DOTTORATO DI RICERCA IN
SCIENZE FARMACEUTICHE

CICLO XXIV

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Design, synthesis and evaluation of novel dualistic molecules provided with UV filtering and scavenging capabilities

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1. Introduction

Characteristics of living organisms are result of gradually evolutive adaptation toward ambient, also humans are exposed to environmental conditions and then during history course they have developed defence mechanisms adapting to habitat.

One of the most variable phenotypes in human is pigmentation; the colour of skin and also of hair and eyes is primarily determined by melanin, a complex group of biopolymers synthesized by specialized cells called melanocytes. Stimulation of melanin synthesis is the main defence against the damaging effects of ultraviolet radiation and skin colour is the principal outcome of adaptation toward UV rays. Melanin acts as an optical and chemical photoprotective filter reducing the penetration of radiation into subepidermal tissues. Skin coloration is strongly correlated with absolute latitude and then with UV radiation levels to which humans are exposed, closer are persons to the equator greater is UV energy that reachs the earth surface and consequently natural selection have favoured in tropical regions dark pigmentation, because highly melanised epidermis affords better protection against UV-induced injuries such as sunburn, skin cancer and sweat glands damages. Lighter skin instead can be explained as adaptation to the lower UV radiation incidence in regions far from the equator and the importance of maintaining UV-induced biosynthesis of vitamin D3, since increasing the melanin in human skin increases the time of exposure to UV light that is needed to maximize synthesis of previtamin D3. Sunlight is not only dangerous for skin, but also degrade some essential nutrients such as folate, a fundamental molecule for nucleotide and, therefore, DNA biosynthesis. Folate deficiency can result in complications during pregnancy and multiple fetal abnormalities, including neural tube defects, such as spina bifida and anencephalus and it was significant cause of perinatal and postnatal mortality in some populations before the introduction of preventive supplementation. Deeply melanised skin protects also folate in the blood from photolysis, another reason that could have favoured positive selection of people with dark skin in areas with high solar intensity together with the lower skin cancer incidence.[1,2]

Dark skin and light skin were adaptations to environments of high ultraviolet light exposure and low ultraviolet light exposure, respectively; light skin is most vulnerable to UV radiation and fair skinned individuals, living in regions with great incidence of UV rays, are at highest risk of developing skin cancer. Although dark skinned persons are less affected by UV radiation, they are not completely safe and can also develop, even though incidence is lower, UV-induced skin cancers. It is important to remember that exposure to UV radiation is dangerous for people all over the world and then to avoid detrimental effects of UV radiation it must avoid UV overexposure, particularly during the time of the day of higher UV incidence, and protect the skin with sunscreen products when it decide to stay in the sun.

1.1 Sunlight

The electromagnetic spectrum of the sun is composed, from shorter wavelengths to higher, by: cosmic rays, gamma rays, X ray, UV radiation (UVC, UVB, UVA), visible light, infrared rays, microwaves and radio waves (Fig.1). Fortunately higher energy rays, such as cosmic rays (below 10^{-16} m), gamma rays (10^{-16}-10^{-11} m), X rays (10^{-11}-10^{-8} m) and UVC rays (100-280 nm), are filtered by the stratospheric ozone layer;[3] while earth's surface is constantly irradiated by light coming from the sun, composed of 56% infrared waves (wavelength, 780-5000 nm), 39% of visible light (400-780 nm), 4,9% of UVA rays (320-400 nm) and 0,1% of UVB light (290-400 nm).[4] Although UVA and UVB rays are a small portion of the total radiation reaching the earth, they are responsible for skin, eyes and hair damages, because of their higher energy content. The following relationship describes the correlation between energy, frequency and wavelength; radiation energy increases proportionally with the frequency and decreases with the increase of wavelength.[3]

$$E = h \cdot \upsilon = \frac{h \cdot c}{\lambda}$$

E = energy

v = frequency (Hertz)

h = Planck'scostant = 6.62 x 10^{-27} erg/s)

c = speedof light = 3.0 x 10^{10} cm/s

λ = lunghezza d'onda in cm o m

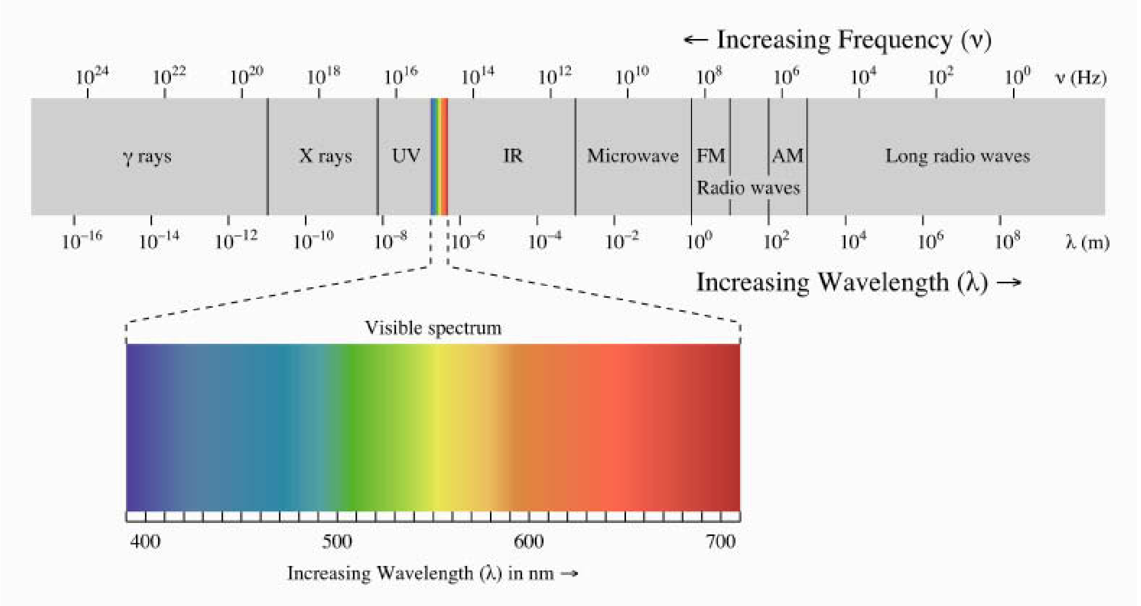


Figure 1.1 Sunlight

Besides energy, it must be considered also the intensity and the composition of UV radiation, since these parameters are not constant but change affected by different factors, such as season, ozone layer, transmission, reflexion, altitude, latitude, cloudiness and inclination of the sun, which varies during the time of the day. The highest irradiance is at higher elevations, because the atmosphere is thinner resulting in an increase of the intensity by 4% every 300m of elevation, and at the equator where the sun is most directly so the UV radiation travels the least distance through the atmosphere and there is less ozone to absorb the UV radiation, since ozone is naturally thinner near the equator. Because UVA rays are of longer wavelength compared with UVB, UVA are less affected by altitude or atmospheric conditions; on the earth's surface the ratio of UVA to UVB is 20:1. Atmospheric agents as fog, haze, clouds, and pollutants can reduce ultraviolet radiation by 10–90% while snow, sand, and metal can reflect up to 90%. Sea water can reflect up to 15% and penetration through water is possible to a depth of 1 m.[5,6]

1.2 The human skin

The skin is the largest organ of the body, covers the entire body surface and is continuous with the mucous membranes. It has several functions involving protection from the environment (against external physical, chemical and biological aggressions), tactile sensation, immunity defence, regulation of body temperature and secretions.

Human skin consists of three layers (fig. 2); a stratified, cellular epidermis and an underlying dermis of connective tissue, below the dermis there is the hypodermis, the panniculus adiposus, a fatty layer usually designated as 'subcutaneous'. This is separated from the rest of the body by a vestigial layer of striated muscle, the panniculus carnosus.
The skin shows considerable regional variations, concerning its thickness (varying from 1 to 4 mm) and distribution of epidermal appendages, so it can be divided in: glabrous skin and hair-bearing skin. Hairless skin is found on palms and soles and is characterized by thick epidermis divided into several well-marked layers, including compact stratum corneum, by the presence of encapsulated sense organs within the dermis and by lack of hair follicles and sebaceous glands. Hair-bearing skin, on the other hand, has both hair follicles and sebaceous glands but lacks of encapsulated sense organs, there is also wide differences between the body sites.[7,8]

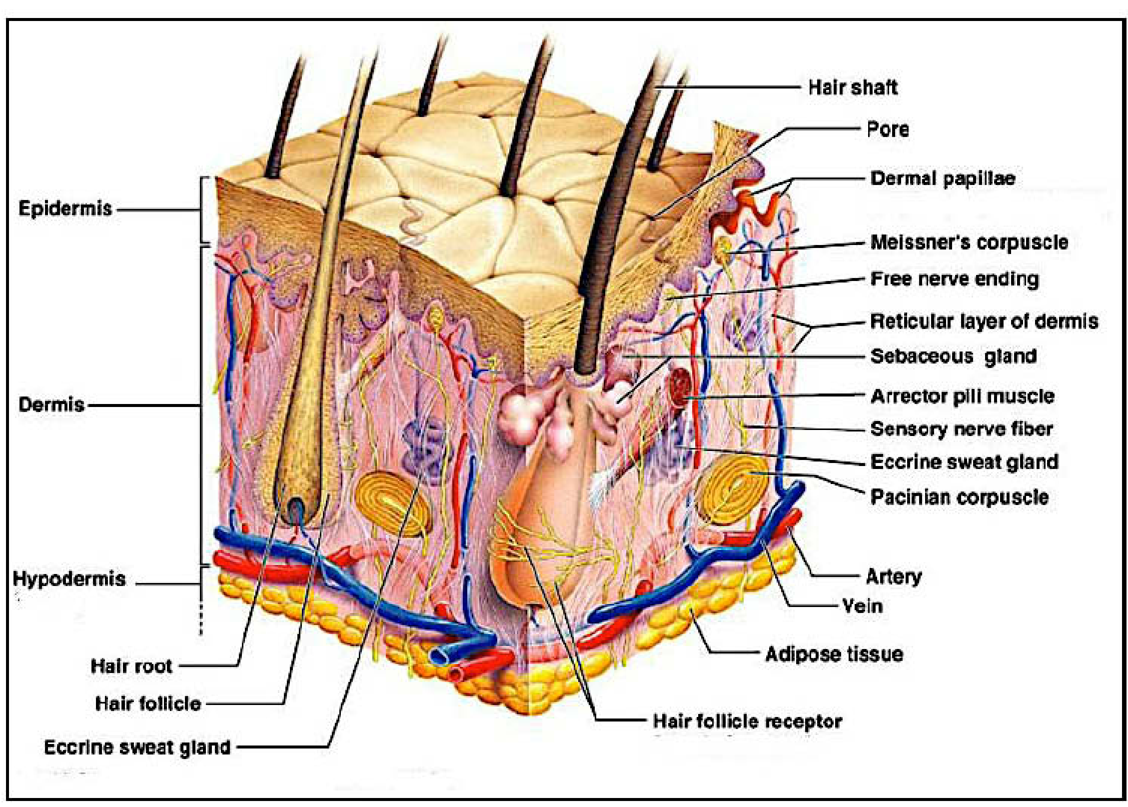


Figure 1.2 Human skin.

The epidermis is a stratified squamous epithelium, made of various cell types, the great number of which (90-95%) are keratinocytes that undergoing a specific differentiation process resulting in the production of flattened, anucleate cells (corneocytes). The remaining 5-10% of epidermal cells are mainly melanocytes, which synthesized melanin,

Langerhans' cells, which have immunological functions and Merkel cells, which seem to function as mechanoreceptors. Keratinocytes moves progressively from the basal layer towards the skin surface, forming several well-defined layers during its transit, so the epidermis can be divided into four distinct layers: *stratum basale* or *stratum germinativum*, *stratum spinosum*, *stratum granulosum* and *stratum corneum*; in some body areas (the palmoplantar region) an additional layer, the *stratum lucidum*, can be seen between the granular and the horny layers.

The stratum basal is a continuous layer, generally described as only one cells thick, but may be two to three cells thick in glabrous skin and hyperproliferative epidermis. The basal cells are small and cuboidal (10–14 nm) and have large nuclei, dense cytoplasm containing many ribosomes and dense tonofilament bundles. Immediately above the basal cell layer, there is the *stratum spinosum*; when a basal cell from the basal layer goes up to the spinous layer begins to differentiate in a keratinocyte. The *stratum spinosum* is succeeded by the *stratum granulosum*, here the cells don't undergo mitotic divisions but produce high quantity of keratohyalin and keratin, basal structural proteins of nails and hairs. The outermost layer of epidermis is the *stratum corneum* where cells (now corneocytes) have lost nucleus and cytoplasmic organelles. These cells are generally already died, flattened, interdigitated and disposed in sheets, they have filamentous keratin matrix and a thick cornified envelope within the plasma membrane.

Epidermal keratinocytes originate from mitotic divisions of stem cells takes 15-30 days to go from basal layer towards the skin surface and during migration undergoes morphological and biochemical differentiation (keratinisation). The cornified cells remaining in the horny layer for about two week, then they are shed from the skin surface.[7,8]

The **dermis** is situated under the epidermis, is a connective tissue compressible and elastic, highly innervated and vascularized. It consists of supporting matrix or ground substance in which polysaccharides and protein are linked to produce macromolecules with a significant capacity in retaining water. Within and associated with this matrix are two kinds of proteins: elastin and collagen fibers, which have great tensile strength and form the main constituent of the dermis. The cells present in the dermis are fibroblasts, mast cells and dermal dendrocytes.

The thickness of the dermis varies considerably with the anatomic location (being much thicker on the back, on palms and soles than on the eyelids) and its fine structure varies depending on depth, it can be distinguish two layer of dermis: superficial or papillary

dermis, reticular or deep dermis. The papillary dermis is made of collagen fibers, arranged in loose bundles, and of thin elastic fibers; it forms conic upward projections (dermal papillae) that increase the surface of contact between dermis and epidermis allowing a better adhesion between these layers; it contains also tactile corpuscles, specialized nerve endings acting as mechanoreceptors. The reticular (deep) dermis is made of denser collagen bundles and the elastic network is also thicker.[7,8]

The **hypodermis** is a fatty tissue, which plays an important role in thermoregulation, insulation, provision of energy (nutritional store) and protection from mechanical injuries. It consists of loose connective tissue and is mainly constituted of adipocytes, large, rounded cells with a high lipid content in cytoplasm (triglycerides, fatty acids). Adipocytes are arranged in primary and secondary lobules, separated by the connective tissue septa containing fibroblasts, dendrocytes and mast cells.[7,8]

1.2.1 Melanocytes and melanogenesis

Melanocytes are dendritic cells residing in the epidermis, hair follicle and eyes; their principal task is to produce the pigment melanin that is responsible for skin hair and eyes pigmentation. In the epidermis, melanocytes are approximately 1–2% of epidermal cells; they are located in the basal layer in contact with keratinocytes. Within melanocyte the pigment melanin is synthesized inside membrane-bound organelles termed melanosomes, which at the maturation move to adjacent keratinocytes through dentritic structures. Within the keratinoctyes, melanosomes are typically aggregated over the nucleus, to provide protection against ultraviolet radiation.[9]

Two kinds of melanin are synthesized within melanosome: eumelanin and pheomelanin; these biopolymers are both deriving from the L-Tyrosine, which is oxidized by the tyrosinase enzyme to dopaquinone, a key intermediate compound of two synthetic pathways, the one leading to eumelanin production and the other to pheomelanin production. Eumelanogenesis involves transformation of dopaquinone by a series of oxidoreduction reactions with production of the intermediates 5,6-dihydroxyindole (DHI) and DHI carboxylic acid (DHICA), that undergo polymerization to form eumelanin consisting of different oxidative states of 5,6-dihydroxyindole (DHI), 5,6-dihydroxyindole-2-carboxylic acid (DHICA) units, and pyrrole units derived from their peroxidative cleavage. Pheomelanogenesis also starts with dopaquinone, here it is conjugated to cysteine to give cysteinyldopa and after further transformation pheomelanin (Fig. 3).[10]

Comparison of the biochemical melanin pathways revealed that eumelanin requires higher concentrations of tyrosine together with higher activity and protein levels of the enzymes tyrosinase and tyrosinase-related proteins, while pheomelanin synthesis requires availability of cysteine and proceeds in the presence of low tyrosine concentrations, low activity and level of tyrosinase and absence of tyrosinase-related proteins; from these results come out that pheomelanin synthesis is less stringent than those of eumelanin and it seems to be the default pathway.[11]

Melanin pigments differ for structure, physical and chemical properties; eumelanin is a black to brown color pigment, whereas pheomelanin show a yellow to reddish colour, the first one is able to scavenge free radical, contrary pheomelanin is photolabile and after irradiation generates hydroxyl radicals and superoxide anions, which might contribute to UV rays oxidative damages; additionally pheomelanin increases the histamine release that contributes to erythema and edema induced after sun exposure. Diverse pigmentary phenotypes vary for the amount and the type of melanin synthesized; darkly pigmented skin present larger and more pigmented melanosomes enriched in eumelanin than lightly pigmented skin, which contains mainly pheomelanins and low concentration of eumelanin, furthermore the melanosomes tend to be less pigmented and smaller in size.[12]

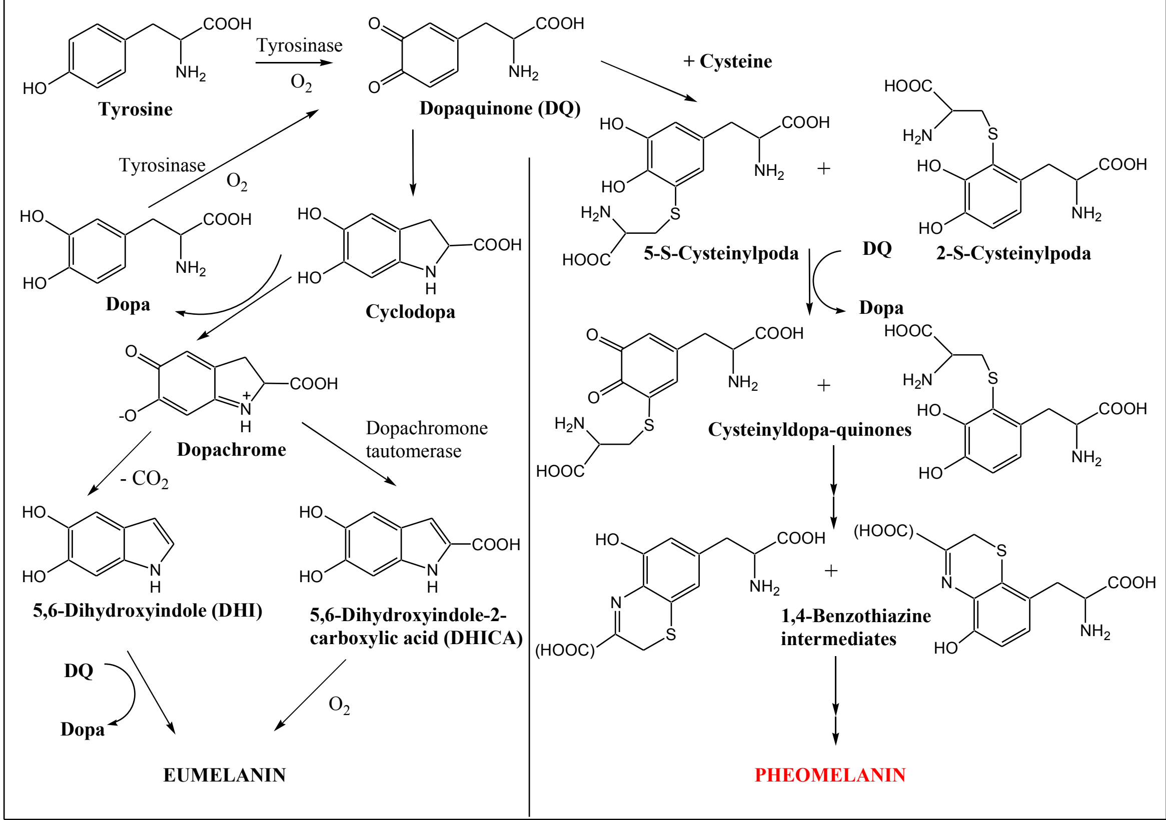


Figure 1.3 Melanin biosynthetic pathways

1.3 Effects of UV rays on the organism

UV rays are the most dangerous radiation reaching the earth surface; the organs mainly affected by UV radiation are skin and eyes, since they are directly exposed to sunlight and because of the presence in the tissues of chromophores, molecules able to absorb the radiation energy, such as melanin, DNA, RNA, proteins, lipids, trans-urocanic acid, and aromatic amino acids, as tyrosine and tryptophan.[13]

In the past only UVB radiation was believed to be dangerous for tissues, while UVA radiation was considered harmless; currently it is known that, although they show different properties, also UVA rays are damaging. UVB rays have higher energy content and are more cytotoxic and mutagenic than UVA, however UVA rays are able to penetrate deeper into the skin reaching the dermis and are responsible for indirect damages by generation of reactive oxygen specie. Human exposure to UV radiation causes different acute and chronic effects on the skin; acute responses include photodamage, erythema, synthesis of vitamin D, tanning and most dangerous immunosuppression and mutation, which are responsible of chronic UVR effects such as photocarcinogenesis.[14]

The most evident effects following exposure to sunlight are appearance of erythema and tanning; the action spectrum for UV-induced tanning and erythema are almost the same, however, UVA rays are more efficient in inducing tanning whereas UVB are in inducing erythema, because the ability of UV to induce erythema decreases going towards longer wavelength, then to produce the same erythemal response its need about 1000 times more UVA dose compared with UVB. UVB-induced **erythema** occurs in about 4 hours after exposure, peaks around 8 to 24 hours and fades generally over a day. The erythema is due to superficial vasodilatation and is associated with the appearance of apoptotic keratinocytes.[6]

Tanning is the main auto-protection mechanism of the skin in response to UV radiation exposure, devised by evolution in order to protect against haemoglobin photo-degradation, and is a process that takes place by step. Within minutes after UVA exposure occurs immediate pigment darkening (IPD), a transient pigmentation not due to new melanin synthesis but to photo-oxidation of pre-existing melanin and to redistribution of melanosomes in a peripheral dendritic location. IPD is followed by a second phase of tanning, the persistent pigment darkening (PPD). PPD appears as brown coloration and is thought to result from the oxidation of melanin (like IPD), occurs within hours after UV exposure and persists at least 3–5 days. The last phase of skin tanning is the delayed

tanning (DT), can be induced by UVB or UVA and becomes apparent 2–3 days after UV exposure. DT is caused by increased tyrosinase activity and then production of new melanin, are involved also increase in the number of melanocytes, melanosomes, and the number of melanosomes transferred to keratinocytes. Tanning is determined directly by the response of melanocytes to UVR, but is also affected indirectly by a complex system of paracrine and autocrine factors such as hormones, cytokines and growth factors, whose synthesis in epidermal cells is influenced by UVR.[14]

Another mechanism of protection against UV rays is **skin thickening**; UV rays exposure is immediately followed by the appearance of keratinocyte apoptotic cells, but 48–72 h later occurs keratinocyte hyperproliferation, that leads to an increase in epidermal thickness and particularly to an increase in the stratum corneum thickness. The production of melanin is the most important defence of the organism against UV rays, but also skin thickness of the stratum corneum or of total epidermis contributes to reduce the radiation amount reaching the epidermis basal layer and the dermis.[15]

A further visible result of sun exposure is **photoaging**, which is due to chronic sun damage and contributes to accelerate the intrinsic ageing process. The injuries of UV radiation exposure varies depending also on ability to block or repair sun induced damages; generally fair–skinned persons are the most affected by photoaging and develop also areas of total depigmentation owing to absence of melanocytes probably destroyed by UV radiation. Generally, photoaged skin appears with deep wrinkles, laxity, a leathery appearance with coarse skin texture, enlarged pores, impaired wound healing and marked telangiectasia with an increase in number and diameter of small blood vessels. Both UVB and UVA radiation contribute to photoaging and then are interested epidermis as well as dermis for direct effects or for the over-stimulated production of reactive oxygen species. UV-generated reactive oxygen species seems responsible for mitochondrial DNA mutations, protein oxidative modifications, within collagen is particularly affected by oxidation and degradation that is carried out by matrix metalloproteases; the synthesis of these enzymes increases following signalling pathways initiated by reactive oxygen species. In addition, the large collagen degradation products inhibit new collagen synthesis and thus, collagen degradation itself negatively regulates new collagen synthesis and then interstitial collagen are reduced and damaged. Photoaged skin is also characterized by a reduced number of anchoring fibrils connecting the epidermis with the dermis and by an increase in the thickness of the horny layer and in general of epidermis and dermis, while intrinsically aged skin is atrophic. A typical feature of photoaging is elastosis, clinically the

skin shows yellow discoloration and coarse surface; histologically the dermis displays an overgrowth of degraded elastic fibers, organized in tangled masses, and an increased amount of ground substance, largely composed of glycosaminoglycans and proteoglycans. In photoaged epidermis keratinocytes can be irregular with a loss of polarity, a disorder know as actinic keratosis, clinically perceived as red, rough, hyperkeratotic patches; actinic keratosis has been demonstrates as the initial lesion that can progress to invasive squamous cell carcinoma.[16,17,18]

Within skin there is the urocanic acid, a chromophore synthesised in keratinocytes by deamination of histidine, which is accumulated in epidermis because here there aren't catabolic enzymes and then it is removed only by monthly cellular skin renewal or by dissolution in sweat. This molecule exists in two isoforms; trans-urocanic acid is the predominant cutaneous isoform, but upon UV exposure isomerizes to cis-urocanic acid and the isomerization ratio increases with UV dose. The **photoisomerization from trans to cis-urocanic acid** is of particular importance because cis-urocanic acid is believed to be responsible for photo-immunosuppression. Urocanic acid sensitizes and reacts with singlet oxygen species and also reacts with biomolecules such as proteins and DNA; the interaction with DNA involves the formation of cyclobutane adducts of urocanic acid with thymine and the production of pyrimidine dimers and strand breaks, that are implicated in immunosuppression.[19,20]

Acute and chronic immunosuppression is caused by both UVB and UVA exposure and is dose dependent. It is believed that the decrease of immune response, observed after UV irradiation, serves to prevent excessive inflammation reaction and damages to the skin following sun exposure. The drawback of this physiological response is the suppression of cell-mediated immunity, result of functional inhibition of Langerhans cells, which leads to an impaired immune defence against neoplastic cells or infectious agent. Furthermore, immunosuppression is not merely an event limited to the skin, but UV radiation suppresses also immune response in internal organ inducing release, by keratinocytes, of immunosuppressive mediators such as cytokines that enter in the circulation.[13,21]

The most detrimental consequence of exposure to sunlight is **skin cancer**; broad-spectrum UV radiation is listed within human carcinogens in the report on carcinogens, while UVB and UVA radiations are classified as "reasonably anticipated to be a human carcinogen".[22]

Many of the adverse effects of UV radiation can be attributed to DNA damage; while UVB, implicated as primary mutagen, is absorbed directly by DNA inducing base structural DNA damage, UVA is mainly responsible for indirect DNA damage by

generation of reactive oxygen species. Photolesions, with potentially mutagenic properties, induced by UVB are dipyrimidine lesions that include cyclobutane pyrimidine dimers (CPD), especially thymine dimers, and pyrimidine (6-4) pyrimidone photoproducts (64PP). The CPD are the most abundant and probably the most cytotoxic lesions as they block transcription and replication; if not repaired, they can lead to misreading of the genetic code, mutations and cell death. UVA rays also can promote the formation of CPD in keratinocytes and melanocytes in vivo, but requires higher doses than UVB, then UVA rays mainly act inducing indirect damage via absorption by other endogenous chromophores that can generate reactive oxygen species with consequently production of DNA strand breaks and mutagenic changes to purines as formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine. Cells have several DNA repair mechanisms to maintain genetic integrity, including nucleotide excision repair, however, the corrections are not always faithful to the original, irreversible defects can occur leading to mutagenesis. Furthermore cells can only manage low amounts of DNA damage, when DNA damage reaches critical levels repair systems can be overloaded and fail. The consequences of such transient or definitive modifications can be the inability to read and transcribe the vital messages (cell death caused by genotoxicity) or to misinterpret genes, the last occurrence can lead to an abnormal behaviour of the cells, such as hyperproliferation. When DNA mutations interest oncogenes and tumour suppressor genes, they can loose their function; in example p53 is a tumour suppressor gene that accumulates and is activated as a transcription factor, in damaged cells, driving a chain of events that culminates in cell cycle arrest or apoptosis. If p53 undergoes mutation, the ability to manage DNA repair and to remove highly damaged cells get lost, facilitating further mutations and enhancing tumour development. Mutation of p 53 is thought to be the first step in induction of nonmelanoma skin cancer.

The three main types of skin cancers are melanoma and nonmelanoma skin cancer that comprises basal cell carcinoma and squamous cell carcinoma. The cutaneous malignant melanoma stems from melanocytes, it is the most aggressive type of skin cancer since it can metastasise very quickly and is the most responsible for skin cancer death; fortunately it is the least widespread types of skin cancer. The nonmelanoma skin cancers, deriving from keratinocytes, are less aggressive than melanoma; however, they can grow invasively and squamous cell carcinoma can also metastasise, event that occurs very rarely for basal cell carcinoma. People who sunburn easily and never tan have the highest risk of develop all three types of skin cancer; amongst white Caucasians basal cell carcinoma is the most common, followed by squamous cell carcinoma and at last cutaneous malignant

melanoma. The incidence rises increasing environmental UV exposure for all cancer types, but, while the occurrence of developing nonmelanoma skin cancer seems increase with the long-term chronic UV exposure, the risk of developing melanoma skin cancer enhances with acute intermittent UV exposure.[23,24,25,26,27] The low incidence of cutaneous malignancies in darker-skinned persons is primarily a result of photoprotection provided by high amounts of melanin, indeed black epidermis transmits 7.4% of UVB and 17.5% of UVA rays, compared with, respectively, 24% and 55% for caucasian epidermis. Dark skin transmits less ultraviolet light thanks to the larger and more melanized melanosomes hat absorb and scatter more light energy than the smaller, less melanized melanosomes of white skin.[28]

Maybe the only useful effect of UV rays is the induction of **vitamin D synthesis**; in fact, humans depend on sun exposure to satisfy their requirements for vitamin D because very few foods naturally contain vitamin D and only few foods are fortified with vitamin D. During exposure to sunlight, 7-dehydrocholesterol, which is present in the skin plasma membranes of both epidermal keratinocytes and dermal fibroblasts, absorbs solar UVB radiation and is converted to previtamin D3 that undergoes thermally induced transformation to vitamin D3 (cholecalciferol). Once formed, vitamin D3 is metabolized in the liver to 25-hydroxy-vitamin-D3 and then in the kidney to its biologically active form, 1α,25-dihydroxy-vitamin-D3 (calcitriol) that regulates mainly calcium homeostasis.[29]

2. UV Filters

In the Cosmetics Directive 76/768/EEC, annex VII, the council of the European communities defines UV filters as substances which, contained in cosmetic sunscreen products, are specifically intended to filter certain UV rays in order to protect the skin from certain harmful effects of these rays and the filters may be added to other cosmetic products within fixed limits and condition.[30]

The sunscreens classification changes worldwide, while in Europe they are included in cosmetic category, in USA, in example, they are categorized as OTC[31] and in Australia the products labelled as protecting the skin from certain harmful effects of the sun's UV rays are regulated as therapeutic goods.[32] Furthermore differ the list of permitted UV filters; currently in Europe 26 molecules are permitted, in Australia 29 actives and in USA only 18 molecules are authorized, also the maximum allowed concentration of active agents shows variation among national regulatory agencies.[30,31,32]

Aside the classification of legislative guidelines, the task of sunscreens remains the same and therefore to shield the skin against acute and long-term UV-induced damages. To provide suitable protection an UV filter should:

- ✓ Absorb radiation in both UVB and UVA range;
- ✓ be photostable;
- ✓ be stable to heat;
- ✓ be chemically stable and inert to other cosmetic ingredients;
- ✓ be photochemically inert;
- ✓ posses a large molar extinction coefficient;
- ✓ not show cytotoxicity and phototoxicity;
- ✓ not penetrate through the stratum corneum but remain on the skin surface[30,33,34]

Generally a single molecule can't satisfy completely the above mentioned requirements, in particular it can't ensure a broad spectrum photoprotection, because most of the molecules are or UVB filter or UVA filter; to overcome this problem sunscreen formulations never contain a single UV filter but a combination of active ingredients, where it can find

associations of organic filters or organic filter and inorganic filters.

2.1 Inorganic UV filters

Inorganic UV filters, also called physical filters, work reflecting or scattering visible, UV, and infrared radiation. Common agents for inorganic sunscreens are zinc oxide, titanium dioxide, silicates and iron oxide, nowadays the most used and authorized by competent authorities are zinc oxide (ZnO) and Titanium dioxide (TiO_2), in Europe only Titanium dioxide are permitted in sunscreen formulations.[30,35]

These actives are photostable, have low allergenic and sensitizing potential, but they are often cosmetically unacceptable because of occlusiveness and production of an opaque and white appearance on skin, due to high refractive index of both ZnO (refractive index = 1.9) and TiO_2 (refractive index = 2.6); the last one with its higher refractive index cause a greater whitening appearance because of higher reflexion of visible light. To reduce reflection of visible light and give at cosmetic formulations a more transparent appearance, the particles of inorganic sunscreens were micronized. Micronized titanium dioxide has an absorption profile greater in UVB than micronized zinc oxide, whereas zinc oxide provided a more effective UVA protection (up to 380 nm) than titanium dioxide; the photoprotection range shifts in function of particle size and micronization causes shift towards smaller wavelengths. The greatest UV absorption/scattering properties of titanium dioxide are afforded with particles size between 20 nm to 30 nm, while zinc oxide particles provide better UV protection in the range from 60 nm to 120 nm.[36]

The particles micronization causes changes in behaviour of the materials, indeed optical, mechanical and electrical properties are different from their conventional-sized counterparts; for this reason there is a growing concern regarding the safety profile of personal care products containing nanomaterials, in particular there are questions regarding the dermal penetration potential, systemic absorption and subsequent toxicity. However, in vitro and in vivo studies using murine, porcine, or human skin have shown that the nano-sized TiO_2 and ZnO don’t go beyond the stratum corneum and the level of penetration are the same of the macrosized counterparts.[36]

The micronized particles not only reflect and spread light but also at the same time absorb the radiation, in this way the electrons of the metallic oxides are mobilized by absorption of UV radiation, leading to the production of reactive oxygen species, causing DNA

damages. To overcome the problem related to photocatalytic activity, titanium dioxide is coated with dimethicone or silica, this reduce the free radical formation and at the same time allows to keep microparticles in dispersion, since, due to electrostatic effects, nanomaterials tend to agglomerate resulting in loss of the efficacy of the formulation. Despite the concern regarding the microparticles, the inorganic sunscreens are considered safer then organic sunscreens and are preferred for the children and in patients with a history of sunscreen allergy. [37,38]

2.2 Organic UV filters

The general structure of organic UV filter consists of an aromatic ring conjugated with an electron-receiving group or a double bond and substituted in *ortho* or *para* position with an electron-releasing group (Fig 2.1).

para-disubstituted

ortho-disubstituted

Figure 2.1 General structures of organic UV filters.

These molecules absorb ultraviolet radiation and tend to delocalize electrons to reach a higher energy state, then the excited molecules returns to the ground state emitting energy lower than that absorbed; if loss of energy is great, the emitted radiation lies in infrared region, whereas if a minor quantity of energy is lost the emitted radiation stays in the visible range and are perceived as either fluorescent or phosphorescent effect.[3,35] Unfortunately, sometimes after energy absorption the UV filters can undergo structural transformation (cis-trans or keto-enol photochemical isomerization with consecutive λ_{max} shift) or even worse degradation, resulting in activity loss; these molecules are defined as photo-unstable. Another undesirable condition is the photoreactivity of the filtering molecules that is the interaction of the molecule in its excited state with oxygen or surrounding biomolecules of the skin, leading to the production of dangerous reactive species.[3,34]

Organic filters absorb radiation energy within a specific range of wavelength, depending on their chemical structure, then on the basis of the lambda maxima and of bandwidth of absorption spectrum they can be divided in UVB, UVA and broad-spectrum filters.

2.2.1 Organic UVB filters

UVB filters mainly absorb radiation in the wavelengths region between 290 nm and 320 nm; several molecules are permitted for use in sunscreen formulation and they can be divided in different groups according to their chemical structure.

- ✓ **PABA and its derivatives** (fig 2.2). Para-aminobenzoic acid (PABA) was one of the first widely available UV filter, it is water-soluble and its peak absorption wavelength is 283 nm. The presence on the aromatic ring of an electro-releasing group ($-NH_2$) in *para* position respect to the carboxylic acid allows an efficient electrons delocalization; however, at the same time the two polar groups in these positions cause problems that have contributed to make the product less attractive for the formulation, because amine and acid tend to form intermolecular hydrogen bond leading to increased association of the molecules and consequent dissolution problem in the cosmetic vehicles. Moreover PABA can make hydrogen bonds with polar emollients too, this solvent effect leads to shift of λ_{max} from 293 nm in non polar solvent to 266 nm in polar solvent.[3] Through hydrogen bonds with the protein of the keratinocytes, PABA also clings to skin cell, this quality makes its an ideal water resistant UV filter; however same problem about PABA have limited its use, actually up to 4% of the population have photoallergic reaction to it and consumers dislike it for staining effects on clothing.[36,38] To overcome the above mentioned problems with PABA and to avoid that pH changes led absorption spectrum variations, due to the presence of free amino and acidic groups, PABA derivatives were designed with both moieties protected.[3] The only PABA ester approved for use by the FDA is Padimate O, or octyl dimethyl PABA (2-ethylhexyl 4-dimethylaminobenzoate), it maintains high UVB filtering activity (λ_{max} 311 nm) and the ability to sticks to keratinocytes, keeping water-resistant properties, like the lead compound, but fortunately with less photoallergenic potential and it is easily to incorporates into cosmetic products.[38] In Europe and Australia another PABA derivative is permitted, the PEG-25 PABA (4-bis(polyethoxy)para-aminobenzoic acid polyethoxyethyl ester), a water soluble UVB filter.[30,32]

PABA
4-Amino-benzoic acid

PADIMATE O
4-Dimethylamino-benzoic acid 2-ethyl-hexyl ester

Figure 2.2 PABA derivatives

✓ **<u>Cinnammates</u>** (fig. 2.3) have an unsaturation between the aromatic ring and the carbonyl portion, members of this class are: Octinoxate (2-ethylhexyl-p-methoxycinnamate), Cinoxate (2-ethoxyethyl-p-methoxycinnamate), Octocrylene (2-cyano-3,3-diphenylacrylic acid 2'-ethylhexyl ester), Amiloxate (isopentenyl-4-methoxycinnamate); they have good UVB filtering capacity and low skin irritancy potential. Octinoxate is the most commonly used cinnamate worldwide, it has a high molar extinction coefficient and being insoluble in water it is suitable for water resistant sunscreen formulation, furthermore its systemic absorption is insignificant after whole-body topical application, estimated nearly 0.002%.[3,38] On the other hand Octinoxate after irradiation undergoes cis-trans isomerisation with consequently loss of UV absorption efficacy in a short time, it is also reported photoinstability of the filter if used together with Avobenzone (UVA filter), it seems that the photoinstability of Avobenzone may cause the photolysis of the Octinoxate affecting the overall UV protection.[36,39] The Octocrylene is the latest molecule approved of this group; it has broad UVB spectrum (peak absorption at 307 nm), but low extinction coefficient so to increase SPF value it is used in association with other UV filter. In the past, this agent was not widely used because of its cost and difficulty in formulation, but the finding that is the best available photostabilizer for Avobenzone has increased the use.[3,36]

OCTINOXATE
2-ethylhexyl-p-methoxycinnamate

AMILOXATE
Isoamyl p-Methoxycinnamate

CINOXATE
2-ethoxyethyl-p-methoxycinnamate

OCTOCRYLENE
2-cyano-3,3-diphenylacrylic acid 2'-ethylhexyl ester

Figure 2.3 Cinnammates

- ✓ **Salicylates** (fig. 2.4) are ortho-disubstituted compounds and their spatial arrangement permit intramolecular hydrogen bond, making thus electrons less available for interactions with biological molecules and with other ingredients of the formulation, this contributes to give stability to the filter and an excellent safe profile. They were the first available UV filters, this group include: Homosalate (3,3,5-trimethylcyclohexyl 2-hydroxybenzoate), Octyl salicylate (2-ethylhexyl salicylate), Trolamine salicylate (triethanolamine salicylate). Their absorption maximum range is between 300 nm and 310 nm, they are weak UVB absorber so it needs high concentrations to achieve a high SPF, despite this, salicylates have the advantages to do not penetrate the horny layer and so have low sensitizing potential. Octyl salicylate and Homosalate are water insoluble and then they still remain on the skin after bathing and perspiration. Trolamine salicylate instead is water soluble and has been used in hair products.[3,38]

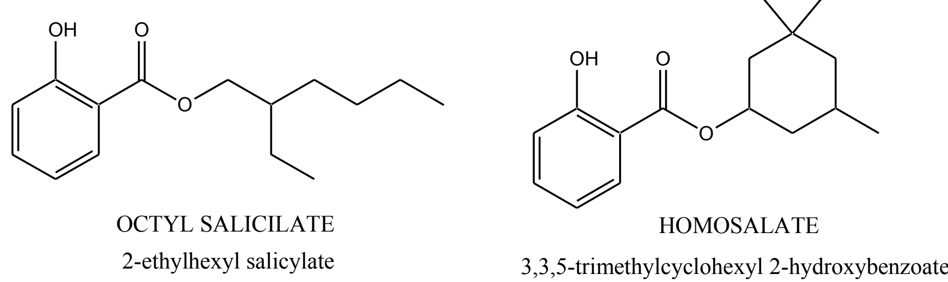


Figure 2.4 Salicylates

- ✓ **Ensulizole** (fig 2.5) or 2-Phenylbenzimidazole-5-sulfonic acid (PBSA) is widely used in sunscreen formulations and because of its strong absorption in the UVB region within 290 nm and 320 nm, it is included in the list of authorized filters in Europe,[30] USA[31] and Australia.[32] PBSA is a water soluble filter, this makes it desirable for the use in cosmetic formulation and if used in association with lipophilic filters achieves a synergic increase of the sun protection factor. It is considered efficient to prevent erythema and safe, because few events are reported of skin irritation, sensitization phototoxicity or photoallergy.[38] Even though PBSA protects skin cells from UVB radiation and is considered photostable, studies have assessed that it can generate reactive oxygen species and cause photoinduced damage to DNA in vitro. PBSA was found to induce the production of singlet oxygen, as demonstrated by the generation of the diagnostic compound 4,8-dihydro-4-hydroxy-8-oxo-2′-deoxy-guanosine when it is irradiated with UVB or natural sunlight in oxygenated solution in the presence of 2′-deoxyguanosine, and to photoinduce the formation of alkali-labile cleavage sites in both single and double-stranded DNA. Although no phototoxic effects have yet been reported in vivo for PBSA, its proven capacity to generate singlet oxygen upon UV irradiation could be a threat of oxidative damage to adjacent skin tissue and to cell membranes, but nuclear DNA molecules would be at risk only if PBSA was able to enter the cells.[40,41]

HO O S O N N H

ENSULIZOLE
2-Phenylbenzimidazole-5-sulfonic acid

Figure 2.5 Ensulizole

- ✓ **Camphor derivatives** have a high molar extinction coefficient (> 20.000) and absorb in the UVB range within 290-300nm, they are considered rather photostable, because upon irradiation they undergo cis-trans photoisomerisation but it is reversible and the two isomer have similar spectra, then photoprotective ability doesn't change significantly.[42] In Europe six camphor derivatives are approved for the use in sunscreen formulation, instead Food and Drug administration has approved only Ecamsule (UVA absorber) and Enzacamene (4-methylbenzylidene camphor) is waiting for approval.[30,31]

ENZACAMENE
4-Methylbenzylidene camphor

Figure 2.6 Enzacamene

2.2.2 Organic UVA filters

UVA filters essentially absorb wavelengths between 320 and 400 nm; most of commercially available filters provide excellent protection against UVB but they are ineffective against UVA and there aren't on market as many molecules effective against UVA; moreover usually these filters absorb in UVA II region (320-340 nm) and only few molecules provide protection in the UVA I range (340-400 nm).[4] Within UVA filters are included different classes of molecules.

- ✓ **Benzophenones** (fig. 2.7) are aromatic ketones with a relative broad absorption profile that goes from 270 to 350 nm, hence they are active against UVB and UVA II rays. There are three benzophenones: Oxybenzone, Sulisobenzone, and Dioxybenzone, Oxybenzone mainly offers protection toward UVA II rays, it is the most commonly used although it presents some drawbacks as high incidence of contact photoallergic dermatitis, systemic absorption and it is also photounstable, initiating free radical production upon UV exposure.[31,43]

OXYBENZONE
(2-Hydroxy-4-methoxy-phenyl)-phenylmethanone

SULISOBENZONE
5-Benzoyl-4-hydroxy-2-methoxy-benzenesulfonic acid

DIOXYBENZONE
(2-Hydroxy-4-methoxy-phenyl)-(2-hydroxy-phenyl)-methanone

Figure 2.7 Benzophenones

- ✓ **Anthranilates** or *ortho*-aminobenzoates are among the oldest available UVA filters, they may absorb up to about 350 nm.[44] The absorption spectrum in the UVA region is principally due to the presence of two functional groups in *ortho*, which can easily

delocalize the electrons, but at the same time the *ortho* disubstitution is responsible of the low molar extinction coefficient of these filters. They are considered stable and safe compounds and in cosmetic formulations don't exhibit significant solvent shift effects. The filter most commonly used of this category is Meradimate (Methyl anthranilate), its lambda maximum is 340 nm in ethanol and it is mainly a UVA II filter.[45]

Figure 2.8 Meradimate

- ✓ **Dibenzoylmethanes** or substituted diketones are a relatively new class of UV filters; only one molecule of this group, Avobenzone (butyl methoxydibenzoylmethane) is permitted for use; in Europe was permitted also 4-isopropyldibenzoylmethane but in 1993 was withdrawn from the market because of high associated incidence of contact and photocontact dermatitis.[44] Avobenzone exhibits absorption properties resulting from *keto-enol* tautomerism, the two structural isomers have their own maximum absorption peak; the keto form absorbs in UVC range from 260 to 280 nm while the enol form absorbs in UVA range within 310 and 400 nm, with peak absorption around 360 nm. The relative amounts of the isomers are solvent dependent but usually in solution and in sunscreen formulations Avobenzone exists predominantly in the enol form and then it can provide good protection against UVA mainly in UVA I range. Unfortunately this filter is photounstable, upon irradiation photoisomerisation occurs from the enol to keto form with consequently absorption loss[46]; in one hour of sun exposure it undergoes 50-60%.photodegradation[37] Avobenzone is also able to generate free radicals after irradiation and in vitro studies proved that it can cause DNA strand breaks and oxidative protein damage.[46] As said above, the photoinstability of Avobenzone can affect the stability of the others filters present in the formulation leading to complete loss of activity of sunscreen formulation; as a consequence, greatly efforts have been made to stabilize this compound and on market there are stabilized

formulation where Avobenzone is formulated with other sunscreen ingredients such as Octocrylene and Oxybenzone with diethylhexyl 2,6- naphthalate (non-filter).[38]

AVOBENZONE
1-(4-*tert*-Butyl-phenyl)-3-(4-methoxy-phenyl)-propane-1,3-dione

Figure 2.9 Avobenzone

- ✓ **<u>Ecamsule</u>** (terephthalylidene dicamphor sulphoic acid) is a camphor derivative, a broad-spectrum UVA filter with an absorption profile ranging from 290 and 390 nm and peak absorption at 345 nm. It is photostable and has low systemic absorption, less then 1% of the compound passes through the horny layer. In keratinocytes irradiated with UV rays it prevents DNA breakage, pyrimidine dimer formation and p53 protein expression compared to unprotected cells and in clinical studies, Ecamsule have demonstrated to prevent photodermatoses, to block UV induced pigmentation and immunosuppression and to preserve skin elasticity slowing down photoaging.[47]

ECAMSULE
{3-[4-(7,7-Dimethyl-3-oxo-4-sulfomethyl-bicyclo[2.2.1] hept-2-ylidenemethyl)-benzylidene]-7,7-dimethy l-2-oxo-bicyclo[2.2.1]hept-1-yl}-methanesulfonic acid

Figure 2.10 Ecamsule

2.2.3 Organic broad-spectrum UV filters

An ideal UV filter should provide a widespread protection against damaging UV rays, however only few of available molecules are able to filter effectively both UVA and UVB rays. These filters are the new generation of UV absorbers and are permitted in Europe and Australia, while in the USA are waiting for approval from the FDA via the Time and Extent Application (TEA) process.

- ✓ **Methylene-bis-benzotriazolyl tetramethylbutylphenol,** known under the trade name of Tinosorb M (Ciba Specialty Chemicals, Basel, Switzerland), is a broad-spectrum UV filter with two absorption peaks at 303 nm and 360 nm. As a result of its molecular structure, that facilitates energy dissipation by intramolecular heat transfer and vibrational relaxation, it is a photostable molecule and helps also to stabilize other UV filters such as Avobenzone. The large size of the molecules minimizes the skin penetration and systemic absorption. It protects from UV rays by absorption, scattering and reflection because, although it is an organic filter, it behaves also like an inorganic one due to the micronized particles that are dispersed in the aqueous phase of sunscreen emulsions.[36,38,48]

TINOSORB M

Methylene-bis-benzotriazolyl tetramethylbutylphenol

Figure 2.11 Tinosorb M

- ✓ **Bis-ethylhexyloxyphenol methoxyphenyl triazine** or Tinosorb S (Ciba Specialty Chemicals, Basel, Switzerland) is an oil-soluble filter with an absorption spectrum that goes from 280 to 380 nm and two absorption peaks at 310 nm and 343 nm. By the presence of two hydroxyl groups in ortho position, which facilitate the return of the molecule to ground stable state by rapid energy release, Tinosorb S is endowed with great stability and then, like Tinosorb M, is used to stabilize other filters.[36,38]

TINOSORB S

Bis-ethylhexyloxyphenol methoxyphenyl triazine

Figure 2.12 Tinosorb S

- ✓ **Drometriazole trisiloxane** (Mexoryl XL, L'Oréal) belongs to the class of hydroxybenzotriazoles, its absorption spectrum covers both UVB and UVA rays with an absorption peak in the UVB range at 303 nm and the other in UVA range at 344 nm. Two structural groups constitute this molecule: the hydroxyphenylbenzotriazole group that provides wide range UV absorption and siloxane chain, which renders the molecule lipophilic. It is a photostable filter and rarely causes intollerances.[38,48]

MEXORYL XL

Drometrizole trisiloxane

Figure 2.13 Mexoryl XL

2.3 Evaluation of sunscreen formulations efficacy

An international standard to measure efficacy of sunscreen is the **Sun Protection Factor (SPF)** that is the value for a product, determined under solar simulated radiation, defined as the ratio of the Minimal Erythemal Dose (MED) on product protected skin to the Minimal Erythemal Dose on unprotected skin of the same subject. MED is defined as the lowest ultraviolet (UV) dose that produces the first perceptible unambiguous erythema with defined borders appearing over most of the field of UV exposure, 16 to 24 hours after

UV exposure. The amount of test product applied to the skin shall be 2 mg/cm^2 before spreading.[49]

$$\text{SPF} = \frac{MED \text{ product protected skin}}{MED \text{ unprotected skin}}$$

Since UVB is approximately 1000 times more erythemogenic then UVA, SPF value gives information on protection towards UVB and UVA II (320-340 nm) but not regarding UVA I (340-400 nm) and therefore SPF is of poor utility in understanding the UVA protection of a sunscreen. Because also UVA rays play a significant role in cellular damaging, it is important to establish, not only SPF value of a sunscreen formulation but, also the UVA protection to ensure a wide defence toward all UV radiation. Commonly used in vivo methods are IPD (immediate pigment darkening) and PPD (persistent pigment darkening), instead, for in vitro determination it is used the critical wavelength.

The **IPD** response occurs during UVA exposure, appears as a transient gray-brown pigmentation and fades within few minutes after the exposure is completed. The threshold dose for the IPD response is used with and without sunscreen protection to assess the UVA protection index; since pigmentation develops relative early after UVA exposure the test response is immediate but pigmentation disappears rapidly after irradiation leading to evaluation errors, along with wide individual variability response.[50]

PPD test is more used than IPD to verify UVA protection, it is also included in European Commission Recommendation to assess UVA protection. PPD is a skin response linearly dependent on the amount of UVA that enters the epidermis and the response is equally sensitive throughout the UVA range. The UVA protection factor of a product is calculated on the Minimal Persistent pigment darkening Dose of protected skin (MPDp) divided by that of unprotected skin (MPDu); MPDu and MPDp are defined as the quantity of radiant energy required to produce the first unambiguous pigmented reaction.

$$\text{UVA protection factor} = \frac{\text{MPDp}}{\text{MPDu}}$$

The test product is applied in amount of 2mg/cm^2, as for SPF test, and the UVA dose required to induce minimal pigmentation (MPD) is greater than 10 J/cm^2 (approximately 40 minutes of midday summer sunlight), thus also the stability of sunscreens is challenged during this test.[51a,b]

The **critical wavelength** method is an in vitro test based on the absorption spectrum of a sunscreen product, measured applying the formulation on a substrate; polymethylmethacrylate plates are a satisfactory substrate for this method because they are UVR-transparent, non-fluorescent, photostable and inert to all potential sunscreen formulation ingredients. The critical wavelength (λ_c) value of a product is defined as the wavelength at which the integral of the spectral absorbance curve reaches 90% of the area under the curve from 290 to 400 nm. It is important to note that the critical wavelength value is based on the inherent shape of the absorbance curve, not on its peak absorbance, therefore is independent by application thickness of the tested sunscreen. The products reaching a critical wavelength of 370 nm or greater are considered broad-spectrum sunscreens.[52,53]

The absorption spectrum measurement by means of UV spectrophotometer is also used to calculate the SPF in vitro.

2.3 Regulatory and labelling

Before became available on the market, sunscreen formulations are tested to determine the filtering capacity and then properly labelled, trying to give clear information on the product efficacy at the consumer; the competent authorities for UV filters define effectiveness tests and labelling, these rule may vary among countries. In USA the Food and Drug Administration (FDA) have recently published the latest guideline (June 2011) for testing and labelling UV filters, where it is given much attention to testing and labelling of UVA protection. In the past there were limited requirement and guideline for assessing UVA protection, and if a sunscreen formulation contained one or more UVA filter it was labelled as “broad spectrum”. In the current FDA rules, instead to measure UVA protection by means of persistent pigment darkening test, the in vitro critical wavelength is adopted as pass/fail test to evaluate the UVA or broad-spectrum protection. To pass the broad spectrum test the amount of UVA protection must increase as the SPF value and only products with a critical wavelength of 370 nm or greater can be labelled as “broad spectrum”. Only sunscreen products that pass the broad-spectrum test and have SPF of 15 or higher can include the statement: “decreases the risk of skin cancer and early skin aging caused by the sun.” Both in USA and in Europe the SPF is estimated by means of an in vivo method; European commission recommends an in vivo method, the persistent

pigment darkening test, also to assess the UVA protection factor in addition to critical wavelength method. In Europe a sunscreen product, to satisfy the minimum efficacy requirements, should provide a minimum UVB and UVA protection and when the SPF increase also the UVA protection should increase; the ratio of UVA protection, measured by persistent pigment darkening test, should be at least 1/3 of the SPF; moreover to ensure a broad protection it is recommended a critical wavelength at least of 370 nm. In USA it is not required in vivo tests method to evaluate the UVA protection, as FDA have identified disadvantages in the persistent pigment darkening test such are: the skin darkening, measured in this test, is mainly due to UVA II (320-340 nm), human subjects are exposed to high dose of UVA radiation, it is expensive and time consuming, exposure to UVA radiation alone does not occur in nature, the method is poor reproducible and also the interpretation of pigmentation can lead to valuation errors.

Regarding labelling, the SPF number have been reduced in recent years to facilitate the comparison between different products, in USA values are expressed in multiples of 5 and about UVA protection, FDA recommends that products, providing UVB and UVA protection, are labelled as broad spectrum and no mention of the word UVA is allowed. In Europe the sunscreens are also divided within categories of protection according to SPF (see Table 2.1) and to attest the UVA protection all sunscreens have to display a simple logo: the letters “UVA” printed in a circular shape.[54,55,56]

Labelled category	*Labelled SPF*	*Measured SPF*	*Transmission of UVR (%) responsible for erythema*	*Recommended minimum UVA protection factor*	*Recommended minimum critical wavelength*	*UVA logo to show regulatory conformance*
No protection	≤ 6	≤ 6	17-100	1/3 of labelled protection factor	370 nm	UVA
Low protection	6	6-9.9	10-17			
	10	10-14.9	7-10			
Medium protection	15	15-19.9	5-7			
	20	20-24.9	4-5			
High protection	25	25-29.9	3-4			
	30	30-49.9	2-3			
Very high protection	50	50-59.9	2			
	50 +	60 ≤	< 2			

Table 2.1 European recommendation 2006

2.4 Sunscreens efficacy and safety

Sunscreens provide effective defence by decreasing the amount of UV radiation to which biomolecules are exposed and therefore preventing both acute and chronic damages due to UV rays. The first visible benefit is the prevention of sunburns but they are useful also to prevent more detrimental consequences; indeed in vitro and in vivo studies have demonstrated that sunscreens protect against UV-induced photoaging, immunosuppression and mutations and then decrease the risk of developing melanoma and non-melanoma skin cancer.[57]

The first step in developing a new sunscreen molecule is to assess the UV filtering capacity and if the molecule has a good absorption profile is then evaluated for the photostability; these assays are performed by means of instrumental analyses (UV spectrophotometer, solar simulator and HPLC analyses). When a molecule display interesting filtering profile and photostability, before becoming available on the market, it undergoes safety and efficacy testing that are very rigorous and comparable to those of dermatological drugs. The safety dossier for a new UV filter includes in vitro as well as in vivo investigation of their potential to produce local toxicity, such are irritation, sensitization, photo-toxicity, photo-genotoxicity, as well as systemic toxicity, such as long-term toxicity, reproductive toxicity, carcinogenicity and photo-carcinogenicity. The first level of safety evaluation consists exclusively of in vitro tests (i.e. MTT test), in order to exclude cytotoxicity on human and/or murine fibroblasts or keratinocytes and then, if they are non cytotoxic, genotoxicity assay, photo-toxicity and photo-genotoxicity challenges, irradiating the cell in presence of the new substance are performed. A new sunscreen should not only be devoid of genotoxic activity, but also demonstrate its potential to protect cells from the genotoxic activity of UV radiation. Another aspect to be verified is the percutaneous absorption of the filtering molecule, because it should remain on the surface of the skin, where it is most effective. Ideally, a sunscreen should impregnate the stratum corneum creating a barrier against UV radiation, but not penetrate into the underlying tissue; UV filters that deeply penetrate the skin are of little value, since they would leave the skin unprotected. Penetration test are performed previously in vitro by means of diffusion cells (Franz cells) using skin of pig, human reconstructed epidermis or human epidermis deriving from surgical interventions. After in vitro studies, the product is tested on animals to determine NOAEL (Non Observable Adverse Effects Levels) for oral or dermal administration in sub-chronic or chronic toxicity studies. If, after these tests, a product is considered

harmless it goes to safety and efficacy trials on humans; these investigations includes confirming studies for absence of skin irritation, photo-irritation, skin sensitization, and photosensitization. Also trials for human systemic exposure dose are required, after topical application of an ultraviolet filter in a typical sunscreen formulation.[58,59]

Although sunscreens available on the market have passed the above-mentioned clinical trials, it doesn't mean that they are completely free of adverse effects or relating problems. They may induce adverse effects such as irritant, allergic contact reactions, photoallergy, and phototoxic effects; contact and photocontact sensitivity to sunscreens has been reviewed for filters such as PABA, padimate O, enzacamene, octinoxate, and ensulizole. Maybe the most important problem to resolve, regarding sunscreen products, is the photostability; during UV exposure some of these molecules may change spectral performance or act as photo-oxidants via generation of free radicals and reactive oxygen species or may degrade producing toxic by-products. Moreover photounstable products give a false sense of safety, because photoprotection is guaranteed only when the UV filters remain stable throughout the entire period of exposure to sunlight, but in labelling the sunscreens there are no informations regarding the photostability of the product. Some filtering molecule currently used suffers of photostability problems that can be overcome adding to the formulation photostable sunscreens or stabilizing agents.

Another undesirable effects is the sunscreens penetration in the underlying tissues, since they can reach the circulatory system.[60,61]

Sunscreen in some cases can display same adverse effects but they offer undeniable protection against dangerous UV rays and the benefit or potential risk in using UV filtering molecules is weighted against the hazard of skin exposure to a carcinogen.

3. Free radicals and antioxidants

The skin, acting as a barrier toward the environment, is the most exposed organ to external agents, including ulraviolet rays. As above mentioned, detrimental effects of UV radiation is also due to production of reactive oxygen species, which are responsible of early ageing, inflammatory disorders, immunosuppression and skin cancers; for these reason free radicals and antioxidants are significant topics in photoprotection.

3.1 Free radical and other oxidant species

Free radicals are very reactive species, capable of independent existence that contain one or more unpaired electrons in the outermost orbital; the energetic situation is responsible for highly instability and short half-life of these substances. To gain stability, free radicals react with surrounding molecules subtracting electrons to form an electrons pair; in this way also the injured molecule becomes a free radical beginning a chain reaction. Usually radical reactions can be divided into three processes; the first is *initiation*, when occurs radical formation; the second is *propagation*, with increase of radicals production because of chain reaction; the last one is *termination*, the radical number decrease because two radicals combine or dismutation occurs with following formation of less reactive species. Human organism is subjected to the action of different kinds of oxidant species known as ROS (Reactive Oxygen Species) an RNS (Reactive Nitrogen Species), which comprise radicals and non-radical species within there are: superoxide anion radical ($O_2^{-}\cdot$), singlet oxygen ($^1O_2$), hydroxyl radical ($OH\cdot$), hydrogen peroxide (H_2O_2), lipid peroxyl radical ($LOO\cdot$), nitric oxide radical ($NO\cdot$), nitrogen dioxide radical ($NO_2\cdot$). Under normal condition, these species are present in small quantity during cellular activity, such as cellular respiration, apoptosis and immune response by macrophages and neutrophils to kill pathogens; unfortunately, in same situations take place an imbalance because of

overproduction of reactive species. Oxidative stress in human body can be enhanced by pathologies or external factors, among which there are:

- ✓ Pathologies as rheumatoid arthritis, cardiovascular diseases, neurodegenerative diseases (Parkinson and Alzheimer) and inflammation in general;
- ✓ Ischemia and reperfusion injury;
- ✓ Excess of transition metals, as iron and copper that promote the production of hydroxyl radical;
- ✓ UV rays and X rays, because of photooxidation;
- ✓ Drugs;
- ✓ Smoke;
- ✓ Alcohol abuse;
- ✓ Environmental pollution;
- ✓ Intense physical exercise, because increases oxygen consumption ;
- ✓ Diet too rich in proteins or saturated fats.[62,63]

3.2 Cellular targets of oxidative stress

At the base of free radicals damages there are biomolecules disruption; the essential cellular constituents impaired are nucleic acids, proteins, lipids and carbohydrates.

3.2.1 Lipids

Oxidative action regarding lipids can initiate a radical chain reaction, the lipoperoxidation that lead mainly to destruction of polyunsaturated fatty acids, which are components also of phospholipids of cellular membrane. Lipoperoxidation proceeds through the three steps; a hydroxyl radical initiate the process taking a hydrogen atom from a saturated carbon of the alchylic chain of the fatty acid, this becomes a radical too (L•) and stabilize its structure through formation of conjugated diene, which are within the first detectable product of lipid peroxidation. In aerobic condition a fatty acid with an unpaired electron reacts with an oxygen molecules to generate a lipoperoxyl radical (LOO•), a very reactive species that can undergo two different kinds of reaction.[64] For molecules like arachidonic and eicosapentanoic acid, peroxylic group can do cyclization producing a cyclic lipoperoxyde that by fragmentation gives aliphatic chain with two carbonyl groups, producing highly reactive compounds such as malondialdehyde (MDA).[65] This molecule

can react with free amine moieties of proteins, phospholipids or nucleic acid forming covalent bonds as Schiff bases; crosslinks proteins-MDA-phospholipid, protein-MDA-protein or phospholipid-MDA-phospholipid cause loss of membrane fluidity.[66]

Another kind of reaction occurs when lipoperoxyl radical (LOO•) takes a hydrogen atom from close fatty acid, producing a hydroperoxide lipid and promoting lipid peroxidation.

Aldehydic products, originating from lipid peroxidation, can also react with functional groups of the proteins, as sulfhydryl moieties causing break of peptidic bonds or formation of disulphide intramolecular bonds; in this way can be inactivated proteins essential for the cell life.

In the last phase of lipoperoxidation take place the formation of stable compounds because of reaction between two radical species or by means of chain breaking substances; these events terminate the radical chain reaction.

3.2.2 Nucleic acids

About cellular injuries caused by reactive oxygen species, damages to DNA are the most harmful. Almost all altered molecules of DNA are replaced and repaired, however this safeguard system can fail and if modified DNA molecules were not corrected, the mutation could be transmitted during replication to daughter cells. Most commonly oxidative alterations of DNA are related to oxygen inclusion in double bond of DNA bases and sugar removing from bases, intermolecular DNA-DNA or DNA-protein binding.[67] Pyrimidines (cytosine and thymine) are most sensitive than purines and can undergo saturation, ring open and therefore loss of aromaticity and planarity with DNA filaments distortion; photocatalized reactions of thymine produce thymine dimers. These DNA transformations are responsible for mutagenesis[68], carcinogenesis[69] and play a role also in aging,[70] diabetes mellitus,[71] inflammatory diseases and liver diseases[72].

3.2.3 Proteins

Damages produced by free radicals to proteins can be distinguish in reversible and irreversible; among reversible there are oxidation of thiolic groups and of methionine, whereas between irreversible there are ring's break of histidine and tryptophan and peptide bond hydrolyzation in presence of proline; the last event affects particularly the collagen because it is rich in proline and hydroxyproline. Oxidation of sulfhydryl moieties of the cysteine to thiyl radical can lead to dimerization or oxidation to sulphide dioxide group;

these changes are responsible for deficiencies in protein structure and function. The most damaged proteins are enzymes, as phosphofructokinases and complex I of mitochondrial respiratory chain that are of fundamental importance for energy production in the cells.[73] Decrease in available energy is responsible for biosynthesis reduction of new macromolecules (proteins, nucleic acids, and phospholipids), causing slowness in repairing processes of cellular constituents.

Oxidation of membrane proteins contribute, beside lipid peroxidation, to decrease the fluidity of cellular membranes that cause reduced activity of enzyme and receptor proteins, changes in permeability and decrease in activity of ATP-ase pump for calcium transport. This last event results in increase of intracellular calcium (Ca^{2+}) and potassium (K^+) that leads to phospholipases actiovation A2, A1, C, D and diglycerides lipases; these are enzymes that increase membrane catabolism and then releasing of easily oxidable fatty acids. Proteins oxidation seems involved in pathologies such as atherosclerosis, ischemia-reperfusion injuries and aging.[74,75]

3.2.4 Carbohydrates

Free radicals react quickly with carbohydrates removing hydrogen atoms; deoxyribose, ribose, proteoglycans and etheropolysaccharide (ialuronic acid) can undergo oxidative degradation. Proteoglycans, high weight molecules of parenchymal tissue, are particularly affected by oxidation and then are fragmented with consequent structural and functional irreversible damages.[75]

3.3 Antioxidants

Antioxidants are substances able to prevent or terminate oxidative stress and then following impairments to biomolecules. To prevent detrimental effects of oxidation, living organisms have different kind of antioxidants that are both endogenous and exogenous substances; the latest are especially helpful when endogenous defence are no more able to counteract oxidative reactions.

On a pharmacological point of view, they can be classified in: enzymatic, preventive and chain-breaking antioxidants, these substances differ for their action mechanism and for distribution in the organism.[62]

The increasing importance of antioxidants is due to the preventive actions that they exert against initiation of pathologies as cancer, inflammatory diseases and cardio-circulatory disorder.[76]

3.3.1 Enzymatic antioxidants

The principal antioxidant enzymes are superoxide dismutase (SOD), catalase and glutathione peroxidase, they are intracellular antioxidant defences.[65]

SOD accelerates about a thousand times the dismutation speed of superoxide anion; this reaction avoids the simultaneous presence of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide H_2O_2, since these two molecules can react producing the hydroxyl radical an highly reactive species that lives only 2 nanoseconds and has a diffusion ray of 2 nanometres.

$$\textbf{SOD} \quad 2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Catalase enzyme promotes the production of water and oxygen from peroxide hydrogen.

$$\textbf{Catalase} \quad 2H_2O_2 \longrightarrow 2H_2O + O_2$$

Glutathione peroxidase is an enzyme containing selenium; it removes hydrogen peroxide and other peroxides thanks to the reducing capacity of glutathione (GSH), a tripeptide formed by glutamic acid, cysteine and glycine.[62]

$$\textbf{Glutathione peroxidase} \quad H_2O_2 + 2GS\text{-}H \longrightarrow 2H_2O + GS\text{-}SG$$

3.3.2 Preventive antioxidants

Iron and copper have a key function in the production of oxidant species, since they are catalysts in reactions, such as Fenton and Haber-Weiss reactions, that lead to the production of the hydroxyl radical (OH•), a very dangerous species.[64]

$$\textbf{Fenton reaction} \quad Fe^{2+} + H_2O_2 \longrightarrow OH\bullet + OH^- + Fe^{3+}$$

$$\textbf{Haber-Weiss reaction} \quad O_2^{\cdot-} + H_2O_2 \longrightarrow OH\bullet + OH^- + O_2$$

Usually transition metals in the organism are bonded with proteins, free metals present consequently to cellular turnover or tissue lesions have to be quickly chelate to prevent the redox activity. Some plasmatic proteins like transferrin, lactoferrin and ceruloplasmin are

preventive antioxidants and have the task of chelating and carrying metals. Also other substances are able to chelate metals, such as polyphenols[77] or deferoxamine, a drug with high chelating capacity used in thalassemia treatment.[64]

3.3.3 Chain-breaking antioxidants

The chain-breaking or radical scavenging antioxidants sacrifice themselves reacting with free radicals before that biomolecules were damaged. The removal of an electron from the antioxidant transforms this molecule in a radical too, however, this event doesn't results in a radical chain reaction because the oxidation product can stabilize its structure delocalizing the electrons and then it is not enough active to initiate a chain reaction.
Radical scavenging antioxidants can be divided in hydrosoluble as ascorbic acid and liposoluble as tocopherol and carotenoids.
Vitamin C or L-ascorbic acid is the main aqueous phase antioxidant in the body and one of the first defence lines against oxidative damages in plasma. It acts by stepwise donation of electrons; the subtraction of one electron leads to formation of ascorbate free radical that is a stable radical and can still works as free radical scavenger donating a second electron. The last resulting oxidation product is dehydroascorbic acid that can be regenerated by dehydroascorbate reductase.[62] Virtually all plants and animals synthesize L-ascorbic acid, human are an exception because they have lost this ability as result of mutation that had lead to function loss of L-gulono-y-lactone oxidase and then humans must take L-ascorbic acid through diet.[78]

Figure 3.1 L-ascorbic acid

Vitamin E is the main antioxidant in lipid phase, with this term is indicated a family of molecules that can be divided into two groups: tocopherols and tocotrienols. The molecules consist of a hydrophobic phytyl chain and a chroman head; the tocopherols have saturated tails whereas tocotrienols have three isolated double bonds in their phytyl chain. Both tocotrienols and tocopherols consist of four isomers α-, β-, γ- and δ-, which differ from one another for the chroman ring that varies in number of methyl substituents and for substituted positions of the phenolic ring; α-tocopherol is considered the most active form.

All stereoisomers have a hydroxyl group on chroman ring in the same position, which is responsible for the antioxidant activity whereas the phytyl chain doesn't seem to have any antioxidant activity but it is thought to be important for proper positioning within bio-membranes. Vitamin E loses hydrogen radical from the hydroxyl group at the 6-position in the chromanol nucleus and it is further oxidized to dopaquinone that is devoid of antioxidant activity. One of the main functions of vitamin E is to prevent lipid peroxidation and then protect polyunsaturated fatty acid and low-density lipoproteins (LDL). In the organism, when vitamin E is in its oxidized form can be regenerated to its reduced form by L-ascorbic acid.[79,80]

α-tocoferolo

Figure 3.2 α-tocopherol

Polyphenols are most abundant and ubiquitous secondary metabolites of higher plants, they are mainly in present in fruits, vegetables, wine, tea, olive oil and chocolate, then people take them with diet.[81] Within polyphenols are included heterogeneous compounds that differ for the complexity of molecular structure and for the number of phenolic hydroxyl moieties, which influence the antioxidant capacity; their high antioxidant power is provided by radical scavenging activity and by metal-chelating potential.[82] They are subdivided by the structure into groups:

1) the phenolic acids with the subclasses derived from hydroxy-benzoic acids such as gallic acid and from hydroxy-cinnamic acid, containing caffeic, ferulic, and coumaric acid;
2) the large flavonoid subclass, which includes the flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols;
3) the stilbenes within there are resveratrol;
4) the lignans and the polymeric lignins.[81]

The antioxidant activity of phenolic acids is proportional to the number of hydroxyl groups in the molecule and hydroxy-cinnamic acids are more effective than hydroxy-benzoic acid, because the electron-withdrawing properties of the carboxylate group in benzoic acids has a negative influence on the H-donating abilities of the hydroxy-benzoic acids.

Caffeic acid   Gallic acid

Figure 3.3 Phenolic acid

Also the flavonoids activity is influenced by hydroxyls position and numbers; particularly important is the 3-hydroxyl group in the C ring, since the glycosylation or removal of this hydroxyl group causes a decrease in antioxidant capacity. Contributes to increase the activity also the presence of two adjacent hydroxyl moieties in ortho arrangement in B ring. Important for the antioxidant activity is also the unsaturation in the C ring that allows electrons delocalization across the molecule and then improves stabilization of generated radical following the action as radical scavenger of the flavonoids. [83]

Figure 3.4 Quercetin (flavonoid)

Polyphenols have many other biological effects that include antibacterial, anti-inflammatory, anti-allergic, antiviral, antineoplastic, antithrombotic and hepato-protective activities, these actions seem to be due to their antioxidant capacity.[84]

3.4 Antioxidants in skin photoprotection

The skin has its own antioxidant defences, which include enzymatic and non-enzymatic antioxidants, to deal with UV-induced oxidative stress; however, excessive and chronic exposure to UV radiation can overcome the cutaneous antioxidant capacity, leading to oxidative stress and then oxidative damage that may result in skin disorders, immunosuppression, premature skin aging and development of melanoma and non-melanoma skin cancers.

Exposure to UV rays influences endogenous antioxidant enzyme levels; after a single low or moderate dose of UV radiation, cultured fibroblasts show an initial decrease in

antioxidant enzyme transcript levels, which lasts for day before enzymes (superoxide dismutase, glutathione peroxidase and catalase) return to basal level. Also antioxidant chain-breaking are depleted and without antioxidant defence the potential damaging effects of UV rays increase leading to an oxidative stress situation, most of all if UV exposures are repeated in a short time period.[85] When endogenous antioxidants undercome by the oxidative stress, can be helpful to use antioxidant supplements. Several studies demonstrate that antioxidant supplements can reduce damages due to UV rays and following reactive oxygen species production. Usually antioxidants are present in sunscreen products; principally there are antioxidants scavenger such as vitamin E, vitamin C and polyphenols.

Ascorbic acid has been shown to prevent erythema and sunburn cell formation after UV exposure, in addition it is widely used as a depigmentation agent due to its inhibitory action on tyrosinase and it is essential for collagen biosynthesis and may inhibit elastin biosynthesis and could, therefore, be useful for reducing the increased elastin accumulation that occurs in photoaged skin.[86] One problem related to ascorbic acid is its high instability, indeed in topical formulation are used derivatives of ascorbic acid (e.g. magnesium ascorbyl-phosphate and ascorbyl-6-palmitate), however, antioxidants properties of derivatives are lower then parent molecule. Also topical application of α-tocopherol has demonstrated a number of protective effects including reduction in erythema, photoaging, photocarcinogenesis, and immunosuppression. Stabilized forms of α-tocopherol, where hydroxyl group on the ring is esterified, include α-tocopheryl acetate and α-tocopheryl succinate but both derivatives are less effective than α-tocopherol in protecting the skin against UV-induced erythema and have lower antioxidant power.[87]

Polyphenols have good antioxidant capacities and display also other activity such as anti-inflammatory and anticarcinogenic effects. Cinnammic acid such as ferulic acid and caffeic acid, applied topically, can protect against UVB-induced erythema in vivo and in vitro.[88] Particularly studied are green tea polyphenols that have demonstrated to reduce UV-induced erythema and sunburn cells formation in human skin, when they are associated with UV filters.[89] Furthermore, experimental data from animal models suggest that green tea polyphenols may reduce the incidence of sunlight-related skin cancer; oral administration at mice of these polyphenols in drinking water resulted in significant protection against UV-induced skin carcinogenesis in terms of tumor incidence, multiplicity and tumor size, compared to mice that were not given green tea polyphenols in

drinking water.[90] Usually, topical antioxidants are inefficient UV filters; therefore, they are used in combination with sunscreens to enhance their protective efficacy. Polyphenols seems also effective in protecting UV filtering molecules from degradation; in example quercetin protects butyl methoxydibenzoylmethane and octyl methoxycinnamate from degradation that proceeds through the initial formation of free radicals and singlet oxygen.[91]

4. Aim

UV radiation that reaches the earth surface is very dangerous and is responsible of detrimental effect such as erythema, photoaging, immunosuppression and skin cancers (paragraph 1.3). The skin is the organ most exposed and affected by UV rays, which can cause direct but also indirect damages to biomolecules. Direct damages are due to absorption of UV radiation by the biomolecules, whereas indirect injuries are caused by production of reactive oxygen species. Since detrimental effects of UV rays are also triggered by over-production of reactive oxygen species, it is important to protect the skin reducing the UV radiation that reaches epidermis and dermis but also trying to scavenge the reactive oxygen species. To this aim, our interest was in developing new compounds provided with both UV filtering and antioxidant capabilities.

To proof the principle an extensive screening of commercial available molecules is necessary in order to start from already active molecules and improve their scavenging and/or filtering activity. Among the screened molecules one of the best candidates was 2-Phenyl-1H-benzimidazole-5-sulphonic acid (PBSA), selected as lead compound for its lack of antioxidant activity, high water solubility and good safety profile.[38] This molecule, commonly used in commercial sunscreen, provides a good protection against UVB rays but lacks of filtering capacity regarding UVA radiation and, not only, it is also devoid of antioxidant activity and, furthermore, it seems to induce the production of reactive oxygen species following irradiation, thus resulting potentially harmfull.[40,41]

Figure 4.1 2-Phenyl-1H-benzimidazole-5-sulphonic acid **(**PBSA)

With the aim to achieve antioxidant activity, maintaining the filtering capacity, PBSA was modified introducing phenolic hydroxyls on the phenyl ring and also substituting the

functional group in position 5 of the benzimidazolic ring to evaluate the influence of this moiety on filtering and antioxidant capabilities. To assess the antioxidant power of the molecules in vitro tests were performed: DPPH, PCL and FRAP assays; then it was necessary incorporate the molecule in a standard cosmetic formulation to evaluate the UV filtering capacity in vitro and also to verify the antioxidant power of the finished formulation. The new molecules have also undergone toxicity and phototoxicity studies to exclude adverse effects of the new products.

Another important issue, concerning UV filters, is the photostability; it is fundamental for photoprotection that a filtering molecule remains active during exposure to sunlight; similar UV filters, available on market, unfortunately suffer of evident photoinstability. To evaluate the photostability of the new molecules, cosmetic formulations containing the study compounds were exposed to solar simulated radiation. Since the aim of the work was production of UV filtering molecules provided with antioxidant capacity, only the molecules that show both good filtering activity and antioxidant capacity and that were devoid of cytotoxicity and phototoxicity were analysed, for photostability. Cosmetic formulations of the best compounds were then tested by accelerated stability studies.

5. Design and Synthesis

In this phase of work it was considered to modify the lead compound 2-Phenyl-1H-benzimidazole-5-sulfonic acid (PBSA) to realize molecules with good antioxidant capacity, but at the same time maintaining the filtering activity. To this purpose, the phenyl ring was modified, introducing hydroxyl functional groups, in different position of the ring, to enhance the antioxidant capacity, also the functional group in position 5 of the benzimidazole ring was changed in order to evaluate the influence of the sulfonic acid moiety or other functional groups on filtering capacity. Three groups of molecules can be identified, which differ for the substituent present in position 5 on the benzimidazole ring. The changes regarding the phenyl ring are the same in the three groups and within a group the compounds differ one from another for the position and the number of hydroxyl moieties on the phenyl ring.

The first group of molecules synthesized maintains the sulfonic acid moiety in position 5 of the benzimidazole ring, whereas the second group shows no functional group on the benzimidazole ring and the third group presents, instead of the sulfonic acid, the carboxylic acid moiety.

The molecules used for the synthesis are shown in scheme 1.

Scheme 1

3,4-Diamino-benzenesulfonic acid | o-Phenylenediamine | 3,4-Diaminobenzoic acid

2-Hydroxybenzaldehyde | 4-Hydroxybenzaldehyde | 2,4-Dihydroxybenzaldehyde

3,4-Dihydroxybenzaldehyde | 2,3,4-Trihydroxybenzaldehyde

The desired molecules were synthesized following procedures described in literature, fitting reaction conditions to improve the reaction yield.

Aldehydes, 3,4-diaminobenzoic acid and o-phenylenediamine were purchased whereas 3,4-diaminobenzenesulfonic acid was synthesized by o-phenylenediamine and sulphuric acid 96% (scheme 2).[92]

Scheme 2

o-Phenylenediamine → (i; 137 °C, 24 h) → 3,4-Diaminobenzenesulfonic acid, sulfate salt **1**

i: H_2SO_4

Different reaction conditions were evaluated to obtain the desired products in good yields and using cheap synthetic procedures. The synthetic procedure described in literature was firstly investigated, the aldehyde was refluxed in DMF with 3,4-diaminobenzenesulfonic acid in presence p-toluene sulfonic acid to catalyze the reaction[93] but this procedure provides very low yield and also, when the reaction was carried out in acetonitrile at room temperature, using HCl and H_2O_2 as oxidant system[94] does not give product in acceptable quantity. Finally it was used sodium bisulfite solution ($NaHSO_3$) to improve the reaction between benzaldehyde and 3,4-diaminobenzenesulfonic acid; the reactions performed in ethanol refluxed for 24 hours[95] give desired products in good yield and purification was simple, by precipitating the product of interest under acidic conditions using hydrochloric acid solution, 1N, and filtering the suspension.

The molecules obtained are summarized in scheme 3

Scheme 3

The same reaction strategy was adopted to synthesize the 2-phenyl-1H-benzimidazole derivatives (scheme 4) and the 2-phenyl-1H-benzimidazole-5-carboxylic acid derivatives (scheme 5), using respectively o-phenylenediamine and 3,4-diaminobenzoic acid, in place of 3,4-diaminobenzenesulfonic acid.

Scheme 4

NH_2 NH_2 + O H R — i, 80 °C → N N H R

o-Phenylenediamina

R = (OH) **7**

R = (OH, OH) **8**

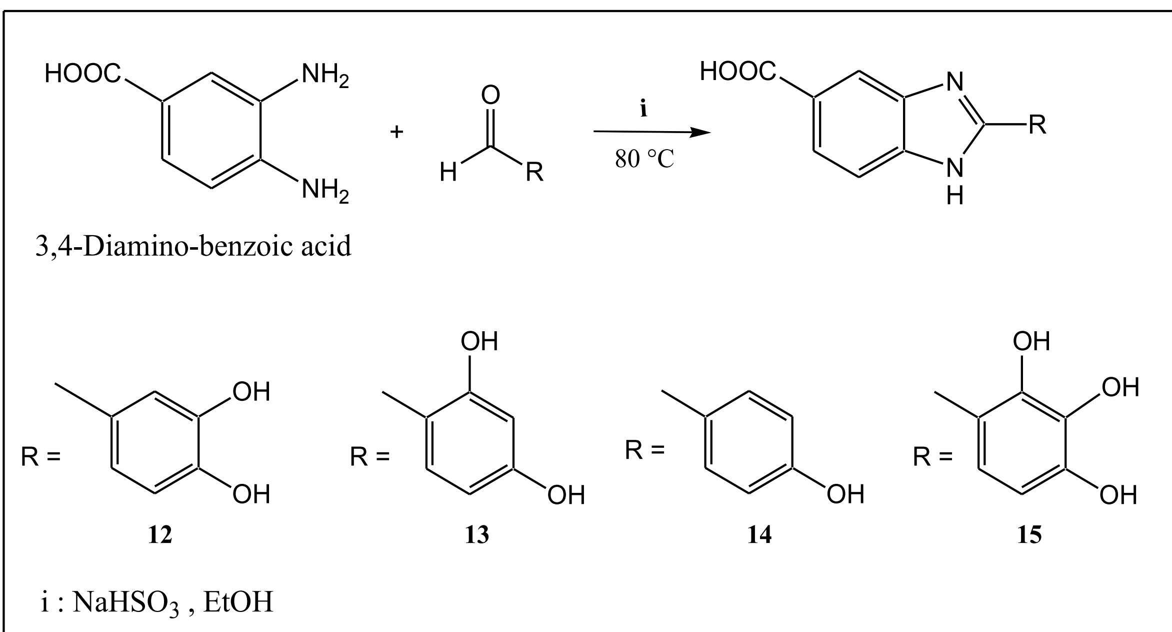
R = (OH, OH) **9**

R = (OH) **10**

R = (OH, OH, OH) **11**

i : $NaHSO_3$, EtOH

Scheme 5



6. Antioxidant measurements

The reactive oxygen species and free radicals display different reactivity and then a compound can be active against an oxidant species and less effective in scavenging another; therefore the synthesized molecules were evaluated by different antioxidant in vitro assays to verify the antioxidant capacity of the molecules against different oxidant species and free radicals, the parent compound PBSA (2-Phenyl-1H-benzimidazole-5-sulfonic acid) was also tested to verify the lack of antioxidant activity.
The molecules were evaluated by means of the following assays: DPPH (1,1-diphenyl-2-picryl-hydrazyl radical), PCL (photochemiluminescence), FRAP (Ferric Reducing Antioxidant Power).

6.1 PCL Test

Photochemiluminescence (PCL) assay joints the photochemical generation of free radicals with the selective detection by using chemiluminescence. The PCL is based on the photo-induced autoxidation of luminol, mediated from the superoxide anion radical ($O^{2\cdot-}$), which is accompanied by intense chemiluminescence; luminol works as photosensitiser as well as detection reagent of oxygen radicals. Antioxidants act as radical traps, in presence of antioxidant molecules the chemiluminescence decrease proportionally with the activity and the concentration of the antioxidant product. It is a sensitive assay, able to measure the scavenging activity of antioxidants against the superoxide radical in the nanomolar range and in few minutes, reducing the activity lost due to the degradation.
PCL is suitable to measure the radical scavenging properties of single antioxidants as well as more complex systems and the assay can be conducted by two different protocols, ACW (Antioxidant Capacity of Water-soluble substance) and ACL (Antioxidant Capacity of Lipid-soluble substance), which permit the measurement, respectively, of the antioxidant capacity in water-soluble and lipid-soluble phase. In the ACL method Trolox (bioactive portion of vitamin E) is used as standard, whereas for the ACW method the standard

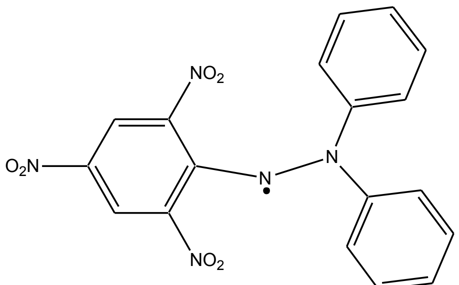
molecule is the ascorbic acid.[96,97,98] The products antioxidant capacity was evaluated employing the ACL method and the results are expressed in micromoles of Trolox per millimoles of sample.

Trolox

Figure 6.1 Trolox

6.2 DPPH Test

DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical is a stable nitrogen-centred free radical, characterized by absorption maximum at 517 nm that decreases in the presence of H-donor molecules. This radical is characterized by a deep purple colour that fades in the presence of an antioxidant agent; the absorbance decrease, produced by reduced DPPH radical, is used to evaluate free radical scavenging capacity of pure compounds or complex mixture. The antioxidant ability of a product is calculated by measuring the inhibition ratio of initial concentration of DPPH radical at 517 nm, after reaction of the radical solution with a solution of antioxidant products. The antioxidant activity of the tested molecules is expressed in micromoles of Trolox per millimoles of sample.[99]



1,1-diphenyl-2-picryl-hydrazyl radical

Figure 6.2 DPPH radical

6.3 FRAP Test

This method was developed by Benzie and Strain to measure plasma antioxidant power;[100] later it was employed also to analyse the antioxidant power of molecules and extracts. FRAP (Ferric Reducing Antioxidant Power) assay is based on the reduction in acid condition of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in presence of TPTZ (2,4,6-tripyridyl-s-triazine). In presence of an antioxidant the ferric-tripyridyltriazine complex is reduced to ferrous complex, the chemical reaction of FRAP method is the following:

$$Fe(TPTZ)_2(III) + ArOH \longrightarrow Fe(TPTZ)_2(II) + ArOH^{\cdot +}$$

The complex with Fe^{3+} show a yellowish colour; when an antioxidant solution is added to the iron (FeIII) complex solution it develops a blue colour due to reduction at ferrous ion, which in complexed form with TPTZ give absorption maximum at 593 nm. Absorbance increase, measured at lambda maxima, is proportional to reducing power of the antioxidant present in solution.[101] Also in this test, the standard used for the calibration curve is Trolox and then results are given as micromoles of Trolox per millimoles of sample.

6.4 Results

The results of the antioxidant assays are presented in table 6.1; the molecules tested are compared for the antioxidant capacity in three different antioxidant assays. The lead compounds PBSA was test too; in DPPH and PCL analyses the activity of PBSA is not evaluable because comparable to values of blank. Only in FRAP assay PBSA presents a minimal activity, but not significative,, therefore it could be confirmed the antioxidant activity lack of this product.

The antioxidant power of the new synthesized molecules varies according to the number and position of hydroxyl moieties on the phenyl ring; changing hydroxyls on the ring changes the order of magnitude of antioxidant capacity, whereas the functional group in position 5 of benzimidazolic ring influences only slightly the antioxidant activity.

Compounds with only one phenolic hydroxyl, in position 2 (molecules **5** and **10**) or 4 (molecules **2**, **7**, **14**) on the phenyl ring, as expected, has the lowest antioxidant capacity in all performed assays.

Table 6.1 Antioxidant assays

Product	**DPPH** **µmolTrolox/mmol** **(P≤0.05)**	**FRAP** **(µmolTrolox/mmol)** **(P≤0.05)**	**PCL** **(µmolTrolox/mmol)** **(P≤0.05)**
PBSA	< LOQ*	0,79 ± 0,06	< LOQ*
2	2,04 ± 0,15	12,23 ± 0,25	11,36 ± 0,08
3	2145,31 ± 45,8	2707,29 ± 29,52	22838 ± 836,26
4	2,03 ± 0,09	16,22 ± 0,89	2,06 ± 0,05
5	21,41 ± 0,43	19,87 ± 0,22	159,1 ± 4,3
6	1362,12 ± 133,96	2713,3 ± 45,17	1924,35 ± 101,82
7	1,67 ± 0,08	21,94 ± 0,12	9,66 ± 0,04
8	1974,58 ± 16,89	2663,21± 32,53	19190,6 ± 443,18
9	16,47 ± 0,54	37,3 ± 0,35	198,53 ± 2,65
10	0,87 ± 0,05	23,04 ± 0,09	10,93 ± 0,05
11	1811,02 ± 61,7	2723,19 ± 35,74	1614,675 ± 19,95
12	1771,82 ± 84,75	2433,28 ± 21,74	13174,31± 240,68
13	32,48 ± 2,38	31,63 ± 1,96	160,26 ± 4,09
14	1,185 ± 0,02	2,62 ± 0,06	6,98 ± 0,27
15	1241,03 ± 9,43	2355,15 ± 52,55	1515,65 ± 75,56

LOQ* limit of quantification

The antioxidant activity increased adding another hydroxyl; products with two phenolic hydroxyls in position 2 and 4 on the phenyl (molecules **5**, **9** and **13**) show activity improvement, but best result are obtained with molecules **3**, **8**, **12** that have hydroxyl moieties in position 3 and 4 of the phenyl ring. The gap, in antioxidant power, of molecules that differ for the position of the two phenolic hydroxyls is very high in all performed tests. Compounds with three hydroxyls on phenyl ring (products **6**, **11**, and **15**) do not demonstrate further improvement in antioxidant activity compared to molecules with two hydroxyls in position 3 and 4 of the phenyl ring; on the contrary, the results for DPPH test are lower then those of molecule **3**, **8** and **12** and significant decrease in antioxidant activity is shown in PCL assay. In FRAP analysis compounds with three hydroxyl moieties give results comparable to that of molecules with hydroxyl in position 3 and 4. Considering the three assays the molecule with better antioxidant profile are those

with two phenolic hydroxyls in position 3 and 4; in all performed tests the compound **3** has the highest antioxidant capacity, followed by compound **8** and **12**. For products **6**, **11** and **15** with three phenolic moieties and compounds **5**, **9** and **13** with two hydroxyls in position 2 and 4 on the phenyl ring, the antioxidant capacity is decreased probably because of hydrogen bond formation between nitrogen of benzimidazole and the phenolic hydroxyl in position 2 on the phenyl ring.

7. UV spectra measurement

Before proceeding with inclusion of the synthesised molecules in a cosmetic formulation, it was measured the UV spectrum of the compounds, because SPF is correlated with the UV spectrum absorption. By UV spectrum it was determined the wavelength of absorption maximum (λ_{max}) and then, using solutions at different concentrations, it was calculated the molar extinction coefficient (ε) of every products.

The UV spectrum of the molecules was analysed in aqueous solution and at controlled pH that was maintained around 7; this value was selected because at pH lower than 7 the lead compound PBSA (2-Phenyl-1H-benzimidazole-5-sulfonic acid) and the new molecules tend to precipitate and on the other hand, while basic conditions are favourable for compounds dissolution, they will not fit with requirement for cosmetic formulation because the skin has acid pH values. Considering that solvent and pH value are responsible for UV spectrum shift and then for UV filtering parameters variation, influencing also the stability of the compounds,[3] the substances were measured at pH 7 because is the same adopted in preparation of cosmetic formulation and is a good compromise between cosmetic tolerability and molecules solubilisation.

The UV spectra were recorded between 270 and 420 nm (20 nm higher and lower then UVA and UVB range) to verify the spectrum profile of the molecules within UVA and UVB region.

In figure 7.1 is shown the UV spectrum of PBSA, it is an UVB filter and presents a λ_{max} of 302 nm, after this peak the absorbance fades going to higher wavelength until almost zero around 325 nm then it provides no absorption in the UVA region.

In figure 7.2, 7.3 and 7.4 are shown the molecules spectra overlapped for category of molecules according to the substituent in position 5 of the benzimidazole ring; in all figures is present the PBSA spectrum for comparison.

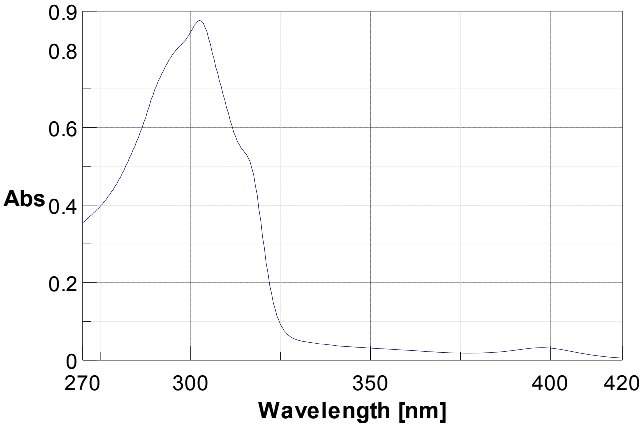


Figure 7.1 PBSA UV spectrum

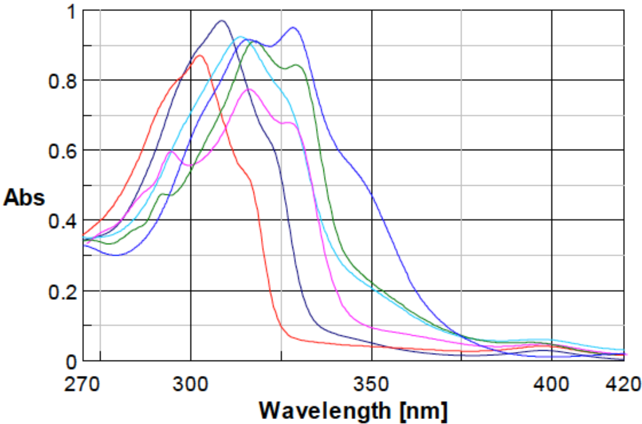


Figure 7.2 Comparison between spectra of PBSA and molecules with sulfonic acid moiety on benzimidazolic ring: **2, 3, 4, 5, 6.**

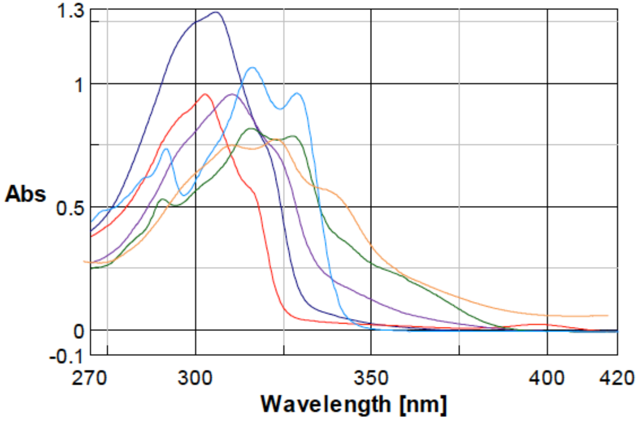


Figure 7.3 Comparison between spectra of PBSA and molecules with no moiety on benzimidazolic ring: 7, 8, 9, 10, 11.

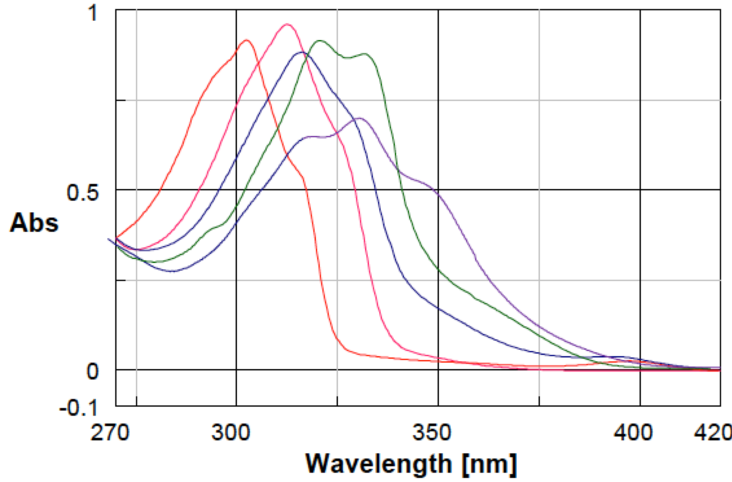


Figure 7.4 Comparison between spectra of PBSA and molecules with carboxylic acid moiety on benzimidazolic ring: PBSA, 12, 13, 14, 15

By observation of UV spectra appears that the lambda maxima (table 7.1) of the new molecules shifts toward higher wavelength and in general the range of absorption curve is wider then PBSA. Within a group of molecules the batochromic shift is more marked for the molecule that have an hydroxyl moiety in position 2 of the phenyl ring (products **4**, **5**, **6**, **9**, **10**, **11**, **13** and **29**); the effect of ortho substituted is known to produce this shift toward higher wave length.[3] Increasing the number of auxochrome groups on phenyl ring an increasing in the absorption range occurs, the products with three hydroxyl on the phenyl ring (**6**, **11**, **15**) have broadest spectrum and their lambda maxima shift at highest wavelength compared to the other compounds. The absorption spectrum of molecules with the same number and position of hydroxyl seems only slightly influenced by the substituent in position 5 of the benzimidazole ring.

After having established the lambda maxima for every compound, solutions at different concentrations were prepared to calculate the molar extinction coefficient by means of linear regression and applying Lambert-Beer equation.

$$A = \varepsilon \cdot c \cdot d$$

A = sample assorbance

ε = molar extintion coefficient

c = concentration (mol/l)

d = light travel (cm)

Table 7.1 Values of λ max and ε

	λ max (nm)	**ε**
PBSA	302	25000
2	308	25000
3	313	23000
4	315	21000
5	318	23000
6	328	22200
7	306	24000
8	311	16000
9	315	14000
10	315	17000
11	325	12000
12	317	21000
13	321	20000
14	312	24000
15	332	17000

Extinction coefficients of new molecule have values nearby the lead compounds, excepted molecules **8**, **9**, **10**, **11** (benzimidazole derivatives) and **15** that have lower molar extinction coefficient.

8. Cosmetic formulation evaluation

The synthesised molecules were included at the concentration of 3% in a cosmetic formulation, to prove the effective filtering capacity and the antioxidant activity of finished formulation. At this purpose the compounds were included in standard formulation oil in water (table 8.1), lacking of antioxidant and filtering activity. The antioxidant capacity of emulsion was evaluated by means of photochemiluminescence assay and the filtering activity was verified by an in vitro method too; before proceeding it was tested the standard emulsion to confirm the absence of antioxidant and filtering capabilities and to exclude the influence of the formulation on antioxidant and filtering power. It was not possible to analyse the compound **7** in cosmetic formulation because it causes emulsion instability.

Table 8.1 Cosmetic formulation O/W.

Ingredients	% p/p
PHASE I	**58.5**
Aqua	52.8
Glycerin	5.0
Phenoxyethanol, Methylparaben, Ethylparaben, Butylparaben, Propylparaben, Isobutylparaben	0.7
PHASE II	**20.5**
Cetyl alcohol, Glyceryl stearate, PEG-75 stearate, Ceteth-20, Steareth-20	6.0
Cetyl alcohol	2.0
Dimethicone	0.5
C12-15 Alkyl Benzoate	6.0
Cocoglycerides	6.0
PHASE III	**21.0**
Active ingredient	3.0
Aqua	15.0
NaOH solution 10%	6.0
TOTAL	**100.0**

8.1 Analysis of filtering parameters

The cosmetic formulations containing the active ingredients were tested by the in vitro method of Diffey and Robson,[102] with the purpose to verify the filtering activity of the new molecules in comparison with the lead compound. This method is based on the measure of spectral transmission of ultraviolet radiation, with and without the sunscreen applied, through an irregular substrate that simulates the skin surface and permits transmission of UV radiation. The spectral monochromatic transmittance is measured, by means of UV spectrophotometer, in the range between 290nm and 400nm and is given by the ratio of the radiation transmitted by the sample to the radiation transmitted by the substrate, spectral monochromatic transmittance (T(λ)) is given by the following relation:

$$T(\lambda) = \frac{Ss(\lambda)}{So(\lambda)}$$

Where:

- Ss(λ) is the transmittance of the substrate with sunscreen applied;
- So(λ) is the substrate transmittance at the wavelength λ.

Measuring every 0,5 nm the transmittance values within UVB and UVA range (290-400 nm), the Sun Protection Factor (SPF) is given by the equation:

$$SPF = \frac{\sum_{290}^{400} E(\lambda)B(\lambda)}{\sum_{290}^{400} E(\lambda)B(\lambda)T(\lambda)}$$

- E(λ) is spectral irradiance of terrestrial sunlight that represents the midday midsummer sunlight for Southern Europe (latitude 40° Nord, solar zenith angle 20°, ozone layer 0,305 cm);
- B(λ) is the relative effectiveness of UVR at wavelength λ (nm) in producing delayed erythema in human skin.

This in vitro test simulates the condition used in SPF in vivo method, both for the applied quantity of sunscreen on substrate (2mg/cm^2) and for the interaction with the substrate. The test is useful to predict SPF in vivo and to screening the potential filtering activity of new molecules that cannot be tested by in vivo method because of lacking of more data concerning safety.

In vitro SPF determination allow also to gain information about the homogeneity of the formulation regarding the filters dispersion and, furthermore SPF values, can be determined also UVA protection factor and UVA/UVB ratio.

Measuring the UV spectrum absorbance of the formulation is also possible to calculate the critical wavelength (see paragraph 2.3) of sunscreen product using the following equation:

$$R = \frac{\int_{290nm}^{\lambda c} A\lambda d\lambda}{\int_{290nm}^{400nm} A\lambda d\lambda}$$

The critical wavelength (λc) is the first value of λc for which R results equal to 0,9.

In table 8.2 are listed the SPF, UVA protection factor (UVAPF), UVA/UVB ratio and critical wavelength of new molecules and PBSA. The standard formulation was also analysed by this method and didn't exhibit any filtering activity.

Table 8.2 Filtering parameters

Formualtion	**SPF** (P ≤ 0.05)	**UVA/UVB** (P ≤ 0.05)	**UVAPF** (P ≤ 0.05)	**λc nm**
PBSA	6.29 ± 1.09	0.31 ± 0.05	1.97 ± 0.25	333
2	6.44 ± 0.15	0.61 ± 0.01	2.57 ± 0.07	346
3	13.36 ± 2.24	0.88 ± 0.04	6.88 ± 1.33	358
4	3.02 ± 0.43	1.14 ± 0.01	2.0 ± 0.23	355
5	2.96 ± 0.64	1.35 ± 0.01	2.3 ± 0.41	370
6	8.14 ± 0.12	1.06 ± 0.03	5.15 ± 0.08	383
8	7.07 ± 1.20	0.93 ± 0.02	3.63 ± 0.50	368
9	4.85 ± 0.73	1.31 ± 0.05	3.58 ± 0.50	380
10	2.67 ± 0.44	1.20 ± 0.02	2.0 ± 0.30	378
11	3.88 ± 0.32	1.07 ± 0.01	2.55 ± 0.1	381
12	9.82 ± 1.30	1.21 ± 0.04	5.75 ± 0.78	370
13	3.88 ± 0.77	1.51 ± 0.02	3.0 ± 0.52	368
14	8.17 ± 2.13	0.83 ± 0.05	3.87 ± 0.91	350
15	5.02 ± 1.4	1.15 ± 0.08	3.55 ± 0.88	382

Evaluating the filtering parameters of new molecules it can be observed that all new molecules have improved UVA filtering parameters, since UVA protection factor, UVA/UVB ratio and critical wavelength are higher than those of lead compound. The molecules with a phenolic hydroxyl in ortho position on the phenyl ring (compound **4**, **5**, **9**, **10** and **13**) have the lower SPF value than parent compound but they have better UVA/UVB ratio and also and higher critical wavelength. Within this group is interesting the molecule **9** that shows a SPF of almost 5 and has a critical wavelength of 380 nm. Formulation containing products **2** and **14**, that have a phenolic hydroxyl in para position on phenyl ring, have higher SPF values, greater UVA protection factor, critical wavelength and also higher UVA/UVB ratio than PBSA but the last two parameters are lower compared to the molecules with a phenolic hydroxyl in ortho on the phenyl ring. Interesting are compounds **3**, **8** and **12**, with hydroxyls in position 3 and 4, which show high SPF values, the best is achieved by product **3** with a SPF double than PBSA. This product shows also great UVA protection factor, UVA/UVB ratio and critical wavelength than PBSA. Molecules **8** and **12** have lower SPF than compound **3**, but higher than PBSA; in comparison to **3**, however, they present higher UVA/UVB ratio and critical wavelength. Regarding compound with three hydroxyls on phenyl ring, they have good UVA filtering parameters, their critical wavelengths are the highest and SPF is higher then PBSA for molecules **6**, while SPF of **15** is slightly lower and decreases further in molecule **11**. Also UV filtering parameters are principally influenced by the number and position of hydroxyl groups on phenyl ring, rather than the substituent in position 5 on benzimidazole ring.

8.2 Antioxidant efficacy of cosmetic formulations

The cosmetic formulations were evaluated also for antioxidant activity by means of PCL test, following the ACL method as for the pure compounds (paragraph 6.1). Besides antioxidant capacity of the single molecules, its is important also to verify the effective antioxidant ability of finished cosmetic formulation, to evaluate the activity of functional ingredient at the concentration employed in the formulation and also to exclude the activity loss because of interaction with the ingredients of the emulsion. The antioxidant power is given in micromoles of Trolox per gram of cosmetic formulation, results are shown in table 8.3; the standard formulation tested with this method did not display any antioxidant activity and as formulation with PBSA.

Table 8.3 Antioxidant activity of formulations

Formulation	**PCL μmol Trolox/g formulation (P ≤ 0.05)**
PBSA	<LOQ
2	0,33 ± 0,03
3	2212,09 ± 68,35
4	0,06 ± 0,02
5	0,95 ± 0,02
6	101,98 ± 1,08
8	2972,107 ± 145,63
9	8,66 ± 0,04
10	0,5 ± 0,02
11	153,35 ± 8,14
12	1373,39 ± 19,43
13	4,97 ± 0,17
14	0,16 ± 0,02
15	86,99 ± 6,95

Results of the antioxidant analysis on the finished formulations correspond to that obtained testing the pure compounds and then formulations containing compounds with only one phenolic hydroxyl (**2**, **4**, **10** and **14**) have poor antioxidant capacity, which slightly increase for formulation having compounds with two phenolic hydroxyl in position 2 and 4 on the phenyl ring (products **5**, **9** and **13**). Formulations with molecules **3**, **8** and **12** present highest antioxidant capabilities; these are followed, but with important decrease in antioxidant capacity, by formulation with products **6**, **8** and **15** that have three hydroxyl moieties on phenyl ring.

9. Cytotoxicity and Phototoxicity tests

To evaluate the safety profile of the new molecules were performed, firstly, cytotoxicity assays to exclude negative effects of the products on cell viability and then phototoxicity tests under UVA and UVB radiation; because filtering molecules cannot show toxicity following irradiation but, on the contrary, they should provide photoprotection.
Since sunscreen products are applied on skin, for testing we used a specific cell line of human keratinocytes (NCTC-2544) to evaluate both cytotoxicity and phototoxity. The evaluation of cell viability, in presence of the filtering molecules, without irradiation and after irradiation was checked by MTT test; this is a colorimetric assay that assesses the cell survival ratio by evaluation of the enzyme activity. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) is a yellow dye that can be absorbed by viable cell and reduced by mitochondrial dehydrogenases enzymes to formazan, producing insoluble purple crystals.

MTT $\xrightarrow{\text{mitochondrial dehydrogenases}}$ MTT formazan

Figure 9.1 MTT reduction

Formazan crystals are solubilized adding a solution of hydrochloric acid in isopropanol, after complete dissolution it is read the absorbance by means of a spectrophotometer, the absorbance is proportional to cell viability since only living cell can produce formazane.[103]
Survival cells ratio is given by:

$$\%\ \text{cell survival} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \cdot 100$$

$Abs_{control}$ represents 100% of survival and it is the absorbance of cell not treated with substance in analysis and not irradiated; Abs_{sample} is the absorbance of cell in contact with substance and for phototoxixity test is the absorbance of cell irradiated and in contact with substance in analysis.

The cytotoxicity was checked by MTT test after 72 hours from the incubation of the keratinocytes with compounds, moreover, the same experiments were performed in the presence of the parent UVB filter PBSA. In table 9.1 are displayed cytotoxicity test results, expressed in IC50 (μM), which is the concentration required to inhibit 50% of cellular growth.

Table 9.1 Cytotoxicity test results

Compounds	IC_{50} (μM)
PBSA	> 50
2	26.4 ± 2.7
3	> 50
4	> 50
5	> 50
6	23.1 ± 2.9
7	26.1 ± 2.4
8	> 50
9	> 50
10	> 50
11	> 50
12	> 50
13	> 50
14	> 50
15	> 50

Most compounds did not show cytotoxicity at the employed concentrations in cell line of human keratinocytes and their IC50 is greater then 50 μM, instead compounds **2**, **6** and **7** have IC50 value in the range between 20 and 30 μM, particularly compound **6** has the lowest IC50 and then is the most cytotoxic of tested compounds.

After cytotoxicity the molecules were verified for photocitotoxicity in the same cell line; the cells were treated with 50 μM solutions of compound and after 30 minutes were irradiated with 20 J/cm^2 UVA or with UVB at two energy amounts: 0.5 J/cm^2 and 1 J/cm^2 UVB. The compounds **2**, **6** and **7** were used at concentration of 20 μM to avoid the antiproliferative effect of these compounds. After irradiation, the solution was replaced with grow medium and the cells were further incubated for 48 hours, then cell viability was assessed by MTT test. Results are presented in table 9.2 and are indicated as percentages of cell survival in comparison with non-irradiated cells (100% cell survival). The product phototoxicity is compared to cell survival ratio of keratinocytes irradiated without any substances (IC = irradiated control).

Table 9.2 Phototoxicity test results

	Compound concentration μM	% CELL SURVIVAL		
		UVA 20 J/cm^2	UVB 0.5 J/cm^2	UVB 1 J/cm^2
IC		69.1 ± 3.2 %	63.1 ± 3.2 %	33.8 ± 3.1 %
PBSA	50	74.5 ± 1.4 %	75.2 ± 4.5 %	64.2 ± 3.1 %
2	20	77.3 ± 2.8 %	78.0 ± 3.5 %	46.7 ± 3.4 %
3	50	77.4 ± 4.0 %	71.5 ± 2.5 %	58.1 ± 2.6 %
4	50	76.9 ± 2.0 %	74.3 ± 3.2 %	37.7 ± 1.7 %
5	50	79.0 ± 6.0 %	70.8 ± 3.9 %	38.6 ± 3.7 %
6	20	18.4 ± 2.3 %	59.3 ± 3.4 %	29.7 ± 2.1 %
7	20	72.8 ± 2.9 %	74.2 ± 1.3 %	44.0 ± 4.1 %
8	50	62.9 ± 4.3 %	72.8 ± 1.0 %	43.9 ± 3.4 %
9	50	3.7 ± 0.5 %	1.1 ± 0.3 %	0.5 ± 0.1 %
10	50	4.8 ± 0.6 %	19.8 ± 2.4 %	3.5 ± 0.3 %
11	50	1.2 ± 0.2 %	45.6 ± 2.6 %	27.0 ± 2.4 %
12	50	74.3 ± 2.0 %	76.3 ± 3.6 %	64.5 ± 3.5 %
13	50	80.7 ± 1.3 %	69.0 ± 0.2 %	57.5 ± 2.4 %
14	50	76.7 ± 3.4 %	74.2 ± 3.4 %	58.4 ± 2.5 %
15	50	12.2 ± 1.3 %	60.1 ± 2.3 %	25.6 ± 2.7%

Both UVA and UVB radiation induced a clear reduction in cell survival of irradiated control; UVA radiation caused a decrease of about 30%, while the lower dose of UVB of about 35% and the higher UVB dose of 65%. When irradiation was carried out in presence of the compounds, some different changes in cell survival were detected; molecules **6**, **9**, **10**, **11** and **15** seemed to have a phototoxic effect as cell survival decrease considerably in

comparison to irradiated control and then these compounds are not suitable as photoprotective agents. All the other compounds, comprising PBSA, increase cell survival after UVA and UVB irradiation in comparison with irradiated control. A great increase in cell viability it is observed after UVB irradiation (1 J/cm^2), particularly, in presence of PBSA and compounds **3**, **12**, **13** and **14** the cell survival is almost double in relation to the control.

Regarding the phototoxicity test the most interesting compounds are **3**, **12**, **13** and **14** that increase cell survival both after UVA and UVB irradiation, therefore these substances seems suitable as filtering molecules.

10. Photostability and Stability studies

Products showing the best features, in term of antioxidants and filtering capabilities, and that demonstrated to be devoid of cytotoxicity and phototoxicity were assayed for stability and photostability in cosmetic formulation. The best candidates, which have all the above-mentioned characteristics, were compounds **3**, **8** and **12**. Photostability and stability studies were performed in the same cosmetic formulation used for the other tests and the active ingredient was at concentration of 3% as for the other assays.

10.1 Photostability studies

Photostability is an important requirement for filtering molecules, since degradation of the filter in sunscreen formulations lead to photoprotection loss and consequently damage to skin, because UV rays can reach skin in large amount and also because photodegradation products can be toxic.[60]

Before proceeding with photostability studies, it was determined the real concentration of filtering molecules in the formulation by HPLC analysis.

A portion of cosmetic formulation containing molecule **3**, **8**, **12** or lead compound PBSA was transferred in a beaker to achieve an amount of sunscreen formulation of $2mg/cm^2$, the same used to test the filtering parameters. The samples were placed under solar simulator device for 1 hour, after exposure the beaker was removed and the irradiated formulation were transferred in a calibrated flask with methanol and the concentration of tested molecules was quantified by HPLC. The photodegradation degree was determined by comparison of areas between irradiated sample and corresponding amount of non-irradiated sample. In table 10.1 are shown analyses results of molecules **3**, **8**, **12** achieved by four independent experiments, results are given as ratio of remaining molecule.

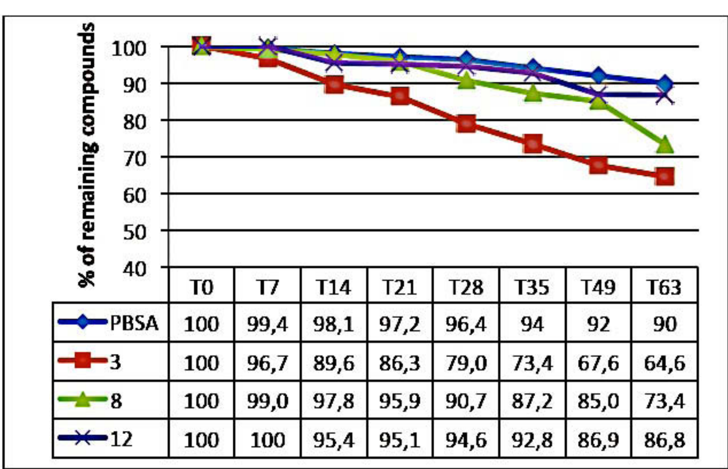
Table 10.1 Remaining amount of products after solar simulated irradiation.

Product	**Residual product (P≤0.05)**
PBSA	96,7% ± 1,8%
(UV3) 3	94,9% ± 0,8%
(UV20) 8	92,9% ± 2,6%
(UV14) 12	98,4 % ± 0,9%

After irradiation procedure the residual amount of new molecules doesn't diverge much from lead compound PBSA; compound **3** degrades a little more than PBSA, whereas product **8** shows a higher degradation rate that, however, is low than 10%. The best result is achieved with compound **12** that exhibits the lowest degradation rate, less than 2% and therefore has better photostability than lead compound.

10.2 Stability studies

Molecules **3**, **8**, and **12** are challenged in accelerated stability studies, in comparison to parent compound. Cosmetic formulations containing the compounds were analysed by HPLC to determine the real concentration of the active molecules before storing the formulations in sealed vessels and put them into oven at 40°C. The formulations were checked at appropriate time intervals; aliquots were taken from the emulsions and dissolved in methanol. In figure 10.1 are displayed results of stability studies.



	T0	T7	T14	T21	T28	T35	T49	T63
PBSA	100	99,4	98,1	97,2	96,4	94	92	90
3	100	96,7	89,6	86,3	79,0	73,4	67,6	64,6
8	100	99,0	97,8	95,9	90,7	87,2	85,0	73,4
12	100	100	95,4	95,1	94,6	92,8	86,9	86,8

Figure 10.1 Stability studies of formulations

In accelerated stability studies the lead compound PBSA shows higher stability than its derivatives; the compound that degrades most quickly is product **3**, followed by product **8**. Within new molecules compound **12** is provided with highest stability and its degradation progression doesn't differ much from the degradation curve of PBSA. It can be expected that molecules with higher antioxidant power could undergo degradation more rapidly than molecules without antioxidant capacity, because of their higher intrinsic reactivity.

11. Conclusions

The finding that harmful effects of UV rays are not only due to direct damages, but also to indirect damages, caused by over-production of radical species, has highlighted the importance of providing a complete photoprotection by screening UV radiation but also by scavenging reactive oxygen species.
Growing interest in sunscreen products, able to ensure photoprotection but also provided with radical scavenging activity, had led research toward production of molecules provided with both sunscreen and antioxidant capabilities. To this purpose, among the newly synthesized molecules, the most interesting were compound **3**, **8** and **12**, that show a good filtering activity and also high antioxidant power, that is maintained in cosmetic formulation. Generally all the new products have improved antioxidant capacity than lead compound. In addiction to antioxidant activity, the introduction of auxochroms on the phenyl ring of PBSA have shifted UV spectrum absorption toward longer wavelengths, indeed new compounds show a better UVA/UVB ratio and higher critical wavelength values then PBSA. UVA/UVB ratio is notably improved in compounds that have a hydroxyl moiety in position two on the phenyl ring, but these molecules exhibit also lower SPF than other compounds, just because of the presence of hydroxyl in ortho position that gives hydrogen bond with nitrogen of benzimidazole ring. Compounds that have hydroxyl in para position instead have a higher SPF than PBSA and also better UVA filtering capacity, but UVA/UVB ratio is lower than that of compounds with an auxochrom in ortho position. The molecules with three hydroxyl moieties on phenyl ring show absorption spectrum widening; considering also the lambda critical values they can be considered broadband UV absorber. Unfortunately, they have antioxidant capacity lower than molecule with two hydroxyl groups in position 3 and 4 on the phenyl ring (compounds **3**, **8** and **12)** and, most of all, these compounds (**6**, **11** and **15**) exhibit phototoxic activity on human keratinocytes; molecule **6** is also cytotoxic in concentration range between 20-30 μM. Because of phototoxicity molecules **6**, **11** and **15** cannot be used as UV filters.

Table 11.1 Filtering and antioxidant capabilities of cosmetic formulations

Cosmetic formualtion	**SPF (P≤0.05)**	**UVA/UVB (P≤0.05)**	**UVAPF (P≤0.05)**	**λc nm**	**PCL μmolTrolox/g formulation (P≤0.05)**
PBSA	6.2 ±1.09	0.31±0.05	1.97±0.25	333	<LOQ*
2	6.44±0.15	0.61±0.01	2.57±0.07	346	0.33±0.03
3	13.36±2.24	0.88±0.04	6.88±1.33	358	2212.09±68.35
4	3.02±0.43	1.14 ± 0.01	2.0±0.23	355	0.06±0.02
5	2.96±0.64	1.35±0.01	2.3±0.41	370	0.95±0.02
6	8.14±0.12	1.06±0.03	5.15±0.08	383	101.98±1.08
(UV20) 8	7.07±1.20	0.93±0.02	3.6±0.50	368	2972.107±14.63
9	4.85±0.73	1.31±0.05	3.58±0.50	380	8.66±0.04
10	2.67±0.44	1.20±0.02	2.0±0.30	378	0.5±0.02
11	3.88±0.32	1.07±0.01	2.55±0.1	381	153.35±8.14

12	9.82±1.30	1.21±0.04	5.75±0.78	370	1373.39±19.43
13	3.88±0.77	1.51±0.02	3.0±0.52	368	4.97±0.17
14	8.17±2.13	0.83±0.05	3.87±0.91	350	0.16±0.02
15	5.02±1.4	1.15±0.08	3.55±0.88	382	86.99±6.95

LOQ* limit of quantification

Molecules **3**, **8** and **12** satisfy the requirements for goodfiltering and antioxidant activity; they are also devoid of cytotoxic and phototoxic effects and, in particular, products **3** and **12** exhibit good photoprotection activity against UVA and UVB radiations, indeed, after irradiation in presence of these compounds it is observed an increase in cellular survival rate.

These three compounds finally were testes also for stability and photostabilty; outcomes of accelerated stability studies suggest that they are less stable than parent compound, this result can be explained by the higher reactivity of molecules provided with high antioxidant power, however, stability of compound **12** does not differ much from PBSA. In photostability studies, under solar simulated condition, products **8** seems the less photostable but its degradation rate is, however, lower than 10%. The best results are achieved by compound **3**, which degrades only slightly more than PBSA, and most of all with product **12** that shows higher photostability than lead compound with a degradation rate lower than 2%.

The substituent in position 5 on benzimidazole ring does not exert significant effect on antioxidant power, which is mainly controlled by phenolic hydroxyls, but differences are observed in stability, photostability, cytotoxicity and phototoxicity of molecules **3**, **8** and **12**, which differ only for the substituent in position 5 on benzimidazole ring. Carboxylic acid seems to provide highest stability, photostability and cell photoprotection; instead, compound **8**, without substituent in position 5 on benzimidazole ring, has the lowest

photostability and provides less photoprotection to irradiated keratinocytes than compound **3** and **12**.

The best UV filter candidates provided with antioxidant activities are compound **3**, **8** and **12**, that are still under study to verify the safety profile. Taking into account all results, i.e., in addiction to filtering and antioxidant capabilities, also cytotoxicity, phototoxicity,stability and photostabilty, the molecule that satisfy all requirement is compound **12.**

12. Materials and Methods

12.1 General

Reactants, solvents and standard samples were purchased from Sigma-Aldrich, Milan, Italy. Reaction course was routinely monitored by thin-layer chromatography on pre-coated silica gel plates (Macherey-Nagel Durasil-25) by detection under a 254-nm UV lamp and/or by spraying the plates with 1% $FeCl_3$ solution in water and using as eluent dichloromethane/methanol (90:10) or butanol/ water/ acetic acid (60:20:20).

The molecular weights of the compounds were determined by ESI (Micromass ZMD 2000), and the values are expressed as [MH]+.

^{1}H-NMR spectra were determined in *d6*-DMSO and recorded on VXR-200 Varian spectrometer and Mercury Plus-400. Chemical shifts are expressed in parts per million (δ) relative to the TMS internal standard.

UV spectrophotometric analyses were carried out on a UV-VIS spectrophotometer (ThermoSpectronic Helios γ, Cambridge, UK).

12.2 Antioxidant Analyses

Photochemiluminescence (PCL) assay was performer by means of *Photochem*® apparatus using PCL Kits purchased by Analytik-Jena AG (Jena, Germany). Production of superoxide anion radicals is sensitized from luminol after exposure to UV light lamp (Double Bore® phosphorus lamp, output 351 nm, 3 mWatt/cm2). The antioxidant activity was measured using ACL method (Antioxidant Capacity of Liposoluble).

A 2.30 mL portion of reagent 1 (HPLC-grade methanol), 0.2 mL of reagent 2 (buffer solution), 25 μL of reagent 3 (photosensitizer), and from 5 μL to 25 μL of standard trolox solution 0.1mM (to obtain the calibration curve) or sample solution proper diluted were mixed and measured, during a time of 130 seconds, by means of Photochem®. The antioxidant capacity of the sample is calculated by comparison with a Trolox standard

curve. The areas under the reaction curves were calculated using the PCLsoft control and analysis software; at greater concentrations of Trolox working solutions correspond a marked reduction in the magnitude of the PCL signal and hence a reduction of the area under the curve. This inhibition was used as a parameter for quantification of antioxidant capacity.

DPPH (1,1-diphenyl-2-picrylhydrazyl) test. To 1.5 mL DPPH methanolic solution 0.5 mM was added 0.750 mL of sample solution proper diluted. Samples absorbance measurements were evaluated with a UV-VIS spectrophotometer (ThermoSpectronic Helios γ, Cambridge, UK) at fixed wavelength of 517 nm. Blank sample was prepared adding methanol to DPPH solution and Trolox was used as standard reference to achieve a calibration curve. The radical-scavenging activity is expressed as inhibition ratio of initial concentration of DPPH radical and is calculated according to the formula: Inhibition percentage (Ip) = $[(A_B-A_S)/A_B] \cdot 100$; where A_B and A_S are, respectively, the absorbance values of blank reaction and of the tested sample.

FRAP method (Ferric Reducing/Antioxidant Power). The reagent for analysis was freshly prepared by mixing the following solutions in the reported ratio 10/1/1 (v:v:v) i) 0.1 M acetate buffer pH 3.6, ii) TPTZ 10 mmol/L in 40 mmol/ HCl, iii) ferric chloride 20 mmol/L. To a 1.9 mL of reagent were added 0.1 mL of sample proper diluted or solvent when blank was performed. Readings at fixed wavelength of the absorption maximum (593 nm) were done after 30 min, using a UV-VIS spectrophotometer; it was evaluated the absorbance increase of sample solution against the absorbance of blank reaction as parameter to calculate the antioxidant activity. The antioxidant activity is given as Trolox activity since this standard was used to perform the calibration curves.

12.3 Evaluation of filtering parameters

Sun protection factor evaluation was performed by means of UV-VIS spectrophotometer (ThermoSpectronic Helios γ, Cambridge, UK) and analytic program Spectra Analysis (Jasco Europe). Test formulation was applied in amount of $2 mg/cm^2$ on Transpore tape, a support transparent to ultraviolet radiations and able to simulate the porosity and texture of human skin, and then the measure of UV transmittance was carried out. The cosmetic

product does not need any preliminary treatment; the sample is applied on the support surface and a glove finger it was used to spread the product and obtain a uniform layer. Before proceeding with the analysis it was recorded the baseline of the support and the UV-VIS spectrophotometer was checked measuring the SPF factor of COLIPA (Cosmetic Europe) standard cosmetic formulation that has to give SPF value between 15 and 18. In table 12.1 is shown composition of COLIPA (Cosmetic Europe) standard cosmetic formulation.

Table 12.1 COLIPA standard formulation

INCI	%
Phase I	**57,6500**
Aqua	53,5700
Phenylbenzimidazole sulfonic acid	2,7800
Aqua, Sodium hydroxide	0,9000
Methylparaben	0,3000
Disodium EDTA	0,1000
Phase II	**21,7500**
Cetearyl alcohol, PEG-40 castor oil, Sodium cetearyl sulfate	3,1500
Decyl oleate	15,0000
Ethylhexyl methoxycinnamate	3,0000
Butyl methoxydibenzoylmethane	0,5000
Propylparaben	0,1000
Phase III	**20,6000**
Aqua	20,0000
Carbomer	0,3000
Aqua, Sodium hydroxide	0,3000
Total	**100,0000**

12.4 Cytotoxicity and Phototoxicity tests

Cytotoxicity and phototoxicity tests are performed in Padova University by research team of Professor Vedaldi Daniela.

Cellular culture. An immortalized, non-tumorigenic cell line of human keratinocytes (NCTC-2544) was grown in a DMEM medium (Sigma-Aldrich Milan, Italy), supplemented with 115 units/mL of penicillin G, 115 µg/mL streptomycin, and 10% fetal

calf serum (Invitrogen, Milan, Italy). Individual wells of a 96-well tissue culture microtiter plates (Falcon, Becton–Dickinson) were inoculated with 100 μL of complete medium containing 5 x 10^3 NCTC-2544. The plates were incubated at 37°C in a humidified (5% CO_2) incubator for 18 h prior to the experiments.

Cytotoxicity. After medium removal, 100 μL of the drug solution, dissolved in DMSO and diluted in DMEM medium, was added to each well, incubated at 37°C for 72 h. Final DMSO concentration never exceeded 0.5 %. Cell viability was assayed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) test as previously described by Mosmann.[103]

Irradiation procedure. Two HPW 125 Philips lamps, mainly emitting at 365 nm, were used for UVA irradiation experiments. The spectral irradiance of the source was 4.0 mW cm^{-2} as measured at the sample level by a Cole-Parmer Instrument Company radiometer (Niles, IL, USA) equipped with a 365-CX sensor. One or two PL-S 9 W/ 12 Philips lamps (280–370 nm; peak at 315 nm) were used for UVB irradiation experiments. To restrict the incident radiation to the range 305– 370 nm, a glass filter (Schott SWG-305) was used. Total energy was detected by the same equipped with a sensor (model CX-312).

Cellular photoprotection experiments. After medium removal, 100 μL of the drug solution, dissolved in DMSO and diluted with Hank's Balanced Salt Solution (HBSS pH = 7.2), was added to each well, incubated at 37°C for 30 min, and then irradiated (20 J/cm^2 for UVA and 0.5 and 1 J/cm^2 for UVB). After irradiation, the solution was replaced with the medium and the plates were further incubated for 48 h. Cell viability was assayed by the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide)] test as previously described by Mosmann.

12.5 HPLC analysis

HPLC analysis was performed using an Agilent 1100 Series HPLC System equipped with a G1315A DAD, autosampler and with a Phenomenex Synergi Hydro-RP C-18 80Å column (4.6 × 150 mm, 4 μm) fitted with a safety guard cartridge (Phenomenex). The column thermostat was manteined at 25°C during all the time of the analysis. Mobile phase consists of solvent A, H_3PO_4 0,01 M in water, and solvent B, H_3PO_4 0,01 M in acetonitrile. For molecule **3** and PBSA the mobile phase was composed by 91% of solvent A and 9% of solvent B at a flow rate of 1mL/min; determination was carried out in

isocratic condition, in 10 minute. Flow rate and run time remain the same for the analysis of molecule **8** and **10** but changes the solvent ratio, in analysis of these compounds the mobile phase was composed by 84% of solvent A and 16% of solvent B. Stability and photostability determinations were monitored with absorbance detection corresponding to λ_{max} of the molecule ± 8 nm. The sample solutions were filtered by a 0.45 μm filter, before be injected into column (HPLC filters were purchased from Chemtek Analitica, Bologna, Italy).

12.6 Photostability studies

Photostability studies were carried out with a solar simulator device (Suntest CPS+; Atlas, Linsengericht, Germany) equipped with a Xenon lamp, an optical filter to cut off wavelengths shorter than 290 nm and an IR-block filter to avoid thermal effects. The solar simulator emission was maintained at 500 W/m^2. A portion of cosmetic formulation containing the sunscreen molecule (3%, w/w) was transferred onto the bottom of a beacker to gain a cosmetic mount of 2mg/cm^2 and then was irradiated for 1h with the solar simulator. After irradiation the beaker was removed and its content quantitatively transferred into a 50 mL calibrated flask with methanol and the remaining sunscreen concentration was determined by HPLC.

All samples were protected from light both before and after irradiation, the degree of photodegradation was evaluated by comparing the areas of the irradiated samples, with those of the unirradiated preparations.

12.7 Synthetic procedures

3,4-Diamino-benzenesulfonic acid, sulfate salt (1)

o-Phenylenediamine (1 g, 9.25 mmol) was added to sulfuric acid 96% (5 mL) at 0°C under stirring for 1 hour, then the solution was heated to 137°C for 24 hours. The reaction mixture was cooled and was added 2-3 mL of cold water to get a white precipitate, which was filtered off, washed with methanol and filtered again to afford 1,77 g of white powder, yield 67%.

^{1}H NMR (DMSO): δ (ppm) 6,85 (d, 1H, Ar, J_{orto}= 8.4 Hz); 7,19 (dd, 1H, Ar, J_{orto}= 8.2Hz, J_{meta}=1.8Hz); 7.37 (1H, Ar, J_{meta}= 2Hz).

ESI+ MS: m/z 188.3 Da $[M + H]^{+}$, $C_6H_{10}N_2O_7S_2$ Mol. Wt.: 286,28

2-(4-Hydroxy-phenyl)-1H-benzimidazole-5-sulfonic acid (2)

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of **1** (100 mg, 0.35 mmol) in ethanol (5 mL) was added a solution of sodium bisulfite 1N in water (0.7 mL, 0.7 mmol) and 4-Hydroxy-benzaldehyde (46 mg, 0.35 mmol); the reaction mixture was heated at 80°C under reflux for 24 hours. The solvent was then evaporated under reduced pressure, the solid was washed with HCl 1N to precipitate the interest product, the suspension was filtered and the solid washed with methanol to afford 60 mg of desired product. Light yellow powder, yield 60%.

^{1}H NMR (DMSO): δ (ppm) 7.09 (d, 2H, aryl, J= 9.2 Hz); 7.73 (d, 1H, benzimidazole, J= 8.4 Hz); 7.78 (d, 1H, benzimidazole, J= 8.4 Hz); 7.91 (s, 1H, benzimidazole); 8.06 (d, 2H, aryl, J= 8.8 Hz); 10,78 (s, broad, 1H, -OH); 13.4-15.8 (s, broad, 2H, -SO_3H, -NH).

ESI+ MS: m/z 290.5 Da $[M + H]^+$; $C_{13}H_{10}N_2O_4S$; Mol. Wt.: 290.30

2-(3,4-Dihydroxy-phenyl)-1H-benzimidazole-5-sulfonic acid (3)

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of **1** (100 mg, 0.35 mmol) in ethanol (5 mL) was added a solution of sodium bisulfite 1N in water (0.7 mL, 0.7 mmol) and 3,4-Dihydroxy-benzaldehyde (48 mg, 0.35 mmol); the reaction mixture was heated at 80°C under reflux for 24 hours. The solvent was then evaporated under reduced pressure, the solid was washed with HCl 1N to precipitate the interest product, the suspension was filtered and the solid washed with methanol to afford 60 mg of desired product. White powder, yield 56%.

^{1}H NMR (DMSO): δ (ppm) 7.05 (d, 1H, aryl, J= 8 Hz); 7.52-7.56 (m, 2H, aryl); 7.68 (d, 1H, benzimidazole, J= 8.4 Hz); 7.75 (dd, 1H, benzimidazole, J_{orto}= 8.4 Hz, J_{meta}= 1.6 Hz); 7.88 (s, 1H, benzimidazole); 9.75 (s, broad, 1H, -OH); 10.4 (s, broad, 1H, -OH); 13.8-15.6 (s, broad, 2H, $-SO_3H$, -NH).

ESI+ MS: m/z 306,7 Da $[M + H]^+$; $C_{13}H_{10}N_2O_5S$; Mol. Wt.: 306.29

2-(2-Hydroxy-phenyl)-1H-benzimidazole-5-sulfonic acid (4)

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of **1** (100 mg, 0.35 mmol) in ethanol (5 mL) was added a solution of sodium bisulfite 1N in water (0.7 mL, 0.7 mmol) and 2-Hydroxy-benzaldehyde (40 µL, 0.35 mmol); the reaction mixture was heated at 80°C under reflux for 24 hours. The solvent was then evaporated under reduced pressure, the solid was washed with HCl 1N to precipitate the interest product, the suspension was filtered and the solid washed with methanol to afford 94 mg of desired product. White powder, yield 70%.

^{1}H NMR (DMSO): δ (ppm) 7.13-7.17 (m, 1H, phenyl); 7.20 (d, 1H, aryl, J= 7.6 Hz); 7.56-7.60 (m, 1H, aryl); 7.75-7.80 (m, 2H); 8.04 (s, 1H, benzimidazole); 8.06 (dd, 1H, J_{orto} = 8 Hz, J_{meta}= 2.4 Hz); 11.9- 14.2 (s, braod, 3H, -OH, -SO_3H, -NH).

ESI+ MS: m/z 290.8 Da $[M + H]^+$; $C_{13}H_{10}N_2O_4S$; Mol. Wt.: 290.30

2-(2,4-Dihydroxy-phenyl)-1H-benzimidazole-5-sulfonic acid (5)

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of **1** (100 mg, 0.35 mmol) in ethanol (5 mL) was added a solution of sodium bisulfite 1N in water (0.7 mL, 0.7 mmol) and 2,4-Dihydroxy-benzaldehyde (48 mg, 0.35 mmol); the reaction mixture was heated at 80°C under reflux for 24 hours. The solvent was then evaporated under reduced pressure, the solid was washed with HCl 1N to precipitate the interest product, the suspension was filtered and the solid washed with methanol to afford 65 mg of desired product. White powder, yield 56%.

^{1}H NMR (DMSO): δ (ppm): 6.57 (dd, 1H, aryl, J_{orto} = 8.8 Hz, J_{meta}= 2.4 Hz); 6.611 (s, 1H, aryl); 7.69-7.75 (m, 2H, benzimidazole); 7.90 (d, 1H aryl, J_{orto} = 8.8 Hz); 7.99 (s, 1H, benzimidazole); 10.66 (s, broad, 1H, -OH); 11.74 (s, broad, 1H, -OH); 13.87 (s, broad, 2H, -SO_3H, -NH).

ESI+ MS: m/z 306.7 Da $[M + H]^+$; $C_{13}H_{10}N_2O_5S$; Mol. Wt.: 306.29

2-(2,3,4-Trihydroxy-phenyl)-1H-benzimidazole-5-sulfonic acid (6)

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of **1** (100 mg, 0.35 mmol) in ethanol (5 mL) was added a solution of sodium bisulfite 1N in water (0.7 mL, 0.7 mmol) and 2,3,4-Trihydroxy-benzaldehyde (54 mg, 0.35 mmol); the reaction mixture was heated at 80°C under reflux for 24 hours. The solvent was then evaporated under reduced pressure, the solid was washed with HCl 1N to precipitate the interest product, the suspension was filtered and the solid washed with methanol to afford 82 mg of desired product. Whitish powder, yield 73%.

^{1}H NMR (DMSO): δ (ppm) 6.64 (d, 1H, aryl, J_{orto} = 8,8 Hz); 7.42 (d, 1H, aryl, J_{orto} = 8,8 Hz); 7.70-7.78 (m, 2H, benzimidazole); 7.99 (s, 1H, benzimidazole); 9.2 (s, broad, 1H, -OH); 10.45 (s, broad, 1H, -OH); 13.8-14.0 (s, broad, 3H, $-SO_3H$, -NH,-OH).

ESI+ MS: m/z 322.4 Da $[M + H]^+$; $C_{13}H_{10}N_2O_6S$; Mol. Wt.: 322.29

2-(4-Hydroxy-phenyl)-1H-benzimidazole (7)

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of o-Phenylenediamine (100 mg, 0.92 mmol) in ethanol (5 mL) was added a solution of sodium bisulfite 1N in water (1.84 mL, 1.84 mmol) and 4-Hydroxy-benzaldehyde (112 mg, 0.92 mmol); the reaction mixture was heated at 80°C under reflux for 24 hours. The solvent was then evaporated under reduced pressure, the solid was washed with HCl 1N to precipitate the interest product, the suspension was filtered and the solid washed with methanol to afford 150 mg of desired product. Light yellow powder, yield 77%.

^{1}H NMR (DMSO): δ (ppm): 7.03 (d, 2H, aryl, J_{orto}= 8.8); 7.38-7.41 (m, 2H, benzimidazole); 7.69-7.7 (m, 2H, benzimidazole); 8.14 (d, 2H, aryl, J= 8.8 Hz); 10.55 (s, broad, 1H, -OH); 14.63 (s, broad, 1H, -NH).

ESI+ MS: m/z Da 211.5[M + H]$^{+}$; $C_{13}H_{10}N_2O$; Mol. Wt.: 210.23

2-(3,4-Dihydroxy-phenyl)-1H-benzimidazole (8)

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of o-Phenylenediamine (100 mg, 0.92 mmol) in ethanol (5 mL) was added a solution of sodium bisulfite 1N in water (1.84 mL, 1.84 mmol) and 3,4-Dihydroxy-benzaldehyde (127 mg, 0.92 mmol); the reaction mixture was heated at 80°C under reflux for 24 hours. The solvent was then evaporated under reduced pressure, the solid was washed with HCl 1N to precipitate the interest product, the suspension was filtered and the solid washed with methanol to afford 132 mg of desired product. Light brown powder, yield 64%.

^1H NMR (DMSO): δ (ppm): 7.06 (d, 1H, J= 8 Hz); 7.49-7.51 (m, 2H); 7.67-7.70 (m, 2H); 7.75-7.77 (m, 2H); 9.7 (s, broad, 1H, -OH); 10.4 (s, broad, 1H, -OH); 15 (s, broad, 1H, -NH).

ESI+ MS: m/z 226.5 Da $[M + H]^+$; $C_{13}H_{10}N_2O_2$; Mol. Wt.: 226.23

2-(2,4-Dihydroxy-phenyl)-1H-benzimidazole (9)

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of o-Phenylenediamine (100 mg, 0.92 mmol) in ethanol (5 mL) was added a solution of sodium bisulfite 1N in water (1.84 mL, 1.84 mmol) and 2,4-Dihydroxy-benzaldehyde (127 mg, 0.92 mmol); the reaction mixture was heated at 80°C under reflux for 24 hours. The solvent was then evaporated under reduced pressure, the solid was washed with HCl 1N to precipitate the interest product, the suspension was filtered and the solid washed with methanol to afford100 mg of desired product. Light brown powder, yield 48%.

1H NMR (DMSO): δ (ppm): 6.57 (dd, 1H, aryl, J_{orto}= 8.8 Hz, J_{meta}= 2.4 Hz); 6.74 (d, 1H, aryl, J_{meta}= 2 Hz); 7.48-7.50 (m, 2H, benzimidazole); 7.79-7.81 (m, 2H, benzimidazole); 8.05 (d, 1H, aryl, J_{orto}=8.8 Hz); 10.76 (s, broad, 1H, -OH); 11.90 (s, broad, 1H, -OH); 14.13 (s, braod, 1H, -NH).

ESI+ MS: m/z 226.6 Da $[M + H]^+$; $C_{13}H_{10}N_2O_2$; Mol. Wt.: 226.23

2-(2-Hydroxy-phenyl)-1H-benzimidazole (10)

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of o-Phenylenediamine (100 mg, 0.92 mmol) in ethanol (5 mL) was added a solution of sodium bisulfite 1N in water (1.84 mL, 1.84 mmol) and 2-Hydroxy-benzaldehyde (96 μL, 0.92 mmol); the reaction mixture was heated at 80°C under reflux for 24 hours. The solvent was then evaporated under reduced pressure, the solid was washed with HCl 1N to precipitate the interest product, the suspension was filtered and the solid washed with methanol to afford 173 mg of desired product. White powder, yield 89%.

^{1}H NMR (DMSO): δ (ppm): 7.0-7.05 (m, 2H); 7.25-7.32 (m, 2H); 7.36-7.41 (m, 1H); 7.61 (d, 1H, J= 7.6); 7.72 (d, 1H, J= 7.6 Hz); 8.06 (dd, 1H, J_{orto}= 7.6 Hz, J_{meta}= 1.6 Hz); 13.16 (s, 1H, -OH); 13.21 (s, broad, 1H, -NH).

ESI+ MS: m/z Da 211.5[M + H]$^+$; $C_{13}H_{10}N_2O$; Mol. Wt.: 210.23

2-(2,3,4-Trihydroxy-phenyl)-1H-benzimidazole (11)

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of o-Phenylenediamine (100 mg, 0.92 mmol) in ethanol (5 mL) was added a solution of sodium bisulfite 1N in water (1.84 mL, 1.84 mmol) and 2,3,4-Trihydroxy-benzaldehyde (142 mg, 0.92 mmol); the reaction mixture was heated at 80°C under reflux for 24 hours. The solvent was then evaporated under reduced pressure, the solid was washed with HCl 1N to precipitate the interest product, the suspension was filtered and the solid washed with methanol to afford 208 mg of desired product. Light brown powder, yield 93%.

^{1}H NMR (DMSO): δ (ppm): 6.66 (d, 1H, aryl, J_{orto}= 8.8 Hz); 7.45-7.50 (m, 2H, benzimidazole); 7.59 (d, 1H, aryl, J_{orto} = 8.8 Hz); 7.77-7.782 (m, 2H, benzimidazole); 9.2 (s, broad, 1H, -OH); 10.4 (s, broad, 1H, -OH fenolico); 14 (s, broad, 2H, -NH,-OH).

ESI+ MS: m/z 242.6 Da $[M + H]^+$; $C_{13}H_{10}N_2O_3$; Mol. Wt.: 242.23

2-(3,4-Dihydroxy-phenyl)-1H-benzimidazole-5-carboxylic acid (12)

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of 3,4-Diaminobenzoic acid (100 mg, 0.66 mmol) in ethanol (5 mL) was added a solution of sodium bisulfite 1N in water (1.32 mL, 1.32 mmol) and 3,4-Dihydroxy-benzaldehyde (91 mg, 0.66 mmol); the reaction mixture was heated at 80°C under reflux for 24 hours. The solvent was then evaporated under reduced pressure, the solid was washed with HCl 1N to precipitate the interest product, the suspension was filtered and the solid washed with methanol to afford 135 mg of desired product. Light brown powder, yield 76%.

^{1}H NMR (DMSO): δ (ppm): 7.06 (d, 1H, J= 8 Hz); 7.69-7.73 (m, 2H); 7.82 (d, 1H, J= 8.4 Hz); 8.05 (dd, 1H, J_{orto}= 8.4 Hz, J_{meta}= 1.6 Hz); 8.25 (s, 1H); 9.8 (s, broad, 1H, -OH fenolico); 10.5 (s, broad, 1H, -OH).

ESI+ MS: m/z 270.7 Da $[M + H]^+$; $C_{14}H_{10}N_2O_4$; Mol. Wt.: 270.24

2-(2,4-Dihydroxy-phenyl)-1H-benzimidazole-5-carboxylic acid (13)

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of 3,4-Diaminobenzoic acid (100 mg, 0.66 mmol) in ethanol (5 mL) was added a solution of sodium bisulfite 1N in water (1.32 ml, 1.32 mmol) and 2,4-Dihydroxy-benzaldehyde (91 mg, 0.66 mmol); the reaction mixture was heated at 80°C under reflux for 24 hours. The solvent was then evaporated under reduced pressure, the solid was washed with HCl 1N to precipitate the interest product, the suspension was filtered and the solid washed with methanol to afford 140 mg of desired product. Light brown powder, yield 78%.

^{1}H NMR (DMSO): δ (ppm): 6.57 (dd, 1H, J_{orto} = 8.8 Hz, J_{meta} = 2.0 Hz); 6.67 (s, 1H); 7.83 (d, 1H, J= 8.8 Hz); 8.03 (dd, 2H, J_{orto}= 8.8, J_{meta}= 1.2 Hz); 8.31 (s, 1H); 10.7 (s, broad, -OH) 12.4-14.6 (s, broad, 3H, -OH, -NH, -COOH).

ESI+ MS: m/z 270.7 Da $[M + H]^+$; $C_{14}H_{10}N_2O_4$; Mol. Wt.: 270.24

2-(4-Hydroxy-phenyl)-1H-benzimidazole-5-carboxylic acid (14)

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of 3,4-Diaminobenzoic acid (100 mg, 0.66 mmol) in ethanol (5 mL) was added a solution of sodium bisulfite 1N in water (1.32 mL, 1.32 mmol) and 4-Hydroxy-benzaldehyde (80.6 mg, 0.66 mmol); the reaction mixture was heated at 80°C under reflux for 24 hours. The solvent was then evaporated under reduced pressure, the solid was washed with HCl 1N to precipitate the interest product, the suspension was filtered and the solid washed with methanol to afford 146 mg of desired product. Light brown powder, yield 87%.

^{1}H NMR (DMSO): δ (ppm): 7.09 (d, 2H, J_{orto}= 8.8 Hz); 7.84 (d, 1H, J_{orto}= 8.4 Hz); 8.06 (d, 1H, J_{orto}= 8.4 Hz); 8.22 (m, 2H); 8.26 (s, 1H); 10.85 (s, broad, 1H, -OH); 14-16 (s, broad, 2H, -COOH, -NH).

ESI+ MS: m/z 254.7 Da $[M + H]^{+}$; $C_{14}H_{10}N_{2}O_{3}$; Mol. Wt.: 254.24

2-(2,3,4-Trihydroxy-phenyl)-1H-benzimidazole-5-carboxylic acid (15)

In a round-bottomed flask (50 mL) equipped with a magnetic stirrer, to a solution of 3,4-Diaminobenzoic acid (100 mg, 0.66 mmol) in ethanol (5 mL) was added a solution of sodium bisulfite 1N in water (1.32 mL, 1.32 mmol) and 2,3,4-Trihydroxy-benzaldehyde (102 mg, 0.66 mmol); the reaction mixture was heated at 80°C under reflux for 24 hours. The solvent was then evaporated under reduced pressure, the solid was washed with HCl 1N to precipitate the interest product, the suspension was filtered and the solid washed with methanol to afford 151 mg of desired product. Light pink powder, yield 80%.

^{1}H NMR (DMSO): δ (ppm): 6.65 (d, 2H, J_{orto}= 8.8 Hz); 7.58 (d, 1H, J_{orto}= 8.8 Hz); 7.82 (d, 1H, J_{orto}= 8.6 Hz,); 8.02 (dd, 1H, J_{orto}= 8.6 Hz, J_{meta}= 1.6 Hz); 8.26 (d, 1H, J_{meta}= 1.6 Hz); 10.2-11 (s, broad, 2H, -OH); 13-14 (s, broad, 3H, -OH -COOH, -NH).

ESI+ MS: m/z 286.4 Da $[M + H]^+$; $C_{14}H_{10}N_2O_5$; Mol. Wt.: 286.24

13. References

1. Nina G. Jablonski and George Chaplin; “The evolution of human skin coloration”; *Journal of Human Evolution*; **2000**, 39: 57–106.

2. Esteban J. Parra; “Human Pigmentation Variation: Evolution, Genetic Basis, and Implications for Public Health”; *Yearbook of Physical Anthropology*; **2007**, 50:85–105.

3. Shaath, NadimA ; “The chemistry of ultraviolet filters”; monograph: “Sunscreens: regulation and commercial development; *Cosmetic science and technology series* **2005**, 218-238.

4. Salvador González, Manuel Fernández-Lorente, Yolanda Gilaberte Calzada; “The latest on skin photoprotection”; *Clinics in Dermatology*; **2008,** 26: 614–626.

5. Stephan Lautenschlager, Hans Christian Wulf, Mark R Pittelkow; “Photoprotection”; *Lancet* **2007**, 370: 528–37.

6. Prisana Kullavanijaya, MD, and Henry W. Lim, MD, “Photoprotection”; *Journal of the American Academy of Dermatology*; **2005**, 52: 937-58.

7. Jean Kanitakis; “Anatomy, histology and immunohistochemistry of normal human skin”; *European Journal of Dermatology*; 2002,12 (4): 309-401.

8. Martini F.H., Timmons M.J., Tallish R.B.; “*Human Anatomy*” 4°edition, **2003**.

9. Colin R. Goding; “Melanocytes: The new Black”; *The International Journal of Biochemistry & Cell Biology*; **2007**, 39: 275–279.

10. John D. Simon, Dana Peles, Kazumasa Wakamatsu and Shosuke Ito; “Current challenges in understanding melanogenesis: bridging chemistry, biological control, morphology, and function” *Pigment Cell Melanoma* Res.; **2009**, 22: 563–579.

11. Zalfa A. Abdel-Malek, James Knittel, Ana Luisa Kadekaro1, Viki B. Swope and Renny Starner; “The Melanocortin 1 Receptor and the UV Response of Human Melanocytes—A Shift in Paradigm”; *Photochemistry and Photobiology*, **2008**, 84: 501–508.

12. Ana Luisa Kedakaro, Renny J. Kavanagh, Kazumasa Wakamatsu, Shosuke Ito, Michelle A. pipitone, Zalfa A. Abdel-Malek; “Cutaneous photobiology. The melanocytes vs. the sun: who will win the final round?”; *Pigment Cell Res*; **2003**, 16: 434-447.

13. J. Longstreth, F.R. de Gruijl, M.L. Kripke, S. Abseckd, F. Arnold, H.I. Slaper, G. Velders, Y. Takizawa, J.C. van der Leun; "Health risks"; *Journal of Photochemistry and photobiology B:Biology*; **1998**; 46: 20-39.

14. Michaela Brenner and Vincent J. Hearing; **"**The Protective Role of Melanin Against UV Damage in Human Skin" *Photochemistry and Photobiology*, **2008**, 84: 539–549.

15. Jonathan L. Rees; "The Genetics of Sun Sensitivity in Humans"; *The American Journal of Human Genetics*; **2004**, 75: 739–751.

16. M. Yaar and B. A. Gilchrest; "Ageing and photoageing of keratinocytes and melanocytes"; *Clinical and Experimental Dermatology*, 2001, 26: 583-591.

17. M. Yaar and B.A. Gilchrest; "Photoageing: mechanism, prevention and therapy"; *British Journal of Dermatology*; **2007,** 157: 874–887.

18. T. Oppel H.C. Korting; "Actinic Keratosis: The Key Event in the Evolution from Photoaged Skin to Squamous Cell Carcinoma"; *Skin Pharmacol Physiol*; **2004**; 17: 67-76.

19. Taj Mohammad, Harry Morrison and Harm HogenEsch; "Urocanic Acid Photochemistry and Photobiology"; *Photochemistry and Photobiology*; **1999**, 69 (2): 115-135.

20. Stefan Beissert and Karin Loser; "Molecular and Cellular Mechanisms of Photocarcinogenesis" *Photochemistry and Photobiology*, **2008**, 84: 29–34.

21. Mary Norval and Gary M. Halliday; "The Consequences of UV-Induced Immunosuppression for Human Health"; *Photochemistry and Photobiology*, **2011**, 87: 965–977.

22. Report on Carcinogens; U.S. Department of Health and Human Services Public Health Service National Toxicology Program; Twelfth Edition **2011**.

23. F.R. de Gruijl; "Skin Cancer and Solar UV Radiation"; *European Journal of* Cancer; **1999**, 35 (14): 2003-2009.

24. Laurent Marrot and Jean-Roch Meunier; "Skin DNA photodamage and its biological consequences"; *Journal of the American Academy of Dermatology*; **2008**, 58 (5): S139-S148.

25. Darrell S. Rigel; "Cutaneous ultraviolet exposure and its relationship to the development of skin cancer"; *Journal of the American Academy of Dermatology*; **2008**, 58 (5): S129-S132.

26. S. Seité, A. Fourtanier, D. Moyal and A.R. Young; "Photodamage to human skin by suberythemal exposure to solar ultraviolet radiation can be attenuated by sunscreens: a review"; *British Journal of Dermatology*; **2010,** 163: 903–914.

27. Deevya L. Narayanan, Rao N. Saladi and Joshua L. Fox; "Ultraviolet radiation and skin cancer"; *International Journal of Dermatology*; **2010**, 49: 978–986.

28. Hugh M. Gloster and Kenneth Neal; “Skin cancer in skin of color”; *Journal of the American Academy of Dermatology*; **2006**, 55: 741-60.

29. Michael F Holick; “Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease”; *The American journal of clinical nutrition*; **2004**; 8: S1678–S1688.

30. Council directive of the European Communities (76/768/EEC), List of permitted UV filters which cosmetic products may contain; Annex VII, **2010**, 011.002: 102-104.

31. Department of Health and Human Services, Food and Drug Administration, PART 352-Sunscreen drug products for over-the-counter human use. Code of federal regulation **2011**-04-01.

32. Therapeutic Goods Administration. Australian regulatory guidelines for OTC medicines (ARGOM). Chapter 10 ‘Sunscreens’; 1 July **2003**;

33. Gerhard J. Nohynek and Hans Schaefer; “Benefit and Risk of Organic Ultraviolet Filters”; *Regulatory Toxicology and Pharmacology*; **2001**, 33: 285–299.

34. Serge Forestier; “Rationale for sunscreen development”; *Journal of American Academy of Dermatology*, **2008**, 58 (5): S133-138.

35. T. Maier H.C. Korting; “Sunscreens – Which and What for?”; *Skin Pharmacol Physiol*; **2005**; 18: 253–262.

36. Steven Q. Wang, Yevgeniy Balagula and Uli Osterwalder; “Photoprotection: a Review of the Current and Future Technologies”; *Dermatologic Therapy*; **2010**; 23: 31-47.

37. Divya R. Sambandan and Desiree Ratner; “Sunscreens: An overview and update”; *Journal of American Academy Dematology*; **2011**; 64 (4): 749-758.

38. Melanie D. Palm and Marianne N. O’Donoghue; “Update on photoprotection”; *Dermatologic Therapy*; **2007**, 20: 360–376.

39. Robert M. Sayre, John C. Dowdy, Andre J. Gerwig, William J. Shields and Roger V. Lloyd; “Unexpected Photolysis of the Sunscreen Octinoxate in the Presence of the Sunscreen Avobenzone”; *Photochemistry and Photobiology*; **2005**, 8: 452-456.

40. Clarke Stevenson and R. Jeremy H. Davies; “Photosensitization of Guanine-Specific DNA Damage by 2-Phenylbenzimidazole and the Sunscreen Agent 2-Phenylbenzimidazole-5-sulfonic Acid”; *Chem. Res. Toxicol.*; **1999**, 12: 38-45.

41. J. Johnson Inbaraj, Piotr Bilski and Colin F. Chignell; “Photophysical and Photochemical Studies of 2-Phenylbenzimidazole and UVB Sunscreen 2-Phenylbenzimidazole-5-sulfonic Acid”; *Photochemistry and Photobiology*, **2002**, 75(2): 107–116.

42. Nils Tarras-Wahlberg, Gunnar Stenhagen, Olle Larkö, Arne Rose´n, Ann-Marie Wennberg and Olof Wennerström; “Changes in Ultraviolet Absorption of

Sunscreens After Ultraviolet Irradiation"; *The Journal of Investigative Dermatology*; **1999**; 113 (4): 547-553.

43. Mark E. Burnett and Steven Q. Wang; "Current sunscreen controversies: a critical review"; *Photodermatology, Photoimmunology & Photomedicine*; **2011**, 27: 58–67.

44. R.Roelandts; "Shedding light on sunscreens"; *Clinical and Experimental Dermatology*; **1998**; 23: 147–157.

45. Andrew Beeby and Allison E. Jones; "The Photophysical Properties of Menthyl Anthranilate: A UV-A Sunscreen"; *Photochemistry and Photobiology*, **2000**, 72 (1): 10–15.

46. Georges J. Mturi, Bice S. Martincigh; "Photostability of the sunscreening agent 4-tert-butyl-4′-methoxydibenzoylmethane (avobenzone) in solvents of different polarity and proticity"; *Journal of Photochemistry and Photobiology A:* Chemistry; **2008**, 200: 410–420.

47. Anny Fourtanier, Dominique Moyal1 and Sophie Seité; "Sunscreens containing the broad-spectrum UVA absorber, Mexoryls SX, prevent the cutaneous detrimental effects of UV exposure: a review of clinical study results"; *Photodermatology, Photoimmunology & Photomedicine*; **2008**, 24: 164–174.

48. C. Antoniou, MG Kosmadaki, AJ Stratigos, AD Katsambas; "Sunscreens – what's important to know"; *Journal of the European Academy of Dermatology and Venereology*; **2008**, 22: 1110–1119.

49. COLIPA guidelines 2006; "International sun protection factor (SPF) test method"; *COLIPA May 2006,* Brussels.

50. C. Cole; "Sunscreen protection in the ultraviolet A region: how to measure the effectiveness"; *Photodermatol Photoimmunol Photomed* **2001**, 17: 2–10.

51. a) Dominique Moyal, Alain Chardon, Nikiforos Kollias; "Determination of UVA protection factors using the persistent pigment darkening (PPD) as the end point (Part 1) Calibration of the method"; *Photodermatol Photoimmunol Photomed*; **2000**, 16: 245–249. b) Dominique Moyal, Alain Chardon, Nikiforos Kollias; "UVA protection efficacy of sunscreens can be determined by the persistent pigment darkening (PPD) method (Part 2)"; *Photodermatol Photoimmunol Photomed*; **2000**, 16: 250–255.

52. Brian L. Diffey, Paul R. Tanner, Paul J. Matts, and J. Frank Nash; "In vitro assessment of the broad-spectrum ultraviolet protection of sunscreen products"; *J Am Acad Dermatol*; **2000**, 43: 1024-35.

53. COLIPA guidelines 2011; "In vitro method for the determination of the UVA protection factor and "critical wavelength" values of sunscreen products"; COLIPA **2011**, Brussels.

54. Department of Health and Human Services, Food and Drug Administration; Labelling and Effectiveness Testing; Sunscreen Drug Products for Over-the-

Counter Human Use; Federal Register/Vol. 76, No. 117; **2011** Rules and Regulations 35620-35665.

55. The commission of the European communities, Commission recommendation of 22 September **2006** on the efficacy of sunscreen products and the claims made relating thereto, 2006/647/EC. Official Journal of the European Union L265, 39–43.

56. COLIPA recommendation N°23; Imoportant usage and labelling instruction for sun protection products; 24 February **2009**.

57. Francis P. Gasparro, Mark Mitchnick and J. Frank Nash; "A Review of Sunscreen Safety and Efficacy"; *Photochemistry and Photobiology*, **1998**, 68 (3): 243-256.

58. Gerhard J. Nohynek, Eric Antignac, Thomas Re, Herve Toutain; "Safety assessment of personal care products/cosmetics and their ingredients"; *Toxicology and Applied Pharmacology*; **2010**, 243, 239–259.

59. Gerhard J. Nohynek and Hans Schaefer; "Benefit and Risk of Organic Ultraviolet Filters"; *Regulatory Toxicology and Pharmacology*; **2001**, 33:285–299.

60. J. Hojerová, A. Medovcíková, M. Mikula; "Photoprotective efficacy and photostability of fifteen sunscreen products having the same label SPF subjected to natural sunlight"; *International Journal of Pharmaceutics*; **2011**, 408: 27–38.

61. M. Lodén, H. Beitner, H. Gonzalez, D.W. Edström, Åkerström, J. Austad, I. Buraczewska-Norin, M. Matssonà and H.C. Wulf; "Sunscreen use: controversies, challenges and regulatory aspects"; *British Journal of Dermatology*; **2011**, 165: 255–262.

62. Maxwell S.R.J.; "Prospects for the use of antioxidant therapies"; *Drugs*, **1995**, 49: 345-361.

63. Hadi Moini, Lester Packer, Nils-Erik L. Saris; "Antioxidant and prooxidant activities of α -lipoic acid and dihydrolipoic acid"; *Toxicology and applied pharmacology*; **2002**, 182: 84-90.

64. Gatè L., Paul J., Nguyen Ba G., Tew K.D., Tapiero H.; **"**Oxidative stress induced in pathologies: the role of antioxidants"; *Biomed & Pharmacother*, **1999**, 53: 169-180.

65. Draper H., Hadley M.; "A review of recent studies on the metabolism of exogenous and endogenous malondialdehyde"; *Xenobiotica* , **1990**, 20: 901-910.

66. Halliwell B., Gutteridge J.M.C.; "Free radicals in biology and medicine", Oxford Science Publications, Clarendon Press; **1989**.

67. Mitscher L.A., Young M., Shankel D., Dou J.H., Steele L., Pillai S.P.; **"**Chemoprotection: A review of the potential therapeutic antioxidant properties of green tea (Camellia sinensis) and certain of its constituents"; *Med Res Rev*, **1997**, 17: 327-365.

68. Ames B.N.; **"**Dietary carcinogens and anticarcinogens – oxigen radicals and degenerative disease"; *Science*, **1983**, 221: 1256-1264.

69. Floyd R.A.; "The role of 8-hydroxyguanine in carcinogenesis"; *Carcinogenesis*, **1990**, 11: 1447-1450.

70. Fraga C.G., Shigenaga M.K., Park J., Deagan P., Ames B.N.; "Oxidative damage to DNA during aging – 8-hydroxy-2'deoxyguanosine in rat organ DNA and urine"; *Proc Natl Acad Sci*, **1990**, 87: 4533-4537.

71. Dandona P., Thusu K., Cook S., Snyder B., Nicotera T.; Oxidative damage to DNA in diabetes mellitus; *Lancet*, **1996**, 347: 444-445.

72. Sipowicz M.A., Chomarat P., Diwan B.A., Anver M.A., Awasthy Y.C., Ward J.M.; "Increased oxidative DNA damage and hepatocyte overexpression of specific cytochrome P450 isoforms in hepatitis of mice infected with Helicobacter hepaticus"; *Am J Pathol*, **1997**, 4: 933-941.

73. Cestaro B.; *Per una vita inossidabile*, Etaslibri – RCS Medicina, **1994**.

74. Berliner J.A., "Heinecke J.W.; The role of oxidized lipoproteins in atherogenesis"; *Free Radical Biol and Med*, **1996**, 20: 707-727.

75. Stadman J.A.; Protein oxidation and aging; *Science*, **1992**; 257: 1220-1224.

76. Scott G. "Antioxidant the modern elixir?" *Chemistry in Britain*, **1995**, 11: 879-882.

77. Rice-Evans C.A., Miller N.J., Paganga G. ; "Antioxidant properties of phenolic compounds"; *Trend in Plant Science*; **1997**, 2: 152-159.

78. Nishikimi M, Fukuyama R, Minoshima S, Shimizu N, Yagi K; "Cloning and chromosomal mapping of the human nonfunctional gene for L-gulono-gamma-lactone oxidase, the enzyme for L-ascorbic acid biosynthesis missing in man"; *The Journal of Biologycal Chemistry*; **1994**, 269:13685-13688.

79. Acker S.A., Koymans L.M.H., Bast A.; "Molecular pharmacology of vitamin E: structural aspects of antioxidant activity"; *Free Radical Biol & Med*; **1993**, 15: 311-328.

80. Meera Rangarajan and Joel L. Zazt; "Skin delivery of vitamin E"; *J. Cosmet. Sci.*; **1999**; 50: 249-279.

81. Han X., Shen T., Lou H.; "Dietary polyphenols and their biological Significance"; *International Journal of Molecular Science*; **2007**, 8: 950-988.

82. Rice-Evans C.A., Miller N.J., Paganga G.; "Antioxidant properties of phenolic compounds"; *Trend in Plant Science*, **1997**; 2: 152-159.

83. Rice-Evans C.A., Miller N.J., Paganga G.; "Structure-antioxidant activity relationships of flavonoids and phenolic acid"; *Free Rradical Biology & Medicine* **1996**, 20 (7): 933-956.

84. Soobratte M.A., Neergheen V.S., Luximon-Ramma A., Aruoma O.I., Bahorun T.; "Phenolics as potential antioxidant therapeutic agent: mechanism and actions"; *Mutation Research*, **2005**, 579: 200-213.

85. Jessica H. Rabe, Adam J. Mamelak, Patrick J. S. McElgunn, Warwick L. Morison and Daniel N. Sauder; "Photoaging: Mechanisms and repair"; *J Am Acad Dermatol*; **2006**, 55: 1-19.

86. Sheldon R. Pinnell; "Cutaneous photodamage, oxidative stress, and topical antioxidant protection"; *J Am Acad Dermatol* **2003**; 48: 1-19.

87. Lin JY, Selim MA, Shea CR, et al.; "UV photoprotection by combination topical antioxidants vitamin C and vitamin E"; *J Am Acad Dermatol*; **2003**: 48: 866–874.

88. Saija A, Tomaino A, Trombetta D, De Pasquale A, Uccella N, Barbuzzi T, et al.; "In vitro and in vivo evaluation of caffeic and ferulic acids as topical photoprotective agents". *Int J Pharm*; **2000**, 199 :39-47.

89. Craig A. Elmets, Divya Singh, Karen Tubesing, Mary Matsui, Santosh Katiyar and Hasan Mukhtar; "Cutaneous photoprotection from ultraviolet injury by green tea polyphenols"; *J Am Acad Dermatol*; **2001**, 44: 425-32.

90. Suchitra Katiyara, Craig A. Elmetsa, Santosh K. Katiyar; "Green tea and skin cancer: photoimmunology, angiogenesis and DNA repair"; *Journal of Nutritional Biochemistry*; **2007,** 18: 287–296.

91. Santo Scalia and Matteo Mezzena; "Photostabilization Effect of Quercetin on the UV Filter Combination, Butyl Methoxydibenzoylmethane–Octyl Methoxycinnamate"; *Photochemistry and Photobiology*, **2010**, 86: 273–278.

92. F. da Silva Miranda, A. M. Signorini, J. Vincente, B. de Souza, J. P. Priebe, B. Szpoganicz, N. sanches Gonçalves, A. Neves; "Synthesis of substituted dipyrido[3,2-a:20,30-c]phenazines and a new heterocyclicdipyrido[3,2-f:20,30-h]quinoxalino[2,3-b]quinoxaline"; *Tetrahedon* (**2008**) 64, 5410-5415.

93. Han Xiangming, Ma Huiqiang and Wang Yulu; "p-TsOH Catalyzed synthesis of 2-arylsubstituted benzimidazoles"; *ARKIVOC*; **2007** (xiii) 150-154.

94. Kiuemars Bahrami, Mohammad Mehdi Khodaei, Iman Kavianinia; "A Simple and Efficient One-Pot Synthesis of 2-Substituted Benzimidazoles"; *Synthesis*; **2007**, 4: 547–550.

95. Kevin J. Duffy, Alan T. Price, Evelyne Delorme,Susan B. Dillon, Celine Duquenne, Connie Erickson-Miller, Leslie Giampa, Yifang Huang, Richard M. Keenan, Peter Lamb, Nannan Liu, Stephen G. Miller,Jon Rosen, Anthony N. Shaw, Heather Smith, Kenneth J. Wiggall, Lihua Zhang, and Juan I. Luengo; "Identification of a Pharmacophore for Thrombopoietic Activity of Small,Non-Peptidyl Molecules. 2. Rational Design of Naphtho[1,2-*d*]imidazoleThrombopoietin Mimics"; *J. Med. Chem.* **2002**, *45,* 3576-3578.

96. Popov I., Lewin G., "Photochemiluminescent detection of antiradical activity; IV: testing of lipid-soluble antioxidants"; *J Biochem Biophys Methods*; **1996**, 31: 1-8.

97. Popov I., Lewin G.; Photochemiluminescent detection of antiradical activity III: a simple assay of ascorbate in blood plasma; *J.Biochem Biophys Methods*; **1994**, 28: 277-282.

98. Popov I., Lewin G.; "Oxidants and Antioxidants Part B – Antioxidative homeostasis: characterization by means of chemiluminescent technique", *Methods in Enzymology*; **1999**, 300: 437-456.

99. Wang M., Li J., Rangarajan M., Shao Y., La Voie E.J., Huang T-C; Ho C-T; "Antioxidative phenolic compound from sage (salvia officinalis)"; J.Agric.Food Chem. **1998**, 46, 4868- 4873.

100. Iris F. F. Benzie and J. J. Strain; "The ferric reducing ability of plasma as a measure of antioxidant power: the FRAP assay." *Analytical Biochemistry*, **1996**, 239: 70-76.

101. Xu et al. ; "Effect of heat treatment on the phenolic compounds and antioxidant capacity of citrus peel extract"; *J. Agric. Food Chem*; **2007**, 55: 330-335.

102. B. L. Diffey and J. Robson; "A new substrate to measures sunscreen protection factors throughout the ultraviolets spectrum"; *J. Soc. Cosmet. Chem,* **1989**, 40: 127-133.

103. Tim Mosmann; "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays"; *Journal of Immunological Methods*; **1983**, 65: 55-63.

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