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The Role of Synthetic and Naturally Derived Isothiocyanates in Experimental Models of Skin Photo-aging and Psoriasis

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ABSTRACT

Isothiocyanates, phytochemicals found in cruciferous vegetables, are known for their health benefits and their antioxidant and anti-inflammatory effect. They are known to act indirectly as antioxidant, through the induction of Nrf2 and phase II enzymes, while they are shown to reduce cytokine production, leading to reduced inflammation. Isothiocyanates are studied on their effects on skin disease, such as dermatitis and photo-aging, and are shown to reduce inflammation and reduce UV damage, respectively. This project aims to provide further insight of the effect of isothiocyanates on photo-aging and psoriasis through models of photo aged HaCaT cells and a novel therapeutic approach using UVB, respectively.

Initially and for the determination of the total isothiocyanates in watercress, a determination method was developed using the cyclocondensation assay. Measurements were taken using the spectrophotometer, while validation of the method was conducted using a hexane extraction method from the literature. The standard curves of five ITCs studied in this work (SFN, IBN, AITC, BITC and PEITC) gave a response factor from 0.028 to 0.035, indicating limited variation in the reaction of ITCs, while the limit of detection (LOD) was 1-60 μg / mL or 1-400 μM or 1-60 mg / g dry matter. Compared to the literature (Y. Zhang et al., 1992), the maximum LOD presented in the current work is significantly higher with an actual confidence level of 98.4%.

During the determination of total ITC content in watercress matrix component interference was examined, revealing a macromolecule interference that was successfully resolved by the addition of a clarification step that includes addition of methanol and centrifugation, improving the reliability of the method by reducing the coefficient of variation from 23.6% to 14% (n = 6). No fibre or metal interference is shown, while plastic is shown to interfere with the quantification and glassware increased recovery of ITCs by 35% giving a 7-fold decrease in the corresponding coefficient of variation from 14% to 2%. The developed

methodology, with an 88.95% recovery, detected 1.54 ± 0.038 μmol / g of total ITCs content in freeze-dried watercress powder, while significantly higher quantities of 20.6 ± 0.31 μmol / g were detected in fresh watercress.

Photo-aging is characterised from physiological changes of the skin and overall increased wrinkles that gradually appear due to sustainably prolonged UVR exposure of the skin. sulforaphane, one of the most studied isothiocyanates, is known to increase viability in photo-aged cells and reduce the effect of UV oxidation in human immortal keratinocyte cells. In this project both phytochemicals had a photo-protective effect on the UVB-induced photo-aging models of 25 and 50 mJ / cm^2 with significantly increased cell viability at 72 hours by at least 10% compared to controls. For the role of ITCs and UVB on psoriasis HaCaT cells were treated with low doses of UVB and post-treated with either of the five isothiocyanates; sulforaphane, Iberin, allyl isothiocyanate, benzyl isothiocyanate and phenethyl isothiocyanate. All but allyl isothiocyanate were shown to further reduce viability and growth, when combined with 5 and 10 mJ / cm^2 UVB, while all phytochemicals mentioned above were shown to induce apoptosis, increase mitochondrial membrane permeability and affect the cell cycle. ROS induction from the combinational treatments was also studied and it was shown that the viability of the cells increased, reversing the effect of the treatment and indicating the main mechanism of the combination therapy. All ITCs but AITC (20 μM) studied showed a significant reduction of viability when combined with 5 mJ / cm^2 UVB at 48h or 10 mJ / cm^2 UVB at 24h, while BITC was shown to be the least potent. HaCaT cell growth was also shown to reduce significantly after their treatment with all ITC combinations but AITC. All ITCs studied showed an increase of apoptotic cells through the evaluation of caspase 3/7 activity, while AITC only showed an increase of apoptotic cells after the treatment with 10 mJ / cm^2 UVB and not with 5 mJ / cm^2 UVB. Moreover, treatments with combinations of UVB and PEITC, AITC, IBN and SFN showed necrotic cells significantly high indicating damage to membranes, while PEITC was shown to be the

most potent. AITC reduced necrotic cells significantly compared to 10 mJ / cm^2 UVB but not with the AITC alone, while BITC combination did not show any increase to necrotic cells compared to the controls. ΔΨm was shown to decrease in all the combinational treatments indicating intracellular ROS induction and mitochondrial dysfunction. SFN and PEITC were the most potent on reducing ΔΨm. ROS induction as the main mechanism of action of the combinational treatment was shown to reverse after the addition of GSH strengthening the hypothesis of reduced viability being mediated by the induction of intracellular ROS. SFN, IBN and BITC were shown to arrest G_2/M phase at 24h / 10 mJ / cm^2 UVB, when compared to the individual controls of UVB or ITC alone, while PEITC significantly arrested G_2/M phase only when compared to UVB control. 48h / 5 mJ / cm^2 UVB only showed a significant G_2/M phase arrest after treatments with IBN and AITC when compared to their corresponding ITC controls. SubG1 phase was arrested by SFN, IBN and BITC combinational treatments at 24h, while similar effect was shown by SFN, IBN, AITC at 48h. No effect on S phase was shown from either ITC, while SFN and PEITC arrested G1 phase at 24h, when compared to SFN alone control and UVB control, respectively. The treatment of 10 mJ / cm^2 UVB in combination with 20 μM of SFN, IBN, BITC or PEITC were the most effective of the two treatments overall, with the most significant effect on cell viability, apoptosis, ΔΨm, cell cycle and ROS induction.

When watercress was used for the treatment of HaCaT cells post-UVB exposure in the optimised combinational treatment it was shown to have a similar effect on cell growth (SRB123 assay), apoptosis (caspase 3/7), mitochondrial membrane permeability (JC-1), cell cycle (PI) and ROS induction (DHR assay and addition of antioxidant glutathione). 20 μM watercress extract expressed in PEITC significantly ($p<0.0001$) reduced cell growth, while its combination with 10 mJ / cm^2 UVB further ($p<0.001$) enhanced the effect. The comparison of the combinational treatments using watercress extract or synthetic PEITC, reveals a similar effect of the two, on caspase 3/7 activation, increasing apoptotic cells

significantly compared to the controls of UVB alone and ITC alone. Significant reduction of the ratio of JC-1 aggregates over JC-1 monomers ($\Delta\Psi m < 6$) on HaCaT cells from watercress extract and synthetic PEITC is shown, while a further enhanced effect ($\Delta\Psi m < 2$) is shown when watercress or PEITC are combined with UVB.

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DECLARATION

I declare that the work contained in my thesis has not been submitted for any other award and that is all my own work. I also confirm that this work fully acknowledges opinions, ideas and contributions from the work of others.

Any ethical clearance for the reseach presented in this thesis has been approved by Northumbria University Ethics Committee.

I declare that the Word Count of this Thesis is 46,381 words.

Name: Vasiliki Lolou

Signature:

Date: May 2021

LIST OF ABBREVIATIONS

AITC	Allyl isothiocyanate
BDT	1,2-benzenedithiol
BITC	Benzyl isothiocyanate
COX2	Cyclooxygenase 2
cQa	caffeoylquinic acid
DAPI	4′,6-diamidino-2-phenylindole
DHR123	Dihydrorhodomanine 123
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ECN	Erucin
EDTA	Ethylenediaminetetraacetic acid
FACS	fluorescence-activated single cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GC/MS	Gas Chromatography – Mass Spectrometry
GSH	Glutathione
GSL	Glucosinolate

GSTP1	glutathione S-transferase pi-1
HaCaT	Human immortal keratinocyte cells
HIV	Human immunodeficiency virus
HO1	Heme oxygenase-1
HPLC	High performance liquid chromatography
IBN	Iberin
IL	interleukin
ITC	Isothiocyanate
JC-1	1,1’,3,3’-tetraethyl-5-5’,6,6’ tetrachloroimidacarbocyanine iodide
LOD	Limit of detection
LOQ	limit of quantification
MBITC	3-methoxybenzyl isothiocyanate
MED	Minimum erythema dose
MEITC	Methyl isothiocyanate
MMPs	Matrix metalloproteinsases
MPACN	3-methoxyphenyl acetonitrile
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
NQO1	NAD(P)H Quinone Dehydrogenase 1
Nrf2	Nuclear factor erythroid 2-related factor 2

PABA	para-aminobenzoic acid derivatives
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffer saline
PEITC	Phenethyl isothiocyanate
PI	Propidium Iodine
PUVA	Psoralen UVA
ROS	Reactive oxygen species
RSD	Relative standard deviation
rt-PCR	Real time - polymerase chain reaction
SD	Standard deviation
SFN	Sulforaphane
SPF	Sun protection factor
SRB	Sulforhodamine B
TCA	Trichloroacetic acid
TGF-β	Transforming growth factor beta
TNF-α	Tumor nuclear factor-α
Tris	trisaminomethane
TβRII	TGF-β type II receptor
UAE	Ultrasound assisted techniques
ΔΨm	Mitochondrial membrane depolarization

1. General Introduction

1.1 Skin structure and function

The main role of human skin is to function as a barrier between internal and external environment. The function of the skin is separated in three categories; the physical, where the outer layer of the skin provides protection from sharp objects and increased pressure due to its mechanical stability, the chemical where the organ acts as a filter and barrier for chemicals to enter the body and immunological where the skin reacts through an inflammatory immune response against stimulants such as pathogens or wounds, causing a series of symptoms, such as swelling or increased production of keratinocytes for wound healing. Homeostatic regulation, prevention of loss of fluids, electrolytes and proteins through the skin, temperature maintenance, sensory perception and immune surveillance are amongst the various roles of skin. Skin is also responsible for the rise of hairs, a mechanism that is supported by muscles surrounding the hair follicle and helps the body maintain its temperature through trapping of small air bubbles at the base of the hair. The skin consists of 4 layers, the epidermis, the dermis, the dermoepidermal junction and the hypodermis (figure 1.1) (Lorencini et al., 2014; Tobin, 2017).

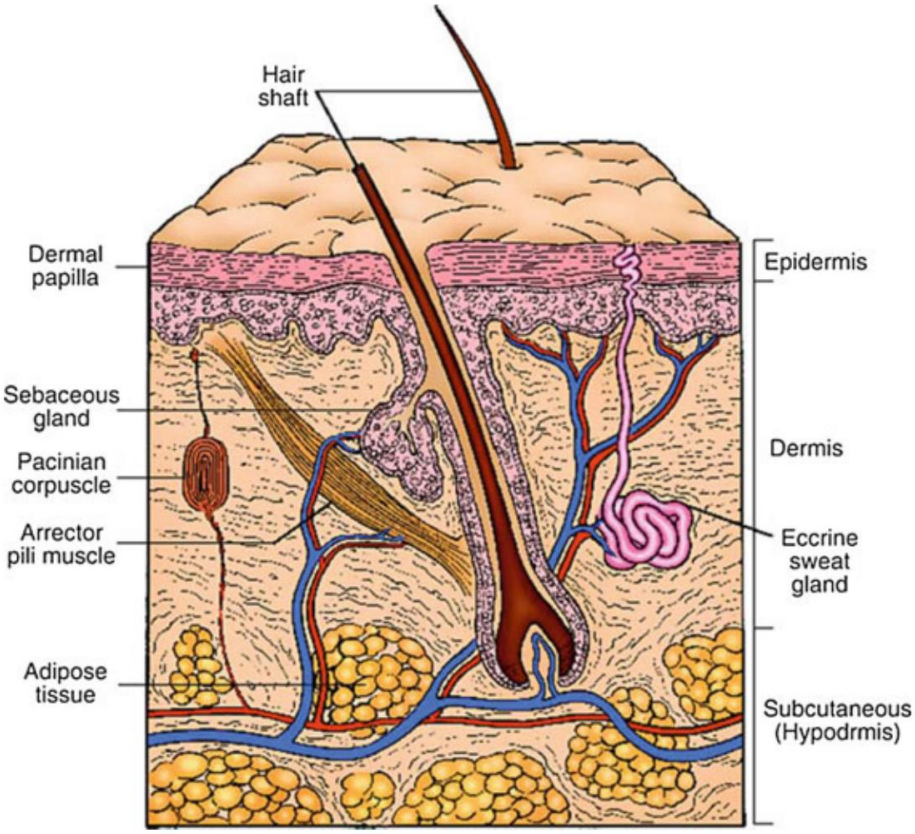


Figure 1. 1 *Skin structure in layers.*

Skin consists of three main layers, the epidermis, the dermis and the hypodermis, while a fourth layer between the epidermis and the dermis acts as a connective layer that plays a role the integrity of the skin. Glands, such as sebaceous glands and eccrine sweat glands are also found in the skin.(Farage et al., 2007)

1.1.1 Epidermis

The epidermis is the external layer of the skin and its thickness varies from 0.05mm on the eyelids to 1.55mm on palms and soles while these measures can differ between humans. Keratinocytes are the main cells that the epidermis consists of (95%), while other cells are also present in this layer, such as melanocytes, Langerhans and Merkel cells. Another important function of the epidermis is the anti-microbial barrier that provides due to the natural flora, such as *Staphylococcus epidermidis* that acts as a growth inhibitor by competitive exclusion of *Propionibacterium acnes* and *Acne vulgaris*, two microorganisms

that are known to cause acne (Berretta et al., 2012; Y. Wang et al., 2014). Moreover, skin microbiota and fatty acid secretions from the sebaceous glands, maintain the low pH of the epidermis inhibiting the growth of pathogens such as *Staphylococcus aureus* (Delanghe et al., 2021). Moreover, hair follicles and sweat glands traverse various layers including the epidermis, while there are no blood capillaries or nerve endings found in this layer.

1.1.1.1 Keratinocytes

Keratinocytes are found in the whole epidermis and their growth, division and cell cycle is initiated at stratum basale (basal layer). There are 4 layers of keratinocytes that are divided based on their stage of life and function. The cells gradually move from the bottom layer of the epidermis, towards the surface of the skin, while this movement is their process of maturing and eventually reaching the stratum corneum (horny layer), the outer layer of the epidermis, in a non-viable form (figure 1.2). The stratum basale, also known as stratum germinativum, is the layer where keratinocytes are differentiated from stem cells and proliferate. Once they reach the final stage of their differentiation, cells enter the stratum spinosum (spinous layer), a layer where cells are metabolically highly active, and their size is increased. Cells take 2 weeks until they are ready to enter the stratum spinosum and another 2 weeks to pass through the latter layer and enter stratum granulosum. At this layer, keratinocytes are flattened and insoluble proteins such as keratin, are produced, also known as keratinization (L. Guo, 2009). Keratin acts as a fibre and is responsible for the bondage of demosomes (cadherin molecules) with epidermal cells, providing stability and resistance in tension and protects the skin from tearing apart (Fenner & Clark, 2016). Lamellar bodies are also formed in this layer secreting lipids that move to the stratum cormeum (horny layer) and maintain the low pH of the skin, protecting the organ from pathogens, while the cells lose intracellular organelles and nuclei (Ng & Lau, 2015). By the time keratinocytes pass the

stratum compactum and reach the stratum corneum (stratum disjunctum), they are non-viable cells that fall from the skin through desquamation.

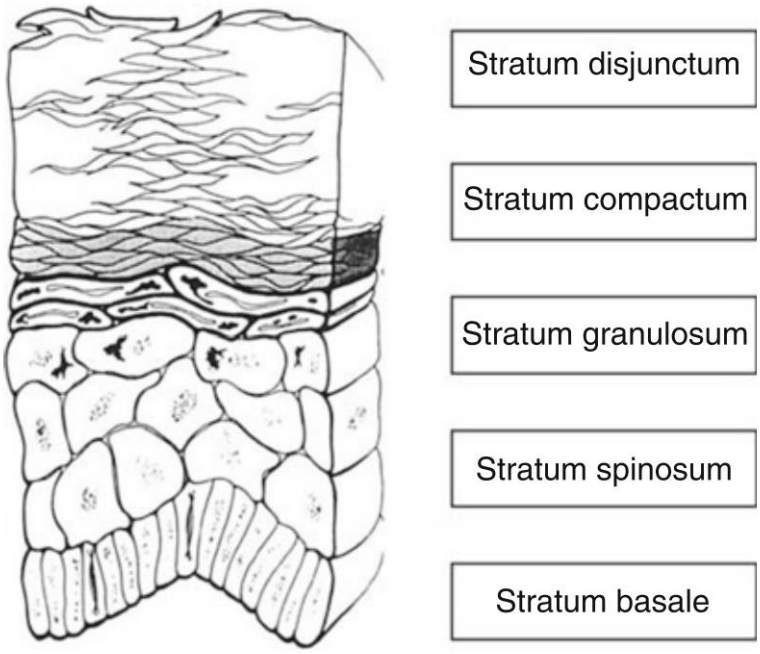


Figure 1. 2 *Schematic representation of epidermis layers.*
Epidermis consists of five layers; stratum basale, stratum spinosum, stratum granulosum, stratum compactum and stratum disjunctum (stratum corneum). (Rawlings, 2017)

1.1.1.2 Melanocytes

Melanocytes are found in the stratum basale and their role is to produce melanin inside melanosomes (Ng & Lau, 2015). Melanosomes are produced from melanocytes and transferred to the main epidermis cells, determining the pigmentation level of the skin and providing photo protection. The increased production of melanin, gives a darker colour to the skin, while vitamin D production is initiated by UVB chemical reactions (Slominski & Postlethwaite, 2015).

1.1.1.3 Langerhans cells

Langerhans cells are dendritic cells resident in the skin. They play an important role in detecting pathogens and migrating to lymphatic tissue where they present potential antigens to T-lymphocytes (Johnston et al., 2000). This presentation is a key initiating step in the activation of the adaptive immune system. T cells play a number of key roles in eliminating infected cells and producing cytokines to direct immune responses, such as the activation of macrophages, while activate B cells that secrete antibodies another vital component of the immune response (Fenner & Clark, 2016). Langerhans cells play a major role in autoimmune skin diseases, such as psoriasis (Rahman et al., 2012) and their mechanism on the disease are mentioned in the next chapters.

1.1.1.4 Merkel cell

Merkel cells are found in the lower layer of the epidermis, amongst keratinocytes, and are associated with the nervous system as they contain hormonal producing particles that are similar to neurosecretory particles (Fenner & Clark, 2016). These cells also provide sensation of pressure, shapes and sharpness to the skin by sending information to the brain and act as tactile sensors. Merkel cells are known to create adrenergic synapsies, which are junction points between the dendrite cell of a nerve to the axon cell of another nerve, through Aβ afferents, while the mechanisms by which Merkel cells activate and communicate with sensory neurons is not well studied, unlike epithelial cells that are shown to activate sensory neuron through B_2-adrenergic receptors (Hoffman et al., 2018).

1.1.2 Dermoepidermal junction

The dermoepidermal junction is the layer that holds the epidermis and the dermis together and strong bonds between fibrils and cells provide further stability to the skin (Rinnerthaler

et al., 2015). Hemidesmosomes are molecules that contain keratin and are found in the basal layer. These molecules connect the epidermis to the dermoepidermal junction layer securing the basal cells to the membrane, while most molecules that are found in the dermoepidermal junction, including collagen IV, are produced by keratinocytes (Tang et al., 2012).

1.1.3 Dermis

The dermis provides the organ with elasticity and firmness (Fenner & Clark, 2016). There are various cells found in the dermis, such as macrophages, T-and B -lymphocytes, mast cells, vascular muscle cells and fibroblasts. The matrix of this layer consists of collagen and elastin fibrils, as well as extrafibrillar matrix that are produced from the fibroblasts. This network is divided in two sections, the papillary and the reticular dermis, the former layer is thinner than the latter. The papillary dermis is located close to the epidermis and it consists of loose collagen fibrils in contrast with the reticular where the network of the collagen and elastin fibrils is denser. The reticular dermis also contains hair follicles, sweat glands, blood vessels and nerve endings (Farage et al., 2007).

1.1.3.1 Collagen

Peptide chains of the types of collagens, also known as procollagens, are mainly produced by fibroblasts before the synthesis of collagen proteins (J. H. Chung et al., 2001). The main collagens found in the dermis extracellular matrix (ECM) is collagen I, which compromises most of the overall collagen found in this layer (70-75%), and collagen III. Keratinocytes produce collagen IV, which is then moved to the dermoepidermal junction and provides stability and strength to the layer. The procollagen of the individual collagens produced are considered biomarkers of the overall collagen produced, since they all lead to mature collagen in the dermis. Once the proteins are expressed, they form triple helices of three

polypeptide chains called “monomers”, and they are exported to the ECM where they form fibrils that connect to the elastin network. Collagen fibrils are held together by molecular bonds between residues of lysine and together with the elastin network, they allow the skin to have elasticity while maintaining its shape and firmness.

1.1.3.2 Elastin

Elastin, similarly to collagen, is produced in fibroblasts and provides the skin and other organs, such as lungs and aorta, with elasticity and durability (Farage et al., 2007; Van Doren, 2015). Elastin forms fibrils and, like collagen, they are found in the ECM forming a network and bonding with collagen fibrils. These molecules are resilient, and they are synthesized in a such way to last for a lifetime (Parks et al., 1993). Although elastin is insoluble, its precursor, tropoelastin, is soluble and exerted in the ECM where elastin fibrils are self-formed by bonding with hydrophobic amino acids found in the ECM, lysine, and extracellular enzyme lysyl oxidase. The hydrophobic parts of elastin are important for the elasticity of the skin and the prevention of body water loss. Tropoelastin expression and elastin synthesis takes place at early life stages and when damage occurs, the fibrils are irreplaceable. The irreplaceability of the elastin fibrils enhances the significance of the need for a protective and preventive method against aging factors. A potential protective and preventive role of phytochemicals against photo-aging will be assessed in this study.

1.1.3.3 Matrix Metalloproteinases

The balance of this extracellular system is controlled by a group of enzymes called Matrix Metalloproteinases (MMPs), that maintain collagen and elastin levels by degrading any excess production from the fibroblasts (Jabłońska-Trypuć et al., 2016). Generally, MMPs take part in various mechanisms in the human body, such as embryogenesis, tissue

restoration and wound healing. In the skin, these enzymes are also produced from fibroblasts, exported to the extracellular matrix (ECM). Their structure separates them in groups, such as collagenases, matrilysins and gelatinases, based on their domain. MMPs have a major role in apoptosis and cancer development, therefore their inhibition is investigated as an anti-cancer drug (Jabłońska-Trypuć et al., 2016). There are 26 MMPs found and most of them are responsible of degrading types of collagens. MMPs that degrade the collagens found in the dermis (collagen I and III) are MMP-1, -2, -8, -13, -14 and MMP-1, -2, -3, -8, -10, -13, -14, -16 respectively, and the enzymes that target elastin are MMP-2, -7, -9, -10, -12, -14, while in the dermoepidermal junction (collagen IV) the enzymes are MMP-3, -7, -9, -10, -11, -12 and -26 (table 1.1) (Chakraborti et al., 2003). The role of MMPs on skin disease is significant due to the degradation of collagens produced by keratinocytes and fibroblasts (section 1.1.3.2) and play an important role of the development of photo-aging.

Table 1. 1 *MMPs that degrade ECM collagen and elastin molecules found in the skin.*

Main collagen and elastin proteins found in the dermis and dermoepidermal junction	MMPs
Collagen I	1, 2, 8, 13, 14
Collagen III	1, 2, 3, 8, 10, 13, 14, 16
Collagen IV	3, 7, 9, 10, 11, 12, 26
Elastin	2, 7, 9, 10, 12, 14

(Chakraborti et al., 2003)

1.1.4 Hypodermis

The hypodermis, also known as subcutaneous fat layer, is the last inner layer of the skin and its main role is to insulate and regulate body temperature, while fat cells store energy (Al-Ghazzewi & Tester, 2012; Farage et al., 2007). It also serves as cushioning and the level of

thickness depends on the part of the body and the individual. In this layer, blood vessels and adipose tissue can also be found, while the latter contains certain fat cells that play a major role in endocrine response, such as adipocyte cells that are shown to increase insulin resistance when they are exposed to sex hormones, such as testosterone (Corbould, 2007).

The structure and function of the skin is significantly affected by external factors, such as sun exposure, that contribute to changes taking place in the epidermis and dermis. Certain proteins of the dermis, such as collagen, elastin and MMPs, have a synergetic role in maintaining a healthy skin and they are shown to be altered on a photo-aged subject and can be considered as biomarkers of this condition (Jariashvili et al., 2012; Ravelojaona et al., 2008).

1.2 Ultraviolet radiation

Solar ultraviolet radiation (UVR) is electromagnetic radiation that is emitted from the sun and its spectrum is separated in three ranges; long-wave UV (UVA) 315-400 nm, medium-wave (UVB) 280-315 nm and short-wave (UVC) 100-280 nm (Orazio et al., 2013). The atmosphere prevents almost 77% of the sun's UVR to reach the earth, while the remaining radiation passed through the ozone layer, a protective layer of the stratosphere which absorbs all UVC, part of UVB and does not absorb UVA at all. Although, UVR is shorter than the human visible light, insects and birds have visibility of near-UV (300-400 nm) that allow them to detect food. UVR can also be produced by specialised lamps and it is widely used for various reasons, such as decoration (black light), cosmetics (tanning) and disease therapy (psoriasis) (Lim et al., 2015; Miller et al., 2012; Singh et al., 2016). Unfortunately, the exposure to such wavelengths can have negative effects, such as skin cancer development, photo-aging and sunburn (Kammeyer & Luiten, 2015; Watson et al., 2013). The above risks depend on the wavelength, the length of the exposure as well as the location of the sun at the

time. UVA is known to cause photo-aging, while it is widely used in combination with UVB for therapies, such as psoriasis, and for indoor tanning. UVB is known to cause DNA damage after overexposure or severe sunburn and increase the risk for skin cancer, and therefore its use for cosmetic or therapeutic purposes is limited (Aufiero et al., 2006; H. J. Choi et al., 2020). UVC causes immediate DNA damage and cell death, and it is lethal for the human skin (Takasawa et al., 2005) it is also used for air-purification and object sterilization, due to its ability to damage DNA in a short period of time. UVA is known to penetrate the epidermis and reach dermis, causing certain molecular changes to the skin, while prolonged exposure to high amounts of UVA can cause photo-aging, a skin disease that is mostly known for its phenotypic changes of the skin and especially the face, due to the sensitive and thin skin of this body part (figure 1.3). On the other hand, UVB only penetrates and reaches the epidermis where it activates melanocytes to produce vitamin D and melanin, causing colour changes of the skin, known as tanning (Slominski & Postlethwaite, 2015). UVB is also known to cause DNA damage, while overexposure of the skin to this radiation can cause malignancies, such as skin cancer and melanoma (Orazio et al., 2013) (figure 1.4). While UVB is more dangerous for the skin health compared to UVA, UVA is not known to cause photo-aging, a disease that is mostly known for the visible facial changes. However, sun exposure of the skin makes both UVA and UVB unavoidable, increasing the risk for both types of disease.

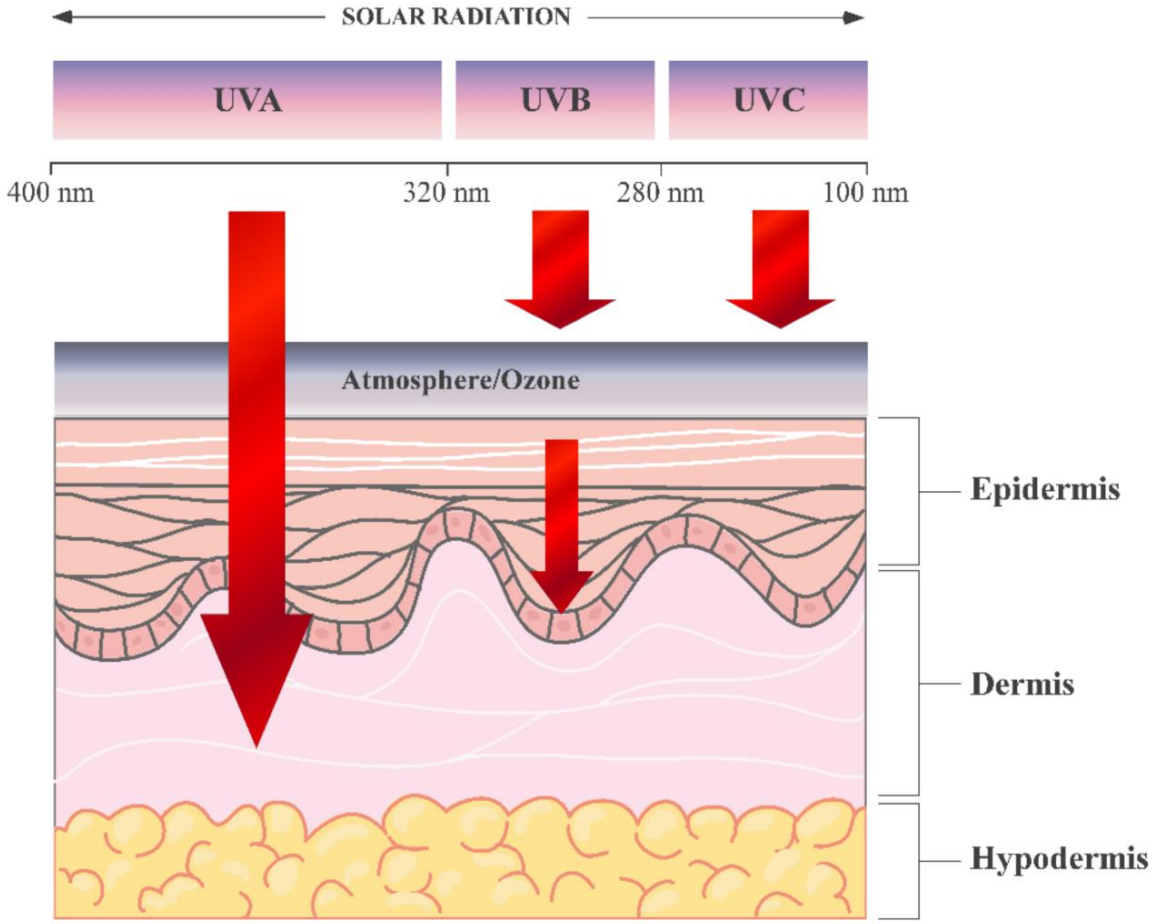


Figure 1. 3 *Filtration of ultraviolet radiation from ozone layer and penetration of UVA and UVB in the skin layers of unprotected skin.*
UVA penetrates the epidermis and dermis, while UVB only reaches the epidermis.

1.3 Photo-aging

Skin aging refers to certain alterations of the skin that appear over time that are not seen in a young healthy skin, while it is divided in intrinsic and extrinsic skin aging (Tobin, 2017). Intrinsic skin aging is the natural aging of the skin that occurs in ages over 80 years old and is characterised by thin expression lines and loss of elasticity and volume (Tobin, 2017). During intrinsic skin aging, the collagen production and the moisture of the skin are reduced, while thin expression lines are visible. Extrinsic aging occurs due to external factors, such as chemicals, lifestyle, air pollution, chronic sun exposure, smoking and diet. The external

factors that mostly contribute to extrinsic skin aging are UVR exposure and smoking. The main path through which the mentioned factors can lead to skin aging, is through the induction of oxidative stress, stimulating various pathways.

Long term exposure to UVR can lead to major structural and molecular changes in the skin, such as collagen breakdown and loss of elastin network stability leading to photo-aging, a condition that is known mostly from its phenotypic characterisation occurring in the skin and more often on the face of patients. Deep wrinkles, excess loss of moisture and volume of the skin, hyperpigmentation and random breakdown of the surface of the skin, are the main phenotypic characteristics observed in photo-aged skin. In contrast with natural aging, photo-aged skin is observed in earlier ages depending on the severity, which depends on the length of the sun exposure over time and the location of the sun. An interesting example is the case of a 69-year-old man, whose profession was a truck driver, and his left side of the face, which was more exposed to the sun, is showing severe signs of photo-aging, in contrast with the other half of his face, as shown in figure 1.5. The structural changes of the skin have caused significant alternations in the features of his face, while hyperpigmentation is also observed.

On a molecular level, it was shown that NB-UVB induces apoptosis in both keratinocytes and T-lymphocytes in a dose and time dependent manner (Aufiero et al., 2006). In a study where naturally aged and photo-aged human skin was analysed from human subjects, it was shown that while the former showed decreased collagen production and increased MMPs, when compared to young skin, the latter showed both collagen production and MMPs to be increased (Chung et al., 2001). In the above study young people with healthy skin and elderly people with diagnosed photo-aging condition were selected. Therefore, it is understood that the balance of collagen and MMPs between natural intrinsic aging and photo-aging are

different. Moreover, it was shown that MMP-1 and MMP-2 was increased in photo-aged skin, compared to the healthy skin (figure 1.6).

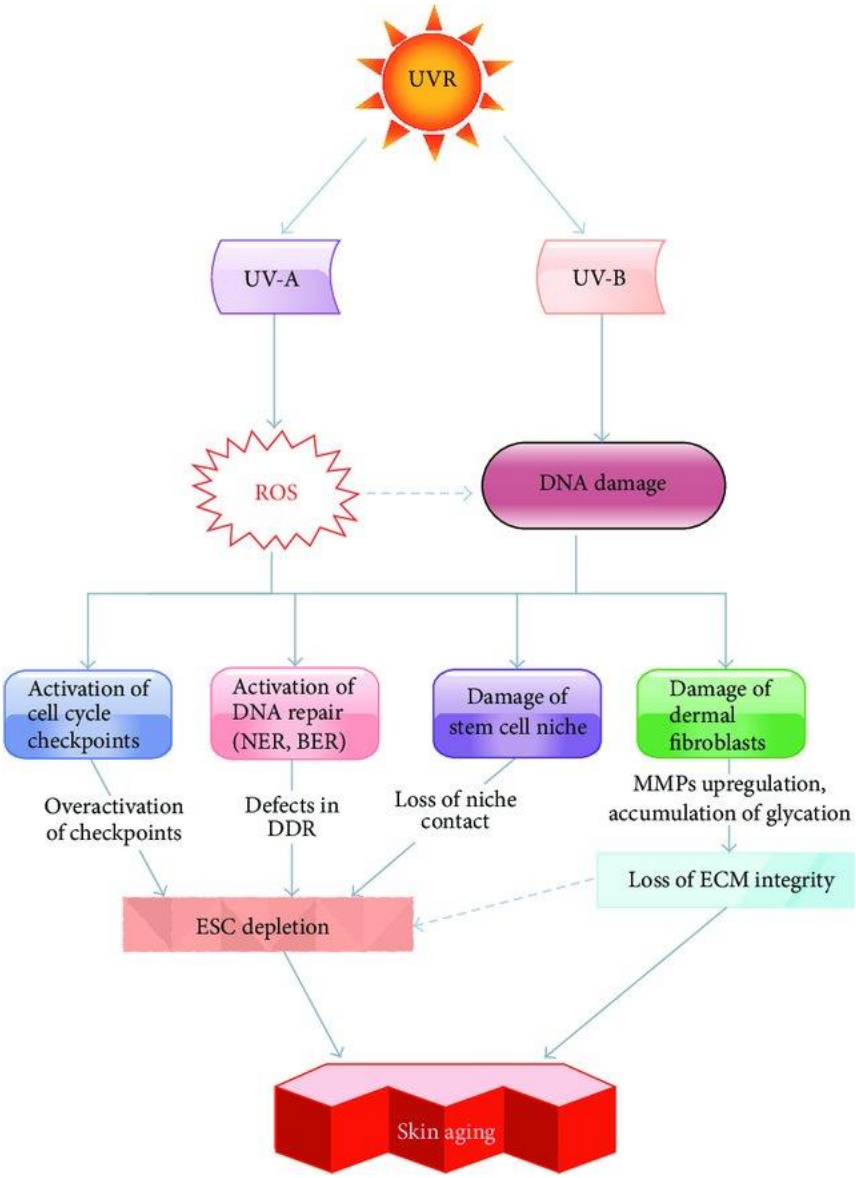


Figure 1. 4 *Effect of UVA and UVB on skin.*

UVA-induced ROS and UVB-induced DNA Damage leads to cell cycle arrest and MMPs overproduction that results to loss of ECM stability and integrity and eventually photo-aging (Panich et al., 2016).

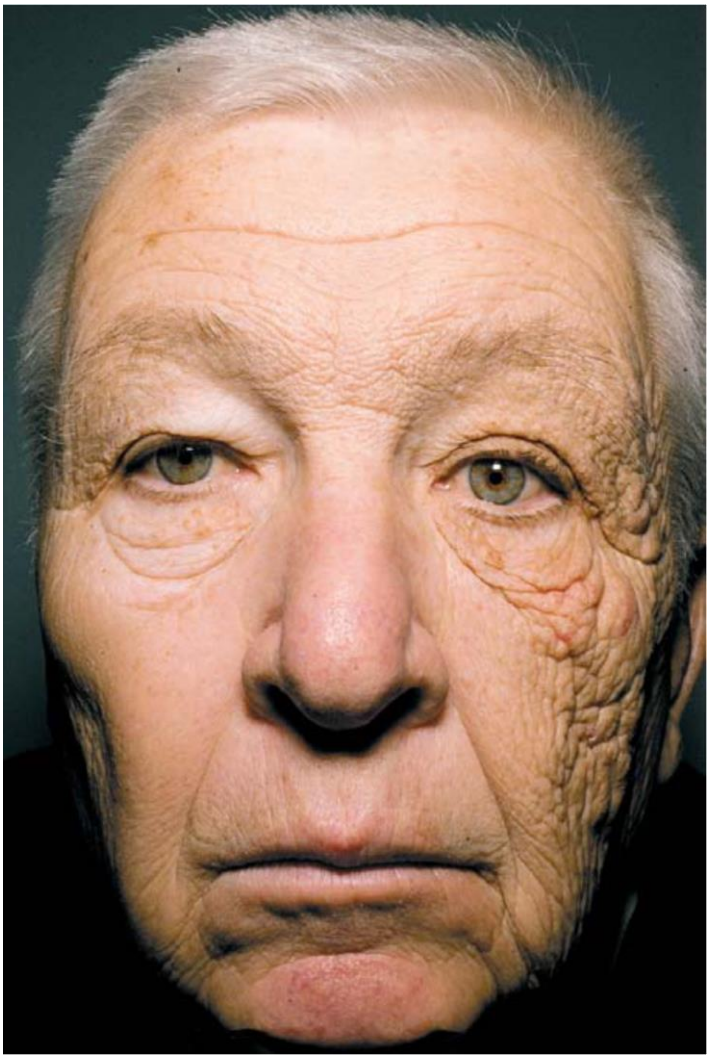


Figure 1. 5 *Photo-aged face of a man.*

The face of a 69-year-old man that his left side is showing heavy signs of photo-aging in contrast with the right side of his face due to the nature of his work as a truck driver. Picture taken from 'The New England Journal of Medicine'.

1.3.1 Photo-aging biomarkers

Certain proteins of the dermis, such as collagen, elastin and MMPs, have a synergetic role in maintaining a healthy skin and they are shown to be altered on a photo-aged subject and can be considered as biomarkers of this condition (Jariashvili et al., 2012; Ravelojaona et al., 2008). Collagen, elastin and MMPs are the main targets of investigation when it comes to photo-aging.

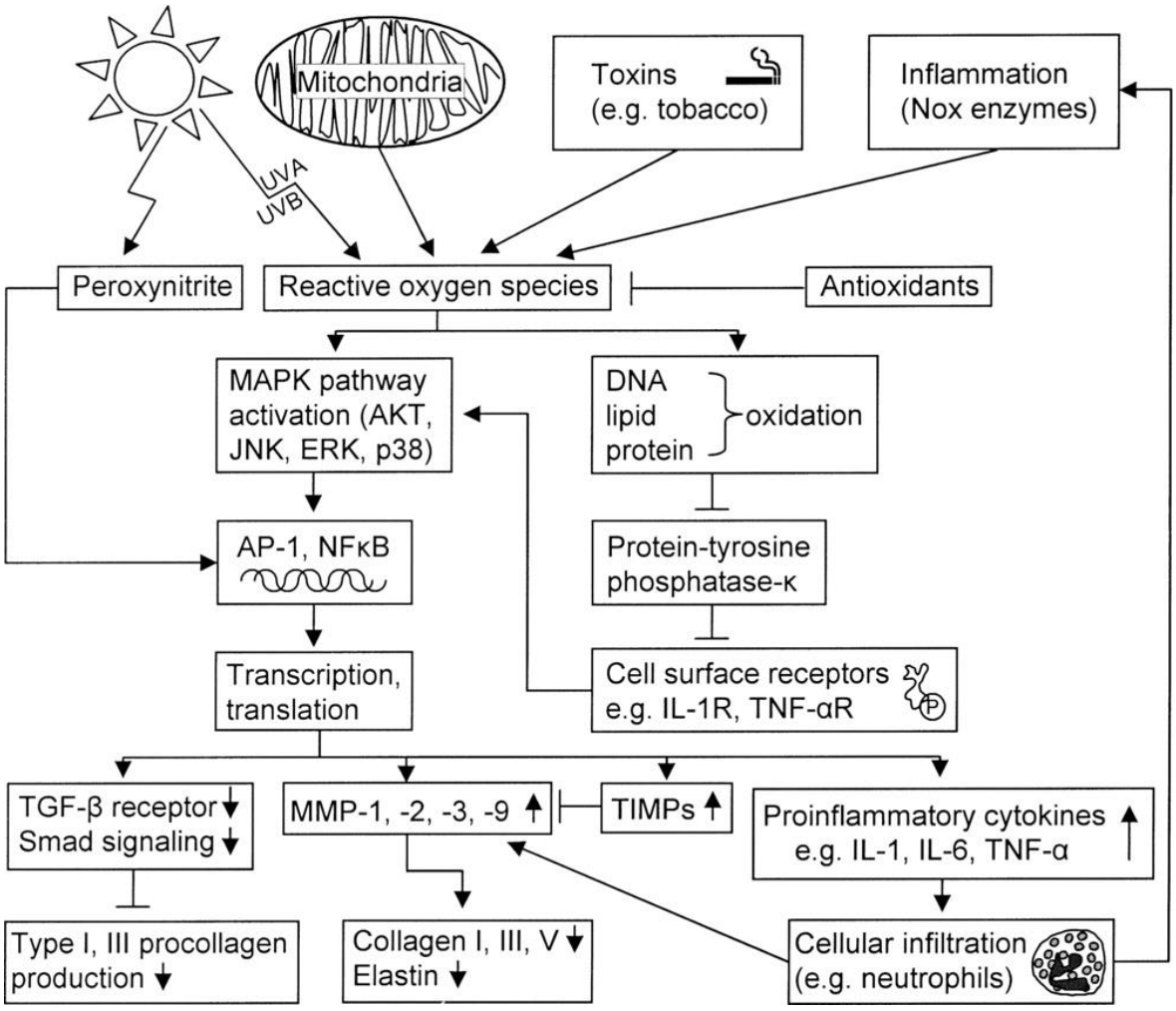


Figure 1. 6 *Role of extrinsically induced ROS in collagen and elastin production.*
ROS through the increase of MMPs, TIMPs and inflammatory cytokines and decrease of TGF-β receptor via the MAPK pathway activation and the oxidation of DNA lipid protein. (Sárdy, 2009)

1.3.1.1 Collagen

As described above, collagen production was shown to increase in photo-aged skin, in contrast with natural aging (J. H. Chung et al., 2001). The effect of UVR on collagen translation and synthesis has been further investigated. The triple helix of collagen I (figure 1.7) contained in solution, was shown to lose the helical content and create a new thermal stability point, following UVC exposure (Jariashvili et al., 2012). A breakdown of collagen

chains and a reduction of their peptides was shown. The mechanism by which the reduction of collagen I (the main characteristic of photo-aging) occurs, was studied by Quan *et. al,.* (2004). UVB (30 mJ / cm^2) – exposed human skin fibroblasts showed changes to transforming growth factor beta (TGF-β) / Smad pathway (Quan et al., 2004). TGF-β pathway is involved in many cellular stages of growth and apoptosis, while Smad are a group of proteins and major regulators of TGF-β receptors. It was shown that a combination of UVB and UVA affects TGF-β/Smad pathway in such way, that reduces the production of TGF-β type II receptor (TβRII). This leads to reduction of collagen I in human skin *in vivo* through downregulation of TβRII blocks Smad 2/3 activity. In another study were sun-exposed and un-exposed samples from healthy human subjects were obtained and compared, UVR was shown to decrease procollagen I in dermis through TGF-β signalling pathway, while MMPs were shown to increase (Kaisers et al., 2017). The denaturation of collagen proteins is caused by MMPs found in the ECM and they are produced by both keratinocytes and fibroblasts in the epidermis and dermis, respectively. The effect of UVR on MMPs and therefore the denaturation of collagen, leading to photo-aging, is described below, at section 1.3.1.3.

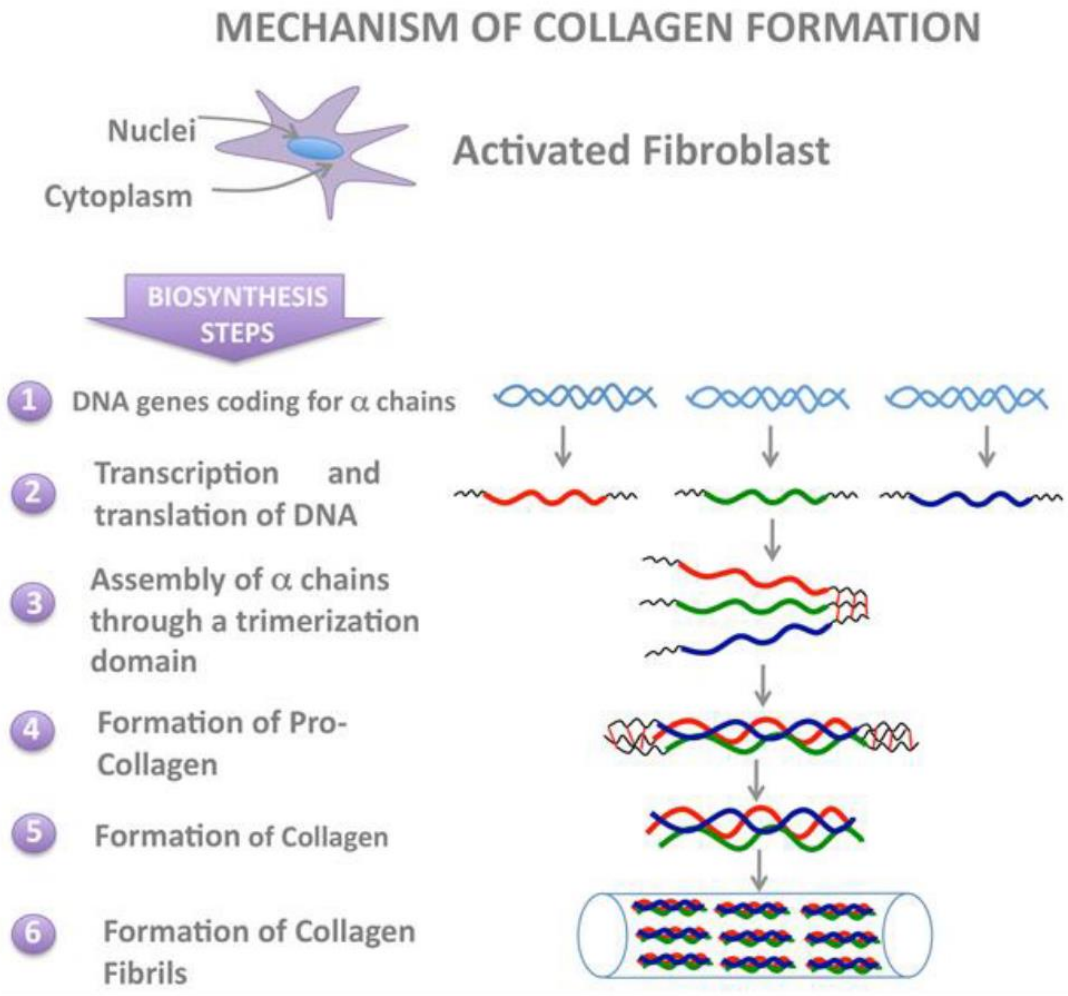


Figure 1. 7 *Mechanism of collagen formation.*

Fibroblasts produce collagen through the α- chains gene expression, transcription and translation. Fibrils are formed after the assembly of α-chains to pro-collagen and the formation of collagen protein (Sibilla et al., 2015).

1.3.1.2 Elastin

The main constituents of elastin fibrils are tropoelastin and fibrillin proteins (Yeo et al., 2012), while changes in their expression can lead to certain conditions that characterise photo-aging disease, such as deep wrinkles and thickening of the skin (Watson et al., 2013). Tropoelastin, the precursor of elastin protein, was shown to increase after UV exposure in a study where normal dermal fibroblasts were treated with 4.5 mJ / cm^2 UVB and tropoelastin production and expression of its mRNA was measured 5 days after the treatment (Schwartz

et al., 1995). Similar observation were made on photo-aged skin where the two proteins were increased 4 days after the UV treatment (Aimes & Quigley, 1995). The increase of elastin production after sun exposure is linked to solar elastosis, which is caused by abnormal production of elastin and its observable characteristic is the deposition of elastic material that creates yellow and deep, thick wrinkles (Kossodo et al., 2004; Watson et al., 2013). This condition is likely to occur due to the destruction of fibrils and the increased production of tropoelastin taking place in the fibroblasts, in combination with the increase of MMPs, that leads to elastogenesis, and eventually solar elastosis (Doyle et al., 2012; Neumann et al., 2008; Yang et al., 2008). Moreover, experiments showed that tropoelastin mRNA was expressed in keratinocytes *in vitro* and *in vivo* in human skin, as well as fibroblasts, revealing an important role of the epidermal cells in the formation of solar elastosis and photo-aging (Seo et al., 2001). Additionally, reactive oxygen species (ROS) generation caused by UVA exposure was shown to lead to tropoelastin degradation, revealing the role of ROS in disorders related to elastosis (Hayashi et al., 1998).

1.3.1.3 MMPs

The role of MMPs is to degrade collagen and elastin proteins found in excess in the ECM. In diseased and stressed conditions, such as photo-aging and extended sun exposure, an increase of those enzymes is observed, reducing the available extracellular collagen and elastin and leading to permanent structural changes in the skin, such as elastin fibril breakdown and reduced collagen in the collagen-elastin network (figure 1.8). UVR is known to induce MMP-1, -3 and -9 in the skin, enzymes that are known to degrade collagen I and II (Pluemsamran et al., 2012; Quan et al., 2009; Van Doren, 2015).Experiments conducted on UVA or UVB exposed human dermal fibroblasts from reconstituted tissue, showed an increase in MMP-1 and -3 production (Kossodo et al., 2004), while the majority of the UVR-

induced MMPs in human skin was produced by keratinocytes in the epidermis, rather than the dermis, indicating the important role of keratinocytes on photo-aging (Quan et al., 2009; Seo et al., 2001). The presence of MMP-1 in the ECM has been specifically studied on human skin obtained from biopsies, 24h after UVB exposure at the minimum erythema dose (MED) of each subject and the analysis of the organ culture fluids showed increased quantities of the enzyme (Brennan et al., 2003). MMP-9 is known to degrade dissolved collagen I and II monomers (Van Doren, 2015), while other MMPs were also shown to have a significant role in photo-aging through abnormal TGF-β signaling and elastolysis (Watson et al., 2013), such as MMP-12 that was shown to lead to elastolysis through the degradation of extracellular proteins, such as chemokines.

Therefore, it is understood that long term sun exposure can lead to indirect damage of collagen and elastin fibrils network through the increase of MMPs, while the balance between the ECM proteins and MMPs is crucial for the healthy function of the skin, its protection from UV radiation and the potential development of skin disease, such as photo-aging.

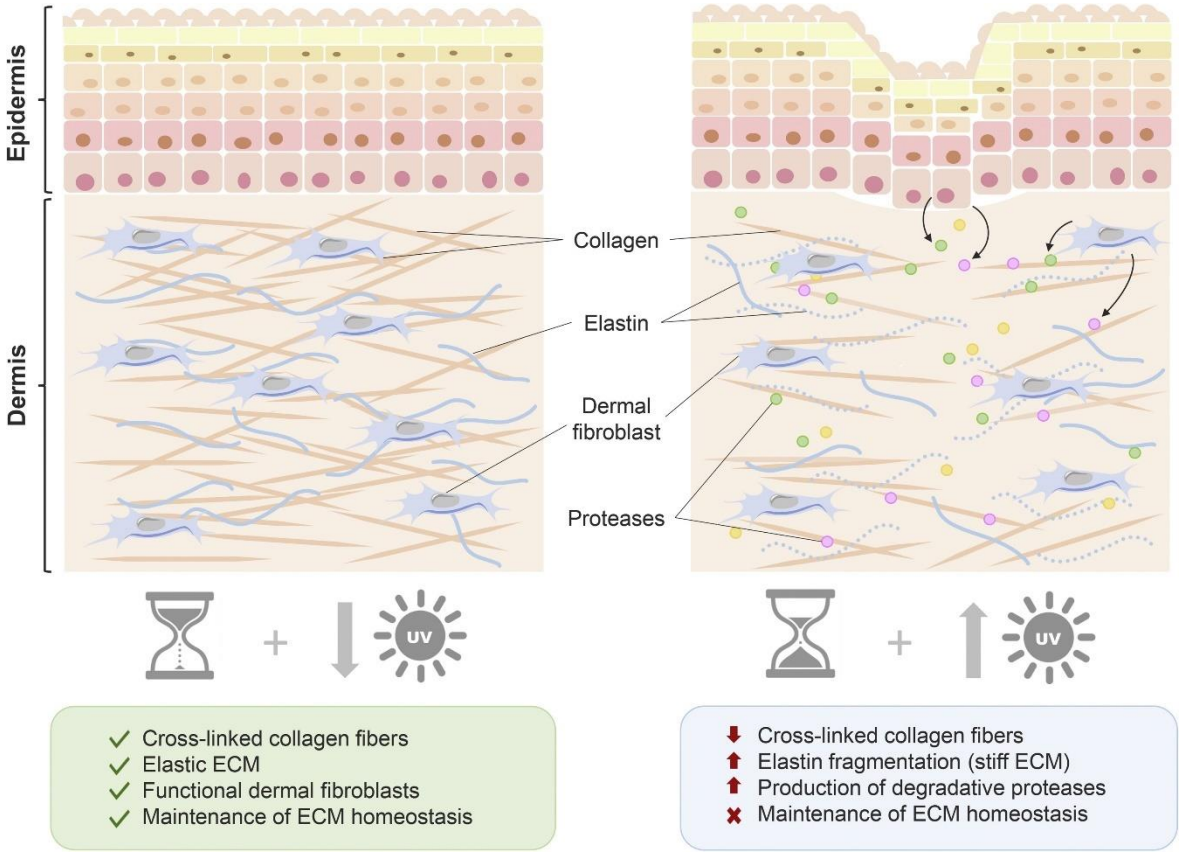


Figure 1. 8 *Changes taking place in the ECM during photo-aging.*
UV radiation causes elastin and collagen network break down through the overexpression of MMPs (degradative proteases) (Freitas-Rodríguez et al., 2017).

1.3.2 Sunscreen

Sun-protective cloths, hats and sunscreens are known to be the most effective means of protection against UVR. Sunscreen creates a protective layer on the skin and, when applied in sufficient quantities, it absorbs / reflect UV light depending on their formula (Mancuso et al., 2017). There is a variety of products and formulas in the market that all aim to protect human skin from the harmful effects of UVR. Sun protection factor (SPF) refers to the protection of a product against UVB (290-320 nm), while UVA filters (320-400 nm) can alter this factor when combined with UVB filters. Sunscreens are divided in organic and inorganic (mineral), with the former category being the most commonly used.

Organic sunscreens absorb both UVA and UVB transforming it in to heat which is then reflected away from the body (Gasparro et al., 1998). There are four types of organic filters: para-aminobenzoic acid derivatives (PABA), salicylates (weak UVB absorbers), benzophenoles, such as oxybenzone, and other, such as cinnamates. Some of the organic filters, such as PABA and oxybenzone, are known to cause photo allergenic reactions and their use is limited, while they are usually combined in order to achieve good absorbance of both UVA and UVB rays (Gasparro et al., 1998; Mancuso et al., 2017).

Inorganic (mineral) filters are non-transparent compounds that absorb and reflect (scatter) UV rays and are mainly zinc oxide and titanium dioxide (Mancuso et al., 2017). These filters are mainly used in nano-particles due to the ability of titanium dioxide smaller particles to absorb more UVB better compared to bigger particles, while zinc oxide absorbance in not altered by the particle size, having flat absorbance curve.

Natural compounds were also studied for their anti-photo-aging and antioxidant effects on skin, such as caffeoylquinic acid (cQa) (Oh, Karadeniz, et al., 2019) and other antioxidant (Pandel et al., 2013) as well as natural products, such as propolis, (Bolfa et al., 2013) and probiotics, such as Lactobacillus (H. M. Kim et al., 2014; Lolou & Panayiotidis, 2019), have also been studied for their potential protective and preventive role on photo-damage and eventually photo-aging.

While UVR is commonly known for its negative effects on skin health, it is also used as a therapy for other skin conditions, such as psoriasis (Singh et al., 2016).

1.4 Psoriasis

Psoriasis is an autoimmune skin disease that is characterised by rapid proliferation of keratinocytes forming excess skin dry patches which are accompanied by inflammation, redness and itchiness (Rahman et al., 2012). Body areas that are affected include the navel area, the shins, the back of forearms, the scalp and the fingernails/toenails, while it can affect the whole body, depending on the type of psoriasis, as explained in section 1.4.3. High income countries mostly in Europe, Australasia and North America are shown to have the highest prevalence of psoriasis, while 81% of the countries lack information on the epidemiology of the condition (Parisi et al., 2020). Moreover, it is reported that a year-long treatment for a mild-severe psoriasis per person, costs £14,255 to the National Health Service (NHS) of United Kingdom (Barker et al., 2021). Therefore, the development of alternative therapies that aim to reduce the cost through use of effective and easily accessible treatments is necessary.

There is a strong immuno-genetic basis to psoriasis and genes involved in the inheritance of the disease include PSORS1, PSORS9, HLA-C, CDSN, CCHCR1, while IL12B, IL23R and chromosome 5q are also shown to be associated with psoriasis (Geisel et al., 2014; Pisani et al., 2021; Rahman et al., 2012). Moreover, medication, such as anti-malarial drugs, beta blockers and lithium has been shown to induce or worsen psoriasis, while psoriatic lesions might persist even after the end of the drug treatment (Balak & Hajdarbegovic, 2017). The drug-induced lesions are shown to differ compared to non-drug induced psoriatic lesions, while treatment includes a combinational therapeutic approach in addition to the conventional treatments (for details see 1.4.3). Psoriasis is also known as a T cell mediated inflammatory disease and its mechanism is divided in three parts (Rahman et al., 2012). Initially (a) Th17 cells are activated and (b) move to the skin lesion, where (c) they release cytokines, while inflammation markers, such as interleukins and TNF-α receptors, increase the proliferation rate of keratinocytes (figure 1.9). CD8+ T and CD4+ T cells are shown to

be the predominant inflammatory mediators in the psoriatic epidermis and dermis, respectively, while genes that are related to psoriasis, such as PSMA6 and ERAPA are located in chromosome 6 (Elder et al., 2010). Other causes of psoriasis are microbial imbalance and infection, other disease, such as HIV, and lifestyle that induces skin stress and inflammation, such as exposure to chemicals and UVR (figure 1.9).

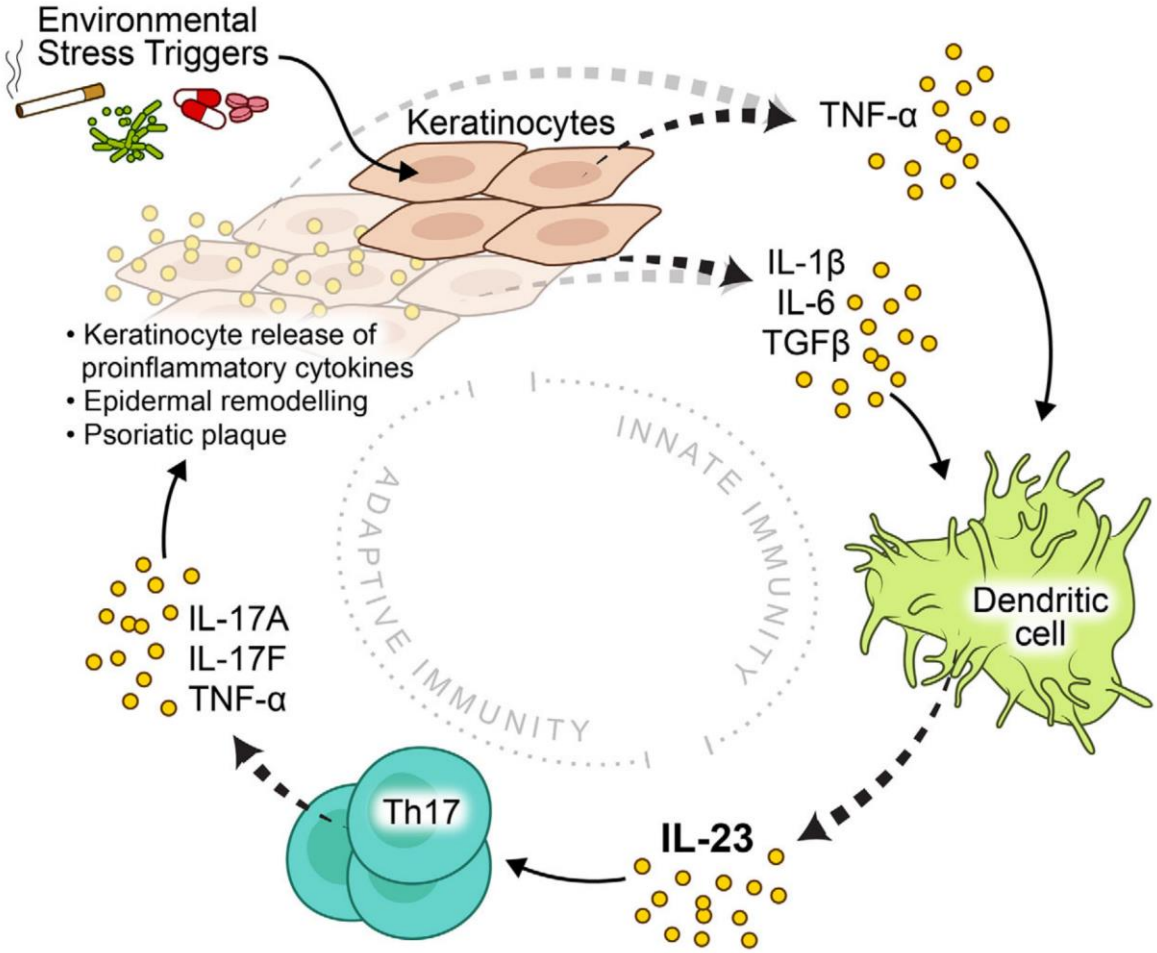


Figure 1. 9 *Psoriasis disease mechanism.*

Following a stimulant, keratinocytes overproduce cytokines and stimulated dendritic cells produce a psoriasis biomarker IL-23 that lead to further production of other interleukins that increase keratinocyte cell proliferation and inflammation (Gooderham et al., 2018)

1.4.1 Types of psoriasis and other related diseases

There are five types of psoriasis, plaque psoriasis, guttate psoriasis, inverse psoriasis, pustular, and erythrodermic psoriasis and are divided based on the visual differences of the lesions and symptoms (figure 1.10). Plaque psoriasis, also known as psoriasis vulgaris, is the most common (80-90% of cases) form of psoriasis and it is characterised by inflamed raised patches covered in white dry skin (Balak, 2015). Guttate psoriasis forms red scaly drop-shaped lesions that are usually spread over the torso, arms, legs and the scalp, while inverse psoriasis is found in skin folds as red patches (Hönigsmann, 2001). Pustular psoriasis forms little blisters that contain pus but are not infectious, while possibly the most severe case of psoriasis is erythrodermic psoriasis which is when other types of psoriasis become widespread and affect the homeostatic mechanism and barrier function, becoming even fatal (figure 1.10). There are other diseases that are linked to psoriasis and affect the overall health of the patient, such as psoriatic arthritis which appears in 30% of patients suffering from psoriasis (Rahmati et al., 2021). Other related diseases are lymphomas, a type of cancer that affect the lymphatic system of the body and is a group of blood malignances that are initiated from lymphocytes, while cardiovascular disease, Crohn’s disease and depression are also seen in psoriasis patients (Elmets et al., 2019).

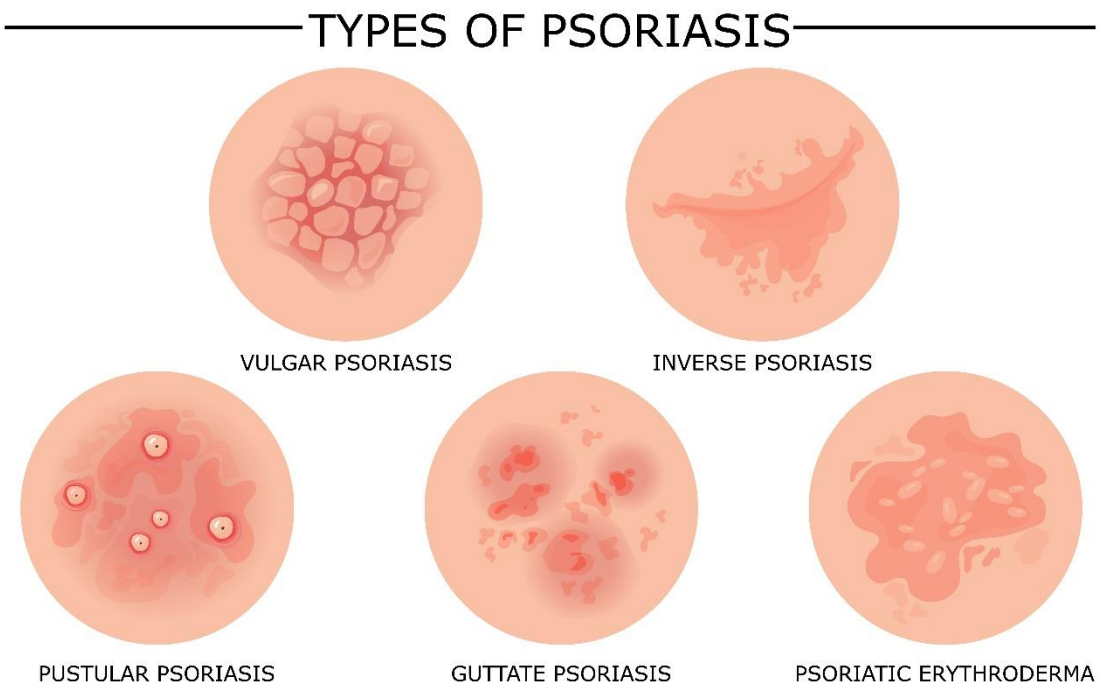


Figure 1. 10 *Types of Psoriasis. Picture taken from www.freepik.com.*

1.4.2 Current treatments

1.4.2.1 Creams

Current treatments for psoriasis are usually combinations of topical treatments and oral drugs, while it depends on the severity and spread of the disease. Initial treatments of mild psoriasis include topical applications with corticosteroid creams and vitamin D creams that reduce inflammation and cell proliferation rate, while they help with itchiness (Balak & Hajdarbegovic, 2017). These creams can be used on every affected area of the body and it is an effective first step for the management of psoriasis symptoms, while the combination of corticosteroid creams and vitamin D creams are shown to be more effective than either treatment alone (Ranjan et al., 2007).

1.4.2.2 UVR treatments

In more severe and prolonged situations where creams are not suppressing the condition, UV light is also used in combination with creams and / or oral medicine (Hönigsmann,

2001). Phototherapy has been widely used in the past, with the exposure of the diseased area to the sunlight. As science progresses these treatments are more controlled in order to achieve more satisfying results and to avoid potential overexposure that could lead to severe burning of the skin and other disease such as skin cancer and photo-aging. There are various treatments when it comes to UVR use, while the MED is usually used in multiple doses over a period. Controlled phototherapy was initially referred to UVB use, while currently either UVA or UVB rays are used, and the intensity of the energy of the treatment depends on the type of skin of the individual and the MED. After evaluation of the MED, patients are treated in weekly (2-5) sessions and initial radiation dose is usually 70% of the MED, while it increases by 20% in every session (Yones et al., 2006). UVA alone does not have an effect on psoriasis, therefore it is used in combination with the oral drug Psoralen UVA (8-methoxypsoralen). Oral medication is supplied 2 hours prior to the treatment to increase the amount of UV light absorbed by the skin (Hönigsmann, 2001; Yones et al., 2006). A common side effect of this medication is nausea which is managed by changing the treatment to 5-methoxypsoralen. The dosage can differ between patients due to the metabolic pathway (first-pass effect) of oral Psoralen from the body, which is metabolised by the liver and discharged through urination in the next 24h after the supplementation (Hönigsmann, 2001). Psoralen-UVA treatment is a well-established form of therapy for psoriasis, while there is a risk of skin cancer and photo-aging in long term exposure. The mechanism through which Psoralen-UVA treats psoriasis is the recurrent phototoxic reactions. UVA exposure of the skin activates oral Psoralen, and the UV rays penetrates the epidermis and reach the papillary dermis. Psoralen enters the double stranded DNA and allows a series of cytokine activation and ROS formation that damage the cell membrane and lead to reduced cell proliferation and cell cycle arrest. Moreover, Psoralen was shown to be a better inducer of apoptosis in lymphocytes, than in keratinocytes, which makes the treatment efficient in reducing T-cells and inflammation (Hönigsmann et al., 2016).

UVB is shown to be very effective on treating psoriasis symptoms without the aid of oral medication and it can be either broadband (BB) UVB (280-320 nm) or narrowband (NB) UVB (311-313 nm), while the latter is considered safer compared to the former due to the damaging effect of UVB rays on skin. BB UVB long term use can cause DNA damage and carcinogenesis and it is therefore not ideal for psoriasis patients that require regular treatements, while NB UVB is safer, emitting light at a shorter wavelength. Thus, NB UVB and Psoralen UVA therapies are preferred over BB UVB due to efficacy difference, as well as the reduced risk of skin cancer and DNA damage. The efficacy between Psoralen UVA and NB UVB treatments have been evaluated by Yones *et. al.*, 2006, where 88 patients were randomly treated with either treatment and results were compared (Yones et al., 2006). Although UVA-treated patients were more likely to develop erythema, compared to UVB-treated patients, who did not show any signs of erythema, Psoralen-UVA treatment showed a longer relapse time of 8 months, compared to the 4 months relapse time of NB-UVB treatment.

As with most treatments, UVR therapy for psoriasis has side effects, such as erythema, blistering, dry skin and in some occasions herpes flare-ups have been reported, while long term effects are photo-aging and carcinogenesis (Hönigsmann et al., 2016). Another side effect that is observed in patients that have been treated with UVR for an extended period of time in their lives, dermatoheliosis is also shown to appear with symptoms of dry skin, changes in pigmentation of the skin, xerosis and actinic keratoses, while hypertrichosis (excess hair growth) and lentiginosis (lentigines in large numbers) is also observed in some cases. In psoriasis patients that UVR treatments are not effective and fail to suppress the symptoms of the disease and clear the lesions, immune system suppression medication (immunosuppression) is often used.

1.4.2.3 Immunosuppression

Immunosuppressive medication, including steroid creams or systemic drugs, such as cyclosporine acitretin and methotrexate, suppress the immune response in order to reduce inflammation and T cell activation (Rahman et al., 2012). Steroid creams are used for local immune suppression and can be used long term, while oral medication suppresses the overall immune system. Systemic immunosuppressant drugs are also known for their side effects affecting the functionality of liver and kidneys, while other side effects have also been shown, such as in the skin organ (Medellín-Luna et al., 2021). Methotrexate is known to suppress bone marrow, cause nausea and hepatic fibrosis, while cyclosporine is known to be toxic to the kidneys. Other systemic drugs include fumaric acid esters and hydroxycarbamide. Hydroxycarbamide is a drug orally supplied and usually used for the treatment of various cancer types, such as leukemia and cervical cancer, or other disease such as thrombocythemia and psoriasis. It is a drug that inhibits cells from synthesizing DNA and repairing DNA damage and therefore, it inhibits cell growth (Ranjan et al., 2007). Fumaric acid esters are known for their role in citric acid cycle, a basic process required for the production of energy from the mitochondria and it is shown that oral supplementation of fumaric acid esters can mediate psoriasis symptoms (Balak, 2015).

1.4.2.4 Other treatments

Other treatments against psoriasis have been studied lately and include anti-TNF-α antagonists and VEGF antagonists, while some of the studies have shown 75% improvement in psoriatic lesions of more than half subjects (Balak & Hajdarbegovic, 2017). Although monoclonal antibodies are widely used on inflammatory disease, such as rheumatoid arthritis and Crohn's disease, as well as psoriasis, they are known to worsen psoriasis symptoms in cases or even cause psoriasis in inflammatory disease that are not related to the skin, such as

inflammatory bowel disease. The cause of this reaction, also known as paradoxal psoriasis, is not fully evaluated in the literature, while it is probably related to dendritic cells producing interferon-α, an inflammatory cytokine. VEGF, a growth factor that regulates neovascularization and a biomarker of psoriasis (Loboda et al., 2005), when inhibited, it was shown to either clear psoriatic lesions from patients or flare up existing lesions (Balak & Hajdarbegovic, 2017). The used VEGF antagonist, sorafenib, is therefore considered a paradoxal medicine, as it is used for the treatment of psoriasis.

1.4.2.5 Natural treatments

Therapies using herbal extracts, such as polyphenolics, steroids and lipids on psoriasis have also been shown to reduce inflammation and suppress the immune system (Rahman et al., 2012). Herbs such as *Eucommia uloides Oliv., Liquorice, Aloe Vera, Ilex paraguariensis* and *Mahonia aquifolium* are known to reduce cell proliferation and skin inflammation either by topical application or oral supplementation (Man et al., 2008). Such herbs are shown to enhance cytokines secretion, such as TNF-α, IL-8 and IL-1β, while *Psoralea corylifolia L.* was shown to inhibit keratinocyte proliferation is psoriasis due to its phenolic glycoside content (Rahman et al., 2012). Curcumin and ginger are also known for their anti-inflammatory properties, while Indigo naturalis, when used as an ointment, was shown to inhibit abnormal cell proliferation and differentiation of keratinocyte cells (J. W. Lee et al., 2005; Liau et al., 2007; Moon et al., 2006; Suzuki et al., 2005) (table 1.2).

Table 1. 2 *Properties of natural products on skin cell proliferation and inflammation.*

Natural products	Properties	Reference
Herbal extracts: polyphenolics, steroids, lipids	Reduction of inflammation and immune system suppression	(Rahman et al., 2012)
Eucommia uloides Oliv.	Reduction of cell proliferation and skin inflammation	(Man et al., 2008)
Liquorice		
Aloe Vera		
Ilex paraguariensis		
Mahonia aquifolium		
Psoralea corylifolia L.	Inhibition of keratinocyte proliferation	(Rahman et al., 2012)
Curcumin	anti-inflammatory	(J. W. Lee et al., 2005; Liau et al., 2007; Moon et al., 2006; Suzuki et al., 2005).
Ginger		
Indogo naturalis	Inhibition of abnormal cell proliferation and differentiation of keratinocyte cells	

1.5 Cruciferous vegetables

Cruciferous vegetables are a group of vegetables that belong in the family of *Brassicaceae*, which contains about 3000 species of plants and includes broccoli, cauliflower and watercress (Dinkova-Kostova & Kostov, 2012; IARC, 1986). In the sulfur-rich cells of this family of vegetables a class, of phytochemicals called glucosinolates (GSLs) is found, which is released after the tissue damage of the plant and reacts with myrosinase, forming isothiocyanates (ITCs) and other by products, such as thiocyanates and nitriles. GSLs role in nature is protect plants from natural enemies, such as insects, birds and mammals, making the compounds instantly toxic for the host and inhibiting their growth and development (Buskov et al., 2002; Halkier & Gershenzon, 2006; Lazzeri et al., 2004; Noret et al., 2005). GSLs were shown to attract or repulse certain insects, while other insects, such as herbivores (beetles and moths) are shown to metabolise the phytochemicals through cleavage of the sulfate with an endogenous sulfatase (Miles et al., 2005; Rojas, 1999).

When the vegetables are chopped or chewed, myrosinase creates cleavage with the thio-glucose bond of the glucosinolate and gives rise to thiohydroximate O-sulfonate, which is then rearranged to various products, such as isothiocyanates, nitriles and thiocyanates, while the former is a well-studied group of phytochemicals that are known for their health benefits (IARC, 1986) (figure 1.11). They are known for their high nutritional value, while they are shown to have various health benefits, such as anti-inflammatory and antioxidant effect and anti-carcinogenic activity (Fung Lung Chung et al., 2000; Fahey et al., 2002; Traka & Mithen, 2009). Sulforaphane (SFN), iberin (IBN), allyl isothiocyanate (AITC), phenethyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC) represent some of the most studied ITCs (figure 1.12). ITCs consist of a common -N=C=S group, while the unique structure of each ITC is shown to determine their biological effect. Their structure can differ in the number of double bonds, the presence of aromatic ring and their oxidation state of sulfur (figure 1.12). In a study where HepG2 cells were treated with various ITCs of different

structures, it was shown that the number of double bonds does not have a significant effect on apoptotic and necrotic cells, ROS generation and cell cycle arrest, while the increased oxidation state of sulfur showed a significant ($p<0.05$) increase of both apoptotic and necrotic HepG2 cells, increased ROS generation compared to SFN ($p<0.05$) and S phase arrest (Pocasap et al., 2018). SFN, an ITC that does not have an aromatic ring in its structure, showed increased apoptotic and necrotic cells, compared to PEITC that has an aromatic ring, while the ROS generation caused in the cells by the exposure of the phytochemicals, was not significant between them. Moreover, when cell cycle was observed, SFN and PEITC showed a G2/M and G1 phase arrest, respectively. Therefore, it is hypothesised that although all ITCs are shown to have similar effect on biological processes and metabolism, some might be more potent from other due to their chemical structure. Moreover, some ITCs are more light-sensitive and volatile than others, which makes them harder to handle and quantify.

Consumption of cruciferous vegetables is linked with various health benefits, such as reduced cases of cancer, due to their anti-carcinogenic effect through apoptosis induction of abnormal cells, when 111μmol of ITCs are administrated (Shapiro et al., 2001; YUAN et al., 2016), while concentrations >10 μM are shown to reduce cell viability of cancer cells by approximately 15%(Mantso et al., 2016). 200mg / day of dried broccoli sprouts are shown to have an indirect antioxidant effect and prevention of oxidative stress, through the induction of phase II enzymes (Traka & Mithen, 2009) and therapeutic effect on inflammatory skin conditions, such as dermatitis and psoriasis, due to their anti-inflammatory effect and their anti-proliferative effect on skin cells in concentrations <10μM. (Guerrero-Beltrán, Calderón-Oliver, et al., 2012; Mantso et al., 2016; Sussan & Sussan, 2012). Topical application of cruciferous plant extracts (15%) are shown to reduce psoriasis lesions (Sussan & Sussan, 2012). Their anti-proliferative effect is shown through cell cycle arrest of normal and diseased cells, while it depends on the concentration of the

phytochemicals. In low concentrations of SFN (0.3 μmol), a 50% reduction of tumor burden is shown, while ITCs are shown to enhance growth and rescue cells that have been exposed to UVR, reducing erythema by 37.7% (Dinkova-Kostova et al., 2006; Saw et al., 2011b; Talalay et al., 2007). Higher concentrations (>10 μM) are shown to further increase apoptotic and necrotic cells and induce ROS production, possibly due to their cleavage with glutathione (GSH), the antioxidant of cells, reducing its availability to the cells and its defense mechanisms against ROS (Mitsiogianni et al., 2021; P Rose et al., 2000) .

Isothiocyanates are metabolised in the human body *via* the mercapturic acid pathway and their cleavage with GSH, giving several dithiocarbamates, while the metabolism of the phytochemicals takes minutes in cells and just a few hours in human subjects (excreted) in 2-6 hours (IARC, 1986).

Table 1. 3 *Studies on the effects of ITC containing extracts on human and rat subjects and cell lines*

Natural Extract	Cell line / subject	Effects	References
Watercress extract	HaCaT cells, endothelial cells	Decrease of cell growth	(Loboda et al., 2005)
Nanoparticles of watercress extract	lung carcinoma cells A549	Decrease in cell growth	(Adlravan et al., 2021)
Watercress extract	Breast cancer cells	cell growth inhibition through alteration of ALDH1 and SOX9 expression	(Abbasi et al., 2018)
Broccoli and watercress extracts	Human breast cancer cells (MDA-MB-231)	Decrease of cell viability, inhibition of MMP9 (0.1 to 1 mg / mL of plant extract)	(Peter Rose, et al., 2005)
Watercress extract	Human colon adenocarcinoma cells and human lung fetal fibroblasts	Anti-proliferative effect, anti-metastatic mode through cycle arrest and inhibition of DNA damage at 50 μL / mL concentration, reducing DNA damage by 28%.	(Boyd et al., 2006)
Watercress extracts	Human colorectal adenocarcinoma cell lines	Cell viability reduction	(Rodrigues et al., 2016)
Moringa extract	Rat paw erythema	Reduction of erythema symptoms by 33%, nitric oxide (NO), IL-1β, IL-6 and Nrf2 target genes.	(Jaja-Chimedza et al., 2017)
Moringa extract	Mice suffering from colitis symptoms, keratinocytes (topical and oral effect)	Reduction of IL-1β, TNF-α expression, colonic inflammation and pro-inflammatory cytokines.	(Y. Kim et al., 2017)
Moringa extract	C57BL/6J mice	Anti-diabetic effects through the reduction of IL-1β and TNF-α levels.	(Waterman et al., 2015)

Broccoli extract	Human skin and mouse models	Reduction of erythema through the increase of phase 2 enzymes.	(Dinkova-Kostova et al., 2007)
Watercress extract	Murine hepatoma Hepa 1c1c7 cells	Inhibition of lipases, α-glucosidase and α-amylase.	(P Rose et al., 2000)
Watercress extract	Human studies	Decrease of TNFα and IL-10 production	(Schuchardt et al., 2019)
Watercress extract	Diabetic rats	Reduction in blood sugar, leptin, total glucose, triglycerides and cholesterol.	(Hadjzadeh et al., 2015)
Cruciferous vegetables extracts	Psoriasis lesions on human subject	Reduction of lesions	(Sussan & Sussan, 2012)

For the ITC profiling of vegetables and the quantification of ITCs in them, various methods have been used in the literature. The most commonly used method of analysis is High Performance Liquid Chromatography (HPLC) and Gas Chromatography – Mass Spectrometry (GC-MS), while the cyclocondensation assay has been widely used for the total quantification of ITCs from plants (Bertelli et al., 1998; Fusari et al., 2015; Pilipczuk et al., 2017; Theresa A Shapiro et al., 1998). ITCs are not stable in aqueous solutions due to their lipophilic and electrophilic nature and are therefore hard to handle in such solutions. Therefore, organic solvents, such as hexane, are usually included in the sample preparation of plants, as well as solid phase extraction and various steps of sample clarification, such as filtration and centrifuge (Bertelli et al., 1998; Pilipczuk et al., 2017; Rodrigues et al., 2016; T A Shapiro et al., 2001).

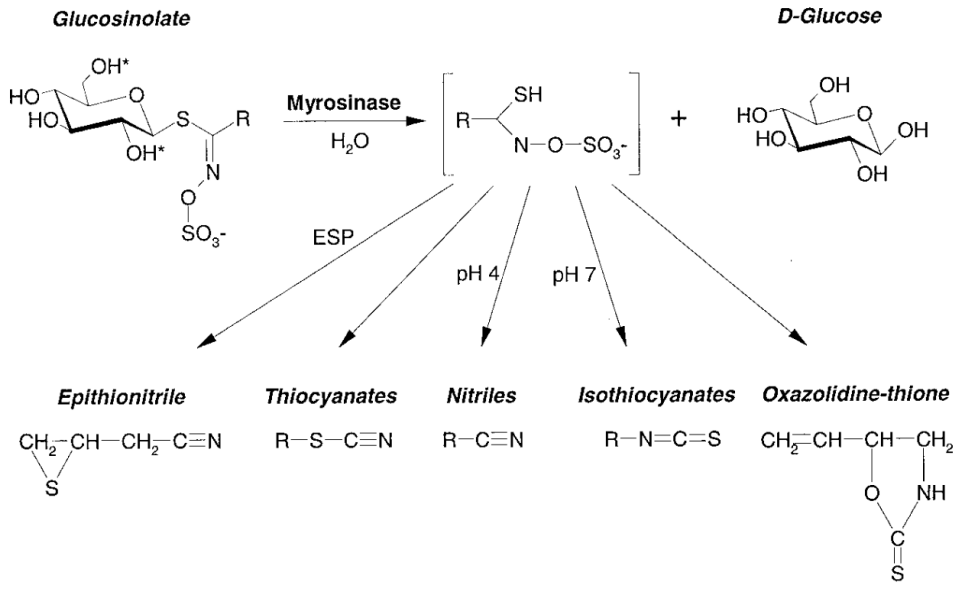


Figure 1. 11 *Hydrolysis of glucosinolates in the presence of myrosinase forming epithionitrile, thiocyanates, nitriles, isothiocyanates and oxazolidine-thione.*
(Rask et al., 2000)

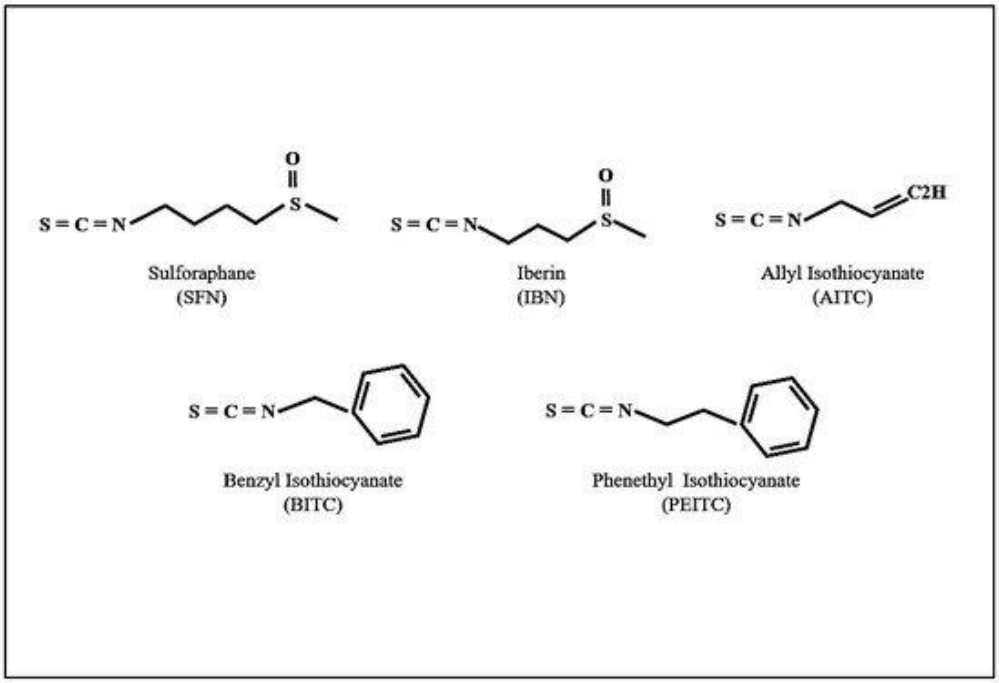


Figure 1. 12 *Structure of isothiocyanates (SFN, IBN, AITC, BITC, PEITC).*
(Mitsiogianni et al., 2019)

1.6 Aims of the thesis

1. To develop rapid and easy sample preparation and analytical procedures for the quantification
2. of isothiocyanates in cruciferous vegetables that do not require expensive equipment and expertise.
3. To investigate the effect of UVB on HaCaT cells in order to develop a UVB induced optimisation model, evaluate the effect of SFN and PEITC on HaCaT cells in the absence of UVB and investigate the effect of SFN and PEITC on a UVB induced photo-aging model using HaCaT cells through observing their dose and time dependent effect on viability levels.
4. To determine the role of five synthetic ITCs (SFN, IBN, AITC, BITC, PEITC) in combination with low doses of UVB on HaCaT cells, as a novel therapeutic approach for psoriasis.
5. To combine low dose UVB and ITCs, with the aim of inducing a strong effect when combined treatment that could negate the risk of skin cancer but lead to a significant improvement of psoriasis symptoms.
6. To validate the findings of aim 4 and determine the role of naturally derived ITCs in combination with low doses of UVB on HaCaT cells, as a novel therapeutic approach for psoriasis.

1.7 Hypotheses of the thesis

1. Isothiocyanates can have a beneficial effect on photo-aging when the phytochemicals are supplemented in low doses.
2. Isothiocyanates, in combination with low doses of UVB, can have a beneficial effect on psoriasis through their biphasic nature.
3. Watercress plant extracts can have a similar effect on psoriasis disease as the commercially available synthetic PEITC.

2. Methodologies and Materials

2.1 Principal of molecular assays used

2.1.1 Resazurin assay

Resazurin assay is based on the ability of cells to metabolise the non-toxic non-fluorescence blue dye (resazurin – alamarBlue dye) that is contained in the assay reagent to resorufin, a fluorescent, pink coloured product. The level of oxidation or reduction is shown through absorbance or fluorescence intensity. For the experiments conducted in this study, data were collected by monitoring absorbance at 570 nm and 600 nm for oxidised and reduced product, respectively (Mantso et al., 2016). This method was used to evaluate cell viability during and after the treatments.

2.1.2 Sulforhodamine B assay

The sulforhodamine B (SRB) assay is a colorimetric method for the determination of cell viability through the ability of Sulforhodamine B to bind to protein basic amino acid of TCA-fixed cells and it measures the cellular protein content of adherent and suspension cells (Orellana & Kasinski, 2016). For this study, the protocol was followed with minor alterations and according to our laboratory's protocol, replacing TCA with Carnoy's solution 3:1 methanol:acetic acid. This alteration aimed to reduce the exposure of the researcher to hazard chemicals. The rest of the protocol was followed as described below. This method was used to evaluate cell growth during and after the treatments.

2.1.3 Caspase 3/7 assay

CellEvent caspase 3/7 Green detection reagent (ThermoFisher Scientific Ltd) is a four-amino acid peptide which is conjugated to nucleic acid-binding dye and in the presence of activated

caspase 3/7 in apoptotic cells, the peptide is cleaved allowing the dye to bind to DNA and have a fluorogenic effect at 530 nm. 4′,6-diamidino-2-phenylindole (DAPI) stain was used for the exclusion of necrotic cells from the analysis.

The response measured through the flow cytometer is presenting the quantified activation of caspase 3/7 and therefore the apoptotic cells, whilst DAPI fluorescent measurements are presenting the necrotic cells. The assay was conducted according to the manufacturer's protocol. This method was used to evaluate cell apoptosis through the induction of caspase 3/7, during and after the treatments.

2.1.4 JC-1 mitochondrial membrane permeability assay

In healthy conditions, mitochondria sustain an interior potential that is higher compared to the exterior potential of the cell and the high inside potential prevents certain molecules to enter into the mitochondria. In pathological conditions, the mitochondrial membrane becomes permeable, leading to a decrease of the interior potential and an increase of the exterior potential. This increased exterior potential allows 1,1',3,3'-tetraethyl-5-5',6,6' tetrachloroimidacarbocyanine iodide (JC-1) stain to enter the mitochondria and form aggregates that have specific absorption and emission characteristics, while monomers are formed in the exterior space with specific absorption and emission characteristics as well. The assay was conducted according to the manufacturer's protocol. This method was used to evaluate mitochondrial membrane permeability of the cells through the induction of caspase 3/7, during and after the treatments.

2.1.5 Cell cycle assay

The assay is based on the different amounts of DNA that exist in each cell cycle stage and the ability of propidium Iodine (PI) to penetrate the cell membrane of only dead cells, binding with the major groove of the DNA (adenine – thymine regions) upon cell death to give fluorescence at excitation/emission 535 / 617 nm, 20-30 fold higher than the unbound dye. Therefore, PI is a good indicator of dead cells and cell cycle stages, as well as the quantification of dead cells. The assay was conducted according to the manufacturer's protocol. This method was used to study the cell cycle, during and after the treatments.

2.1.6 Dihydrorhodomanine 123 (DHR123) assay

DHR123 assay is used for the indication and quantification of ROS in live cells through its rapid reaction with them and its conversion to rhodamine 123, a fluorescent compound the levels of which quantitatively indicate the presence of ROS. Moreover, in order to exclude the dead cells from the analysis, DAPI, a dye that penetrates only dead cell's membrane and binds with the major groove of the DNA, was used as a quantitative indication of dead cells. The assay was conducted according to the manufacturer's protocol. This method was used to investigate and quantify ROS induction in cells during and after the treatments.

2.2 Materials and methods for the development of a novel rapid determination of total ITCs in Watercress

2.2.1 Materials and chemicals

Watercress was provided by The Watercress Company (TWC) and kept in the freezer at -18 °C until further use. Reference compounds sulforaphane (SFN) (>98% purity) and iberin (IBN) (> 95% purity) were purchased from Cayman Chemical Company. Allyl isothiocyanate (AITC) [99.7% purity], phenethyl isothiocyanate (PEITC) (99% purity), benzyl isothiocyanate

(BITC) (>=98.5% purity), methanol, hexane (99.9% HPLC gradient grade) and the cyclocondensation assay reagent benzene 1-2 dithiol were purchased from Sigma-Aldrich. The spectrophotometric measurements were carried out using a Varian Cary 50 Bio UV-Visible Spectrophotometer (Agilent (Varian), Stockport, Cheshire, UK) and operated with the Varian Cary Win UV software. Plastic UV-transparent plastic disposable cuvettes (1 cm) were used during spectrophotometry.

2.2.2 Sample and reference compound preparation

Frozen watercress was kept in the freezer at -18° C until further use. For the preparation of the freeze-dried watercress, leaves and stalks were taken from the frozen material and were immediately placed in the freeze-drier for 72 hours at -40 °C. The frozen plant tissue was carefully and quickly processed to avoid thawing and potential myrosinase activation. The dried material was then milled to a thin powder, using IKA A11 basic analytical mill (IKA-Werke GmbH, Staufen, Germany) and stored in a cool and dry place, in glass containers, until further use. Fresh watercress was purchased from a local store and processed immediately. Briefly, watercress leaves and stems were chopped and mixed with distilled water for further analysis (sections 2.2.3 and 2.2.4). For the commercially available ITC, dilutions (1 µg / mL, 2 µg / mL, 4 µg / mL, 8 µg / mL, 15 µg / mL, 30 µg / mL, 60 µg / mL) of 5 ITCs (SFN, IBN, AITC, PEITC and BITC) were prepared in methanol and stored at -18 °C.

2.2.3. Aqueous extraction of ITCs

Watercress powder (0.5 g) was vortex mixed with 10 mL distilled water and the samples were ultrasonicated for 5 minutes in ultrasonic water bath under ice and then incubated for 2 hours at 37 °C in a shaking water bath. Samples were then centrifuged (4000 rpm, 10 minutes) and 0.2 mL of the supernatant was mixed thoroughly with 0.8 mL of methanol

followed by a second centrifuge step (14000 rpm, 5 minutes). Absorbance of the samples was measured at 365 nm following the cyclocondensation assay.

2.2.4. Solvent extraction

ITC extraction was performed, following modifications of the method proposed by Rodrigues (2016). Aliquots (0.5 g) of dried watercress were humidified in 10 mL distilled water and incubated at 37° C for 2 hours. After incubation, 10 mL of *n*-hexane was added to the aliquots and extractions were performed for 2 hours under magnetic stirring. Filtration and concentration of the extract was done in a rotary evaporator under at 40 °C under reduced pressure, whilst the dried matter was reconstituted in 3 mL methanol. For the measurement of the total ITC content, samples were prepared for the cyclocondensation assay as described below.

2.2.5 Cyclocondensation assay

The quantification of the total ITC content was conducted through the cyclocondensation assay, as described by Zhang et. al. (Y. Zhang et al., 1992, 1996). Watercress extracts and reference compound solutions (0.2 mL) were mixed with 0.8 mL potassium phosphate buffer (100 mM, pH 8,5) and 1 mL 1,2-benzenedithiol (BDT) in methanol (8 mM) to a final volume of 2 mL in 12 mL glass vials. Samples were kept at 65 °C for 1 hour, transferred to cuvettes and absorbance was measured at 365 nm using a Varian Cary 50 Bio UV-Visible Spectrophotometer (Agilent (Varian), Stockport, Cheshire, UK) operated with the Varian Cary Win UV software. For the evaluation of heavy metal interference, Ethylenediaminetetraacetic acid (EDTA) was added to cyclocondensation assay samples substituting 0.1 mL of the buffer volume used for the assay, while control samples contained 0.1 mL of distilled water instead. For the spiked standard curve samples PEITC was used in 4 concentrations (7.5, 3.75, 2, 1 µg / mL).

2.3 Materials and methods for the evaluation of the effect of isothiocyanates on a photo-aging experimental model

2.3.1 Cell culture

Cell recovery

HaCaT cell line (Sigma – Aldrich) was used for both experimental models of photo-aging and psoriasis. Cells were stored at -150 ° C in a liquid nitrogen freezer in cryovials containing a minimum of $5x10^5$ cells / mL in DMSO-containing fetal bovine serum (FBS) 10% (v/v). Upon use, cells were removed from the liquid nitrogen freezer and quickly defrosted and resuspended in 5 mL at 37 ° C medium. The complete DMEM was preprepared mixing Dulbecco's Modified Eagle Medium (DMEM) (Labtech International Ltd, East Essex, UK) high glucose with 2 mM *L*-Glutamine (Labtech) (1% final concentration), 10 mL fetal bovine resume heat-inactivated (Labtech) (10% final concentration) and 1% (v/v) penicillin/streptomycin mix (1% final concentration). The cell-containing media was centrifuged at 2000 rpm for 2 minutes and the formed pellet was resuspended in warm media and transferred in a $25cm^3$ cell culture flask. Cells were cultured and used for no more than 20 passages until new stocks were used, while they were kept in an incubator for growth in conditions of 5% atmospheric CO_2 at 37 ° C with high humidity. Before use, cells were observed daily regarding the normality of their morphology and growth rate.

Subculture

The subculture and expansion of HaCaT cells was conducted through trypsinization. The cells were initially washed with 5 mL warm PBS following the discharge of the growth media and then washed with 1 mL trypsin-EDTA (1x) (trypsin-EDTA 0.25% in phosphate buffer saline (PBS) w/o Mg^{2+}, w/o Ca^{2+}) . 2 mL of fresh trypsin-EDTA was added and the flask was incubated for 12-15 minutes and until all cells were detached. For the deactivation

of trypsin-EDTA, 5 mL of media was added in the flask while the mix was used to aid the cells fully detach from the flask and in to the media. Cells were then centrifuged (2000 rpm for 2 min) and supernatant was discharged and resuspension of cells in certain volumes of complete DMEM depending on the pellet of cells followed. $5x10^5$ cells were resuspended in 10-15 mL of complete DMEM and added to the new flask, following counting of the cells using the hematocytometer.

Cell plating

Cells were left to grow in the flask to between 80 - 100% confluence before they were used for plating. For the plating process, cells were collected *via* trypsinization as described above and centrifuged at 2000 rpm for 2 minutes after their resuspension in media. To initiate plating cells were resuspended in 5 - 10 mL and in accordance to the size of the pellet. For the determination and calculation of the cell number of the sample, equal amounts (30 µL) of trypan blue solution 0.4% (v/v) (HyClone Inc, South Logan, USA) and cell-containing media were added to a centrifuge tube. The mix was vortexed and 10 µL of the sample was loaded in a hemocytometer (Neubauer). Cells were counted under the microscope and the calculations were conducted using the equation as shown below:

$$\boldsymbol{Number\ of\ cells = \frac{N}{4} * dilution\ factor * 10^4}$$

Where N is the number of cells that were counted, and the dilution factor is 2 and it is set according to the dilution made while mixing the media with trypan blue solution. 10^4 refers to the conversion factor that is calculated based on the dimensions of the squares of the hemocytometer. After the calculation of the number of the cells contained in the media, the appropriate amount was added to the wells of 96-well plates, 12-well plates or in 60 mm plates depending on the experiment.

2.3.2 Treatment of HaCaT cells with ITCs

Cells were seeded in 12-well plates (0.3 x 10^6, 0.15 x 10^6 and 0.1 x 10^6 for 24, 48 and 72 hours, respectively) and left to adhere overnight. ITC containing-media was added to the wells / plates in concentrations of 0.15, 0.3, 0.6, 1.24, 2.5, 5 μM, depending on the experiment, and was allowed in the wells / plates until the timepoint that measurements were taken. For the dilutions, stock of 100 μM ITC that was kept in the freezer, was used and calculation were made based on the following simple formula:

$$\mathbf{C_1 * V_1 = C_2 * V_2}$$

Where C_1 is the concentration of the starting solution, V_1 is the volume of the starting solution, C_2 is the concentration of the final solution, V_2 is the volume of the final solution.

2.3.3 Treatment of HaCaT cells with UVB

For the optimisation of the photo-aging model, propagation and seeding of HaCaT cells was conducted as described below. For the UVB treatments, CL-1000 Crosslinkers (Ultra-Violet Products Ltd) were used. The range of UVB doses used for the following experiments were based on minimum erythema doses (MED) that were found in the literature (Rodríguez-Granados et al., 2017). Treatments' conditions were set up for exposures based on energy emitted rather than time. Time was automatically adjusted from the UV crosslinkers after appropriate calibration from the manufacturer. Cells were seeded in 12-well plates (0.3 x 10^6, 0.15 x 10^6 and 0.1 x 10^6 for 24, 48 and 72 hours, respectively) and left to adhere overnight. They were then washed with PBS w/o Mg^{2+}, w/o Ca^{2+} following the removal of the media. 500 μL of fresh PBS w/ Mg^{2+}, w/ Ca^{2+} in room temperature was added to the wells as the salts in PBS promote of cell adhesion and prevent cell detachment under low temperature conditions. Plates were placed in UVB chambers and exposed to narrow band (NB) UVB irradiation 5, 10, 25, 50 and 100 mJ / cm^2 under ice, by placing the plates on a

thin layer (0.5 cm) of ice on a thin plastic tray. Ice was used to prevent damage-repair enzymatic activation, while control plates were not exposed to UVB (Feller & Gerday, 1997; Gerday et al., 2000). After the exposure, plates were immediately removed from the ice and PBS was replaced with warm media. Plates were placed in an incubator (37 ° C) until the measurements of the first timepoint (24, 48 and 72 hours).

2.3.3.1 Viability determination of UVB exposed HaCaT cells

For the determination of the viability of the cells, the resazurin assay (Alamar Blue dye) was utilized (Mantso et al., 2016). 0.3 x 10^6, 0.15 x 10^6 and 0.1 x 10^6 cells for 24, 48 and 72 hours, respectively, were seeded in 12-well plates and left to adhere overnight at 37 ° C. Treatment with five UVB intensities (2.5, 10, 25, 50 and 100 mJ / cm^2) was conducted under ice by placing the plates on a thin layer (0.5 cm) of ice on a thin plastic tray, following washing of the cells with PBS and replacing with fresh PBS. Immediately after the treatment, PBS was replaced with fresh complete DMEM (Labtech International Ltd, East Sussex, UK). At the timepoint, 500µL of resazurin in PBS (1 mg / mL) was added to the wells following the removal of the old media. The plates were then incubated for 4 hours at 37 ° C and absorbance was measured at 570 nm and 600 nm using Tecan plate reader. Results were expressed as percentage of viability at 24, 48 and 72 hours. The plates were incubated for 4 hours at 37 ° C and the absorbance was measured at 570 nm and 600 nm using Spark multimode plate reader (Tecan, Switzerland). The percentage of control cells was used to express the cell viability.

2.3.3.2 Apoptosis determination of UVB induced HaCaT cells

For the determination of apoptosis of the photo-aging model and the optimisation of the final model used for the experiments of this chapter, CellEvent Caspase 3/7 Green flow cytometer

assay kit (ThermoFisher Scientific Ltd) was used along with DAPI (Sigma Aldrich Ltd) which was used to exclude necrotic cells from the analysis (Mantso et al., 2016). Briefly, 0.35 x 10^6 (24h) and 0.5 x 10^6 (48h) cells were seeded in 60 mm plates and left to adhere overnight. Treatment with four UVB intensities (10, 25, 50 and 100 mJ / cm^2) was conducted under ice after washing of the cells with PBS and replacing with fresh PBS. Complete DMEM (Labtech International Ltd, East Sussex, UK) was added immediately after the treatment and the removal of PBS and plates were incubated (37 ° C) until the timepoint indicated (see individual figure legends). At the indicated timepoints cells were collected in tubes with the assistance of cell scrapers, after trypsinisation with 2 mL trypsin-EDTA [0.25% in phosphate buffer saline (PBS) w/o Mg^{2+}, w/o Ca^{2+} (1x)] for 12 minutes and washed twice with PBS. 0.5 µL of cellEvent Caspase 3/7 Green detection reagent was added into 500 µL single cell suspension sample (10^6 cells / mL) and incubated at 37 ° C for 30 minutes before the addition of DAPI (1µM) (Sigma Aldrich Ltd) and the further incubation in room temperature and dark environment for another five minutes. Samples were analysed using fluorescence-activated single cell sorting (FACS) Canto II flow cytometer (BD Biosciences, San Jose, CA, USA) and data acquisition was set at 20,000 events. Cell apoptosis was indicated as caspase 3/7 positive, whilst necrotic cells were stained with DAPI and indicated as DAPI positive.

2.3.3. Treatments of HaCaT cells with ITCs

For the ITC treatments, complete DMEM was used for the dilutions of two ITCs, PEITC and SFN as described in section 3.2.2. The phytochemicals were diluted in complete DMEM in six concentrations 0.15, 0.3, 0.6, 1.25, 2.5 and 5 µM. The effect of each ITC on cells was measured at timepoints 24, 48 and 72 hours. At the timepoint, the treatment media was

removed, and viability of cells was determined using the resazurin assay as described in section 3.2.3.1. Data are presented as percentage of viability.

2.3.4 Viability determination of ITC-treated and UVB-exposed HaCaT cells

The optimised photo-aging model was pre-treated with PEITC and SFN and the cell viability was determined as described in section 2.3.2. Specifically, HaCaT cells were seeded in 12-well plates and left to adhere overnight. PEITC was prepared in dilutions of 0.15, 0.3, 0.6, 1.24, 2.5 and 5 μM, and 1 mL of the prepared dilutions replaced the old media. 24 hours after the treatment, cells were exposed at 25 and 50 mJ / cm^2 UVB as described in section 3.2.1, as indicated from the optimised model. After the UVB exposure and at the timepoints 24, 48 and 72 hours, viability was determined using resazurin assay. The same protocol was followed for the pre-treatment of the model with SFN.

2.3.5 Apoptosis determination of ITC-treated and UVB-exposed HaCaT cells

Cells were seeded in 60 mm plates and treated as described above until 24 hours after the UVB exposure. HaCaT cells were treated with the optimum conditions of UVB exposure indicated from the previous experiments, with 25 mJ / cm^2 UVB exposure following a pre-treatment of 0.5, 2 and 5 μM SFN. The effect of SFN pre-treatment on UVB exposed cells was assessed using the CellEvent Caspase 3/7 Green flow cytometer assay kit as described in section 3.2.3.2. Measurements were taken at 24 hours timepoint.

2.4 Materials and methods for the evaluation of the role of synthetic isothiocyanates in combination with low dose UVB on HaCaT cells as a therapeutic approach for psoriasis

For the optimisation of the model, propagation and seeding of HaCaT cells was conducted as described in section 2.3.1. For the UVB treatments, a CM-1000 Crosslinker (Ultra-Violet Products Ltd) was used, emitting narrow band UVB at 302nm. The range of UVB doses used for the following experiments were based on minimum erythema doses (MED) that were found in the literature (Rodríguez-Granados et al., 2017). It is shown that the MED for phenotype I is < 7 mJ/cm^2 UVB, while for phenotype II, III and IV is < 19 mJ/cm^2 UVB, < 27 mJ/cm^2 UVB and < 38 mJ/cm^2 UVB respectively.

2.4.1 Effect of UVB on HaCaT cells

For this section, the data presented in chapter 4, section 4.2.1.1, was used for the optimisation of this chapter's model. Method was as described in section 2.3.3. For the determination of the viability of the cells, the resazurin assay was as described in section 2.3.4, while for the determination of apoptosis was as described in sections 2.3.5

2.4.2 Treatments of HaCaT cells with SFN and resazurin assay

Cells were seeded in 12-well plates (3 x 10^5 and 0.15 x 10^6 for 24 and 48 hours, respectively) and left to adhere overnight. SFN containing-media was added to the wells / plates in concentration of 5 and 20 µM until the timepoints studied, 24 and 48h. The calculations were made as described in section 2.3.1. At each timepoint, the treatment serum was removed, and the viability of cells was determined using the resazurin assay as described in section 2.3.4. Data is presented as percentage of viability relative to controls of untreated cells.

2.4.5 Viability and growth determination of combinational treatments of HaCaT cells with ITCs and UVB

Experiments were initiated using resazurin assay for the determination of viability of the cells, while SRB (Sulforhodamine B) assay was utilized for the observation of cell growth. 3×10^5 and 0.15×10^6 cells / well were seeded in 12-well plates and left to adhere overnight. They were then washed with PBS w/o Mg^{2+}, w/o Ca^{2+} following the removal of the media. 500 μL of fresh PBS w/ Mg^{2+}, w/ Ca^{2+} at room temperature was added to the wells as the salts in PBS promote of cell adhesion and prevent cell detachment under low temperature conditions. Plates were placed in the UVB crosslinker and exposed to 5 and 10 mJ / cm^2 UVB under ice as described in section 2.3.3. Control plates were not exposed to UVB. After the exposure, plates were immediately removed from the ice and PBS was replaced with warm ITC-containing complete DMEM. 20μM of either of the 5 ITCs, SFN, IBN, AITC, BITC and PEITC, was added in the wells, while control wells contained complete DMEM. Plates were placed in an incubator (37°C) until the measurements of the first timepoint (24 and 48 hours). At the timepoint, media was removed from the plates and a solution of resazurin-containing PBS (1 mg / mL) is added at volumes 100 μL – 500 μL depending on the experiment and the plates used. The plates were incubated for 4 hours at 37 ° C and the absorbance was measured at 570 nm and 600 nm using Spark multimode plate reader (Tecan, Switzerland). The percentage of control cells was used to express the cell viability.

2.4.5.1 SRB (Sulforhodamine B) Assay

Cells were prepared as described above until the timepoint indicated (24 and 48h) where the below protocol was followed for the SRB assay:

Fixation of cells with Carnoy's solution

At the timepoint, 250 mL of Carnoy's solution were added for every 1 mL of complete DMEM in each well. Plates were left to fix for 1 hour at 4 °C and then washed 4-5 times with tap water immediately after the incubation period. After drying the plates for 2 hours at 60 °C, they were kept at 4 °C until next step.

Staining of cells

After achieving room temperature, 250 µL of 0.4% SRB in 1 % acetic acid was gently added to each well and left to stain for 30 minutes at room temeperature. After the removal of the dye solution and the rinsing of the wells with 1% acetic acid (5 times), the plates were dried at 60 °C. 250 µL of 10 mM trisaminomethane (Tris) pH 10.5 was added to each well and absorbance was read at 570nm after a 20-minute solubilization of the stain on a plate shaker (mark 4). The absorbance (nm) was taken using Spark multimode plate reader (Tecan, Switzerland). The percentage relative to control cells was used to express the cell growth.

2.4.6 Apoptosis determination of combinational treatments of HaCaT cells with ITCs and UVB

For the preliminary evaluation of apoptosis of UVB and SFN-exposed HaCaT cells, 0.8 x 10^6 cells were seeded in 60mm plates and left to adhere overnight. Cells were exposed to 10 and 25 mJ /cm^2 UVB and 5 and 20 µM of SFN in complete DMEM were used for the preliminary treatments of the cells, immediately after the UVB exposure. The preliminary intensities were selected based on the aim of the study, which is to achieve a strong combinational effect using mild conditions. Chapter 3 showed that exposure at 25 and 50 mJ /cm^2 UVB is effective to reduce viability in HaCaT cells, an effect opposite to the aim of

this chapter. At 24h apoptosis was determined using flow cytometry (FACS) and CellEvent Caspase 3/7 Green detection reagent, as described in section 2.3.5.

For the determination of apoptosis of the optimised combinational treatments, 0.5 x 10^6 and 0.8 x 10^6 cells were seeded in 60 mm plates and left to adhere overnight. Cells were treated with 20μM of either of the 5 ITCs, SFN, IBN, AITC, BITC and PEITC, following the UVB exposure at 5 and 10 mJ /cm^2 and apoptosis was determined at 24 and 48h, using the same protocol as above.

2.4.7 Mitochondrial membrane permeability determination of combinational treatments of HaCaT cells with ITCs and UVB

0.5 x 10^6 and 0.8 x 10^6 cells were seeded in 60 mm plates and left to adhere overnight. Cells were then exposed to 5 mJ /cm^2 (48h) and 10 mJ /cm^2 (24h) UVB and immediately treated with 20 μM of either of the 5 ITCs studied, SFN, IBN, AITC, BITC, PEITC or the equivalent concentration of total ITC content from the natural compound depending on the experiment. At the indicated timepoints cells were collected in tubes with the assistance of cell scrapers, after trypsinisation with 2 mL trypsin-EDTA [0.25% in phosphate buffer saline (PBS) w/o Mg^{2+}, w/o Ca^{2+} (1x)] for 12 minutes and washed twice with PBS. Cells were resuspended in 300 μL PBS and 0.3 μL of JC-1 stain (0.1 mg / mL) was added to the suspension. After a 30- minute incubation at 37 °C, cells were centrifuges (1000 rpm for 5 minutes) and fresh PBS was used for the resuspension of the cells. Samples were injected in the flow cytometer and data was acquired through acquisition of 10,000 cell event for each sample. For the analysis and representation of the data, the ration of JC-1 aggregates over JC-1 monomers was used.

2.4.8 Cell cycle determination of combinational treatments of HaCaT cells with ITCs and UVB

0.5×10^6 and 0.8×10^6 cells were seeded in 60 mm plates and left to adhere overnight. Cells were then exposed to 5 mJ /cm^2 (48h) and 10 mJ /cm^2 (24h) UVB and immediately treated with 20μM of either of the 5 ITCs studied, SFN, IBN, AITC, BITC, PEITC. At the indicated timepoints cells were collected as described above and washed twice with PBS. Fixation of the cells (approx. 0.5×10^6) using cold 70% ethanol was initiated with the gradual addition of the ethanol under very low vortex and incubation of the cells for 1h at 4 °C, until further processing. After washing of the cells with PBS (x2) to remove the ethanol, cells were suspended in FxCycle PI/RNase staining solution and remained in room temperature and dark environment for 30 minutes. Samples were injected in the flow cytometer and data was acquired through acquisition of 10,000 cell events for each sample. Debris was excluded from the gating, while the graphs were set at DAPI and FITC dies.

2.4.9 ROS induction determination of combinational treatments of HaCaT cells with ITCs and UVB

Two assays were utilized to determine ROS induction. Experiments were initiated using DHR (Dihydrorhodomanine) 123 assay, according to the manufacturer's instructions (Sigma Aldrich Ltd), and results were compared with the Glutathione assay.

DHR 123 (Dihydrorhodomanine 123) assay

0.5×10^6 and 0.8×10^6 cells were seeded in 60 mm plates and left to adhere overnight. Cells were then exposed to 5 mJ /cm^2 (48h) and 10 mJ /cm^2 (24h) UVB and immediately treated with 20μM of either of the 5 ITCs studied, SFN, IBN, AITC, BITC, PEITC. At the indicated

timepoints cells were collected as described above and washed twice with PBS. Five minutes incubation (37 °C) followed after 10μM of DHR 123 were added to single cell suspensions (10^6 cells / mL). After the incubation, 1μM DAPI was added to the samples, to exclude dead cells from the analysis, and a further 5-minute incubation followed before the samples were injected in the FACS and acquisition run at 10,000 events.

Glutathione Assay

For the determination of ROS induction through the glutathione assay, 0.08 x 10^6, 0.1 x 10^6 and 0.15 x 10^6 cells / well were seeded in 12-well plates and left to adhere overnight. They were then pre-treated with 1mM glutathione in complete DMEM for 4 hours at 37 °C. After the incubation UVB exposure was conducted as per the optimised model (5 mJ /cm^2 for 48h and 10 mJ /cm^2 for 24h UVB) and ITC treatment followed with and without the addition of glutathione (1 mM). For this experiment the same conditions as above were used. Plates were placed in an incubator (37 ° C) until the measurements of the first timepoint (24 and 48 hours). At the timepoint, media was removed from the plates and a solution of resazurin-containing PBS (1 mg / mL) is added at volumes 100 μL – 500 μL depending on the experiment and the plates used. The plates were incubated for 4 hours at 37 ° C and the absorbance was measured at 570 nm and 600 nm using Spark multimode plate reader (Tecan, Switzerland). The percentages used were relative to control cells.

2.5 Materials and methods for the evaluation of the role of total isothiocyanates derived from watercress powder in combination with low dose UVB

2.5.1 Watercress extract preparation for use on HaCaT cells

For the preparation of the watercress extract, 0.5 g of freeze-dried watercress powder were diluted in 10 mL of milli-Q water. After the mix was thoroughly homogenised, it was incubated at 37 °C for 2h in a shaking water bath, in order to provide the necessary environment for the glucosinolates and myrosinase to hydrolyze and form isothiocyanates. After the incubation period, the mix was centrifuged (4000 rpm for 10 minutes) twice and the supernatant was filtered using a 0.22 µm filter, in a sterile environment. For the calculations of the amount of watercress extract that is required to achieve a final concentration of 20 µM of total ITCs, the findings from chapter 2 were used. 559 µL of watercress extract were diluted in 8 mL of complete DMEM and the mix was thoroughly vortexed. The treatment mix was always prepared fresh to minimize denaturation of ITCs in the aqueous solution and kept in the hood for no more than 30 minutes before its use on cells.

2.5.2 Growth determination of combinational treatments of HaCaT cells with watercress extract and UVB.

1.5 x 10^5 cells / well were seeded in 12-well plates and left to adhere overnight. They were then washed with PBS w/o Mg^{2+}, w/o Ca^{2+} following the removal of the media. 500 µL of fresh PBS w/ Mg^{2+}, w/ Ca^{2+} in room temperature was added to the wells as the salts in PBS promote cell adhesion and prevent cell detachment under low temperature conditions. Plates were placed in the UVB crosslinker and exposed to 10 mJ / cm^2 UVB under ice as described in section 3.2.3. Control plates were not exposed to UVB. After the exposure, plates were immediately removed from the ice and PBS was replaced with warm DMEM containing

watercress extract. Preparation of the extract was as described in section 5.2.1. Plates were placed in the incubator (37 ° C) for 24h and the SRB assay was utilized for the determination of cell growth of the experimental model as described in section 2.4.5.

2.5.3 Apoptosis determination of combinational treatments of HaCaT cells with UVB and either watercress extract or synthetic PEITC.

8 x 10^5 cells were seeded in 60mm plates and left to adhere overnight. Cells were then exposed to 10 mJ /cm^2 UVB as described in section 3.2.3, and 20 μM of PEITC or 20 μM of total ITCs in watercress extract (final concentration), in complete DMEM were used immediately after the UVB exposure. The watercress extract was prepared as described in section 2.2.2. 24h after the treatment, apoptosis was determined using flow cytometry (FACS) and CellEvent Caspase 3/7 Green detection reagent, as described in 2.3.5.

2.5.4 Mitochondrial membrane permeability determination of combinational treatments of HaCaT cells with UVB and either watercress extract or synthetic PEITC.

8 x 10^5 cells were seeded in 60 mm plates and left to adhere overnight. Cells were then exposed to 10 mJ /cm^2 UVB as described in section 2.3.3 and 20 μM of PEITC or 20 μM of total ITCs in watercress extract (final concentration), in complete DMEM were used immediately after the UVB exposure. 24h after the treatment, cells were collected in tubes with the assistance of cell scrapers, after trypsinisation with 2 mL trypsin-EDTA [0.25% in phosphate buffer saline (PBS) w/o Mg^{2+}, w/o Ca^{2+} (1x)] for 12 minutes and washed twice with PBS. Cells were resuspended in 300 μL PBS and 0.3 μL of JC-1 stain (0.1 mg / mL) was added to the suspension. After a 30- minute incubation at 37 °C, cells were centrifuged (1000 rpm for 5 minutes) and fresh PBS was used for the resuspension of the cells. Samples were injected in the flow cytometer and data was acquired through acquisition of 10,000 cell

event for each sample. For the analysis and representation of the data, the ratio of JC-1 aggregates over JC-1 monomers was used.

2.6 Statistical Analysis

Statistical analysis of the data of this thesis was carried out using GraphPad Prism 8.

For the statistical analysis in chapter 3, Wilcoxon signed-rank test was used for the comparison of the standard curves of the individual ITCs analysed. The significance and confidence level were set at 0.05 (95% confidence interval). The distribution of the data was tested using Shapiro-Wilk normality test. One-way ordinary Anova was used for the rest of the analysis in chapter 3 and for multiple comparisons statistical hypothesis Tukey test was used. For the quantification of total ITCs in watercress, t-test was used and experiments were conducted at least three independent times. Each experiment had at least 6 replicates, whilst the number of replicates was selected based on the level of repeatability.

For the statistical analysis of the data for photo-aging model (chapter 4), one-way Anova and two-way Anova was used, and the parameters were adjusted for multiple comparisons using statistical hypothesis testing (Tukey test). The significance and confidence level were set at 0.05 (95% confidence interval). Each P value was adjusted to account for multiple comparisons.

For the psoriasis experimental model (chapter 5), two-way Anova analysis was used for apoptosis, cell cycle and JC-1 experiments, adjusting for multiple comparisons, using statistical hypothesis Tukey testing, while ROS induction data (DHR assay data) were analysed using two-way Anova, adjusting for multiple comparisons, using statistical hypothesis Dunnett testing, as suggested from the statistic guide. The significance and

confidence level were set at 0.05 (95% confidence interval). Each P value was adjusted to account for multiple comparisons.

For the statistical analysis of chapter 6, one-way anova was used and the parameters were adjusted for multiple comparisons using statistical hypothesis Tukey testing, as well as two-way Anova, using statistical hypothesis Dunnett testing. The significance and confidence level were set at 0.05 (95% confidence interval). Each P value was adjusted to account for multiple comparisons.

The selection of the statistical hypothesis testing was in accordance with the software's guide and there was no interaction detected between Tukey and Dunnett statistical hypothesis testing results.

3. A Novel Rapid Determination of Total Isothiocyanates in Watercress

3.1 Introduction

Glucosinolates (GSLs) are an important class of phytochemicals naturally found in the localized sulfur-rich cells of the *Brassicaceae* family of vegetables (Dinkova-Kostova & Kostov, 2012) which contains about 3000 species of plants (IARC, 1986) and includes important agricultural crops such as cabbage, broccoli, cauliflower, watercress and Brussels sprouts, commonly named cruciferous vegetables, and white mustard. GSLs have also been identified in other families, in the order Capparales, which is the only order that GSLs have been reported (Halkier & Gershenzon, 2006). The role of GSLs in plants has been associated with protection mechanisms against phytophagic animals, such as mammals, birds, and insects, making the compounds instantly toxic for the host and by inhibiting the development and nourishing pathways (Buskov et al., 2002; Halkier & Gershenzon, 2006; Lazzeri et al., 2004; Noret et al., 2005). Moreover, observations made on insect behaviour patterns indicate either attraction or repulsion to GSLs depending on the insect (Miles et al., 2005; Rojas, 1999), while herbivores, such as moths (*Plutella xylotella*) and flea beetles (*Phyllotreta)* probably metabolise GSL to a form not metabolised from myrosinase through cleavage of the sulfate with an endogenous sulfatase. The flying and landing behaviour of the cabbage moth (*Mamestra brassicae*) on allyl isothiocyanate (AITC), the hydrolised product of sinigrin GSL, was significantly preferred from female insects (Rojas, 1999). GSLs are the precursor of isothiocyanates (ITCs), a group of phytochemicals characterised by the functional -N=C=S group (Jimenez-garcia et al., 2018), metabolised through the mercapturic acid pathway with an initial conjugation of glucosinolates with glutathione and enzymatic degradation by myrosinase (thioglucoside glucohydrolase), when the latter

becomes available from myrosin cells, following tissue disruption. This rapid hydrolysis through the cleavage of GSL's glucose and formation of unstable aglycones is completed with the formation of isothiocyanates (figure 3.1). Sulforaphane (SFN), iberin (IBN), allyl isothiocyanate (AITC), phenethyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC) represent some of the most studied ITCs. Although cruciferous vegetables contain a variety of ITCs, most of those plants have a dominant ITC. SFN is widely known to be found in broccoli and broccoli sprouts (Clarke et al., 2008), while erucin (ECN) is also found in these vegetables, as well as rocket, kohl rabi, turnip and rutabaga (Vermeulen, 2009). AITC is the dominant ITC in cabbage, horseradish, mustard, wasabi and kale, BITC in garden cress and Indian cress, PEITC in watercress and horseradish, while methyl isothiocyanate (MEITC), a less known ITC is dominant in cauliflower and capers (table 3.1) (Fung Lung Chung et al., 2000; Sávio et al., 2015; Wu et al., 2009). The carbon center of ITCs is highly electrophilic that makes them react with nucleophiles, such as thiol, amino and hydroxyl groups, that give dithiocarbamates, thioureas and *O*-thiocarbamates, respectively (Kaschula & Hunter, 2016). Although ITCs have a similar structure, the side group of each phytochemical determines their electrophilicity and hydrophobicity, with PEITC being more hydrophobic when compared to SFN, making it react with cysteines in hydrophobic crevice of proteins (Kaschula & Hunter, 2016; Y. Zhang et al., 1996).

Isothiocyanates have exhibited strong anti-carcinogenic activity via modulation of key carcinogenesis regulators on rat models (Fung Lung Chung et al., 2000; Fahey et al., 2002; Gerhäuser et al., 1997; Hu et al., 2006; Sugie et al., 1994; Y. Zhang et al., 1994). SFN induced phase 2 enzymes and blocked chemical carcinogenesis (Dinkova-Kostova et al., 2007; Y. Zhang et al., 1994), inhibited the effect of UVB on human dermal fibroblasts (S. Y. Lee & Moon, 2012) and reduced erythema/inflammation and inflammation markers on UVR exposed rat and human skin (Talalay et al., 2007). BITC inhibited tumor induction in intestinal tract in rats and decreased colon tumors (Sugie et al., 1994) while PEITC reduced

p65 levels and inhibited nuclear factor-kappa B (NF-κB) activation on human prostate cancer PC-3 cells (Xu et al., 2005). Modulation of Nuclear factor erythroid 2-related factor 2 (Nrf2) response (Benedict et al., 2012; Guerrero-Beltrán, Calderón-Oliver, et al., 2012), reduction in cytokines, such as tumor necrosis factor (TNF-α), Cyclooxygenase (COX-2), interleukin 6 (IL-6), interleukin 1β (IL-1β) and iNOS, reducing the signaling pathways of NF-κB and decreasing p65 translocation (Prawan et al., 2009; Srivastava & Singh, 2004; Xu et al., 2005) are amongst demonstrated biological effects of ITCs. Their electrophilic and nucleophilic character give a variable role on ROS, with high concentrations (> 10 μM) of ITCs increasing ROS generation through either reduction of glutathione (GSH), direct generation of intracellular ROS or mitochondrial leakage of ROS through mitochondrial protein binding (Pocasap et al., 2018; Sestili & Fimognari, 2015) and low concentrations reacting with other proteins, such as cysteines, to decrease ROS through the induction of Nrf2 (Keap1) (Dinkova-Kostova et al., 2002).

Table 3. 1 *ITC profile of cruciferous vegetables.*

Dominant ITC	Cruciferous vegetables	Reference
AITC	Cabbage, horseradish, mustard, wasabi, kale	(Sávio et al., 2015; Vermeulen, 2009; Wu et al., 2009)
BITC	Garden cress, Indian cress	(Vermeulen, 2009)
ECN	Rocket, broccoli, arugula, kohl rabi, rutabaga, turnip	(Vermeulen, 2009)
PEITC	Watercress, horseradish	(Fung Lung Chung et al., 2000; Peter Rose, Yen, et al., 2005; Vermeulen, 2009)
SFN	Broccoli, broccoli sprouts	(Clarke et al., 2008)
MEITC	Cauliflower, capers	(Vermeulen, 2009)

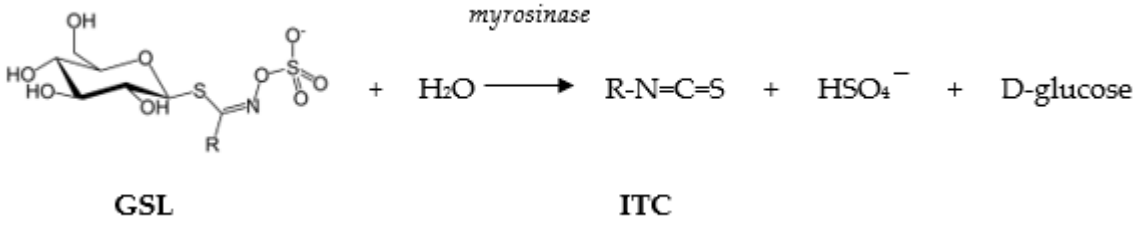


Figure 3. 1 *The hydrolysis of glucosinolates and the formation of isothiocyanates by myrosinase*

Y. Zhang (1992) was the first to develop a methodology for the quantification of isothiocyanates in cruciferous vegetables via a cyclocondensation assay and subsequent analysis using High Performance Liquid Chromatography (HPLC) and spectrophotometry (Y. Zhang et al., 1992, 1996) (table 3.2). The cyclocondensation assay is based on the quantitative reaction of the isothiocyanate's carbon atom (R–N=C=S) with the sulfydryl group of 1-2 benzene dithiol, leading to the formation of a five membered 1,3-dithiole-2-thione and the amine R-NH_2 (Li Tang et al., 2013) (figure 3.2). The absorbance of the main end-product (1,3-dithiole-2-thione) is measured at λ_{max} 365 nm and α_m 23,000 M^{-1} cm^{-1}. The methodology has been widely used in the detection and quantification of isothiocyanates, and modified accordingly to accommodate a wide range of samples (M. M. F. Choi et al., 2004; Song et al., 2005; Ye et al., 2002b). The limitations of this method is on the detection of ITCs that have a tertiary linking carbon of the R group which have an incomplete reaction (Bertelli et al., 1998; M. M. F. Choi et al., 2004; Jiao et al., 1998; Rodrigues et al., 2016; T. A. Shapiro et al., 2001; Theresa A Shapiro et al., 1998; Song et al., 2005; Li Tang et al., 2013; Ye et al., 2002a). Sample preparation procedures for the analysis of total ITC in cruciferous vegetables include use of organic solvents, such as acetonitrile (Theresa A Shapiro et al., 1998; Y. Zhang et al., 1992), ethyl acetate (Ye et al., 2002b), methylene chloride (Bertelli et al., 1998), hexane (Rodrigues et al., 2016) and mix of solvents (T A

Shapiro et al., 2001) followed by sample purification and HPLC determination, whilst for the analysis of blood and urine samples a more complex preparation that includes multiple levels of purification is required (Conaway et al., 2000; T A Shapiro et al., 2001; Ye et al., 2002b). However, even plant samples preparation and extraction process differ between studies depending on the nature of the samples and method of analysis. Extraction of ITC was also performed by supercritical CO_2 (Rodrigues et al., 2016) and solid phase extraction (SPE) (Pilipczuk et al., 2017), techniques that are time consuming and expensive. Simpler methods with water extractions (M. M. F. Choi et al., 2004; Fusari et al., 2015; Jiao et al., 1998; Prestera et al., 1996) have also been reported while ultrasound assisted techniques (UAE) have been used by various researchers (Fusari et al., 2015; Soares Melecchi et al., 2006; T. Wang et al., 2011).

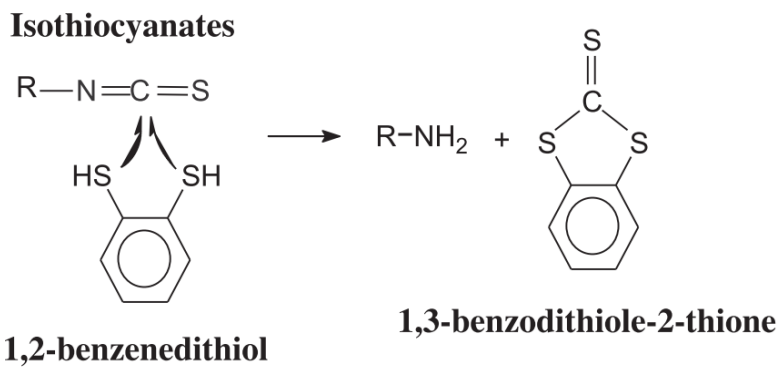


Figure 3. 2 *Cyclocondensation assay reaction of isothiocyanates with 1,2-benzenedithiol leading to the formation of 1,3-benzodithiole-2-thione.*
Picture taken from Tang et, al. 2013 (Tang et al., 2013).

The most common analytical method for the qualitative and quantitative characterization and determination of ITCs is HPLC (Fusari et al., 2015; Pilipczuk et al., 2017; Rodrigues et al., 2016; Theresa A Shapiro et al., 1998; Y. Zhang et al., 1996), whilst gas chromatography – mass spectrometry (GC-MS) has also been used (Bertelli et al., 1998; C. Chen et al., 1998;

M. M. F. Choi et al., 2004). However, these methods of analysis require expensive equipment and skilled personnel for the operation, analysis and maintenance. On the other hand, spectrophotometric analysis, following a cyclocondensation assay, is a simpler and faster analytical method that does not require expensive equipment and maintenance and is more suitable for rapid quality control tests at the site of manufacturing/primary production and when there is no requirement for detailed characterization of samples.

Thus, the purpose of this investigation was to develop rapid and easy sample preparation and analytical procedures for the quantification of isothiocyanates in cruciferous vegetables that do not require expensive equipment and expertise. For the preparation of the samples, a simple water/methanol extraction process was employed using sonication, followed by the cyclocondensation assay and spectrophotometric analysis.

Table 3. 2 *Total isothiocyanate content and main glucosinolate/isothiocyanate in the most common cruciferous vegetables.*

Common Vegetables	**ITC content (μmol / 100 g)**	**GLS/ITC**	**Extraction method/solvent**	**Method of Analysis**	**References**
Mustard greens	61.3 (0.4 - 137.9)	Sinigrin / AITC	Water extraction	HPLC	(Tang et al., 2013; Verkerk et al., 2009)
Broccoli	4.9 (2.0 – 7.5)	Glucoraphanin / SFN	Water and triple solvent extractions: DMSO, dimethylformamide, and acetonitrile	HPLC	(Jiao et al., 1998; Shapiro et al., 1998)
Brussel Sprouts	6.9 (2.6 – 18.1)	Glucoraphanin / SFN	Water extraction	HPLC	(Tang et al., 2013)
Cabbage	31.7 (0.5 – 77.9)	Glucotropaeolin / BITC	Water extraction	HPLC	(Tang et al., 2013)
Cauliflower	1.5 (0.7 – 2.7)	Sinigrin / AITC	Water extraction	HPLC	(Tang et al., 2013)
Kale	3.7 (0.4 – 12.9)	Glucoraphanin / SFN	Water extraction	N/A	(Tang et al., 2013)
Lesquerella	1.38 ± 0.07	Glucoiberin / IBN	Hexane	GC - MS	(Vaughn & Berhow, 2005)

3.2 Results and discussion

3.2.1 Plant preparation and matrix interference using dried watercress

For the determination of ITCs in plant tissues, as well as blood and urine, commonly extraction methods utilize organic solvents, such as acetonitrile (Franco et al., 2016) and methylene chloride, whilst combinations of solvents have also been used. Extraction methodologies reported in the literature for the determination of the Total ITC content in plants as well as blood and urine samples as shown in table 3.3. Aqueous extraction of isothiocyanates was chosen in this study as a more environmentally friendly extraction protocol, thus minimizing the use of organic solvents. Due to the hydrophilic nature of glucosinolates (Kaschula & Hunter, 2016; Y. Zhang et al., 1996), aqueous extractions are commonly used and complemented by simple clarification steps such as cloth filtration and centrifugation of the plant samples. Choi et al. (2004) followed 3 stages of dilution of 10g plant sample with distilled water, filtering the water samples with cloth, before passing 10 mL of the final dilution through a 0.45μm filter. A similar preparation was followed by Conaway et al. (2000) and Jiao et al. (1998), while Fusari et al. (2015) centrifuged and filtered the samples after the cyclocondensation assay took place. For an optimum ITC extraction from watercress samples, disruption of the plant cell walls through sonication was employed for our experiments and whilst limited applications (Fusari et al., 2015; T. Wang et al., 2011) for the extraction of ITC from plants have been reported, this method is widely used for extractions and improvement of enzymatic reactions (Bhat & Goh, 2017; Millward-Sadler et al., 1996; Şener et al., 2006; Soares Melecchi et al., 2006; T. Wang et al., 2011) while ultrasounds have been used for the extraction of the GLS sinigrin from mustard seeds (T. Wang et al., 2011). Fusari et al. (2015) ultrasonicated the homogenised samples, before they hydrolysis stage using a sonication bath, while Wang et al. (2011) used an ultrasonic cell pulverizer, where a cylindrical probe was introduced to the sample for 5 minutes. In most applications mentioned above the temperature of the sonication process was not

controlled, however, the addition of ice in the sonication bath was necessary in order to prevent myrosinase inactivation due to increased temperature locally (Özbek & Ülgen, 2000).

Interferences by matrix components during the extraction of ITCs such as metals and macromolecules, such as proteins and fibre, was investigated by the addition of inulin (soluble fibre) or cellulose (insoluble fibre) as well as the use of EDTA and methanol in the extraction media. Moreover, as the resulting ITCs are hydrophobic in nature, interferences from plastics were investigated by performing the extraction process using glassware instead of plasticware. A simple pre-clarification step including the addition of methanol and centrifugation was also introduced, prior to the cyclocondensation assay to allow for the removal of proteins and other macromolecules present in the crude aqueous extracts. This step significantly improved the reliability of the method by reducing the coefficient of variation of analytical results from 23.6% to 14% (n = 6). The cyclocondensation assay is not limited on the detection of isothiocyanates but also reacts with dithiocarbamates, xanthanes and thiourea by-products (Y. Zhang et al., 1996) as well as urine metabolites of allyl isothiocyanate, N-acetyl-S—cysteine (Bhattacharya et al., 2012; Ye et al., 2002b), thus necessitating some form of sample pre-treatment before analysis.

Table 3. 3 *Extraction and analytical platforms used in the determination of ITCs in plant and other biological tissue.*

Sample origin	Extraction method	Method of analysis	Reference
Broccoli	Solvent: methylene chloride, SPE	GC-MS, HPLC	(Bertelli et al., 1998)
Various	Solvent: water	GC-MS	(M. M. F. Choi et al., 2004)
AITC, PEITC	Solvent: methylene chloride	GC-MS, GC, HPLC, LC-MS	(C. Chen et al., 1998)
Broccoli, human	Solvent: water	HPLC	(Conaway et al., 2000)

blood and urine			
Mouse and human skin	Solvent: ethyl acetate	HPLC	(Dinkova-Kostova et al., 2007)
Various	Solvent: ethanol, Teflon vessels with MARS system	HPLC-ES-MS/MS	(Franco et al., 2016)
Various	Solvent: water	HPLC	(Fusari et al., 2015)
Various	Solvent: water	HPLC	(Jiao et al., 1998)
Various	NAC, SPE	HPLC DAD MS	(Pilipczuk et al., 2017)
Watercress	Solvent: ethanol and supercritical CO_2, hexane	Ultraviolet spectrophotometry, HPLC, GC-MS	(Rodrigues et al., 2016)
Broccoli and human urine	Triple solvent: DMSO, dimethylformamide, and acetonitrile	Paired-Ion Chromatography	(T A Shapiro et al., 2001)
Various	Solvent: water	HPLC	(Li Tang et al., 2013)
Dried roots of *A. rusticana*	Solvents: cooking oil, petroleum, dichloromethane, diethyl ether and ethyl acetate, supercritical CO_2	N/A	(Wu et al., 2009)
Human blood and urine	Filtrations and water	HPLC	(Ye et al., 2002b)
Broccoli	acetonitrile	Ultraviolet spectrophotometry, HPLC	(Y. Zhang et al., 1992)
Broccoli	Solvent: water	NMR spectroscopy, Paired-Ion Chromatography, MS	(Prestera et al., 1996)
Various	Triple solvent: DMSO, dimethylformamide, and acetonitrile	HPLC	(Theresa A Shapiro et al., 1998)

Addition of small amounts of methanol coupled with a high centrifugal force centrifugation step assisted the purification process and enabled the more precise determination of ITCs. The necessity of this step was determined after the turbidity of the samples was gradually increasing during the cyclocondensation assay. Many variations of intermediate filtrations

before and after the cyclocondensation assay were tested, however, the precipitation was an insisting issue. There was no fibre interference detected when spiked samples of inulin (soluble) and cellulose (insoluble) solutions were passed through the methodology nor heavy metal interference detected after the addition of EDTA to watercress samples (Appendix C). Interestingly, when plastic (polypropylene) equipment was removed from the method, there was an approximately 35% increase in total ITC detection.

The effect of plastic equipment and consumables was investigated further. For the evaluation of potential interaction between polypropylene and isothiocyanates during aqueous extractions, the samples were processed as described earlier, while half of the sample were prepared, using polypropylene tubes during the initial preparation of the samples and incubation time and the rest of the samples were prepared using all glass equipment. Using all glassware had a significant impact on both the recovery of the analytes as well as the precision of the measurement with plasticware method detecting 0.99 ± 0.14 μmol / g total ITC content and glassware method detecting 1.54 ± 0.04 μmol / g total ITC content, a 35% increase in the recovery of ITCs and 7-fold decrease in the corresponding coefficient of variation (from 14% to 2%).

Moreover, citrate phosphate buffer was used in place of water during the first step of the methodology in order to investigate potential improvement on ITC recovery and detection and further response optimisation, as described from the literature (Q. Guo et al., 2013; Luang-In & Rossiter, 2015). Although, there was a slight elevation of total ITC content detected in samples containing the citrate phosphate buffer, the difference between them and the samples prepared with water was not significant ($p = 0.7518$).

3.2.2 Determination of linearity and limits of detection

The response factors from the reference standard curves of five ITCs (SFN, IBN, AITC, PEITC, BITC) (1.5 - 92 µmol/mL) ranged from 0.028 for AITC to 0.035 for BITC (figure 3.3) indicating limited variation in the reactivity of the ITCs studied and/or molar absorptivity of the 1,3-benzodithiole-2-thione derivatives of the different ITCs. The limits of detection (LOD) for PEITC were 0.7 µmol / mL (final concentration), RSD 9.4%, limit of quantification (LOQ) 1.5 µmol / mL (final concentration) and an actual confidence level 98.4%. The comparison and confidence level of the standard curves were evaluated using Wilcoxon test, whilst all ITCs passed the normality Shapiro-wilk test (alpha = 0.05).

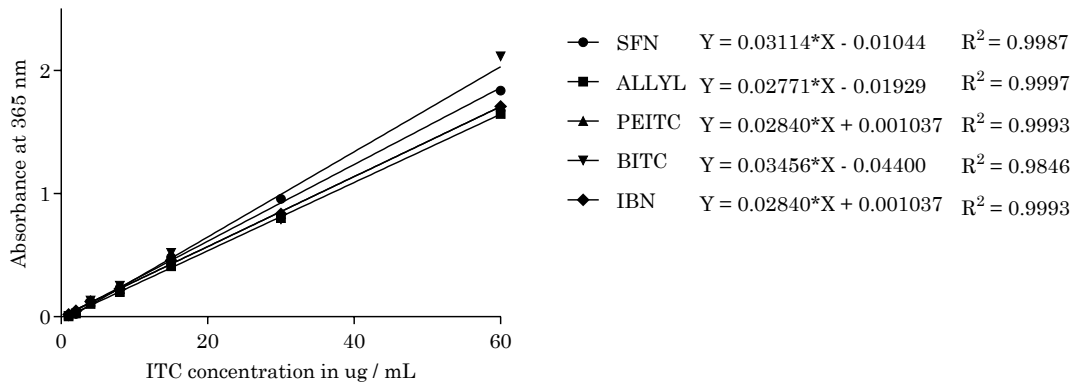


Figure 3. 3 *Standard curves of ITCs.*

Sulforaphane (SFN) iberin (IBN), allyl isothiocyanate (AITC), phenethyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC) as determined by cyclocondensation assay and absorbance measurements at 365 nm. Wilcoxon signed-rank test was used for the comparison of the standard curves of the individual ITCs analysed. The significance and confidence level were set at 0.05 (95% confidence interval). The distribution of the data was tested using Shapiro-Wilk normality test. Actual confidence level 98.4%. Data shown are representative of three independent experiments.

The spectrophotometric LOD according to Zhang (1992) (Y. Zhang et al., 1992) was as low as 1 nmol / mL, while typical standard curves were constructed at concentrations of 1.25 –

40 nmol / mL phenyl isothiocyanate, in a 2 mL reaction volume. In this present study linearity was observed at concentrations of 0.25 - 15 μmol/mL (final concentration) corresponding to 0.25 - 15 mg/g dry matter (figure 3.3). In contrast with the methods described in the literature (Y. Zhang et al., 1992) where phenyl-NCS and propyl-NCS (artificial ITCs) were used for the standard curve, in this study the choice of ITCs was based on their biological effects and bioavailability and they are naturally found in cruciferous vegetables. The phytochemical used for the typical standard curve of our plant experiments is PEITC based on its bioavailability in watercress, the plant studied which its total GSL content consists mostly of gluconasturtiin, converting to PEITC when hydrolysed (F. L. Chung et al., 1992). However, as shown in figure 3.3, five ITC of three different chemical structures, respond similarly using the cyclocondensation assay, with an actual confidence level of 98.4%. Therefore, it is shown that the standard curve of any of the five ITCs can be used for the detection of total ITC content in vegetables, through the assay. This is because 1,2 benzene dithiol reacts quantitively with the N=C=S group of the ITC giving the end product 1,3-dithiole-2-thione that absorbs ultraviolet light at 365 nm.

The recovery of the developed methodology (glassware) (figure 3.4) was determined by spiking of watercress samples with 0.44 mg of synthetic PEITC and analysed as described earlier, giving a recovery of 88.95% (figure 3.5).

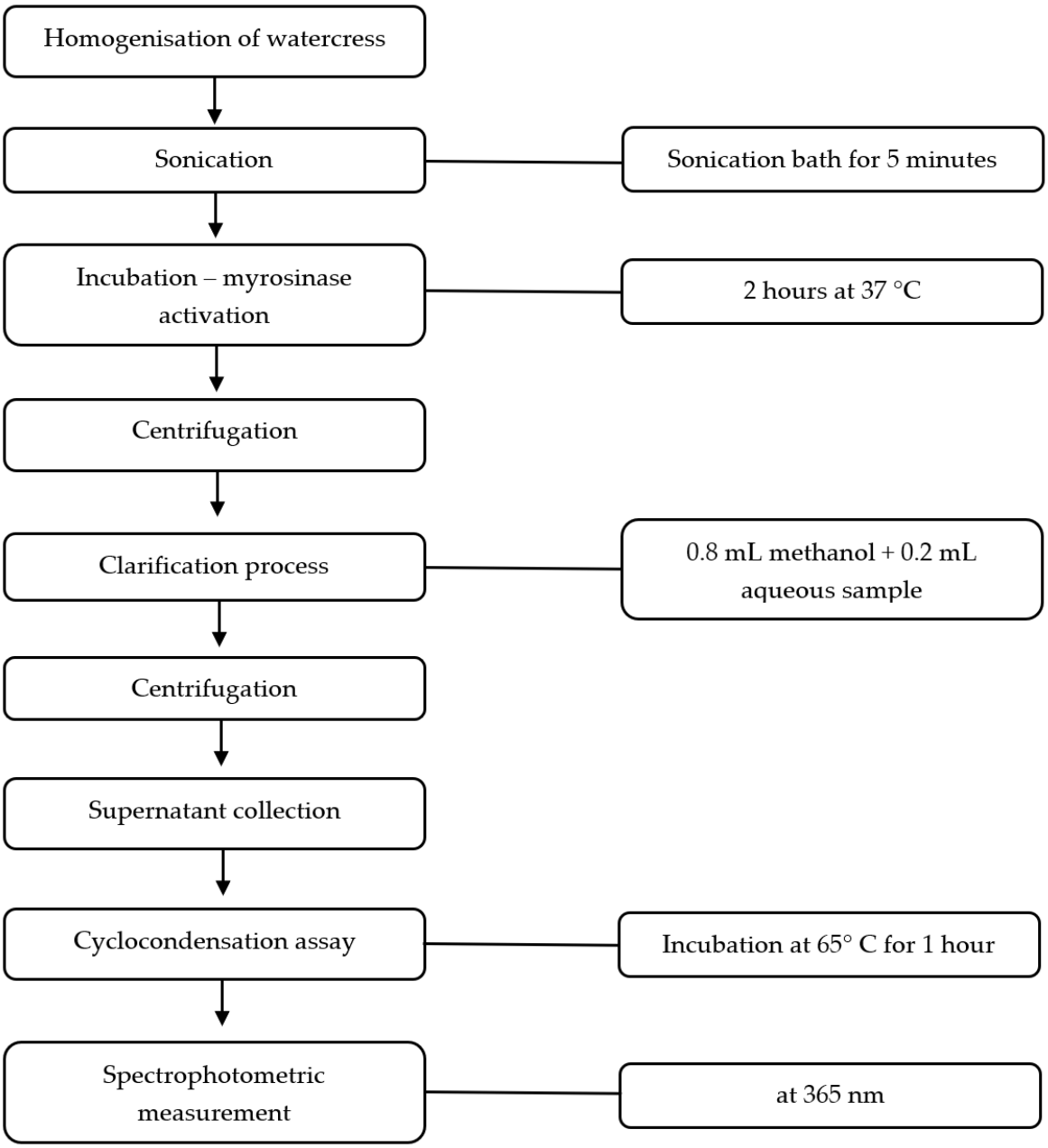


Figure 3. 4 *Schematic representation of the developed methodology for the determination of total ITC content in cruciferous vegetables.*

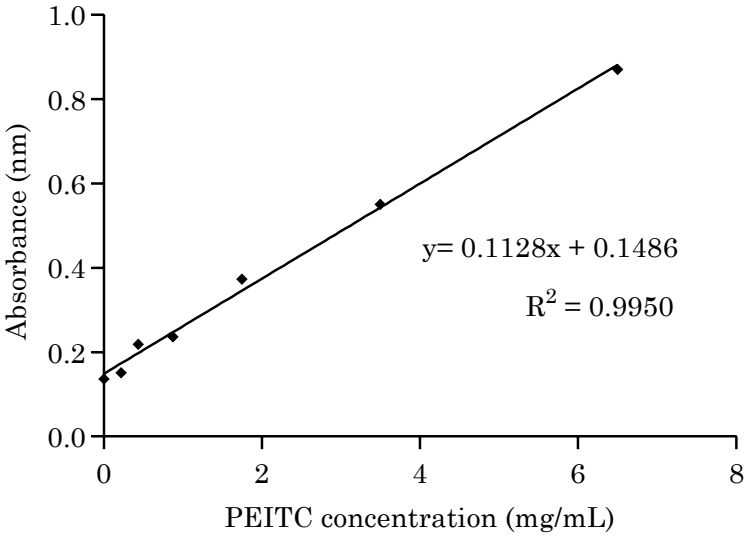


Figure 3. 5 *PEITC standard addition curve in watercress samples.*
Samples were spiked with 0.44 mg of PEITC with a recovery of 88.95%. Graph was created by linear regression analysis of data.

3.2.3 Comparison of novel aqueous extraction method with organic solvent extraction using dried and fresh watercress

The novel, rapid methodology was compared against the methodology suggested by Rodrigues (2016) with minor alterations, as described earlier using both powdered and fresh watercress (Rodrigues et al., 2016). The two methods resulted in almost identical total ITC values, for both powdered and fresh watercress. In the first instance detecting 1.54 ± 0.04 μmol / g versus 1.48 ± 0.04 μmol / g of total ITC content using the modified method of Rodrigues (2016), while much higher and similar content between the two methods was determined in fresh watercress. The discrepancy is attributed to the possible destruction of myrosinase during drying and / or storage or to possible loss of ITCs during the same processes. Freeze-dried watercress contained only 7.5% of total ITCs compared to the fresh watercress samples (20.6 ± 0.31 μmol / g) as determined by the aqueous extraction protocol, while similar results were obtained when the modified extraction process using hexane was

employed confirming our findings (19.7 ± 0.39 μmol / g) (table 3.4). To further investigate the hypothesis of destruction of myrosinase during drying and / or storage, exogenous myrosinase was added to both dried and fresh watercress. The addition of enzyme (1 mL of exogenous myrosinase at 2 mg / mL in 0.1 M potassium phosphate buffer, pH 6.6) (Getahun & Chung, 1999; Jiao et al., 1998) showed a 10-times increase in the total ITC content detection when using dried watercress, with a concentration of 10.05 μmol / g, whereas the addition of the enzyme in fresh watercress did not show a significant difference ($p > 0.05$) when compared with samples before the enzyme addition. The moisture content of the fresh watercress was found to be 92.74%.

Table 3. 4 *Total ITCs in freeze-dried and fresh watercress using the novel aqueous extraction and the modified organic solvent method by Rodrigues (2016)*

Method	Samples	Total ITC content detected (μmol / g)	Total ITC content in watercress found in the literature (μmol/g)
Aqueous extraction	Freeze-dried watercress	1.54 ± 0.038	25.1 ± 4.2 (Rodrigues et al., 2016)
Modified from Rodrigues (2016)	Freeze-dried watercress	1.48 ± 0.035	17.98 ± 4.31 (Gill et al., 2007)
Aqueous Extraction	Fresh watercress	20.6 ± 0.31	5.7 ± 0.7 (Rodrigues et al., 2016)
Modified from Rodrigues (2016)	Fresh watercress	19.7 ± 0.39	

[1]Novel methodology using methanol clarification step; [2] Hydration and hexane extraction modified from Rodrigues (2016) (Rodrigues et al., 2016).

Data were processed using t-test and are means of 6 replicates.

Due to the hydrophobic nature of isothiocyanates it is hypothesised that during the first incubation period (hydrolysis of GLS), part of the ITC content was attached to the pulp and/or walls of the plastic tubes used. Therefore, the methodology was followed using only glass equipment. The total ITC content was determined spectrophotometrically following the cyclocondensation assay. Ultraviolet spectrophotometry is a less complicated and less time-consuming method compared to chromatography and other advanced methods of analysis, although it has limitations on the characterisation of the ITCs contained in the samples. Moreover, it does not require expensive equipment and / or consumables, while the training for the operation of the equipment is comparatively easier. Therefore, the choice of the method of analysis for the suggested methodology, makes the process approachable for industrial use. For the spectrophotometric measurements, plastic disposable cuvettes (1 cm) were used. The watercress powder that was used, contained 1.54 ± 0.038 μmol / g total ITC, amount that is significantly lower than the total ITC content found on the literature (Gill et al., 2007). Myrosinase could have been either lost or inactivated during processing and/or storage. Most researchers have followed a myrosinase deactivation step and introduction of additional myrosinase to initiate their extraction processes. Although this study has assessed the effect of the addition of exogenous myrosinase, for the further use of this methodology it was decided to not include the addition of enzyme, in order to provide a method that can quantify total ITCs in crude samples with no further additions and to determine the natural potential nutritional value of the vegetable as consumed. Although it is found that intestinal microflora produces small amounts of myrosinase, the activity can vary from each individual (Angelino et al., 2015). In a recent study, where the sensitivity of mysorinase in stomach acids was evaluated by the supplementation of glucoraphanin along with additional enzyme, a 28% bioavailability increase of the formed ITC was observed, when coated and uncoated capsules where supplemented (Fahey et al., 2019). This suggests an effect of the stomach acids to myrosinase and to the overall bioavailability of ITC after consumption.

Possible experimental errors were dismissed by validating the results obtained using a modified reference protocol, (hexane) extraction process, as described previously by Rodrigues (2016) (Rodrigues et al., 2016). The comparative method detected similar amounts of total ITC (1.48 ± 0.035 μmol / g) and it confirms the accuracy of the suggested extraction process. In order to investigate the low content of ITCs in the freeze-dried watercress powder, both methodologies were repeated using fresh watercress. The significantly higher amounts of phytochemicals found in the plant (20.6 ± 0.31 μmol / g with novel methodology and 19.7 ± 0.39 μmol / g with the hexane extraction), as well as the myrosinase addition experiments, confirms the earlier hypothesis that myrosinase is being inactivated or lost during freeze-drying process. Comparing the data collected from the two methods, it is shown that the novel methodology benefits compared to the hexane method on the detection of total ITCs. Moreover, the organic solvent use in the former method is eliminated and it is less expensive, friendly to the environment and less time consuming.

3.3 Conclusions

A low cost, green, reliable and accurate methodology to determine the ITC content in cruciferous vegetables is of use to both academic research as well as for the industry since such compounds have been widely studied for their beneficial properties on human health and disease. The proposed aqueous methodology suggested in this study provides a rapid methodology for the detection of total ITC content in watercress with low RSD (2%), similar to other methodologies utilizing more complex processes, and the method was validated with hexane extraction as described from Rodrigues et al 2016. Moreover, the total ITC found in fresh watercress agrees with the literature and the content found in freeze-dried watercress when myrosinase is added. In contrast, very low quantities of ITCs were detected in the freeze-dried watercress powder, suggesting a potential inactivation of naturally occurring myrosinase during the freeze-drying process. Various ITCs reference standards tested

demonstrated little variance in reactivity and/or molar absorptivity during the cyclocondensation assay making it suitable for the determination of the Total ITC content in various plant tissues of different origin and of different ITC profile.

4. The effect of isothiocyanates on a photo-aging experimental model

4.1 Introduction

The skin is the largest organ on the human body and it is the first protective barrier that protects us from external factors, such as extreme temperature changes, harmful chemicals and pathogens. It has various mechanisms and levels of defense, such as homeostasis for temperature control as well as an acidic environment for microbiological defense, while it hosts beneficial microorganisms that maintain that pH balance of the external layer of the skin and prevent pathogens to colonise and enter the body (Y. Wang et al., 2014). The skin consists of three main layers, the epidermis, the dermis and the hypodermis, while there is a department, called the dermoepidermal junction found between the epidermis and dermis (section 1.1, figure 1.1, 4.1) (Lorencini et al., 2014; Tobin, 2017).

The epidermis is the first layer of defense of the skin and it consists mainly of keratinocytes (section 1.1.1.2). The main role of those cells is to protect the skin from pathogens and other extrinsic factors, such as UVR, chemicals and extreme temperature changes. Melanocytes are also found on the epidermis and are responsible for the production of vitamin D when activated from UVB exposure. Other cells that form part of the epidermis, are immune cells that respond when inflammation is triggered, such as Langerhans cells, as well as epithelial cells (Merkel) (L. Guo, 2009). As mentioned above, the epidermis is also hosting a variety of normal flora that are part of the defense mechanisms of the skin and work against pathogens through competitive exclusion. In the main functions and roles of epidermis is the production of keratin that provides the skin layer with strength, durability, and elasticity through the bonds between keratinocytes that is supported by desmosomes (section 1.1.1). Between the epidermis and the dermis, a dermoepidermal junction makes the organ more

resistant on damages and tears through bonding of the epidermal basal cells with the basement layer by collagen, hemidesmosomes and other molecules (section 1.1.2). It also consists of molecules, such as various types of collagen , that contribute to hold the two layers (epidermis and dermis) together (Fenner & Clark, 2016). This layer is attached to dermis, the second layer of the skin, which consists mainly of fibroblasts that produce collagen, elastin and other proteins, such as MMPs and maintain the balance of this network. MMPs are found in the dermis matrix maintaining healthy levels of collagen by degrading the latter when in excess, whilst TIMPs are also found in the matrix. Finally, the hypodermis is the subcutaneous tissue that is mainly used for fat storage consisting of subcutaneous fat, along with blood and lymphatic vessels. This layer also provides protection of the organs through cushioning.

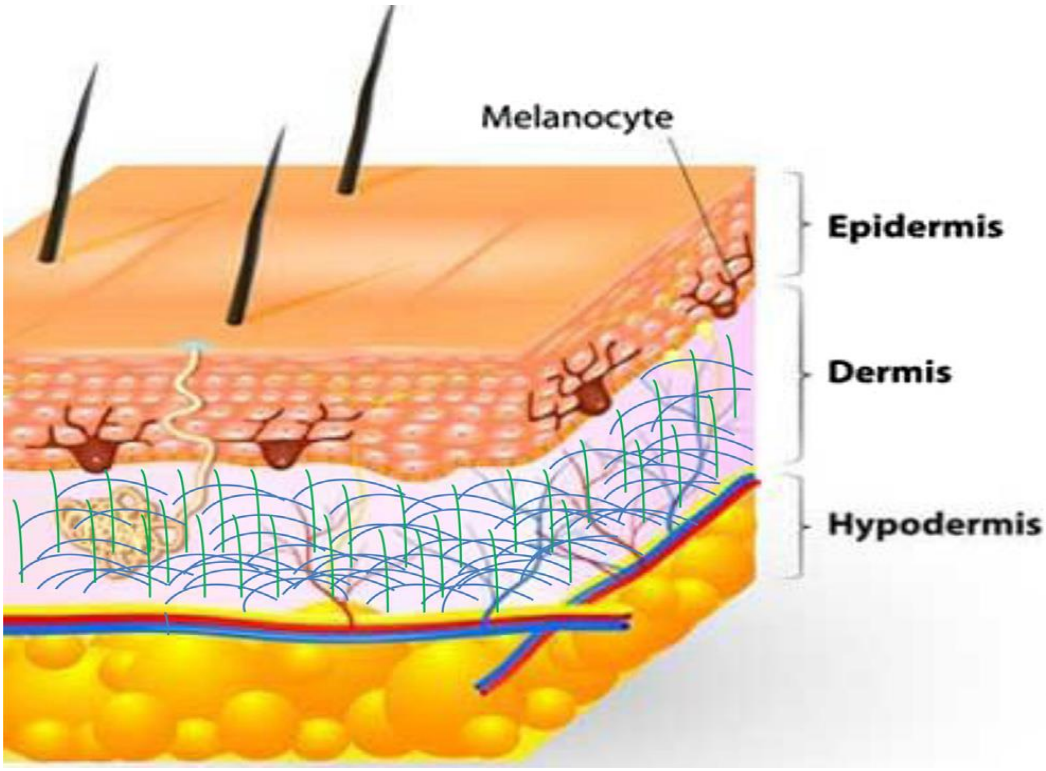


Figure 4. 1 *The layers of the skin.*
The skin consists of 3 main layers, the epidermis (top layer), the dermis and the hypodermis (Lephart, 2016).

Photo-aging is a skin condition that is characterised by early aging of the skin through extensive and long-term exposure of the organ to UV and its degeneration depends on the intensity and length of exposure as well as the skin pigmentation that provides protection from UVR (Kammeyer & Luiten, 2015; Watson et al., 2013). UV irradiation that reaches the skin is UVA and UVB, with the former penetrating the first two layers of the skin with UVB only reaching the epidermis activating melanocytes and causing erythema and malignancies *via* excess Deoxyribonucleic acid (DNA) damage. Although, UVA is mainly responsible for the photo-aging of the skin, both UVRs are known to cause photo-damage on the organ resulting in various harmful effects such as sunburn, or disease, such as malignant melanoma. The visual characteristics of photo-aging differ from the natural aging of the skin and are shown as deep wrinkles of the skin (other than expression lines), overall breakage of the surface of the skin, loss of elasticity and thinner skin. The structural molecular changes that are observed in a photo-aged skin include loose elastin network, reduced collagen production and breakage of the collagen network (Fenner & Clark, 2016).

Certain proteins of the dermis, such as collagen, elastin and MMPs, have a synergetic role in maintaining a healthy skin and they are shown to be altered on a photo-aged subject and can be considered as biomarkers of this condition (Jariashvili et al., 2012; Ravelojaona et al., 2008). Collagen, elastin and MMPs are the main targets of investigation when it comes to photo-aging. Collagen type I and III are the main collagen molecules that are produced from the fibroblasts, and their expression is shown to be reduced by UVR (Ravelojaona et al., 2008). MMP1 and MMP3 expression is shown to increase, resulting in further denaturation of collagen proteins and reduction of their overall availability in the dermis matrix (S. Y. Lee & Moon, 2012). Photo-aged skin is also characterised by increased production of ROS and oxidative stress (Kammeyer & Luiten, 2015; Lephart, 2016).

Various cosmetic products, such as creams and masks, claim to have a preventive role on photo-aging, with one type of chemical sun protecting product (sunscreen) shown to protect from the damage caused from UV. Sunscreen creates a protective layer on the skin and when it is applied in sufficient quantities, it absorbs UV light and transforms it in to heat that is then reflected away from the body (Gasparro et al., 1998). Natural compounds such as caffeoylquinic acid (cQa) (Oh, Karadeniz, et al., 2019) and other antioxidant (Pandel et al., 2013) as well as natural products, such as propolis, (Bolfa et al., 2013) and probiotics, such as Lactobacillus (H. M. Kim et al., 2014; Lolou & Panayiotidis, 2019), have also been studied for their potential protective and preventive role on photo-damage and eventually photo-aging. A widely studied naturally found group of phytochemicals are isothiocyanates (ITCs) which are shown to have an anti-cancer (Fung Lung Chung et al., 2000; Dinkova-Kostova et al., 2006; Gerhäuser et al., 1997), antioxidant (Benedict et al., 2012; Brown & Hampton, 2011) and photo-protective effect (Shibata et al., 2010; Sikdar et al., 2016). ITCs are generally known to have a variable effect depending on the concentration and the type of cell line or tissue. It is shown that low concentrations (<10μM) of ITCs do not have an effect on human immortal keratinocyte (HaCaT) cells or other cell lines, such as A375 skin cancer cells, whilst higher concentrations of the mentioned phytochemicals show an antioxidant and inhibiting role of anti-apoptotic mechanisms through suppression of NF-κB and stimulation of phase 2 enzymes (Mantso et al., 2016; Mitsiogianni, Mantso, et al., 2020). ITCs have been shown to reduce inflammation though the activation of antioxidants, such as IL-6 and COX2, induce apoptotic pathways through the caspase 3 activation and inhibitions of NF-kB and reduce ROS and oxidative stress (Sikdar et al., 2016; Talalay et al., 2007; Y. Zhang et al., 2005; Zhu et al., 2004). Moreover, SFN's antioxidant activity is shown through activation of transcriptional factor AP-1 and inhibition of NF-κB on HaCaT cells, and therefore maintaining collagen levels and providing an anti-wrinkle effect (Sikdar et al., 2016). Through the inhibition of NF-κB pathway, the same phytochemical inhibits

MMP-1 and MMP-3, proteins that degrade collagen proteins in the extracellular matrix (section 1.1.3.4). PEITC is also known to be a strong antioxidant compound and as being the main ITC in watercress, both pure PEITC and plant extracts have been studied on their antioxidant and chemo preventive effects, while the most effective concentration are between 0.12μM to 14μM (Gupta et al., 2014). The phytochemical inhibited anti-apoptotic pathways on various clinical trials and models through inhibition of MMP-9 action in breast cancer cells, in doses of 0.1 to 1 mg / mL of plant extract, (Peter Rose, Huang, et al., 2005), oxidative stress-induced DNA damage inhibition and delay of cell cycle of colon cancer cells at 50 μL / mL concentration, reducing DNA damage by 28% (Boyd et al., 2006). As mentioned above, photo aged skin is characterised from high levels of oxidative stress and increased production of ROS and the mentioned phytochemicals have been widely studied on their antioxidant properties. Therefore, it is hypothesised that both SFN and PEITC can have a beneficial effect on photo-aging. Although there is research on the former compound and its photo protective and antioxidant effect, little research has been conducted on PEITC and its anti-photo-aging effects.

The aim of this chapter was to investigate the effect of UVB on HaCaT cells in order to develop a UVB induced optimisation model, evaluate the effect of SFN and PEITC on HaCaT cells in the absence of UVB and investigate the effect of SFN and PEITC on a UVB induced photo-aging model using HaCaT cells through observing their dose and time dependent effect on viability levels.

4.2 Results and Discussion

4.2.1 Effect of UVB on HaCaT cells

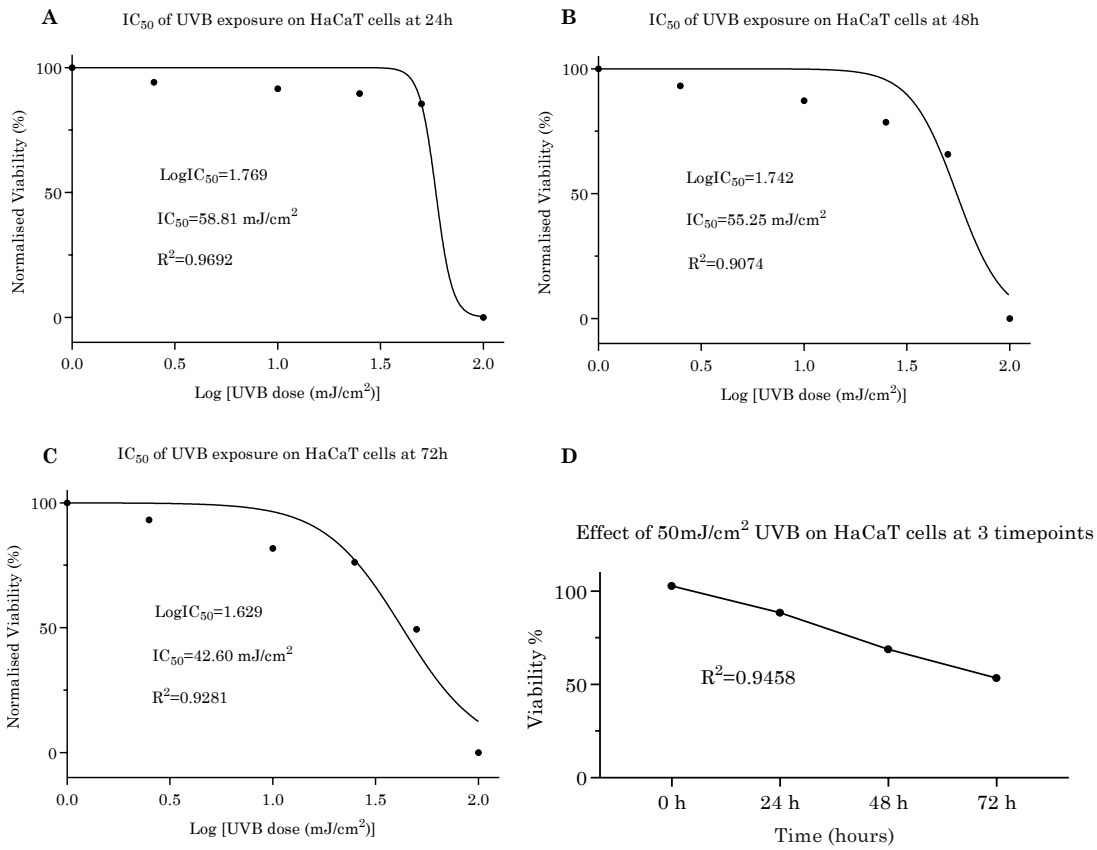
4.2.1.1 Cell Viability determination of UVB on HaCaT cells

During initial optimisation both 96-well plates and 12-well plates were used. 96 well plates gave more scope for replicates however it was observed that results were less consistent compared to the 12-well plates and the data collected showed a high variation in viability. Indeed, six and twelve-well plates are preferred in similar studies (Benedict et al., 2012; Kannan & Jaiswal, 2006; Talalay et al., 2007; Zhao et al., 2013; Zhu et al., 2004).. We hypothesised that the narrow walls of the wells of the 96-well plates may prevent the uniform exposure of the cells. Therefore, 12-well plates were preferred for the experiments and all results shown are from the experiments conducted with these plates. The number of replicates was determined based on the repeatability.

A remarkable reduction in cell number (> 60%) was observed after treatment, when PBS w/o Mg^{2+}, w/o Ca^{2+} was used. It is hypothesised that during the low temperature conditions of the treatment, cells detached from the bottom of the wells and were eventually discarded with the removal of the PBS w/o Mg^{2+}, w/o Ca^{2+}. PBS w/ Mg^{2+}, w/ Ca^{2+} was therefore used with little cell loss noted. It is well established that salts have an important role in cell adhesion with Ca^{2+} creating bridges between anions on cells, binding them together, while Mg^{2+} supports the connections between cells and the bottom of the wells (Takeichi & Okada, 1972). The optimisation of the experimental model was initiated with exposure of HaCaT cells in eight doses of UVB: 2.5, 10, 25, 50, 100, 250, 500 and 1000 mJ / cm^2 and the evaluation of their viability after 24, 48 and 72 hours was completed using resazurin assay and absorbance was measured at 570 and 600 nm as described earlier. It was observed that 250, 500 and 1000 mJ / cm^2 inhibited the cell proliferation significantly and the assay failed to detect any viability, with OD (570-600 nm) < 0.1 absorbance. A dose dependent viability

reduction is observed at 24h, 48h and 72h on all UVB exposures when compared to the controls. Also, UVB treatments within the range 10-50 mJ / cm^2 show a viability reduction of a time dependent manner, while no significant changes are shown at 2.5 mJ / cm^2 UVB exposure, which remains stable through the time studied (figure 4.2). 100 mJ / cm^2 reduced viability to <20% ($p<0.0001$) at 24h, while 48h and 72h show a further 50% viability reduction, an observation that makes this intensity unsuitable for our experiments, as the aim of the optimisation is to select levels of UVB that the damage caused would be reversible and would enable the effects of ITCs to increase viability. The suitable UVB range for this study, based on the viability test is shown to be 25 and 50 mJ /cm^2 with reduction of viability percentage at 91.68 ± 0.634 % (24h), 80.51 ± 0.936 % (48h) and 78.14 ± 1.845 % (72h), and 88.39 ± 1.289 % (24h), 68.84 ± 0.686 % (48h) and 53.42 ± 1.690 % (72h), respectively (table 4.1). This range was decided based on current studies on MED (details below), while a range the two intensities selected provide a range of viability reduction through time starting from mild viability reduction of 10% at 25 mJ /cm2 at 24h and 50% at 50 mJ /cm2 at 72h. In a study investigating the MED in various phenotypes, it was shown that NB UVB < 7 mJ / cm2 can cause erythema in phenotype I, which is the minimum erythema dose investigated, whilst the maximum erythema dose was found to be < 38 mJ / cm2 for patients that have phenotype IV (Rodríguez-Granados et al., 2017). In other studies, 15 mJ / cm2 is shown to be the MED to cause oxidative stress without causing significant damage and mortality to the cells (Miller et al., 2012; Oh, Karadeniz, et al., 2019; Oh, Seo, et al., 2019), something that is also observed on the viability levels of this study (figure 4.2). However, when photo-damage rescue was studied from Ryu et al. (2019), 40 mJ / cm2 was used for the experimental model reducing viability of HaCaT cells by approximately 50 % (table 4.1), an effect that is observed in this study 72 hours after the UVB exposure. In similar studies where UVB was used to treat human dermal fibroblasts in order to determine photo-damage (S. Y. Lee & Moon, 2012) and photo-oxidative stress (Benedict et al., 2012), 25 and 20 mJ / cm2

was used respectively, conditions that agree with UVB intensities selected for this study's optimised model. Considering all the above, it is concluded that the effect of UVB on HaCaT cells varies between studies and it is depended on the model of each study. In most studies where photo damage was studied, MTT assay is used for the evaluation of cell viability (E. Choi & Lee, 2018; S. Y. Lee & Moon, 2012; Oh, Karadeniz, et al., 2019; Oh, Seo, et al., 2019), while resazurin, the assay used in the present study, is shown to be more sensitive method compared to colorimetric methods, such as WST and MTT assays (George et al., 2010; Präbst et al., 2017) and it is also widely used as a method of determination of cell viability (Jaisin et al., 2020; Mantso et al., 2016; Medellín-Luna et al., 2021; Mitsiogianni, Trafalis, et al., 2020). On the other hand, resazurin is known to cause cellular stress and induce apoptosis due to production of ROS, when higher concentration of the dye are used and / or the cells are incubated for an extensive time (Erikstein et al., 2010; O'Brien et al., 2000). For the present experiments, resazurin was used under the laboratory's optimised conditions (section 2.1.1) and the assay was used as an end point measurement. Another parameter that differs between studies is the UVB chambers / lamps used, such as Bio Sun UV lamps (Oh, Karadeniz, et al., 2019; Oh, Seo, et al., 2019) and cross linker CL-508M (S. Y. Lee & Moon, 2012), while a variety of NB and BB lamps are used, such as SF20UVB (NB) (Zhu et al., 2004), FS72T12-UVB-HO (BB) (Benedict et al., 2012) and Baarlin Spectra lamps FS24HO (NB and BB) (Aufiero et al., 2006). For the experiments of this study, CL-1000 Crosslinkers (Ultra-Violet Products Ltd) were used, chambers that have the flexibility in UVR choice and the time of the exposure is automatically adjusted from the chambers depending on the energy settings (section 2.3.3).



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UVB dose mJ / cm²	Timepoints (hours) / Viability %			
	0	24	48	72
Control	100 ± 0.587	100 ± 0.4688	100 ± 1.454	100 ± 0.846
2.5	97.45 ± 0.883	95.28 ± 1.073	93.77 ± 1.080	93.74 ± 0.980
10	95.65 ± 1.448	93.22 ± 1.054	88.35 ± 0.410	83.26 ± 0.725
25	98.93 ± 0.716	91.68 ± 0.634	80.51 ± 0.936	78.14 ± 1.845
50	102.71 ± 0.436	88.39 ± 1.289	68.84 ± 0.686	53.42 ± 1.690
100	100 ± 0.480	20.34 ± 1.070	9.9 ± 0.450	8.32 ± 0.991

Figure 4. 2 *Effect of UVB on HaCaT cells.*

Cells were treated with five UVB intensities (2.5, 10, 25, 50 and 100 mJ / cm²) for a duration automatically adjusted from the UV crosslinker and measurements were taken at 24, 48 and 72h using the resazurin assay. Viability of cells is presented (A, B, C) in non-linear regression curves [log(inhibitor) vs. normalised response – Variable slope] to calculate IC_{50} of UVB and (D) the effect of 50 mJ/cm² UVB on cells is shown in linear regression at all 3 timepoints. (E) The viability percentages and standard deviations for all timepoints and intensities of UVB used.

Table 4. 1 *Studies on photo-damage and apoptosis caused by UVB on HaCaT cells*

Model	UVB type	UVB dose	Study	Viability Assay	Reference
HaCaT cells	-	40 mJ / cm^2	Photo-damage and rescue of cells from prunin	WST-1	(Ryu et al., 2019)
HaCaT cells	BB UVB (280 – 320 nm)	100, 150, 200, 300 J / m^2	The Production IL-21 and VEGF in UVB-irradiated HaCaT	N/A	(H. Kim et al., 2010)
HaCaT cells	312-nm UVB	15 mJ / cm^2	Protective effect of 3,5-dicaffeoyl-epi-quinic acid against UVB-induced photoaging model	MTT	(Oh, Karadeniz, et al., 2019)
HaCaT cells	312-nm UVB	15 mJ / cm^2	Anti-photoaging effects on UVB-stressed human keratinocytes	MTT	(Oh, Seo, et al., 2019)
HaCaT cells	-	50 mJ / cm^2	Antioxidant and anti-aging properties of rice extracts	MTT	(E. Choi & Lee, 2018)
Primary human keratinocytes	NB UVB	NB-UVB at 1000 mJ / cm^2 and BB-UVB at 125 mJ / cm^2	UVB apoptosis induction	N/A	(Aufiero et al., 2006)

4.2.1.2 Cell apoptosis determination of UVB on HaCaT cells

To achieve optimum conditions on the photo-aging model, cell apoptosis was also studied for the four indicated intensities (10, 25, 50, 100 mJ / cm^2) at 24h. This timepoint was preferred over the other two timepoints as it is shown that apoptosis and caspase 3,7 activity peaks at 24h when low concentrations of chemicals used, as shown in a study where tamoxifen was used to access the apoptosis time (Sundquist et al., 2006). Moreover, 48 and 72 hours are shown to further reduce viability indicating that cells fail to repair and therefore, apoptosis might be less indicative for the optimisation due to potential deterioration of apoptotic cells to necrotic or debris (cell material), that represents death of cells and indicates that apoptosis and / or necrosis has already occurred, which would not be detectible from the assay. The lowest dose (2.5 mJ / cm^2) was excluded from this experiment due to the minimal viability reduction shown in figure 4.2. Briefly, HaCaT cells were cultured as described in

3.2.1.2 in 60 mm plates and left to adhere overnight before they were treated with UVB in the intensities indicated, following the protocol as described in section 2.3.3. At the timepoint, cells were collected, and CellEvent Caspase 3/7 Green flow cytometer assay kit was used for the determination of cell apoptosis of the model. Generally, apoptotic cells were shown to increase with the increase of UVB exposure at 10mJ / cm2: 15.4 ± 1.0%, 25 mJ / cm2: 20.633 ± 1.518%, 50 mJ / cm2: 38.7 ± 1.47% compared to the control: 5.13 ± 0.71%, whilst necrotic cells remained at consistent increased levels on all UVB doses used at 10mJ / cm2: 19.86 ± 1.10%, 25 mJ / cm2: 21.6 ± 1.0%, 50 mJ / cm2: 21.36 ± 1.02% and 100 mJ / cm2: 21 ± 1.0% ($p<0.0001$) when compared to the control group: 11.5 ± 1.5% (figure 4.3). Also, an increase in debris was observed during the data collection of the experiment, showing a significant increase at 100 mJ / cm^2. The debris was excluded from the data and it is suspected that increased cell debris may explain the reduction in apoptotic cells at 100 mJ / cm^2: 31.8 ± 3.215% without an increase of necrotic cells as described above (figure 4.3A).

A wide range of UVB doses have been used in previous studies investigating potential protective effects of ITCs with 20 mJ / cm^2 (Benedict et al., 2012) and 25 mJ / cm^2 (Al-barnawi, 2018; S. Y. Lee & Moon, 2012) being the most common conditions in the literature, 50 mJ / cm^2 has also been used in similar experimental models (Shibata et al., 2010). The above findings of viability and apoptosis indicated 25 and 50 mJ / cm^2 the most suitable conditions for the HaCaT cells model.

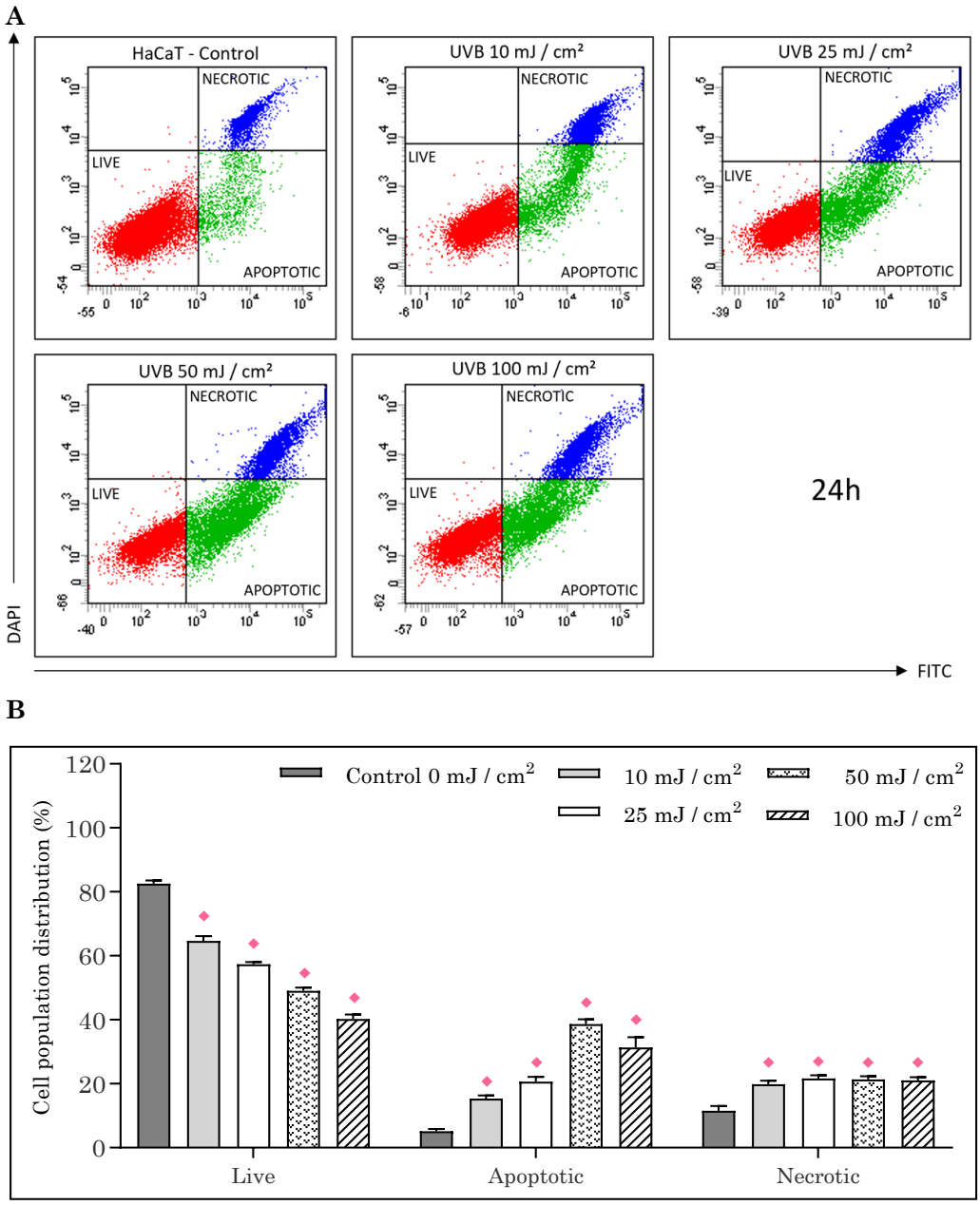


Figure 4. 3 *Cell apoptosis of photo-aging model*

*HaCaT cell apoptosis and necrosis was measured following the UVB exposure of four doses (10, 25, 50 and 100 mJ / cm^2). CellEvent Caspase 3/7 Green flow cytometer assay kit was used along with DAPI and live, apoptotic and necrotic cells are shown as (A) cell events (20,000 events/second) in scatter plots (B) percentage of events. Statistical significance was set as *p < 0.0001 relative to corresponding controls. Data shown are representative of three independent experiments. For the analysis of the data two-way Anova was performed adjusting for multiple comparisons.*

4.2.2 Effect of ITCs on HaCaT cells in the absence of UV

The determination of the effect of ITCs on HaCaT cells was required for the selection of ITC doses that are not toxic to the cells in order to be used in combination with UV. To evaluate this effect in the absence of UV, HaCaT cells of normal cellular growth were pre-treated with 6 concentrations of either of the two phytochemicals for 24, 48 and 72h and the resazurin assay was utilized to determine the viability levels (figure 3.4). It is shown that SFN is inhibiting cell proliferation, when applied on healthy growing cells, at 48h and 72h at 2.5 (48h: 91.1 ± 4.7 %, $p<0.05$, 72h: 86.1 ± 4.9 %, $p<0.001$) and 5 μM (48h: 83.6 ± 3.9%, $p< 0.0001$, 72h: 84.5 ± 6.4% $p<0.0001$), whilst PEITC is shown to have a stronger cytotoxic effect on cells, with reduced viability levels at 24h [0.6 μM (88.1 ± 4.8%), 1.25 μM (84.3 ± 5.5%) and 5 μM (92.7 ± 2.3)] and 48h [0.15 μM (84.4 ± 10%), 0.3 μM (83 ± 5%), 0.6 μM (82.5 ± 6.9%), 1.25 μM (78.9 ± 79 ± 4.8%), 5 μM (86 ± 4.2%)]. The inhibiting effect of PEITC is shown to reverse at 72 hours where cell proliferation is increased at 0.15μM: 10.3 ± 0.93%, 0.3 μM: 104.8 ± 2%, 0.6 μM: 106 ± 1.1%, 1.25 μM: 106.2 ± 1.2%, 2.5 μM: 106.8 ± 2.6%, 5 μM: 106.6 ± 1% when compared to the control but this effect is not significant ($p > 0.05$). Similar observations were made when the same concentration of phytochemicals was used on PC-3 C4 cells and the cell viability was assessed, PEITC was shown to be a more potent growth inhibitor than SFN (Xu et al., 2005). On the other hand, when Mantso *et. al.* (2016) treated HaCaT and human malignant melanoma cells with 5 μM of either SFN or PEITC and investigated their proliferative and apoptotic effect , SFN was found to be a more potent phytochemical on the reduction of cell viability, using the resazurin assay, while lower concentration had no effect on HaCaT cells at either end point (24h and 48h) (Mantso et al., 2016). The potency of the phytochemicals to induce apoptosis and reduce cell viability varies between cells lines, with HaCaT cells being more resistant to ITCs when compared to A375 melanoma cells (Mitsiogianni, Trafalis, et al., 2020), whilst the two studied compounds have been shown to be less potent on HL60/S and 8662/S cells than other ITCs

when compared to HaCaT cells (Y. Zhang et al., 2003). Although the reason for this is still unknown, we hypothesis that it might be because of the doubling time of HaCaT cells compared to A375. Another possible reason for this variation is the density of the cells, since HaCaT cells are growing faster when denser compared to A375, as observed in our laboratory group. In contrast with our findings, when 5 μM PEITC were used for the treatment of HaCaT cells and apoptosis was investigated, showing no significant effect on caspase 3 after 24 and 48 hours (Kleszczyński et al., 2013).

Although our findings suggest a mild toxic effect of both phytochemicals when used on their own, rather than a beneficial role on their cell growth, other studies have shown that when ITCs are used on a diseased model, such as photo damaged (Saw et al., 2011a; Sikdar et al., 2016) or cancer model (Clarke et al., 2008; Xu et al., 2005), is shown to have a beneficial effect through reduction of Nrf2 (Kleszczyński et al., 2013) or suppression of NF-κB (Xu et al., 2005), respectively.

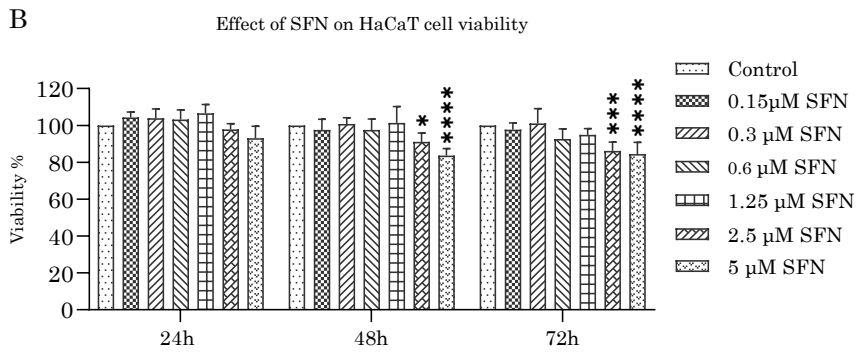


Figure 4. 4 *The effect of isothiocyanates on HaCaT cell viability*
*Cells were treated with 6 concentrations (0.15, 0.3, 0.6, 1.25, 2.5, 5 μM) of either SFN or PEITC and their viability at 24, 48 and 72 hours was evaluated using resazurin assay. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001,* ***ns*** *for not significant, relative to corresponding controls. Data shown are means of ±SD of three replicates from 3 independent experiments with 5 replicates per sample. For the analysis of the data two-way Anova was performed adjusting for multiple comparisons.*

SFN alone was shown to activate this nuclear factor in rat kidneys and prevent inflammation and cell death in nephropathy (Guerrero-Beltrán, Mukhopadhyay, et al., 2012). PEITC alone is shown to moderately inhibit the activation of NF-κB by downregulating its signaling pathways on human colon cancer cells with IC_{50} = 21.24 ± 6.24 μM (Prawan et al., 2009), while another ITC with similar chemical structure, BITC, was shown to have the same effect on human pancreatic cancer cells with IC_{50} ~8 μM. Although this is beneficial for disease, such as cancer or psoriasis, the opposite effect is expected for photo-aging. Lee and Moon 2012, showed that SFN and PEITC induce NF-κB in low concentrations of 2 and 5 μM and 2 μM on PC-3C4 cells, respectively, but this observation was not significant. Whereas, higher concentration of the phytochemicals was shown to inhibit NF-κB. Therefore, it is hypothesised that ITC can have a beneficial effect on a photo-aged model using HaCaT cells, despite their mild effects on viability.In this chapter we aimed to study the potentially beneficial effect of PEITC and SFN pre-treatment in low concentrations, on a UVB-induced photo-aging model using HaCaT cells, through utilising methods of viability and apoptosis determination.

4.2.3 Effect of SFN and PEITC on UVB-exposed HaCaT cells

For the investigation of the effect of ITCs on the photo-aging model, two doses of UVB, 25 and 50 mJ / cm^2 were indicated as suitable during the optimisation of the experimental model. Although, our study showed that 5 μM of either of the ITCs inhibit the cell proliferation in most timepoints, it was decided to include this condition in the experimental model in order to investigate a potential reverse effect of the combination of ITCs and UV. Although, other studies have shown ITCs to inhibit cell growth in a time and dose dependent manner (Mantso et al., 2016; Mitsiogianni, Trafalis, et al., 2020), our findings reveal a more complex effect when the phytochemicals are applied to a UVB induced model.

For the 25 mJ / cm^2 photo-aging model, SFN is shown to significantly increase cell viability at 24h [0.15 μM (102 ± 8%), 0.3 μM (101.9 ± 6.5%), 0.6 μM (102.5 ± 8.9%), 1.25 μM (102.2 ± 4.5%) $p<0.05$] and 72h [0.15 μM (97.9 ± 6.5%), 0.3 μM (102.8 ± 6.1%), 0.6 μM (100.7 ± 4.2%), 1.25 μM (102.9 ± 7.2), 2.5 μM (96.7 ± 7.6%) $p< 0.0001$] when compared to the relative UVB controls, whilst the 50mJ / cm^2 model is showing increased cell growth at the same timepoints at 0.15 μM (101.7 ± 3.1%, $p<0.05$) and 0.3 μM (101.6 ± 5.5%, $p<0.05$), and 0.15 μM (85.4 ± 5.9, $p<0.0001$), 0.3 μM (91.3 ± 5.1%, $p<0.0001$), 0.6 μM (86.3 ± 3.7%, $p<0.0001$), 1.25 μM (80.3 ± 1.8%, $p<0.001$) and 2.5 μM (87.2 ± 5.4%, $p<0.0001$), respectively. On the other hand, PEITC did not show protective effect at the early timepoints, but reduced viability levels further at 24h (5 μM: 80.4 ± 3.2%, $p<0.0001$), 48h (0.15 μM: 77.6 ± 7.5%, $p<0.05$) and 72h (5 μM: 82.7 ± 3.2, $p<0.01$) for the 25 mJ / cm^2 model and at 48h (0.3 μM: 76 ± 7.4% and 0.6 μM: 75.8 ± 6.1%, $p<0.05$) for the 50 mJ / cm^2 model. A protective effect is however seen with PEITC at 72h at both photo-aging models, with 25 mJ / cm^2 showing an significant increase in cell growth at 0.3 μM (96.4 ± 2.9%, $p<0.05$) and with 50 mJ / cm^2 at 0.15 μM: 78.5 ± 2.3%, $p<0.05$, 0.3 μM: 80.3 ± 3.1%, $p<0.001$, 0.6 μM: 80.4 ± 2.1%, $p<0.001$, 1.25 μM: 82.8 ± 1.2%, $p<0.0001$, 2.5 μM: 86.8 ± 3.9%, $p<0.0001$ and 5 μM: 81.4 ± 7.3%, $p<0.001$. This study showed that both ITCs can have a significantly beneficial effect on a UVB-induced photo-aging model, through either protective (SFN) and / or repairing effect (SFN and PEITC) when the model is pre-treated for 24 hours with low doses 0.15, 0.3, 0.6, 1.25, 2.5, 5 μM of the phytochemical. In a study where DNA damage through oxidative stress was investigated on keratinocytes and dermal fibroblasts using 20 mJ / cm^2 BB UVB and 20 J / cm^2 UVA a protective effect of SFN against UVA through the induction Nrf2 pathway genes, was observed, while damage caused due to UVB exposure remained unaffected. (Benedict et al., 2012). Another study, where the photo-protective effect of the SFN on HaCaT cells was investigated, cells were pre- and post- treated with SFN and four doses of UVB were used (25, 50, 100 and 200 mJ / cm^2)

(Al-barnawi, 2018). Although, post-treatments with SFN did not show significant protective effect, increased cell proliferation was observed on pre-treated cells after 24 and 48 hours. These findings agree with the findings presented in this study, where the photo-protective effect of SFN is shown 24h hours after the UVB exposure. There is little research conducted on the role of PEITC as a photo-protective compound, possibly due to its strong cytotoxic character (table 4.3). Kleszczyński *et. al.* (2013) studied the same two phytochemicals and their photo-protective effect looking at apoptosis and oxidative stress, and both ITCs were shown to reduced sunburn cells on human skin tissue, an observation that its mechanism was evaluated through experiments on HaCaT cells and showed induction of antioxidant Nrf2 activity and reduction of caspase-3 activation (Kleszczyński et al., 2013). Moreover, although a previous study did not show 5 μM of SFN to have a cytotoxic effect on healthy cells (Shibata et al., 2010), our findings revealed a significant inhibition of cell viability of this concentration with this effect being reversed at 72h of both photo-aging models, while no effect is shown at 48h. PEITC keeps its cytotoxic character on both healthy and photo-aged cells in certain concentrations (figure 4.4). Moreover, in the mentioned study the same dose of UVB on a UVB-induced inflammation model was used on the same cell line and the anti-inflammatory effect of SFN was shown through increased proliferation in concentration up to 5μM, in line with the data shown in figure 4.4 (Shibata et al., 2010).

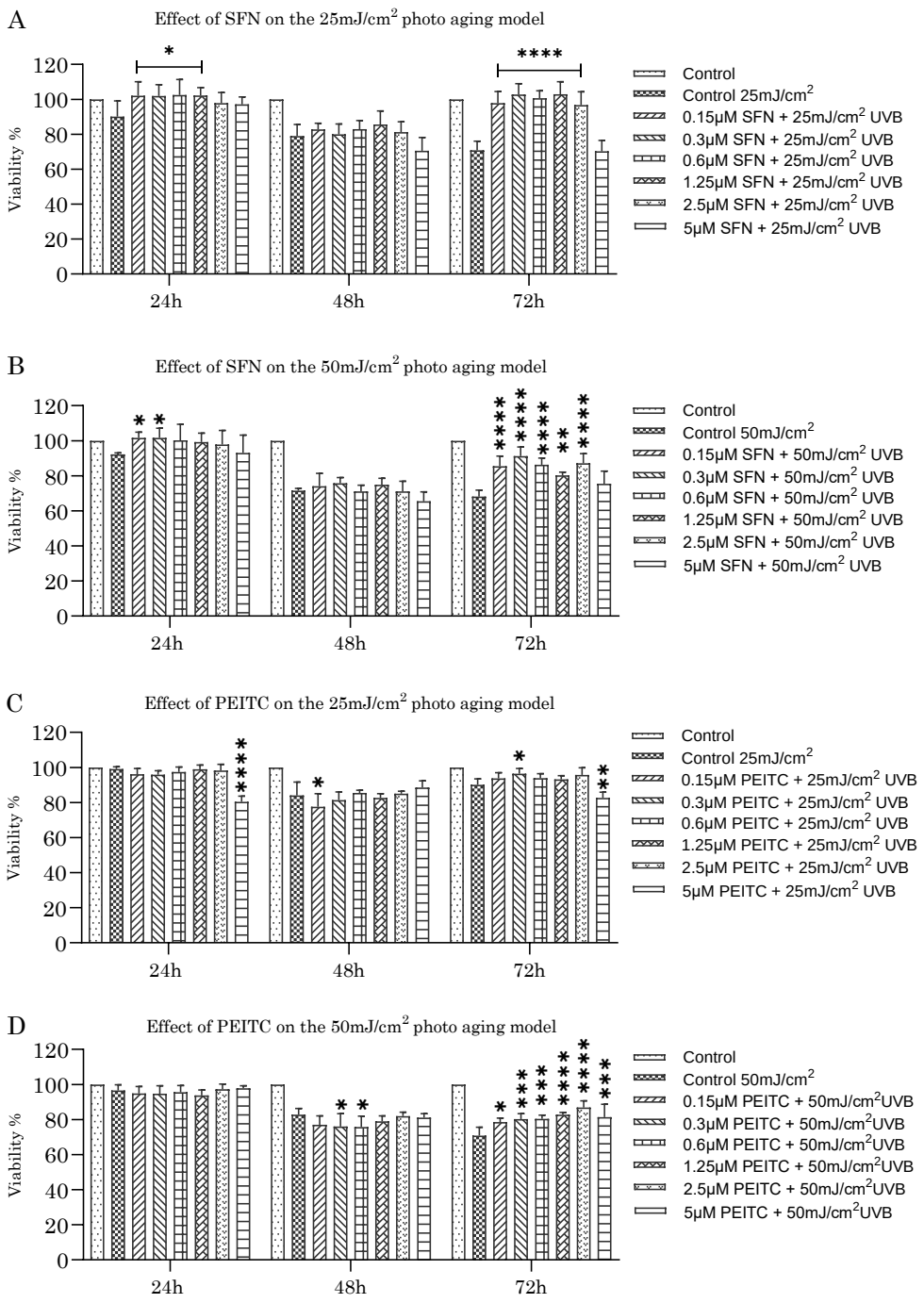


Figure 4. 5 *Isothiocyanate effect on HaCaT cell viability of UVB-induced photo-aging model at 25 and 50 mJ / cm2.*

*The experimental models were treated with 6 concentrations of either SFN or PEITC (0.15, 0.3, 0.6, 1.25, 2.5, 5 μM) and their viability was evaluated at 24h, 48h and 72h. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001 relative to corresponding controls. Data shown are representative of three independent experiments with 5 replicates per sample.*

Similar observations were made from Carpenter *et al.* 2018 when human primary epidermal keratinocytes were treated with 3-methoxybenzyl isothiocyanate (MBITC) and 3-methoxyphenyl acetonitrile (MPACN) after the treatment of 10 mJ / cm^2 UVB and the combinational treatment further inhibited cell viability (Carpenter et al., 2018). Although there is not extensive literature on the role of ITCs on the prevention of photo damage and photo-aging of the skin, research so far has shown that main mechanisms of action of specific ITCs are through suppression of UVB-induced inflammatory response and oxidative stress. Specifically, SFN is shown to enhance the activity of Nrf2 (Kleszczyński et al., 2013; Saw et al., 2011a), while suppresses the production of inflammatory markers, that are induced by UV irradiation, such as IL-6 and cyclooxgenase-2 (COX-2) and therefore reduce inflammatory response. All the above could give a possible explanation on the variant effect of the phytochemicals on the current model with increased viability in lower concentrations indicating an increase of NF-κB and therefore cell rescue, and decreased viability of higher concentrations indicating an inhibition of NF-κB and therefore cell viability reduction. Moreover, the effect of ITCs should not only be linked with NF-κB but also with the anti-inflammatory role of the phytochemicals as described above.

Table 4. 2 *Studies on photo-protective effect of various ITCs*

Model	UVB type	UVB dose	ITC studied	ITC concentrations	Study findings	Reference
Fibro-blasts	UVB cross-linker 312 nm	25 mJ / m^2	SFN	1, 2, 5, 10, 20 µM	Prevention of MMPs expression through inhibition of NF-kB.	(S. Y. Lee & Moon, 2012)
HaCaT cells	-	200, 100, 50 and 25 mJ / cm^2	SFN	0.5, 1, 2.5, 5, 10 µM	increased rate of cell proliferation at 0.5µM (122.0%), 1.0µM (130.0%), 2.5µM (136.0%), 5.0µM (141.0%) and 10.0µM (111.0%).	(Al-barnawi, 2018)
HaCaT cells	EL lamp (peak at 302 nm)	50 mJ / cm^2	SFN	0 – 25 µM	COX-2 protein expression and IL-6 production suppression.	(Shibata et al., 2010)
primary keratino-cytes and dermal fibro-blasts	BB UVB	20 mJ / m^2	SFN	1 µM	Protection from UVA through induction of Nrf2 genes.	(Benedict et al., 2012)
Keratino-cytes	BB UVB	20 mJ / cm^2	SFN	1 µM	photo-oxidative stress	(Benedict et al., 2012)
HaCaT cells and tissue	-	Cells: 10 mJ/cm^2, tissue: 60 mJ / cm^2	MPACN , MBITC	Cells: 50–0.5 µM, tissue: 10, 25, 50 µM	ITCs failed to protect from UVB damage	(Carpenter et al., 2018)
Human and mouse subjects	NB UVB (311 nm)	Humans: 100–800 mJ / cm^2 in 100 mJ / cm^2 increments, mice: 30 mJ / cm^2	SFN	Broccoli sprouts extracts	Reduction of UVR-induced erythema by 37.7%.	(Talalay et al., 2007)
Skin and keratino-cytes	UVR-source of 3.3 mW/cm^2	Human skin: 300 mJ / cm^2 UVR	SFN, PEITC	5, 10, 25 µM	Increase of caspase 3 activation in concentrations of 5 µM and 10 µM.	(Kleszczyński et al., 2013)
HaCaT cells	313 nm	250 J / m^2	SFN	0 – 10 µM	Increase of phase II enzymes and GSH levels do not inhibite UVB-induced AP-1 activation.	(Zhu et al., 2004)

SFN, PEITC, MPACN and MBITC were used for the treatment of human epidermal cells (either primary keratinocytes or HaCaT cells), human and mouse skin and human fibroblasts on UVB- induced models and the photo-protection and antioxidant role of the phytochemicals was evaluated.

The present results are significant in at least two major respects. From the two phytochemicals studied in this chapter, SFN is shown to be highly potent to enhance cell growth and increase viability levels at 24 and 72 hours after the UVB exposure. and therefore, may be an indicator of SFN mediated recovery from UV exposure. In the contrary, PEITC is shown to inhibit cell viability at the first two timepoints studied, while a protective effect is shown at 72h, increasing cell proliferation by 10-15%.

Contrary to expectations, this study has also shown that although ITCs have a protective role on a photo-damage model in low concentrations through the increase of cell viability, higher concentrations (5 μM) of ITCs, when combined with UVB, are showing the opposite effect, than when used alone on HaCaT cells (figure 4.4). Although this is observed only for PEITC, there is an inconsistency on the potency and on the concentrations on which the phytochemical reduces growth. Hence, it could conceivably be hypothesised that certain concentrations of ITCs, when combined with UVB can provide an effect that is beneficial on disease that the reduction of cell viability and the increase of apoptotic cells is desirable. All this can reflect an effect on NF-κB that appears to have a more complex role when it comes to photo-aging. Specifically, it was shown the UV light induced NF-κB leads to an increase in MMPs and, therefore to collagen degradation and eventually skin wrinkle formation (S. Y. Lee & Moon, 2012). On the other hand, when NF-κB is inhibited, apoptosis is promoted through the stimulation of TNF-a.

More research needs to be conducted on the mechanisms of protection of both phytochemicals, as well as the repair processes that possibly take place from the time of exposure and 72 hours after this point, by which the viability levels are shown to significantly increase. In future investigations, it might be possible to use a different cell line, such as human dermal fibroblasts, as well as UVA, the irradiation that penetrates the epidermis and reaches dermis, causing the breakage of elastin and collagen network, the main biomarkers

of photo-aging. Further investigation of the findings of this study could include experiments that look at apoptosis markers, such as caspase 3/7, and oxidative stress. Furthermore, the epigenetic mechanisms of the effect of ITCs on the suggested UVR -induced experimental models and their protective mechanisms, could be analysed through gene (real time polymerase chain reaction (rt-PCR)) and protein expression (western blots) assays. With further investigation of the findings of this study, the naturally found compounds, are promising, active ingredients on potential cosmetic and care products that could protect from severe photo-damage and potentially decrease the symptoms of photo-aging.

4.3 Conclusions

The current chapter showed that both phytochemicals had a photo-protective effect on the UVB-induced photo-aging models of 25 and 50 mJ / cm^2 with significantly increased cell viability at 72 hours by at least 10% compared to controls. Although there is abundant room for further progress in determining the exact effect of ITCs on photo-aging, this study provides two significant findings:

1) The significant cell viability enhancing effect of SFN and PEITC in low concentrations (< 5 μM) on the studied photo-aging model of 25 mJ /cm^2 and 50 mJ / cm^2.

2) The cytotoxically enhanced combination of higher concentration of the phytochemicals with UVB and its potential use as a therapeutic approach for psoriasis.

The second significant finding of this chapter will be further studied in the next chapter as a combinational treatment of ITCs with UVB as a novel therapeutic approach for Psoriasis, using HaCaT cells.

5. The role of synthetic isothiocyanates in combination with low dose UVB as a novel therapeutic approach for psoriasis

5.1 Introduction

Psoriasis is an autoimmune skin disorder that is usually developed at a young age (13-20 years old) (Rahman et al., 2012). The condition worsens after a skin injury or when another autoimmune disease already exists. The key mechanism of pathogenesis is through increased keratinocyte cell proliferation, which takes place every 3-5 days, instead of the normal rate of 28-30 days. This is triggered by the underlying immune dysfunction seen in these patients. Briefly, dendritic cells, macrophages and T cells stimulate keratinocyte cell proliferation by moving to the epidermis and secreting cytokines, such as IL12B and IL23R (Elder et al., 2010) (section 1.4). Psoriatic skin is characterised by patches of abnormal inflamed skin with signs of dryness, itchiness, and redness, and it can affect the whole body or parts of the skin, which is usually the elbows, the knees, the scalp, the navel area, and the fingernails / toenails. There are five types of psoriasis, plaque psoriasis, also known as psoriasis vulgaris, pustular psoriasis that is characterised by small non-infectious pus-filled blisters, guttate psoriasis with drop-shaped lesions and erythrodermic psoriasis, when the other types of psoriasis spread throughout the body and can be fatal. 30% of patients that suffer from psoriasis, might also suffer from psoriatic arthritis, while other related diseases are lymphoma, cardiovascular disease, Crohn's disease and depression (Chimenti et al., 2013; Egeberg et al., 2016; Rahmati et al., 2021). Although the reason this auto-immune disease exists is still unknown, psoriasis may be inherited, evidence shows that there are certain genes involved in the development of psoriasis such as PSORS1, PSORS9, IL-12β and IL-23R which are pro-inflammatory cytokines and chromosome 5q, a cytokine cluster-rich region (Geisel et al., 2014; Pisani et al., 2021). Moreover, psoriasis can be triggered by changes in the lifestyle of

the patient, such as smoking, other infections such as human immunodeficiency virus (HIV), microbial infections as well as medication, such as antimalarial drugs, beta blockers and lithium (Balak & Hajdarbegovic, 2017; Tsankov et al., 2000).

Current treatments include conventional and natural treatments, while the choice of treatment depends on the stage and severity of the disorder (section 1.4.2). Steroid creams and vitamin D3 creams are shown to be effective on psoriasis by suppressing skin inflammation and improving the symptoms of the disease (figure 5.1). In more severe and prolonged situations where creams are not suppressing the condition, UV light is also used in combination with creams or oral medicine, known as Psoralen (Hönigsmann, 2001). As described in section 1.4.2.2, UVA and UVB light is currently used in multiple doses. UVA alone is not effective in treating the symptoms of the disorder but when combined with oral treatment of Psoralen (PUVA), it is used for the suppression of inflammation and keratinocyte overproduction. BB UVB and NB UVB is also used in sessions, while the former is responsible for the carcinogenic effects on skin, which depend on the length of the exposure and the number of treatments (Aufiero et al., 2006; Markham et al., 2003). Although these treatments are shown to be very effective in dealing with the symptoms of the disease, it is an approach that cannot be repeated often due to its carcinogenic effects, while it is also an unpleasant procedure for the patients due to the erythema caused by UVB (Pasker-De Jong et al., 1999). As the effect of these treatments are therefore limited, they are usually combined, while NB UVB is usually used in situations where a full body treatment is required (Talalay et al., 2007; Yones et al., 2006). NB UVB is shown to cause less erythema compared to BB UVB and it is considered a better option for treatments (section 1.4.2.2). Another therapeutic approach for psoriasis, is the use of immunosuppressants, such as methotrexate (Balak & Hajdarbegovic, 2017; Medellín-Luna et al., 2021).

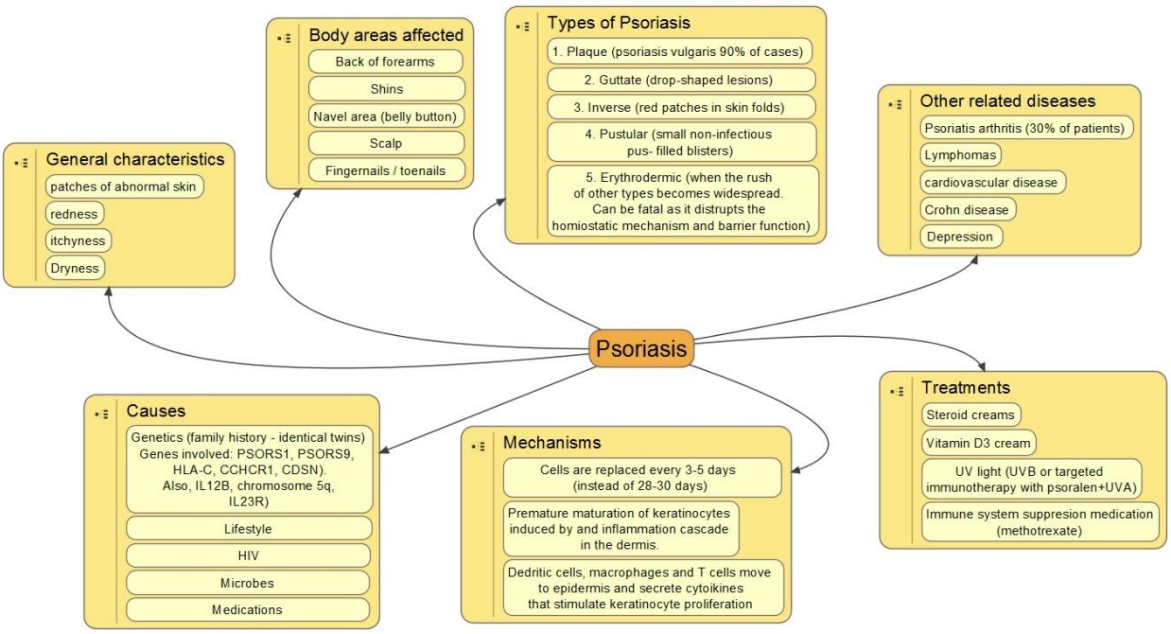


Figure 5. 1 *An overview of psoriasis.*
Psoriasis is presented in sections of types of psoriasis, general characteristics and areas affected, causes, mechanisms, other related disease and current treatments(Chimenti et al., 2013; Egeberg et al., 2016; Elder et al., 2010; Kemény et al., 2010; Singh et al., 2016).

While there are various therapeutic approaches based on medicine and UV light, natural remedies and compounds have also been shown to help and treat the symptoms of the disease. Turmeric, ginger, *Asparagus cochinchinensis Merrill (Liliaceae), Linné, Monordica charantiai and Rhizoma coptidis* due to their rich content of berberine that exerts an anti-inflammatory effect are only a few of the herbs and natural compounds that are known to have an effect on psoriasis and other inflammatory disorders (Peral et al., 2009; Tse et al., 2006).

Although ITCs have not been widely used in the treatment of psoriasis, they are shown to have beneficial effects on inflammatory conditions and autoimmune disorders due to their indirect anti-oxidant effect (Kubo et al., 2017), through induction of Nrf2 target genes and

inhibition of cytokines such as IL-12 and IL-23 in dendritic cells (Geisel et al., 2014). *In-vivo* experiments have shown that ITCs decrease inflammatory activity and reduce psoriatic patches, through the reduction of the production of cytokines, related to psoriasis, such as IL-12/23 and TNF-α (Yehuda et al., 2012). Moreover, SFN is shown to increase ROS and deplete GSH leading to inhibition of inflammatory response of T-cells, a mechanism that could be beneficial for many autoimmune conditions and cancer (Liang et al., 2018). In a patent where plant extract or 1-20μM of ITCs were used on patients suffering from psoriasis, it was shown that 10μM of the phytochemicals was the preferred concentration that successfully treated the patches of the disease topically (Sussan & Sussan, 2012). However, and to the best of our knowledge, the combination of ITCs with low dose UVB, as a therapy for this autoimmune disorder, has never been studied. The above information alongside the findings from chapter 4, where 5μM of ITCs, when combined with UVB, were shown to further decrease HaCaT cell viability that indicates a potential use as a psoriasis treatment which we went on to investigate.

The aim of this chapter was to determine the role of five synthetic ITCs (SFN, IBN, AITC, BITC, PEITC) in combination with low doses of UVB on HaCaT cells, as a novel therapeutic approach for psoriasis.

<u>Objectives of this chapter:</u>

To determine the role of five synthetic ITCs (SFN, IBN, AITC, BITC, PEITC), alone and in combination with low doses of UVB on HaCaT cell's:

1. Measure of viability using resazurin assay and observation of growth using SRB assay.
2. Apoptosis through caspase 3/7 activity.
3. Evaluation of the effect of the treatment on mitochondrial membrane permeability, looking at the levels of JC-1 aggregates over JC-1 monomers ration (ΔΨm).

4. Determination of the effect of the treatment on life cycle and potential cell cycle arrest.
5. Evaluation of ROS induction using two different methods – DHR assay and addition of antioxidant (GSH).

5.2 Results and Discussion

5.2.1 Cell viability of UVB on HaCaT cells

For the preliminary evaluation of UVB on HaCaT cells for the therapeutic approach on psoriasis, the results of the UVB exposure conducted in chapter 4 were taken into account (section 4.2.3). Briefly, it was shown that when HaCaT cells are exposed to various UVB intensities, in a UVB crosslinker that emits radiation at 302nm, viability of the cells is decreased in a dose and time dependent manner. Specifically, after the exposure of the cells to 2.5, 10, 25, 50 and 100 mJ / cm^2, the cell viability decreased significantly in all intensities and for the photo-aging model, 25 and 50 mJ / cm^2 were selected as the most suitable for the experiments of chapter 4. However, the selection of the suitable UVB intensity for the combinational treatments on psoriasis, aims to provide a significant cell viability decrease with reversable effects, while causing minimal damage, since it is hypothesised that the addition of ITCs will reduce viability. Intensities of 25 and 50 mJ / cm^2 UVB were not suitable for chapter 4 since the purpose of this chapter is to provide an alternative therapeutic approach for psoriasis that will benefit on the low UVB damage, an asset that differs from the current treatments that include exposure on MED or higher, depending on the skin phenotype, and in weekly treatments (section 1.4.2.2). Therefore, for the preliminary determination of the suitable conditions for the treatments 10 and 25 mJ / cm^2 was used. Current therapies for this disease, using UVB, are initiated with 75-100% of the MED and it differs based on the skin type, while treatments are usually done in doses, in order to

minimize skin damage (Hönigsmann, 2001). As described in section 1.4.2.2, UVB therapy includes BB UVB that comes with the risk of skin cancer development, while NB UVB is used in whole body therapies and is suitable during pregnancy (Singh et al., 2016)

5.2.2 Cell Viability determination of SFN on HaCaT cells

For the initial optimisation of the therapeutic model, SFN was selected for the treatment on HaCaT cells. The selection was based on the observations that were made on effect of the phytochemical on the cell line in chapter 4 (figure 4.3). Moreover, SFN is a widely used compound known for its ability to reduce cell viability in high concentrations. Moreover, SFN as was preferred over PEITC, which was also used in chapter 3, due to the effect of the latter on cell viability at 24 and 48h, where PEITC was shown to be a more potent phytochemical compared to SFN, as shown in figure 5.3 section 5.3.2. Therefore, the selection of a milder phytochemical was preferred in order to establish the most effective concentration in mild conditions. However, in this chapter, the effect of five ITCs on the therapeutic model is evaluated. For the purpose of this preliminary optimisation, the highest concentration (5 μM) studied in chapter 4 was selected as a minimum dose, while a 4-fold higher concentration (20 μM) was also included as studies have shown that ITCs reduce viability at concentrations >10 μM (Mantso et al., 2016). Moreover, as this is a novel therapeutic approach, 5 μM were selected as a minimum dose in order to evaluate the mechanisms through which the combinational treatment reduces cell viability. Viability was observed at 24 and 48 hours after the treatment with 5 and 20 μM SFN and it was shown that 5 μM of the phytochemical, significantly reduced cell viability by 17% drop at 83.6 ± 3.9% ($p<0.0001$) 48h after the exposure, while 20 μM significantly reduced viability in both timepoints at 86.5 ± 3.7% after 24h and at 76.6 ± 3.3% ($p<0.0001$) after 48h of exposure, when compared to the controls (figure 5.2).

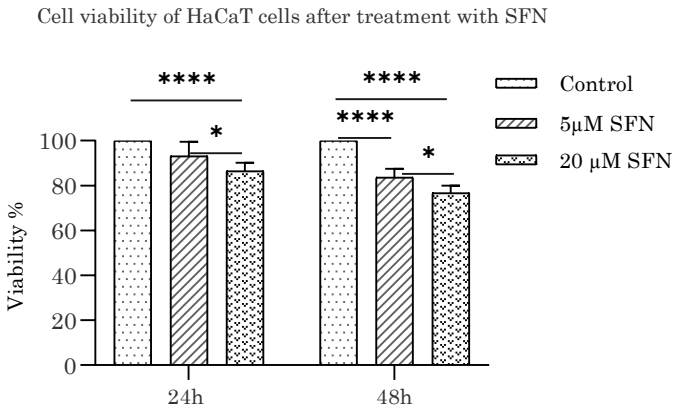


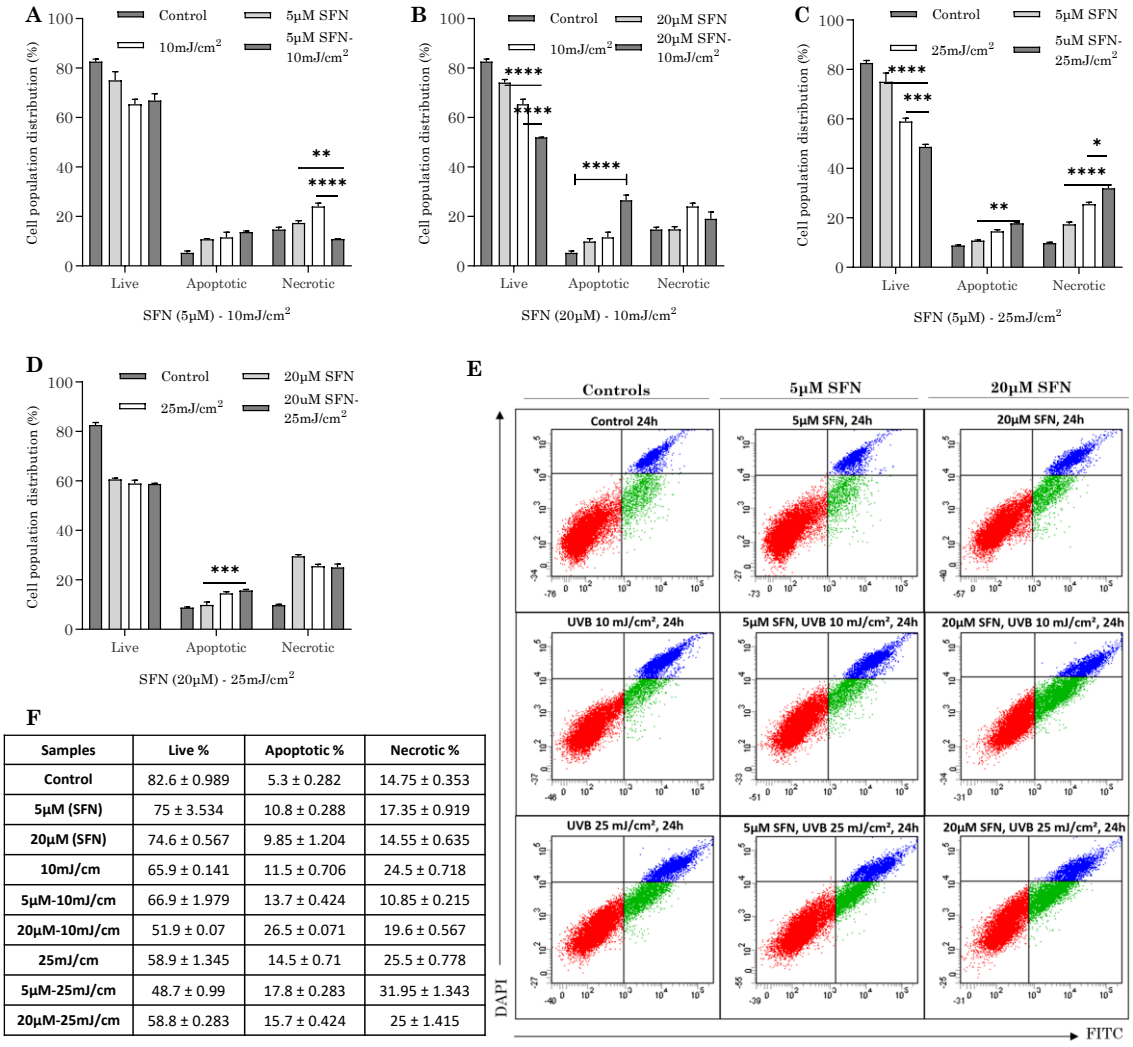
Figure 5. 2 *Preliminary evaluation of the effect of SFN on HaCaT cell viability. Cells were treated with either 5 or 20μM of SFN and their viability was observed at 24 and 48h. . Statistical significance was set as *p < 0.05 and ****p < 0.0001, relative to corresponding controls. Data shown are representative of three independent experiments.*

Based on the above results, it is shown that both SFN concentrations were able to significantly reduce viability, with 20 μM showing an earlier and greater effect. Current therapies for psoriasis are aiming to reduce topical inflammation and reduce cell viability through apoptosis, using UVR exposure in skin type-dependent doses. These results come in contrast with other studies that do not show a significant reduction of cell proliferation at concentrations < 10 μM (Kleszczyński et al., 2013; Mantso et al., 2016). To better investigate this mechanism and determine the effect of this therapeutic approach, it is essential to study the effect of the combinational treatment on apoptotic pathways, such as induction of caspase 3,7.

5.2.3 Preliminary evaluation of apoptosis of preliminary combinational treatments of SFN and UVB on HaCaT cells

For the better understanding of the effect of the suggested therapeutic approach, the evaluations of the effect of the preliminary treatment conditions on apoptosis was required. Although *in-vitro* experiments are very early stages of a possible therapy, in order to mimic a more realistic approach of this treatment, cells were first exposed to UVB, as it would happen in the current UV therapies and ITCs were used as a post-treatment. Moreover, the aim of this optimisation was to achieve an effect that would be significantly stronger than the UVB alone. As introduced above, combinations of 5 and 20 μM of SFN and 10 and 25 mJ / cm^2 UVB were used for the evaluation of caspase 3/7 activation. It was shown that 5 μM of SFN when combined with 10 mJ / cm^2 UVB, increase the apoptotic cells when compared with the control sample, but this observation is not significant when compared to the other controls: SFN and UVB. However, the combination showed a decrease in the necrotic cells at 10.85 ± 0.215%, compared to the UVB: 24.5 ± 0.718%. 20 μM of SFN significantly increased apoptotic cells at 26.5 ± 0.071% ($p<0.0001$) when compared to the relative controls. When the same concentration of SFN was combined with 25 mJ / cm^2 UVB, it was observed that the UVB exposure was overlapping the effect of the ITC, with apoptotic cells between the combination: 17.8 ± 0.283% and the UVB alone: 14.5 ± 0.71%, not being significantly different, while necrotic cells of the combination are significantly increased at 31.95 ± 1.343% ($p<0.05$), when compared to the UVB alone: 25.5 ± 0.778% (figure 5.3 C). Apoptotic cells at 20 μM SFN and 25 mJ / cm^2 UVB: 25 ± 1.415% (figure 5.3 D) are not shown to have a significant difference when compared to the UVB alone: 25.5 ± 0.778%. However, the opposite is observed when compared to the SFN alone: 9.85 ± 1.204%, indicating that UVB is mainly responsible for effect of the combination, rather than the ITC. The combination of 20 μM SFN and 10 mJ / cm^2 UVB, although it is showing

significantly lower apoptotic cells: 14.5 ± 0.071%, when compared to UVB: 18.5 ± 0.706% ($p<0.01$), it is hypothesised that this decrease is related to the significantly decreased live cells: 51.9 ± 0.07% and the increased debris, indicating that cells have passed through the apoptotic and necrotic phase and were eventually excluded from the analysis, leading to a drop in live and apoptotic cells. Overall, it is observed that 25 mJ / cm^2 UVB is an intensity that has a strong effect on apoptosis of cells, masking the effect of SFN, while 10 mJ / cm^2 UVB are shown to enhance the effect of the combination when it comes to live cells (figure 5.3 B). From the four combinations above, 10 mJ / cm^2 UVB and 20 μM SFN is shown to be a more promising combination for this therapeutic approach.



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Samples	Live %	Apoptotic %	Necrotic %
Control	82.6 ± 0.989	5.3 ± 0.282	14.75 ± 0.353
5μM (SFN)	75 ± 3.534	10.8 ± 0.288	17.35 ± 0.919
20μM (SFN)	74.6 ± 0.567	9.85 ± 1.204	14.55 ± 0.635
10mJ/cm	65.9 ± 0.141	11.5 ± 0.706	24.5 ± 0.718
5μM-10mJ/cm	66.9 ± 1.979	13.7 ± 0.424	10.85 ± 0.215
20μM-10mJ/cm	51.9 ± 0.07	26.5 ± 0.071	19.6 ± 0.567
25mJ/cm	58.9 ± 1.345	14.5 ± 0.71	25.5 ± 0.778
5μM-25mJ/cm	48.7 ± 0.99	17.8 ± 0.283	31.95 ± 1.343
20μM-25mJ/cm	58.8 ± 0.283	15.7 ± 0.424	25 ± 1.415

Figure 5. 3 *Cell apoptosis of the combinational treatments for psoriasis – preliminary optimisation.*

*HaCaT cells were treated with either 10 or 25 mJ / cm^2 UVB on a thin layer of ice. Immediately after the exposure, cells were treated with either 5 or 20 μM of SFN. The apoptotic and necrotic cell population distribution was observed at 24h using CellEvent Caspase 3/7 Green flow cytometer assay kit, along with DAPI and live, apoptotic and necrotic cells are shown as (A, B, C, D, F) percentage of events and (E) cell events in scatter plots. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001,* ***ns*** *for not significant, relative to corresponding controls. Data shown are representative of three independent experiments. FACS images are representative.*

From the current information found in the literature and as mentioned in section 5.2.3 as well as in chapter 4, ITCs have a very complex effect on cells. ITCs are known for their indirect antioxidant effect through induction of phase II enzymes (Y. Zhang et al., 2005) and it was shown that phytochemicals, such as SFN, BITC and PEITC, suppress UVB induced inflammation on HaCaT cells (Kleszczyński et al., 2013; Shibata et al., 2010). It is shown that the concentration of BITC that are shown to have an antioxidant effect are also inducing oxidative stress. On the other hand, AITC, BITC and PEITC is shown to activate caspase 3, 8 and 9 on HaCaT cells after 6 hours of exposure (Y. Zhang et al., 2003). Although, 25 µM is shown to inhibit ROS formation (superoxide), 2-10 µM of the same phytochemical is shown to induce mitochondrial damage (Abbaoui et al., 2012; Y. Zhang et al., 2003). Therefore, it is clear that the effect of these phytochemicals strongly depends on the duration of exposure and the cell line used.

Based on the observations made so far and for the determination of the effect of UVB in combination with ITCs, a more extensive study is required using more ITCs, such as IBN, AITC, BITC and PEITC. Moreover, the effect of the most suitable combination of ITC concentration and UVB is required to be observed in a later timepoint (48h) in order to understand and evaluate the effect and its long-term effects. 72 hours were not considered for this chapter since the effect on apoptosis is detected in the first 48 hours after the exposure.

5.2.4 Cell viability and growth determination of combinational treatments of 5 ITCs with UVB.

Five ITCs (SFN, IBN, AITC, BITC and PEITC) were used for the study of the combined effect of ITCs with UVB, while in addition to the optimised conditions determined in section 5.3.3, 5 mJ / cm^2 UVB were also used. The addition of this condition was considered as it is

hypothesised that this intensity might have a similar effect on cells at 48h, as the effect of 10 mJ / cm^2 UVB at 24h. This hypothesis is based on the long-term effect of UVB and the ability to gradually reduce viability at 48 and 72 hours as presented in this study at section 4.2.1.1. Intensities less than 7 mJ / cm^2 NB UVB are shown to cause erythema on people with phenotype I (Rodríguez-Granados et al., 2017) and therefore, the minimum possible dose should also be considered. It is also hypothesised that the effect will be enhanced with the addition of ITCs, as shown so far in this study.

For the cell viability and growth determination of the combinations above, two assays were utilised, a metabolic assay using resazurin and a colorimetric assay (SRB123). Overall, the two assays gave similar response with variations at the p value. 10 mJ / cm^2 UVB, when combined with either of the four ITCs: SFN, IBN, BITC and PEITC, is shown to significantly reduce viability and growth (figures 5.4., 5.5, 5.6, 5.7, 5.8), with the exception of BITC at 24h where the resazurin assay does not show any effect of the phytochemical or the combination. This comes in contrast with the SRB assay where the effect of the combinational treatment: 63.16 ± 2.74% is significantly stronger than BITC alone: 76.8 ± 4.54% ($p<0.05$) and UVB alone: 95 ± 2.45% ($p<0.0001$) (figure 5.7).

Specifically, resazurin assay showed that the effect of 20 μM SFN + 5 mJ / cm^2 UVB on cell viability is stronger at 24h: 93.2 ± 4.45% ($p<0.05$) and at 48h: 80.1 ± 1.62 ($p<0.0001$), than UVB alone at 24h: 102.09 ± 5.33% and 48h: 96.6 ± 4.66%, similarly, the SRB assay also showed a significant difference at 24h: 62.4 ± 1.866% ($p<0.05$) with SFN alone: 76.738 ± 2.31%. The effect of 20 μM SFN+ 10 mJ / cm^2 UVB on cell viability is shown to be stronger than the 5 mJ / cm^2 UVB at 24h: 82.65 ± 0.859% ($p<0.0001$) and at 48h: 55 ± 4.39% when compared to UVB at 24h: 96 ± 1.87% and 48h: 84.3 ± 3.956%, and SFN at 48h: 86.88 ± 2.27%, ($p<0.0001$). For the same combination, the SRB assay showed a stronger response with the combination at 42.827 ± 1.68% at 24h, when compared to UVB at 24h: 95 ± 2.45%,

and SFN at 24h: 69.65 ± 2.82%, while 48h are showing the same effect with 46.1 ± 1.53% compared to the relative controls ($p<0.0001$).

IBN was studied in the same conditions and resazurin assay showed that when combined with 5 mJ / cm^2 UVB, cell viability significantly decreased at 76.17 ± 1.763%, when compared to UVB at 24h ($p<0.0001$) and IBN alone at 24h: 87.55 ± 1.378% ($p<001$). Similarly, at 48h the combination's viability was at 40.81 ± 2.86%, which is significantly lower than UVB ($p<0.0001$) and IBN: 80.26 ± 4.065% ($p<0.0001$). SRB assay did not agree with resazurin assay, where IBN alone: 73.1 ± 0.898% was not different ($p>0.05$) than the combination 63.4 ± 3.65%. For the combination of IBN with 10 mJ / cm^2 UVB, both assays agree on the response and the significance ($p<0.0001$) of the effect, between the combinational treatment and the relative controls. However, the effect shown by the SRB assay is significantly stronger than the effect shown by the resazurin assay, where the combination cell viability was at 41.53 ± 1.96% at 24h and 43.95 ± 0.294% at 48h, and cell growth at 70.53 ± 1.735% at 24h and 25.42 ± 2.51% at 48h ($p<0.0001$), respectively. The difference observed between the two assays at 48h, is significant and shows that the treatment is a good growth inhibitor, which indicates multifactorial effect on cell growth and viability. When the data were taken into account with the pictures taken using ZOE imaging (figures 5.4E, 5.5E, 5.7E, 5.8E), the data collected using the resazurin assay appear to reflect with the image of the cells, with the 24h showing enough cells being attached on the bottom of the plate, while 48h present the severity of the effect.

There was no effect of AITC when combined with UVB or alone on cell viability in both assays (figure 5.6). The same observations were made when viability was determined using resazurin assay at the combination of BITC with 5 mJ / cm^2 UVB at both timepoints. In the contrary, SRB assay showed a significant reduction of cell growth after the combinational treatment at 24h: 73.3 ± 4.18%, when compared to UVB ($p<0.0001$) or BITC alone: 76.8 ±

4.54% ($p<0.001$) and at 48h: 79.6 ± 3.67% when compared to the relative UVB control ($p<0.01$). The combination of 20 μM BITC and 10 mJ / cm^2 UVB showed a significant effect on cell viability at 48h: 78.1 ± 6.61% with either of the relative controls ($p<0.0001$) when using the resazurin assay, while SRB assay showed a stronger effect of the phytochemical with the combination reducing cell viability at 24h: 63.16 ± 2.74%, when compared to the controls, UVB ($p<0.0001$), BITC: $p<0.05$) and at 48h: 69.5 ± 1.2% , when compared to the UVB ($p<0.01$) and the BITC ($p<0.0001$) (figure 5.7).

Finally, PEITC is shown to have an effect in all conditions and assays used. In detail, when the phytochemical was combined with 5 mJ / cm^2 UVB and the cell viability was determined using the resazurin assay, a decrease of viability was observed at 24h: 89.73 ± 4.176% ($p<0.001$), compared to the UVB control, and at 48h: 69.64 ± 4.589% ($p<0.0001$), compared to the relative controls, UVB and PEITC alone: 87.74 ± 2.1%. SRB assay, agreed with the effect of the combination with viability at 24h: 62.47 ± 6.31% ($p<0.0001$) when compared to the relative UVB control and at 48h: 71.6 ± 3.98% ($p<0.001$) when compared to the relative UVB control. The effect of PEITC alone: 69.73 ± 3.944% (24h), 81.45 ± 0.34% (48h) was not significantly different than the combination with 5 mJ / cm^2 UVB, indicating that either the phytochemical's effect is overlapping with the effect of UVB intensity or that the latter is not effective enough, to enhance the effect of the former. This hypothesis is confirmed when 10 mJ / cm^2 UVB were combined with PEITC and a significant difference was observed using the resazurin assay at 24h between the combination: 76.88 ± 1.888% and the relative UVB sample ($p<0.001$) and the PEITC control: 92.9 ± 2.39% ($p<0.0001$) and at 48h: 54.84 ± 3.684% ($p<0.0001$). SRB assay showed that the proposed combination reduced viability at 24h at 43.036 ± 2.1% ($p<0.0001$), when compared to the relative controls, and at 48h at 62.7 ± 3.47% ($p<0.001$). Based on the results of the metabolic assay, PEITC alone and in combination with 10 mJ / cm^2 UVB is shown to be less effective on cell viability at 24h and the results, when compared to the SRB assay, are significantly different.

Also, when the results are compared with the visual image of the cells after the treatment (figure 5.4 5E), the SRB assay results appears to be agree more with the visual image, rather than the resazurin assay results. This is possibly due to an increase of cell proliferation rate, linked to increase of ROS that could lead to increased metabolism of the dye and therefore false positive results. However, the difference of the results between the two assays is observed in all ITCs used and especially in BITC + 20 mJ / cm^2 UVB at 24 hours were resazurin assay did not show a significant effect, while SRB assay showed an approximately 40% drop in growth, while BITC alone effect is also significantly different as described above (figure 5.8). For the combinational treatments, when the results of the two assays were compared, resazurin assay is shown to give overall higher values by approximately 30%. Specifically, the effect of SFN, BITC and PEITC in combination with 5 mJ / cm^2 UVB, and SFN, IBN, BITC, PEITC in combination with 10 mJ / cm^2 UVB at 24h, was stronger by approximately 30% when using the SRB assay, while IBN in combination with 5 mJ / cm^2 UVB at 48h and in combination with 10 mJ / cm^2 UVB at 24h, showed a stronger effect by approximately 20% when using the resazurin assay. This difference between the two assays indicates the potential effect of the treatment on cell cycle, since SRB assay measures cell growth, which could be inhibited but not necessarily escalate to apoptosis. Moreover, it is possible that the metabolism of the cells has increased due to stress and therefore increase the metabolism of resazurin to resorufin, giving false positive results. Further studies using CalcuSyn software are required in order to measure whether the effect of UVB and ITCs on HaCaT cells is synergistic.

It is widely known that UVB alone induces ROS formation and leads to reduction of cell viability. In a study where HaCaT cells were exposed to four intensities of UVB (5 ,10, 20, 30 mJ / cm^2) a significant reduction of approximately 20-25% in cell viability was observed in the two highest doses, while the lower doses agree with our findings (Mammone et al., 2000). A significant effect was shown, when HaCaT cells and fibroblasts were exposed to

10, 20 and 30 mJ / cm^2 UVB, the viability dropped at 13%, 29% and 33% in fibroblasts and 19.5%, 33% and 51% in HaCaT cells (Farrukh et al., 2014). The latter study presented a drop of cell viability, significantly higher than our findings and the previous study by approximately 15%. Also, ITCs are known to have a biphasic effect on cell viability, as shown in a study where the effect of SFN was evaluated it was shown to increase cell viability in low concentration up to 10μM, while having the opposite effect on UVB models of 25 and 50 mJ / cm^2 UVB at 24h (Shibata et al., 2010). Wanger 2010 exposed HaCaT cells in a wide range of SFN concentrations and the phytochemical was shown to be toxic over 25 μmol / l (Wagner et al., 2010). In another study where the same cell line was treated with SFN and IBN, cell viability showed a significant decrease at concentrations over 10μM at 24 and 48h, with EC50 for SFN at 24h: 21.6 ± 2 μM and at 48h: 23.3 ± 2.4 μM, and for IBN at 24h: 23.56 ± 4.4 μM and at 48h: 23.6 ± 1.9 μM (Mitsiogianni, Trafalis, et al., 2020). Similarly, when SFN, PEITC and BITC were used for the treatment of HaCaT cells at various concentrations, a significant decrease in cell viability was shown at concentrations over 10 μM for SFN and PEITC and over 5 μM for BITC at 24 and 48h. 20 μM of BITC was used for the treatment of the same cell line in another study, and a significant decrease in 24h (approximately 10-20%) and 48h (approximately 20-30%) was shown (S. H. Huang et al., 2012), findings that agree with the results of this study. In the same study of Huang et al., allyl isothiocyanate was not shown to have any effect of HaCaT cell viability at 24h and 48h at concentrations 2.5 – 50 μM, a study that agrees with our findings. In a study where the protective effect of SFN was studied in a UVB induced model (200 mJ / cm^2 UVB), it was shown to increase cell viability at 24h at concentrations 0.5, 1, 2.5, 5 and 10 μM and at 48h at concentrations 1, 2.5, 5 and 10 μM. The cells in this study were pre-treated with SFN instead of post-treatment that is used in our experiments (Al-barnawi, 2018) This is a parameter that could significantly change the effect of ITCs on cells and it is possible that extended ITC-exposure of the cells might lead to increased cytotoxicity. Finally, when this

cell line was exposed to PEITC, an IC_{50} of 40 μM was shown at 24h and 48h. Overall, it is clear that the conditions selected for the experiments of the current study are both mild conditions that do not cause severe viability reduction to the cells, while their combination is showing an either enhanced effect or an additive effect on cell viability.

As hypothesised, the combination of ITCs with 5 mJ / cm^2 UVB has a milder effect at 24h, compared to 48h, while the effect shown at 48h is similar to the effect of ITCs with 10 mJ / cm^2 UVB at 24h. Moreover, the effect of the latter combination on cell viability remains stable between 24h and 48h, without showing any further reduction in cell viability through time. Due to the above observation, it was considered necessary to keep two conditions for the further experiments of this chapter: ITCs + 10 mJ / cm^2 UVB at 24h and ITCs + 5 mJ / cm^2 UVB at 48h, for reasons of comparison and better understanding of the mechanisms by which the effect of UVB, when combined with ITCs is more enhanced. Generally, it is shown that isothiocyanates have a variable effect on cell viability that depends on the duration of the treatment and the cell line (Y. Zhang et al., 2005). Short treatment of cells with low doses of ITCs are shown to have a beneficial effect on cell proliferation by protecting cells from oxidative stress that could lead to cell cycle arrest, while it is shown that prolonged exposure of cells to ITCs arrest cell cycle and induce ROS formation leading to a reduction in cell proliferation. Although, HaCaT cells are generally shown to be more resistant to the effect of ITCs compared to other cell lines (Mitsiogianni, Trafalis, et al., 2020), they are the most suitable cell line for the preliminary experimentation on psoriasis, a disease characterised from increased proliferation of keratinocyte cells.

For further investigation of the mechanisms of the above effect, apoptosis was also investigated through the activation of caspase 3/7. Although AITC was not shown to affect cell viability of HaCaT cells, it was not excluded from our experiments as current literature suggests an effect on viability on human prostate cancer cells (Xiao et al., 2003) and human

cervical cancer HeLa cells (Qin et al., 2018), increase of G_2/M cell cycle on human prostate and bladder cancer cells, induction of apoptosis in bladder carcinoma cells, mitochondrial dysfunction and cell cycle arrest (Geng et al., 2011; Savio et al., 2014; Xiao et al., 2003). Therefore, we considered it necessary to observe the phytochemical's effect on apoptotic pathways and cell cycle, along with the effect of the combination of it with UVB.

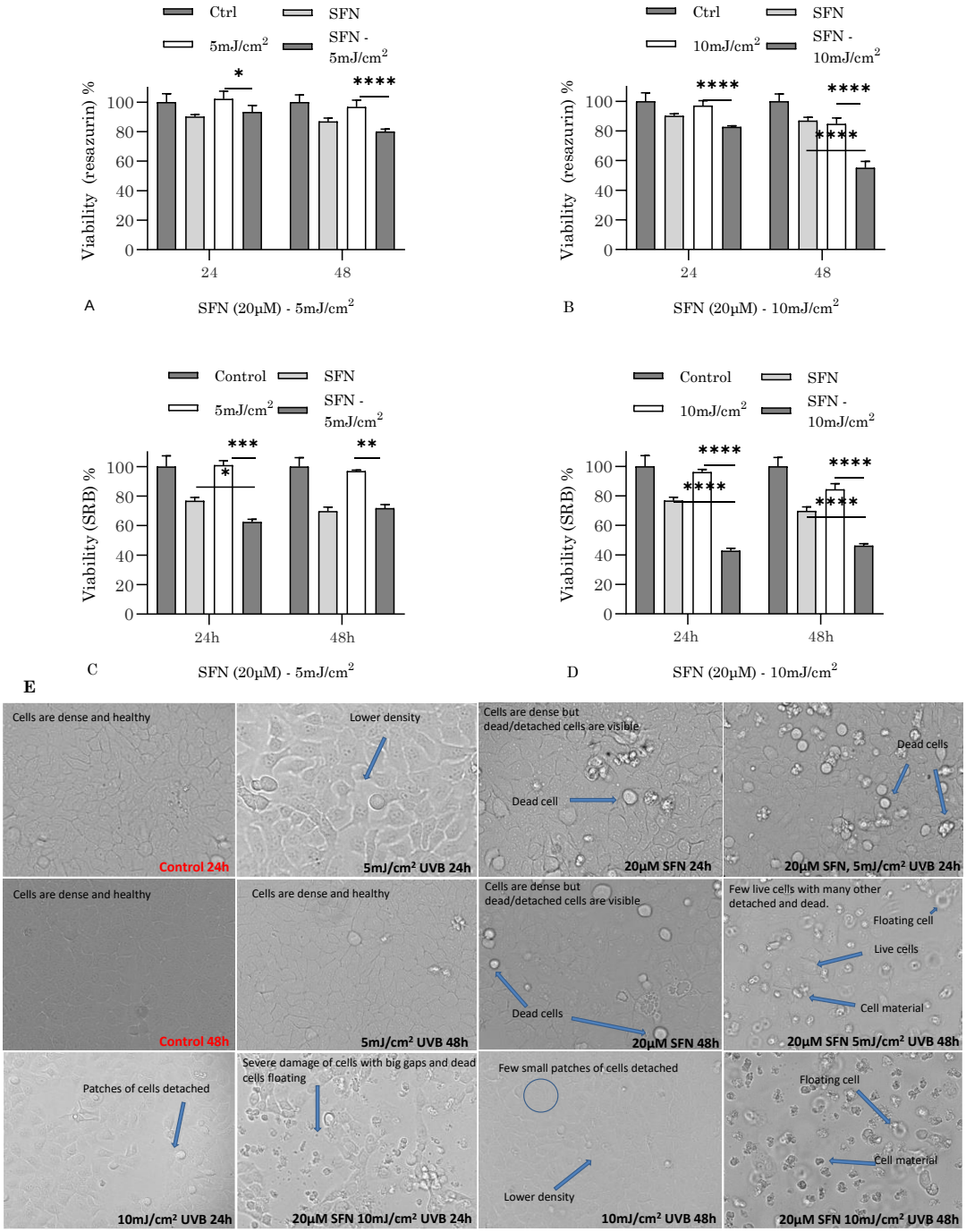


Figure 5. 4 *Cell viability and growth determination of SFN and UVB on HaCaT cells. Cells were exposure to either 5 or 10 mJ / cm2 UVB on a thin layer of ice and treatment with 20µM of SFN followed immediately after the UV exposure. Cell viability was measured using (A, B) the resazurin assay and growth using (C, D) the SRB assay at 24h or 48h, respectively. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001, ns for not significant, relative to corresponding controls. Data shown are means of ±SD of three replicates from 3 independent experiments. E: Microscopic representation of cells using ZOE Fluorescent Cell Imager.*

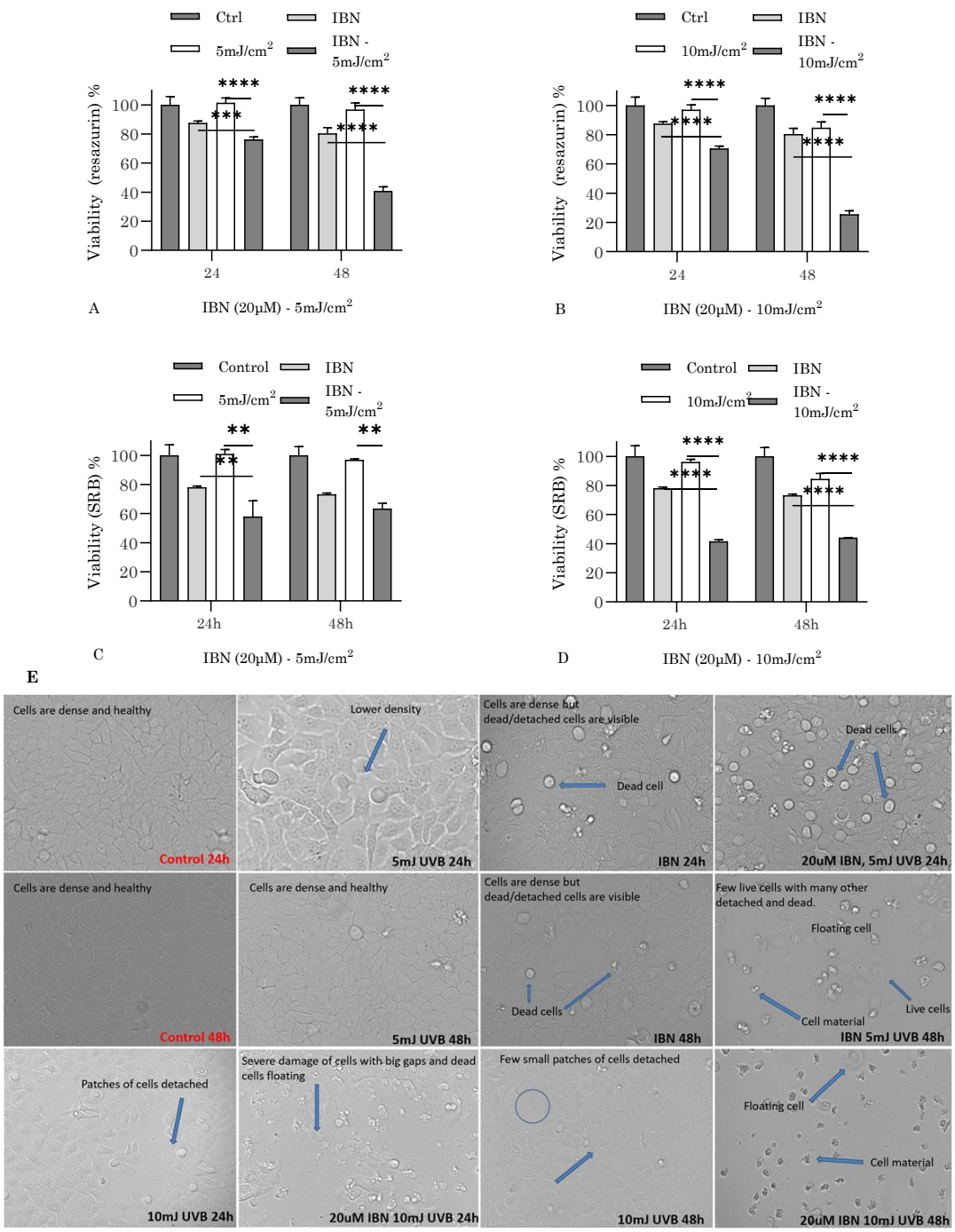


Figure 5. 5 *Cell viability and growth determination of IBN and UVB on HaCaT cells. Cells were exposure to either 5 or 10 mJ / cm2 UVB on a thin layer of ice and treatment with 20µM of IBN followed immediately after the UV exposure. Cell viability was measured using (A, B) the resazurin assay and growth using (C, D) the SRB assay at 24h or 48h, respectively. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001, ns for not significant, relative to corresponding controls. Data shown are means of ±SD of three replicates from 3 independent experiments. E: Microscopic representation of cells using ZOE Fluorescent Cell Imager.*

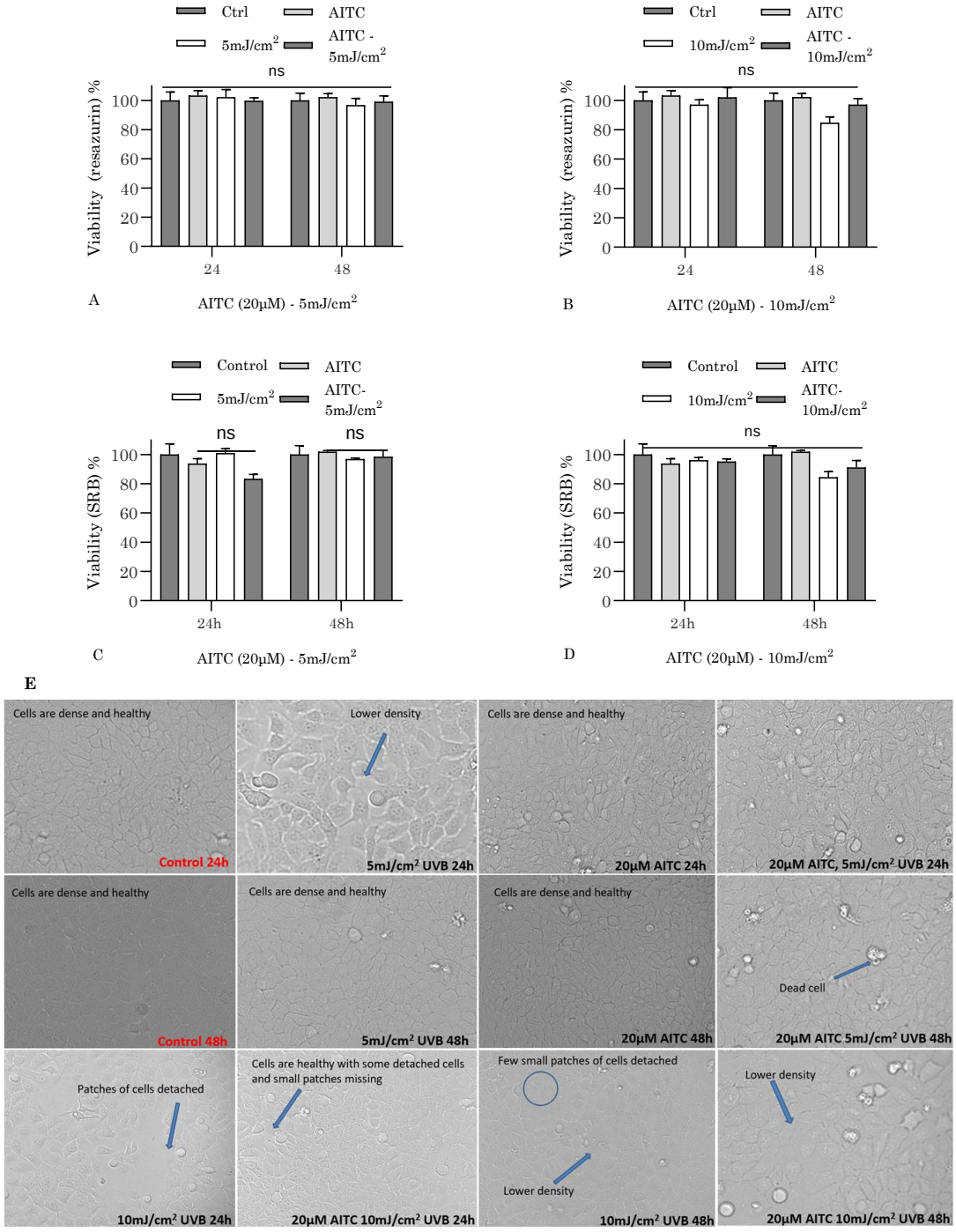


Figure 5. 6 *Cell viability and growth determination of AITC and UVB on HaCaT cells Cells were exposure to either 5 or 10 mJ / cm2 UVB on a thin layer of ice and treatment with 20µM of AITC followed immediately after the UV exposure. Cell viability was measured using (A, B) the resazurin assay and growth using (C, D) the SRB assay at 24h or 48h, respectively. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001, ns for not significant, relative to corresponding controls. Data shown are means of ±SD of three replicates from 3 independent experiments. E: Microscopic representation of cells using ZOE Fluorescent Cell Imager.*

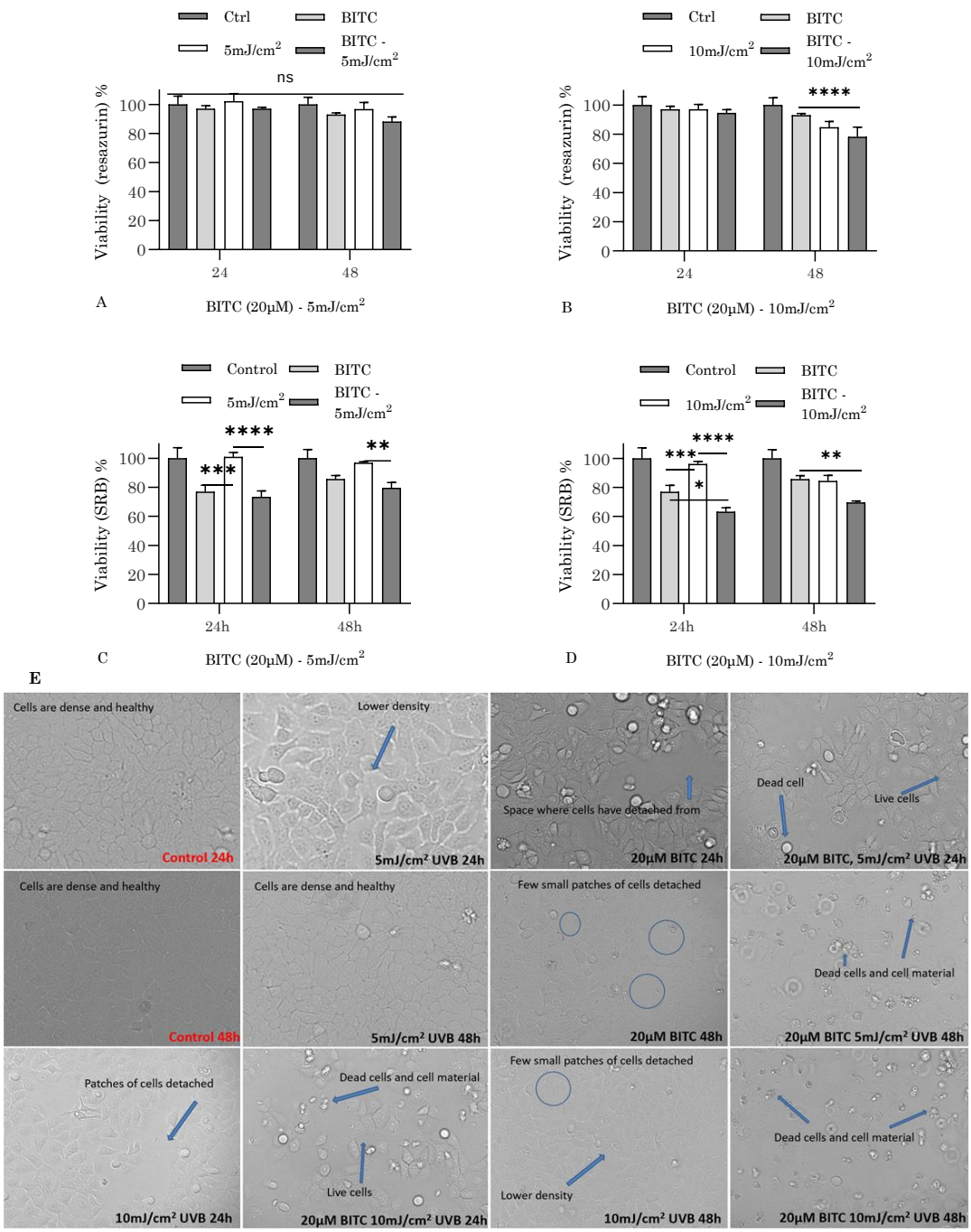


Figure 5. 7 *Cell viability and growth determination of BITC and UVB on HaCaT cells Cells were exposure to either 5 or 10 mJ / cm2 UVB on a thin layer of ice and treatment with 20µM of BITC followed immediately after the UV exposure. Cell viability was measured using (A, B) the resazurin assay and growth using (C, D) the SRB assay at 24h or 48h, respectively. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001, ns for not significant, relative to corresponding controls. Data shown are means of ±SD of three replicates from 3 independent experiments. E: Microscopic representation of cells using ZOE Fluorescent Cell Imager.*

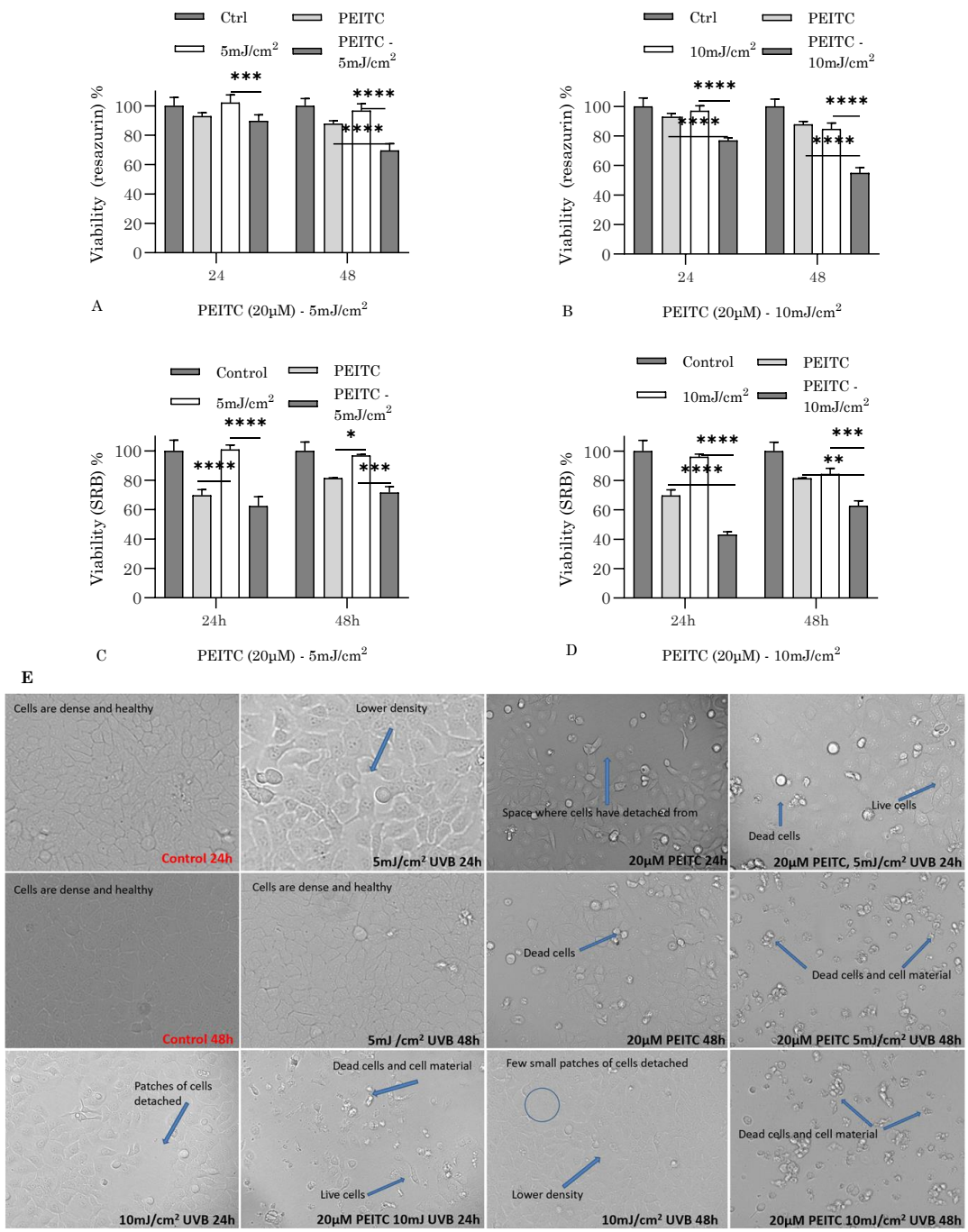


Figure 5. 8 *Cell viability and growth determination of PEITC and UVB on HaCaT cells Cells were exposure to either 5 or 10 mJ / cm2 UVB on a thin layer of ice and treatment with 20µM of PEITC followed immediately after the UV exposure. Cell viability was measured using (A, B) the resazurin assay and growth using (C, D) the SRB assay at 24h or 48h, respectively. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001, ns for not significant, relative to corresponding controls. Data shown are means of ±SD of three replicates from 3 independent experiments. E: Microscopic representation of cells using ZOE Fluorescent Cell Imager.*

5.2.5 Apoptosis determination of combinational treatments of 5 ITCs with UVB

Two combinational treatments of 20 μM ITCs and two intensities of UVB at two different timepoints were selected for the determination of apoptosis. After the treatment of HaCaT cells with 10 and 5 mJ / cm^2 UVB and 20 μM of either of the 5 ITCs, apoptosis was evaluated at 24h and 48h, respectively. Overall, four out of five ITCs had an effect on apoptotic cells when combined with either 5 or 10 mJ / cm^2 UVB, and on necrotic cells for some ITCs at both conditions.

Specifically, it was shown that when SFN was combined with 10 mJ / cm^2 UVB, apoptotic cells were significantly higher: 25.64 ± 5.33% ($p<0.0001$) than all relative controls (cells alone, UVB alone and ITC alone), with UVB at: 10.1 ± 2.12% and SFN alone at: 10.2 ± 3.3%. Similar pattern is observed at 5 mJ / cm^2 UVB at 48h, where apoptotic cells of the combination with SFN are higher: 23.3 ± 3.96% ($p<0.0001$) when compared with UVB: 9.8 ± 4% and SFN alone: 13.87 ± 3.02%, and necrotic cells: 41.375 ± 4.73% ($p<0.0001$), when compared with UVB: 24.4 ± 4.95% (figure 5.9).

The combination of IBN with 10 mJ / cm^2 UVB showed an effect on apoptotic cells: 30.50 ± 1.27% ($p<0.0001$), when compared to the controls, UVB and IBN alone: 14.6 ± 0.71% and the combination with 5 mJ / cm^2 UVB showed an effect on apoptotic cells: 24.65 ± 65% ($p<0.0001$), when compared to UVB and IBN alone: 15.45 ± 1.77%, and on necrotic cells: 46.15 ± 5.87% ($p<0.0001$), when compared to either of the controls, with IBN alone at: 36.65 ± 0.49% (figure 5.10).

When AITC was combined with 10 mJ / cm^2 UVB, it showed an effect on apoptotic cells: 18.55 ± 2.47% ($p<0.05$), when compared with AITC alone: 7.3 ± 1.84% and necrotic cells: 20.75 ± 1.2% ($p<0.05$), when compared with UVB: 25.54 ± 3.53%. There was no significant effect of AITC and 5 mJ / cm^2 UVB when combined, on either apoptotic or necrotic cells, which follows the pattern seen in viability determination in section 5.2.4 (figure 5.11).

The combination of BITC with 10 mJ / cm^2 UVB, showed a significant effect on apoptotic cells: 25 ± 0.14% ($p<0.0001$), when compared to BITC alone: 10.95 ± 3.61%, and on necrotic cells: 29.7 ± 1.13% ($p<0.01$), when compared to BITC alone: 16.85 ± 2.62%. Although the same effect is observed with the other intensity: 5 mJ / cm^2 UVB measured at 48h for apoptotic cells: 21.85 ± 2.05% ($p<0.0001$), with BITC alone at: 13.8 ± 1.27%, necrotic cells are not shown to be increased, but this observation is not significant (figure 5.12).

PEITC is shown to have the strongest effect ($p<0.0001$) of all ITCs, when combined with either intensities of UVB. At PEITC + 10 mJ / cm^2 UVB, apoptotic cells are shown to increase at: 22.8 ± 2.8%, when compared with PEITC alone: 8.9 ± 2.12%, and necrotic cells at: 44.8 ± 3.3%, when compared with PEITC alone: 30.65 ± 4.60%. At PEITC + 5 mJ / cm^2 UVB, apoptotic cells are shown to increase at: 21.5 ± 2.7%, when compared with UVB and PEITC alone: 28.65 ± 2.19% (figure 5.13).

It is generally observed that apoptotic cells between the two controls, PEITC alone and UVB, are not statistically different ($p>0.05$). However, the combination is shown to work at least additively and enhance the effect of the individual conditions. Moreover, when the combinations were compared, AITC + 10 mJ / cm^2 UVB apoptotic cells were significantly ($p<0.001$) lower than IBN + 10 mJ / cm^2 UVB ($p<0.001$) apoptotic cells, while PEITC + 10 mJ / cm^2 UVB necrotic cells were found significantly higher ($p<0.0001$), when compared with either ITC combination; SFN + 10 mJ / cm^2 UVB, IBN + 10 mJ / cm^2 UVB, AITC + 10 mJ / cm^2 UVB and BITC + 10 mJ / cm^2 UVB. Overall, the effect of the combination of BITC and PEITC with 10 mJ / cm^2 UVB is shown to be stronger, with necrotic cells being higher than the other ITC combinations.

AITC + 5 mJ / cm^2 UVB apoptotic cells were significantly lower than SFN + 5 mJ / cm^2 UVB ($p<0.05$) and IBN + 5 mJ / cm^2 UVB ($p<0.05$). However, AITC + 5 mJ / cm^2 UVB

apoptotic cells were not significantly less than BITC + 5 mJ / cm^2 UVB and PEITC + 5 mJ / cm^2 UVB apoptotic cells, an observation that comes in contrast with the results presented above. Considering that AITC had no effect on cell viability as described in section 5.2.4, the mild effect in apoptotic and necrotic cells shown above, as well as the strong effect of BITC and PEITC, alone or in combination with UVB, it is hypothesised that the last two phytochemicals have such an effect that cells have already gone through the apoptosis and necrosis phase and became cell material (debris) that was excluded from the analysis. This hypothesis was also confirmed from the results collected from the analysis from the flow cytometer, where debris is shown to be increased compared to AITC combinations. This is also seen from the significant increase of necrotic cells observed in PEITC combinations. Notably, both UVB intensities at their timepoint, significantly enhanced the effect of ITCs on caspase 3/7 activity, while necrotic cells are also significantly affected. SFN + 5 mJ / cm^2 UVB and IBN + 5 mJ / cm^2 UVB necrotic cells were significantly higher than AITC + 5 mJ / cm^2 UVB ($p<0.0001$) and BITC + 5 mJ / cm^2 UVB ($p<0.05$ and $p<0.0001$ respectively) necrotic cells. PEITC + 5 mJ / cm^2 UVB effect increased necrotic cells significantly, when compared with all other combinations: SFN + 5 mJ / cm^2 UVB ($p<0.0001$), IBN + 5 mJ / cm^2 UVB ($p<0.01$), AITC + 5 mJ / cm^2 UVB ($p<0.0001$) and BITC + 5 mJ / cm^2 UVB ($p<0.0001$).

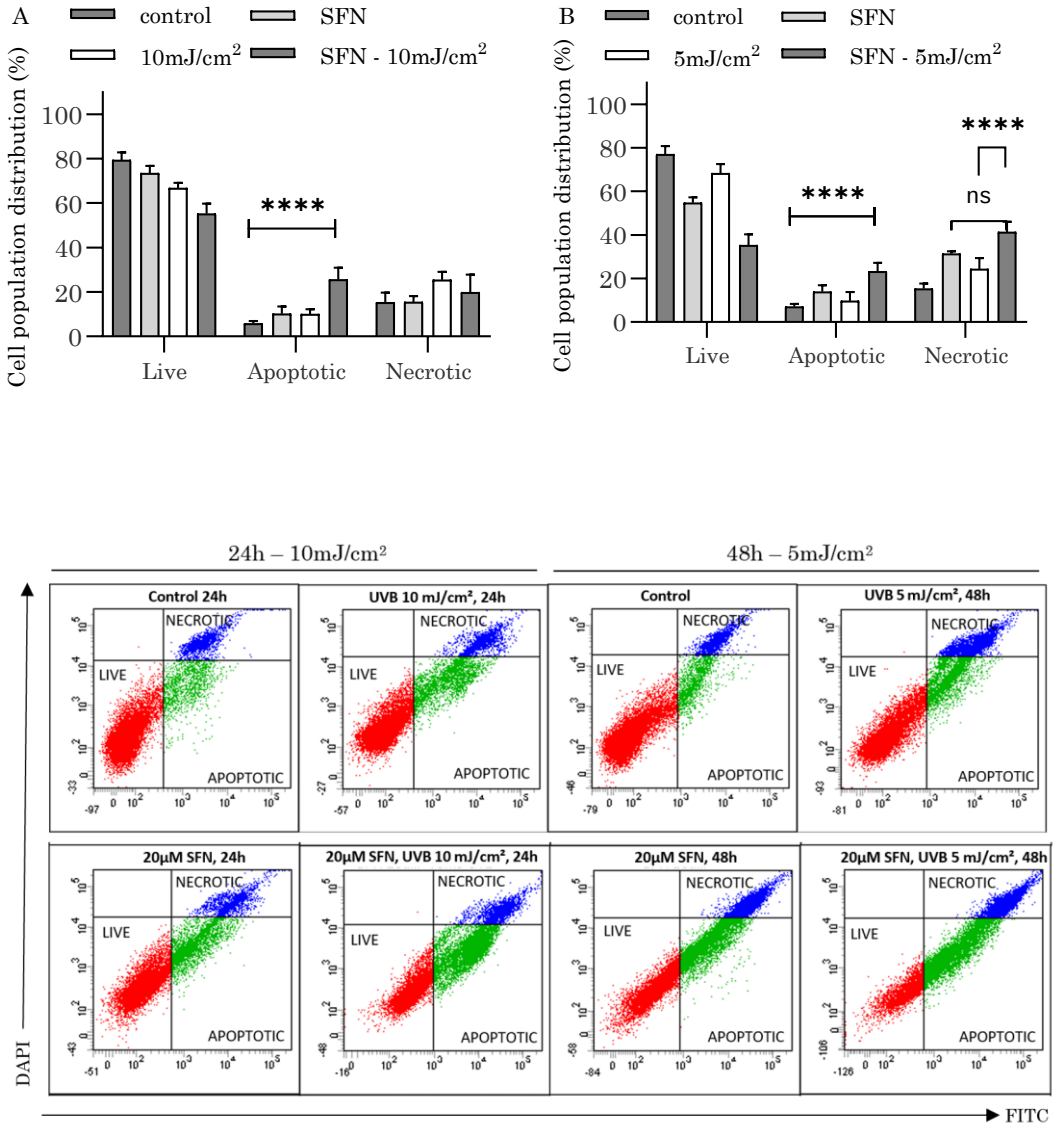


Figure 5. 9 *Cell apoptosis of the combinational treatments with SFN for psoriasis HaCaT cells were treated with either 5 or 10 mJ / cm^2 UVB on a thin layer of ice. Immediately after the exposure, cells were treated with 20 µM SFN. The apoptotic and necrotic cell population distribution was observed at using CellEvent Caspase 3/7 Green flow cytometer assay kit, along with DAPI and live, apoptotic and necrotic cells are shown are shown as percentage of events (A: 10 mJ / cm^2 , B: 5 mJ / cm^2) and (C) cell events (20,000 events/second) in scatter plots. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001,* ***ns*** *for not significant, relative to corresponding controls. Data shown are means of ±SD of three replicates from 3 independent experiments. FACS images are representative.*

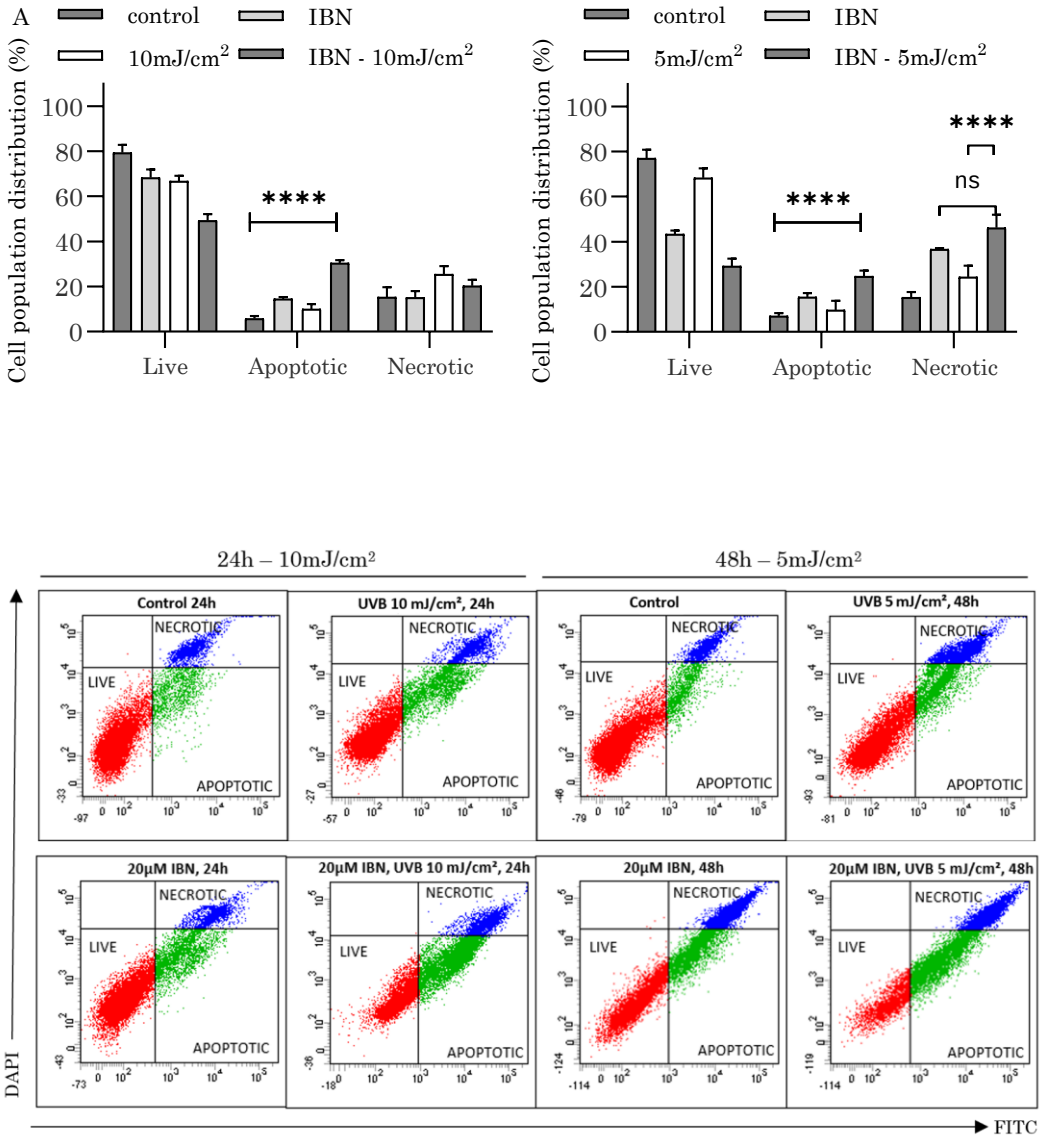


Figure 5. 10 *Cell apoptosis of the combinational treatments with IBN for psoriasis HaCaT cells were treated with either 5 or 10 mJ / cm^2 UVB on a thin layer of ice. Immediately after the exposure, cells were treated with 20 µM IBN. The apoptotic and necrotic cell population distribution was observed at using CellEvent Caspase 3/7 Green flow cytometer assay kit, along with DAPI and live, apoptotic and necrotic cells are shown are shown as percentage of events (A: 10 mJ / cm^2 , B: 5 mJ / cm^2) and (C) cell events (20,000 events/second) in scatter plots. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001,* ***ns*** *for not significant, relative to corresponding controls. Data shown are means of ±SD of three replicates from 3 independent experiments. FACS images are representative.*

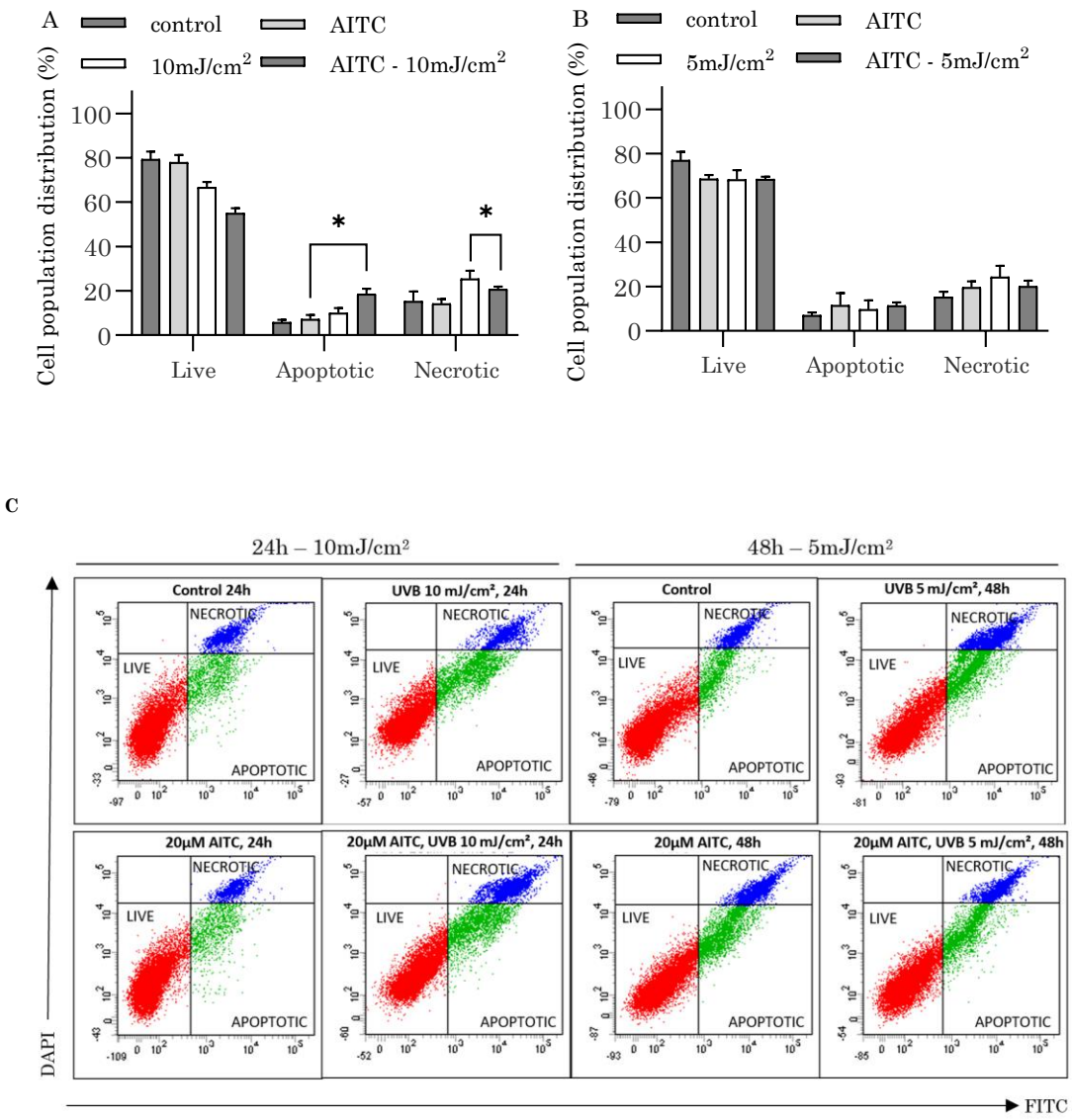


Figure 5. 11 *Cell apoptosis of the combinational treatments with AITC for psoriasis HaCaT cells were treated with either 5 or 10 mJ / cm^2 UVB on a thin layer of ice. Immediately after the exposure, cells were treated with 20 µM AITC. The apoptotic and necrotic cell population distribution was observed at using CellEvent Caspase 3/7 Green flow cytometer assay kit, along with DAPI and live, apoptotic and necrotic cells are shown are shown as percentage of events (A: 10 mJ / cm^2 , B: 5 mJ / cm^2) and (C) cell events (20,000 events/second) in scatter plots. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001,* ***ns*** *for not significant, relative to corresponding controls. Data shown are means of ±SD of three replicates from 3 independent experiments. FACS images are representative.*

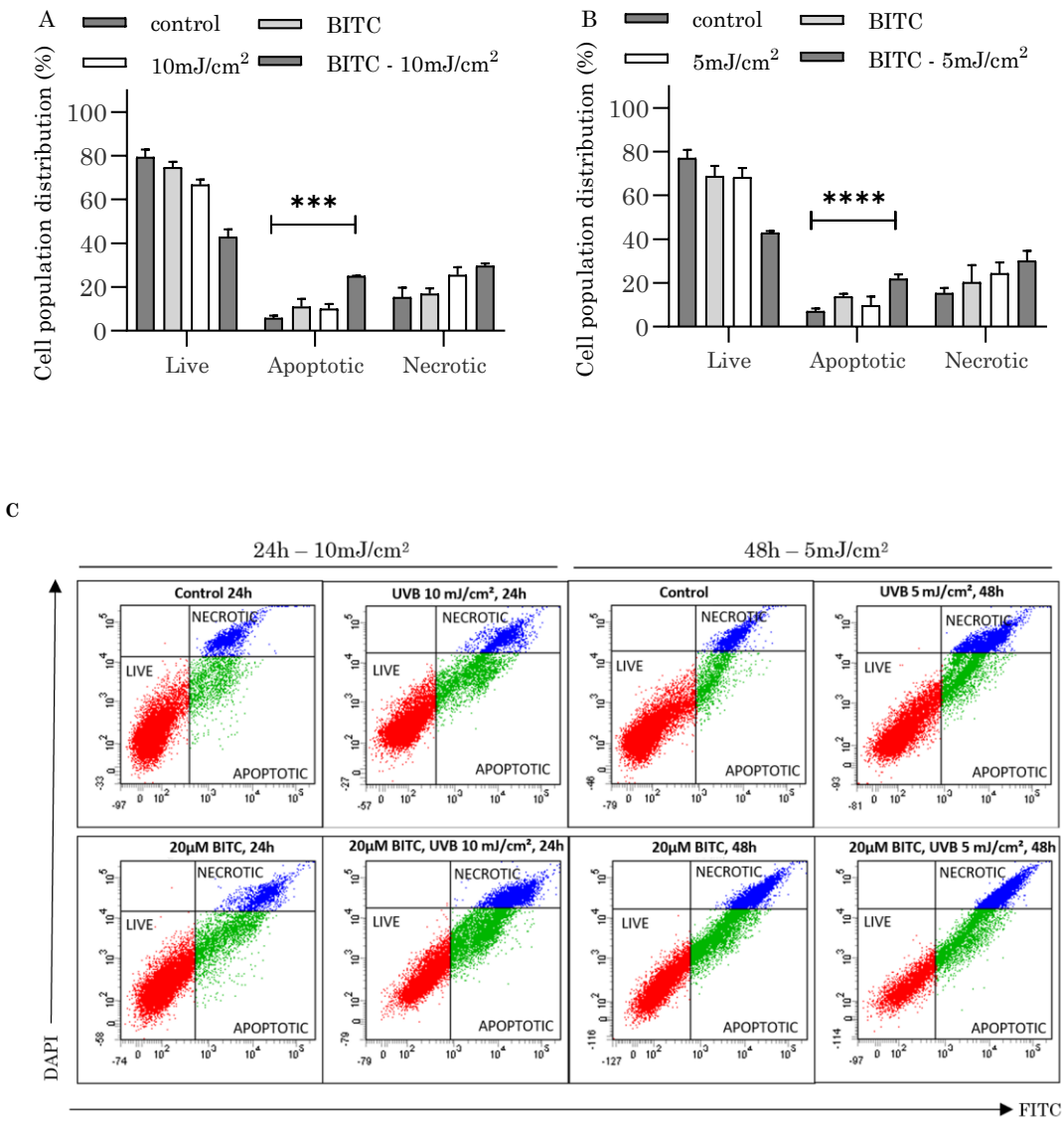


Figure 5. 12 *Cell apoptosis of the combinational treatments with BITC for psoriasis HaCaT cells were treated with either 5 or 10 mJ / cm^2 UVB on a thin layer of ice. Immediately after the exposure, cells were treated with 20 µM BITC. The apoptotic and necrotic cell population distribution was observed at using CellEvent Caspase 3/7 Green flow cytometer assay kit, along with DAPI and live, apoptotic and necrotic cells are shown are shown as percentage of events (A: 10 mJ / cm^2 , B: 5 mJ / cm^2) and (C) cell events (20,000 events/second) in scatter plots. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001,* ***ns*** *for not significant, relative to corresponding controls. Data shown are means of ±SD of three replicates from 3 independent experiments. FACS images are representative.*

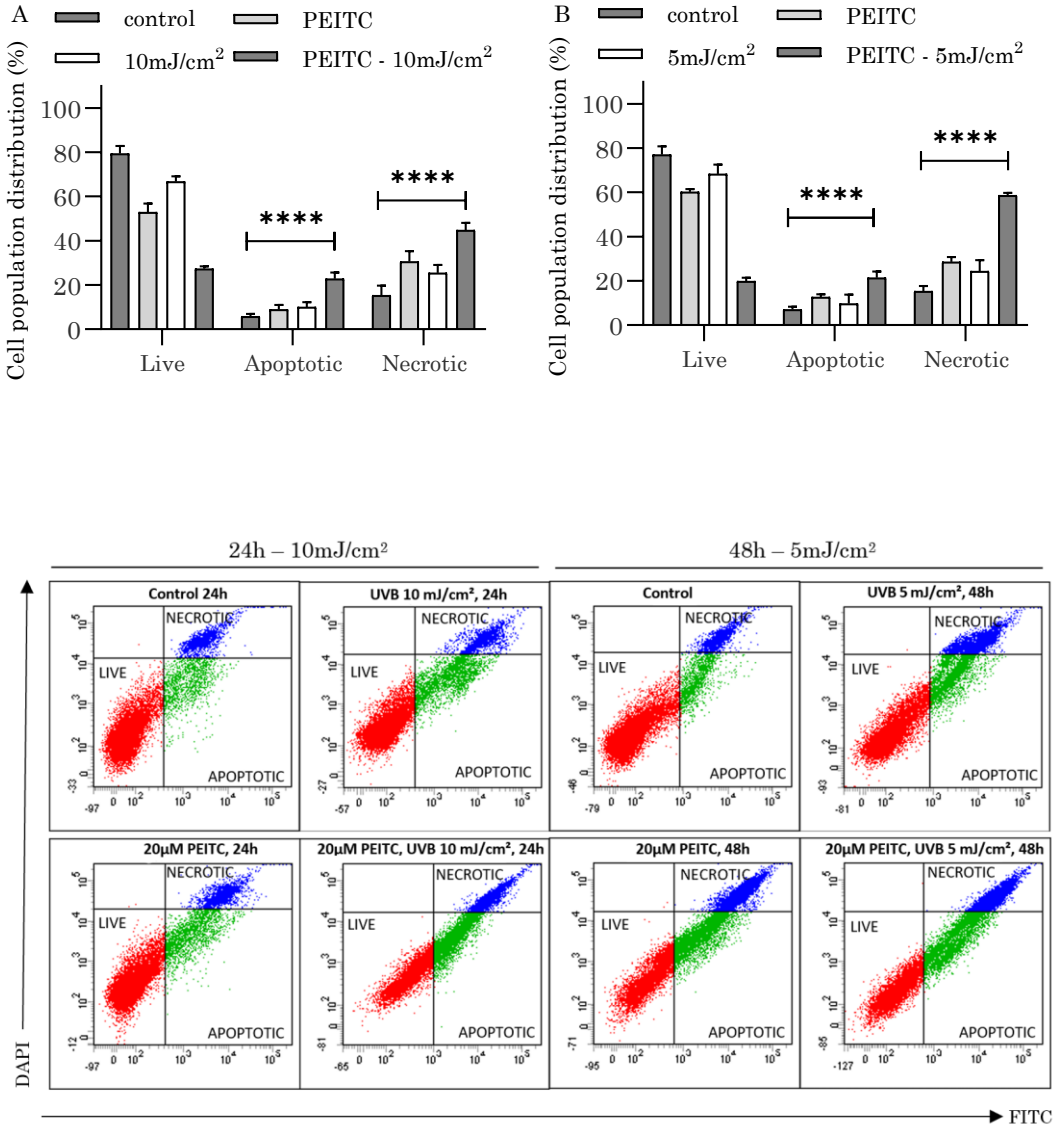


Figure 5. 13 *Cell apoptosis of the combinational treatments with PEITC for psoriasis HaCaT cells were treated with either 5 or 10 mJ / cm^2 UVB on a thin layer of ice. Immediately after the exposure, cells were treated with 20 µM PEITC. The apoptotic and necrotic cell population distribution was observed at using CellEvent Caspase 3/7 Green flow cytometer assay kit, along with DAPI and live, apoptotic and necrotic cells are shown are shown as percentage of events (A: 10 mJ / cm^2 , B: 5 mJ / cm^2) and (C) cell events (20,000 events/second) in scatter plots. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001,* ***ns*** *for not significant, relative to corresponding controls. Data shown are means of ±SD of three replicates from 3 independent experiments. FACS images are representative.*

UVB alone is shown to induce apoptosis (A. Chen et al., 2015) through ROS induction that activates p38, MAPK and BAX, that lead to increase of mitochondrial potential and eventually an increase in caspase 9 and 3 that are the late apoptosis biomarkers (Van Laethem et al., 2004). However, as UVB induces apoptosis *via* multiple pathways, caspase 3/7 activation is an indicator of late apoptosis and is not specific. In a study, 30 mJ / cm^2 of UVB were shown to increase apoptosis in various ways, such as Poly [ADP-ribose] polymerase 1 (PARP) cleavage or suppression and FLICE activity reduction (protein markers of apoptosis) (Mammone et al., 2000). PARP are proteins that are involved in DNA repair and are cleaved by caspases (Chaitanya et al., 2010), while FLICE are inhibitory proteins that modulate caspase activation. A study that involved exposure of HaCaT cells in 5, 10, 20 and 30 mJ / cm^2 UVB, showed the complex effect of UVB with the lowest doses (5 and 10 mJ / cm^2 UVB) decreased PARP activity, while the opposite was observed for the highest doses (Mammone et al., 2000). Moreover, FLICE activity was reduced at the lowest doses, while Fas receptor (receptor that leads to apoptosis) was only increase at 5 mJ / cm^2 UVB and reduced from that intensity on-wards. Therefore, it is hypothesised that one pathway of apoptosis observed, after the combinational treatment, is through Fas increase, at least for the lower dose of 5 mJ / cm^2 UVB used in this chapter. Moreover, the above doses of UVB are shown to decrease Keratin K1, K10 and involucrin expression, while doubling of the apoptotic cells was observed at 2-10 mJ / cm^2. Another study where 500 J / m^2 UVB were used on HaCaT cells, showed that this intensity increased caspase 3, 8 and 9 activation, cytochrome C and fragments of PARP, while experiments of the mentioned study also showed that caspase 8 and 9 are activated directly by cross linkage of death receptors (Takasawa et al., 2005). In a study where the effect of SFN and PEITC on a UVB model (300 mJ / cm^2 UVB) using HaCaT cells, was evaluated, it was shown that depending on the dose of isothiocyanate used, the effect on apoptosis differed (Kleszczyński et al., 2013). Specifically, 5 μM of SFN increased caspase 3 at 24h and 48h, while 10 μM and 25 μM

reduced caspase 3 at 24h and 10 μM reduced caspase 3 at 48h as well. PEITC only increase caspase 3 at 24h at 25 μM, while 48h showed a decrease in all concentrations. Notably, the two isothiocyanates did not affect the activation of caspase 3 when used on the cells with the absence of UVB, an observation that does not agree with our findings. Moreover, BITC is shown to give rise to caspase 8,9 and 4 when used on A375 human melanoma cells and PEITC to increase caspase 9, while SFN was shown to have no effect on caspases (Mantso et al., 2016). In the same study, western blot analysis showed that all three isothiocyanates reduced procaspase 3 expression levels, which agrees with our findings, while only PEITC reduced procaspase 7 expression levels. From the three phytochemicals, BITC was shown to be more potent, followed by PEITC and SFN, while our findings show PEITC and IBN to be the most potent of the five isothiocyanates studied. Although these results suggest difference between the current work and the literature, it is important to note that ITCs are shown to have variable effect depending on the cell line. When various cells, including HaCaT and skin cancer cells, were treated with ITCs, HaCaT were shown to be more resistant to the treatment (Mantso et al., 2016; Mitsiogianni et al., 2019). When A375 cells were exposed to 10 μM BITC for 24h, an increase in apoptosis, DNA damage and chromatin condensation was observed as well as induction of caspase 3 (S. H. Huang et al., 2012). In other cell lines, PEITC was shown to activate caspase 9 and 3 in malignant glioma cells and have no such effect on immortalized normal glial HEB cells (T. Zhang et al., 2016). Although, the current study shows no effect of allyl isothiocyanate on the cell viability and some significance of the combinational treatment of the phytochemical with UVB, on apoptotic cells, it is shown in the literature that the isothiocyanate (<1 μM) increased necrotic and apoptotic cells 48 hours after the treatment of bladder cancer cells (Savio et al., 2014). It is therefore hypothesised that AITC selectively leads to apoptosis depending on the model.

5.2.6 Mitochondrial membrane permeability determination of combinational treatments of 5 ITCs with UVB

For further evaluation of the effect of the suggested combinational treatments of UVB with 20 μM ITCs on apoptosis, intrinsic apoptosis was also studied. The effect on the mitochondrial membrane permeability through depolarization ($\Delta\Psi m$) was studied using JC-1 stain and the mitochondrial dysfunction was expressed as a ratio of JC-1 aggregates over JC-1 monomers.

All combinational treatments with 20 μM ITC and 10 mJ / cm^2 UVB at 24h and 20 μM ITC and 5 mJ / cm^2 UVB at 48h, were shown to significantly induce depolarization of mitochondria: SFN + 10 mJ / cm^2 UVB, AITC + 10 mJ / cm^2 UVB, BITC + 10 mJ / cm^2 UVB, PEITC + 10 mJ / cm^2 UVB: $p<0.0001$ and IBN + 10 mJ / cm^2 UVB: $p<0.001$, when compared to all relative controls, and SFN + 5 mJ / cm^2 UVB, IBN + 5 mJ / cm^2 UVB, AITC + 5 mJ / cm^2 UVB, BITC + 5 mJ / cm^2 UVB, PEITC + 5 mJ / cm^2 UVB: $p<0.0001$ when compared to all relative controls (figures 5.14, 5.15, 5.16, 5.17, 5.18). The above results reveal the induction of intrinsic apoptosis from the suggested therapeutic approach for all ITCs and the indication of ROS production from the mitochondria. AITC is shown to induce equally significantly mitochondrial depolarization as the other ITCs studied in this chapter, which comes in contrast with the viability and apoptosis results in sections 5.2.4 and 5.2.5, respectively (figure 5.16). Finally, both UVB intensities are shown to have a similarly significant effect on intrinsic apoptosis at their timepoints.

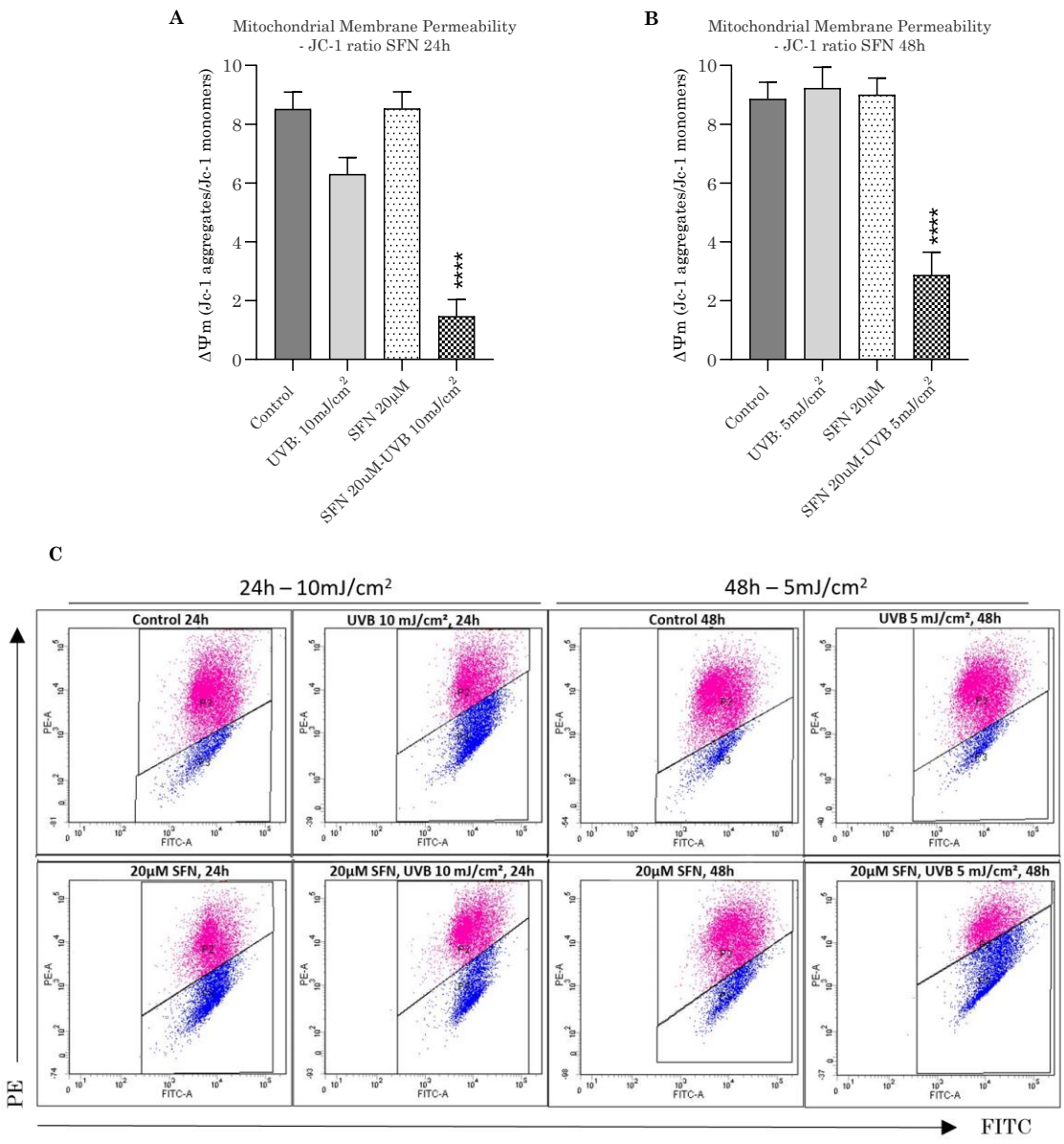


Figure 5. 14 *Mitochondrial membrane permeability of HaCaT cells after the combinational treatment with UVB and SFN*

Cells were treated with either 5 or 10 mJ /cm^2 UVB and 20μM SFN immediately after the UV exposure, mitochondrial membrane permeability was studied at 24h and 48h, respectively using flow cytometry. The number of JC-1 monomers and aggregates were quantified as (A) and (B) the ration of JC-1 aggregates over JC-1 monomers and (C) cell events in a scatter plot. Statistical significance was set as ***$p < 0.001$, ****$p < 0.0001$, *relative to corresponding controls. Data shown are means of ± SD of 3 replicates from 3 independent experiments. FACS images are representative.*

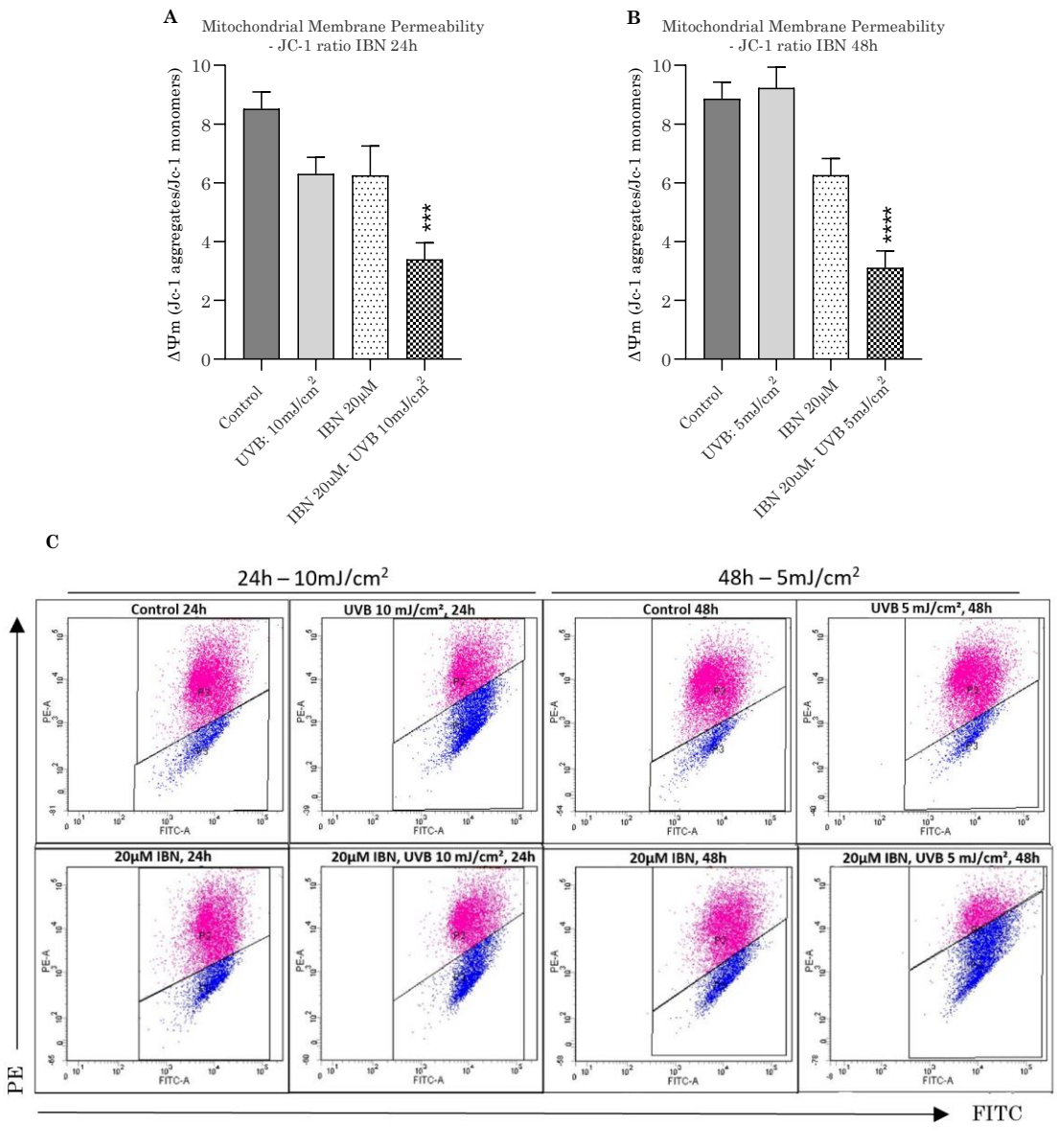


Figure 5. 15 *Mitochondrial membrane permeability of HaCaT cells after the combinational treatment with UVB and IBN*

Cells were treated with either 5 or 10 mJ /cm^2 UVB and 20μM IBN immediately after the UV exposure, mitochondrial membrane permeability was studied at 24h and 48h, respectively using flow cytometry. The number of JC-1 monomers and aggregates were quantified as (A) and (B) the ration of JC-1 aggregates over JC-1 monomers and (C) cell events in a scatter plot. Statistical significance was set as ***$p < 0.001$, ****$p < 0.0001$, *relative to corresponding controls. Data shown are means of ± SD of 3 replicates from 3 independent experiments. FACS images are representative.*

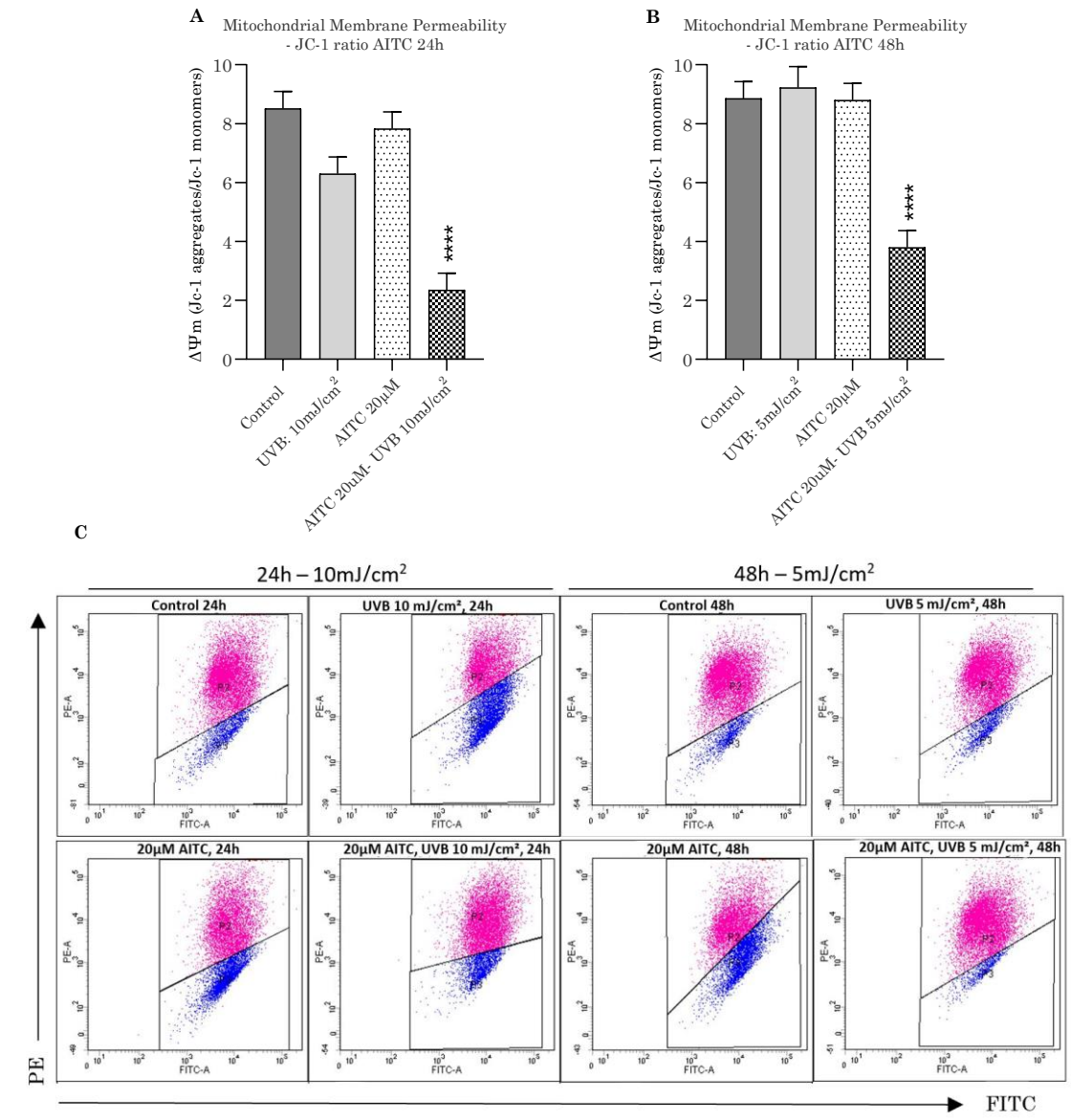


Figure 5. 16 *Mitochondrial membrane permeability of HaCaT cells after the combinational treatment with UVB and AITC*

Cells were treated with either 5 or 10 mJ /cm^2 UVB and 20μM AITC immediately after the UV exposure, mitochondrial membrane permeability was studied at 24h and 48h, respectively using flow cytometry. The number of JC-1 monomers and aggregates were quantified as (A) and (B) the ration of JC-1 aggregates over JC-1 monomers and (C) cell events in a scatter plot. Statistical significance was set as ***$p < 0.001$, ****$p < 0.0001$, *relative to corresponding controls. Data shown are means of ± SD of 3 replicates from 3 independent experiments. FACS images are representative.*

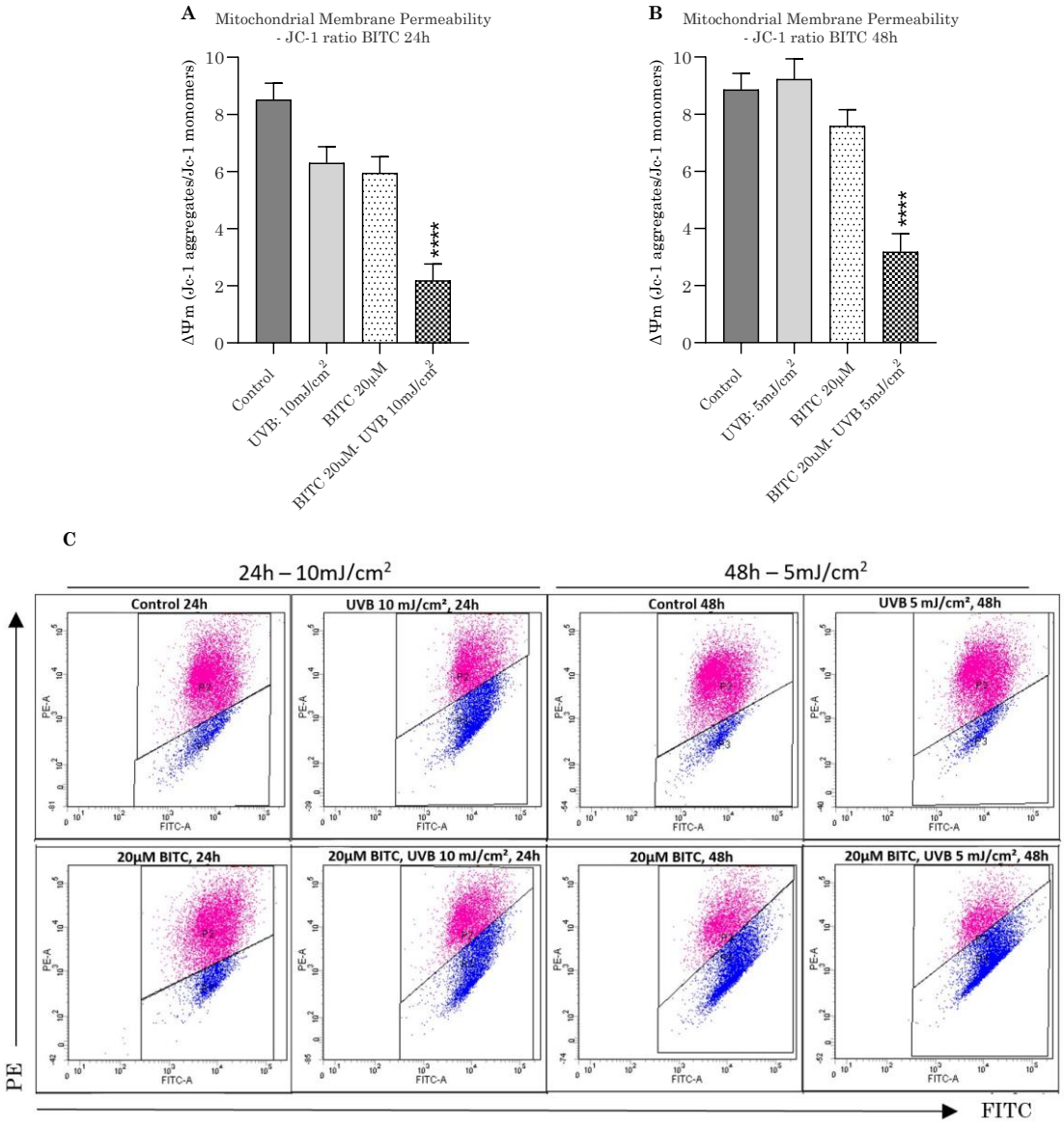


Figure 5. 17 *Mitochondrial membrane permeability of HaCaT cells after the combinational treatment with UVB and BITC*

Cells were treated with either 5 or 10 mJ /cm^2 UVB and 20μM BITC immediately after the UV exposure, mitochondrial membrane permeability was studied at 24h and 48h, respectively using flow cytometry. The number of JC-1 monomers and aggregates were quantified as (A) and (B) the ration of JC-1 aggregates over JC-1 monomers and (C) cell events in a scatter plot. Statistical significance was set as ***$p < 0.001$, ****$p < 0.0001$, *relative to corresponding controls. Data shown are means of ± SD of 3 replicates from 3 independent experiments. FACS images are representative.*

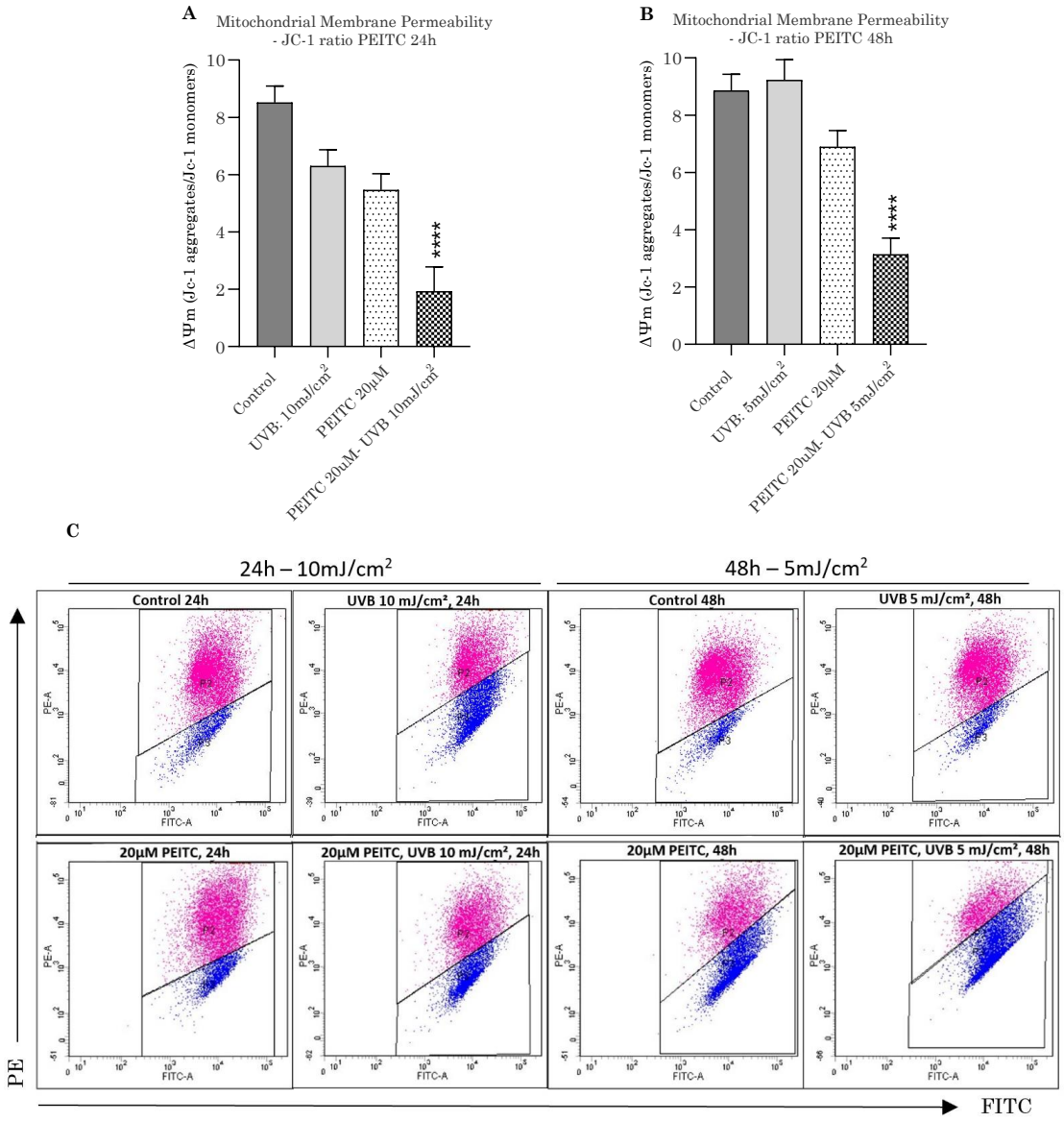


Figure 5. 18 *Mitochondrial membrane permeability of HaCaT cells after the combinational treatment with UVB and PEITC*

Cells were treated with either 5 or 10 mJ /cm^2 UVB and 20μM PEITC immediately after the UV exposure, mitochondrial membrane permeability was studied at 24h and 48h, respectively using flow cytometry. The number of JC-1 monomers and aggregates were quantified as (A) and (B) the ration of JC-1 aggregates over JC-1 monomers and (C) cell events in a scatter plot. Statistical significance was set as ***$p < 0.001$, ****$p < 0.0001$, *relative to corresponding controls. Data shown are means of ± SD of 3 replicates from 3 independent experiments. FACS images are representative.*

Mitochondrial membrane permeability, when increased, is an indicator of intrinsic apoptosis and malfunction of mitochondria. It is known that ROS induction increases MAPK and p38 protein levels that reduce ΔΨm and therefore increase mitochondrial permeability that leads to an increase of cytochrome C, caspase 9 and procaspase 3 and eventually lead to apoptosis. Also, as mentioned earlier, UVB's main mechanism of action on the cells is through ROS induction in the cytosol that eventually affects apoptotic markers in various pathways. In a study where 50 mJ / cm^2 BB UVB were used for the exposure of HaCaT cells, an increase in mitochondrial membrane permeability was increase with a peak timepoint at 24h (Paz et al., 2008). Similarly, when the same cell line was exposed to 60 mJ / cm^2 UVB, the same observations were made (Van Laethem et al., 2004). Lower intensities of 10, 20 and 30 mJ / cm^2 UVB were used by Farrukh *et. al* 2014, on HaCaT cells and fibroblasts reducing ΔΨm and the intracellular apoptotic pathways were confirmed with the addition of antioxidant that lead to damage restoration (Farrukh et al., 2014). On the other hand, ITCs are also shown to affect apoptotic pathways in various ways, as it has already been mentioned in section 5.2.5. Although not many research has been done on the cell membrane permeability of ITC induced HaCaT cells, BITC is shown to decrease levels of ΔΨm (S. H. Huang et al., 2012). Our results agree with the literature on the effect of UVB on mitochondrial membrane permeability, while a similar effect is observed when ITCs alone were used on HaCaT cells. The effect of the combinational treatments is shown to have at least an additive effect of the two parameters together.

5.2.7 Cell cycle effect of combinational treatments of 5 ITCs with UVB

For the determination of the effect of the suggested therapeutic approach on cell cycle distribution and its ability to induce cell cycle growth arrest, FxCycle PI/RNase staining was used. The present results reveal a significant elevation of subG1 phase on SFN, IBN and BITC in combination with 10 mJ / cm^2 UVB (24h), when compared to either of the relative controls (cells alone, UVB alone and ITC alone) ($p<0.0001$), while AITC and PEITC in combination with 10 mJ / cm^2 UVB remain unaffected (figure 5.19). Cell cycle growth arrest at G1 phase is also shown at the combination of PEITC with 10 mJ / cm^2 UVB, when compared with UVB ($p<0.001$) and a reversed effect of SFN with 10 mJ / cm^2 UVB, when compared with SFN alone ($p<0.05$). The above results are followed by a significant reduction of phase G2/M for all combinational treatments: SFN, IBN, BITC, PEITC ($p<0.0001$), AITC ($p<0.01$) and 10 mJ / cm^2 UVB, when compared with UVB and a reduction of SFN ($p<0.01$), IBN ($p<0.001$), BITC ($p<0.001$) and 10 mJ / cm^2 UVB when compared with the relative ITC controls. AITC and PEITC do not reduce phase G2/M, when compared with the relative ITC controls. Finally, phase S remained unaffected overall (table 5.1).

A milder cell cycle arrest is shown when ITCs were combined with 5 mJ / cm^2 UVB, with phases G1and S remaining unaffected, while a significant elevation of subG1 phase on SFN ($p<0.01$), IBN ($p<0.0001$) and AITC ($p<0.001$) when combined with 5 mJ / cm^2 UVB is shown, when compared with the relative controls of UVB and ITC (figure 5.20). There was no effect of the treatments with BITC and PEITC on this phase due to high standard deviation (table 5.2). Finally, IBN and 5 mJ / cm^2 UVB and AITC and 5 mJ / cm^2 UVB reduce phase G2/M, when compared to their relative ITC controls ($p<0.01$). Therefore, these two combinations are looking at the effect of the UVB alone.

In the literature, 10 μM of BITC are shown to increase G_2/M phase at 24, 36 and 48 hours by 12-50%, while G_0/G_1 and S phases were reduced (S. H. Huang et al., 2012). Moreover, protein changes that are associated with G_2/M phase arrest were also observed in the same study, showing an increase in Cyclin A, CDK1 and CDC25C proteins and a decrease in Chk1 and Wee1. Also, it was shown that the isothiocyanate enhanced the levels of BAX, cytochrome C, Apaf-1, AIF, FasL, Fas, caspase 8, 9, 3 and Eydo G, while reduced levels of Bid and Bcl2 that are known to take part in cell apoptosis. All the above affected biomarkers of apoptosis show that BITC promotes apoptosis through caspase 3, Fas and mitochondria dependent ways. In another study, where fibroblasts and HaCaT cells were exposed to 10, 20 and 30 mJ / cm^2 UVB, a G_2/M phase arrest and G_1/S phase arrest was shown, respectively at 24h (Farrukh et al., 2014; Geng et al., 2011). It is therefore clear that both ITCs and UVB, when applied in certain concentrations, affect the cell cycle of the cells and therefore it is understood that both parameters act independently on the cells.

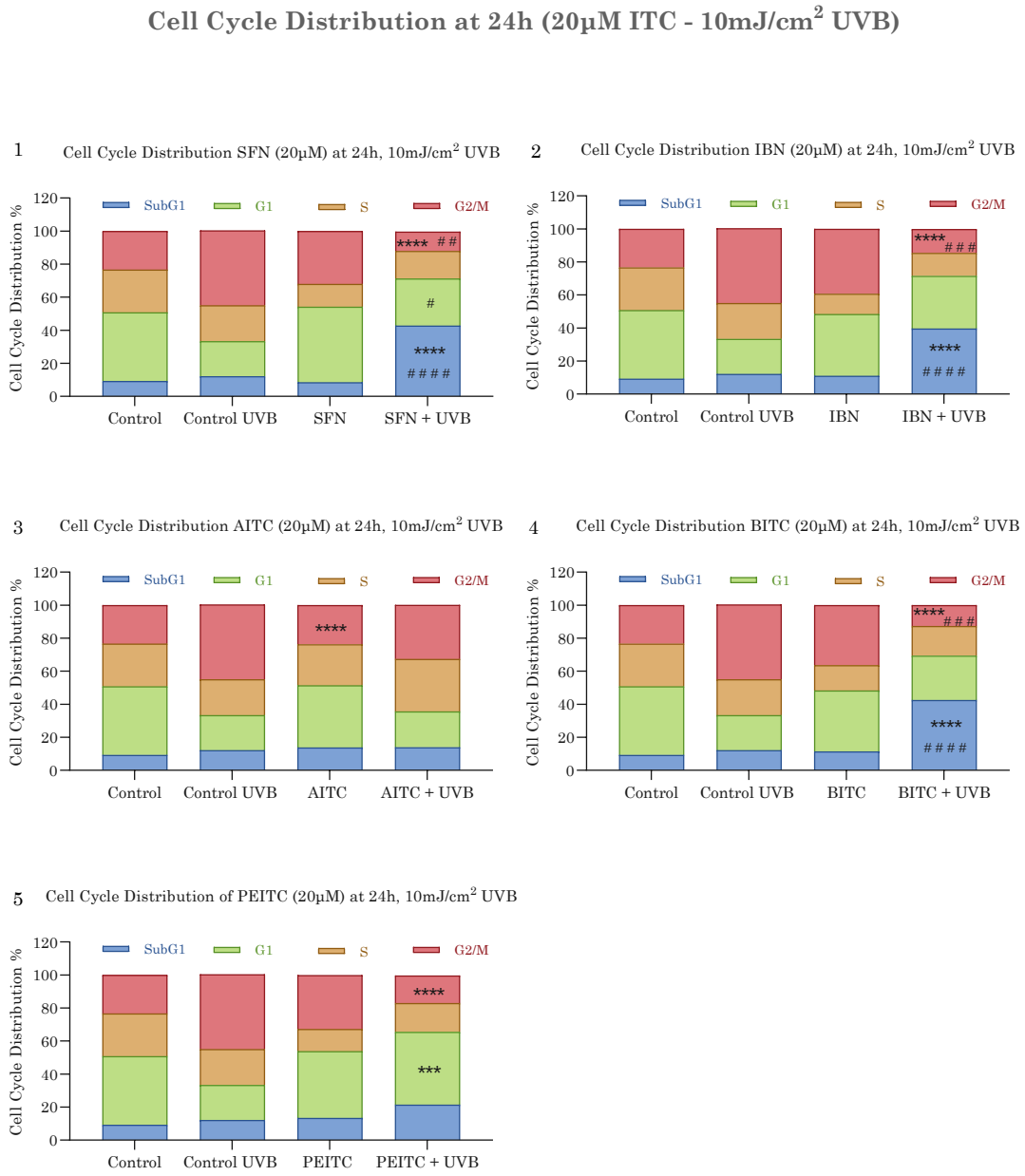


Figure 5. 19 *Cell cycle of HaCaT cells after the combinational treatment with 10 mJ /cm2 UVB and ITCs*

*Cells were treated with 10 mJ /cm^2 UVB and 20µM of either ITC: (1) SFN, (2) IBN,(3) AITC, (4) BITC, (5) PEITC immediately after the UV exposure, was measured at 24h and 48h, respectively, using flow cytometry. Cells were recorded at each stage and quantified as percentage of total DNA cellular content distributed at each phase of the cell cycle. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001, for significance with the UVB controls and set as $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 $^{\#\#\#}$p < 0.001, $^{\#\#\#\#}$p < 0.0001, for significance with the corresponding ITC controls. FACS pictures are representative.*

24h – 10mJ/cm^2 UVB – 20 µM ITC

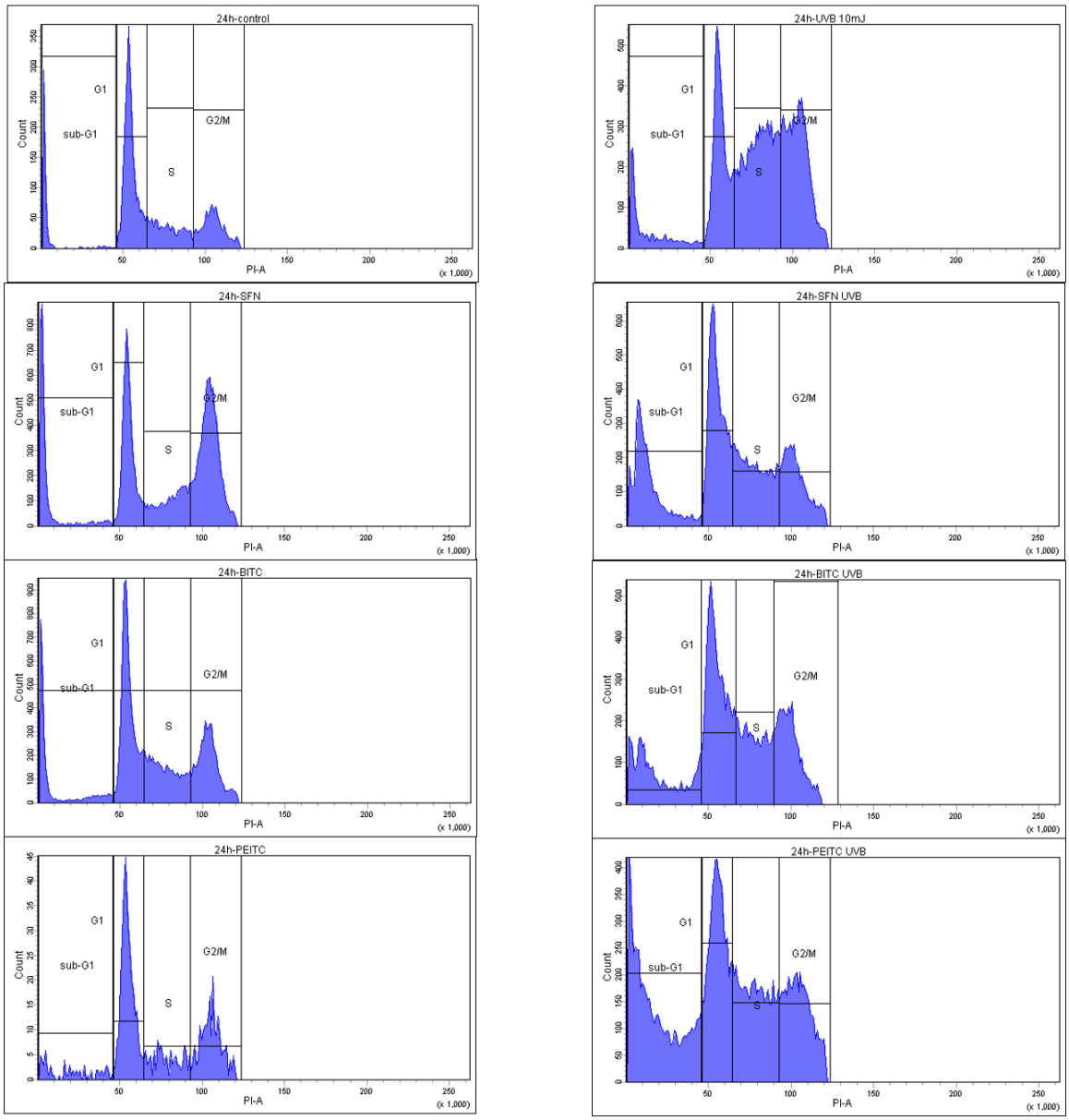


Figure 5. 20 *FACS graphs on cell cycle of HaCaT cells after the combinational treatment with 10 mJ /cm2 UVB and ITCs*

Cells were treated with 10 mJ /cm^2 UVB and 20µM of either ITC: (1) SFN, (2) IBN,(3) AITC, (4) BITC, (5) PEITC immediately after the UV exposure, was measured at 24h and 48h, respectively, using flow cytometry. Cells were recorded at each stage and quantified as percentage of total DNA cellular content distributed at each phase of the cell cycle. Statistical significance was set as ***$p < 0.05$, ****$p < 0.01$ *****$p < 0.001$, ******$p < 0.0001$, *for significance with the UVB controls and set as* $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$, *for significance with the corresponding ITC controls. FACS pictures are representative.*

Cell Cycle Distribution at 48h (20μM ITC - 5mJ/cm^2 UVB)

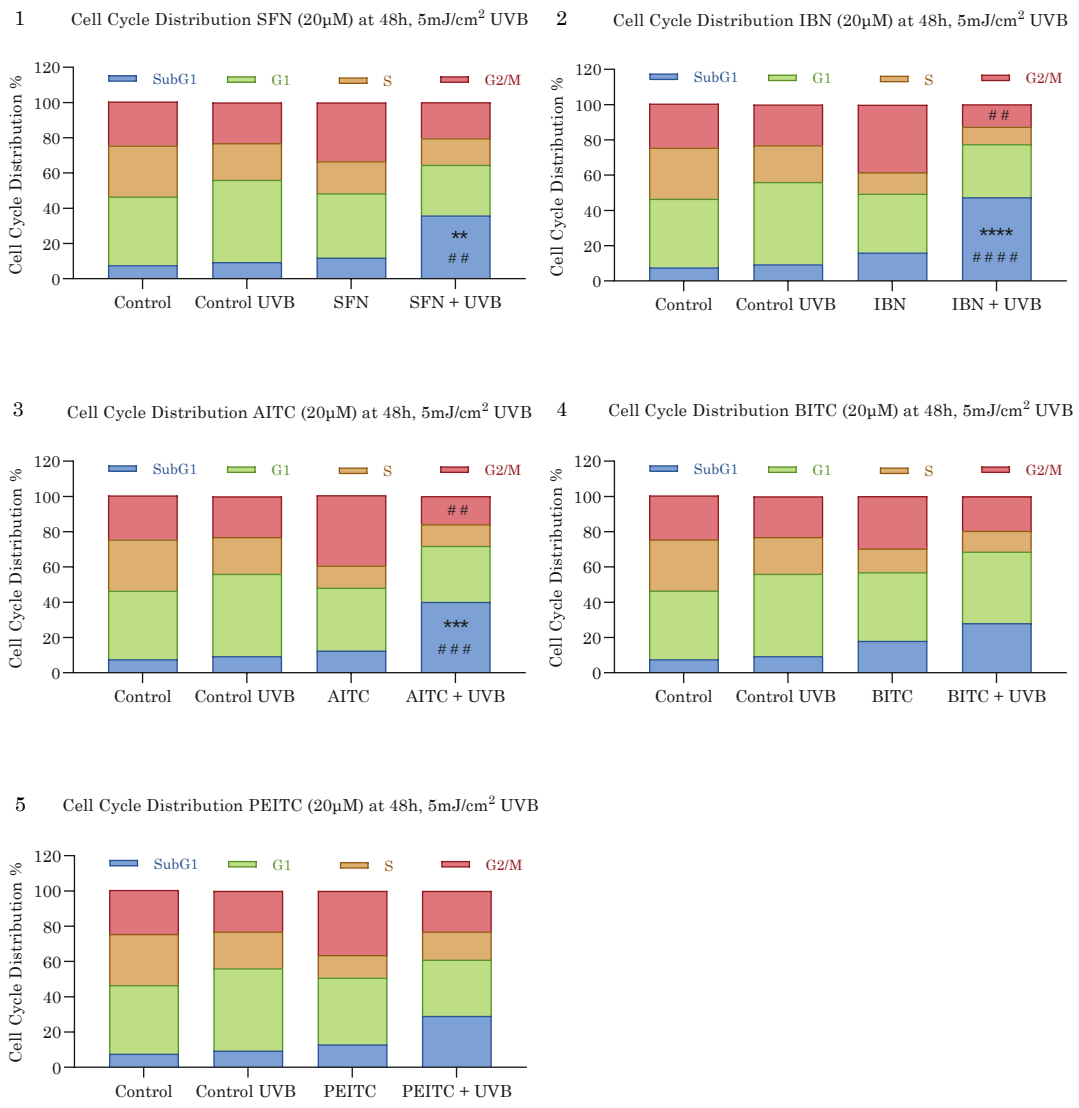


Figure 5. 21 *Cell cycle of HaCaT cells after the combinational treatment with 5 mJ /cm2 UVB and ITCs*

*Cells were treated with 5 mJ /cm^2 UVB and 20μM of either ITC: (1) SFN, (2) IBN,(3) AITC, (4) BITC, (5) PEITC immediately after the UV exposure, was measured at 24h and 48h, respectively, using flow cytometry. Cells were recorded at each stage and quantified as percentage of total DNA cellular content distributed at each phase of the cell cycle. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001, for significance with the UVB controls and set as #p < 0.05, ##p < 0.01 ###p < 0.001, ####p < 0.0001, for significance with the corresponding ITC controls. FACS pictures are representative.*

48h – 5mJ/cm² UVB – 20 µM ITC

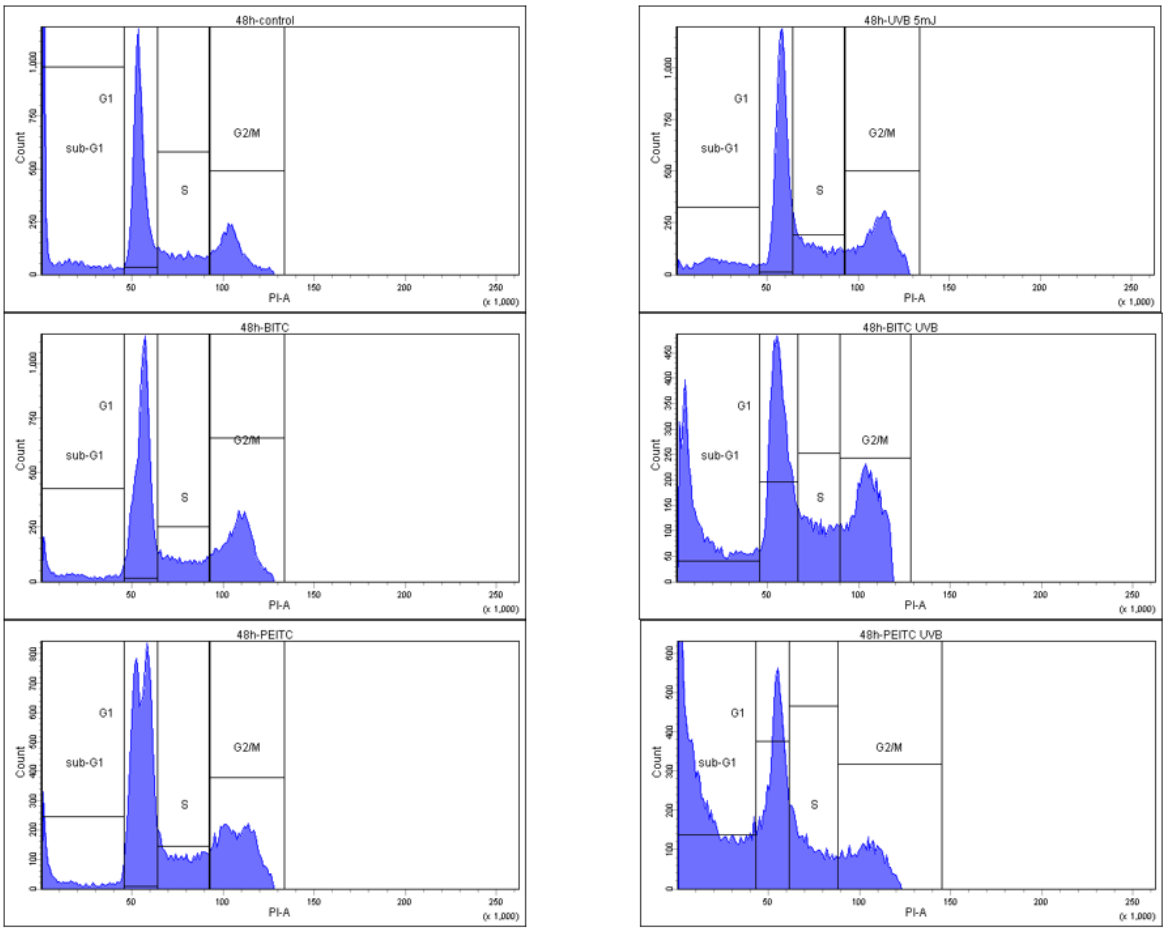


Figure 5. 22 *FACS graphs on cell cycle of HaCaT cells after the combinational treatment with 5 mJ /cm2 UVB and ITCs*

*Cells were treated with 5 mJ /cm² UVB and 20µM of either ITC: (1) SFN, (2) IBN,(3) AITC, (4) BITC, (5) PEITC immediately after the UV exposure, was measured at 24h and 48h, respectively, using flow cytometry. Cells were recorded at each stage and quantified as percentage of total DNA cellular content distributed at each phase of the cell cycle. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001, for significance with the UVB controls and set as #p < 0.05, ##p < 0.01 ###p < 0.001, ####p < 0.0001, for significance with the corresponding ITC controls. FACS pictures are representative.*

Table 5. 1 *Cell cycle percentages and standard deviation of the combinational treatment with 10 mJ /cm2 UVB and ITCs*

Samples 24h /phases	sub-G1 %	sub-G1 STDEV	G1 %	G1 STDEV	S %	S STDEV	G2/M %	G2/M STDEV
Control	9.25	8.98	41.55	5.30	25.85	12.80	23.30	1.56
Control UVB 10mJ/cm	12.15	2.90	21.20	3.25	21.75	7.57	45.25	6.72
SFN (20µM)	8.50	4.95	45.65	5.59	13.85	8.27	31.95	8.84
IBN (20µM)	10.95	3.32	37.40	7.07	12.30	0.42	39.30	3.25
AITC (20µM)	13.7	4.37	37.7	5.32	24.9	6.18	23.7	2.4
BITC (20µM)	11.30	1.41	36.95	1.34	15.35	2.05	36.40	1.98
PEITC (20µM)	13.40	6.36	40.40	1.41	13.40	0.57	32.60	7.50
SFN/UVB 10mJ/cm	42.75	12.66	28.45	5.02	16.70	8.91	11.75	0.78
IBN/UVB 10mJ/cm	39.65	14.50	31.85	7.00	13.90	6.08	14.40	2.40
AITC/UVB 10mJ/cm	13.9	5	21.7	5.47	31.8	5.89	32.7	2.67
BITC/UVB 10mJ/cm	42.45	6.58	26.90	4.67	17.95	9.12	12.65	2.90
PEITC/UVB 10mJ/cm	21.45	2.19	44.05	2.19	17.40	3.25	16.75	3.75

Percentage of total DNA cellular content distribution at each phase of the cell cycle and standard deviations of HaCaT cells after the combined treatment with 10 mJ /cm^2 UVB and 20µM of either ITC: SFN, IBN, AITC, BITC, PEITC immediately after the UV exposure, was measured at 24h using flow cytometry.

Table 5. 2 *Cell cycle percentages and standard deviation of the combinational treatment with 5 mJ /cm2 UVB and ITCs*

Samples 48h /phases	sub-G1 %	sub-G1 STDEV	G1 %	G1 STDEV	S %	S STDEV	G2/M %	G2/M STDEV
Control	7.65	4.74	38.80	7.64	29.10	6.08	24.90	5.66
Control UVB 5mJ/cm	9.30	3.25	46.70	9.19	20.85	12.66	23.10	6.65
SFN (20µM)	11.80	0.00	36.55	1.91	18.20	4.81	33.40	6.79
IBN (20µM)	16.05	3.89	33.40	3.68	12.10	3.96	38.35	4.03
AITC (20µM)	12.47	3.96	35.67	3.32	12.57	3.58	39.88	4.73
BITC (20µM)	18.05	1.91	38.85	5.44	13.45	3.46	29.70	5.88
PEITC (20µM)	12.85	3.46	37.93	5.76	12.75	3.61	36.50	5.66
SFN/UVB 5mJ/cm	35.85	10.01	28.75	12.13	15.00	2.97	20.45	5.16
IBN/UVB 5mJ/cm	47.40	13.63	30.05	13.69	10.00	3.39	12.60	9.48
AITC/UVB 5mJ/cm	40.08	11.70	31.83	10.67	12.21	3.22	15.96	7.49
BITC/UVB 5mJ/cm	28.10	13.81	40.55	14.30	11.70	2.97	19.65	10.54
PEITC/UVB 5mJ/cm	29.10	12.02	31.80	10.18	16.00	0.99	23.05	2.76

Percentage of total DNA cellular content distribution at each phase of the cell cycle and standard deviations of HaCaT cells after the combinational treatments with 5 mJ /cm^2 UVB and 20µM of either ITC: SFN, IBN, AITC, BITC, PEITC immediately after the UV exposure, weas measured at 48h using flow cytometry.

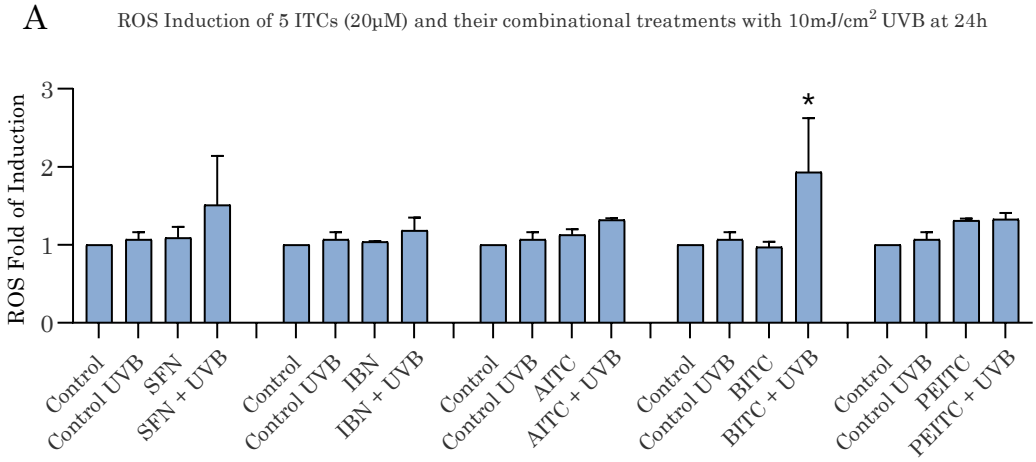
5.2.8 ROS induction of combinational treatments of 5 ITCs with UVB

5.2.8.1 DHR assay (Dihydrohodamine)

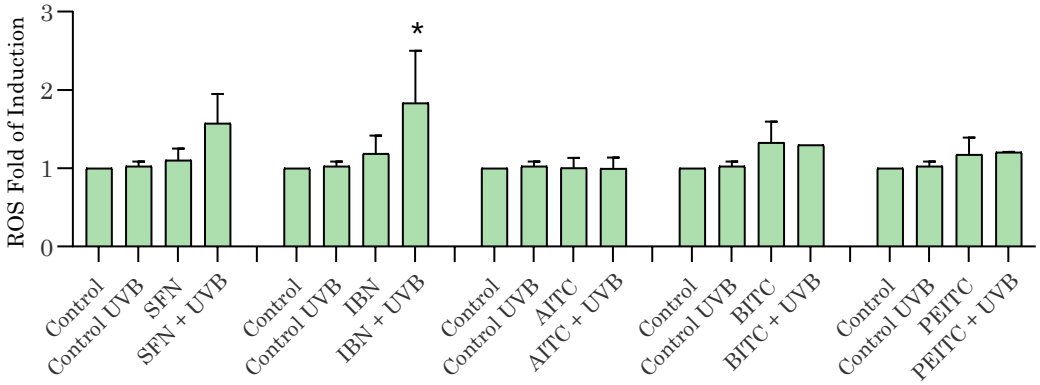
For the further evaluation of ROS induction, DHR123 assay was utilized for the same conditions as described above. The data show that only the combinations of BITC + 10 mJ

/ cm^2 UVB (24h) and IBN + 5 mJ / cm^2 UVB (48h) revealed the presence of ROS in the mitochondria, an observation that does not agree with the findings of section 5.2.6 where the ratio of JC-1 aggregates to JC-1 monomers indicate depolarisation of the mitochondrial membrane and therefore the production of ROS. However, it is known from the literature, that UVB alone significantly induces ROS formation in the cytosol and as shown in all the above experiments, UVB has a significant effect on viability, apoptosis and cell cycle through ROS induction. At a first glance on the results shown in figure 5.8, where not significant ROS induction was shown, it is suspected that ITC have possibly protected cells from ROS. However, as shown in a study where 50 mJ / cm^2 UVB were used on HaCaT cells a peak of ROS induction was observed at 24h (Paz et al., 2008). The determination on whether the increased Ca^{2+} levels (Ca^{2+} stimulates an activity that leads to increased levels of ROS), observed after the exposure of fibroblasts and HaCaT cells to 10, 20 and 30 mJ / cm^2 UVB, by the addition of an antioxidant mix of Troxol and ascorbic acid, confirmed that the effect of UVB is caused by intracellular ROS induction (Farrukh et al., 2014). On the results shown in figure 5.21, it is observed that the combinational treatments slightly increase the ROS fold induction, but this observation is not significant, except for BITC at 24h and IBN at 48h, which have a large standard deviation showing a concerning variation to the samples. Although the results shown are from 3 independent experiments, it is hypothesised that an unknown possible technical issue might have occurred during the assay, such as an interaction of the assay with the control groups leading to false non-significant difference between the controls and the combinational treatment samples. For this reason, the addition of the antioxidant glutathione (GSH) to evaluate whether the effect presented so far is caused from intracellular activities and ROS generation, was required.

A


ROS Induction of 5 ITCs (20µM) and their combinational treatments with 10mJ/cm2 UVB at 24h
ROS Fold of Induction
0
1
2
3
*
Control
Control UVB
SFN
SFN + UVB
Control
Control UVB
IBN
IBN + UVB
Control
Control UVB
AITC
AITC + UVB
Control
Control UVB
BITC
BITC + UVB
Control
Control UVB
PEITC
PEITC + UVB

B


ROS Induction of 5 ITCs (20µM) and their combinational treatments with 5mJ/cm2 UVB at 48h
ROS Fold of Induction
0
1
2
3
*
Control
Control UVB
SFN
SFN + UVB
Control
Control UVB
IBN
IBN + UVB
Control
Control UVB
AITC
AITC + UVB
Control
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BITC
BITC + UVB
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Control UVB
PEITC
PEITC + UVB

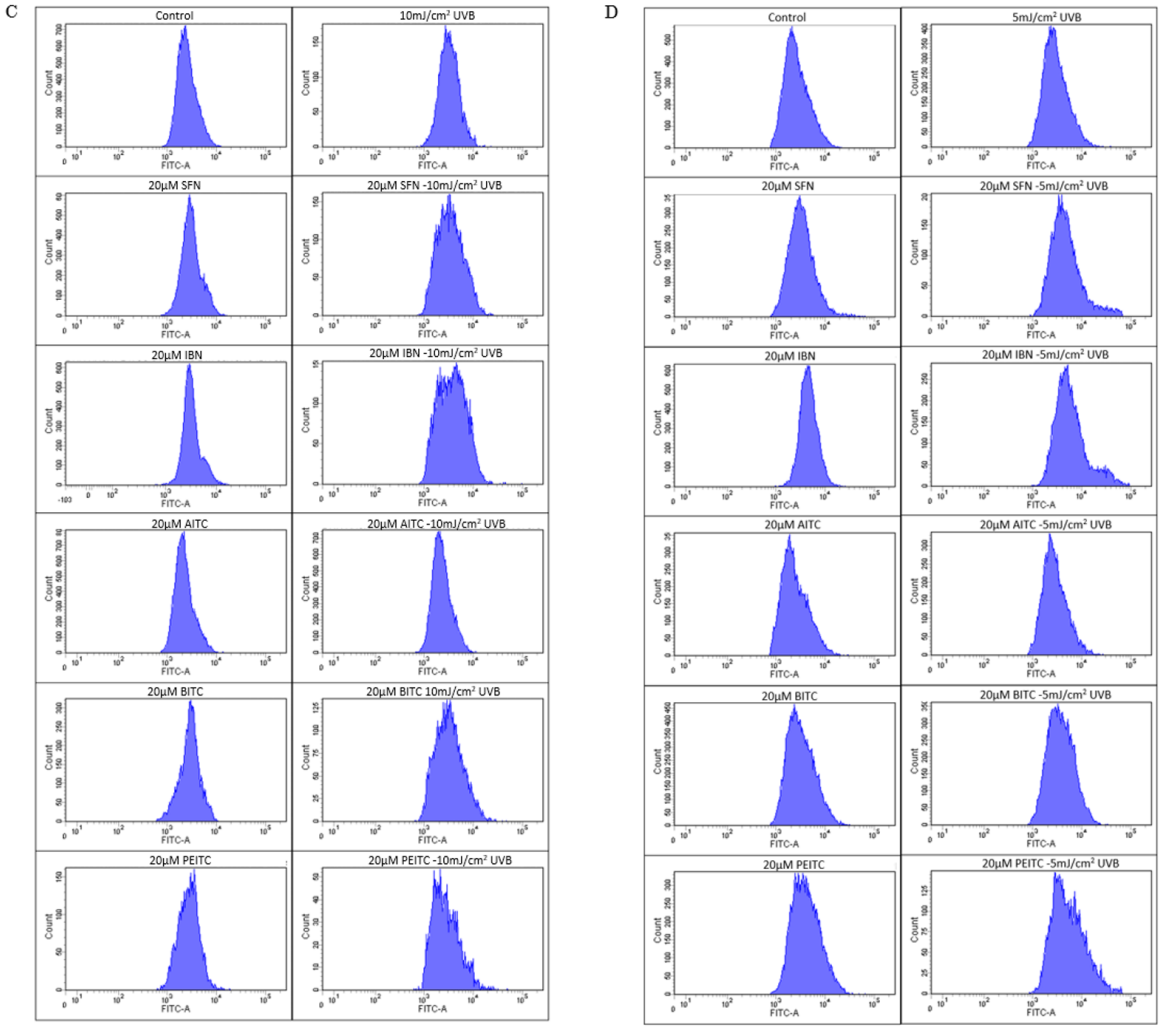


Figure 5. 23 *ROS induction of HaCaT cells after the combinational treatments*

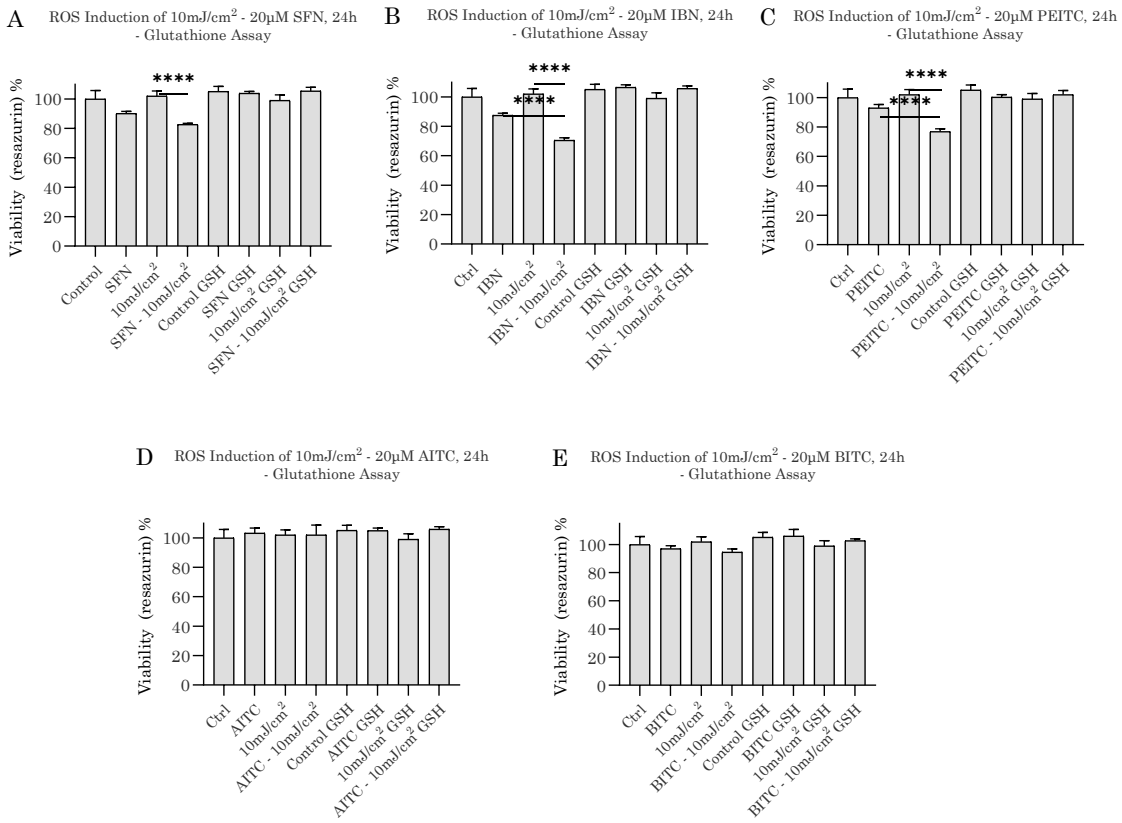
ROS fold of induction of HaCaT cells after the combinational treatments with either 10 or 5 mJ /cm^2 UVB and 20μM of either ITC: SFN, IBN, AITC, BITC, PEITC immediately after the UV exposure, was measured at (A,C) 24h and (B,D) 48h, respectively, using flow cytometry and DHR assay. Statistical significance was set as *******$p < 0.05$*, relative to corresponding controls. Data shown are from 3 independent experiments. FACS images are representative.*

5.2.8.2 Glutathione Assay

For the validation of our findings on the ROS inductive effect of the ITCs in combination with UVB, the glutathione assay was utilized. Viability was measured at the indicated timepoints depending on the conditions (24h for 10 mJ / cm^2 UVB and 48h for 5 mJ / cm^2 UVB). It is shown that glutathione mitigated the damage caused from treatment-induced ROS and the cell viability levels increase from either treatment when cells were pre-treated and post-treated with the antioxidant (figure 5.22). The cell viability reductive effect of the ITCs, UVB and their combinations, is absent in samples that glutathione was added. As described above, this indicates the main apoptotic pathways through intracellular ROS production. ITCs are known to have an indirect antioxidant activity through the induction of phase II enzymes, while at the same time inducing apoptosis in higher concentrations. In the study conducted from Kleszczyński *et al.* 2014, where SFN and PEITC were used on HaCaT cells, an upregulation of Nrf2 target genes (γ-GCS, HO-1, NQO1) was shown using RT-PCR at 5 μM of both isothiocyanates. On the other hand, 25 μM of the same isothiocyanates did not have the same effect (Kleszczyński et al., 2013). Similarly, 5μmol / l of SFN increased mRNA levels of γ-GCS and HO-1 by 150% when applied on the same cell line (Wagner et al., 2010). The same isothiocyanate (1-10 μM) reduced the AP-1 activation caused by 250 J / m^2 UVB and an increase of GSH and QR-1 (quinone reductase 1 reduces quinones to semiquinones and is marker of phase 2 enzymes) was observed at 24h (Zhu et al., 2004). BITC at 10 μM, is shown to increase ROS levels in a time dependent manner, in A375 cancer cells (S. H. Huang et al., 2012). Not much research is done on similar combinational models of ITCs and UVB on HaCaT cells. However, when SFN (5 and 10μM) studied for its potential protective effect on a UVB induced model (50 mJ / cm^2 UVB) an anti-inflammatory effect was revealed through the reduction of IL-1β, IL-6 and COX-2 in HaCaT cells (Shibata et al., 2010). A similar effect was observed on fibroblasts, when 2 and 5 μM of SFN blocked UVB-induced (25 mJ / cm^2) NF-κB activation and MMP expression,

in a dose dependent manner (S. Y. Lee & Moon, 2012). In the contrary, the same phytochemical (3 µM) did not protect HaCaT cells from direct UVB (20 mJ / cm^2) damage (Benedict et al., 2012). Therefore, it is clear that the effect of each parameter depends on the concentration / intensity and the current study reveals that the combinational treatments of 20 µM of either ITC and UVB at 5 mJ / cm^2 UVB (48h) and 10 mJ / cm^2 UVB (24h) shows a significant damage on HaCaT cells.

i)



ii)

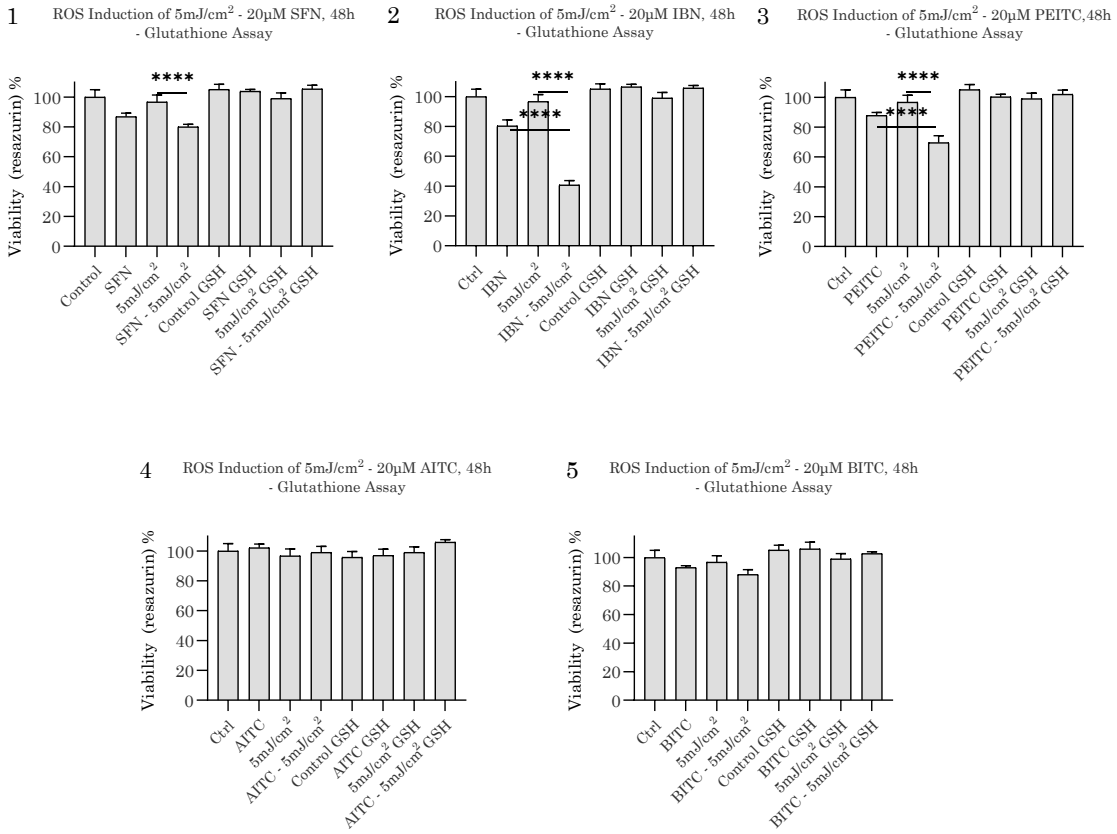


Figure 5. 24 *Viability of HaCaT cells after the combinational treatments – GSH addition Cells were treated with either (i) 10 or (ii) 5 mJ /cm^2 UVB and 20µM of either (A,1)SFN,(B,2) IBN, AITC, BITC, (C,3) PEITC immediately after the UV exposure, was measured at (A) 24h and (B) 48h, respectively, with and without the addition of glutathione. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001, relative to corresponding controls. Data shown are from 3 independent experiments.*

5.3 Conclusions

In the current chapter, where HaCaT cells were treated with two intensities of UVB in two different timepoints (5 mJ / cm^2 UVB for 48h and 10 mJ / cm^2 UVB for 24h), a promising therapeutic approach has revealed a significant effect of certain ITCs, such as IBN and PEITC, that can be utilised for the therapy of inflammatory skin disease, such as psoriasis. This study provides the below significant findings:

1. Viability and growth: All ITCs but AITC (20 μM) studied in this chapter showed a significant reduction of viability when combined with 5 mJ / cm^2 UVB at 48h or 10 mJ / cm^2 UVB at 24h, while BITC was shown to be the least potent. HaCaT cell growth was also shown to reduce significantly after their treatment with all ITC combinations but AITC.
2. Apoptosis: All ITCs studied in this chapter showed an increase of apoptotic cells through the evaluation of caspase 3/7 activity, while AITC only showed an increase of apoptotic cells after the treatment with 10 mJ / cm^2 UVB and not with 5 mJ / cm^2 UVB. Moreover, treatments with combinations of UVB and PEITC, AITC, IBN and SFN showed necrotic cells significantly high indicating damage to membranes, while PEITC was shown to be the most potent. AITC reduced necrotic cells significantly compared to 10 mJ / cm^2 UVB but not with the AITC alone, while BITC combination did not show any increase to necrotic cells compared to the controls.
3. Mitochondrial membrane permeability: $\Delta\Psi m$ was shown to decrease in all the combinational treatments indicating intracellular ROS induction and mitochondrial dysfunction. SFN and PEITC were the most potent on reducing $\Delta\Psi m$.
4. ROS induction as the main mechanism of action of the combinational treatment was shown to reverse after the addition of GSH strengthening the hypothesis of reduced viability being mediated by the induction of intracellular ROS.

5. Cell Cycle: SFN, IBN and BITC were shown to arrest G_2/M phase at 24h / 10 mJ / cm^2 UVB, when compared to the individual controls of UVB or ITC alone, while PEITC significantly arrested G_2/M phase only when compared to UVB control. 48h / 5 mJ / cm^2 UVB only showed a significant G_2/M phase arrest after treatments with IBN and AITC when compared to their corresponding ITC controls. SubG1 phase was arrested by SFN, IBN and BITC combinational treatments at 24h, while similar effect was shown by SFN, IBN, AITC at 48h. No effect on S phase was shown from either ITC, while SFN and PEITC arrested G1 phase at 24h, when compared to SFN alone control and UVB control, respectively.
6. The treatment of 10 mJ / cm^2 UVB in combination with 20 μM of SFN, IBN, BITC or PEITC are shown to be the most effective of the two treatments overall, with the most significant effect on cell viability, apoptosis, $\Delta\Psi m$, cell cycle and ROS induction.

To the best of our knowledge this is the first time that this combination has been studied as a therapeutic approach for psoriasis, aiming for further enhancement of the effect of the individual parameters. Further research needs to be conducted to evaluate the exact role of the suggested treatment and whether the effect is additive or synergistic. Validation of the findings of the current study using RT-PCR assay for gene expression of certain markers of apoptosis, such as caspase 8,9 and 3, and western blot for protein expression, such as Fas, that indicates intracellular ROS induction, PARP cleavage and FLICE expression, biomarkers that are shown to be affected by UVB. Moreover, further research on the effect of the combinational treatment on Nrf2 target genes is required in order to evaluate whether ITCs, as indirect antioxidants, maintain their effect on a UVB exposed model. Furthermore, the use of a different cell line and / or reconstituted tissue and / or human psoriatic skin substitute, would provide a better and more complete understanding on the effect and

mechanisms of the suggested therapy. *In-vivo* experiments, as a model that provides inflammatory response, would also provide a more realistic effect of the treatment.

6. The role of total ITCs derived from watercress powder in combination with low dose UVB as a novel therapeutic approach for psoriasis

6.1 Introduction

Psoriasis, an autoimmune disease characterised by inflamed skin with excess keratinocyte cell production, has been looked at from a more natural and environmentally healthy perspective using plant extracts or naturally derived compounds, as described in the introduction of chapter 5. Moreover, UVB exposure is a known and widely used treatment for psoriasis that although it has a good response, it increases the risk for skin cancer as described in chapter 4 and 5. On the other hand many plant extracts and their compounds have been studied for their anti-inflammatory (table 1.2) and antioxidant effects on cells of relevant skin conditions, such as caffeic acid (Pluemsamran et al., 2012), shikonin (Jing et al., 2016) and the common grape vine (Marabini et al., 2020). Amongst cruciferous vegetables, garden cress, a 'close relative' of watercress and rich in BITC, is shown to reduce inflammation on HaCaT cells through a decrease of the psoriasis biomarkers VEGF, TNFα and 5 aplha-reductase type 2 (Türkoğlu et al., 2018). Broccoli extracts are shown to reduce erythema caused from UVB on human skin and mouse models through topical application and through the increase of phase 2 enzymes, the indirect antioxidant pathway of ITCs (Dinkova-Kostova et al., 2007; Talalay et al., 2007).

Watercress has also been studied for its beneficial effects on human health and disease. This cruciferous vegetable that belongs in the family of Brassicaceae is also known with the botanical name *Nasturtium officinale* (Giallourou et al., 2016). It is grown in areas that are rich in water sources since their leaves float in water and it is categorized as an aquatic

flowering plant. This plant is well known for its composition and high content in glucosinolates that form isothiocyanates when the leaves are chopped or chewed (Esteve, 2020). As described in chapter 3, watercress is high in gluconasturtiin the precursor of phenethyl isothiocyanate that is a known anti-cancerous and anti-inflammatory phytochemical (Hecht, 1995). Watercress is also known for its rich content in carotenoids, flavonoids, vitamins, such as pro-vitamin A, C and K, and for its antioxidant and anti-inflammatory properties (Gill et al., 2007), while it is usually consumed raw or stir fried.

Watercress extracts have been shown to inhibit MMP9 in Human MDA-MB-231 breast cancer cells in doses of 0.1 to 1 mg / mL of plant extract (Rose et al., 2005), increase apoptosis of cancer cells and act in an anti-tumor and anti-metastatic mode through cell cycle arrest and inhibition of DNA damage at 50 μL / mL concentration, reducing DNA damage by 28% (Boyd et al., 2006). Moreover, ITCs contained in watercress extracts are shown to act as indirect antioxidants through an increase in the detoxification enzymes expression, such as phase II enzymes leading to reduction of free radicals, inhibition of lipases, α-glucosidase and α-amylase as well as decrease of TNFα and IL-10 production (Rose et al., 2000; Schuchardt et al., 2019). The anti-diabetic effects of watercress have also been studied and a reduction in blood sugar, leptin, total glucose, triglycerides and cholesterol has been observed after the introduction of watercress extracts on diabetic rats (Hadjzadeh et al., 2015; Schuchardt et al., 2019). Psoriasis lesions have been treated with cruciferous plant extracts containing ITCs and a significant improvement of the disease was shown (Sussan & Sussan, 2012).

The aim of this study was to combine low dose UVB and ITCs, with the aim of inducing a strong effect when combined treatment that could negate the risk of skin cancer but lead to a significant improvement of psoriasis symptoms. Moreover, the aim of this chapter was to validate the findings of chapter 5 and determine the role of naturally derived ITCs in

combination with low doses of UVB on HaCaT cells, as a novel therapeutic approach for psoriasis.

Objectives of this chapter:

To determine the role of naturally derived ITCs alone and in combination with low doses of UVB on HaCaT cells growth, apoptosis and mitochondrial membrane permeability. Specifically:

1. To evaluate the role of the therapeutic approach on HaCaT cell growth using the SRB assay.
2. To evaluate the role of the therapeutic approach on HaCaT cell apoptosis through caspase 3/7 activity.
3. To evaluate the role of the therapeutic approach on HaCaT cell mitochondrial membrane permeability, looking at the levels of $\Delta\Psi m$.

6.2 Results and Discussion:

6.2.1 Effect of combinational treatments of HaCaT cells with watercress extract and UVB, and comparison with synthetic PEITC on cell growth.

The combinational treatment of 10 mJ / cm^2 UVB and 20 µM of ITCs for 24h was selected from chapter 5 as the most suitable condition for the treatment of HaCaT cells using watercress extract instead of synthetic ITCs. HaCaT cells were treated with 20 µM of total ITCs from watercress extract, with or without the exposure at 10 mJ / cm^2 UVB. It is shown that the treatment with the combination significantly reduced cell growth at 43.04 ± 2.1% when compared to watercress samples alone: 50.4 ± 5.021% ($p<0.01$) or UVB alone: 88.68 ± 3.757% ($p<0.0001$) (figure 6.1A). The morphological changes caused by the watercress

extract to the cells are characterised by large patches of detached cells and cell material floating in the wells, while the combination showed a more severe damage with very few attached cells on the bottom of the wells and large patches of apoptotic and necrotic cells floating in the media (figure 6.1B). These observations were compared to the results of PEITC on HaCaT cells, and in combination with the same intensity of UVB, shown in chapter 5. When comparing the effect of PEITC alone and watercress extract alone, a significant difference between the samples is observed with the latter further reducing cell growth by approximately 20% ($p<0.0001$) (table 6.1). Therefore, it is hypothesised that the further reduction of growth is due to other unknown compounds in the matrix of the watercress extract, with PEITC being the major ITC in watercress. Moreover, it is important to note that the watercress extract was not altered or adjusted chemically in order to fit the cell growth environment, because the aim of this chapter was to provide a therapy approach that does not require processing of the material but simple and natural processes for the extraction and application. However, it is possible that the plant extract altered the pH of the growth media, while other compounds, such as plant proteins or minerals might have affected cell growth, leading to cell growth reduction. PEITC was selected for the comparison of the results collected from the SRB assay, since this ITC is the most dominant from the total ITCs found in watercress (F. L. Chung et al., 1992). When the combination of the UVB with watercress extract was compared with the one with PEITC, an approximately 10% further reduction is shown by the former ($p<0.0001$). It is generally seen that both the synthetic PEITC and the natural extract have a significant effect on HaCaT cell's growth either alone or in combination with 10 mJ / cm^2 UVB, 24h after the treatment. (figure 6.1)

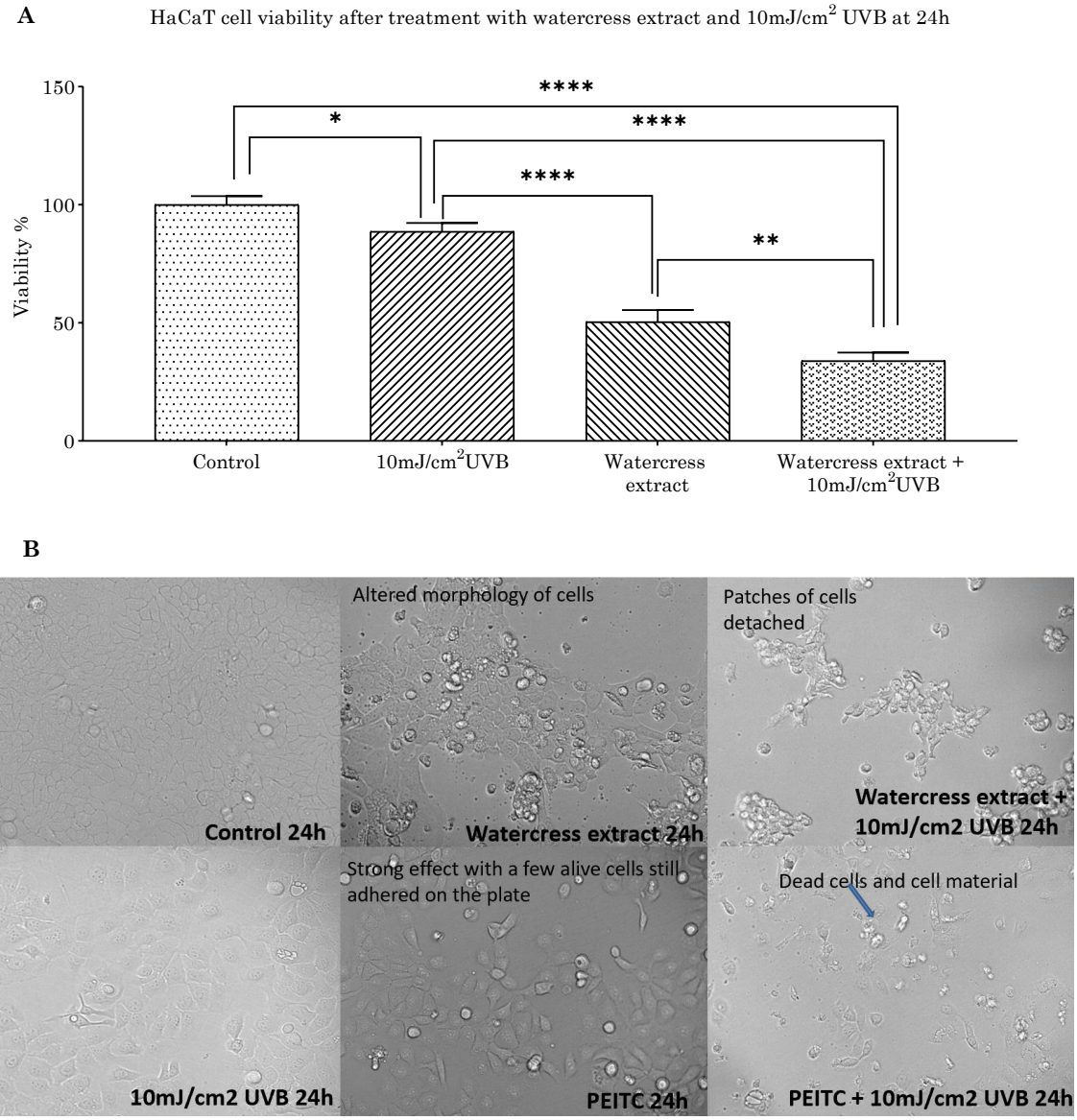


Figure 6. 1 *Effect of watercress extract on HaCaT cells and of its combination with 10 mJ / cm2 UVB*

*Cells were treated with UVB and watercress extract - containing complete DMEM was added immediately after the exposure. Cells were incubated for 24h and cell growth was measured using the SRB assay (A). B: Microscopic representation of cells and comparison of watercress extract with synthetic PEITC using ZOE Fluorescent Cell Imager. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001, relative to corresponding controls. Data shown are means of ±SD of three replicates from 3 independent experiments.*

Table 6. 1 *HaCaT cell growth reduction caused by the suggested combination of 10 mJ / cm2 UVB and 20 µM of either watercress extract or synthetic PEITC*

Sample	Growth (%)	±SD	N
Control	100	3.65	3
10 mJ/cm^2 UVB	88.68	3.757	3
W. extract	50.40	5.021	3
W. extract + 10 mJ/cm^2 UVB	33.94	3.44	3
PEITC (20µM)	69.73	3.944	3
PEITC (20µM) + 10 mJ/cm^2 UVB	43.04	2.1	3

Data for synthetic PEITC were taken from chapter 5.

It is possible that the effect of the plant extract has a more variable effect compared to the synthetic PEITC, due to other ITCs present in the extract, and the overall composition of the matrix of the plant. When watercress was used on HaCaT cells and other cell lines, such as human microvascular endothelial cells, in a study where the angiogenic effect of the plant extract was evaluated, a decrease in cell growth was shown, an observation that agrees with our findings (Loboda et al., 2005). After the treatment of lung carcinoma cells A549 with nanoparticles that carry the plant extract, a decrease in cell growth was shown with IC_{50} at 80 µg / mL (24h), 70 µg / mL (48h) and 40 µg / mL (µg of plant extract) (72h) (Adlravan et al., 2021). Watercress extracts have not been widely studied on HaCaT cells, but studies on breast cancer cells show a cell growth inhibiting effect of the plant extract through alteration of ALDH1 (a marker of normal and malignant human breast cells) and SOX9

expression (Abbasi et al., 2018). SOX9 is a protein that is involved in the formation and progression of tumors, and its inhibition shows anti-cancerous effects. When broccoli and watercress extracts were applied on breast cancer cells (MDA-MB-231), both plants were shown to reduce viability (Rose et al., 2005). Although the mentioned studies did not include an incubation step for the hydrolysis of GSLs to ITCs, LC-MS analysis showed the presence of ITCs in the plant matrixes, indicating a very rapid ITC formation immediately after the homogenisation of the leaves. Similar effect was shown when watercress was applied on other cells lines, such as human colon adenocarcinoma cells and human lung fetal fibroblasts confirming the anti-proliferative effect of watercress (Boyd et al., 2006). Various extractions were utilised for the extraction of total ITCs from watercress and the material was applied on human colorectal adenocarcinoma cell lines, reducing cell viability with an IC_{50} of 50 μM expressed in PEITC (Rodrigues et al., 2016). Conversely, no changes on cell viability were shown when watercress extract was applied on peripheral blood cells. Other plants extracts containing ITCs, such as meadowfoam, have also been used on human primary epidermal keratinocytes, where cells were exposed to UVB and MPACN was shown to fail to protect cells from viability reduction, while MBITC further reduced cell viability (Carpenter et al., 2018).

6.2.2 Apoptotic effect of combinational treatments of HaCaT cells with UVB and watercress extract.

For the evaluation of the effect of watercress extract on apoptosis of HaCaT cells, both the synthetic and the natural extract were used. Apoptosis was evaluated through the caspase 3/7 activity that indicates late apoptosis. It is shown that the watercress extract has the same effect on apoptosis with apoptotic cells at 26 ± 2.47% and necrotic cells at 41 ± 3.85% when compared with synthetic PEITC apoptotic cells: 22.8 ± 2.83% and necrotic cells: 44.8 ±

3.25% ($p>0.05$). It is observed that the natural extract has slightly more apoptotic cells and less necrotic cells, but this difference is not significant (figure 6.2A). The two combinations of either the synthetic or the natural extract are shown to significantly induce caspase 3/7 activation compared to the relative controls ($p<0.0001$) (table 6.1). Moreover, it is necessary to note that the watercress extract had more debris that had to be excluded from the data and we hypothesise that this is the reason the growth assay is showing more reduced results when compared to the synthetic PEITC, while results on apoptosis are similar.

Little research has been conducted on the apoptotic effect of watercress extracts on HaCaT cells. However, in a study where nanoparticles that carried watercress extract were applied on lung carcinoma A549 cells, a significant increase of apoptotic cells was observed at 63.3% for 40 μg / mL and at 72.3% for 60 μg / mL, concentrations significantly higher than in our study with use of 0.0033 μg / mL of total ITCs, while the expression of p53, BAX and caspase 3 apoptotic biomarkers was also increased (Adlravan et al., 2021).

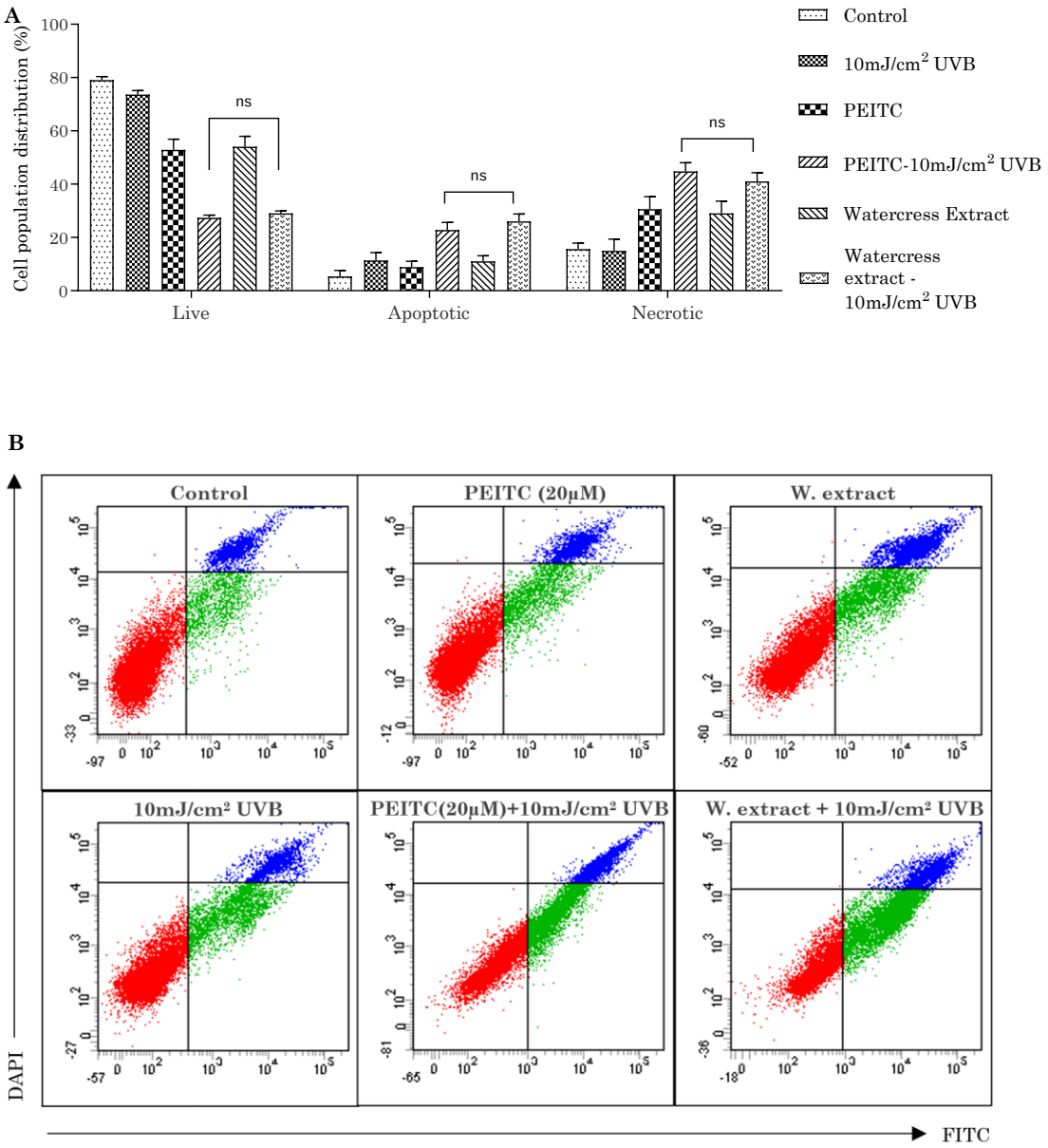


Figure 6. 2 *Cell apoptosis of the combinational treatments for psoriasis using PEITC or watercress extract on HaCaT cells*

*Cells were treated with 10 mJ / cm^2 UVB on a thin layer of ice. Immediately after the exposure, cells were treated with 20 µM of PEITC or watercress extract. The apoptotic and necrotic cell population distribution was observed at using CellEvent Caspase 3/7 Green flow cytometer assay kit, along with DAPI and live, apoptotic and necrotic cells are shown are shown as percentage of events (A) and (B) cell events (20,000 events/second) in scatter plots. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001, relative to corresponding controls. Data shown are means of ±SD of three replicates from 3 independent experiments.*

Table 6. 2 *Percentages and standard deviation of live, apoptotic and necrotic cells from the combinational treatments of 10 mJ / cm2 UVB and either 20 µM PEITC or 20 µM of total ITCs in watercress extract*

Sample	Live cells			Apoptotic cells			Necrotic cells		
	%	±SD	N	%	±SD	N	%	±SD	N
Control	79	1.41	3	5.4	2.26	3	15.65	2.19	3
UVB	73.65	1.48	3	11.35	2.9	3	14.95	4.45	3
PEITC	52.9	3.96	3	8.9	2.12	3	30.65	4.6	3
PEITC+ UVB	27.4	0.99	3	22.8	2.83	3	44.8	3.25	3
W. extract	54	3.96	3	11	2.12	3	29	4.6	3
W. extract+ UVB	29	0.79	3	26	2.47	3	41	3.85	3

Statistical analysis was done using two - way ANOVA.

Similar observations were made when the pure form of PEITC, the dominant ITC in watercress, was applied on tumor promoter-sensitive mouse epidermal cells and apoptosis, along with p53 protein expression, were induced (C. Huang et al., 1998). When watercress extracts were used on various cancer cells, such as human lung fetal fibroblasts, a G_1 and S phase arrest was observed, along with a reduced viability indicating inhibition of carcinogenesis after treatment with 20 µM of watercress extract (Boyd et al., 2006). The reduced cell count observed in the mentioned study also indicates potential effect of the plant extract on apoptotic pathways that lead to reduced cell count, either through DNA damage

or necrosis. Similarly, G_1 and S phase arrest was observed on human prostate cancer cells, when mice with the xenografted tumors were fed with a PEITC metabolite and an increase of apoptosis through cleavage of poly ADP-ribose polymerase (Chiao et al., 2004). Poly ADP-ribose polymerases are a family of proteins that are involved in programmed cell death which are inactivated when caspase 3 is cleaved and apoptosis takes place. On the other hand, watercress was shown to reduce DNA damage on cancer cells through the increase of carotenoids, when the extract was introduced as a supplement on healthy adults (Gill et al., 2007). Based on the above, watercress extract and synthetic PEITC, (as also shown in chapter 5), induce apoptosis in various pathways and therefore, the evaluation of the effect of their combination with UVB on the mitochondria is essential. It is suspected that the combinational treatment acts synergistically and PEITC enhances the effect of UVB in various ways, such as intracellular stress. To test this hypothesis, the mitochondrial membrane permeability of HaCaT cells following the treatment of either watercress extract or synthetic PEITC, in combination with 10 mJ / cm^2 UVB was also studied.

6.2.3 Effect of combinational treatments of HaCaT cells with UVB and watercress extract on mitochondrial membrane permeability.

For further evaluation of the effect of the watercress extract on HaCaT cells apoptosis, when combined with 10 mJ / cm^2 UVB, intrinsic apoptosis was also studied. The effect on the mitochondrial membrane permeability through depolarization ($\Delta\Psi m$) was studied using JC-1 stain and the mitochondrial dysfunction was expressed as a ratio of JC-1 aggregates over JC-1 monomers. Once again, as in section 5.2.2, synthetic PEITC was also included. It is shown that both the synthetic PEITC and the watercress extract equally increase the mitochondrial membrane permeability, through the reduction of $\Delta\Psi m$, when combined with

UVB, as shown in figure 6.3, while the effect is milder, but significant, with the absence of UVB.

As mentioned in chapter 5, mitochondrial membrane depolarization is caused by intracellular ROS induction through Fas protein expression (Farrukh et al., 2014) which leads to release of cytochrome C and the production of caspase 9 and procaspase 3, and therefore it is possible that the watercress extract is inducing apoptosis in such pathways. As mentioned in section 5.2.5, watercress extract induces apoptosis through various ways, such as caspase 3 activation and cell cycle arrest, with a 63.3% for 40 μg / mL and at 72.3% for 60 μg / mL of watercress extract and 20 μM of watercress extract in another study (Adlravan et al., 2021; Boyd et al., 2006). Although the mechanism by which UVB is inducing apoptosis is well known and described in various parts of this thesis (chapter 4 and 5), the mechanism by which the combinational treatment is inducing cell apoptosis have not been studied before for the treatment of psoriasis. It is possible that the synergistic effect of the combination is enhancing multiple pathways to apoptosis. PEITC is shown to act as an anti-inflammatory compound on various cells, an effect that comes in contrast with our findings, since the phytochemical, either synthetic or naturally derived, it failed to reduce oxidative stress caused by UVB, based on the results of this study.

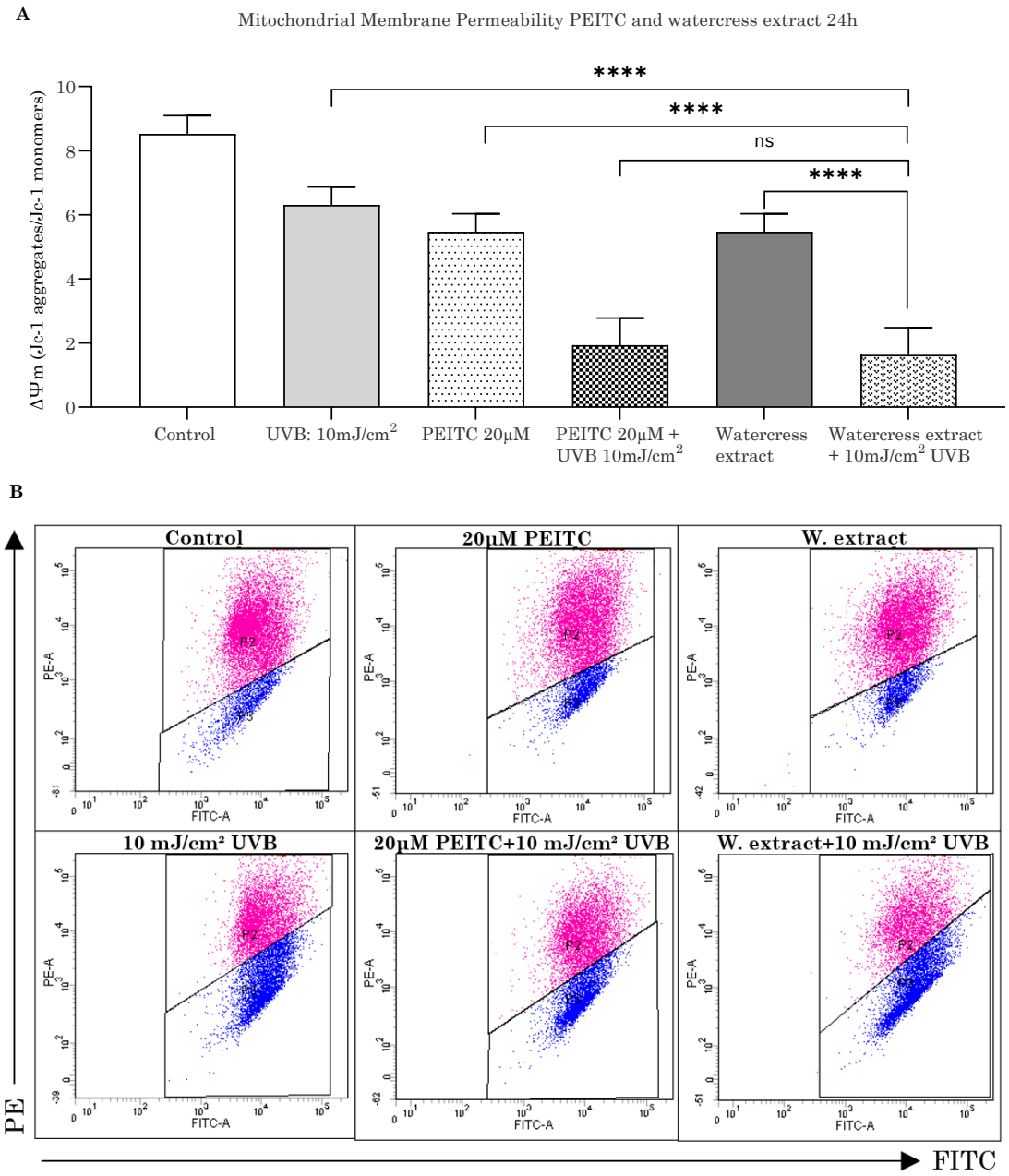


Figure 6. 3 *Mitochondrial membrane permeability of HaCaT cells after the combinational treatments with 10 mJ /cm2 UVB and 20µM of PEITC or 10 mJ /cm2 UVB and watercress plant extract*

*Measurements were taken at 24h using flow cytometry. The number of JC-1 monomers and aggregates were quantified as (A) the ration of JC-1 aggregates over JC-1 monomers and (B) cell events in a scatter plot. Statistical significance was set as *p < 0.05, **p < 0.01 ***p < 0.001, ****p < 0.0001,* ***ns*** *as not significant, relative to corresponding controls. Data shown are means of ±SD of three replicates* from 3 independent experiments.

The potentially differentiated effect of the watercress extract on cell apoptosis and mitochondrial function needs to be further explored by looking at apoptotic genes, such as pro-caspase 9, and proteins expression, such as caspases 3, 8 and 9, as well as oxidative and inflammatory markers, such as cytokines (interleukins), through utilization of PCR and western blots. Gill *et al.* 2007, showed that watercress extract, when supplemented, acts as antioxidant through the increase of carotenoids and decreases DNA damage, tested with comet assay, in healthy adults, reducing the risk of cancer (Gill et al., 2007). The anti-inflammatory effect of the same plant extract was shown in a study where watercress leave extract was applied in mice, on which psoriasis was induced using croton oil (Camponogara et al., 2019). The post-treatment of the diseased subjects with the extract reduced oedema, cytokine levels, and NF-κB protein expression by 100%, revealing the significant anti-inflammatory effect of watercress. In another study where the botanical complex HAT1, that contains watercress and it is used for its anti-inflammatory effect, was used on patients with Psoriasis a reduction of the disease was observed through the improvement of psoriasis area and severity index by 75% (Alex et al., 2020). Moreover, the mentioned extract is shown to inhibit VEGF synthesis in HaCaT cells, a growth factor that regulates neovascularization and it a known marker of psoriasis (Loboda et al., 2005). Therefore, the anti-inflammatory effect of the plant extract on the diseased skin is a major indicator of the beneficial role of watercress on psoriasis. However, it is still unknown whether the combination with UVB maintains this anti-inflammatory effect and whether the post-treatment with the extract can inhibit the carcinogenic effects of UVB but maintain the apoptotic effect. Moreover, ITCs are known to for their antioxidant indirect effect through the induction of phase 2 enzymes (Rose et al., 2000) and specifically the Nrf2 target genes, such as NAD(P)H Quinone Dehydrogenase 1 (NQO1), Heme oxygenase-1 (HO1) and glutathione S-transferase pi-1 (GSTP1). Studies on Moringa extracts, a plant rich in ITCs, have shown the anti-inflammatory properties of the compounds on rat paw erythema by reducing the symptoms

by 33%, nitric oxide (NO) (1 μM), IL-1β, IL-6 (5 μM of total ITCs in Moringa) and Nrf2 target genes (10 μM of total ITCs in Moringa) (Jaja-Chimedza et al., 2017) and on mice suffering from colitis symptoms, reducing IL-1β, and TNF-α expression, while reducing colonic inflammation and pro-inflammatory cytokines derived from the keratinocytes (Y. Kim et al., 2017). From the latter study it is also shown that the effects of ITCs can affect keratinocytes not only when applied topically but also *via* oral supplementation. The same plant extract was used on C57BL/6J mice to study the anti-diabetic effects of the compounds contained in Moringa, and similar observations were made on levels of IL-1β and TNF-α (Waterman et al., 2015).

6.3 Conclusions

The combinational treatment of watercress extract, naturally containing 20 μM of PEITC with 10 mJ / cm^2 UVB studied in this chapter, to the best of our knowledge, is a novel approach for the treatment of psoriasis. Although the two parameters have been shown to have effects on the disease in various ways, such as anti-inflammatory, antioxidant, anti-proliferative effect of the watercress extract and apoptotic and stress inducing effect of UVB leading to reduction of cell viability, the current study has shown a cell viability reduction, apoptotic effect and $\Delta\Psi m$ decreasing effect of them alone or combined. Moreover, this chapter validates our findings from chapter 3 and the total ITCs found in watercress, based on the similarity of effect of the natural extract when compared to the effect of the synthetic PEITC. However, due to the complexity of the plant matrix, further experiments are required to evaluate the mechanisms by which the watercress extract in combination with UVB, affects psoriasis biomarkers, such as VEGF. Experiments including the expression of genes and proteins that are involved in inflammation, such as interleukins and cytokines, and Nrf2 signaling pathways, to evaluate the effect on ROS induction are required in order to

determine whether ITCs enhance or inhibit the effect of UVB on ROS induction and whether the damage caused from the combination of them is due to oxidative stress-induced apoptosis or other pathways are also activated.

This study provides the following significant findings:

1. HaCaT cell growth: 20 μM watercress extract expressed in PEITC significantly ($p<0.0001$) reduced cell growth, while its combination with 10 mJ / cm^2 UVB further ($p<0.001$) enhanced the effect (figure 5.1).
2. HaCaT cell apoptosis: The comparison of the combinational treatments using watercress extract or synthetic PEITC, reveals a similar effect of the two, on caspase 3/7 activation, increasing apoptotic cells significantly compared to the controls of UVB alone and ITC alone (figure 6.2).
3. Mitochondrial membrane permeability: Significant reduction of the ratio of JC-1 aggregates over JC-1 monomers ($\Delta\Psi m < 6$) on HaCaT cells from watercress extract and synthetic PEITC is shown, while a further enhanced effect ($\Delta\Psi m < 2$) is shown when watercress or PEITC are combined with UVB.

7. Final Discussion

7.1 Discussion on findings

Skin diseases are often characterised by inflammation of the skin either topically or in the whole body and are accompanied by other symptoms, more specific to each disease. Phytochemicals have been widely studied and used in the treatment of skin disease, such as dermatitis, photo-aging, allergic reactions, and psoriasis, mostly due to their anti-inflammatory and antioxidant properties. The preference of phytochemicals over drugs for the treatment of such skin conditions is usually based on their low side effects on the patient. Both topical and oral application of phytochemicals, either in a supplementation form or through nutrition has been widely used and studied either for the prevention or supportive therapy of disease. Vegetables are well known for their health benefits and support of the immune system. Their absorbance, bioavailability and function is well studied. Cruciferous vegetables, known for their high content in ITCs, have been studied for their effects on skin cancer, photo-aging and other inflammatory conditions (Prawan et al., 2009; Shibata et al., 2010; Yehuda et al., 2012). Although they are well known for their antioxidant and cancer preventive properties, their effect on cells and diseased skin is more complex. Their biphasic effect on cell function and survival, such as the induction of phase II enzymes increasing the antioxidant effect of cells and the GSH cleavage, reducing the available antioxidants in cells, is crucial on the development of a treatment. The above mechanisms depend on the ITC concentration and the length of the exposure of cells to the phytochemicals. It is shown that low concentrations of ITCs act as antioxidants in stressed cellular environment, such as after UVR exposure, while their anti-inflammatory effects are shown to reduce such response from cells (Dinkova-Kostova et al., 2007; Saw et al., 2011b; Xu et al., 2005). On the other hand, the anti-proliferative effect and their ability to induce apoptosis on cancer cells makes the phytochemicals good candidates for the treatment of skin cancer or other diseases that

the targeted reduction of cell viability or arrest of cell growth is desired (Conaway et al., 2000; Guerrero-Beltrán, Mukhopadhyay, et al., 2012).

In this study, the biphasic character and effect of ITCs was studied on human immortal keratinocyte (HaCaT) cells, initially for their preventive effect on UVB-induced damage as a preventive mechanism against photo-aging, while their anti-proliferative effect and induction of apoptosis, in combination with low dose UVB, is studied as a novel therapeutic approach for psoriasis.

To understand the bioavailability of ITCs in vegetables, the quantification of total ITC content in watercress was studied (chapter 3). Although current methods for the quantification of isothiocyanates in cruciferous vegetables are well established, they are utilising either expensive methods of analysis, environmentally and time-consuming methods of extraction or both. In the current work, an environmentally friendly, low cost and simple method was developed, aiming to provide an industrially approachable methodology that requires little training and expertise. The standard curves of five ITCs studied in this work (SFN, IBN, AITC, BITC and PEITC) gave a response factor from 0.028 to 0.035, indicating limited variation in the reaction of ITCs, while the limit of detection (LOD) was 1-60 μg / mL or 1-400 μM or 1-60 mg / g dry matter. Compared to the literature (Y. Zhang et al., 1992), the maximum LOD presented in the current work is significantly higher with an actual confidence level of 98.4%.

For the determination of total ITC content in plants, watercress was selected amongst the vegetables of its family, due to its high content in PEITC and the challenging volatile nature of the ITC. Matrix component interference was examined, revealing a macromolecule interference that was successfully resolved by the addition of a clarification step that includes addition of methanol and centrifugation, improving the reliability of the method by reducing the coefficient of variation from 23.6% to 14% (n = 6). No fibre or metal interference is

shown, while plastic is shown to interfere with the quantification due to the hydrophobic nature of ITCs. The use of a glassware increased recovery of ITCs by 35% and gave a 7-fold decrease in the corresponding coefficient of variation from 14% to 2%. The developed methodology, with an 88.95% recovery, detected 1.54 ± 0.038 μmol / g of total ITCs content in freeze-dried watercress powder, while significantly higher quantities of 20.6 ± 0.31 μmol / g were detected in fresh watercress. To validate those findings, a hexane extraction protocol was following according to the literature (Rodrigues et al., 2016) and similar detection was shown (table 3.3).

To further investigate the significant difference of detection between the freeze-dried and the fresh watercress, exogenous myrosinase was added to the freeze-dried sample and a 10-fold increase in ITC content was detected, while samples of fresh watercress did not show a significant increase in ITC content with the addition of the enzyme. As most researchers have used exogenous myrosinase to initiate their extraction processes and detected higher levels of ITCs, compared to our findings (Gill et al., 2007), it is hypothesised that freeze-drying process and / or storage of watercress powder resulted in either loss or inactivation of myrosinase.

Current preventive methods of photo-aging are sunscreens that are separated into organic and inorganic sunscreens and are widely used either solely for sun protection or in other cosmetic products to provide a solar protection factor (Gasparro et al., 1998). The effect of plants or compounds found in nature and their application on potential products have been studied (Kostyuk et al., 2018). The aim of this work was to determine the effect of ITCs on a UVB-induced model, in a concentration and time manner.

For the determination of the effect of ITCs on photo-aging, SFN and PEITC were used a UVB-induced photo-aging model and cell viability was observed (chapter 4). SFN has been widely studied for its antioxidant and anti-inflammatory effect and many studies have shown

that SFN protects HaCaT and human dermal fibroblasts from the UVB damage, while limited research is conducted on the effects of PEITC on a photo-aging model. This is probably due to the known toxicity of PEITC and its volatile nature that makes it more sensitive while handling and analysing. The two phytochemicals differ in their chemical structure and while they are both shown to have the biphasic effects of ITCs, our findings reveal a different response between the two.

For the optimisation of the photo-aging model, various intensities of UVB (2.5, 10, 25, 50, 100 mJ / cm^2) were used for the exposure of the cells, with IC_{50} 58.81 mJ / cm^2 for 24h, 55.25 mJ / cm^2 for 48h and 42.60 mJ / cm^2 for 72h. 25 and 50 mJ / cm^2 UVB was considered the two most suitable intensities for further experiments on this model, with 6 concentrations (0.15, 0.3, 0.6, 1.25, 2.5, 5 μM) of either of the two ITCs used for the pre-treatment of the photo-aging model (figure 4.1). SFN is shown to increase cell viability in the photo-aged HaCaT cells in most concentrations, and both UVB intensities, while PEITC is shown to have no effect on cell viability at most low concentrations but does significantly increase cell viability at 72h in the 50 mJ / cm^2 UVB model, indicating a reparative effect. On the other hand, 5 μM of PEITC was shown to further reduce cell viability at the 25 mJ / cm^2 UVB model, indicating an opposite effect of UVB and PEITC compared to SFN (figure 4.4). Although, ITCs are known to be toxic and have an anti-proliferative effect in high concentrations, studies have shown that this effect is observed in concentrations > 10 μM (Mantso et al., 2016; Mitsiogianni, Trafalis, et al., 2020). In this study we showed even when ITCs were used for the treatment of cells without UVB, 0.6, 1.25 and 5 μM of PEITC reduced cell viability at 24 and all concentrations at 48h, while SFN reduced cell viability only at 2.5 and 5 μM (figure 4.3). Interestingly, this effect is reversed when the same concentration was used on the UVB-exposed model, increasing cell viability when compared to the UVB control (figure 4.4). Although SFN and PEITC are shown to have a different response on the two photo-aging models of 25 and 50 mJ / cm^2 UVB, they are both promising

compounds for the prevention of UVB damage and their potential use in cosmetics and products that aim to protect from UVB. However, the effect shown is not as strong as expected and when compared to the literature. To evaluate this even further, shorter timepoints were included in our experiments and no effect was shown at 2h and 4h after the UVB exposure (Appendix B.2). Moreover, post-treatments of the UVB-exposed cells were conducted but no effect was shown. It is hypothesised that the length of the treatment of the cells with ITCs has a crucial role on the effects of the phytochemicals on cell function and that shorter treatment times could have a more protective effect on cells. However, this was not studied in the current work.

The effect of the two phytochemicals in combination with UVB on cell viability on HaCaT cells, has also been evaluated as a novel therapeutic approach for psoriasis (chapter 5). Current treatments for the management of the symptoms of psoriasis include use of steroid creams, vitamin D creams, UVB and UVA/Psoralen treatments and immunosuppression (Hönigsmann, 2001; Rahman et al., 2012). Although these treatments can be effective, there are many limitations when it comes to duration or repeatability of the treatment (Leon Carrion et al., 2014). Cream treatments can lack efficacy on severe cases, while UVR treatments are limited due to the risk of cancer development and the discomfort of the potentially burnt skin following the treatment. Immunosuppression, as the last and most drastic treatment, is not an ideal solution for patients suffering from psoriasis, because of the drug side effects and the risk of infections due to suppressed immunity and therefore, alternative and natural therapies are desired. Current natural remedies for the improvement of the symptoms of psoriasis include plant extracts and natural compounds that are shown to reduce inflammation of the skin and have an antioxidant effect, leading to the reduction of the symptoms (Rahman et al., 2012). However, these natural treatments are rarely sustainable and effective to a degree that provides a satisfactory treatment and are used as a side topical therapy in current established treatments, such as UVR and oral medication.

The aim of this work was to use mild conditions of specific parameters (ITCs and UVB) that have a negligible or mild effect on their own, but in combination are very effective. To the best of our knowledge, this is the first time that this approach has been presented, to investigate the anti-proliferative effect of ITCs in combination with low UVB, to enhance damage for use on psoriasis cells.

For the determination of cell viability after the combinational treatment of HaCaT cells with 20 μM of either of the five ITCs (SFN, IBN, AITC, BITC, PEITC) and 5 or 10 mJ / cm^2 UVB, two assays were utilised; a metabolic (resazurin) and colorimetric (SRB) assay. Results of both assays showed a reduction of cell viability and growth (approximately 15-20%) respectively, when using either of the ITCs alone, with the combinational treatment of UVB and ITC significantly reducing cell viability (20%-80%) and growth (20%-60%) further depending on the UVB intensity and timepoint, when compared to the relative ITC and UVB control (figures 5.4, 5.5, 5.6, 5.7, 5.8). From the five ITCs used for this work, IBN and PEITC are shown to be the most potent phytochemicals when used either alone or in combination with UVB, while BITC is also shown to have a mild effect (figure 5.7). On the other hand, AITC is shown to have no significant effect on cell viability or growth (figure 5.6). Overall, the resazurin assay showed a less severe effect of the treatments, when compared to the SRB assay where the difference between them was significant as mentioned above. Resazurin assay, as a method of measuring viable cells, indicates a good efficacy of the novel therapy in reducing cells, while the SRB assay, as a method of measuring growth (proliferation) indicates an even stronger efficacy of the therapy on slowing down the progress of the disease and reveal promising mechanisms that could successfully treat psoriatic lesions without severe side effects. In the case of our experiments the results were repeatable with relatively low standard deviation. 5 ITCs with 5 mJ / cm^2 UVB had an overall lower and not significant effect on cell viability at 24h and the effect at 48h was similar to the effect of 10 mJ / cm^2 at 24h (figures 5.4, 5.5, 5.6, 5.7, 5.8). The selection of the following

conditions was considered suitable for the determination of apoptosis: 20 μM ITCs and either 5 or 10 mJ ITCs with 5 mJ / cm^2 at 48h and 10 mJ / cm^2 at 24h.

Apoptosis through the activation of caspase 3/7 by the selected treatments, showed a significant increase in apoptotic cells with IBN and PEITC being the most potent ITCs in line with viability assay. Interestingly, although AITC did not show a significant effect on cell viability and growth on either of the assays (figure 5.6), an increase in apoptotic cells was observed ($p<0.05$) at 24h and 10 mJ / cm^2 UVB (figure 5.11). Intrinsic apoptosis through the mitochondrial membrane depolarisation leading to increased permeability, was studied on the same conditions, showing a decrease of $\Delta\Psi m$ for all combinations (figures 5.14, 5.15, 5.16, 5.17, 5.18). Once again AITC showed a significant ($p<0.0001$) increase of mitochondrial membrane permeability, an effect that was not shown during cell viability assessment (figure 5.16). UVB is known to induce ROS formation and cause oxidative stress to the cell leading to apoptosis, while ITCs are known for their indirect antioxidant effect, through the induction of phase II enzymes. Conversely, ITCs are shown to create cleavage with GSH and therefore reduce the available GSH to act as an antioxidant during cellular stress. This is possibly one of the mechanisms that ITCs enhance the effect of UVB, along with an additive effect of the phytochemicals apoptotic inducive effect, in the case of this work. Moreover, UVB is shown to increase Fas receptors in cells, leading to apoptosis through the caspase 8 activation, and induce ROS formation through BAX and BAK pathways leading to cytochrome C release from the mitochondria to the intracellular matrix and eventually activate caspase 9 and procaspase 3 that lead to apoptosis (Djiadeu et al., 2017; Farrukh et al., 2014; Paz et al., 2008; Van Laethem et al., 2004). All the above mechanisms are hypothesised to work at least additively and create this enhanced effect observed in the combinational treatments presented in this work.

The effect of the suggested therapeutic approach was further investigated, looking at potential cell cycle arrest, and subG_1 phase arrest is shown at combinations UVB with either SFN, IBN or BITC, while the increase of this phase is also shown when the PEITC combination was used but this was not significant, at 24h and 10 mJ / cm^2 UVB (figure 5.19) A decrease of G_2/M phase is shown from combinations with SFN, IBN, BITC, while only combination with PEITC reduced this phase significantly when compared to the UVB control only. 48h and 5 mJ / cm^2 showed a milder effect on cell cycle with combinations of UVB with SFN, IBN and AITC, increasing subG_1 phase significantly, when compared to both relative controls (figure 5.20). In the literature, 10 μM of BITC are shown to increase G_2/M phase at 24, 36 and 48 hours by 12-50%, while G_0/G_1 and S phases were reduced in human melanoma A375.S2 cells (S. H. Huang et al., 2012). It is shown that this cell cycle arrest was conducted through multiple pathways of apoptosis and ROS induction. In another study, where fibroblasts and HaCaT cells were exposed to 10, 20 and 30 mJ / cm^2 UVB, a G_2/M phase arrest and G_1/S phase arrest was shown, respectively at 24h (Farrukh et al., 2014; Geng et al., 2011).

To further investigate the cause of cell cycle arrest and apoptosis observed so far, the presence of oxidative stress through ROS induction was studied. The DHR123 assay showed a significant ROS induction at combinational treatments of BITC (24h - 10 mJ / cm^2 UVB) and IBN (48h - 5 mJ / cm^2 UVB), while the rest of the combinations also showed an induction, but it was not significant (figure 5.21). In a study where higher intensity of UVB was used (50 mJ / cm^2 UVB) on HaCaT cells, a peak of ROS induction was observed at 24h (Paz et al., 2008). The determination on whether the increased Ca^{2+} levels (Ca^{2+} stimulates an activity that leads to increased levels of ROS) that were observed after the exposure of fibroblasts and HaCaT cells to 10, 20 and 30 mJ / cm^2 UVB, by the addition of an antioxidant mix of Troxol and ascorbic acid, confirmed that the effect of UVB is caused by intracellular ROS induction (Farrukh et al., 2014). In order to validate the above findings and further

investigate the potential ROS induction caused from the combinational treatments, the addition of GSH antioxidant was necessary. The presence of GSH is shown to reverse the effect of all treatments, including the effect caused by the controls alone, confirming that the apoptotic effect is possibly caused by ROS induction. GSH is shown to mitigate the damage caused from ROS and the cell viability levels remain unaffected from either treatment, when cells were pre-treated and post-treated with the antioxidant (figure 5.22). Once again, the biphasic effect of ITCs was shown in the literature where in a study conducted from Kleszczyński *et al.* 2014, SFN and PEITC were used on HaCaT cells, and an upregulation of Nrf2 target genes (γ-GCS, HO-1, NQO1) was shown using RT-PCR at 5 μM of both isothiocyanates. On the other hand, 25 μM of the same isothiocyanates did not have the same effect (Kleszczyński et al., 2013). Similarly, 5μmol / l of SFN increased mRNA levels of γ-GCS and HO-1 by 150% when applied on the same cell line (Wagner et al., 2010). The same isothiocyanate (1-10 μM) reduced the AP-1 activation caused by 250 J / m^2 UVB and an increase of GSH and QR-1 (quinone reductase 1 reduces quinones to semiquinones and is marker of phase 2 enzymes) was observed at 24h (Zhu et al., 2004). BITC at 10 μM, was shown to increase ROS levels in a time dependent manner, in A375 cancer cells (S. H. Huang et al., 2012). Not much research is done on similar combinational models of ITCs and UVB on HaCaT cells. However, when SFN (5 and 10μM) was studied for its potential protective effect on a UVB induced model (50 mJ / cm^2 UVB) an anti-inflammatory effect was revealed through the reduction of IL-1β, IL-6 and COX-2 in HaCaT cells (Shibata et al., 2010). A similar effect was observed on fibroblasts, when 2 and 5 μM of SFN blocked UVB-induced (25 mJ / cm^2) NF-κB activation and MMP expression, in a dose dependent manner (S. Y. Lee & Moon, 2012). In the contrary, the same phytochemical (3 μM) did not protect HaCaT cells from direct UVB (20 mJ / cm^2) damage (Benedict et al., 2012).

The findings of chapter 5 were validated in chapter 6 using natural aqueous extract derived from watercress powder and results were compared using synthetic PEITC. The effect of

watercress extract was studied on cell viability and extrinsic and intrinsic apoptosis through caspase 3/7 activation and mitochondrial membrane permeability. The extract is shown to have a similar effect when compared to the synthetic PEITC. However, due to the observed differentiated morphology of the cells, when treated with the natural extract, the exact pathways and effect on cells, in combination with UVB, need to be further investigated.

7.2 Study limitations and suggested future experiments

Further research is required to establish the therapeutic efficacy of the suggested combination therapy on psoriasis that is presented in this study and the potential reparative effect of PEITC for the prevention of photo-aging.

7.2.1 Total ITC content quantification

1. Although the suggested methodology was developed using a volatile ITC, it is still to be confirmed whether the same method can be applied on other cruciferous vegetables. Use of other cruciferous vegetable extracts, such as broccoli, cauliflower, and Brussel sprouts, to confirm suitability of the method for a variety of plants. It is unknown whether this method has the same efficiency on the quantification of total ITCs on plants with harder stems and leaves. This is possibly due to the chemical structure of ITCs that varies depending on the phytochemical. BITC and PEITC are shown to have similar chemical structure with similar effects on cell viability, as shown in this study (chapter 5). Moreover, challenges such as the volatility of the phytochemicals needs to also be studies and considered for further evaluation and validation of the method developed.
2. Further validation of findings using GC-MS, taking in to account the rapid degradation of the cyclocondensation end-product that required specific storage

conditions. Although experiments using GC-MS have been conducted for the validation of our findings (appendix A), the tested method failed to detect any ITCs possibly due to the low storage temperature.

7.2.3 Photo-aging

1. Use of other cell lines, such as pig keratinocyte cells or human dermal fibroblasts would provide a rich evaluation of the effect of ITCs on the disease and therefore a limitation of this study is the use of only one cell line. Fibroblasts produce collagen and elastin proteins that create the protective network of the skin, while they are the main markers of photo-aging. Moreover, the production of MMPs from the same cell line is also an important biomarker of photo-aging and its increase from UVB is known to cause a further collagen degradation, resulting to a less robust extracellular network. The effect of ITCs in the above biomarkers are important to be evaluated in order to provide a better understanding of the role of ITCs on gene expression.
2. RT-PCR analysis for the expression of the above biomarkers (collagen, elastin, MMPs) and western blot analysis for the expression of the proteins will provide a better understanding on the effect of ITCs on photo-aging and their potential use for the prevention of the disease.
3. Use of reconstituted tissue would provide a more complete model, as well as the inflammatory response that HaCaT cells do not provide. Moreover, tissue will provide a better understanding of the distribution of UVB and a more realistic approach of the effect of ITCs. Reconstituted tissues have the benefit of inflammatory response, a significantly important factor that can change and enhance the findings of this project, through evaluation of inflammatory markers, such as

Interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-alpha) and C-reactive protein (CRP).

7.2.4 Psoriasis therapy using synthetic ITCs

1. Further analysis on the induction of ROS by the evaluation of antioxidant Nrf2 target gene expression or downregulation, such as γ-GCS, HO-1 and NQO1. A study conducted from Kleszczyński *et al.* 2014, SFN and PEITC were used on HaCaT cells, and an upregulation of Nrf2 target genes (γ-GCS, HO-1, NQO1) was shown using RT-PCR at 5 μM of both isothiocyanates. On the other hand, 25 μM of the same isothiocyanates did not have the same effect (Kleszczyński et al., 2013). Similarly, 5μmol / l of SFN increased mRNA levels of γ-GCS and HO-1 by 150% when applied on the same cell line (Wagner et al., 2010). The same isothiocyanate (1-10 μM) reduced the AP-1 activation caused by 250 J / m^2 UVB and an increase of GSH and QR-1 (quinone reductase 1 reduces quinones to semiquinones and is marker of phase 2 enzymes) was observed at 24h (Zhu et al., 2004).
2. Extend the analysis to a psoriatic reconstituted tissue which has the benefit of inflammatory response, a significantly important factor that can change and enhance the findings of this project, through evaluation of psoriasis markers, such as CD4, CD8 and VEGF. Watercress extracts are shown to inhibit VEGF synthesis in HaCaT cells, a growth factor that regulates neovascularization and it a known marker of psoriasis (Loboda et al., 2005). Therefore, the anti-inflammatory effect of the plant extract on the diseased skin is a major indicator of the beneficial role of watercress on psoriasis.

3. RT-PCR and western blots for the evaluation of the effect of the combinational treatment on HaCaT cells on VEGF expression, a main biomarker of psoriasis, as well as inflammatory cytokines, such as IL-1β, IL-6, COX2.

7.2.5 Psoriasis using watercress extract

1. Further analysis on the induction of ROS using either the addition of antioxidant, similar to chapter 5 and by the evaluation of antioxidant Nrf2 target gene expression or downregulation, such as γ-GCS, HO-1 and NQO1.
2. Use of different total ITC concentration on cells and comparison with the suggested treatment. For a better evaluation of the effect of the combinational treatment, the experimentation of multiple concentrations of ITCs with UVB on cells / reconstituted tissue is necessary.
3. Extend the analysis to a psoriatic reconstituted tissue that provide inflammatory response and the cells can activate their repairing mechanisms. Watercress extracts are shown to inhibit VEGF synthesis in HaCaT cells, a growth factor that regulates neovascularization and it a known marker of psoriasis (Loboda et al., 2005). Therefore, the anti-inflammatory effect of the plant extract on the diseased skin is a major indicator of the beneficial role of watercress on psoriasis.
4. Investigation and analysis of the extract's matrix and its potential effect on cells due to potential pH variations. The chemical profile of the extract could potentially alter the significance of the effect shown in this work.
5. Examine the effect of shorter treatment timepoints. Due to novelty of the combinational treatment suggested in this study and the complexity of the plant matrix, it is necessary that the effect of the therapy is evaluated in shorte timepoints providing a better understanding on the effect of the treatment on the cells / reconstituted tissue.

7.3 Concluding remark

This thesis has provided a novel methodology for the preparation of cruciferous vegetables and the quantification of total ITC content using the cyclocondensation assay as described from Zhang et al.,1996. The novel method benefits from rapid and eco-friendly sample preparation and relatively low-cost total ITC quantification. Evidence for the potential of PEITC and SFN, to rescue UVB-photo-aged HaCaT cells is also shown. Novel preliminary evidence is presented in this work, for the effect of the combinational treatment of HaCaT cells, with low dose UVB and ITCs, as a novel therapeutic approach for psoriasis, while this effect was validated using natural aqueous watercress extract on cells. The total ITC quantification method was also validated through the treatment of HaCaT cells with the extract and compared with known concentration of synthetic PEITC. The impact of ITCs and the relevant plant extracts on photo-aging, and the effect of their combination with UVB on psoriasis, should be further explored, for the development of a potentially improved treatment of skin disease and in support of the current promising findings (figure 7.1).

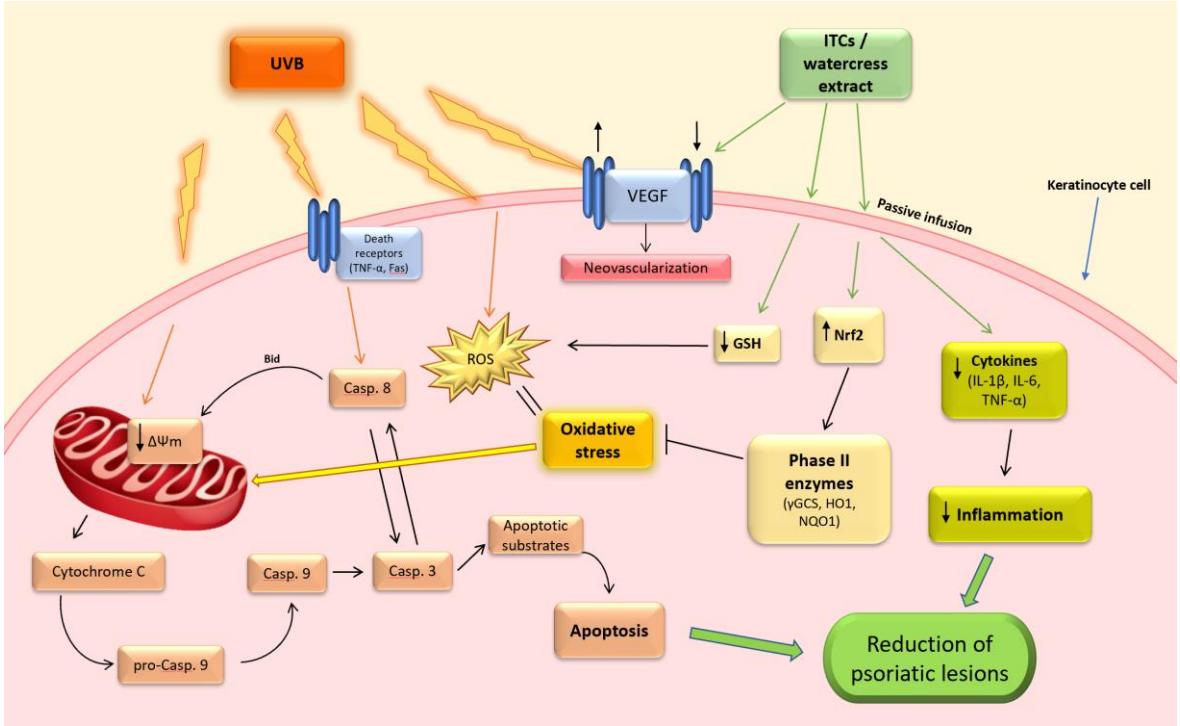


Figure 7. 1 *Tentative model of the novel therapy for psoriasis*

UVB induces ROS productions that leads to oxidative stress and increase of mitochondrial permeability. Damaged mitochondria induce cytochrome C and pro-caspase 9, that produce caspase 9, 3 and 8, leading to apoptosis. Caspase 8 is also induced by UVB-stimulated death receptors, and affects the mitochondria through Bid protein production, leading to cytochrome C production and eventually apoptosis. Isothiocyanates contained in watercress extract cleave glutathione (GSH), reducing the antioxidant capacity of the cell. On the other hand, isothiocyanates increase phase II enzymes (γGCS, HO1, NQO1) through the increase of Nrf2, while they reduce inflammation through the suppression of cytokines. UVB induced stress and apoptosis, in combination with the cleavage of GSH and the reduction of inflammation, could result in significant reduction of psoriatic lesions, as a novel therapy for psoriasis.

Appendix A

A.1 Clarification of watercress samples using solid phase extraction (SPE)

Solid phase extraction was utilised as a clarification step during the optimisation of the watercress extraction process. Preliminary experiments showed a high variation between samples of the same experiments, as well as between experiments (figure A.1). C18 cartridges were used to pass centrifuged watercress samples and 80% or 100% methanol solution was used for the elution of isothiocyanates. This method showed similar recovery when compared with the same methodology without the SPE, while 80% methanol/water solution was shown to successfully recover the synthetic PEITC, during recovery experiments (table A.1). Therefore, we concluded that the additional step of SPE does not provide an improvement on sample clarification, while the method itself is expensive and laborious.

Figure A. 1 *Concentration of total ITC content in watercress using solid phase extraction. Data shown are representative*

Table A. 1 *SPE recovery using 30 µg / mL PEITC*

SPE RECOVERY CALCULATIONS USING 30ug/ml OF PEITC									
rf	Intercept								
0.0285	-0.1393								
	ABS (365nm)	**ug/mL**	**AVRG**	**STDEV**	**Theoretical (ug**	**Recovery**	**AVRG**	**STDEV**	**RSD**
SPE (80%)	0.8507	29.99	30.70	1.11	30	99.96%	102.32%	0.0370	3.6%
SPE (80%)	0.8546	30.13			30	100.42%			
SPE (80%)	0.9074	31.98			30	106.59%			
SPE (100%)	0.0497	1.88	1.98	0.09	30	6.28%	6.60%	0.0029	4.3%
SPE (100%)	0.0534	2.01			30	6.71%			
SPE (100%)	0.0543	2.04			30	6.82%			
Total			32.68						

Data shown are representative of the SPE recovery experiments.

A.2 Validation of ITC quantification methodology using Gas Chromatography - Mass Spectrometry

For the validation of the developed methodology, watercress extract was added to GC/MS column (Supelcosil LC-18-T, 15 x 3 cm, 3 µm, flow rate 0.2 m L/ min, injection volume 5 µL, temperature: ambient, UV set at 365 nm, elution solution 65/35% methanol/water) in order to analyse and quantify the individual ITCs contained in the samples before and after the cyclocondensation assay. However, the instability of the PEITC in crude samples gave many variable results. In order to improve the handling process of the samples and get better results, cyclocondensation assay samples were also used for the GC/MS methodology. All experiments conducted in chapter 3, were repeated using GC/MS or HPLC as a method of analysis of ITCs in the watercress samples. However, the end product of the cyclocondensation assay is only stable in temperatures over 25 degrees, while in lower temperature the product is rapidly degradated (figure A.3, A4). It is hypothersised that the environment between the end of the cyclocondensation assay and the running time of each sample, has altered the data collected due to the sensitivity of the chemical, detecting significantly lower quantities of PEITC in the samples with a great variability. Further

optimisation of the analysis method was required in order to ensure stable conditions for the samples. Instead, an alternative method was used for the validation of our findings using a hexane extraction method, as described in chapter 3.

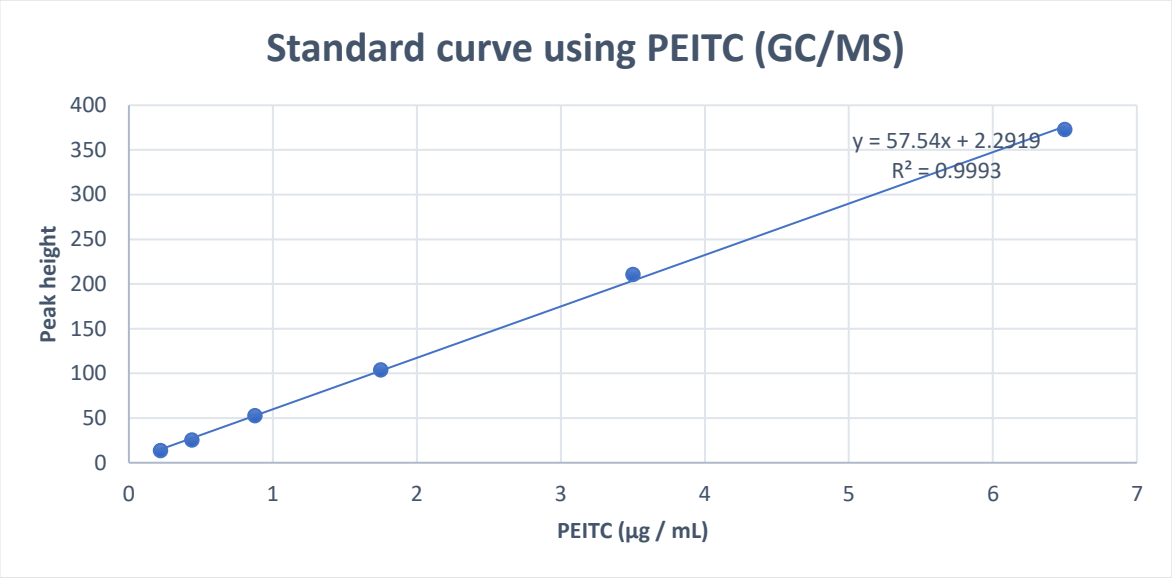


Figure A. 2 *Standard curve using PEITC and GC/ MS.*
Six concentrations of synthetic PEITC were diluted in methanol and passed through a GC / MS column. For the statistics of this curve Microsoft excel was used.

Table A. 2 *Concentration of PEITC found in watercress samples*

Date of experiment	Sample name	y- peak area	m	C	x - concentration ug/mL
07/09/2018	watercress sample 1	44.90723	104.96	-3.3675	0.459934546
07/09/2018	watercress sample 2	41.74604	104.96	-3.3675	0.429816502
07/09/2018	watercress sample 3	29.37465	104.96	-3.3675	0.311948838
12/09/2018	watercress sample 1	39.10817	112.64	-5.2201	0.393539329
12/09/2018	watercress sample 2	35.75702	112.64	-5.2201	0.363788352
12/09/2018	watercress sample 3	40.10204	112.64	-5.2201	0.402362749
12/09/2018	watercress sample 4	41.77222	112.64	-5.2201	0.417190341
12/09/2018	watercress sample 5	36.26489	112.64	-5.2201	0.368297141
12/09/2018	watercress sample 6	35.70066	112.64	-5.2201	0.363287997
16/08/2018	watercress sample 1	22.90836	57.54	2.2919	0.35829788
16/08/2018	watercress sample 2	19.78963	57.54	2.2919	0.304096802
16/08/2018	watercress sample 3	20.39192	57.54	2.2919	0.314564129

Watercress extracts were passed through the preliminary methodology (no methanol clarification step) and samples were analysed using GC / MS. Results show high viariability and low detection, when compared to the cyclocondensation assay results (chapter 3).

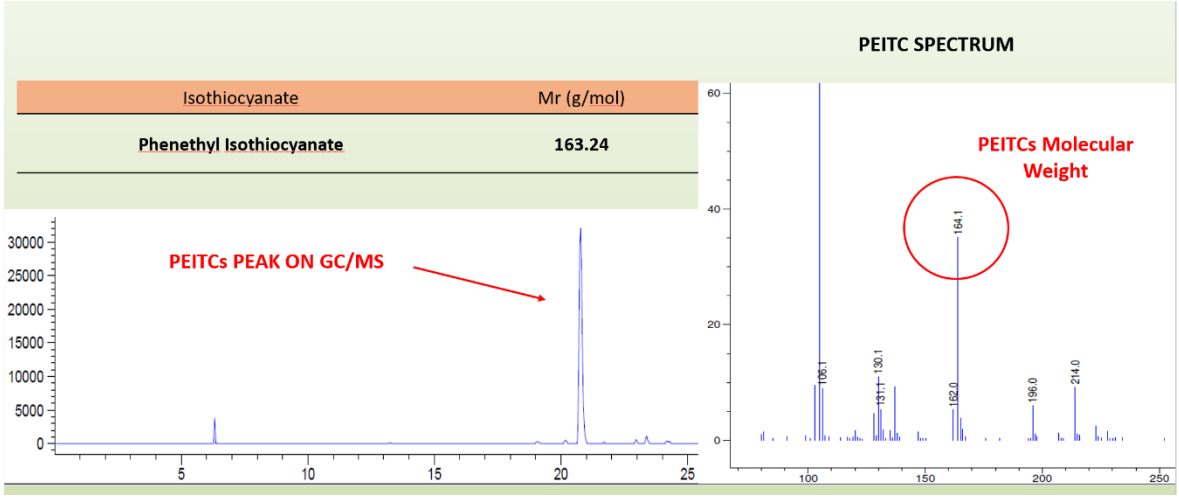


Figure A. 3 *PEITC peak and spectrum obtained from GC/MS*

Graphs are representative of the data.

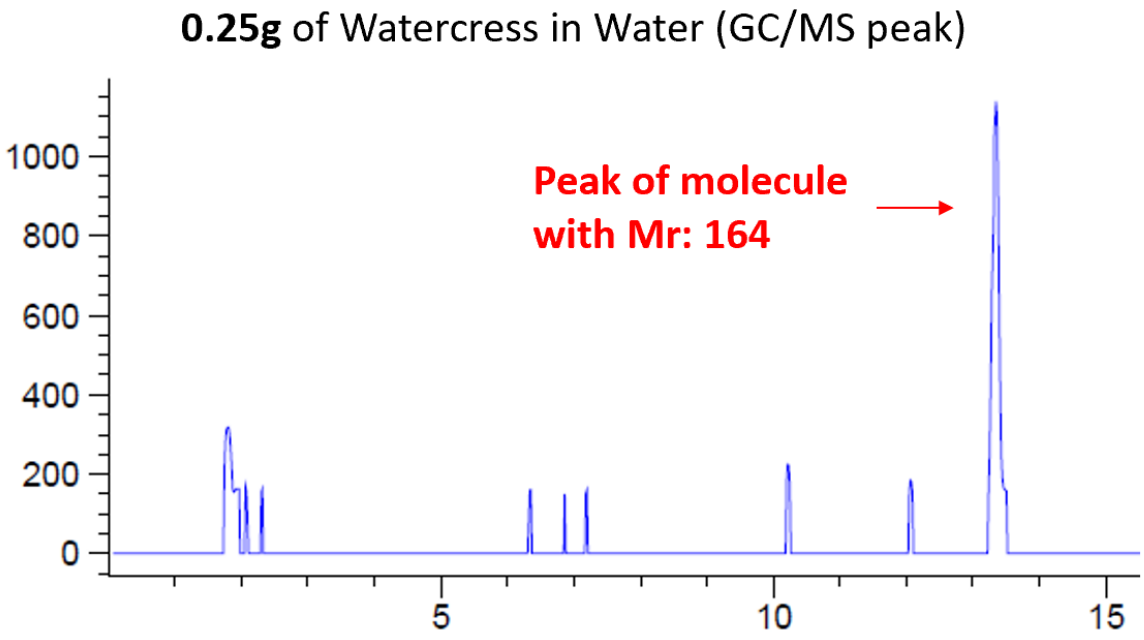


Figure A. 4 *PEITC peak shown in 0.25g watercress sample*

Graph is representative of the data.

Appendix B

B.1 Matrix Interference - Chapter 3 supplementary

B.1.1 Heavy metal interference

For the evaluation of heavy metal interference, Ethylenediaminetetraacetic acid (EDTA) was added to cyclocondensation assay samples substituting 0.1 mL of the buffer volume used for the assay, while control samples contained 0.1 mL of distilled water instead. For the spiked standard curve samples PEITC was used in 4 concentrations (7.5, 3.75, 2, 1 µg / mL). Statistical analysis of the data was carried out using GraphPad Prism 8. No heavy metal interference was detected after the addition of EDTA to watercress samples (figure B.1, B.2).

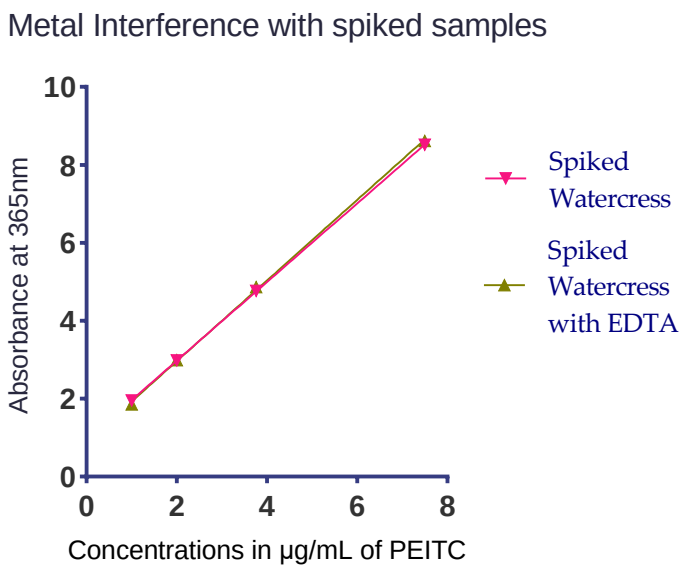


Figure B. 1 *Metal interference standard curve*

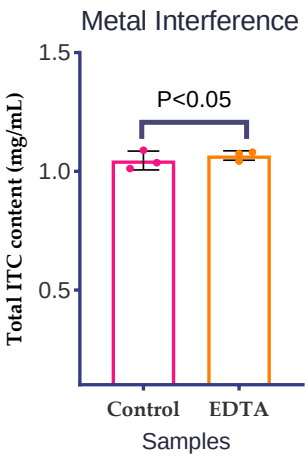


Figure B. 2 *Metal interference pre- and post- EDTA addition*

B.1.2 Fibre Interference

Interferences by fibre during the extraction of ITCs was investigated by the addition of inulin (soluble fibre) or cellulose (insoluble fibre). No fibre interference was observed when 0.021 mg / mL of PEITC was added in the inulin and cellulose solutions (figure A.7).

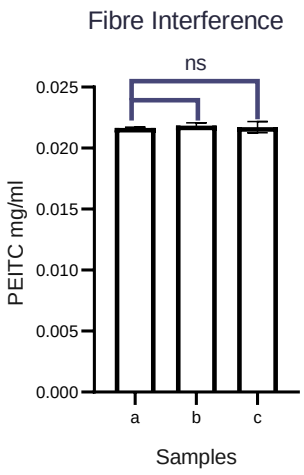


Figure B. 3 *Fibre Interference*

Soluble and insoluble fibres in PEITC-containing solution were added and the samples were passed through the cyclocondensation assay. Measurements were taken using the spectrophotometer.

B.1.3 Macromolecule interference

A simple pre-clarification step including the addition of methanol and centrifugation was also introduced, prior to the cyclocondensation assay to allow for the removal of proteins and other macromolecules present in the crude aqueous extracts. This step significantly improved the reliability of the method by reducing the coefficient of variation of analytical results from 23.6% to 14% (n = 6). The visual changes of the samples pre- and post- the clarification step is shown in figure B.4.

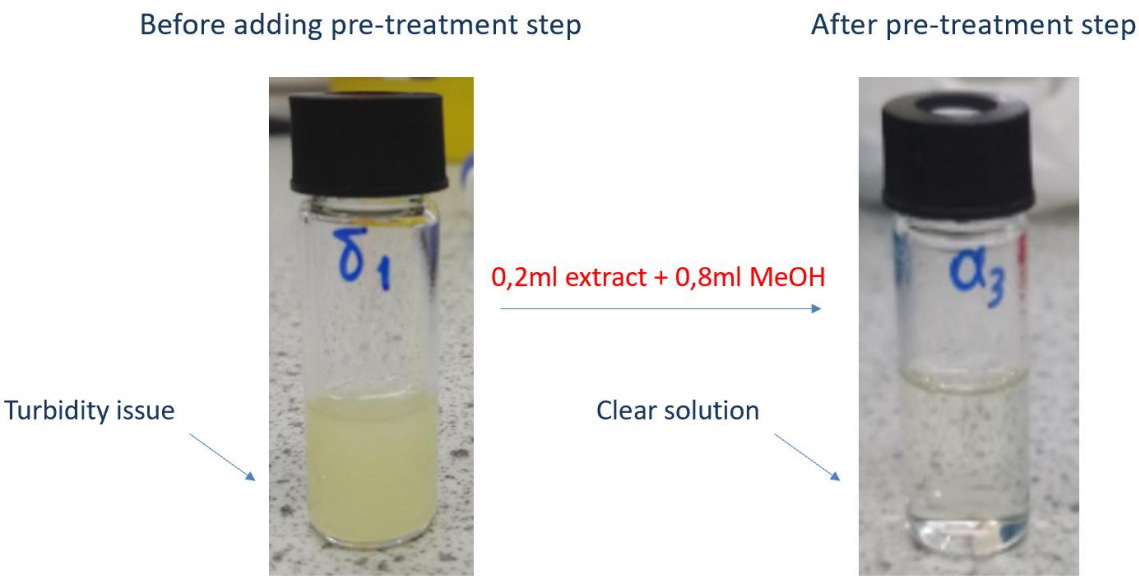


Figure B. 4 *Visual changes pre- and post- clarification step with methanol*

B.2 The effect of SFN and PEITC on UVB treated HaCaT cells in shorter timepoints

To evaluate the effect of SFN and PEITC on UVB (25 and 50 mJ / cm^2) treated HaCaT cells, shorter timepoints were included in our experiments and no effect was shown at 2h and 4h after the UVB exposure. Moreover, post-treatments of the UVB-exposed cells were conducted but no effect was shown. It is hypothesised that the length of the treatment of the cells with ITCs has a crucial role on the effects of the phytochemicals on cell function and that shorter treatment times could have a more protective effect on cells. However, this was not studied in the current work. HaCaT cells were pre-treated with SFN in concentrations of 0.15, 0.3, 0.6, 1.25, 2.5 and 5μM (table B.1).

Table B. 1 *Absorbances observed at shorter timepoint of 4 hours*

Control samples	**0.697**	**0.671**	**0.628**	**0.645**	**0.667**	**0.603**	**0.620**	**0.597**	**0.609**	**0.647**
0.15μM - no UVB	0.641	0.618	0.581	0.575	0.544	0.539	0.597	0.597	0.590	0.647
0.3μM - no UVB	0.689	0.655	0.572	0.525	0.483	0.534	0.495	0.484	0.523	0.569
0.6μM - no UVB	0.649	0.648	0.524	0.550	0.545	0.514	0.514	0.526	0.530	0.560
1.25μM - no UVB	0.625	0.532	0.506	0.572	0.493	0.458	0.460	0.527	0.506	0.529
2.5μM - no UVB	0.542	0.584	0.536	0.535	0.525	0.514	0.498	0.467	0.527	0.549
5μM - no UVB	0.450	0.422	0.417	0.415	0.500	0.466	0.360	0.224	0.324	0.292
25 mJ/cm UVB 1	0.681	0.652	0.638	0.569	0.610	0.605	0.570	0.629	0.632	0.630
0.15μM – UVB 1	0.651	0.633	0.592	0.589	0.554	0.600	0.576	0.565	0.614	0.613
0.3μM – UVB 1	0.626	0.594	0.583	0.499	0.594	0.579	0.574	0.594	0.629	0.630
0.6μM – UVB 1	0.611	0.605	0.640	0.592	0.500	0.512	0.541	0.565	0.591	0.669
1.25μM – UVB 1	0.592	0.603	0.575	0.580	0.545	0.535	0.495	0.560	0.574	0.490
2.5μM – UVB 1	0.563	0.564	0.571	0.540	0.478	0.456	0.505	0.508	0.476	0.566
5μM – UVB 1	0.370	0.449	0.512	0.497	0.470	0.467	0.525	0.508	0.591	0.548
50 mJ/cm UVB 2	0.529	0.647	0.610	0.621	0.629	0.540	0.584	0.571	0.603	0.545
0.15μM – UVB 2	0.576	0.632	0.595	0.627	0.548	0.507	0.597	0.565	0.548	0.543
0.3μM – UVB 2	0.628	0.638	0.593	0.586	0.588	0.509	0.550	0.555	0.553	0.690
0.6μM – UVB 2	0.479	0.533	0.423	0.577	0.526	0.636	0.575	0.573	0.557	0.541
1.25μM – UVB 2	0.600	0.518	0.568	0.537	0.426	0.503	0.482	0.510	0.488	0.501
2.5μM – UVB 2	0.610	0.555	0.524	0.441	0.483	0.399	0.473	0.492	0.510	0.496
5μM – UVB 2	0.501	0.527	0.470	0.472	0.596	0.469	0.498	0.510	0.527	0.533

Viability resazurin assay absorbances of 25 and 50 mJ / cm^2 UVB-exposed HaCaT cells and treated with 0.15, 0.3, 0.6, 1.25, 2.5 and 5μM of SFN at 4 hours timepoint.

Appendix C

Publications

fermentation

MDPI

Review

Functional Role of Probiotics and Prebiotics on Skin Health and Disease

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Abstract: Scientific and commercial interest of probiotics, prebiotics and their effect on human health and disease has increased in the last decade. The aim of this review article is to evaluate the role of pro- and prebiotics on the normal function of healthy skin as well as their role in the prevention and therapy of skin disease. *Lactobacilli* and *Bifidobacterium* are the most commonly used probiotics and thought to mediate skin inflammation, treat atopic dermatitis (AD) and prevent allergic contact dermatitis (ACD). Probiotics are shown to decolonise skin pathogens (e.g., *P. aeruginosa*, *S. aureus*, *A. Vulgaris*, etc.) while kefir is also shown to support the immunity of the skin and treat skin pathogens through the production of antimicrobial substances and prebiotics. Finally, prebiotics (e.g., Fructo-oligosaccharides, galacto-oligosaccharides and konjac glucomannan hydrolysates) can contribute to the treatment of diseases including ACD, acne and photo aging primarily by enhancing the growth of probiotics.

Keywords: probiotics; prebiotics; skin health; skin disease; dermatitis; skin infections

1. Introduction

Fermented food has been part of our diet, in addition to being used for therapeutic purposes, as early as 7000 BC from Egyptians, Greeks and Italians [1–3]. Some of the most ancient fermented foods used in history is wine, bread and milk products such as yoghurt. In fact, it is documented that Georgians were using wine in their diet as early as 6000 BC, whilst fermented dairy products were used for the treatment of diarrhea and other gastroenteric infections [4,5]. The relationship between human health and microbiota was first mentioned in 1907, by Elie Metchnikoff, when the enhanced longevity due to the intentionally present bacteria in yogurt was described [6]. In addition, fermented food became famous after Werner Kollath first introduced the term "Probiotic". The food industry has used probiotics in their products as an aiding ingredient and/or as a preservative means since 1989 [7]. With the evolution of food processing and preservation and the consumer's interest for a healthier and more balanced diet, probiotics became one of the most marketable ingredients. According to the World Health Organization (WHO), probiotics are live microorganisms that "when administered in adequate amounts, confer a health benefit on the host" [8]. Most common species of probiotics belong in the families of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* [9] with the first two families being mostly used in studies related to human health [10]. As these microorganisms are naturally found in the gut microbiota, most studies are focused on their effects in the context of the natural function in the gut and as preventive or therapeutic agents against disease development [11–18]. To this end, probiotics have been used for the study and treatment of intestinal diseases such as gastroenteritis [19], intestinal hyperpermeability [20], urinary tract infection [21], intestinal dysbiosis [22], irritable bowel syndrome [23], Crohn's disease [24], colon cancer [25,26], ulcerative colitis [27,28] and peptic ulcer [23]. In particular, many studies have shown their involvement in regulating signaling molecules like

NFκB, MAPK, PPARγ, HSP, etc. by either activating or inhibiting their expression profile depending on the microorganism studied. Such effect(s), in turn, can trigger other signaling events including perturbations in the (i) phosphorylation content of IκBα, (ii) activation status of p38, (iii) inhibition of nuclear binding by p65 as well as (iv) induction of PPARγ mRNA levels [29–61]. In addition, probiotics have been extensively utilized in the context of intervention studies towards prevention and/or treatment of a number of human diseases including those of the skin like atopic dermatitis [AD] [62–69], allergic rhinitis [66,70,71] and wound healing [72–79] being some of the major ones (Figure 1).

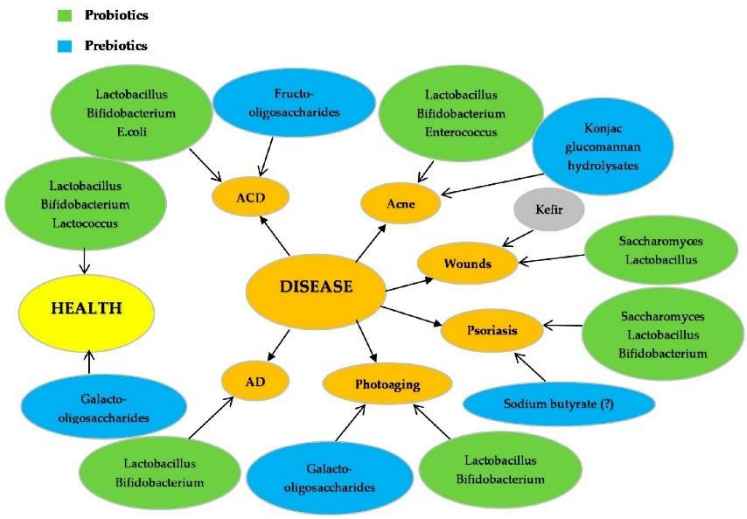

Figure 1. The role of probiotics and prebiotics on skin health and disease including Allergic Contact Dermatitis (ACD), Acne, Wounds, Psoriasis, Photoaging and Atopic Dermatitis (AD).

On the other hand, with the term "prebiotics" we refer to specific fermented components that enhance changes in the composition and the activity of the gut microflora in favor to the host [80]. Prebiotics are characterized by low dosage activity, absence of side effects and persistence through the gut [81]. The most commonly known prebiotics are oligosaccharides (OS; e.g., glycans), fructans (inulin-type), sugar alcohols and complex polysaccharides (e.g., β-glucans, cellulose) [82,83]. The available literature on prebiotics and their effect on human health is limited, compared to the probiotics, and it is often included in several probiotic studies. These non-digestible compounds are known for their bifidogenic effect, which varies depending on the type of prebiotic. This is based on the fact that long-chain OS are fermented in the entire gut whereas the short-chain ones are only processed in the ascending colon and the caecum. Breast milk mostly consists of prebiotic OS and as being the first food for infants; it provides the initial intestinal microbiota whose growth is supported by these OS. Furthermore, recent studies have shown the ability of prebiotics to enhance calcium absorption and have an effect on bone structure as well [82]. Moreover, these compounds are shown to affect the immune system by increasing IgA, CD4+ cells, INF-γ and IL-4 in spleen and mesenteric lymph nodes [84–86]. Additionally, other studies on healthy participants have shown a decrease of toxic fermentation metabolites in the colon (e.g., [H_4] tyrosine and lactose-[N]ureide) after consumption of pro- (e.g., *L. casei*) and prebiotics (e.g., n9; lactulose) [87].

Finally, the skin represents the largest organ in the human body and as such, its main function is to act as a barrier to extrinsic factors including physical, chemical and microbial threats. In this

context, a strong symbiotic relationship between microorganisms exists that constitutes its microbiota. This natural microflora supports the immune system in various ways including the production of natural antimicrobial compounds (e.g., lactic acid) as well as activation of various signaling pathways and modulation of the inflammatory response [88,89]. In this review article, we aim to focus on the beneficial role of pro- and prebiotics on skin health as well as their therapeutic and/or preventive role on specific skin diseases.

2. Probiotics and Prebiotics on Skin Health

There is a rather small number of studies on healthy subjects to show a beneficial effect of probiotics on skin health (Figure 1) [18,61,90–92]. In one such study, when the *L. lactis* strain; H61 was supplemented on middle-aged women, daily for eight weeks, an improvement on skin elasticity and body characteristics were observed (e.g., skin appeared more hydrated and the hair follicles had improved) [92]. Similarly, in another such study, oral intakes of *L. plantarum*; HY7714 from a group of subjects aged 41–59 years old also confirmed the effect of probiotics on increasing skin moisture, decreasing the depth of existing wrinkles and improving the overall skin gloss and elasticity [61]. Moreover, other studies have shown that when probiotic and para-probiotic *L. reuteri* were administrated orally, for 12 weeks, an increase in melanin and a decrease in Trans-Epidermal Water Loss (TEWL) were observed [91]. Such effects are in agreement with studies utilizing other probiotics (e.g., *L. rhamnosus*, *B. breve* Strain Yakult, *L. lactis*, *S. thermophilus*) and prebiotics (e.g., galacto-oligosaccharides; GOS) (Figure 1) all of which have indicated (i) improved levels of skin hydration and cathepsin-L-like activity levels (an indicator of keratinocyte differentiation and a marker of skin barrier function) as well as (ii) reduced urine and serum phenol levels (e.g., toxic by-products formed by gut bacteria) [90,93].

3. Probiotics and Prebiotics on Skin Disease

3.1. Dermatities

3.1.1. Atopic Dermatitis

Atopic Dermatitis (AD), also known as atopic eczema, is a skin inflammatory disease that is observed in early stages of life and is linked with allergic rhinitis, food allergies and asthma, all of which are more prevalent in children suffering from this disease. One of the most common symptoms of eczema, apart from itchiness, is the reduction of barrier function that leads to allergen exposure and overall reduction of the TEWL, leading to dry skin [94]. In an AD model, allergens can penetrate the stratum corneum, which is altered by the epidermal epithelium deformities. Moreover, symptoms include the presence of pathogenic microorganisms, such as *S. aureus*, that colonize and infect the subjects. Another significant aspect of AD is its relationship with the gut microbiota. More specifically, the balanced microbial profile of the mucosa can promote the production of immunoglobulin A (IgA) which supports the defensive mechanisms of the gut membrane, whilst enhancing the expression of the Transforming Growth Factor (TGF) [95]. A relationship between the gut microflora and the development of AD was also observed in infants at high risk for developing AD showing an increased number of clostridia compared to control, disease free infants [96].

Specific probiotic microorganisms are shown to have a preventing role on AD and mediate the symptoms of the disease (Figure 1). They appear to do so by influencing a number of biological processes not only in AD but rather in a wide range of skin diseases (e.g., acne, psoriasis, photo aging, wounds, etc.) (Table 1 and Figure 2). More specifically, in a recent study, supplementation with *L. rhamnosus* in combination with *L. reuteri* improved the severity of eczema by 56% in children suffering from AD [65]. Moreover, in another study, *L. rhamnosus* was utilized as a supplemented probiotic, to women four weeks before delivery and six months postnatal, demonstrating to significantly reduce the risk of children developing AD during their first seven years of age [66]. Finally, when infants at high risk of developing AD were supplemented with a mix of probiotic microorganisms (e.g.,

L. acidophilus, *B. bifidum* and *B. lactis*), during pregnancy and after birth, they showed a reduction of immunoglobulin E (Ig-E) associated eczema by 40% [62].

Table 1. Probiotics and their effect on skin diseases.

Probiotics	Disease	Function	Reference
L. rhamnosus	AD [1]	Improvement of severity of eczema, reduction of risk of AD development in infants	[65,66]
L. reuteri	AD Infections (*S. aureus*)	Improvement of eczema. Blocks integrin, Reduces cell death due to *S. aureus* infection	[65,97]
L. delbrueckii subspecies bulgaricus	Acne	Improvement of Acne symptoms (Acne Vulgaris)	[98]
L. sporogenes	Psoriasis	Improvement of symptoms, reduction of blood sugar levels and fever	[99]
L. plantarum	Photoaging	Inhibition of MMP-1, MMP-2, MMP-9 and MMP-13 [2], enhancement of procollagen expression, inhibition of phosphorylation of Jun N-terminal kinase, increase of palmitoytransferase mRNA levels, decrease of ceramide mRNA levels, reduction of wrinkles and epidermal thickness	[100,101]
L. fermentum	Infections (wounds)	Production of gNO [3], increases productions of IL-1 [4] and TGF-β [5] cytokines	[102,103]
L. acidophilus	AD ACD [6] Infections (*S. aureus*) Acne	Reduction of Ig-E [7], reduction of eczema, Increase of TGF-β, Foxp3 [8], IFN-γ [9] and IL-10 [10] expression, Inhibition of *S. aureus* infection, reduction of acne symptoms	[62,98,104,105]
L. casei *L. salivarius*	ACD Infections (MRSA) [11]	Reduction of skin inflammation, inhibition of IFN-γ, $CD8^+$ T cells, increase in IL-10 production, activation of $CD4^+CD25^+$ T cells, inhibition of MRSA	[105–107]
B. bifidum	AD Acne	Reduction of Ig-E, reduction of development of AD in infants, reduction of Acne Vulgaris symptoms	[62,98]
B. lactis	AD	Reduction of Ig-E, reduction of development of AD in infants.	[62]
B. pseudolongum	ACD	Reduction of allergic reaction on mice	[108]
B. longum	Photoaging	Prevention of TEWL [12], reduction of skin erythema, increase of mRNA expression of CD44, TIMP-1[13] and Col1[14].	[109]
B. breve strain Yakult	Photoaging	Prevention of loss of elasticity, suppression of elastase, activation of IL-1β	[38,110]
B. infantis	Psoriasis	Reduction of plasma TNF-α[15], increase of IL-6	[111]
S. epidermidis	Acne	Growth inhibition of Propionibacterium acnes and Acne Vulgaris by competitive exclusion	[112]
E. faecalis	Acne	Reduction of inflammation areas, production of bacteriocins	[113]
E. coli Nissle 1917	ACD	Increase of TGF-β, Foxp3, IFN-γ and IL-10 expression	[114]
Kefir grains	Infections	Production of antimicrobial substances (lactic acid, acetic acid, hydrogen peroxide, bacteriocins), Healing of *P. aeruginosa* infected wounds, Inhibition of *S. aureus*, *S. salivarius*, *S. pyogenes*, *P. aeruginosa*, *C. albicans*, *S. tympimurium*, *L. monocytogenes* and *E. coli* growth	[115,116]

[1] Atopic Dermatitis; [2] Matrix Metalloproteinases (MMPs)-1,-2,-9,-13; [3] Nitric Oxide; [4] Interleukin 1; [5] Transforming Growth Factor β; [6] Allergic Contact Dermatitis; [7] Immunoglobulin E; [8] Forkhead box P3; [9] Interferon gamma; [10] Interleukin 10; [11] Methicilin Resistant Staphylococcus aureus; [12] Trans Epidermal Water Loss; [13] Tissue inhibitor of metalloproteinases 1; [14] Collagen 1; [15] Tumor Necrosis Factor.

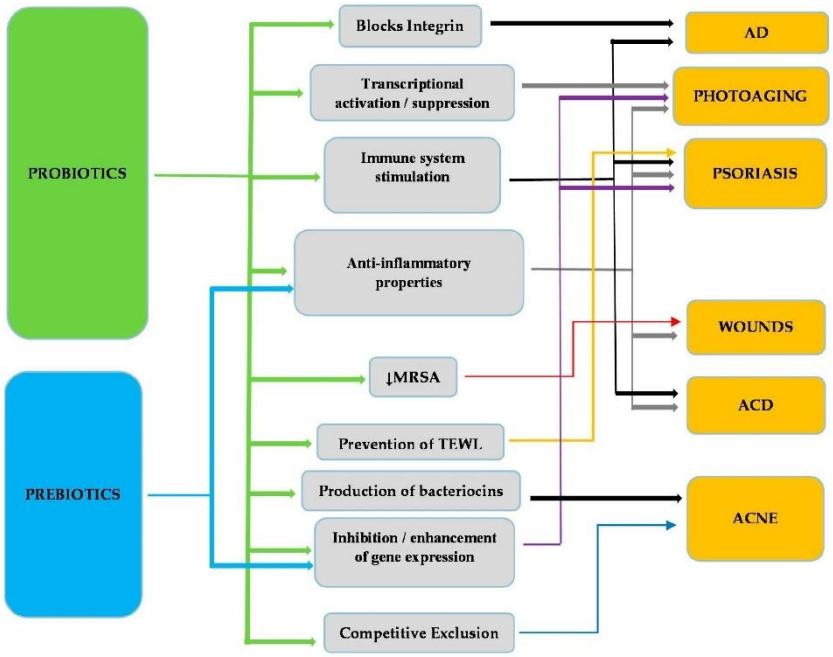

Figure 2. Linkage of various skin diseases with their respective mode of action through which pro- and prebiotics exert a beneficial effect. Methicilin Resistant Staphylococcus aureus (MRSA); Trans Epidermal Water Loss (TEWL).

3.1.2. Allergic Contact Dermatitis

Allergic contact dermatitis (ACD), also known as eczema, is caused after the skin comes in contact with an allergenic substance capable of causing an allergic reaction. Symptoms vary but include skin inflammation, itchiness, dry skin, blisters, etc. The allergic reaction is regulated by $CD4^+$ T cells in a manner where peptides derived from allergens activate Th2-type cytokines (produced by these $CD4^+$ T lymphocytes) including interleukins 4, 5 and 13 [117]. Overall, pro- and prebiotics are shown to have a preventing role on ACD and consequently mediate its symptoms (Figure 1).

L. casei is found to reduce skin inflammation either by targeting the inhibition of INF-γ (responsible in producing $CD8^+$ effector T cells) [106] or via mechanisms that include the involvement of regulatory $CD4^+$ T cells [107]. In addition, the microorganism has also been shown to increase the production of IL-10 by promoting the activation of CD^+4CD25^+ Tregs thus further supporting its specific mode of action against skin inflammation [107] (Table 1 and Figure 2). On the other hand, *E. coli* Nissle 1917 (EcN) is another probiotic microorganism shown to prevent ACD by means of increasing the number of $Foxp3^+$ cells (suppress antigen priming of lymphocytes) as well as the expression of TGF-β, IFN- γ and IL-10 (regulatory cytokine network) thus suggesting an immunomodulatory function against allergen-induced dermatitis [114] (Table 1 and Figure 2). Similar observations were made in the case of the para-probiotic *L. acidophilus* strain L-92 which was also shown to induce the activation of $CD^+4CD25^+3^+$ Tregs and consequently suppress ACD [104] (Table 1 and Figure 2).

Finally, in another study, consumption of the prebiotic fructo-oligosaccharide resulted in suppressed skin inflammation due to a favorable change in the population of the intestinal microbiota by means of increasing the population of *B. pseudolongum*. This, in turn, has led to reduced contact

hypersensitivity associated with proliferation of *B. pseudolongum* in the intestinal tract of the mice [108] (Table 2).

Table 2. Prebiotics and their effect on skin disease.

Prebiotics	Disease	Function	Reference
Fructo-oligosaccharides	ACD	Reduction of allergic reaction.	[108]
Konjac glucomannan hydrolysates (GMH)	Acne	Inhibition of Acne Vulgaris and P. acnes, growth enhancement of lactic acid bacteria.	[118,119]
Galacto-oligosaccharides	Photoaging	Prevention of [1] TEWL, reduction of skin erythema, increase of mRNA expression of CD44, [2]TIMP-1 and [3]Col1.	[109]
Sodium Butyrate (?)	Psoriasis	Increases Fas, [4] TGF-β and p52	[120–123]
Oligo-saccharides	Photoaging	Modulation of the expression of elastase-type proteases through elastin receptors	[124,125]

[1] Trans Epidermal Water Loss; [2] Tissue inhibitor of metalloproteinases 1; [3] Collagen 1; [4] Transforming Growth Factor β.

3.2. Skin Infections

3.2.1. Wounds

Most skin infections are initiated when an opening of the skin is infected with a pathogen. Briefly, when the cohesion of the skin is disrupted (either accidentally or as an effect of a disease) it forms a wound which is characterized by torn skin or by a hematoma of the tissue. In the case of a torn tissue, there are four stages descriptive of the healing process: (i) stopping the blood flow to the damaged blood vessels (hemostasis); (ii) initiating an inflammatory response which prevents potential pathogenic microorganisms to infect the wound and maintains the microbial balance of the skin; (iii) stimulating production of growth factors causing (iv) proliferation of fibroblasts and production of extracellular matrix proteins (e.g., hyaluronan and collagen) [126]. Furthermore, these stages are characterized by the involvement of other events including generation of oxidative stress [127].

There is a great scientific interest regarding the role of skin microflora in the process of wound healing as it has been shown that the absence of microbiota can decrease the healing time [128]. On another note, wound infections occur when exogenous bacteria become dominant over the systemic and local factors of host resistance. Therefore, it is only when a balance is achieved between bacteria and host that allows for the normal processes of wound healing to proceed [129]. Over the years, scientists have turned their interest to topical application of specific probiotic microorganisms in order to evaluate their effectiveness in preventing wound inflammation as well as improving on the speed of the healing process itself. In one such study, when burn wounds were treated with *Saccharomyces cerevisiae* an overall improvement on the healing process was observed [130]. More specifically, an increase in the expression levels of collagen type 1 and transcription growth factor beta 1 (TGF-β1) were observed accompanied by improved morphological and biomechanical characteristics of the healing wounds [130].

Meticillin-resistant *Staphylococcus aureus* (MRSA) is one of the most widely known pathogens with the ability to infect wounds [131]. A number of studies have shown the capacity of specific probiotics (e.g., *L. acidophilus* and *L. casei*) to act as antibacterial agents against MRSA [105] (Table 1 and Figure 2). More specifically, the growth of the pathogen was found to be inhibited and eliminated by 99% after 24 h at 37 °C incubation [105]. Moreover, in another study, three different probiotics (e.g., *L. reuteri*, *L. rhamnosus* and *L. salivarius*) were tested against *S. aureus* infection on epidermal keratinocytes [97]. Overall, it was found that *L. reuteri* and *L. rhamnosus* (but not *L. salivarius*) reduced the ability of the pathogen to induce keratinocyte cell death. This observation was directly associated with the ability of *L. reuteri* to inhibit the adherence and invasion of the pathogen to keratinocytes while *L.*

salivarius did not. Furthermore, the degree of protection was greater in *L. reuteri* than *L. rhamnosus* [97] (Table 1). To conclude, given that *S. aureus* adheres with the epidermal keratinocyte cells via the α5β1 integrin, it was suggested that both of the protective probiotics reduce keratinocyte cell death by competitively excluding the pathogen from the integrin's binding sites on these skin cells [97]. Finally, antibiotic properties of probiotics have been also documented in experimental settings where wounds, infected with *S. aureus*, were treated with patches of *L. fermentum*. In these experiments, it was shown an increased wound closure concomitant with production of nitric oxide (gNO) induced by the probiotic [102] (Table 1 and Figure 2). In general, gNO is known to mediate the process of wound healing through promoting the production of IL-1, TGF-β and cytokines all of which play a major role in immune response and inflammation [103].

In addition, a number of other studies have focused on topical applications of kefir and other fermented products because of their well-known anti-microbial and healing properties. Kefir is the product of milk fermentation that contains grains characterized by specific starter cultures used in the fermentation process [132]. These grains include (i) *L. kefiri*, (ii) species of the genera *Leuconostoc*, *Lactococcus* and *Acetobacter*, (iii) lactose fermenting (e.g., *K. marxianus*) as well as (iv) non-lactose fermenting (e.g., *S. unisporus*, *S. cerevisiae* and *S. exiguous*) yeasts [132]. However, there are many more microorganisms found in Kefir grains including the species *Lactobacilli*, *Streptococci*, *Lactococci*, *Enterococci*, *Bacillus*, etc. The composition of kefir grains varies depending on their origin and the microorganisms they contain [133]. Another aspect that can change the effect and the composition of kefir is the fermentation time and conditions [134–136]. Collectively, the antimicrobial activity of kefir is the result of the composition of the product that is high in lactic acid, acetic acid, hydrogen peroxide and bacteriocins all of which can have an effect on the growth of pathogens [137] (Table 1 and Figure 1). Consequently, the complexity of the kefir grains (and kefir itself) has raised the scientific interest in the context of exploring any potential effect on the growth of existing microorganisms in the human body. To this end, when *B. bifidum* PRL2010 (a dominant microorganism in the human gut) was cultured in the presence of kefir and/or kefiran (the polysaccharide produced by kefir), it was shown that the glycans present in kefir had a beneficial role on the growth of the bacteria (perhaps due to the increased transcriptional activation of genes related to the metabolisms of glycans) [138]. Furthermore, a few studies have documented a protective effect of kefir on the wound healing process [79,115,137,139]. To this end, one of the biggest challenges in wound healing is the infection of burn wounds from the antibiotic resistant pathogen *P. aeruginosa*. As a result, this pathogen is responsible for complications on serious illnesses such as hospital acquired infections and sepsis syndromes [73–75]. Experiments on burn wounds (after contamination with *P. aeruginosa* and then treatment with kefir) showed a reduction of their size accompanied by reduced healing time when kefir was administered alone than in the co-presence of silver sulfadiazine (a common topical antibiotic used for the treatment of *P. aeruginosa* on burn wounds). Such findings highlight the potential pharmaceutical use of kefir on the treatment of burn wounds [115]. Finally, in another study, burn wounds were contaminated with 8 different pathogens (e.g., *S. aureus*, *S. salivarius*, *S. pyogenes*, *P. aeruginosa*, *C. albicans*, *S. tympimurium*, *Listeria monocytogenes* and *E. coli*) and when kefir and/or kefiran were applied to the subject's infected areas the growth of these pathogens was considerably reduced [116].

3.2.2. Acne

Although not many studies have been conducted on the effect of pro- and prebiotics in acne, a number of them suggest a potential preventive role of pro- and prebiotics on acne thereby mediating its symptoms (Figure 1). More specifically, in a study utilizing a mixture of probiotics (*L. acidophilus*, *B. bifidum* and *L. delbrueckii*), the side effects of minocycline administration (an antibiotic used for the treatment of *A. Vulgaris*) were reduced while still being effective in exerting a synergistic anti-inflammatory effect. These results suggest a potential use of the probiotic mixture as an alternative treatment option against *A. Vulgaris* in addition to being capable of reducing adverse side effects after chronic systemic antibiotic use [98]. Acne is enhanced in the presence of the bacterium *P. acnes*.

On the other hand, *S. epidermidis* is naturally found on skin and has been shown to antagonize *P. acnes* thus highlighting its therapeutic potential against acne [112] (Table 1 and Figure 2). In another study, the therapeutic role of *E. faecalis* SL-5 on acne was also evaluated with results demonstrating that bacteriocin (CBT SL-5; an antimicrobial compound produced by *E. faecalis*) was capable of reducing inflammation suggesting the use of *E. faecalis* as an alternative approach to acne therapy thereby avoiding the extensive use of antibiotics [113] (Table 1 and Figure 2).

Finally, despite the lack of literature on the effect of prebiotics to skin disease, konjac glucomannan hydrolysates (GMH) have also been shown to inhibit *A. Vulgaris* and *P. acnes* by stimulating the growth of probiotic microorganisms including *lactobacilli*. To this end, it is noteworthy that lactic acid bacteria show selectivity towards a mannose, a glucose substrate (found in GMH), because of the nature and accessibility of these sugars as carbon sources [118,119] (Table 2 and Figure 2).

3.3. Psoriasis

Psoriasis is a skin condition that causes a variety of symptoms including flaky skin (patches), itchiness and redness of the area. It is a non-contagious disease and it can affect individuals of any age [140]. There are different types of the disease including pustular psoriasis, psoriatic arthritis and plaque. Even though the literature on the effects of probiotics to skin inflammation and dermatitis is extensive, little is known on their effects on psoriasis. Nevertheless, a number of studies have been conducted on the effect of pro- and prebiotics in psoriasis suggesting a potential preventive role of their action by means of mediating the symptoms of the disease (Figure 1).

In general, studies on the role of the human epidermal microbiome in psoriasis and other skin diseases revealed that *S. epidermidis* (although a permanent member of the normal human microbiota) is second most prevalent staphylococcal species only to *S. aureus* [141]. To this end, a recent study was shown that *S. aureus* was at significantly higher levels on diseased skin as opposed to *S. epidermidis* and *P. acnes* both of which were shown to be in abundance on healthy skin thereby suggesting that psoriasis is highly associated with the microbial load of the skin [142]. To this end, another study has shown that the abundance of *S. cerevisiae* is decreased in psoriasis patients and that treatment with dimethylfumarate (DMF) successfully restored its levels, a finding of utmost importance given the well-known and beneficial immunomodulatory properties of this yeast species [143]. Moreover, extensive research indicates a strong link between potential mediators of T cell activation and the development of the disease. In particular, $CD4^+$ T cells are linked with the development of psoriatic arthritis whilst probiotics regulate T cells and reduce skin inflammation and dryness of the skin [144] (Table 1 and Figure 2). In a recent case report, the probiotic microorganism *L. sporogenes* was successfully used for the treatment of pustular psoriasis as evident by an overall improvement of the appearance of lesions and patient's general condition [99] (Table 1). A year later, Groeger et al., 2013 studied the immuno-regulatory effects of *B. infantis* in patients with ulcerative colitis, chronic fatigue syndrome and psoriasis. In the case of psoriasis, reduced plasma levels of C-reactive protein (CRP) and TNF-α were observed thus highlighting the ability of *B. infantis* to reduce systemic pro-inflammatory biomarkers and thus to act as a potential therapeutic approach in treating psoriatic disease [111] (Table 1 and Figure 2).

Sodium butyrate is produced by the gut microflora [145] and it is known for its effect on cell cycle [120], tumor growth factors (TGF-β) [121] and protease enzymes [122]. In various studies utilizing human keratinocyte (HaCaT) cells it was shown that exposure to sodium butyrate induced apoptosis by 50% through up-regulation of death receptor Fas with concomitant activation of caspases 8 and 3. In addition, increased expression levels of p52 and TGF-β were also shown suggesting the involvement of cell proliferation and terminal differentiation as well [121]. Finally, a combined treatment protocol with sodium butyrate and PD153035 (an epidermal growth factor receptor inhibitor) was shown capable of enhancing keratinocyte differentiation [123]. Collectively, data suggest that sodium butyrate can act as a potential additional approach to the management of hyperproliferative skin diseases (including psoriasis) by modulating key cellular processes like apoptosis, proliferation

and differentiation (Table 2 and Figure 2). To this end, a recent study examining the gut microbial composition in psoriatic patients revealed that a reduction of butyrate microbiota producers may have an impact on the established anti-inflammatory role of this short chain fatty acid [146] and thus explain, at least partially, its preventive role in psoriasis (among other disorders) [110]. In fact, *F. prausnitzii* (one of the most common microbial inhabitants of the large intestine) serves as an important source of butyrate which, in turn, (i) provides energy for colonocytes, (ii) reduces oxidative stress and (iii) exerts anti-inflammatory action (by triggering regulatory T cells) thereby conferring immune tolerance that goes beyond the GI tract [38,100]. Finally, another study has shown that psoriatic patients possess a substantially reduced number of *F. prausnitzii* when compared to healthy controls [101].

3.4. Photoaging

Skin aging is considered in the context of being either extrinsic or intrinsic. Extrinsic skin aging is caused by a number of environmental factors like UVR exposure (photo aging), smoking and life style habits (diet). In particular, photo aging is characterized by a specific phenotype that includes excessive loss of skin moisture, formation of deep and thick wrinkles, age spots, discoloration, loss of collagen and overall breakdown of the elastin network of the dermis, resulting in loss of skin elasticity [109]. To date, there are few studies investigating into the effects of probiotics/prebiotics to photo aging (Figure 1). In one such study, when hairless mice were administrated probiotic-containing fermented milk together with para-probiotic *B. breve* strain Yakult, and then subjected to UVB irradiation, it was shown an improvement in elasticity and appearance of the skin [124] together with suppression of elastase and IL-1β activity levels [125] (Table 1). These findings are in agreement with another study where administration of *L. plantarum* HY7714 to hairless mice and human epidermal fibroblasts was followed by UVB exposure and inhibition of MMPs-1,-2,-9 and -13 was recorded indicating rescued procollagen expression accompanied by inhibition of Jun N-terminal kinase phosphorylation and c-Jun expression levels. In addition, wrinkles formation and epidermal thickness were also reduced [147] (Table 1 and Figure 2). Moreover, *L. plantarum* HY7714 was shown to increase the mRNA levels of palmitoyl transferase (SPT) while reducing those of ceramide in human epidermal fibroblasts [148] (Table 1 and Figure 2). Furthermore, Galacto-oligosaccharides (GOS; one of the main prebiotics found in fermented food) were evaluated either alone or in the presence of probiotics (e.g., *B. longum*) in order to assess their effects on skin disease and inflammation. It was shown that the combination of probiotics and prebiotics prevented TEWL and reduced skin erythema whilst increasing the mRNA expression of CD44, TIMP-1 and Col1 [149] (Table 2 and Figure 2). Finally, in other studies, oligo-saccharides were also shown to prevent skin aging by modulating the expression of elastase-type proteases (through elastin receptors) [150] and/or prevent damage to the skin immune system [151].

4. Conclusions

Scientific and commercial interest on probiotics and prebiotics as well as their effect on human health and disease has increased in the last decade. The aim of this minireview article was to evaluate the role of pro- and prebiotics on the normal function of healthy skin as well as their role in the prevention and therapy of skin disease. Whilst a number of studies have determined the mechanisms by which some of these individual microorganisms can affect specific processes involved in the pathophysiology of skin disease, others have focused on more complex natural products (e.g., kefir) known to contain a mixture of probiotics but nevertheless also capable of exerting a potent beneficial effect. Overall, our manuscript favours the idea of the utilization of probiotics as a means of prevention and/or treatment options in skin disease. Such an alternative approach can have a huge impact in the context of therapy as it will aim to reduce the use of antibiotics and thus also reduce the side effects associated with their chronic usage. However, in order to do so, the precise mechanism of their action remains to be fully elucidated, whilst further studies need to explore their benefit in managing the outcome(s) of skin disease(s) at the clinical setting.

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Review

The Role of Probiotics and Synbiotics on Hirsutism

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Abstract: Probiotics and synbiotics are known to have beneficial effects on human health and disease. Hirsutism, a disorder that is characterised by the presence of coarse terminal hairs in a male-like pattern, is usually caused by elevated androgen levels in blood plasma. This disorder is usually observed in PCOS women and it is linked to insulin resistance (IR). Although idiopathic hirsutism (IH) is not shown to have excess androgen production from the ovarian and adrenal glands, increased 5α-reductase in peripheral tissues and insulin resistance are common observations. The effect of probiotics and synbiotics have been recently studied on PCOS women; androgens were also included in the hormonal groups that were investigated. Only a few studies focus on hirsutism and the potential effect of the beneficial microbes mentioned, whereas the increasing interest on insulin resistance and synbiotics indicate a potential beneficial effect on hirsutism through the management of insulin resistance.

Keywords: probiotics; synbiotics; hirsutism; androgens; insulin resistance; PCOS

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1. Introduction

Fermented food, such as yoghurt, bread, bear and wine, has been widely known since ancient times, and some have been used since then, for therapeutic purposes [1–3]. Fermented dairy products (yoghurt, kefir) were used to treat diseases like diarrhea and other infections of the intestinal tract [4,5]. Interaction between microorganisms and human health was first reported in 1907, where yoghurt microflora was described by Elie Metchnikoff [6]. Fermented food is still used as an additional treatment for disease, such as wounds [7–9], gastroenteric disorders and infections [7,10], as well as an overall support for health [7,8,10–12]. The term probiotic was introduced by Werner Kollath [13] and according to the World Health Organisation (WHO), this term refers to the microorganisms that "when administered in adequate amounts, confer a health benefit on the host" [14]. Probiotics are found to have multiple beneficial roles on human health and disease, through restoration of gut microbiota (GM) [15,16], symptom improvement on intestinal disease, such as irritable bowel syndrome (IBS) [17], modulation of hormonal profile in animals and humans [18–22] and skin infections and healing [23–25]. Along with the beneficial microorganisms, prebiotics, which are fermented compounds that support and promote modifications in the activity and composition of GM [26], have also been studied. It is shown that a controlled combination of probiotics and prebiotics, called synbiotics, can provide a more enhanced beneficial effect on human health and disease [16,27–29].

Although the effect of probiotics and synbiotics are extensively studied, there has been limited research conducted on their effect on sex hormones and sex hormone imbalances, such as hirsutism, a disorder that is mainly characterised by elevated androgen levels in women. The aim of this review is to focus on the role of probiotics and synbiotics and their metabolic process on hirsutism.

1.1. Probiotics

Lactobacillus, *Streptococcus*, *Bifidobacterium*, *Lactococci* and *Saccharomyces* are some of the probiotic species that are known [30]. Microorganisms that are mostly used as treatments

are *Lactobacillus*, *Streptococcus*, *Bifidobacterium* and *Saccharomyces*, whilst most of them are naturally present in the human GM [31–37]. There has been an increase in research on the effect of probiotics on health and disease, whilst they are shown to have both beneficial effects on healthy subjects and therapeutic effect on various diseases and conditions, such as intestinal disease [38–46], skin disease [10,24,47–52] and wound healing [53–57]. Probiotics have also been used for the restoration of disturbed GM [58]. Dysbiosis of gut microbiota (DOGMA) is the alteration of gut microflora that can occur due to a variety of reasons, such as antibiotic use [59,60] and diet [61,62]. The human gut, also described as the "second brain" of the human body, plays an important role in human health and disease [63,64]. The GM is known to have a significant role in the functionality of the bowel, maintaining the gut mucosa through their role on gut homeostasis [63–65]. The probiotic *E. coli* Nissle 1917 protects and prevents against inflammatory response via TLR-4 and TLR-2-dependent pathways, whilst lower counts of *Firmicutes prausnitzii* are linked with potential inflammatory bowel disease pathogenesis due to its anti-inflammatory effects [65,66]. The impact of the metabolic processing of the gut microflora in the gut is extended outside of the bowel and can impact other functionalities of the human body, reaching the skin [10,24,67–69]. *L. casei* is shown to reduce skin inflammation through the regulation of $CD8^+$T cells, which initiate inflammatory response [47]. The role of probiotics on endocrinology has also been reported and GM was shown to affect the production of hormones, such as leptin [62,70,71], stress hormones [72], insulin [73,74] and sex hormones [75,76] in the intestinal tract. Additionally, the disturbance of gut microbiota can affect the levels of endogenous hormones, such as estrogens, as well as administrated steroids, such as megestrol acetate, medroxyprogesterone acetate, norethisterone and others, suggesting that the use of antibiotics can cause hormonal imbalance and reduce the absorption of contraceptive hormones, which are used as a method of birth control and/or treatment for metabolic syndromes [75]. An altered microbiota during the early life of the diabetic mouse (Type 1) can determine sex hormones and cause metabolic changes, such as increased testosterone levels [76]. Administration of probiotics can restore the imbalanced microorganisms that live in the human intestine and improve certain conditions through their metabolic processes [58]. Additionally, DOGMA is also observed in patients suffering from inflammatory bowel disease, colitis [77], Crohn's disease, IBS [63], polycystic ovary syndrome (PCOS) [78] and other conditions.

1.2. Synbiotics

Synbiotics are the controlled combination of probiotics and prebiotics and their supplementation aims to provide a more enhanced health benefit on human health and disease. Known prebiotics are fructans (e.g., inulin), complex polysaccharides, oligosaccharides and sugar alcohols [16,79] and in combination with probiotics, they have been used for the treatment of conditions and disease [18,27,28,80,81]. Synbiotics containing *L. acidophilus NCC90*, oligofructose and acacia gum can have a preventive role on bone mineral loss after ovariectomy in rats [16]. It has been reported that synbiotic treatments showed beneficial effects on non-alcoholic fatty liver disease (NAFLD), reducing fibrosis and hepatic steatosis in humans [82,83], total necrosis factor α (TNF-α), total nuclear factor κ-B (TNF-κB) and other NAFLD biomarkers, such as high-sensitivity C-reactive protein [83]. Moreover, synbiotics can bring improvements on Crohn's disease through reduction in TNF-α production [84], IBS [85] and delayed Alzheimer's disease in *Drosofila melanogaster* [86]; improvement of thyroid function was observed after 8 weeks of synbiotic supplementation in hypothyroid patients, decreasing thyroid stimulating hormone (TSH) levels and increasing tri-iodothyronine (FT3), whilst overall increasing FT3/TSH ratio [18]. *Bifidobacterium Longum* in combination with inulin, reduced levels of TNF-α and IL-1α in patients suffering from ulcerative colitis [77]. Another study on patients with ulcerative colitis showed that *B. breve* and galacto-oligosaccharide (GOS)-containing beverage (Yakult) managed to clinically improve the condition of the patients, through reduction of UC markers, such as myeloperoxidase, and by lowering the faecal pH [87].

1.3. Hirsutism

Hirsutism is a condition that appears in 5–10% of women and it is recognized by the presence of coarse terminal hairs in a male-like pattern [88]. Excess hair growth that consists of terminal hairs are present in areas were women normally have thinner hair. The clinical diagnosis of hirsutism is completed based on Ferriman and Gallwey criteria (Figure 1) [88] and the areas that are scored include the face, chest, thighs, upper arms, abdomen and back. Scores (m-FG) from 1–4, with 1 describing minimal terminal hair and 4 describing frank virilization, are given to the mentioned areas whist total scores less than 8 are considered normal [88].

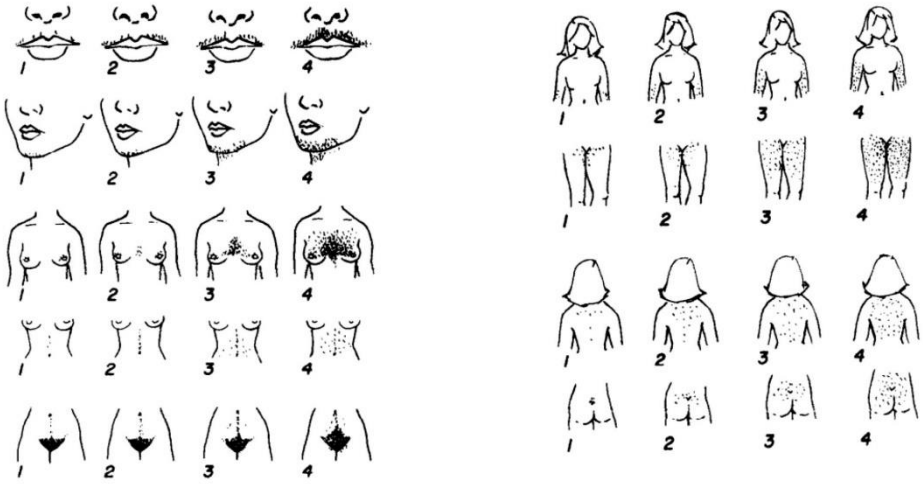

Figure 1. Hirsutism scoring system presenting scores ranking from 1—minimal hirsutism, to 4 virilization in 9 body parts. A total score of less than 8 is considered normal, whereas higher scores indicate mild to severe hirsutism [88].

Hirsutism is a quite complex condition that can occur due to other syndromes or disease, such as PCOS (Figure 2). Although the main reason for this condition is the presence of excess androgens, such as testosterone (T) and dihydrotestosterone (DHT), IH shows normal levels of androgens [89,90]. The most common reason for the excess androgen levels is PCOS and 60–80% of PCOS women suffer from hirsutism [91]. The diagnosis of this condition is conducted according to the Rotterdam criteria, by which two of the three criteria have to be met: (a) evidence of hyperandrogenism, (b) oligo- and/or anovulation and (c) polycystic ovaries [92]. Other clinical features are ovarian enlargement and IR, which is found to be very common amongst PCOS women, whilst the syndrome has also been linked with cardiovascular disease and inflammation. The heritability of this disorder has been studied and it is possible that genetic changes on the androgen receptor gene can lead to hirsutism [90]. During this study, a repeatability of the trinucleotide CAG in exon 1 was observed and more frequent repeats in the N-terminal domain of the androgen gene were linked with the development of the disorder. However, other researchers have not shown a significant role of CAG in the pathogenesis of hirsutism [93,94]. Hirsutism has also been linked with IR (Figure 3) [91,95–101], whilst excess insulin production leads to hyperinsulinemia, which increases luteinizing hormone (LH) through insulin receptor stimulation [97]. LH, along with follicle-stimulating hormone (FSH), is a hormone that regulates androgen production through the secretion of them from ovarian and adrenal glands. Moreover, high levels of insulin inhibit sex hormone-binding globulin (SHBG), a hormone that binds with plasma T and is considered its major determinant along with 17-β hydroxysteroids from plasma [102]. Consequently, both

effects of IR are directly linked with hirsutism through excess androgen production. This relationship between IR and hirsutism was studied in healthy women, where adipose tissue was collected and in-vitro treatments with testosterone and/or anti-androgens were conducted on insulin stimulated adipose cells [100].

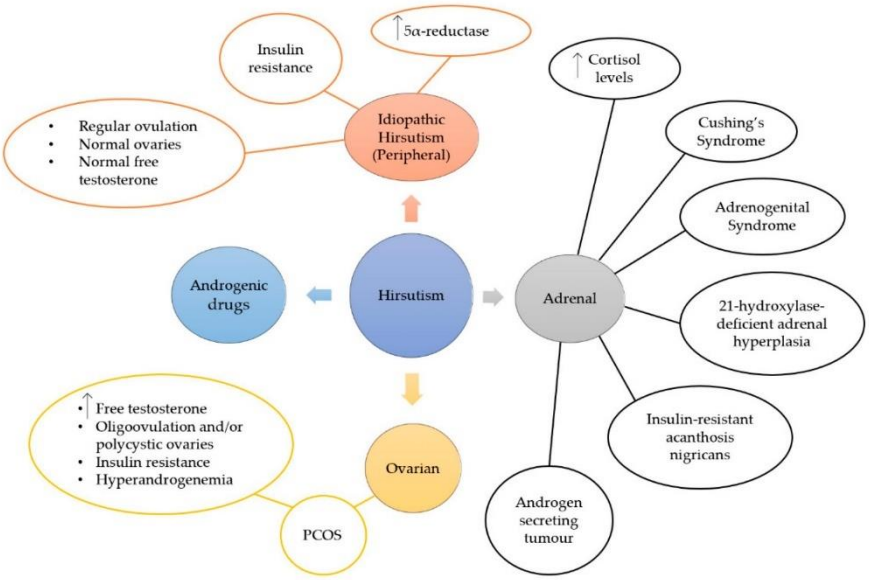

Figure 2. Hirsutism can originate from adrenal or ovarian disorders, or both. Various syndromes and disorders related to the adrenal glands, such as Cushing's syndrome, adrenogenital syndrome, 21-hydroxylase-deficient adrenal hyperplasia, insulin-resistant acanthosis nigricans and androgen secreting tumour, show high levels of cortisol hormone and hirsutism. Ovarian hirsutism is caused by PCOS that is characterised from high levels of free testosterone and/or polycystic ovaries, insulin resistance and hyperandrogenemia. Idiopathic hirsutism is caused by peripheral increase of androgens, and women that suffer from it show increased levels of 5α-reductase and insulin resistance, whilst they do not show any abnormalities in their ovarian or adrenal function.

Adipose tissue contains adipocytes, also known as fat cells and are responsible for fat energy storage. This study showed that the exposure of these cells to testosterone led to insulin resistance, suggesting there is a link between hirsutism, androgen presence and insulin resistance development.

Other disorders that are known for the increased production of androgens are hyperandrogenic insulin-resistant acanthosis nigricans syndrome [103,104], 21-hydroxylase-deficient non-classic adrenal hyperplasia [90,105], androgen-secreting tumor [106], and rarely androgenic drug intake [107]. On the other hand, the causes of IH are not well known. However, when hormonal profiles of PCOS women and IH women were compared, IR was found to be significant in both groups compared to the control group. IR and hyperinsulinemia increase the insulin-like growth factor (IGF) which affects hair follicles [95,96], and therefore it is suggested that IR is potentially a cause of IH. This disorder is also characterised as an increase in 5α-reductase in peripheral tissues, an enzyme that converts T to DHT, and is generally responsible for the metabolism of steroids [95]. Potential causes of IH include increased sensitivity of hair follicles to androgens, and androgen receptor gene polymorphism [96].

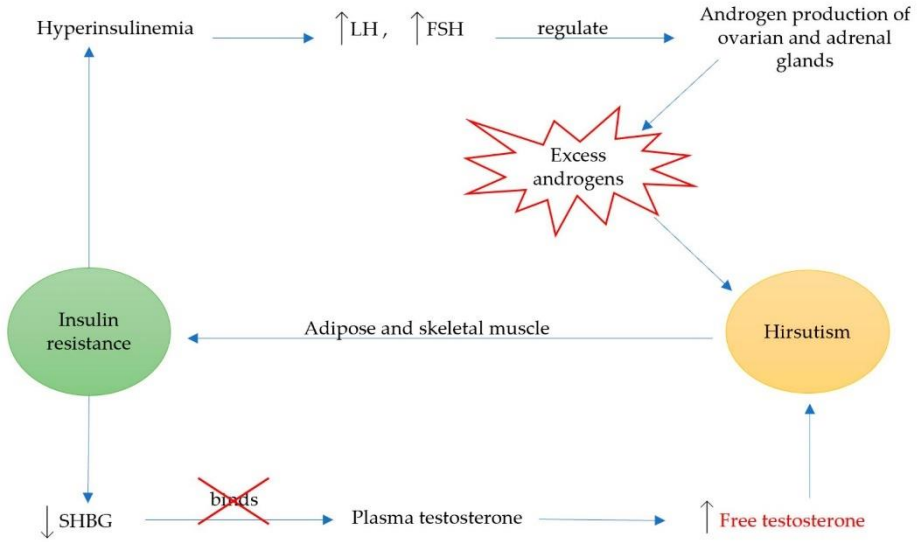

Figure 3. The relationship between insulin resistance and hirsutism. Hyperinsulinemia is caused when insulin resistance is left unmanaged, causing an increase in LH and FSH, which regulate the production of ovarian and adrenal androgens. This increase results in excess androgens, and eventually hirsutism. Moreover, insulin resistance reduces SHBG, which leaves plasma testosterone unbound leading to increased free testosterone and hirsutism. Chronic androgen exposure leads to adipose and skeletal muscle insulin resistance. The graph above presents the circular relationship between the two conditions, leading to a vicious cycle of effects.

2. Therapeutic Index

Hirsutism treatments are usually combinational treatments, and they include peripheral androgen blockage, androgen suppression and cosmetic intervention. Cosmetic and mechanical interventions are more effective and long lasting when used in combination with either androgen suppression treatment or peripheral androgen blockage. Moreover, alternative treatments have been investigated in recent years and naturally derived additional treatments have been used for the improvement of hirsutism, as well as live organisms that are shown to have a significant role in regulating and reducing androgen production on PCOS women.

2.1. Chemically Derived Therapies

Current drug therapies for hirsutism include androgen suppression and peripheral androgen blockage, also known as anti-androgens. For a more effective treatment these drugs are usually administered in combination [108–112].

Androgen suppression treatments include insulin sensitisers, long acting gonadotrophin releasing hormone (GnRH) analogue and estrogen-progestin oral contraceptive (OC) pills [98,112–114]. OC action can differ in composition, but it is not shown to significantly influence the effectiveness of the treatment. Their compositions can be monophasic, biphasic or triphasic and the action of the OC is through the suppression of LH and FSH and therefore a reduced androgen production from the ovaries [90]. Although this treatment is shown to reduce SHBG and hyperandrogenemia in general, it does not have an effect in low-grade inflammation and other hormonal disorders. Third generation OCs are not as effective at reducing the levels of SHBG when compared to older combined OC [115]. OCs containing sex hormones progestin and estrogen are shown to have significant effect on the hormonal profiles of hirsute women, reducing the levels of androgens, such as T, dehy-

droepiandrosterone sulfate (DHEAS) and DHT [116]. GnRH is a hormone produced in the hypothalamus and it stimulates the production of LH/FSH which stimulates the ovaries to produce androgens [97]. GnRH analogues are antagonists of GnRH and, when used on PCOS women, were shown to reduce the total and free T, DHEAS and androstenedione sex hormone. This treatment is usually combined with OCs. Moreover, the combination of insulin sensitisers with OC has also been studied. Insulin sensitisers, such as metformin and thiazolidenediones, are shown to reduce androgen production through the regulation of blood glucose and the increase of insulin sensitivity [108,112,117,118]. Metformin has been widely used, alone or in combination with other OCs or anti-androgens, for the treatment of PCOS as it is shown to decrease levels of LH, testosterone, and androstenedione, and improve the ovulation and decrease IR [108,109,112,113,119–122]. Metformin is shown to be equally but moderately effective on reducing the hair diameter in PCOS women when compared to anti-androgen combined with OC [112]. Although there was no decrease of androgen levels, the effect is suggested to appear due to the reduction of hyperinsulinemia. This suggests that managing insulin resistance and increasing insulin sensitivity might be a better therapy treatment and approach, than administrating anti-androgens. Overall, the combination of metformin with other drugs appears to be more effective on the treatment of hirsutism, decreasing m-FG scores, levels of androgens and further drop of free androgens through the enhancement of SHBG [109].

Peripheral androgen blockage drugs include cyproterone acetate, spironolactone, flutamide and bicalutamide. 5α-reductase inhibitors, such as finasteride, have also been used for the treatment of hirsutism [123]. These drugs bind to the androgen receptors in selective tissues and therefore reduce the synthesis of androgens [124–126]. Cyproterone acetate is shown to be effective in treating excessive hair growth and reducing androgen levels [100,110,112,123,124,127,128], although side effects, such as weight gain, tiredness, loss of libido and adrenal disfunction were reported [127–129]. The effect of this progestogenic drug is through the inhibition of gonadotropin response to reduce T levels, decrease hair growth and androgen levels [127,128,130]. Spironolactone was originally used as a diuretic drug. However, it was shown to reduce T, through cytochrome P450 inhibition, without having any effect on SHBG or free T [131]. It is shown to be an effective and tolerable drug for the treatment of hirsutism, especially when combined with OC [132–134]. Flutamide is reported to be a better choice of treatment for the disorder studied when compared to the previously mentioned drugs [134]. It is shown to have a significant effect on hirsutism, acne and male-pattern hair loss in hirsute women. However, severe side effects are reported, such as embryotoxicity, thus it is suggested to use in combination with other drugs, such as insulin sensitisers or OC [98,108,111,122,134]. Bicalutamide was shown to have similar effectiveness with the above drugs on reducing m-FG scores and lower androgen levels, although it did not have any effect on insulin resistance [135–137]. Despite the effectiveness of these drugs, many subjects are sceptical when it comes to the administration of such treatments due to the potentially severe side effects. Thus, the potential therapeutic effect of naturally derived therapies is also studied.

2.2. Naturally Derived Therapies

Many natural remedies have been studied and used for the management of hirsutism and acne, however, extensive research has not been conducted for many of them [138–141]. Some of the naturally derived anti-androgens are spearmint tea, green tea, licorice, Chinese peony, marjoram, and red reishi [141]. In a study where the effects of spearmint tea as an anti-androgen were investigated, forty two patients were asked to consume two cups of spearmint tea every day for thirty days [142]. Their gonadotrophin levels and androgen hormones were tested before and after the treatment and a significant reduction of total and free T was observed. Increased LH and FSH were also observed after short administration with spearmint tea [140]. Marjoram is shown to significantly reduce levels of DHEAS and improve IR, and therefore inhibit the production of androgens [143]. Diet has also been investigated on the role of hormonal imbalances especially since the production of

androgens is related to IR. Studies are still at early stages, looking at the potential effect of dairy and starch on the hormonal profile of PCOS women [144–146]. Studies where women were asked to follow a low starch and low dairy diet presented mixed results and the role of these foods on hormonal profiles requires more research. In addition to plant-based remedies and diet for the treatment of hirsutism and other hormonal imbalances, live organisms have also been studied on their potential effect on the hormonal profile of hirsute women [147–151].

2.2.1. Probiotics and Hirsutism

Microbes have been widely used for the production of various chemicals in the pharmaceutical world. In a recent study, after metabolically engineering *Rhodococcus ruber Chol-4*, it was shown to produce high amounts (61%) of testosterone through the conversion of 4-androstene-3,17-dione (AD) to the mentioned hormone [152]. There is increasing literature on how probiotics can affect the hormonal profiles in animals and humans and how probiotic supplementation can be used for the management and improvement of metabolic disorders and conditions. When the GM taxa of boards and gilts was analysed, host sex hormones were shown to significantly interact with the GM, which indicates the relationship between hormones and microbes [20]. Another research study that presents the relationship between the gut microbiota and the hormonal balance, is when the androgen levels of normal GM mice were compared to germ-free mice and findings showed exceptionally low unconjugated DHT detected in the later subject's distal intestine in contrast with the former subjects [153]. Interestingly, in the same study, human male feces samples with normal GM were tested and similar findings were observed compared to the normal GM mice samples. For the mice samples, male and female subjects were included. It is therefore proposed that treatment with probiotics could potentially affect the levels of DHT and T.

As mentioned earlier, PCOS is the most common cause of Hirsutism and most women that suffer from this syndrome show symptoms of increased hair growth in a male-like pattern. There is growing literature on the role of probiotics on PCOS and how the mentioned microbes are potentially involved in the management of this condition. Tremellen and Pearce in 2012 presented a detailed hypothesis on the development of PCOS starting from dysbiosis of gut microbiota (DOGMA) and how this causes a series of effects that eventually leads to hirsutism along with other symptoms of PCOS [154]. More specifically, it is hypothesised that the disruption of the balance of GM, and in combination with a diet high-in- sugar and saturated fat, leads to increased gut mucosa permeability. This allows the gram-negative bacteria to enter the systemic circulation and stimulate the immune system causing mild chronic inflammation, leading to damage in insulin receptors that further develops into IR and hyperinsulinemia in the long term. The latter condition affects the ovaries to produce excess androgens and disrupts their normal function, causing further disturbance in the development of follicles. Therefore, it is possible to hypothesise that these conditions are less likely to occur if a well-balanced gut microbiota is maintained. The above hypothesis was further investigated a few years later, where the potential gut permeability, and the level of inflammation and GM in PCOS women was studied [155]. Even though the GM was indeed found to be altered, compared to healthy subjects, there was no significant changes in gut permeability and function suggesting that "leaky" gut is only potentially a symptom in women with PCOS. In another study where the relationship of PCOS and gut microbiota was investigated, PCOS rats' GM was compared to control groups and a decreased number of *Lactobacillus*, *Clostridium* and *Ruminococcus* were detected, whereas *Prevotella*, a gram-negative bacteria, was found in higher numbers than for the healthy subjects [156]. The rat's PCOS was induced using an aromatase inhibitor called letrozole and the subjects showed increased levels of androgens along with symptoms of abnormal hormonal levels and cycles with their ovaries morphologically altered. To further investigate this relationship, faecal microbiota and *Lactobacillus* were transplanted in the gut of PCOS rats and restoration of the gut microbiota was observed along with improvement of PCOS symptoms. Interestingly, the androgen levels were reduced, and

the ovaries' morphology was improved. Such effects are in agreement with more recent studies where androgen levels and hirsutism are shown to improve after supplementation with probiotics [148,149,157]. Possibly the first investigation on hormonal profiles of PCOS women after the supplementation of probiotics, was presented in 2018 in a study where 60 women participated [157]. Probiotics *Lactobacillus* and *Bifidobacterium*, two of the most common species used in probiotic treatments, were supplied to the subjects for 12 weeks after which time significant increase in SHBG was observed, whilst m-FG scores and total testosterone were decreased. Combinational treatments of vitamin D and probiotics also showed a significant response when it comes to androgen level reduction [149]. Although this suggests that probiotics might be more efficient on managing hirsutism when combined with vitamin D, other researchers have not shown any significant effect of the vitamin alone to the referred condition [158–161], despite its significant anti-inflammatory and insulin sensitivity effect [162–164]. Therefore, it is possible that the effects of the combinational treatment are due to the improvement of IR from vitamin D, along with the probiotics effect [149]. It is also reported that PCOS-rats showed improvement in reproductive function after the treatment with probiotics [165]. Although only a few studies have looked at the effects of probiotics on hirsutism, the literature found on the role of the former on IR and sensitivity has been explored in several studies [27,165–173]. Moreover, it is now well established from a variety of studies that hirsutism is linked with IR [21,95–97,104,174–177]. As described earlier IR and hyperinsulinemia enhances the production of androgens such as T and DHT, whilst reduces SHBG levels leading to increased free testosterone levels [102].

Considering the above it is necessary to briefly present the role of probiotics on IR through which a potential improvement of hirsutism can occur. Research has been conducted on PCOS subject as well as prediabetics and healthy participants. In 2015, Shoaei et al. studied PCOS women for 8 weeks while the subjects were under probiotic supplementation, observing decreased FBS, insulin levels and IR [173]. The same observations were made in another study conducted on PCOS women where probiotics were supplemented for 12 weeks [167]. Glucose levels decreased during pregnancy and insulin sensitivity increased during the 12 month postpartum period, in a study where subjects were treated with *Lactobacillus* and *Bifidobacterium* [168]. Lower glucose levels were also observed when post-menopausal women were provided with *Lactobacillus plantarum*-containing beverages [170]. It is reported that *Lactobacillus salivarius UBL22*, when supplemented on healthy subjects, showed a significantly reduced fasting insulin compared to the control group (placebo) [27]. Another study conducted on prediabetic participants showed a reduction in fasting glucose and fasting insulin levels after treatment with probiotics (Table 1) [166].

Table 1. Probiotic treatments and the hirsutism-relevant outcomes.

Treatment	Study Population	Relevant Outcomes	Reference
Lactobacillus	PCOS [1] rats	Improvement on estrous cycles, reduction of androgen biosynthesis, normalization of ovaries, GM [2] restoration, reduction of *Prevotella*	[156]
L. acidophilus, *L. casei*, *B. bifidum*	PCOS women	Reduction of total testosterone, increase of SHBG [3], decrease of m-FG scores	[157]
L. acidophilus, *L. reuteri*, *B. bifidum* and Vitamin D	PCOS women	Reduction of total testosterone and hirsutism	[149]

Table 1. *Conts.*

Treatment	Study Population	Relevant Outcomes	Reference
E. faecali, *L. reuteri*, *Bifidobacterium*	PCOS rats	Improvement in reproductive function and GM restoration	[165]
L. acidophilus, *L. casei*, *B. bifidum*	PCOS women	Reduced fasting plasma glucose, insulin concentrations and insulin resistance. Increased insulin sensitivity	[167]
L. acidophilus, *L. casei*, *L. rhamnosus*, *L. bulgaricus*, *L. breve*, *B. longum*, *S. thermophiles*	PCOS women	Reduced FBS [4], reduced insulin levels and reduced insulin resistance	[173]
L. plantarum	Postmenopausal women	Reduced glucose levels	[170]
L. rhamnosus, *B. lactis*	Postpartum period women	Reduced blood glucose concentrations, improved glucose tolerance, reduced insulin concentrations and increased insulin sensitivity	[168]

[1] polycystic ovary syndrome; [2] gut microbiota; [3] sex hormone-binding globulin; [4] fasting blood sugar.

2.2.2. Synbiotics and Hirsutism

In reviewing the literature, very few studies were found on the association between synbiotics and hirsutism. A recent study looked at the effects of synbiotics on PCOS women using a mix of pomegranate juice, inulin and *Lactobacillus* [178]. Testosterone levels were significantly reduced after the treatment with synbiotics, compared to the control group. These findings were accompanied with an increase in insulin sensitivity and improved anthropometric measurements. Inulin is a prebiotic that has been shown to improve IR and has a beneficial effect on SHBG [147,179,180]. In a 12 week study, *Lactobacillus bifidum*, *Lactobacillus acidophilus*, *Lactobacillus casei* and inulin were used for the treatment of PCOS women and their hormone levels were studied along with other biomarkers, such as serum high sensitivity C-reactive protein [19]. Despite the statistical significance of the decrease of m-FG scores, the clinical picture of the subjects showed little improvement and longer treatment with prebiotic inulin was suggested. Selenium, known for its prebiotic activities [181–183] was also used in a combinational treatment with probiotics on women and the hormonal profiles were studied [150,184,185]. Although initial studies have not shown an effect of the treatment on free [184] and total [185] testosterone, a decrease on m-FG scores was observed [184]. On a 12-week study conducted two years later, 60 PCOS subjects were treated with *Lactobacilllus*, *Bifidobacterium* and selenium-containing synbiotics and, in contrast with the above findings, a statistically significant reduction of total testosterone and hirsutism was shown [150]. There is abundant room for further progress in determining the exact effect of synbiotics on hirsutism, whilst there is an increasing literature on the beneficial effects of synbiotics on IR on PCOS women.

Brief investigation of the role of synbiotics on IR is necessary to understand how synbiotics could potentially improve hirsutism through the improvement and management of IR. A year after Nasri et al. (2018) published their work as described earlier, Samimi et al. (2019) investigated the effects of the same synbiotics supplementation on biomarkers of IR and sensitivity along with cholesterol and triglycerides on PCOS women [180]. In this study, it was shown that *Lactobacillus bifidum*, *Lactobacillus acidophilus*, *Lactobacillus casei* and 0.8 g of inulin, significantly improved insulin metabolism markers but not FBS, after 12 weeks of treatment, suggesting that metabolic disorders can benefit from the mentioned

treatment while managing symptoms. Longer treatment periods (24 weeks) were also investigated and prediabetic adults were treated with *Lactobacillus* and *Bifidobacterium* in a inulin-containing prebiotic [166]. There was no significant improvement on fasting insulin levels and plasma glucose between the two studied time points, 12 and 24 weeks. The use of a single probiotic along with inulin is found in a study that was conducted on diabetic rats and their insulin markers were observed [80]. The synbiotic containing *Lactobacillus plantarum* and inulin managed to increase insulin sensitivity. All the above studies described were conducted on subjects that were either diagnosed as prediabetics or suffered from conditions (PCOS) for which IR is known to be a symptom. However, the beneficial effect of synbiotics was also observed when healthy subjects were treated with *Lactobacillus salivarius UBL S22* and FOS and the treatment was shown to improve insulin sensitivity significantly more compared to the probiotics alone [27]. Interestingly, despite the higher variation of probiotic bacteria in the synbiotic mix (*Bifidobacterium breve*, *Lactobacillus casei*, *Lactobacillus bulgaricus*, *Lactobacillus rhamnosus*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Streptococcus thermophilus*), in combination with the fructo-oligosaccharide inulin on PCOS women, it does not show a significant effect on the FBS and insulin sensitivity [179]. *Lactobacillus* synbiotics and 200 µg/day selenium [186] were used for the treatment of PCOS subjects for 12 weeks and it was found to improve IR and insulin levels, findings that were not observed when selenium alone was used for the treatment of diabetic participants [187]. Other studies show similar observations on fasting plasma glucose [188] and insulin levels, IR and insulin sensitivity (Table 2) [189].

Table 2. Synbiotic treatments and the hirsutism-relevant outcomes.

Treatment	Study Population	Relevant Outcomes	Reference
Lactobacillus and pomegranate juice	PCOS [1] women	Reduced testosterone levels, increased insulin resistance and improved anthropometric measurements	[178]
L. bifidum, *L. acidophilus*, *L. casei* and inulin	PCOS women	Reduced m-FG scores but did not affect the clinical picture of hirsutism	[19]
Lactobacillus, *Bifidobacterium* and Selenium	PCOS women	Reduced total testosterone and hirsutism	[150]
L. bifidum, *L. acidophilus*, *L. casei* and inulin	PCOS women	Improved insulin metabolism markers but did not influence FBS [2]	[180]
Lactobacillus, *Bifidobacterium* and inulin	Prediabetics	Decreased fasting insulin levels and fasting plasma glucose	[166]
L. plantarum and inulin	Diabetic rats	Improved insulin resistance, hypothalamic levels of insulin and leptin	[80]
L. salivarius UBL S22 and fructo-oligosaccharides	Healthy participants	Improved insulin sensitivity	[27]

Table 2. *Conts.*

Treatment	Study Population	Relevant Outcomes	Reference
L. bulgaricus, *L. acidophilus*, *L. casei*, *L. rhamnosus*, *B. longum*, *B. breve*, *S. thermophilus* and inulin	PCOS women	No effects on fasting insulin levels	[179]
L. acidophilus, *L. reuteri*, *L. fermentum*, *B. bifidum* and selenium	PCOS women	Reduced insulin levels and insulin resistance. Increased insulin sensitivity	[186]
L. acidophilus, *L. casei*, *B. bifidum* and inulin	Gestational diabetics	Decreased insulin levels and insulin resistance. Increased insulin sensitivity	[189]

[1] polycystic ovary syndrome; [2] fasting blood sugar.

3. Conclusions

Probiotics and synbiotics have been widely used for the treatment of several conditions and diseases [54,155,186,190–192]. The potentially healing properties of natural products are presented more often in recent years and people tend to turn to more natural ways of treating symptoms or even aiming towards them providing a reversing effect. On the other hand, endocrinology is a very complex area of study. Although hirsutism, a disorder characterised of excess terminal hair in a male-like pattern [88,193], has been generally studied, not much research was conducted on the effects of probiotics/synbiotics on this condition. The complexity of the disorder is also shown through its relationship with lipid metabolism [194]. Main markers for the determination of lipid metabolism, such as low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglycerides are shown to be altered in women suffering from PCOS and other related disorders [195]. Elevated levels of triglycerides and lower levels of LDL, a group of lipoproteins that are the main source of cholesterol, are observed in hirsute women. Moreover, insulin is known to increase the activity of tissue lipoprotein lipases (LPL), an enzyme that takes part in the metabolism of lipoproteins and is regulated by plasma hormones. IR, on the other hand, can lead to the elevation of triglycerides, an observation that indicates an indirect relationship of hirsutism with high levels of lipids through the increase of IR through the elevated levels of androgens and vice versa. However, this complex connection between hormones and lipids appears to be independent, as shown in a study where hirsute women were treated with GnRH and, despite the unaffected IR and lipid metabolism of the subjects, estrogens and androgens were decreased [195]. The vicious circle between increased androgens and IR, as shown in Figure 3, supports the hypothesis that hirsutism can affect the metabolic process of lipoproteins and the related enzymes, through the enhancements of IR. Therefore, treatments that provide an overall improvement to IR and steroid levels could potentially alter the metabolic process of lipids and enzymes. Although there is an extensive research on the effects of androgens on cardiovascular disease risk on men [196–199], not many studies exist on the effects of hirsutism on lipid metabolism. There is a mixed association between the effects of elevated androgen levels on women and their independent relationship with lipids and cardiovascular disease [200]. The purpose of the current study was to investigate the role of probiotics and synbiotics on hirsutism.

The limited literature found on this topic, shows that probiotics can decrease the production of androgens, and specifically total testosterone, and improve m-FG scoring on hirsute women, increasing the levels of SHBG that bind and regulate free testosterone [102,153,201]. Moreover, synbiotics are shown to have a similar effect with probiotics but more enhanced [27].

Therefore, it is suggested that a combination of probiotics and prebiotics can improve and help on the management of hirsutism and other conditions. Additionally, considering the direct relationship of hirsutism with IR [91,95,96,174], the effects of the beneficial compounds on IR was also examined. Growing literature shows a reduction of FBS, insulin levels, insulin resistance and insulin sensitivity after treatments with probiotics and/or synbiotics supports the beneficial effects on hirsutism through the mentioned mechanisms [178–180,202]. Taken together, these findings suggest a role for probiotics and synbiotics in reducing androgens and decreasing m-FG scores, directly and through managing and decreasing IR. Moreover, treatments with probiotics and/or synbiotics are shown to improve the lipid metabolism in hirsute women, reducing serum triglycerides levels [27,167,203], reducing levels of LDL and increasing HDL [27,186,203], although it is not clear whether hirsutism is independently correlated with lipoprotein elevation. The majority of studies found in the literature refer to research conducted on PCOS women, since most hirsute women also suffer from this metabolic syndrome. Current drug therapies on hirsutism including insulin sensitisers, anti-androgen monotherapies and OCPs, can have severe side effects [98,112–114] and a potential therapeutic role of probiotics along with prebiotics, can be a significant relief for women suffering from the disorder.

The effect of the studied microorganisms on the metabolic profile of women is shown to be significant and interestingly, correlated with many abnormalities. The combinational effect of them on IR, steroids and lipids profiles, shows a multifunctional character of these treatments that could potentially support the metabolic processes to such degree that conventional treatments with drugs could be replaced, especially when combined with lifestyle changes, such as exercise and diet. Although there is evidence for the beneficial role of probiotics and synbiotics on hirsutism and the related disorders, more studies need to be conducted on their direct effects, mechanisms, length of treatment and dosage.

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