 7.0	*6.1 ± 2.7

5.3.3 Protection by extracts against H_2O_2-induced oxidative stress on human skin cells

Oxidative stress-induced cell killing by 300 µM H_2O_2 (with no pre-treatment) was significantly different from the untreated control in every individual experiment. The relative survival values for each experimental series ranged from 5–35%, indicating that the oxidative stress challenge induced cell killing (untreated control + H_2O_2; Figs. 5.1 and 5.3). However, the level of cell killing by H_2O_2 varies due to the instability of ROS (Fenech et al. 1999). Pre-treatment with the C5 and C6 extracts resulted in protection against oxidative stress (free radical (H_2O_2)-induced cell killing). Quercetin, the positive control, offered protection at all time-points for the tested dose (16 µg/ml). The four hour pre-treatment with the C5 and C6 extracts demonstrated dose-dependent protection against oxidative stress, with 2% (v/v) and 5% (v/v) doses offering significant protection against H_2O_2 -induced cell killing (Figure 5.1). This is evident as there is greater relative survival at these doses after the H_2O_2 challenge (unfilled bars, Figure 5.1) compared to H_2O_2 without any extract pre-treatment (bars with horizontal stripes, Figure 5.1). Significant protection against oxidative stress was also observed after 24 and 48 hours of pre-treatment with 1% (v/v) of the C5 and C6 extracts (Figure 5.2). At lower doses (0.125–1% (v/v)), relative cell survival following pre-treatment and the H_2O_2 challenge was significantly higher than that observed after the H_2O_2 treatment without any extract pre-treatment (Figure 5.3). The protection offered by the extracts was time-dependent. The 48 hour pre-treatments offered more protection against oxidative stress-induced cell killing for HaCaT skin cells than either

the four or 24 hour treatments. This could be because the longer incubation time offers more time for the cellular uptake of bioactives in the extracts. This time-course could reflect low diffusivity across the cell membrane; that is, it takes longer for the antioxidants to enter and protect the cells. This shows that not only is it safe to expose cells to low doses for a longer period of time, but also that low doses over a longer period time offer the best protection against oxidative stress-induced cell killing. Ahmad et al. (2012) demonstrated that calendula extracts offer *in vitro* antioxidant protection by screening for nitric oxide free radical scavenging, and proposed that this could be used to slow the aging process. The current study is consistent with this theory, in particular the theory that a 48-hour pre-treatment with lower, non-toxic doses acts to protect cells.

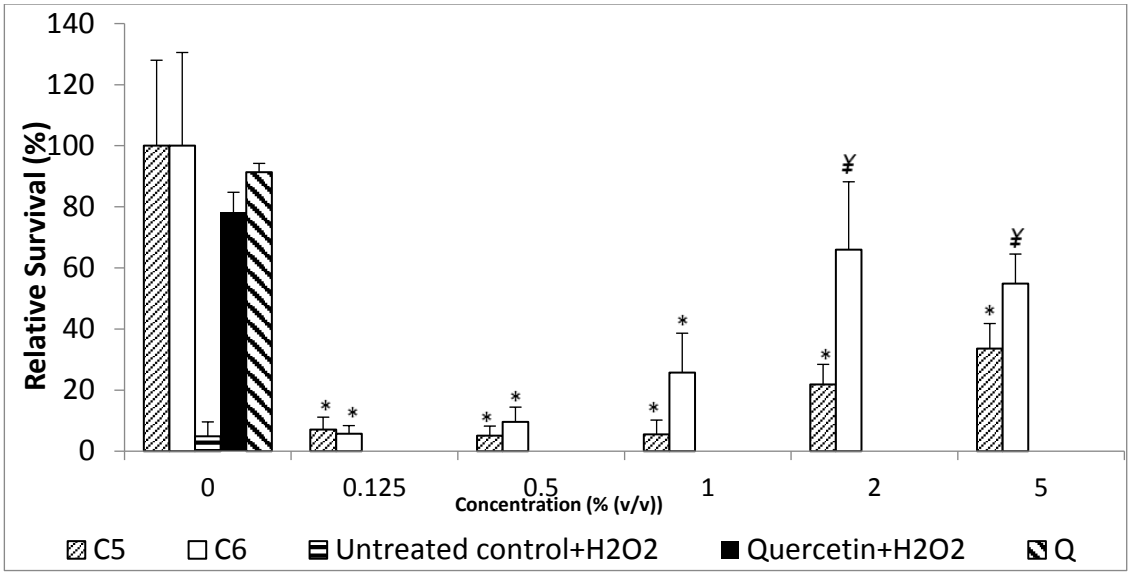

Figure 5.1: Survival relative to the untreated control, determined by the MTT assay. The cells were plated in microplates at 10,000 cells/well then treated for 4 hours with C5 or C6, then for one hour with 300μM H_2O_2. Q = populations of cells treated with the control antioxidant quercetin, H_2O_2= hydrogen peroxide = oxidative stress, + = challenged with H2O2. ¥ = significantly different from the untreated control + H_2O_2, P < 0.05. * = significantly different from Q + H_2O_2, P < 0.05. The data are shown as mean ± SEM; n = 3. Quercetin = 0.016 mg/ml; 1% C5 = 0.88 mg dry weight equiv/ml; 1% C6 = 0.84 mg dry weight equiv/ml.

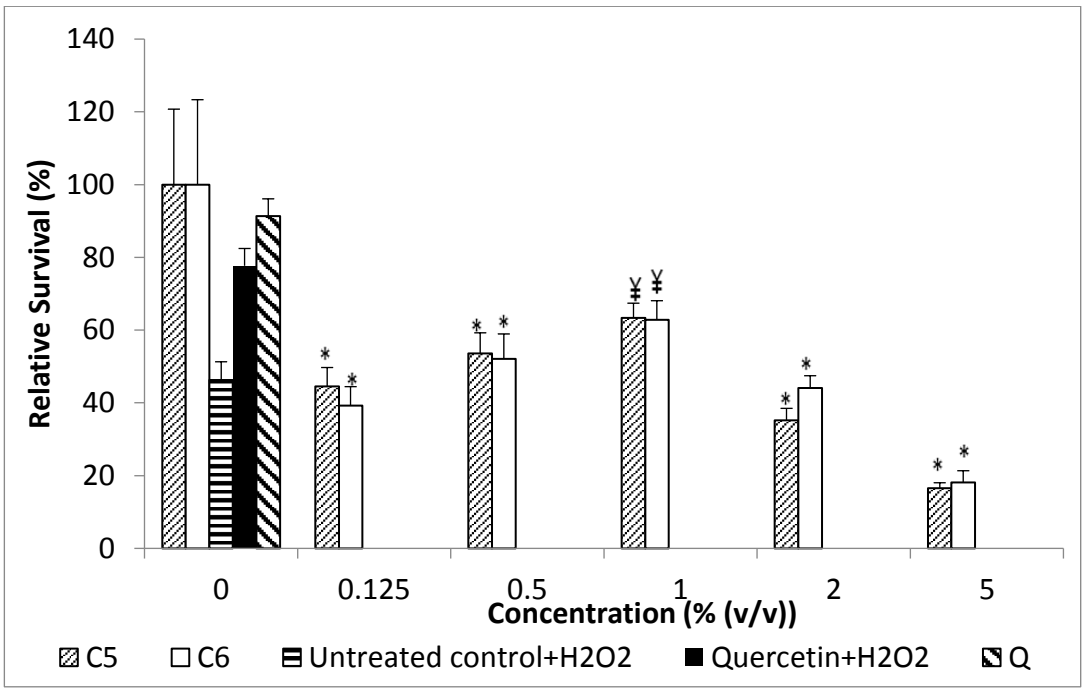

Figure 5.2: Survival relative to the untreated control, determined by the MTT assay. The cells were plated in microplates at 10,000 cells/well then treated for 24 hours with C5 or C6, then for one hour with 300μM H_20_2. Q = populations of cells treated with the control antioxidant quercetin; H_2O_2 = hydrogen peroxide = oxidative stress; + = challenged with H_2O_2. ¥ = significantly different from the untreated control + H202, P < 0.05. * = significantly different from Q + H_2O_2, P < 0.05. The data are shown as mean ± SEM; n = 3. Quercetin = 0.016 mg/ml; 1% C5 = 0.88 mg dry weight equiv/ml; 1% C6 = 0.84 mg dry weight equiv/ml.

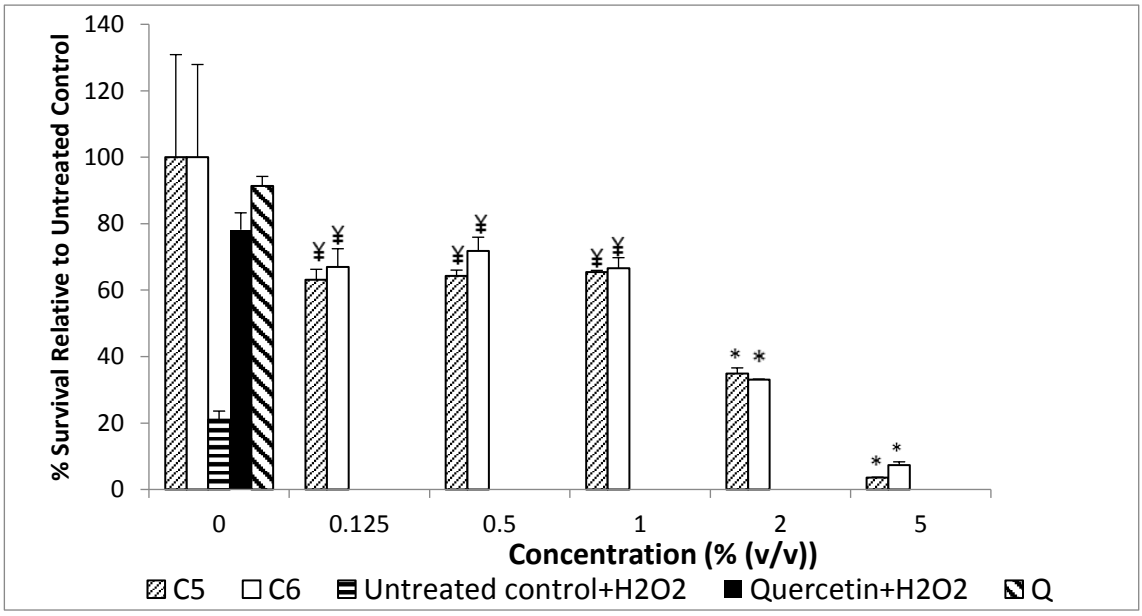

Figure 5.3: Survival relative to the untreated control, determined by the MTT assay. The cells were plated in microplates at 10,000 cells/well then treated for 48 hours with C5 or C6, then for one hour with 300μM H_20_2. Q = populations of cells treated with the control antioxidant quercetin; H_2O_2 = hydrogen peroxide = oxidative stress; + = challenged with H_2O_2. ¥ = significantly different from the untreated control + H202, P < 0.05. * = significantly different from Q + H_2O_2, P < 0.05. The data are shown as mean ± SEM; n = 3. Quercetin = 0.016 mg/ml; 1% C5 = 0.88 mg dry weight equiv/ml; 1% C6 = 0.84 mg dry weight equiv/ml.

The DPPH assay revealed that C5, C6 and the positive control quercetin, all exhibited free radical inhibition in a linear dose-dependent manner for a 10-minute reaction time (Figure 5.4).

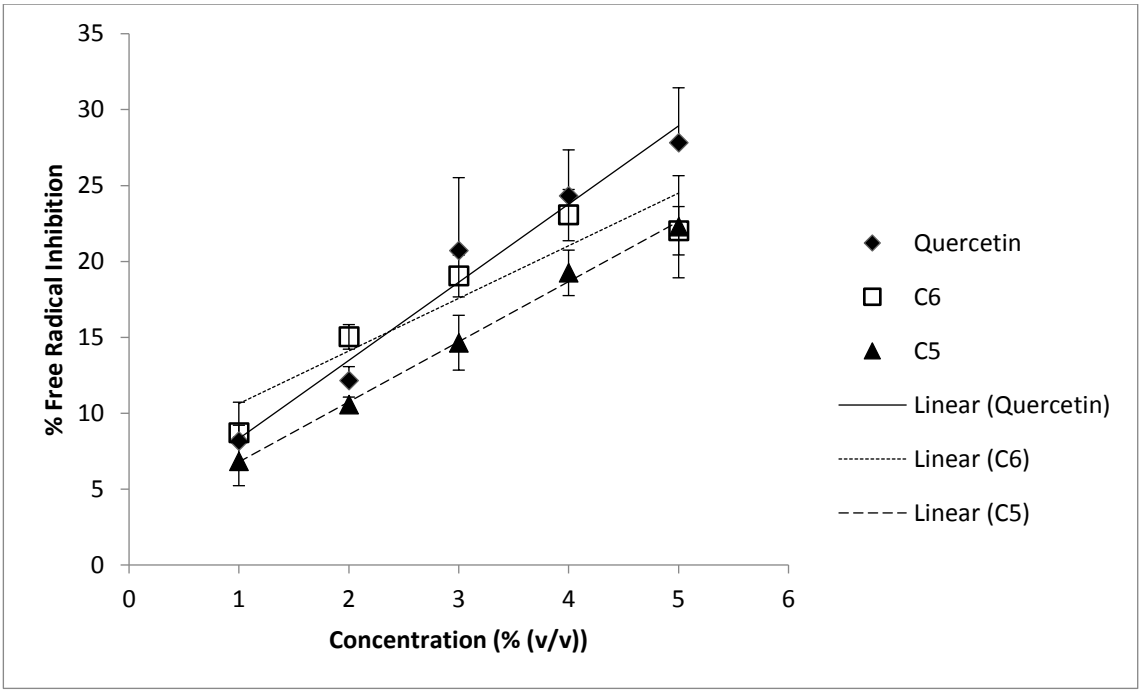

Figure 5.4: The % free radical inhibition of the quercetin, C6 and C5 solutions, including the linear line of best fit between antioxidant concentration and free radical scavenging ability. 1% quercetin = 0.016 mg/ml; 1% C5 = 0.88 mg dry weight equiv/ml; 1% C6 = 0.84 mg dry weight equiv/ml. The level of antioxidant activity was determined by a DPPH assay. Each value represents the mean ± SEM; n = 3.

Table 5.3: Free radical inhibitory concentrations (ICs) determined by a DPPH assay for quercetin (Q) and the C5 and C6 extracts. Shown is the dose inhibiting 20% of free radicals (IC20), the protective dose for these agents based on performance in the MTT oxidative challenge bioassay and the IC level (predicted from the DPPH assay) equivalent to the protective dose at 24 hours (Figure 5.1).

		24 hours extract pre-treatment	
Sample	IC_{20}	Dose significantly protecting against H_2O_2	Predicted IC of protective dose
Q	52 µg/ml	16 µg/ml	IC_8
C5	4.3% (v/v)	1% (v/v)	IC_7
C6	3.7% (v/v)	1% (v/v)	IC_{11}

The IC_{20} values calculated from the graph are summarised in Table 5.3, and show that 4.3% (v/v) of C5 and 3.7% (v/v) of C6 had the same antioxidant activity (IC_{20}) as 52µg/ml of quercetin. Also shown in Table 5.3 are the predicted IC values for the concentrations of quercetin, C5 and C6 that showed protective effects in the 24-hour

cellular challenge bioassay. The IC values are similar. Extrapolating from the DPPH assay IC values, it can be predicted that protection would occur at a level similar to quercetin for the 1% (v/v) concentrations of the extracts. However, with regard to the 48-hour pre-treatment, cellular antioxidant protection at 0.1% (v/v) is equally as effective as 16 µg/ml of quercetin (Figure 5.3). The DPPH assay results did not predict that the lower concentrations would show such strong activity. Thus, both assays are needed to gain an understanding of the extracts' antioxidant potential.

In conclusion, calendula flower extracts protect HaCaT skin cells against an oxidative stress challenge in the form of H_2O_2. Such oxidative stress can result from a normal metabolism giving rise to charged particles, including OH and ROS. This can lead to the oxidation of cellular components, which in turn can lead to inflammation, skin aging or cancer. *Calendula officinalis* dried flower extracts could function as antioxidants to protect against such damage. Such extracts are potentially useful as product additives that offer protection against oxidative stress damage. However, further work is needed to fully characterise the protective action of these extracts, including studies on human skin fibroblasts.

CHAPTER 6: CALENDULA OFFICINALIS EXTRACTS PROTECT AGAINST H_2O_2-INDUCED CHROMOSOMAL DAMAGE ON HACAT HUMAN SKIN CELLS

This chapter is a reproduction of the article published as Alnuqaydan, AM, Lenehan, CE, Hughes, RR & Sanderson, BJS 2015, 'Calendula officinalis extracts protect against H2O2 induced chromosome damage on HaCaT human skin cells', *Journal of Carcinogenisis and Mutagenesis*, vol. 6, no. 2.

6.1 Introduction

The alteration of genetic material can lead to cancer and can include chromosome breakage and loss, di-centric chromosomes and DNA damage, including mutations and gene amplification. The number of chromosome alteration is correlated with the tumour process and malignant potential of the cancer (Loeb & Loeb 2000). DNA damage occurs from cellular sources (endogenous) and environmental agents (exogenous) (Loeb & Loeb 2000). Exogenous agents, such as chemicals or radiation, can interact with genetic material and induce genetic damage (genotoxicity), chronic toxicity and/or cell death (cytotoxicity) (Wurgler & Kramers 1992). Exogenous agents can promote the formation of free radicals, for example, UV light can lead to the formation of single oxygen (O) and other ROS in the skin. Other atmospheric pollutants, such as ozone and nitrogen peroxide, lead to radical formation and may cause respiratory disease. Cigarette smoke also contains free radicals (Fusco 2007; Murray et al. 1954). Oxidative stress arises from an imbalance between free radicals, including ROS and antioxidant defences. ROS can damage cellular components including lipids, proteins and nucleic acids (Young & Woodside 2001a). Oxidative damage to proteins or nucleic acids often increases the variety of damage products due to modifications in amino acids and nucleotides (de Zwart et al. 1999). Oxidative damage is involved in many conditions, such as the aging process, cancer, atherosclerosis, inflammatory conditions, viral infections, trauma, effects of cigarette smoke and interaction with environmental pollution (Ashok & Ali 1999; Frankel 2007; Hecht 1999; Rosenfeld 1998). An antioxidant is a substance that can inhibit or delay oxidative damage and can therefore potentially protect against the damaging effects of ROS. Many studies have examined the chemical nature of fruits, vegetables, herbs and grains for their natural antioxidant activity (Atoui et al. 2005; Young & Woodside 2001a) The majority of antioxidants isolated from higher plants are polyphenols, and their main biological activity identified as anti-inflammatory, anti-carcinogenic, anti-bacterial and anti-viral (Atoui et al. 2005; Larson 1988; Shahidi 2000). The activity of different calendula extracts includes anti-tumoural, anti-inflammatory and anti-viral activity and anti-HIV properties (Frankel 2007; Jiménez-Medina et al. 2006; Kalvatchev et al. 1997a; Katalinic et al. 2006). Calendula officinalis extracts contain antioxidant compounds, including phenolic, tocopherols and carotenes (Re et al. 2009a). Individual components of relevance to this study are quercetin, vitamin E, beta-carotene and amino acids (Alnuqaydan et al. 2015b). The extracts tested in the current study have been shown to protect human cells *in vitro* against ROS-induced cytotoxic activity (Alnuqaydan et al. 2015b). We tested four calendula extracts for protection against chromosome damage caused by free radicals using an *in vitro* human skin cell model.

6.2 Materials and methods

6.2.1 Materials

The materials used were RPMI-1640 media and FBS; these were purchased from Gibco® Cell Culture. Cytochalasin B (Cyt-B) solution was purchased from Sigma™. SDS (approximately 99%) and PBS was purchased from Sigma™. H_2O_2 and quercetin were purchased from Sigma™. The Folin-Ciocalteu reagent was purchased from Sigma™. Diff-Quik stains were purchased from Lab Aids (Australia). The media prepared for growth and treatment were the RPMI medium supplemented with 10% FBS.

6.2.2 Plant extracts

The plant extracts were prepared and characterised as previously published (Alnuqaydan et al. 2015b) (see subsection 5.2.2). In brief, an industry partner (Jurlique International, Mount Barker, South Australia) supplied dried calendula flowers from biodynamically grown plants. Four different extracts were prepared independently. Extracts A (C5) and B (C6) were prepared as water/ethanol-based proprietary extracts, and extracts C and D as proprietary aqueous extracts. When required, the extracts were diluted in media to prepare the treatment solution. Total phenolic content, dry weight and density determination were used to characterise the plant extracts. The determination of density for all extracts was carried out at 20°C from the average of 10 independent measurements. Table 6.1 presents the data as mean ± SD, with errors calculated using the propagation of uncertainty equation (density = mass/volume). The percentages of the total dry weights of all extracts (A (C5), B (C6), C or D) were determined after the extract samples were freeze-dried at -56°C at 3.4 Pa for 24 hours. The total dry solids were weighed once all volatiles were removed from the samples.

6.2.3 Cell line and cell cultures

The cells were cultured as described in subsection 4.2.4

6.2.4 Cell treatment with calendula extracts

The cells were treated *in vitro* to expose them to the extracts as part of the genetic protection assay. Six wells of a 6-well plate were seeded with 3×10^5 cells/well at a volume of 2 ml and incubated for 19 hours at 37°C in 5% CO_2 to allow the cells to adhere. The media were aspirated and replaced with 2 ml of the treatment solution per well. The cells were treated for 48 hours prior to the CBMN assay analysis at doses derived from the MTT assay results for extracts A, B, C and D (See Chapter 5 for the toxicity of calendula extracts A (C5) and B (C6), as well as the supplemental

data below for the toxicity of calendula extracts C and D). The treatment solutions included A (C5) and B (C6), C or D at 0, 0.125, 0.5 and 1 (v/v). The positive control compound used was quercetin (16µg/ml final concentration), which has a known antioxidant capacity. Media were used as a negative control.

6.2.5 Induction of oxidative stress by hydrogen peroxide to determine chromosome protection activity

After incubation with or without extracts, oxidative stress was induced using H_2O_2 as described in subsection 5.2.5 and published previously (Alnuqaydan et al. 2015b).

6.2.6 Folin-Ciocalteu assay

To prepare the calendula extracts, they were diluted 50-fold in 10 mL water. Gallic acid aqueous standards were prepared by adding 5.8, 12.8, 16.4, 22.8 and 27.8 mg in 250 mL of water. The Folin-Ciocalteu assay was then carried out as previously described (Alnuqaydan et al. 2015b; Atanassova & Georgieva 2010). For the assay, the mixtures were incubated for two hours at room temperature. The absorbance of each solution at 765 nm and 750 nm was measured using a UV-Visible spectrophotometer. A calibration curve of gallic acid concentration versus absorbance was constructed from the gallic acid aqueous standards. The GAEs of the extracts were then determined in triplicate.

6.2.7 The DPPH assay

The DPPH assay was performed to determine the extracts' antioxidant activities via radical scavenging ability using a stable DPPH radical. A methanolic stock solution of quercetin (1.6 mg/mL) was the positive control with dilutions in methanol (5, 4, 3, 2 and 1% (v/v)) used to determine the linear range of the DPPH assay. Calendula extracts were prepared and analysed as previously reported (Alnuqaydan et al. 2015b; Politeo et al. 2006). The decrease in absorbance at 517 nm was determined with a Thermo Scientific Evolution Array UV-Visible spectrophotometer after 15 minutes for all samples.

6.2.8 Cytokinesis-block micronucleus assay

The CBMN assay was carried out as previously described (subsection 3.2.7)

6.2.9 Statistical analysis

Triplicate assays were performed for all experiments, and the data are presented as the mean ± SEM. A one-way ANOVA (SPSS software, version 19.0) was used, followed by Tukey's HSD post, and a T-test for independent samples. Differences were considered statistically significant when the value of $P < 0.05$.

6.3 Results

6.3.1 Characteristics of calendula extracts

The physico-chemical properties of extract A (C5) and B (C6), C and D were characterised (Table 6.1). The Folin-Ciocalteu assay was used to estimate polyphenol composition and is expressed using per dry weight determinations and GAEs (Table 6.1). The DPPH assay characterisation is also included (Table 6.3).

Table 6.1: Characteristics of different calendula extracts. The table shows the results of density determination, total dry weight after freeze-drying and GAE results derived from the Folin-Ciocalteu assay.

Characteristic	Extract A (C5)	Extract B (C6)	Extract C	Extract D
Density at 20 ºC (mean ±SD)	1.003 g/ml	1.002 g/ml	1.036	0.973
Total dry weight	8.80%	8.40%	13.97%	14.65%
GAE (mg GAE/g dry weight)	28	26	10.83	11.73

6.3.2 Genotoxicity of calendula plant extracts on human skin cells HaCaT

The *in vitro* genotoxicity and protective effects of extracts A (C5), B (C6), C and D on HaCaT cells were performed after incubating cells with the extracts for 48 hours. No genotoxicity was induced by the extracts themselves, as shown in (Figures 6.1, 6.2, 6.3 and 6.4). At no dose of any single extract is the frequency of MNi greater than that of the zero-dose control.

6.3.3 Protection induced by extracts against hydrogen peroxide-induced oxidative stress on human skin cells

Oxidative stress-induced MNi frequency by 300-µM H_2O_2 with no pre-treatment was significantly higher than the zero dose (untreated control) ($P < 0.05$) in all of the experiments. The extracts A (C5), B (C6), C and D protected against H_2O_2-induced MNi, as shown in (Figures 6.1, 6.2, 6.3 and 6.4), This occurred at all doses and was statistically significant ($P < 0.05$) for human skin cells when treated with each of the extracts for 48 hours followed by 1 hour of treatment with 300-µM H_2O_2. This is shown for all of the extracts in (Figures 6.1, 6.2, 6.3 and 6.4), where the solid lines (ROS challenge series of experiments) show a significant difference ($P < 0.05$) between the cells in the absence of the extract but in the presence of H_2O_2 (0 dose of extract) and all other doses of extracts in the presence of H_2O_2. The positive control of a known antioxidant, quercetin, significantly protected against H_2O_2-induced MNi (Table 6.2, $P < 0.05$). The level of protection offered by all of the extracts was similar to that offered by quercetin against H_2O_2-induced oxidative stress genetic damage.

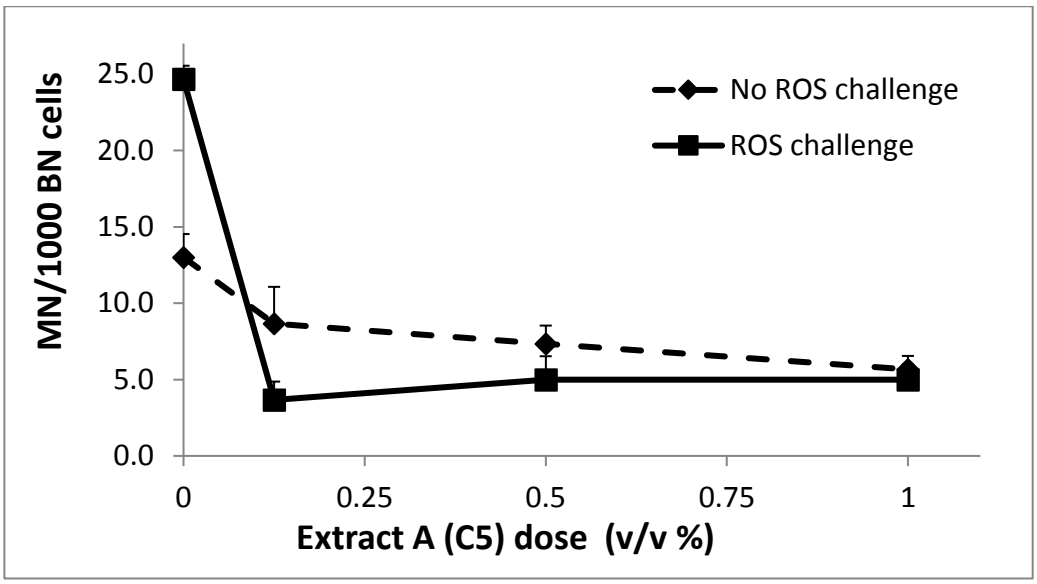

Figure 6.1: Dose-response effect of extract A on MNi frequency. HaCaT cells were plated as described in the materials and methods section and treated with calendula extract A (C5) for 48 hours. They were then treated for one hour without H_2O_2 (no ROS challenge) or for one hour with 300µM H_20_2 (ROS challenge). The frequency of MNi per 1000 BN cells was determined using the CBMN assay (as described in section 2.8). Data are shown as the mean ± SEM; n = 3; 1% (v/v) A = 0.88mg dry weight equiv/ml.

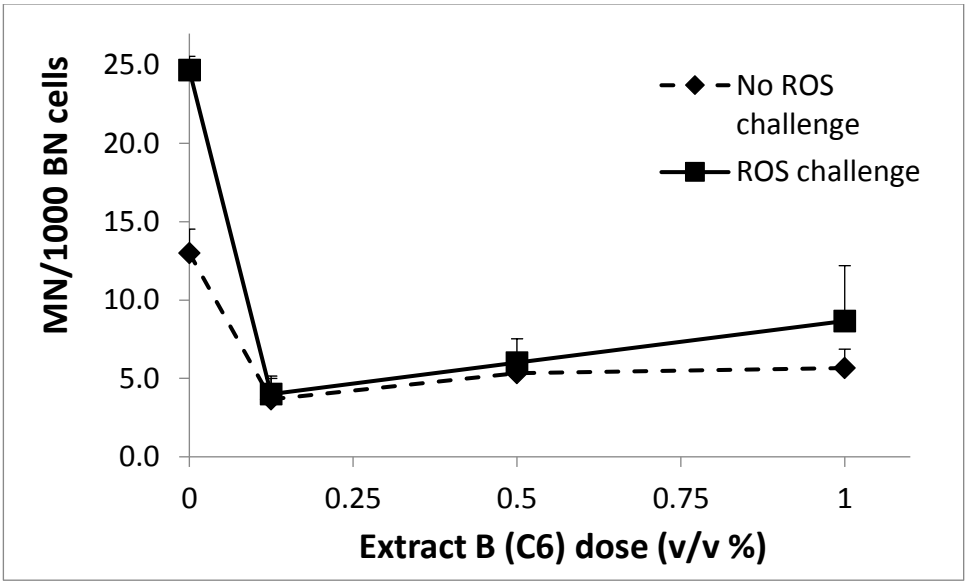

Figure 6.2: Dose-response effect of extract B on MNi frequency. HaCaT cells were plated as described in the materials and methods section and treated with calendula extract B (C6) for 48 hours. They were then treated for one hour without H_2O_2 (no ROS challenge) or for one hour with 300µM H_20_2 (ROS challenge). The frequency of MNi per 1000 BN cells was determined using the CBMN assay (as described in section 2.8). Data are shown as the mean ± SEM; n = 3; 1% B = 0.84mg dry weight equiv/ml.

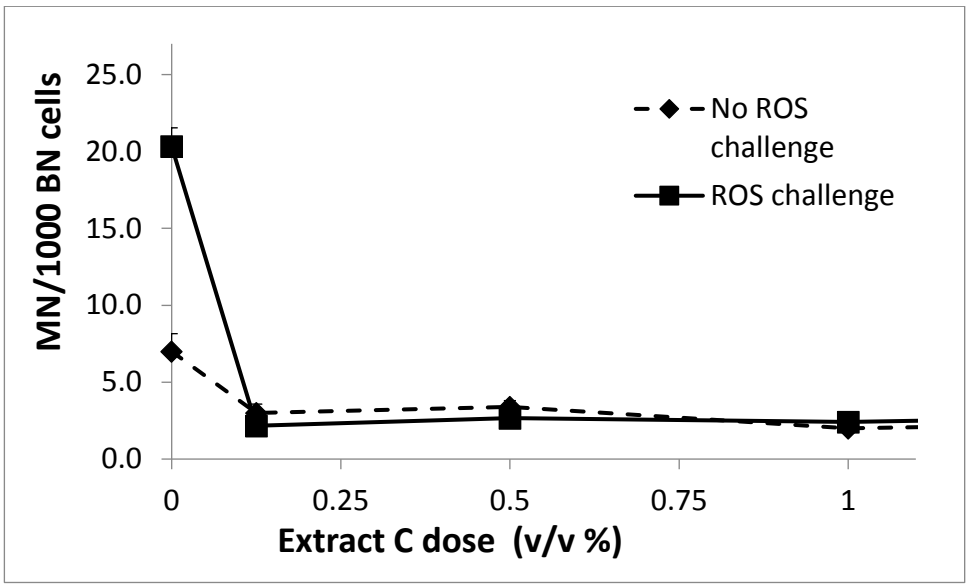

Figure 6.3: Dose-response effect of extract C on MNi frequency. HaCaT cells were plated as described in the materials and methods section and treated with calendula extract C for 48 hours. They were then treated for 1 hour without H_2O_2 (no ROS challenge) or for one hour with 300μM H_20_2 (ROS challenge). The frequency of MNi per 1000 BN cells was determined using the CBMN assay (as described in section 2.8). Data are shown as the mean ± SEM; n = 3; 1% (v/v) C = 1.4 mg dry weight equiv/ml.

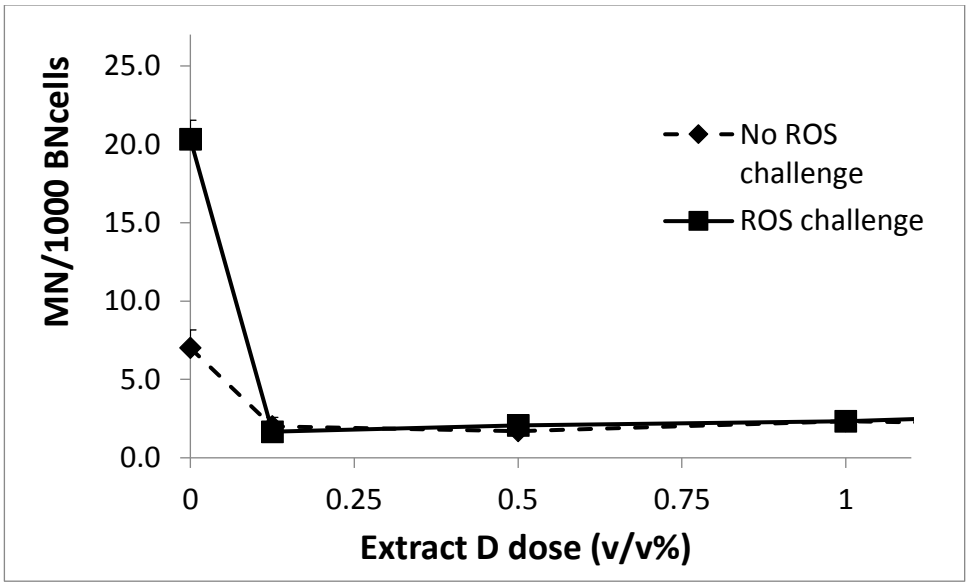

Figure 6.4: Dose-response effect of extract D on MNi frequency. HaCaT cells were plated as described in the materials and methods section and treated with calendula extract D for 48 hours. They were then treated for one hour without H_2O_2 (no ROS challenge) or for one hour with 300μM H_2O_2 (ROS challenge). The frequency of MNi per 1000 BN cells was determined using the CBMN assay (as described in section 2.8). Data are shown as the mean ± SEM; n = 3; 1% (v/v) C = 1.47 mg dry weight equiv/ml.

Table 6.2: Effect of quercetin pre-treatment and the media control on MNi/1000 BN cells. Shown is the number of MNi/1000 BN control and post-quercetin treatment cells with or without H_2O_2. Each value represents the mean ± SEM; n = 3.

Pre-treatment	ROS challenge	Experiment series 1[a]	Experiment series 2[b]
Control	0	13.0 ±1.5	7.0 ± 1.2
	300 μM	24.7 ± 0.9	20.3 ± 1.2
Quercetin	0	6.0 ± 0.6	4.3 ± 0.3
	300 μM	8.7 ±1.2	9.3 ± 0.9

[a]Experiment series 1 was conducted to investigate extracts A (C5) and B (C6).

[b]Experiment series 2 was conducted to investigate extracts C and D.

Table 6.3: Free radical inhibition of extracts A (C5), B (C6), C and D; quercetin and methanol. The calendula extracts and quercetin (stock 1.61 mg/ml in methanol) were tested using a DPPH assay. The extracts were added neat to a 2ml DPPH solution. Each value represents the mean ± SEM.

Sample	Conc. (%)	Dry weight equivalent (mg/ml)	% radical inhibition at 517 nm (mean ± SEM)
A (C5)	2.5	2.2	41.0 ± 2.0
B (C6)	2.5	2.1	44.4 ± 1.4
C	2.5	3.5	47.7 ± 9.1
D	2.5	3.8	28.7 ± 4.7
Quercetin	2.5	-	55.0 ± 1.6
Methanol	2.5	-	0.1 ± 0.1

6.4 Discussion

A previous study demonstrated that calendula extracts protected against H_2O_2-induced cell killing (Alnuqaydan et al. 2015b). Further work was required to characterise additional aspects of the extracts' protective action. The current study investigated the protection offered against chromosomal damage by four calendula extracts on HaCaT cells *in vitro*. Based on the cytotoxicity data for extracts A (C5) and B (C6), C and D (see Chapter 5 for the toxicity of calendula extracts A (C5) and B (C6), as well as the supplemental data below for the toxicity of calendula extracts C and D), non-toxic doses were chosen for use in the CBMN assay. Firstly, the study showed the safety of calendula extracts, as has been shown for medicinal plants (Alnuqaydan et al. 2015b; Jiménez-Medina et al. 2006; Ramos et al. 1998; Re et al. 2009b). No extract was individually genotoxic at any dose tested on HaCaT skin cells. Total phenolics in the extracts were determined by the Folin-Ciocalteu assay. The

values for extracts A (C5) and B (C6) were 28mg GAE/g dry weight and 26 mg GAE/g dry weights; these values were 10.83 mg GAE/g dry weight for C and 11.73 mg GAE/g dry weights for D (Table 6.1). The levels of polyphenols obtained are equal to or greater than that obtained by others for calendula extracts and other medicinal plants (Atanassova & Georgieva 2010; Miliauskas et al. 2004). Similarly, the DPPH assay demonstrated that the extracts and the positive control quercetin all exhibited free radical inhibition at a 2.5% dose ranging from 29% to 55% (Table 6.3). This study explored the genotoxicity protection of HaCaT skin cells against chromosome damage caused by ROS *in vitro* by pre-treating cells with calendula extracts for 48 hours, then challenging them with H_2O_2. The calendula extracts were negative in the Ames salmonella/microsome mutagenicity assay, and the extracts contained carotenes and flavonoids, indicating high antioxidant activities (Ramos et al. 1998). Laser calendula extracts have shown significant inhibition of tumour growth and tumour cell proliferation when tested on human and murine tumour cell lines; this indicates that calendula extracts could be used to treat cancer (Jiménez-Medina et al. 2006). The findings of this study were consistent with the previously observed antioxidant activity of calendula extracts, as the four extracts protected against chromosomal damage from oxidative stress induced by H_2O_2, which is known to induce free radicals, including ROS. Genetic damage from oxidative stress induced by 300µM H_2O_2 differed significantly from the background level of MNi in the negative control (untreated control). The number of MNi/1000 BN in the untreated control populations ranged from 7–13 MNi/1000 BN, n = 3. H_2O_2, however, induced up to 24 MNi/1000 BN (range = 20–24 MNi), n = 3. The four extracts offered protection against genetic damage induced by ROS, with significant decreases in the number of MNi/1000 BN when HaCaT cells treated with extracts were challenged with H_2O_2 for one hour post-treatment. These results are similar to those of quercetin, the positive control for protection, which reduced genetic damage to only 8.7 MNi/1000 BN, as shown in Table 6.2. The bioactivity level of the extracts appears high enough that there is no significant difference between any of the treatment doses for each extract. Extracts A (C5), B (C6), C and D offered protection against MNi induction at all doses tested (Figures 6.1, 6.2, 6.3 and 6.4), which is consistent with the DPPH and Folin-Ciocalteu assay results. The extracts were also characterised for Folin-Ciocalteu, and extract D was also consistently lower than extracts A (C5) and B (C6) for this test as well. The performance of extract C varied in these two assays, indicating its composition of bioactives differs from that of the other extracts. This requires further investigation, as the protection offered by the extracts did not fully reflect the chemical characterisation results.

Increases in the total number of MNi per 1000 BN cells indicates changes at the chromosomal level, reflecting H_2O_2 –induced damage due to oxidative stress from free radicals. Free radical-induced damage to DNA and chromosomes by a cellular redox imbalance from oxidative stress can result in cancer (Dizdaroglu 1991; Valko et al. 2006). MNi arise from either whole chromosome losses or a-centric chromosome fragments. To investigate the specific type of chromosome damage responsible for the increases of MNi, it is necessary to use molecular techniques in combination with the CBMN assay (e.g., probe for the presence of centromeres in MNi using fluorescence probes) (Fenech 2007). NPB and NBUDs were not observed after the cells were exposed to H_2O_2. NPB can be due to di-centric chromosomes and NBUDs can arise from gene amplification. Cancer develops due to the long-term accumulation of mutations in human genes (Lengauer et al. 1998). Some cancers are also diagnosed as having a genetic instability; of these, most are observed as having an instability at the chromosomal level, which can be chromosome breakage or whole chromosome losses (Lengauer et al. 1998). The calendula extracts examined in this study offered protection against chromosomal damage induced by oxidative stress. Preventing the induction of chromosomal damage using calendula extracts could reduce the risk of cancer. Interestingly, although it is not being used for protection, calendula is being used versus trolamine in the third phase of a randomised trial of calendula for breast cancer (Pommier et al. 2004).

In conclusion, H_2O_2 damage can lead to oxidative stress and cause human cancer via chromosomal breakage or loss and DNA damage (Fenech 2007). Calendula extracts protect HaCaT skin cells against oxidative stress induced (by H_2O_2) chromosome damage by decreasing the frequency of MNi. Further work on calendula extracts should include studying the protection offered against UV light-induced chromosome and mutation damage on human skin cells *in vitro* (Fonseca et al. 2010). The extracts tested in this paper have potential applications as protectants due to their antioxidant properties, including bioactives that act against oxidative stress-induced damage to human cells.

6.4.1 Additional data (not included in the publication)

A) Toxicity and toxicity protection of calendula extracts C and D

The toxicity of calendula extracts A and B refers to section 5.3.2. The toxicity of calendula extracts C and D on HaCaT human skin cells is shown in Figures 6.5 and 6.6. Toxicity was induced by the highest dose (5%) of all extracts after the HaCaT cells were incubated for 48 hours. Extracts C and D offered significant protection for HaCaT cells against H_2O_2 (Figures 6.7 and 6.8).

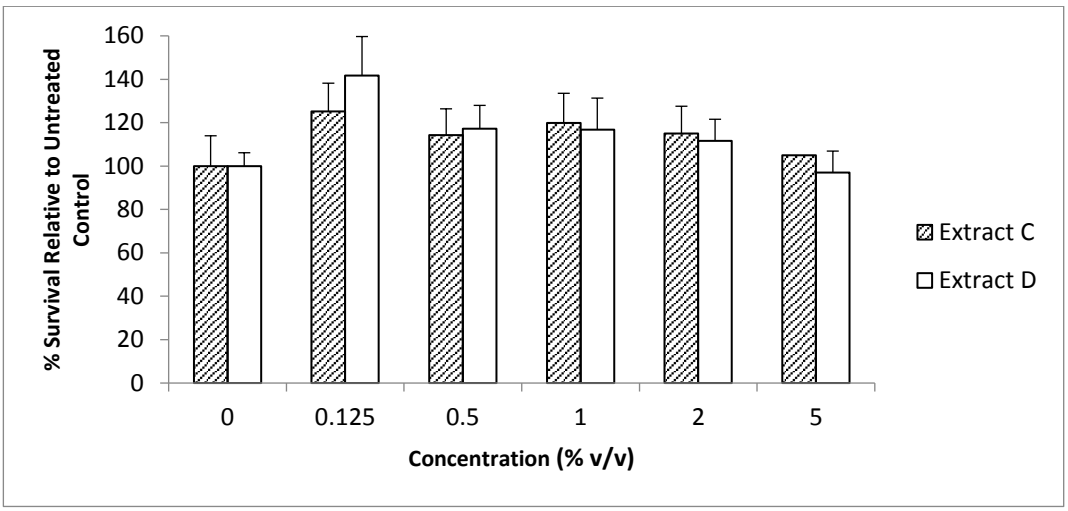

Figure 6.5: Relative survival at 24 hours for extracts C and D. HaCaT cells were treated for the time indicated, then assayed by MTT assay. The data are shown as mean survival relative to the untreated control ± SEM; n = 3.

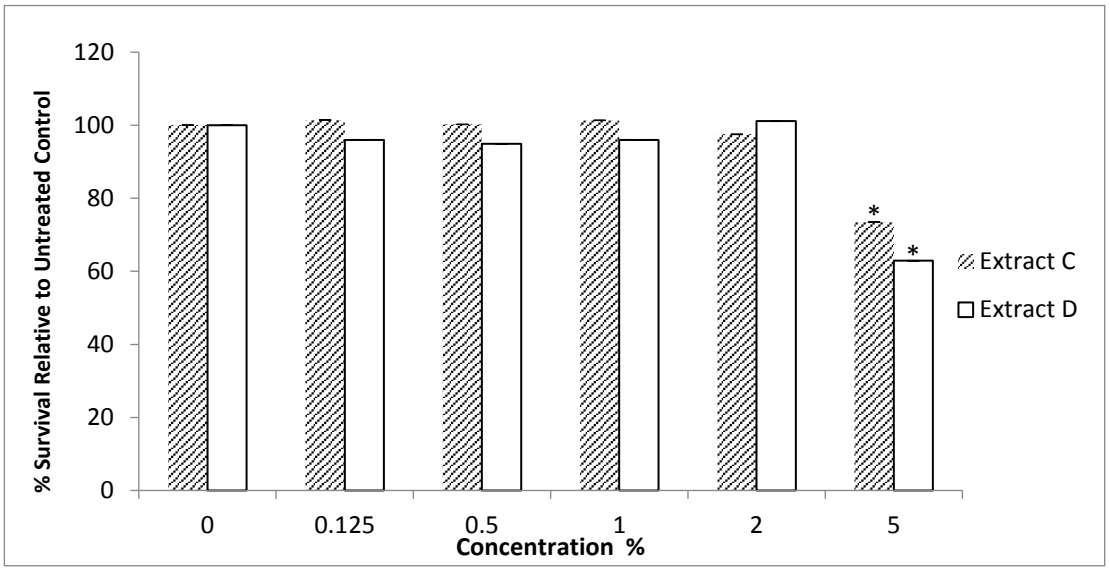

Figure 6.6: Relative survival at 48 hours for extracts C and D. HaCaT cells were treated for the time indicated, then assayed by MTT assay. The data are shown as mean survival relative to the untreated control ± SEM; n = 3.

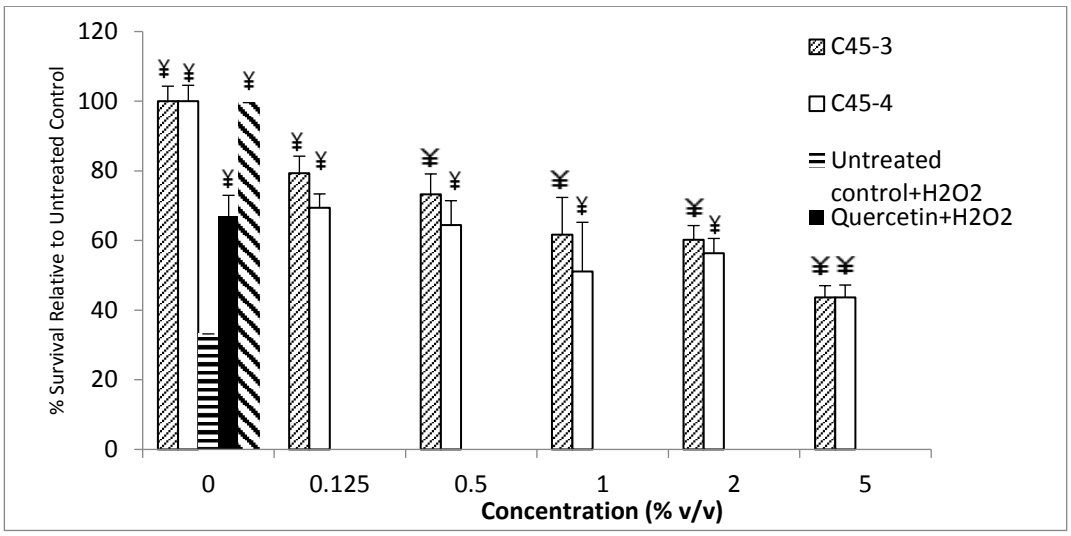

Figure 6.7: Survival relative to the untreated control, determined by the MTT assay. The cells were plated in microplates at 10,000 cells/well, then treated for 24 hours with extracts C and D, then for one hour with 300μM H_2O_2. Q = populations of cells treated with the control antioxidant quercetin; H_2O_2 = hydrogen peroxide = oxidative stress; + = challenged with H_2O_2. ¥ = significantly different from untreated control + H_2O_2, P < 0.05. * = significantly different from Q + H_2O_2, P < 0.05. Data are shown as mean ± SEM; n = 3. Quercetin = 0.016 mg/ml; 1% C = 1.4 mg dry weight equiv/ml; 1% D = 1.47 mg dry weight equiv/ml.

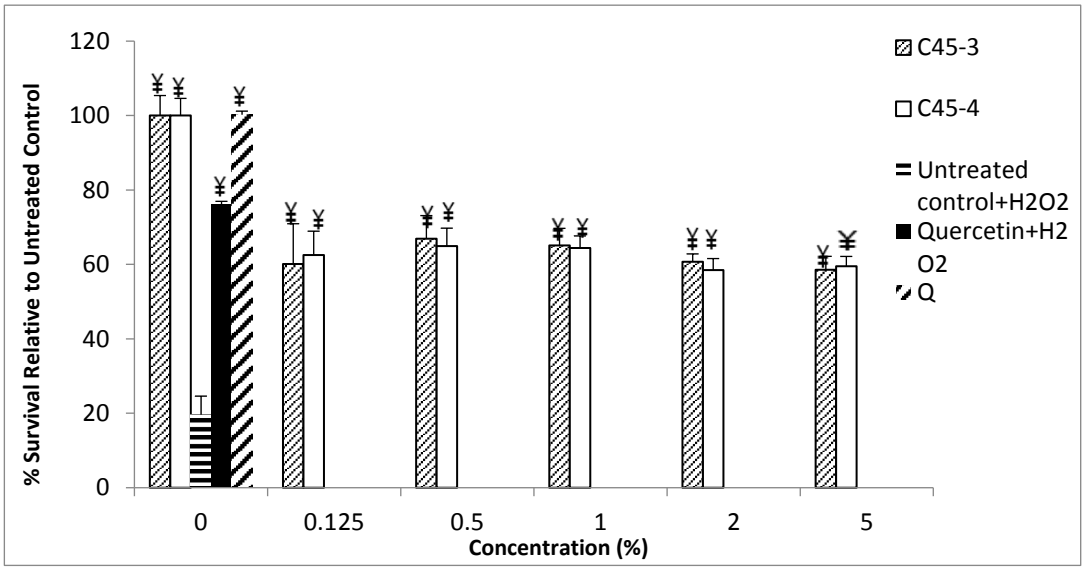

Figure 6.8: Survival relative to the untreated control, determined by the MTT assay. The cells were plated in microplates at 10,000 cells/well, then treated for 48 hours with extracts C and D, then for one hour with 300μM H_2O_2. Q = populations of cells treated with the control antioxidant quercetin; H_2O_2 = hydrogen peroxide = oxidative stress; + = challenged with H_2O_2. ¥ = significantly different from the untreated control + H_2O_2, P < 0.05. * = significantly different from Q + H_2O_2, P < 0.05. Data shown as mean ± SEM.; n = 3. Quercetin = 0.016 mg/ml; 1% C = 1.4 mg dry weight equiv/ml; 1% D = 1.47 mg dry weight equiv/ml.

B) The mechanism of cell killing of all calendula extracts

The NDI is a method used to measure the general toxicity of viable cells (Fenech 2007; Ionescu et al. 2011). Table 6.4 shows the NDI results of all calendula extracts examined in this study. Calendula extracts significantly increased the NDI value, protected the cell cycling of HaCaT cells against H_2O_2, decreased the NDI value and affected the cellular division. The design of cell death (apoptosis or necrosis) after HaCaT cells were treated with calendula extracts A (C5), B (C6), C and D for 48 hours with or without H_2O_2 is shown in Table 6.4.

Table 6.4: The mechanism of cell killing for all calendula extracts determined by the CBMN assay. Data are shown as the mean of three observations ± SEM. *Significant difference from untreated control at *P < 0.05. ¥ = significantly different from the untreated control + H_2O_2, P < 0.05. Data are shown as the mean ± SEM; n = 3.

Extracts	Apoptosis		Necrosis		NDI	
	No H_2O_2	With H_2O_2	No H_2O_2	With H_2O_2	No H_2O_2	With H_2O_2
zero control	5.3± 2.58	9.6 ± 5.0	4 ± 2.0	4 ± 2.0	1.6 ± 0.12	1.2 ± 0.03
Quercetin	3.3 ± 2.0	4 ± 2.6	3.3 ± 3.2	2.3 ± 1.5	1.5 ± 0.08	¥1.7 ± 0.05
A (C5) 0.125%	5.6 ± 3.0	5 ± 3.2	2.7 ± 2.1	3 ± 1.7	1.6 ± 0.03	1.5 ± 0.03
A (C5) 0.5%	4 ± 2.0	5.6 ± 3.5	3.7 ±.05	3.5 ± 1.5	1.6 ± 0.09	1.5 ± 0.02
A (C5) 1.0%	*10 ± 0.1	6.7 ± 1.15	5.7 ± 2.5	5 ± 2.0	1.6 ± 0.02	¥1.6 ± 0.04
B (C6) 0.125%	2.3 ± 1.5	7 ± 2.5	1 ± 0.5	4 ± 0.1	1.7 ± 0.15	¥1.6 ± 0.12
B (C6) 0.5%	3.3 ± 2.5	4 ± 1.7	2.7 ± 2.0	3.1 ± 1.5	1.6 ± 0.12	¥1.8 ± 0.21
B (C6) 1.0%	5.7 ± 1.5	6.3 ± 3.1	2.6 ± 3.0	4 ± 2.6	1.6 ± 0.10	¥1.6 ± 0.11
C 0.125%	7 ± 2.6	8 ± 1.0	3 ± 7.8	2 ± 1.0	1.6 ± 0.05	¥1.7 ± 0.02
C 0.5%	4 ± 1.0	4.3 ± 1.5	2.6 ± 1.5	2.9 ± 1.5	1.5 ± 0.02	¥1.7 ± 0.01
C 1.0%	6 ± 2.6	5 ± 1.5	2.8 ± 0.5	5 ± 0.0	1.5 ±.04	¥1.6 ± 0.03
D 0.125%	5.6 ± 4.0	4 ± 1.7	3 ± 1.0	2.3 ± 0.5	1.5 ± 0.01	1.5 ± 0.04
D 0.5%	3.6 ± 0.5	5 ± 1.0	2 ± 1.0	3.3 ± 0.5	1.5 ± 0.01	¥1.6 ± 0.07
D 1.0%	6.3 ±4.9	3.3 ± 1.15	2.6 ± 0.5	1.3 ± 0.6	1.6 ± 0.11	¥1.6 ± 0.01

CHAPTER 7: CALENDULA OFFICINALIS PROTECTS AGAINST THE CYTOTOXICITY EFFECTS OF PERSONAL CARE PRODUCTS ON HACAT HUMAN SKIN CELLS

7.1 Introduction

Human skin exposed to ionizing and UV radiations or drugs and/or personal care products such as head lice treatments or beauty products that could generate ROS in vast quantities could oxidise degrading pathways (Bickers & Athar 2006). Uncontrolled ROS can be implicated in many aspects of pathogenesis, such as human skin disorders, for example cutaneous neoplasia (Bickers & Athar 2006; Black 2004). There are many agents that can produce oxidative stress in human skin including industrial sources, UV radiation, food contaminants, additives, drugs or cosmetic products (Athar 2002; Bickers & Athar 2006). RNS can also be formed from exposure to environmental agents like xenobiotics (Bickers & Athar 2006). Oxidative stress interacts with the process of a variety of skin diseases (Bickers & Athar 2006). Calendula extracts have been shown to protect cells from being killed and to fight chromosomal damage induced by H_2O_2 in HaCaT cells (Alnuqaydan et al. 2015a; Alnuqaydan et al. 2015b). Calendula was shown to contain a number of compounds with antioxidants and free radicals with scavenging potential. Calendula was used as a skin conditioning agent in cosmetics due to its anti-inflammatory properties (Re et al. 2009a). Other uses include treatment for first degree burns and rashes (Re et al. 2009a). However, chemical head lice treatments and chemical beauty products can induce toxicity and genotoxicity in human skin cells (see Chapter 3 and 4) (Alnuqaydan & Sanderson 2016). The hypothesis of this study is that personal care products such as chemical head lice treatments and beauty products induce toxic effects by producing oxidative stress in human skin cells.

This study examined two head lice treatments (Lice Breaker treatment (permethrin 1% w/w) and Organix pyrethrum treatment (4g/L pyrethrins plus 16g/L piperonyl butoxide)), and two beauty products, (NVAW face moisturiser (containing a mixture of chemical ingredients) + TiO_2 and NVAW face moisturiser (containing a mixture of chemical= ingredients) (without TiO_2)). This study aimed determine the ability of the calendula flower extract to protect human skin cells against any detrimental effects of these personal care products on human skin cells *in vitro.*

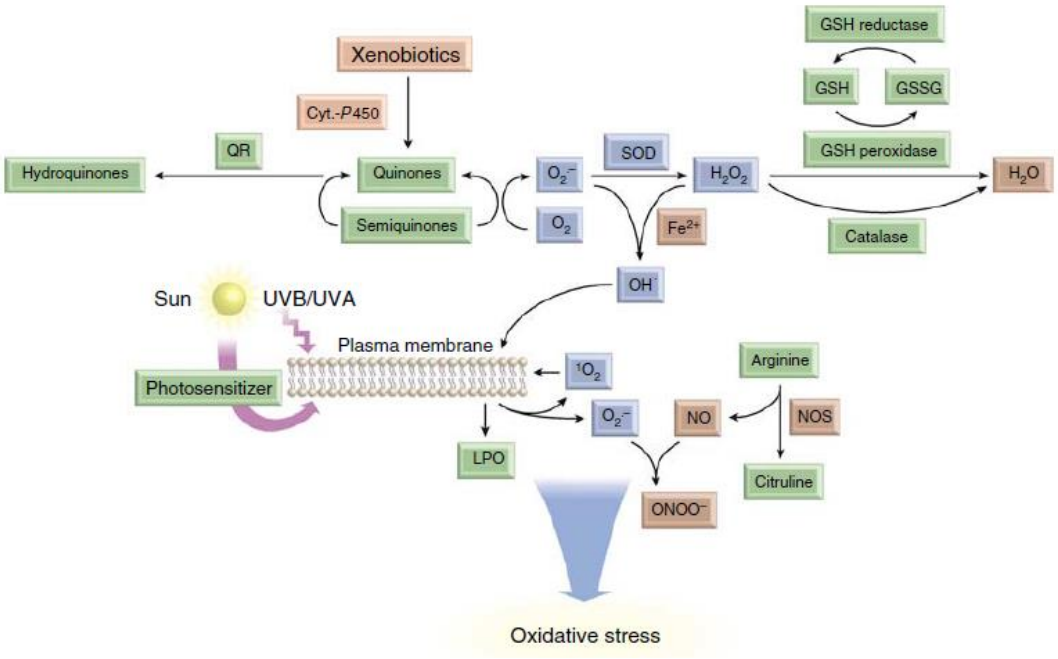

Figure 7.1: Generation of ROS and antioxidant defense in skin cells. Source: Bickers and Athar (2006).

7.2 Material and methods

7.2.1 Materials

All of the reagents used in this chapter were purchased from Sigma-Aldrich unless otherwise stated.

7.2.2 Plant extract and characterisation

The characterisation of calendula extract C and its ability to protect HaCaT cells against H_2O_2-induced oxidative stress cell killing and genetic damage was described in Chapter 6 and published previously (Alnuqaydan et al. 2015a). In brief, an industry partner (Jurlique International, Mount Barker, South Australia) supplied dried flowers of *Calendula officinalis* from biodynamically grown plants. Calendula extract C was prepared as a proprietary aqueous extract and diluted in RPMI media to make the treatment solution. The determination of density for extract C (1.036) was carried out at 20°C by averaging 10 independent measurements. The dry weight of extract C was 1.4 mg/ml as determined after the extract samples were freeze-dried at -56 °C at 3.4 Pa for 24 hours. The Folin-Ciocalteu assay was used to estimate polyphenol composition, and the GAE of extract C was 10.83 mg GAE/g dry weight. The DPPH assay was used in previously published work to perform the free radical inhibition of extract C. Extract C showed 47.7% radical inhibitions at a concentration of 2.5% (3.5 mg/ml dry weight equivalent).

7.2.3 Cell culture

The cells were cultured as described in Chapters 5 and 6.

7.2.4 Cell treatment

HaCaT cells were treated to expose them to toxicity before the protection assay was conducted. A 96-well, flat-bottom microplate was seeded with 10^4 cells/well and incubated for 19 hours at a temperature of 37°C in 5% CO_2 to allow the cells to adhere. The media were aspirated and replaced with 200µl of the treatment solution, which was 0.125, 0.5, 1.0, 2.0 and 5% (v/v) extract C per well for 48 hours. Then follow-up was conducted with 100µl of the product treatments (head lice treatments, TiO_2 or beauty products) for one hour before MTT assay analysis. The negative or zero control was media.

7.2.5 The MTT cell viability assay

A MTT colorimetric assay was conducted to measure cell viability (Alnuqaydan et al. 2015a; Mosmann 1983; Young et al. 2005). This treatment assay was carried out as described in section 3.2.5.

7.2.6 Statistical analysis

The experiments were done in triplicate and the data are presented as the mean ± SEM. A one-way ANOVA and Tukey's posthoc test were carried out using SPSS software, version 22. Differences were considered to be statistically significant when the P value was less than 0.05.

7.3 Results

The toxicity of calendula extract C on HaCaT cells *in vitro* was explored by incubating cells with the extract for 48 hours. Figure 7.1 showed the toxicity of extract C. Extract C showed toxicity only at a high dose (5%) after being treated for 48 hours. At the lowest doses, the extract (0.125, 0.5, 1.0 and 2.0 % (v/v)) was not toxic to HaCaT skin cells for any of the treatment periods. This effect could involve a period of contact between these components and the cells, such that these toxic components would be taken up by cells, thus reducing the cellular viability. In contrast, lower concentrations of these extracts would be non-toxic if incorporated into products at a safe dose, which is difficult to ascertain on human skin. The toxicity of the calendula extracts at higher doses found in this study needs to be translated into a recommendation for the use of calendula extract on human skin.

Head lice treatments can induce cell killing as apoptosis or necrosis (Chapter 4) (Alnuqaydan & Sanderson 2016). Figure 7.2 showed the toxicity results of personal

care products on HaCaT human skin cells. HaCaT human skin cells were pre-treated for 48 hours with 1% calendula extract C followed by one hour of treatment with personal care products (beauty products or head lice treatments). Permethrin doses of 10% and 100%, pyrethrum doses of 10% and 50% and NVAW at doses of 0.3% and 3% with or without TiO_2 proved to be significantly toxic to HaCaT cells when measured by MTT assays.

The lower concentration of extract C (1% v/v) was chosen as a pre-treatment for HaCaT cells due to its safety at all treatment periods. Calendula extract showed a significant ability to protect HaCaT cells against the toxic effects of personal care products including TiO_2 after the cells were treated with extract C for 48 hours, as illustrated in Figure 7.2. Treatment with calendula for 48 hours offers more protection than a 24 hour treatment period (data not shown). Table 7.1 shows the untreated and positive controls of TiO_2 in the experiments. When the cells were incubated for 48 hours with calendula extract C alone, there was a mean increase in relative survival of 62%. This is likely due to some cell growth during the incubation period. It is important to ascertain whether that the protection observed as being offered by the personal care products is real, because the positive control TiO_2 challenge still results in cells being killed and only offers a low level of protection. Notably, treatment with calendula alone led to an increase in cell numbers. This was not used as the untreated control as the correct control was the untreated cells.

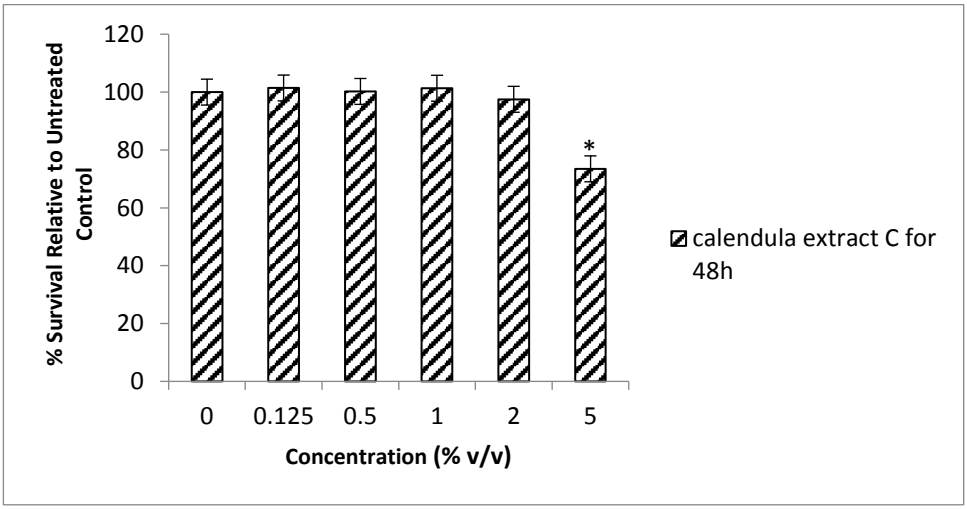

Figure 7.2: Relative survival at 48 hours of exposure to the calendula extract. HaCaT cells were treated for the time indicated, then assayed by an MTT assay (see section 7.2.4). Data are shown as mean survival relative to the untreated control ± SEM; n = 3. * = treatments are significantly different from the 0 untreated control at $p < 0.05$.

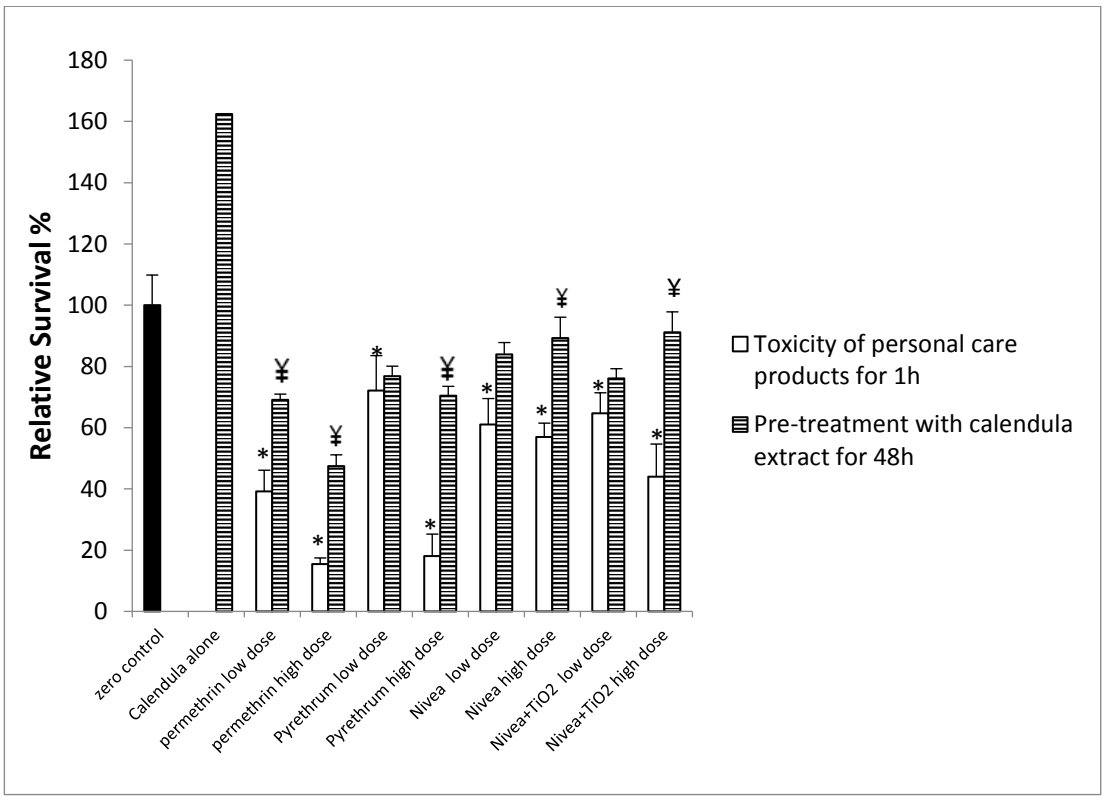

Figure 7.3: Toxicity and calendula protection of personal care products on HaCaT human skin cells, as measured by MTT assays. Permethrin (low dose, 10%; high dose, 100%), Pyrethrum (low dose, 10%; high dose, 50%), NVAW without TiO_2 (low dose, 0.3%; high dose, 3%) and NVAW + TiO_2 (low dose, 0.3%; high dose, 3%). Data are shown as mean ± SEM, n = 3. * = treatments are significantly different from the 0 untreated control at p < 0.05. ¥ = pre-treatment with calendula extract significantly different from products toxicity (protection).

Table 7.1: Untreated control and TiO_2 = the positive control of beauty products. Data are shown as the mean ± SEM, n = 3. * = treatments are significantly different from the zero untreated control at p < 0.05. ** = treatment is significantly different from the TiO_2 dose at P < 0.03 one-tailed T-Test.

Sample	Toxicity for 1 hour	Pre-treatment with calendula extract for 48 hours
Medium control	100 ± 1.3	100 ± 1.5
TiO_2 low dose (150µg/ml)	*67.8 + 1.4	73.6 ± 10.9
TiO2 high dose (200µg/ml)	*58.2 + 3.2	**73.3 ± 5.2

7.4 Discussion

The human body is exposed to a variety of pro-oxidants in the environment including drugs, chemicals, mixtures, radiation, pollutants and cosmetics. These agents can target lipid-rich membranes, cellular DNA or proteins to produce toxicity. Personal care products such as beauty products can generate oxidative stress in human skin cells (Bickers & Athar 2006). The increases in ROS play a role in a variety of skin diseases and carcinogenesis (Bickers & Athar 2006; Klaunig & Kamendulis 2004). The beauty products and head lice treatments examined in this study induced significant toxicity in HaCaT human skin cells. The hypothesis of this study was that personal care products such as beauty products or head lice treatments could generate oxidative stress in human skin cells. As a result, significant toxicity was induced after treating HaCaT cells with these products for one hour. The toxicity results of personal care products on HaCaT cells were measured by the MTT assay which is a relative survival assay. Calendula showed protective activity against H_2O_2-induced oxidative stress cell killing and chromosomal damage (Alnuqaydan et al. 2015a; Alnuqaydan et al. 2015b). Therefore, HaCaT cells treated with calendula extract C to produce bioactivity antioxidant compounds did protect against the toxicity of personal care products (beauty products and head lice treatments) that caused oxidative stress cell killing. The protection offered by extract C was time-dependent as explored previously (Alnuqaydan et al. 2015a;Alnuqaydan et al. 2015b). It was noted that significantly higher protection occurred after pre-treatment with calendula extract with compounds that displayed higher toxicity when induced by the treatment of compounds alone. This was seen in the protection offered against high doses of pyrethrum, NVAW and NVAW with TiO_2. Further work needs to be done to understand the mechanism of action of the calendula plant's components and how they work to protect human skin cells. TiO_2 was classified as a carcinogenic nanoparticle used in cosmetics. TiO_2 plays a role in the induction of apoptosis as well as oxidative stress (Bhattacharya et al. 2009; Park et al. 2008; Reeves et al. 2008). One study indicated that TiO_2 can affect the mitochondria (Tucci et al. 2013).

Calendula extract contains bioactives and free radical scavenging compounds that can interact with ROS to either eliminate them or minimise their deleterious effects (Bickers & Athar 2006). These antioxidants or bioactive compounds include ascorbic acid, vitamin E, vitamin C, alpha-tocopherol, quercetin, beta-carotene and amino acid (Bickers & Athar 2006; Alnuqaydan et al. 2015b). Oxidative stress also drives the production of oxidation products (e.g., 4-hydroxy-2-nonenal or malonaldehyde), that can denature proteins and alter apoptosis or influence the release of pro-inflammatory mediators such as cytokines in inflammatory skin diseases (Bickers & Athar 2006).

Moreover, ROS in the induction of many biological responses can act as second messengers, such as in the generation of cytokines (Briganti & Picardo 2003). The alterations in cellular proteins or peroxidation of lipid-rich membranes caused by ROS can contribute to a range of skin diseases or cancer (Bickers & Athar 2006). Experimental evidence supports the role played by ROS in the cancer process (Bickers & Athar 2006; Klaunig & Kamendulis 2004).

Increases of ROS in the cell, through either endogenous or exogenous factors, contribute to the carcinogenesis process. This could occur via genotoxic effects resulting in oxidative DNA adducts or via the modification of gene expression (Klaunig & Kamendulis 2004). The MTT assay employed in this study as a colorimetric assay for mammalian cell growth and survival depends on the ability of viable cells to metabolise the yellow, water-soluble tetrazolium salt in the mitochondria of living cells. It can be used for mitochondrial dysfunction (Brand & Nicholls 2011; Mosmann 1983). Mitochondrial damage is linked to the induction of mutations (Tamura et al. 1999). Moreover, mitochondrial DNA mutation and alteration in gene expression (mutation in the gene encoding for complexes I, II, IV and V) has been identified in many types of cancers and human tumours (Tamura et al. 1999). The mutation rate in mitochondria is reported to be greater than in nuclear DNA (Wang et al. 1997).

In conclusion, it is evident that the beauty products and head lice treatments examined in this study induced significant toxicity in HaCaT human skin cells, perhaps by generating oxidative stress in skin cells. This process contributes to skin disorders such as inflammation and carcinogenesis or cancer. In addition, the positive control of the beauty product containing TiO_2, which is a carcinogen nanoparticle, does play a role in the oxidative stress by damaging the mitochondria in the cell (Bhattacharya et al. 2009; Park et al. 2008; Reeves et al. 2008; Tucci et al. 2013). Finally, this study indicated that *Calendula officinalis* extract C does protect HaCaT human skin cells against the toxicity caused by beauty products and head lice treatments.

CHAPTER 8: MAJOR FINDINGS AND FUTURE DIRECTIONS

8.1 Major findings and future directions

Skin is the largest external organ in the body, and it is the first organ exposed to environmental hazards, including hazardous compounds, pharmaceuticals or consumer products that are intentionally applied. Toxic substances can pass through the skin. The amount of skin absorption depends on the contact of the surface area (intact or broken skin) and the lipid solubility of the toxic agent (Olson 2014). The skin's layers play an important role in protecting the body against toxic agents. For example, the epidermis (keratinocytes HaCaT cell line) acts as a lipid barrier; the stratum corneum provides a protective barrier against toxic substances and the dermis (human dermal fibroblast cells CCD-1046) is permeable to various toxic agents (Olson 2014; Zeliger 2008b). Absorption also can be enhanced or affected by many factors, such as using gloves or wearing clothes. Hydrated skin is more permeable than dry skin, and thick skin is more resistant than thin skin (Olson 2014). For example, the thick skin on the palms of the hands is more resistant than the thin skin on the face, neck or scrotum (Olson 2014). Moreover, injured skin, such as burns, abrasions or dermatitis, is vulnerable to absorbing vast quantities of toxic substances (Olson 2014). Tissue injuries can be affected by the pH of the substance; for example, highly acidic substances cause coagulation-type necrosis with some damage. However, highly alkaline substances cause liquefactive necrosis to occur, with extensive deeper damage in the tissue (Olson 2014; Zeliger 2008b).

In vitro methods are important as a first step for estimating the effect on the skin (permeation, skin irritation and sensitisation) and valuable for predicting the carcinogenicity of products that can induce toxicity in the skin (ATSDR 2009; Middleton 1989). The advantages of *in vitro* studies are as follows: they are inexpensive with regard to cost and time; no ethics approvals are needed and the methodology and assays are well-validated.

This study's original contribution to knowledge is that testing products on human skin cells *in vitro* is an important step to predict the carcinogenicity of tested products. In exploring this issue, this study examined different commercial products (beauty products, head lice treatments and calendula plant extracts used in cosmetics). Normal human skin cell lines were used as *in vitro* models.

This important topic seeks to evaluate the safety and assess the risk of all kinds of compounds that make contact with human skin.

This study used MTT and CV assays to evaluate the cytotoxic and toxic effects of these beauty products and head lice treatments on human skin cells. However, there

were some differences in the results obtained from the two assays. The CV assay potentially overestimated the relative cell number, as it is based on the detection of all of the adherent cells, including dead cells (Chiba et al. 1998).

Further, the mechanism of cell death of these treatments was detected using an FITC Annexin V apoptosis detection assay followed by flow cytometry. In this study, the apoptosis detection assay was optimised to measure apoptosis on adherent human keratinocytes, although adherent cell lines are not recommended by the manufacturer. The rationale for this was that specific membranes could be damaged during harvesting (Pharmingen™ 2008). The result of the trypan blue assay showed that harvesting using the TrypLE express enzyme did not damage the membrane of the adherent cells. Therefore, harvesting did not affect the result of apoptosis detection measured by flow cytometry. The result of this optimisation indicated that adherent cell lines can be used to detect apoptosis using the FITC Annexin V apoptosis detection assay followed by flow cytometry.

Four beauty products (NVAW + TiO_2, NVAW without TiO_2, FMCGB and glycerol) were examined for their effects on two human skin cell lines. The consequence of exposing the cells to these products indicated that, although none of these chemicals are known to be carcinogenic individually, the mixtures are carcinogenic. The chemicals in these mixtures may work synergistically to be carcinogenic. The current study indicated that NVAW + TiO_2 induced a significant level of toxicity and genotoxicity in the HaCaT cell line. A new Nivea Visage product without TiO2, but with all the other ingredients ('improved' formula) induced a significant level of genotoxicity on the human fibroblast CCD-1064SK cell line (see Table 8.1).

In comparing the two products by examining them on the HaCaT cell line, it was clear that NVAW + TiO_2 differed significantly from NVAW without TiO_2. There was a significant rise in the number of MNi with the NVAW+TiO_2, indicating that TiO_2 manifests as chromosomal damage. The general mechanism of toxicity measured by the NDI indicated that a significant low NDI value (1.4 ($P < 0.05$)) was measured at a dose of 0.3% in both of the Nivea products. The mechanism of cell killing, which the two different Nivea formulae inflicted on the HaCaT cells, was measured by the CBMN assay. Significant levels of apoptosis and necrosis were induced by both products on human skin cells.

The FMCGB product, which is a mixture of natural ingredients, showed less toxicity in this study than NVAW. It demonstrated no significant toxicity on HaCaT or CCD-1064SK cells, but low NDI and significant levels of necrosis were observed in HaCaT

cells. Finally, glycerol, which used as a negative control, showed no toxicity or genotoxicity on human skin cells *in vitro.*

Using two cell lines of human skin cells provides extra information to link cell types, such as *in vivo* fibroblasts. This study examined doses that are lower than what the general public would have been exposed to with daily use. Further work is needed to correlate this *in vitro* data with *in vivo* results initially with an *in vivo* animal model. This could be the next phase in this research.

The effects of five currently used head lice treatments on human keratinocytes in culture have also been examined and presented in Chapter 4. The finding of this study confirmed that significant toxic hazards are posed by some of the ingredients of head lice treatments, such as pyrethrum and pure TTO at the concentration used to kill head lice (10%). Pure lavender oil (100%) proved to be significantly toxic to HaCaT cells. However, the concentration of lavender oil used in head lice treatments (1%) is safe and less toxic *in vitro*. Permethrin (1% v/v) and maldison (malathion) (0.5% w/w) were evaluated as being genotoxic *in vitro* and induced wide-ranging toxic outcomes through necrosis and apoptosis. This type of genetic damage has been linked to carcinogenesis (see Table 8.1) (Fenech 2007; Ionescu et al. 2011). Further work could be done on maldison to observe the cells microscopically for phenotypical changes and utilise a live/dead assay. Future work might also study the action of TTO further using additional genotoxic endpoints and extra replicates for the CBMN assay.

Table 8.1: Order of the highest dose toxicity (1/100 dilution of origin commercial concentration) of all of the products examined on human skin cells

Product	*Cell killing on keratinocytes cells	Cell killing on fibroblast cells	Chromosomal damage MNi/1000 BN
Beauty products			
NVAW day moisturiser + TiO_2	92%	Not done	+
NVAW day moisturiser without TiO_2	Not done	75%	+
FMCGB	33%	23%	-
Glycerol BP	0	40%	
Head lice treatments			
Permethrin (1% commercial concentration)	35%	Not done	
Maldison (malathion) (commercial concentration 0.5% w/w)	32%	Not done	+
Head lice treatment concentration of TTO (10%)	70%	Not done	-
Head lice treatment concentration of lavender oil (1%)	0	Not done	-
Head lice ingredients			
Pyrethrum	100%	Not done	-
Pure TTO 100%	96%	Not done	-
Pure lavender oil 100%	98%	Not done	-

*The toxicity (cell killing) of beauty products on keratinocytes (HaCaT) cells was presented in Alnuqaydan 2011.

Calendula officinalis dried flower extracts could function as antioxidants to protect against H_2O_2-induced oxidative stress cell killing of human skin cells. Four different calendula extracts were examined for their ability to protect HaCaT cells against oxidative stress induced by 300μM H_2O_2. Two calendula extracts C and D offered significant protection with all doses at 24 and 48 hours of treatment. The DPPH assay showed the antioxidant activity of calendula extracts in terms of radical scavenging ability. The calendula extracts examined in this study contained the same or greater levels of polyphenols than those obtained by others for calendula extracts or other medicinal plants (Atanassova & Georgieva 2010; Miliauskas et al. 2004).

The CBMN assay indicated that all four calendula extracts protected against H_2O_2-induced MNi. This was significant ($P < 0.05$) at all doses for human skin cells after treatment with each of the extracts for 48 hours followed by one hour of treatment with 300-µM H_2O_2. The protection level of all calendula extracts examined was similar to the protection level of the positive control (quercetin) against H_2O_2-induced oxidative stress genetic damage. However, further work is needed to fully characterise the protective action of these extracts, including studies on human skin fibroblasts. To investigate the specific type of chromosomal damage induced by H_2O_2 due to oxidative stress from free radicals responsible for increases in MNi, it is necessary to use molecular techniques in combination with the CBMN assay (e.g., probe for presence of centromeres in MNi using fluorescence probes).

The problem with commercial products and beauty products in particular is that millions of people apply beauty products to their skin every day for long periods of time (Middleton 1989). The hypothesis of this study was that commercial products such as beauty products or even head lice treatments generate oxidative stress that could produce ROS-induced cell death and chromosomal damage in the form of apoptosis or necrosis. The mechanism of cell death via oxidative stress through apoptosis or necrosis has been studied by Higuchi (2003).

Calendula extract is known to exhibit antioxidant, anti-tumoural, anti-inflammatory, anti-viral and anti-HIV properties (Jiménez-Medina et al. 2006; Kalvatchev et al. 1997b). Moreover, different extracts of the calendula plant have demonstrated the ability to protect HaCaT cells against oxidative stress-induced cell death and chromosomal damage. The ability of the calendula plant to protect against oxidative stress is time- and dose-dependent.

Chapter 7 showed the ability of calendula extract to prevent the toxicity caused by personal care products that induce oxidative stress (ROS) cell killing in HaCaT human skin cells. TiO_2 is a nanoparticle classified as a carcinogenic that is used in cosmetics. It is known to induce programmed cell death through oxidative stress (Bhattacharya et al. 2009; Park et al. 2008; Reeves et al. 2008). Calendula extract does protect HaCaT skin cells against TiO_2 after a 48-hour period of pre-treatment with the extract. This also applies to the two beauty products (NVAW + TiO_2 and NVAW (without TiO_2). Chapter 7 illustrated the ability of calendula extract to prevent the toxicity of head lice treatments with one active chemical ingredient such as Lice Breaker treatment, which has 1% w/w of permethrin, and pyrethrum treatment (4g/l pyrethrin plus 16g/L piperonyl butoxide).

Additional studies need to be performed to investigate the ability of calendula extract to protect human keratinocyte cells against the potential genetic damage caused by personal care products using the CBMN assay.

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APPENDICES

Appendix I: Cell culture medium for human skin cell lines

RPMI-1640 medium (1L)

RPMI-1640 medium (10.44 g/L, with L-Glu and Phenol Red) was used to grow the HaCaT cells. Weigh out 10.4 g/l (HyQ RPMI-1640 medium, Cat. No. SH30011.03), 0.592 g/l (G8540) L-glutamine, 10ml/L (Hyclone) penicillin and 890 ml Baxter water. The medium solution was adjusted to 7, then 100 ml of 10% heat-inactivated FBS and 7.8 ml/L sodium bicarbonate ($NaHCO_3$) (Pfizer, WA, Australia) was added. The medium was sterilised by 0.22 µm filter and stored at 4°C.

Iscove's Modified Dulbecco's Medium

Iscove's Modified Dulbecco's medium powder IMDM, Sigma Cat. No. 17633-1L; 17.86 ml/L sodium bicarbonate (Pfizer, WA, Australia); L-glutamine (0.592 g/L) or 10 ml; 10 ml penicillin/streptomycin (Gibco®, Hyclone, Utah, US) and 10% heat-inactivated FBS (Hyclon, Victoria, Australia) were added. Volium was made up to 1L using sterile Milli-Q water (Baxter). The medium was the adjusted to pH 7.4 and sterilised by 0.22 µm filter. The medium was sterilised by 0.22 µm filter and stored at 4°C.

Appendix II: Preparation of stock solutions

1X Phosphate-buffered saline

8g of NaCL, 0.2g of KCl, 1.44g of $Na2HPO_4$ and 0.24g of $NH2PO_4$ were dissolved in 900ml of Milli-Q water. PH was adjusted to 7.4, and volume increased to 1L. The stock solution was then autoclave sterilised or filtered through a 0.22µm filter and stored at room temperature.

Methylthiazol thiazolyl tetrazolium

The MTT cell-culture-stock solution (250 mg) was dissolved in 50 ml of sterile 1X PBS for a final concentration of 5 mg/ml. This MTT stock solution was filtered using a sterile 0.22 µm filter and aliquots (to avoid repeat thaw/freeze) into sterile 1.5 mL Eppendorf tubes and stored at -20°C until required.

Preparation of 20% SDS in 0.02M HCl

To prepare 20% SDS, 200 ml of the solution was prepared by adding 40 g SDS into 0.02 M HCl (100 ml) while stirring and applying heat to dissolve the powder. 0.02 M of HCl was added to produce a final volume of 200 ml.

Crystal violet solution (0.5%)

CV stain (0.5g; Aldrich) was dissolved in a 50% methanol solution (Merck; 100 ml) and stored at room temperature in a fume hood.

Acetic acid solution (33%)

33 ml of acetic acid solution (APS Chemicals) was added to 67 ml of reverse osmosis (RO) water and stored in a 100 ml sterile glass schott bottle at room temperature.

Trypan blue solution

A 0.2% dose of trypan blue (0.2 g) was dissolved in a 0.9% NaCl saline solution (100 ml), then filtered and stored at 4°C.

Trypsin diluent

One aliquot 1:5 was diluted in 1X PBS to make the final solution 0.05% trypins/EDTA. The aliquot was stored at -20°C.

0.1% Sodium azide trypan blue solution (0.2%)

0.1 ml of sodium azide (S8032) was added to 90 ml of 1X PBS, then mixed by stirring. The solution was then increased to 100 ml with PBS and stored in a glass Pyrex bottle at -20°C.

6-Thioguanine (HPRT mutation assay)

6-TG stock solution was used within six hours of preparation. 4 ml of stock was prepared with a final concentration of 1 mg/ml. 4 mg of 6-TG was prepared, then 0.4 ml of 0.1 M NaOH was added (0.1 M NaOH was prepared from 1 M of stock). 3.6 ml media was added and filtered through a 0.22µm filter and stored at room temperature.

Cytochalasin B

Cytochalasin B was dissolved in dimethyl sulfoxide at a final concentration of 1.8mg/ml, the filter sterilised and stored at -20°C.

Appendix III: Chemicals, materials and equipment list

Chemical/Material/Equipment	Company
Baxter water	Baxter Healthcare, NSW, Australia
CV stain	Sigma-Aldrich, MO, US
CCD-1064SK fibroblast cell line	ATCC ®
Culture flasks (T75 & T25)	Thermo scientific (Nunc), Roskilde, Denmark
Flat bottom 96-well microplates	Thermo Scientific (Nunc), Roskilde, Denmark
FITC Annexin V apoptosis detection kit	BD pharmingen cat# 556547
HaCaT cell line	ATCC (Department of Hematology, Flinders University)
RPMI-1640	Sigma-Aldrich, MO, US
IMDM medium	Sigma-Aldrich, MO, US
FBS	Hyclone, Australia
Penicillin/streptomycin	Gibco, Utah, US
L-glutamine	Sigma-Aldrich, MO, US
Sodium chloride (NaoH)	Merck, Australia
Trypan blue (TB)	Sigma-Aldrich, MO, US
Trypsin-EDTA	Hyclone, Utah, US
Thiazolyl blue tetrazolium (MTT)	Sigma-Aldrich, MO, US
Sodium bicarbonate ($NaHCO_3$)	Pfizer, WA, Australia
SDS	Sigma-Aldrich, US
Sodium azide	Sigma-Aldrich, US
Hydrochloride acid (HCL)	AJAX Chemicals, Australia
Triple express	Sigma-Aldrich, MO, US
Methanol	Sigma-Aldrich, MO, US
Round-bottom 96-well microplates	Thermo Scientific (Nunc), Roskilde, Denmark
Tubes (sterile, 50ml)	Geriner Bio-One (cell start)
Tissue culture dish	Geriner Bio-One (cell start)
Cytocentrifuge	Shandon Scientific Ltd., Astmoor, Runcorn, UK
Centrifuge 5804	Eppendorf
Microscope	Lieitz Wetzler
Spectrophotometer	Bio-Tek Intruments, Inc.
Laminar hoods	HWS Series (HWS-120,BHA-120)
Fume hoods	Southern Cross
Inverted microscope	Olympus
Haemocytometers	Noubauer Improved
Co_2 incubator	Laboratory Supply Pty Ltd

Appendix IV: Commercial products list

Product	**Company**
Beauty products	
NVAW + TiO_2	Nivea
NVAW without TiO_2	Nivea
FMCGB	Grown Alchemist ®
Glycerol BP	Pharmaceuticals Pty Ltd
Head lice treatments	
Lice Breaker (Permethrin 1% w/w)	Pyrifoam Lice Breaker™
Pyrethrum	Amgrow Organix Organic Xtra
Pure TTO	Bosisto's Australia
Pure lavender oil	Bosisto's Australia

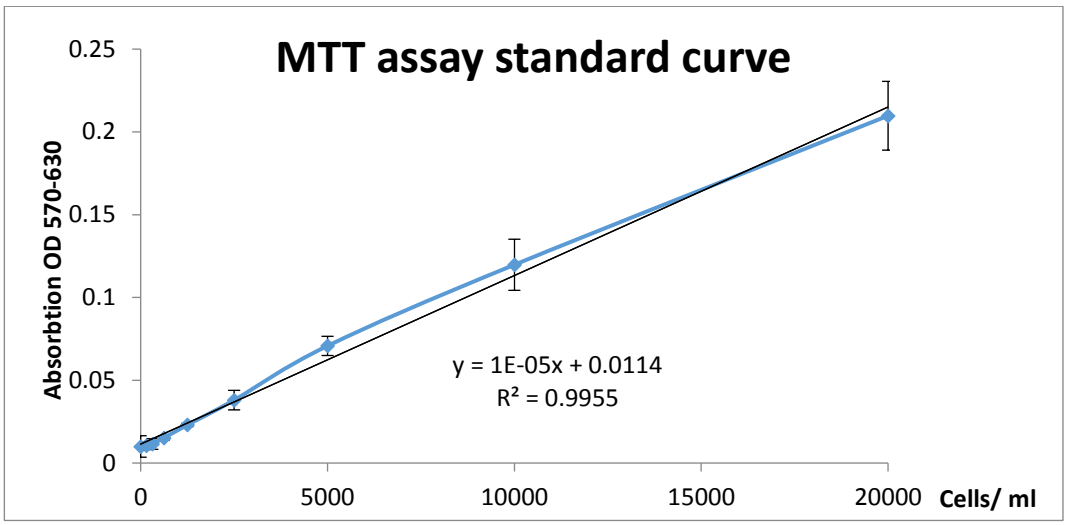
Appendix V: Raw data and data processing of MTT assay

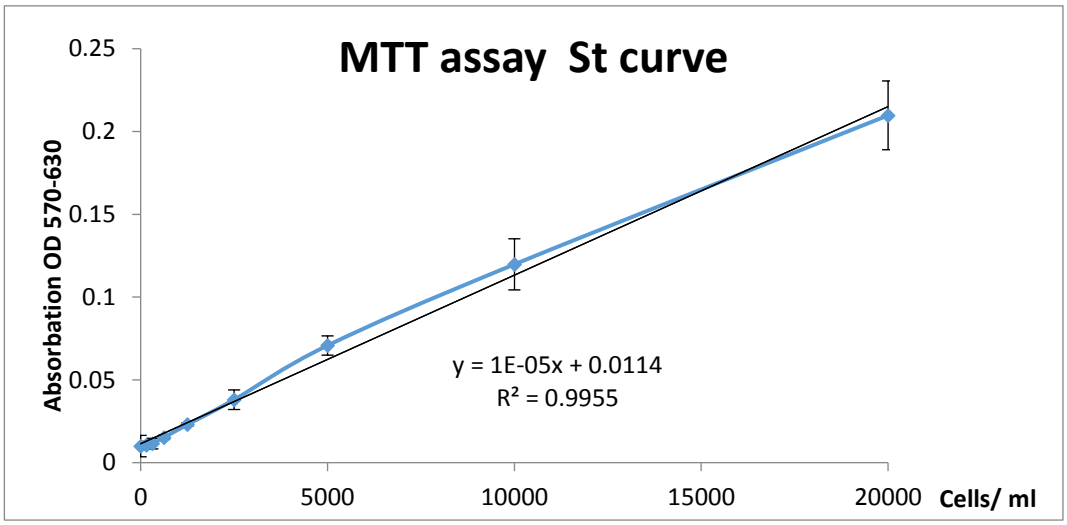
Table A5.1: The ODs value of the MTT standard curve measured by spectrophotometer at an absorption of 570–630.

1	2	3	4	5	6	7	8	9	10
0.009	0.01	0.01	0.013	0.022	0.036	0.069	0.111	0.216	0.36
0.01	0.012	0.013	0.015	0.022	0.038	0.07	0.117	0.192	0.29
0.01	0.013	0.008	0.016	0.024	0.037	0.072	0.124	0.207	0.408
0.011	0.007	0.015	0.017	0.024	0.041	0.072	0.127	0.224	0.385

Table A5.2: The average, standard deviation and concentration of variations of MTT standard curve values

cell no	0	156	312	625	1250	2500	5000	10000	20000	40000
wells no	1	2	3	4	5	6	7	8	9	10
Mean	0.01	0.0105	0.0115	0.01525	0.023	0.038	0.07075	0.11975	0.20975	0.36075
St.Dev	0.000816	0.002646	0.003109	0.001708	0.001155	0.00216	0.0015	0.007182	0.01372	0.051078
C.variatio	8.164966	25.19763	27.03588	11.19885	5.020437	5.68486	2.120141	5.997623	6.541322	14.15871





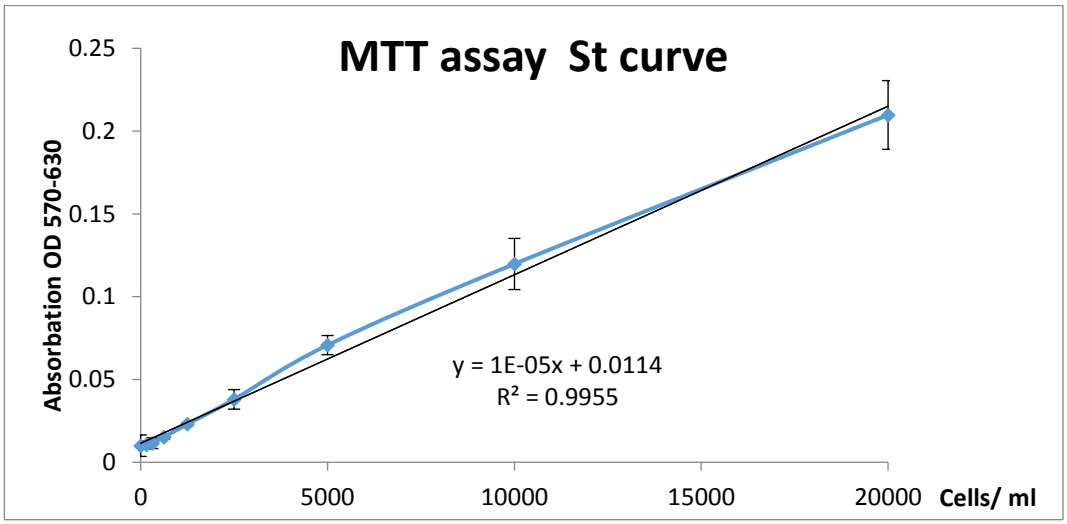


Figure A5.1 MTT standard curve showing the calendula extracts C and D toxicity experiment that was prepared as in Chapters 5 and 6. The X axis shows the concentration of cells/wells until 20 × 10^4. He Y axis shows the absorbance at OD 570–630.

Table A5.3: The ODs values of calendula extract C treatment measured by MTT assay. It used the mean, standard deviation and concentration of variation to find out the cell number and cell viability.

Dose %	Just media/no cells	Untreated control zero	0.125	0.5	1	2	5
	0.108	0.721	0.687	0.662	0.701	0.598	0.458
	0.088	0.736	0.728	0.699	0.72	0.653	0.53
	0.085	0.779	0.694	0.718	0.73	0.624	0.547
	0.084	0.774	0.718	0.711	0.667	0.688	0.553
Mean	0.09125	0.7525	0.70675	0.6975	0.7045	0.64075	0.522
Standard deviation	0.011295	0.028455	0.019414	0.024933	0.027743	0.03869	0.043765
Concentration of variation	12.37839	3.781348	2.746989	3.574659	3.937951	6.038238	8.384008
Cell number		74110	69535	68610	69310	62935	51060
Cell viability		90.50068	84.91384	83.78426	84.63908	76.85414	62.35278

Table A5.4: The ODs values of calendula extract D treatment measured by MTT assay. It used the mean, standard deviation and concentration of variation to find out the cell number and cell viability.

Dose %	Just media/no cells	Untreated control zero	0.125	0.5	1	2	5
	0.085	0.759	0.713	0.724	0.693	0.661	0.491
	0.094	0.746	0.692	0.756	0.714	0.638	0.44
	0.09	0.749	0.67	0.722	0.679	0.631	0.44
	0.129	0.216	0.272	0.368	0.381	0.418	0.2
Mean	0.0995	0.6175	0.58675	0.6425	0.61675	0.587	0.39275
Standard deviation	0.020008	0.267724	0.210566	0.183662	0.157823	0.113393	0.13073
Concentration of variation	20.10888	43.35617	35.88692	28.5855	25.58954	19.3174	33.28572
Cell number		60610	57535	63110	60535	57560	38135
Cell viability		90.61296	86.01578	94.3505	90.50083	86.05315	57.01246

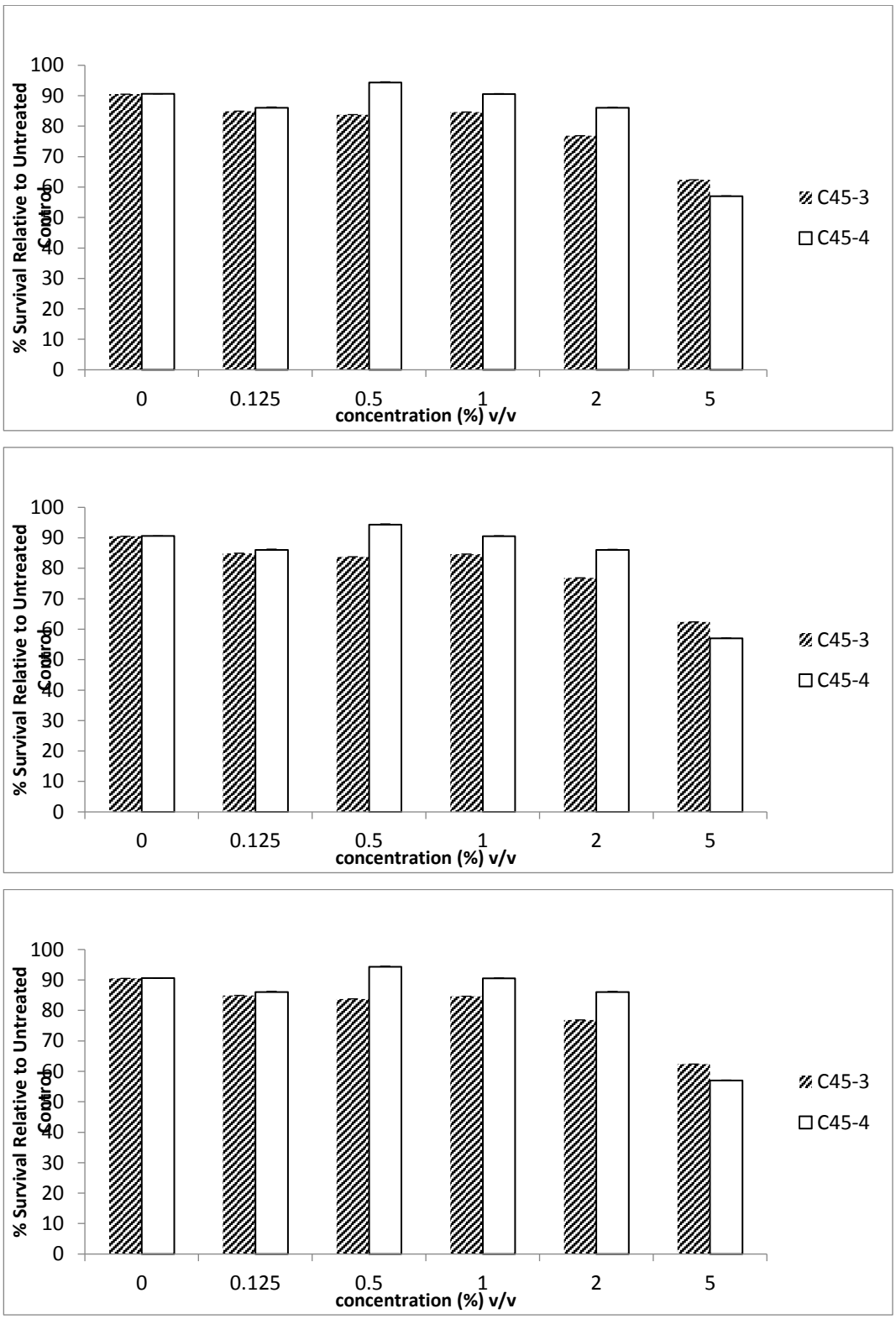


Figure A5.2 Relative survivals at 48 hours for calendula extracts C and D. HaCaT cells were treated for the time indicated, then assayed by MTT assay. Data are shown as mean survival relative to the untreated control ±.

Appendix VI: Raw data and data processing of the crystal violet assay

Table A6.1: The ODs value of the MTT standard curve measured by spectrophotometer at absorption of 570–630

1	2	3	4	5	6	7	8	9	10
0.025	0.029	0.022	0.027	0.039	0.063	0.108	0.17	0.293	0.508
0.029	0.03	0.024	0.032	0.04	0.063	0.102	0.173	0.259	0.479
0.024	0.021	0.024	0.032	0.035	0.056	0.103	0.172	0.295	0.499
0.024	0.026	0.028	0.033	0.038	0.051	0.11	0.179	0.286	0.454
0.026	0.026	0.029	0.031	0.038	0.053	0.094	0.161	0.231	0.474
0.03	0.026	0.032	0.034	0.047	0.056	0.092	0.161	0.285	0.491

Table A6.2: The average, standard deviation and concentration of variation of MTT standard curve values

cell no	0	156	312	625	1250	2500	5000	10000	20000	40000
wells no	1	2	3	4	5	6	7	8	9	10
Mean	0.026333	0.026333	0.0265	0.0315	0.0395	0.057	0.1015	0.169333	0.274833	0.484167
St.Dev	0.002582	0.003141	0.003782	0.002429	0.004037	0.00502	0.007259	0.007118	0.025047	0.019364
C.variatio	9.805021	11.92832	14.26994	7.711084	10.22108	8.806948	7.152194	4.203574	9.113628	3.999461

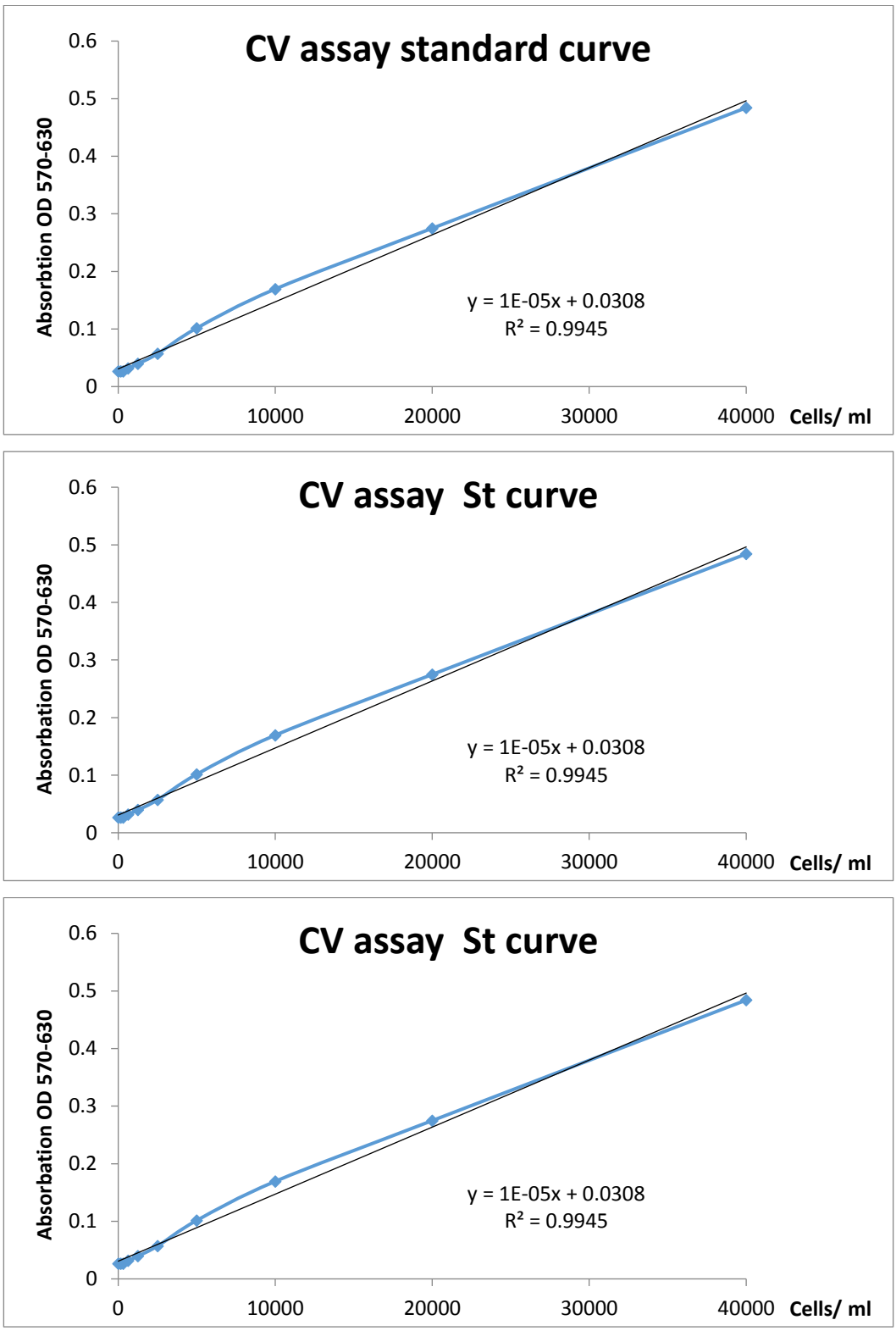


Figure A6.1 MTT standard curve NVAW (no TiO_2) toxicity experiment that was prepared as in Chapter 3. The X axis shows the concentration of cells/wells until 20 × 10^4. The Y axis shows the absorbance at OD 570–630.

Table A6.3: The ODs values of NVAW (no TiO_2) treatment measured by CV assay. It used the mean, standard deviation and concentration of variation to find out the cell number and cell viability.

Dose %	Untreated control zero	0.00003	0.0003	0.003	0.03	0.3	3
	0.192	0.179	0.259	0.242	0.23	0.212	0.14
	0.234	0.247	0.26	0.218	0.208	0.133	0.09
	0.243	0.228	0.226	0.284	0.263	0.197	0.107
	0.205	0.338	0.404	0.253	0.253	0.165	0.125
Mean	0.2185	0.248	0.28725	0.24925	0.2385	0.17675	0.1155
Standard deviation	0.023979	0.066488	0.07942	0.027391	0.024583	0.035141	0.021703
Concentration of variation	10.97444	26.80972	27.64849	10.98924	10.30742	19.88197	18.79007
Cell number	18770	21720	25645	21845	20770	14595	8470
Cell viability	100	115.7166	136.6276	116.3825	110.6553	77.75706	45.1252

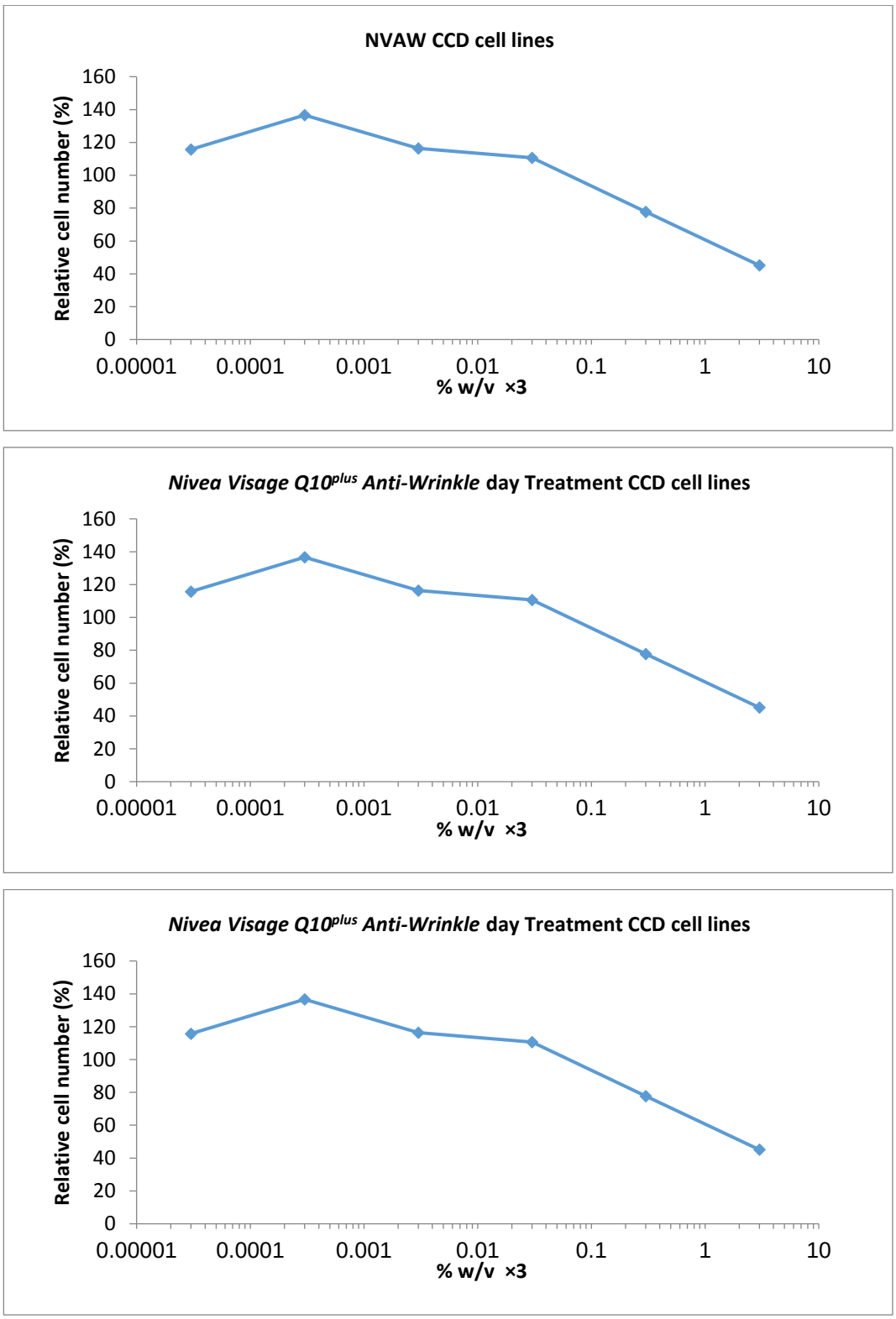


Figure A6.2 Relative cell number after treatment of CCD-1064 fibroblast cells with NVAW for one hour. Relative cell number was measured by CV assay. Data are shown relative to the untreated control.

Appendix VII: Raw data of the flow cytometry and apoptosis detection assay

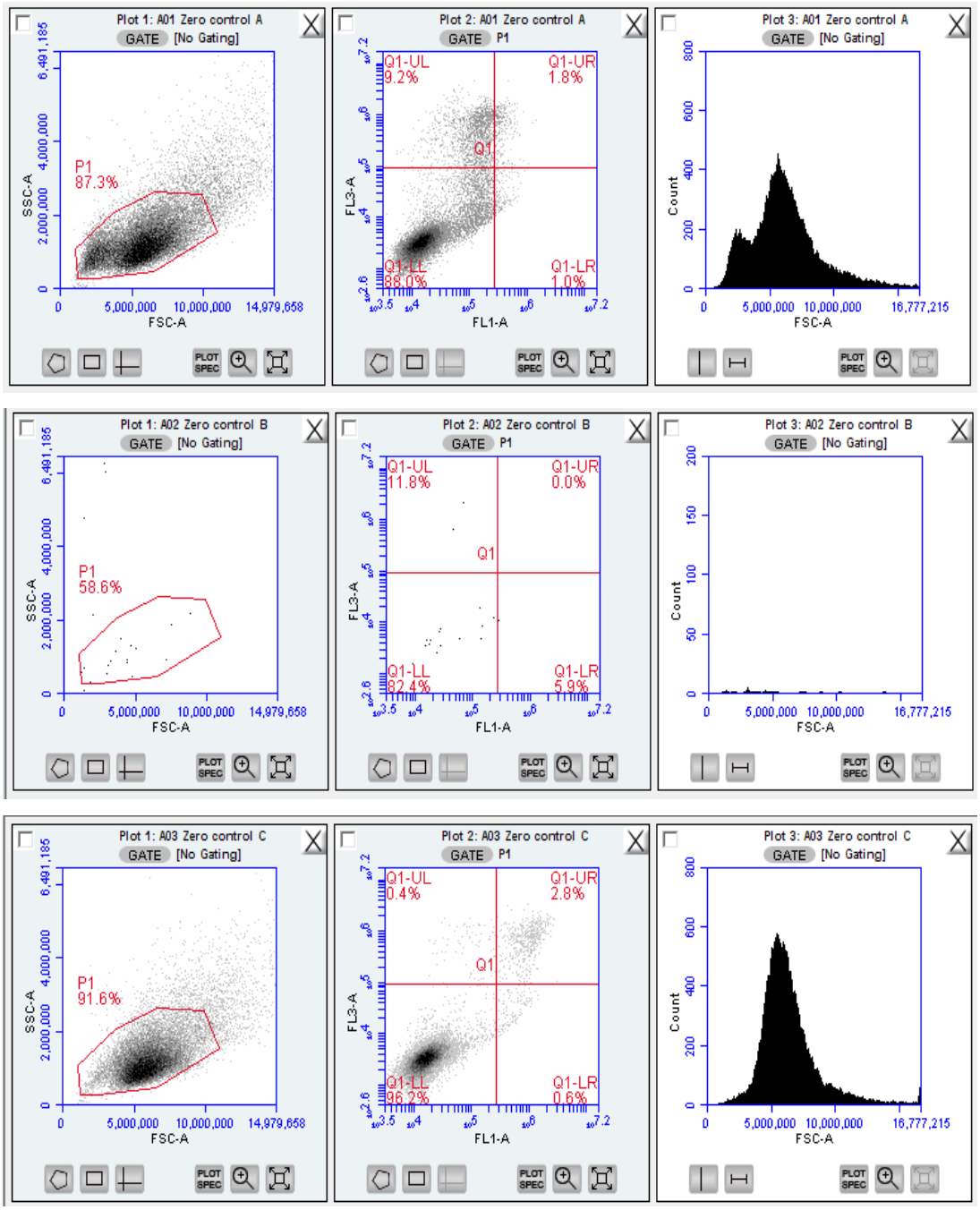


Figure A7.1: Untreated control of HaCaT cells were stained as per section 2.2.4 and analysed using flow cytometry. The left panel shows cell population; the middle panel shows distribution of viable cells, apoptotic cells and necrotic cells and the right panel compares two peaks of cells population. A is a medium and HaCaT cells sample without supernatant. B is only the supernatant sample. C is the combination of media, cells and the supernatant.

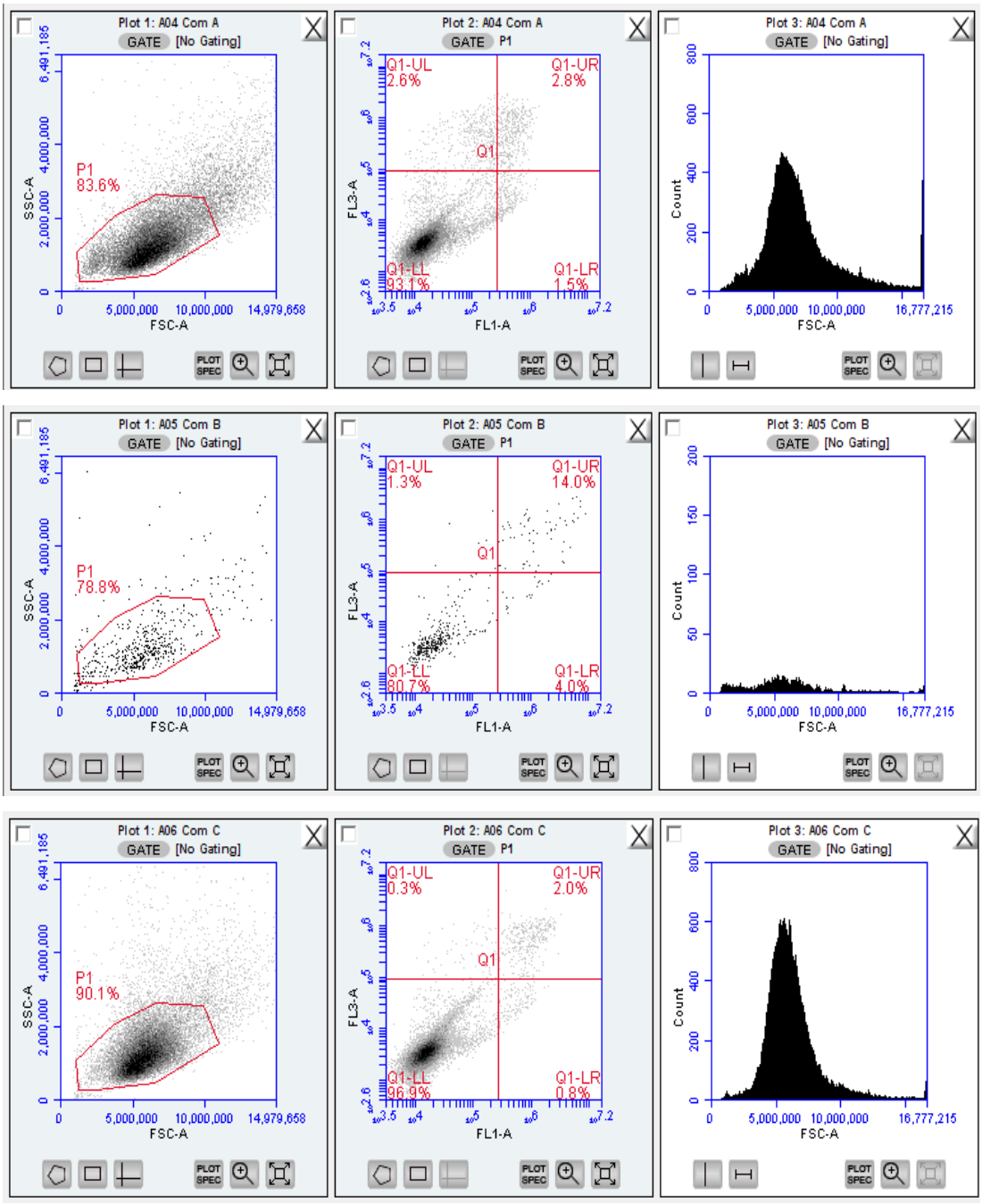


Figure A7.2 The mechanism of HaCaT cell killing after treatment with 25 µM Campothecin for 1. Data were obtained from 20,000 events and early apoptotic cells (annexin positive; PI negative) and late apoptotic cells (necrotic cells) (annexin positive/PI positive) are presented as a percentage of total cells analyse. The left panel shows cell population, the middle panel shows distribution of viable cells, apoptotic cells and necrotic cells and the right panel shows compare two peaks of cells population. A is a media and HaCaT cells sample without supernatant. B is only supernatant sample. C is the combination of media and cells and the supernatant.

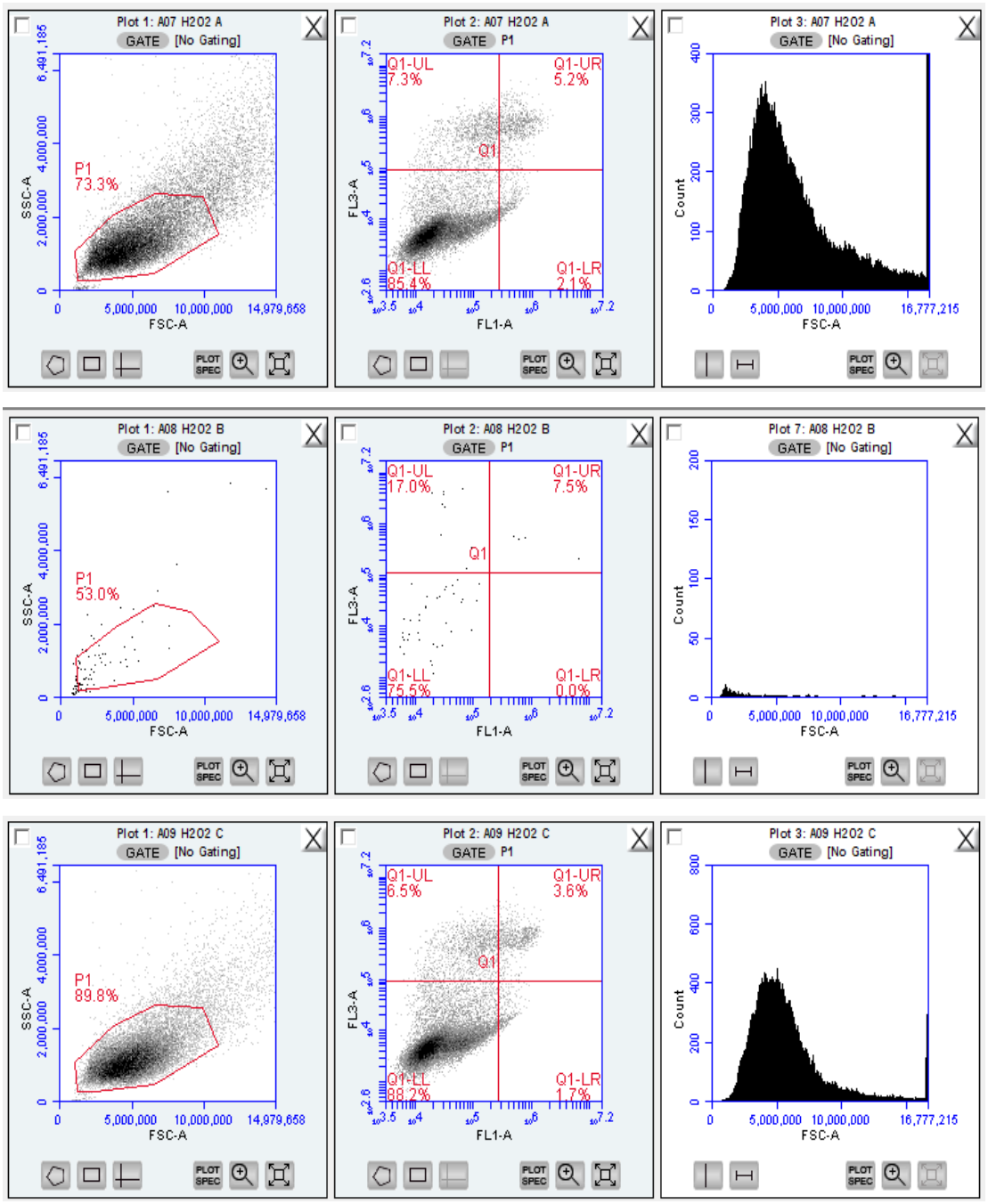


Figure A7.3 The mechanism of HaCaT cell killing after treatment with 300300 µM of campothecin for one hour. Data were obtained from 20,000 events; early apoptotic cells (annexin positive; PI negative) and late apoptotic cells (necrotic cells) (annexin positive/PI positive) are presented as a percentage of total cells analysed. The left panel shows cell population; the middle panel shows the distribution of viable cells, apoptotic cells and necrotic cells and the right panel compares two peaks of cell population. A is a media and HaCaT cells sample without supernatant. B is only a supernatant sample. C is the combination of the media, cells and the supernatant.

Appendix VIII: Row data processing of the cytokinesis-block micronucleus assay

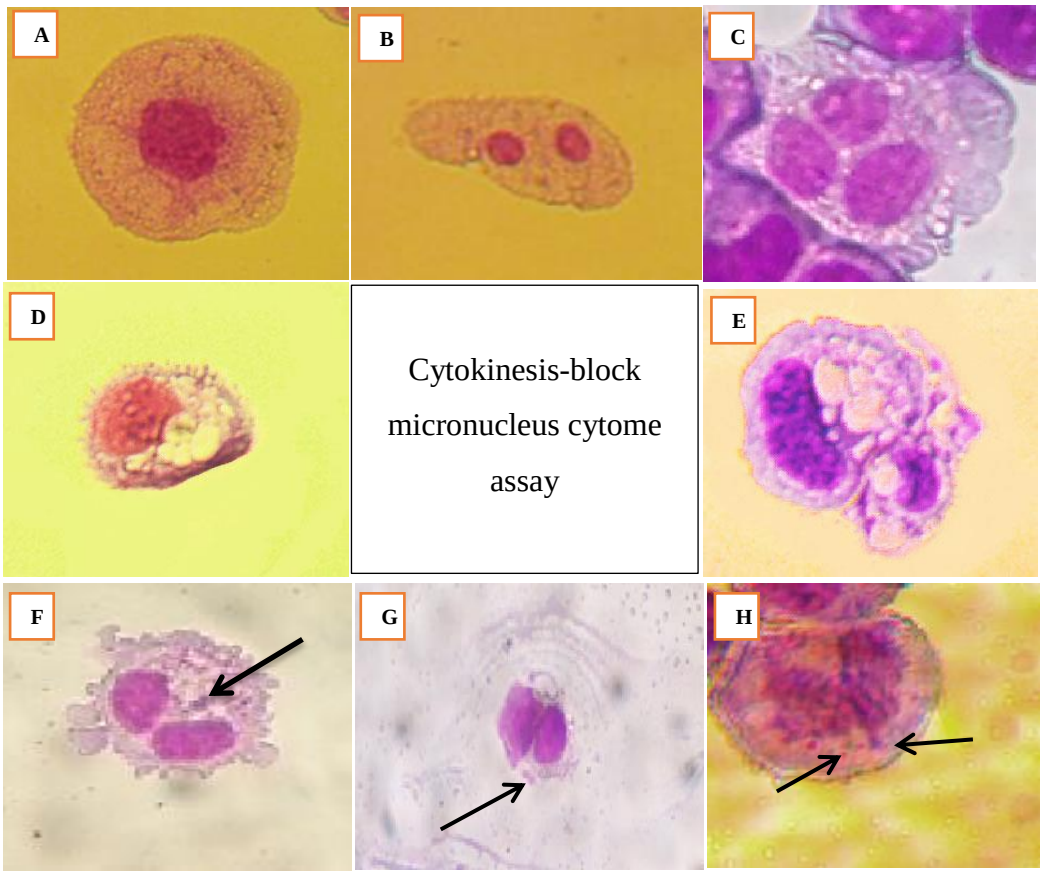


Figure A8.1 Photomicrograph of the cells scored in the cytom assay (CBMN assay). The slides were scored at a magnification of 250X or 40X as described in Fenech (2007). A is a mononucleated cell. B is a BN cell. C is a multinucleated cell. D is an early necrotic cell. E is a late apoptotic cell. F is a BN cell containing one MNi. G is a BN cell containing two MNi. H is a BN cell containing NBUDs.

Table A8.1 Row data of CCD-1064SK fibroblast skin cells treated with beauty products (NVAW (improved formula, without TiO_2), Glycerol BP and FMCGB (a natural product) for one hour. Scoring toxic cells (mononucleated cell, BN cell; multinucleated cell, apoptotic cell, necrotic cell) or genotoxic cells (BN cell containing one or more MNi, BN cell containing an NPB (and a MNi), BN cell containing NBUDs).

Slide	Mono	BN	Multi	Apopt	Necro	Scored	NDI	BNs	Total # MN	#BNs	BNs
	A	BN	C	D	E	400 cells		With MN	in BNs	with NPB	with Nbud
Nivea 0.3%	216	170	5	5	4	400	1.460358	20	1	0	0
Nivea 0.3%	205	176	6	9	6	400	1.485788	37	1,3	0	0
Nivea 0.3%	227	173	3	1	1	400	1.444169	28	1	0	0
Average	216	173	4.666667	5	3.666667	400		28.3333333		0	0
SD	11	3	1.527525	4	2.516611	0		8.50490055			
SEM	6.350853	1.732050808	0.881917	2.309401	1.452966			4.91030662			
NDI											
Nivea 0.03%	205	170	3	15	7	400	1.465608	23	1	0	0
Nivea 0.03%	173	168	24	20	21	400	1.591781	25	1	0	0
Nivea 0.03%	210	169	8	9	20	400	1.478036	26	1	0	0
Average	196	169	11.66667	14.66667	16	400		24.6666667		0	0
SD	20.07486	1	10.96966	5.507571	7.81025			1.52752523			
SEM	11.59023	0.577350269	6.333333	3.179797	4.50925			0.8819171			
Facial Natural 5%	177	202	7	10	3	400	1.559585	13	1	0	1
Facial Natural 5%	210	177	7	3	7	400	1.484772	15	1	0	0
Facial Natural 5%	210	169	8	9	20	400	1.478036	12	1	0	0
Average	199	182.6666667	7.333333	7.333333	10	400		13.3333333			
SD	19.05256	17.21433511	0.57735	3.785939	8.888194			1.52752523			
SEM	11	9.938701011	0.333333	2.185813	5.131601			0.8819171			
Facial Natural 0.5%	169	210	10	5	6	400	1.59126	8	1	0	0
Facial Natural 0.5%	135	234	13	11	7	400	1.680628	7		0	0
Facial Natural 0.5%	177	200	13	4	8	400	1.579487	10	1,2	0	0
Average	160.3333	214.6666667	12	6.666667	7	400		8.33333333			
SD	22.30097	17.4737899	1.732051	3.785939	1	0		1.52752523			
SEM	12.87547	10.0884973	1	2.185813	0.57735			0.8819171			
Glycerol 10%	222	167	1	7	4	400	1.433333	11	1	0	0
Glycerol 10%	116	265	14	2	1	400	1.741772	9	1	0	0
Glycerol 10%	143	231	19	2	5	400	1.684478	7	1	0	0
Average	160.3333	221	11.33333	3.666667	3.333333	400		9			
SD	55.08478	49.75942122	9.291573	2.886751	2.081666	0		2			
SEM	31.80321	28.72861524	5.364492	1.666667	1.20185			1.15470054			
Glycerol 1%	194	202	1	5	1	400	1.513854	10	1	0	0
Glycerol 1%	145	232	19	2	6	400	1.681818	7	1	0	0
Glycerol 1%	124	268	7	1	3	400	1.706767	9	1	0	0
Average	154.3333	234	9	2.666667	3.333333	400		8.66666667			0
SD	35.92121	33.04542328	9.165151	2.081666	2.516611			1.52752523			
SEM	20.73912	19.07878403	5.291503	1.20185	1.452966			0.8819171			
Zero control	194	180	24	2	0	400	1.572864	6	1	0	0
Zero control	164	214	13	6	3	400	1.613811	8	1	0	0
Zero control	189	183	19	7	2	400	1.565217	9	1	0	0
Average	182.3333	192.3333333	18.66667	5	1.666667	400		7.66666667			
SD	16.07275	18.82374387	5.507571	2.645751	1.527525			1.52752523			
SEM	9.279607	10.86789359	3.179797	1.527525	0.881917			0.8819171			

Appendix IX: Processing data of the DPPH assay

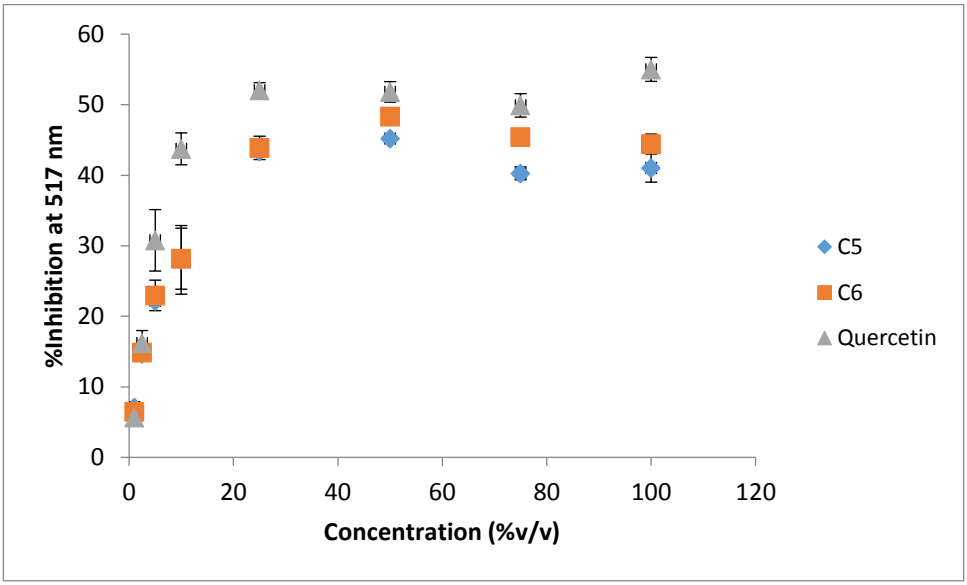


Figure A9.1 The % free radical inhibition of quercetin, A (C5) and B (C6) solutions. 1% quercetin = 0.016 mg/ml; 1% A (C5) = 0.88 mg dry weight equiv/ml; 1% B (C6) = 0.84 mg dry weight equiv/ml. The level of antioxidant activity was determined by DPPH assay at 517 nm % inhibition. Each value represents the mean ± SEM; n = 3.

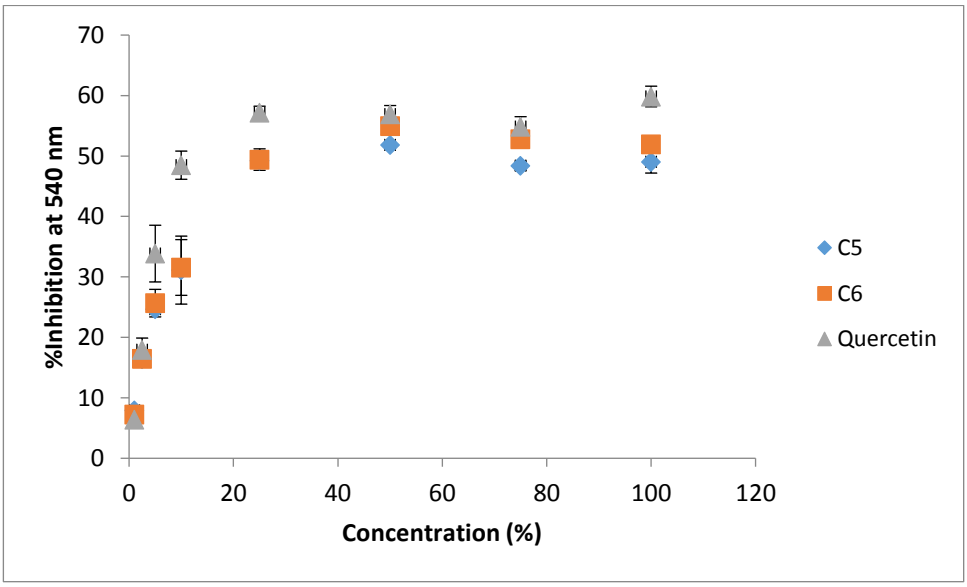


Figure A9.2 The % free radical inhibition of quercetin A (C5) and B (C6) solutions. 1% quercetin = 0.016 mg/ml; 1% C5 = 0.88 mg dry weight equiv/ml; 1% C6 = 0.84 mg dry weight equiv/ml. The level of antioxidant activity was determined by DPPH assay at 540 nm % inhibition. Each value represents the mean ± SEM; n = 3.

Appendix X: Calendula extracts matrix dose response

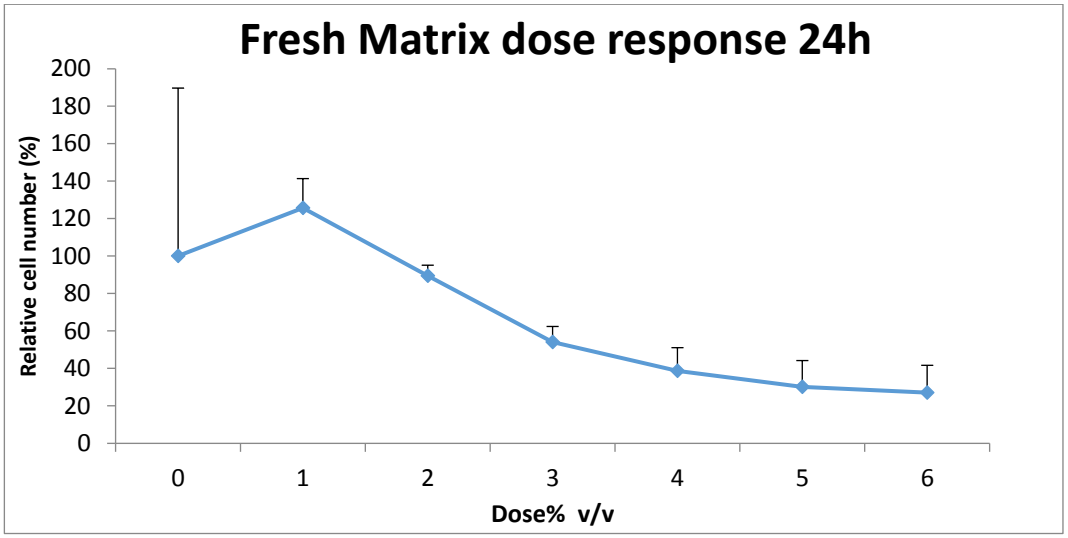


Figure A10.1 Relative survivals at 24 hours for the fresh matrix (used for calendula extracts). HaCaT cells were treated with serial dilutions of the fresh matrix for the time indicated, then assayed by MTT assays. Data are shown as the mean survival relative to the untreated control ±.

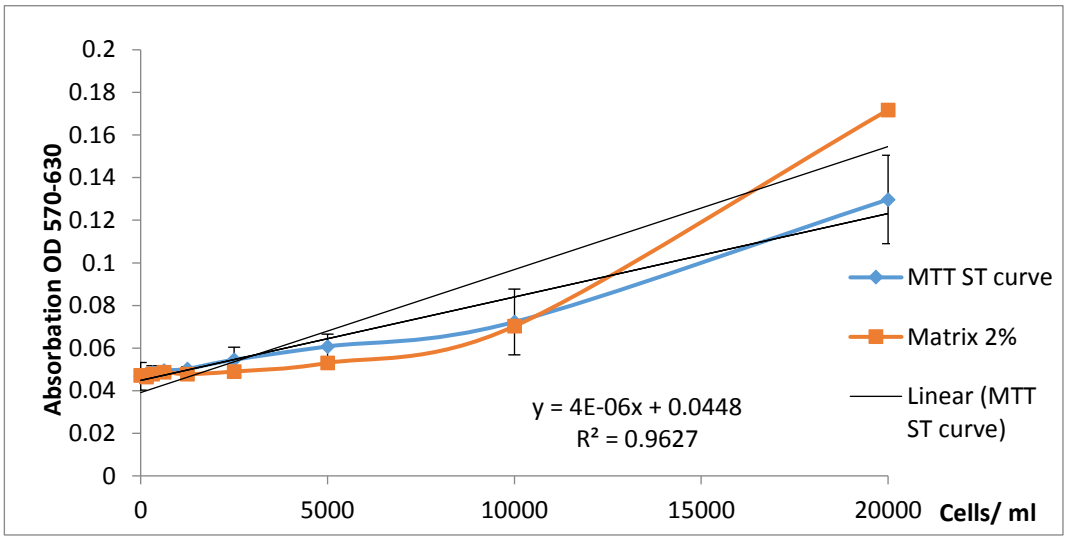


Figure A10.2 Relative survivals at 24 hours for the fresh matrix (used for calendula extracts). The MTT standard curve on HaCaT cells compared with the 2% fresh matrix standard curve of the fresh matrix for the time indicated, then assayed by MTT assays.

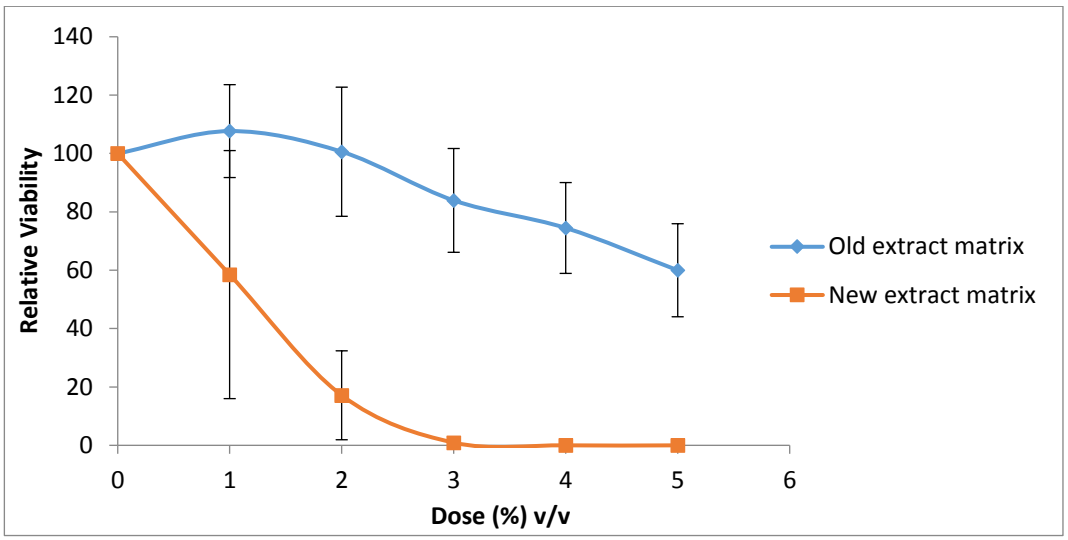


Figure A10.3 Relative survivals dose response at 24 hours for fresh (recently made) and old (It's Left in the fridge for approximately 5 months) matrixes (used for calendula extracts) on HaCaT cells, then assayed by MTT assay. The result indicated that the new (fresh) extract matrix induced toxicity in HaCaT human skin cells.