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Research and development of botanicals obtained from local medicinal plant, potentially active in skin aging by air pollution

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INDEX

1 ABSTRACT

Environmental air pollution encompasses various particulate matter (PM) consisting in solid and/or liquid particles of different sizes and compositions, including mineral dust, metals, metalloids, sea salts, ammonium nitrate and sulfate, organic compounds, elemental carbon. The increasing of the environmental PM caused by human activity is documented to be associated with morbidity and mortality worldwide, presenting one of the most severe pollution related problems.

Recent epidemiological studies on the impact of PM showed that its high level is closely related to the development of inflammatory skin diseases and respiratory problems. PM also induces oxidative stress through the production of reactive oxygen species (ROS) and the secretion of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6. In addition, the increased production of ROS such as superoxide and hydroxyl radicals by PM exposure increases the activity of metalloprotease (MMP) and of other extracellular matrix degrading enzymes.

These processes increase inflammatory skin impairments and accelerated skin aging; the first line of intervention are drugs and functional cosmetics topically administered. Anti-oxidant and anti-inflammatory drugs are primarily considered for the treatment of skin diseases induced by PM.

In the last years, phytotherapy has focused its research on the field of anti aging and aging-related skin diseases, proving to be a particularly useful discipline in the management and prevention of skin health.

This interest in herbal products in the field of skin protection is also very active in Italy, but it is amazing how in our country there is still a little regard to the ethnobotanical tradition and the knowledge of species typical of the area.

The results of bibliographic and ethnobotanical research allowed us to identify four interesting and novel species: *Calluna vulgaris* (L.) Hull. and *Cistus creticus* L. subsp. *eriocephalus* (Viv.) Greuter & Burdet, used in Tuscany for skin care, but currently scarcely investigated, and *Juglans regia* L. and *Castanea sativa* Mill., very interesting for the potentiality of their by-products, namely leaves collected after fruit harvesting.

Optimized extracts, with the highest yield in polyphenols were obtained after having taken into account the collecting time and extraction procedures.

A multistep experimental protocol, consisting in cell free and cell-based tests was set up and validated in order to investigate the biological activities of selected extracts in stress models that mimed all the main skin impairments related to aging and pollution exposure.

All the selected extracts proved to be interesting candidates for a development in the field of dermatology for skin protection.

By-products, *C. sativa* and *J. regia* leaves hydroethanolic extracts, as well as *C. vulgaris* flowering tops water extract showed more likely specific skin protective activities, whereas *C. creticus* aerial parts hydroethanolic extract resulted effective in all performed tests.

C. sativa leaves extract proved to be the most effective extract for its activity in counteracting urban dust-induced MAPK activation.

J. regia leaves extract proved to be effective in protecting fibroblasts from immunosuppressive lowered viability and keratinocytes from dehydrative stress probably related to the promotion of occluding expression.

C. vulgaris flowering tops extract shared the most important biological characteristic with *J. regia* extract but it exerted a markedly higher protective activity in UVA-induced keratinocytes damage and in acute and slow oxidative stress.

Tuscan *C. creticus*, taken into account mostly being a species of ethnobotanical interest very scarcely investigated in the field of skin protection, provided the most unexpected and positive results. Indeed, the extract, rich in ellagitannins and flavonols, exerted the best cell-free antiradicalic/antioxidant profile and a strong activity against urban dust-induced keratinocytes stress, both oxidative and inflammatory, accompanied by the best inhibitory effect on elastase, collagenase and hyaluronidase activity.

The very positive results obtained in *in vitro* tests prompted us to perform a small clinical trial on *J. regia* extract and a formulative development on *C. sativa* and *C. vulgaris* extracts.

2 AIM OF THE STUDY

The highly anthropized and industrialized environments typical of our time have been causing strong repercussions on human health. Being the most exposed organ to air pollution, skin undergoes to tissue and cellular changes due to an excess of pro-inflammatory and pro-oxidant elements.

The aim of this research project is to apply a large, multistep experimental protocol, in order to investigate and develop new natural products derived from Italian and in particular Tuscan species, with biological characteristics suitable for their use as agents for skin aging, and skin oxidative and inflammatory impairments induced by air pollution.

Beside Italian species well-known for their consolidated activity and with supporting scientific literature, we also considered spontaneous species of the Tuscan flora with long and proven tradition use and by-products of their processing (for farmers a problem of disposal) but which may be important sources of active ingredients; these need a scientific validation through modern and advanced methodologies in order to correctly use.

The implementation of this project will have important consequences in the field of phytotherapy and phytocosmetics, as the results obtained will allow the development of herbal preparations that can be used in an area that is of great interest today, it is skin aging caused by pollution.

3 ORGANIZATION OF WORK

A comprehensive description of the state of the art of skin aging caused in particular by air pollution is provided in the first part of this work, followed by an introduction on the role of herbal products in the skin aging-related impairment

The conceptualization and plan of this scientific research was based on an extensive multistep experimental protocol:

STEP 1: Selection of the species and their by-products traditionally used and in accordance with the Italian ethnobotanical literature, with particular attention to the spontaneous Tuscan species; extraction optimization and chemical analyses.

STEP 2: cell-free antiradicalic and antioxidant evaluation; optimization of cell-free and cell-based *in vitro* models set up to investigate the efficacy of selected extracts: immunosuppressive, osmotic, photo-induced, acute and chronic oxidative cellular - dosage of barrier proteins and tight junctions - dosage of pro-collagen I and elastin - extra-cellular matrix degrading enzymes activity inhibition.

STEP 3: Evaluation of the activity of selected extract in an *in vitro* model of air pollution cellular stress by means of stimulation of human keratinocytes with urban dust: cell viability, intracellular ROS dosage, cytokines release (IL-6, IL-8, IL-1β and TNF-α), MAPK activation (ERK 1/2, JNK and p38).

STEP 4: Laboratory development and prototyping of new topical formulations containing the most promising products from the result obtained in the previous steps.

4 INTRODUCTION

4.1 Air Pollution

Today, about 54% of the world's population lives in cities where environmental pollution is still a health challenge, especially in developing countries.

The World Health Organization (WHO) defines pollution as contamination of the indoor or outdoor environment by any chemical, physical, or biological agent that changes the natural characteristics of the atmosphere (WHO's Ambient Air Pollution Database). "Pollution" is an umbrella term that covers a wide variety of chemicals, inorganic and organic, present in the environment (Drakaki et al. 2014).

Outdoor pollution comes from stationary, generally industrial and mobile sources, such as road and air traffic. These sources produce primary and secondary pollutants can be divided into two main groups: particulate matter (PM) and volatile organic compounds (VOCs), which consist of gases (CO_2, CO, SO_2, NO, NO_2, NOx_2) and low molecular weight hydrocarbons.

Secondary pollutants are defined as those compounds such as ozone (O_3) and peroxy-acetyl nitrates (PANs) that are generated under certain atmospheric conditions by photochemical reactions between primary pollutants, heat and UV radiation. These pollutants remain low in the atmosphere (troposphere) and settling, both in urban and rural areas, form what is typically known as smog.

The level of pollution and pollutant concentration varies throughout the day and seasons, and is related to geographic location and level of human activity. In particular, ozone (O_3) levels increase during summer, when strong sunshine leads to ozone production through photochemical reactions among primary pollutants.

4.1.1 Particulate Matter

Particulate matter is commonly referred to as "fine dust" which is divided into fine particles (PM2.5) and coarse particles (PM10). These are terms that identify the set of substances suspended in the air in the form of atmospheric aerosol, with an average aerodynamic diameter of less than 2.5 and 10 µm respectively. Fine dusts are generally produced by combustion and are suspended by the wind.

Regarding the chemical composition of particulate material, three main classes can be identified: inorganic ions (sulfates, nitrates, ammonium ions), carbonaceous fraction (formed by organic carbon and elemental carbon) and crustal material that can be associated with atmospheric dust (Si, Ca, Al...) or heavy elements in trace form (Pb, Zn...).

It is evident that among air pollutants, airborne particulate matter (PM) represents a threat to which millions of human beings are exposed. Air pollution is a growing environmental health hazard and is obviously associated with adverse effects on human health.

These harmful effects occur in the skin, which functions as the critical interface between the body and the external aggressors attacking it. The adverse effects of PM on human health are currently a serious concern, and it has been shown that the most serious adverse effects can certainly include an increased risk of cancer, pneumonia, and cardiovascular disease (Beelen et al., 2008; Castano-Vinyals et al., 2008).

The effects of environmental PM exposure on human skin in general, and on skin aging in particular, have yet to be thoroughly and comprehensively studied; however, an important mechanism by which environmental PM exerts adverse effects on the skin through the generation of oxidative stress has been established (Donaldson et al., 2005), which is responsible for an important contribution to extrinsic skin aging (Schroder et al., 2006).

Small particles such as nano-particles, particularly those from traffic-related pollution, are considered among the most environmentally damaging components of PM. These nano-particles cause oxidative stress because their physical properties, i.e., small size but large surface area per unit mass, make them highly reactive with biological surfaces and structures (Donaldson et al., 2005). It has also been postulated that these particles can serve as receptors for organic chemicals and heavy metals that are able to dilute in mitochondria and generate highly toxic reactive oxygen.

These environmental pollutants are the focus of important toxicological concerns as they can be pro-oxidants, mutagens, and carcinogens; in addition, some of them also react with sunlight thus exhibiting photo-toxicity (Krutmann et al., 2017; McDaniel et al., 2018).

Smaller particles have a very large specific active surface area, which increases their toxic potential. PM2.5, UFP, and PAH undoubtedly have a non-negligible interaction with the skin of polluted city dwellers.

4.2 Skin: Structure And Functions

Human skin protects the body from air pollutants, but exposure to pollutants over a prolonged or repeated period can have a serious negative impact on the skin itself (Mancebo and Wang, 2015; – Parrado et al., 2019).

The skin is the largest organ in the human body, accounting for about 10∼15 % of the body weight (Jabłońska-Trypuć et al., 2018).

It represents a protective sheath for the organism in relation to the external environment, towards which it acts as a barrier against adverse mechanical, physical and chemical forces (Montagna and Celleno, 2008).

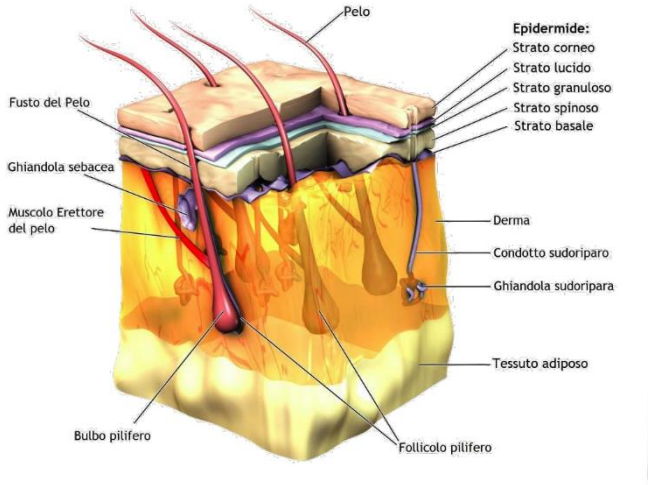


Figure 1: Three-dimensional representation of human skin.

The skin, together with the cutaneous adnexa, such as nails, hair and glands, performs numerous general functions, ranging from protection against mechanical, thermal and light, biological and chemical insults, to thermoregulation, secretion, excretion, absorption and sensory perception. The skin is a tough, elastic membrane, the thickness of which varies from individual to individual, depending on gender, body area and exposure to mechanical stress (Castano et al., 2006).

the skin has a surface area of approximately 1.5 m^2 and a thickness that varies depending on the body region, from 0.5 mm at the level of the eyelids to 4 mm at the sole of the foot, and on the sex, thicker in the male than in the female (Castano et al., 2006). It changes colour depending on the area, physiological condition, exposure to external agents and race. It may present more or less deep furrows, folds and ridges. On the fingertips, on the palms of the hands and partly also on the anterior extremity of the soles of the feet, furrows and ridges take on particular and typical aspects of the subject, called dermatoglyphs or imprints (Seeley, 2005).

Hypodermis supports the skin and acts as an intermediary between it and the underlying muscle and bone tissues; it is through the hypodermis that blood vessels and nerves reach the skin (Seeley et al., 2005). It provides a good energy reserve and also acts as an excellent insulator, facilitating neurovascular thermoregulation mechanisms (Montagna et al., 2012).

The skin consists of two closely connected layers lying on a subcutaneous lipid layer called the panniculus adiposus: the epidermis and the dermis (Amirlak, 2013). Between these two layers is the dermo-epidermal junction (GDE), consisting mainly of type IV collagen, which plays a key role in mechanically supporting the adhesion of the epidermis to the dermis and regulating the exchange of metabolic products between the two layers (Kanikatis, 2002).

The skin is a complex organ that covers the entire surface of the body, acting as a protective barrier to the external environment. It opposes the passage of micro-organisms, absorbs and blocks radiation, contributes to the regulation of body temperature, with loss of water through the epidermis (*perspiratio insensibilis*), plays an important immunological role and contains a dense network of nerves that gives it the function of an organ of relationship. The skin is made up of several layers that, listed from the surface to the depths, are: epithelium (epidermis), connective tissue (dermis) and adipose tissue (subcutaneous). The last two layers contain skin adnexa, vessels and nerves (Cainelli et al., 2000).

Epidermis

The epidermis is a stratified, squamous epithelium that varies in thickness between 0.06 and 0.8 mm, is highly cellular and contains no blood vessels. It provides a physical barrier between the individual and the external environment, prevents water loss and protects against infection, acting both as a physical barrier and through immune cells (Fuchs, 2009).

Histologically, the epidermis is divided into five layers:

- Basal layer: this is the deepest and consists mainly of keratinocytes, cylindrical cells that are at a progressively increasing state of differentiation from the innermost to the outermost layer, and are specialised in keratin synthesis. This layer also contains melanocytes and Merkel cells, which are involved in skin sensitivity (Kolarsick, 2011).
- Spiny layer: this consists of 8-10 layers of polymorphic cells, which flatten out as they approach the surface. At the level of the desmosomes, protein junctions that weld the cytoskeletons together, giving the skin resistance to traction and other physical shocks, the keratin filaments look vaguely

like thorns, hence the name. This layer also contains Langerhans cells, which derive from a precursor in the bone marrow and are involved in the immune response (Seeley et al., 2005).

- Granular layer: consists of 2-5 layers of flattened polygonal cells. The name of this layer comes from the membrane-free granules of keratohyalin protein, a highly phosphorylated acidic protein with high molecular weight and rich in histidine (Micali et al., 2014), which accumulate in the cytoplasm of the cells. The nuclei show signs of degeneration, the cells are less viable but continue to produce keratin, which accumulates in the cell itself making it less permeable (Seeley et al., 2005).
- Glossy layer: only present in thick skin, i.e. on the palm of the hand and soles of the feet. It consists of many layers of dead cells with indistinct boundaries, keratin fibres are present, but no keratohyalin is visible (Seeley et al., 2005).
- Stratum corneum: is the uppermost layer, consisting of approximately 25 or more layers of flattened dead cells held together by desmosomes (Seeley et al., 2005). The cytoplasm is filled with keratin, and the plasma membrane is made up of proteins, including involucrin, which becomes insoluble through specific enzymes, creating a horny envelope immediately inside the plasma membrane, and loricrin, which fixes the keratin macrofibrils with the horny coating, giving some resistance to the skin surface (Cainelli et al., 2012).

Between the corneocytes are intercellular lipids, such as ceramides, cholesterol and fatty acids organised in discs within small cytoplasmic organelles surrounded by a membrane, the so-called lamellar bodies. This layer is made up of 70% proteins, 15% lipids and the remaining 15% water, hence the great importance of the skin's state of hydration.

Hygroscopic substances are also present: Natural Moisturizing Factors (NMF) such as amino acids and their derivatives; it is assumed that filaggrin, a protein synthesised during the keratinisation process, once it has fulfilled its task of forming the tertiary structure of keratin, is attacked by proteinases and thus gives rise to the above-mentioned molecules (Rawlings and Harding, 2004).

Calcium plays a crucial role in the keratinisation process, far from an 'on-off' concept: the ion regulates gene expression in a dose-dependent manner. A low dose of calcium serves for the renewal and division of stem cells that will give rise to keratinocytes, while higher concentrations are functional for their differentiation. Moving from the basal to the stratum spinosum to the stratum granulosum, there is an increasing calcium gradient, which stops abruptly at the stratum corneum made up of dead cells (Rinnerthaler et al., 2015).

An increase in calcium concentration not only causes the release of the lipid-rich lamellar bodies contained in the keratinocytes of the stratum spinosum and stratum granulosum, but also induces

the release of keratohyalin granules, due to which profilaggrin undergoes proteolytic processing leading to the formation of filaggrin monomers (FILament AGGRegation proteinIN), a filamentous protein in the stratum corneum rich in proline and sulphur amino acids.
Filaggrin in stratum corneum cells binds to the keratin that forms the intermediate filaments of the cytoskeleton, causing them to collapse, so that the keratinocytes flatten out and become squamous cells. This forms a smooth compact structure that makes the skin smooth and even as well as well-hydrated (Sandilands et al., 2009).

Derma

The dermis is a tissue of mesenchymal origin, consisting of fibroblasts, collagen fibres, elastic fibres and basic substance, within which are contained the blood and lymph vessels, nerves and skin adnexa. It is a resistant tissue that supports and provides nourishment to the epidermis and skin adnexa, protecting the body from external mechanical stimuli. Two types of dermal compartments can be distinguished: the papillary dermis and the reticular dermis (Cainelli et al., 2000).
The dermis supports the organ, housing the vascular, lymphatic and neuronal systems. It is a lamina of connective tissue 0.3-4 mm thick and consists of two layers: the papillary layer, made up of collagen fibres and elastic fibres arranged perpendicularly to the skin surface and containing projections extending towards the epidermis and a large number of blood vessels, and the reticular layer, in which the collagen fibres form a dense network arranged mainly parallel to the skin surface (Castano et al., 2006).
The cells that make up the dermis are: fibroblasts, responsible for the synthesis of procollagen, from which collagen is derived through the action of proteolytic enzymes, elastic fibres and components of the basic substance, mast cells, important for the inflammatory response because they contain histamine, and cells migrated from the blood, such as macrophages and mononuclear cells (Amiralk, 2013). Fibroblasts are the main cells of the dermis, they are the producers of the other components: basic substance, collagen fibres and elastic fibres (Castano et al., 2006). Cells of the immunocompetent system such as mast cells, lymphocytes and histiocytes are also present.
Fibroblasts are variable, spindle-shaped or star-shaped cells located between the fibre bundles. Under quiescent conditions they are small, with few granules and are called fibrocytes; under active conditions, such as during reparative processes or growth, their cytoplasm is abundant and rich in both wrinkled endoplasmic reticulum and Golgi complexes (Castano et al., 2006).

The dermis is composed of a complex extracellular matrix (ECM) that gives the skin its strength and elasticity (Theocharis et al., 2016).

The ECM is the environment in which the body's cells are immersed and with which they must continually interact for their vital functions. It consists of two main classes of macromolecules that are produced locally, where they are continuously degraded and resynthesised: glycosaminoglycans (hyaluronic acid) and other proteins (elastin and collagen) (Theocharis et al., 2016).

The glycoproteins are linked together with hyaluronic acid and form a dense network. According to recent studies, the extracellular matrix not only performs a structural support function, giving organs their shape and consistency, but also enables the uninterrupted flow of molecules (nutrients, chemical mediators, drugs and waste substances) between the blood and cell compartments. Over the years, the matrix progressively loses its morpho-functional integrity: various factors chronically activate matrix metalloproteases (MMPs), leading to ECM degradation. As a result, metabolic exchanges slow down, communication between cells is impaired and toxic residues from cellular activities accumulate, triggering a mechanism that accelerates the signs of ageing (Theocharis et al., 2016).

The precursor of collagen is procollagen, whose chain undergoes hydroxylation of specific proline and lysine residues, thus becoming procollagen α-chains. Three of these polypeptide chains (two α1 chains and one α2 chain) wrap around to form a triple helix, which is stabilised by hydrogen bonds between hydroxylated amino acids and cross-links between lysine molecules.

After glycosylation, the helix is secreted into the intercellular space where it is converted by peptidases into tropocollagen, the structural unit of collagen. The tropocollagen molecules organise into fibrils, which are arranged in bundles to form fibres. Collagen fibres are the skin scaffold responsible for the mechanical strength of the skin (Kanitakis, 2002).

The main types of collagen found in the dermis are types I and II (Daly, 1982). Elastin fibres make up less than 1% by weight of the dermis (Amirlak et al., 2013), but, forming a large mesh network, they give the skin its characteristic plastoelasticity.

Collagen is the most abundant protein in the human body and 28 different types and about 46 distinct polypeptide chains have been identified. Two or more different types of collagen are present in the ECM. All collagen types share a trimeric structure, consisting of three polypeptide chains, called α chains, wrapped around each other to form a triple helix. Collagen is very rich in

glycine and other rare amino acids that are almost absent in other proteins, such as hydroxyproline and hydroxylysine (Theocharis et al., 2016).

The most abundant types of fibrillar collagen in the dermis are types I and II, which form extensive arrays of fibrils (Daly, 1982).

The stability of collagen fibres is reinforced by hydrogen bridges involving the hydroxyls of the hydroxyproline and hydroxylysine residues of the α-chains; these hydrogen bridges form cross-links both within and between individual collagen molecules in a fibril. From these characteristics it is understood how collagen provides tensile strength (Blume-Peytavi et al., 2016; Ritz-Timme et al., 2003; Shapiro et al., 1991). Elastic fibres are macrostructures of the ECM and provide deformability to tissues that are subjected to repeated mechanical stress, such as skin. Elastic fibres in young individuals are very stable with little or no turnover. In adults, however, damaged elastic fibres are often improperly repaired and do not function normally. The major constituent of elastic fibres is elastin, while another is microfibrils. The precursor of elastin is tropoelastin, a 60-70 kDa protein that contains hydrophobic domains alternating with cross-linking domains (Theocharis et al., 2016).

The basic, or amorphous, substance covers the space between the fibres and the dermal cells and is particularly abundant in the papillary layer of the dermis. It consists of glycosaminoglycans (GAGs), long polymers of variable molecular size, depending on the type and tissue of origin, and substituted with sulphate groups in various positions. GAGs have a strong ability to bind water and therefore keep the skin soft and hydrated (Puizina-Ivic, 2008).

Hyaluronic acid is the only one of the GAG class that is not replaced by sulphate groups, is able to retain water molecules up to 1000 times its molecular weight and acts as a shock absorber and space filler. It is mainly synthesised by fibroblasts, which have a production rate ten times higher than epidermal keratinocytes. Crosslinking of hyaluronic acid with matrix proteins, such as the collagen network, results in the formation of supra-molecular structures and increases tissue stiffness (Theocharis et al., 2016).

Hyaluronic acid also reacts with its own receptors such as CD44, a transmembrane glycoprotein with no intrinsic enzymatic activity that also acts as a receptor for MMPs (Theocharis et al., 2016), RHAMM, a receptor for hyaluronan-mediated motility, and LYVE-1, an endothelial receptor 1 of lymphatic vessels, in order to regulate cell proliferation, differentiation and migration (Lee et al., 2016).

When there is a need for rapid tissue proliferation, regeneration and repair the content of hyaluronic acid increases (Sivamani et al., 2015).

Nerve endings, hair follicles, smooth muscle cells, glands and lymphatic vessels are also found in the dermis. Free nerve endings represent receptors for pain, itching, tickling and temperature changes, those around the hair follicle are receptors for touch.

Hypodermis

This is the last, deepest and thickest layer of the skin, which lies between the dermis and the underlying muscle tissue. It is also the skin layer through which blood vessels and nerves reach the skin (Amirlak et al., 2013). The hypodermis is made up of loose connective tissue, in which collagen shoots and elastic fibres delimit lobes filled with fat cells.

The main cell populations are fibroblasts, adipocytes and macrophages.

Approximately half of the fat (adipose) in the body is located in the hypodermis and the amount and location vary with age, sex, nutritional status and hormonal influences of the individual (Borellini, 2013).

Fat provides an energy reserve to draw on in times of need and plays a protective role in internal organs.

Finally, the hypodermis is an important thermal insulator for the body and therefore contributes to maintaining body temperature (Seeley, 2005).

4.3 Environmental Polluttion, Particulate Matter And Its Harmful Effects On The Skin

People with skin barriers impairment are more susceptible to PM mainly because of an increased cutaneous absorption (Bakke et al., 2012; Jin et al., 2018). PM can damage the skin barrier itself as well, enhancing the subsequent absorption of pollutants (Pan et al., 2015).

PM can cause oxidative damage to nucleic acids, proteins, and lipids inside cells (Piao et al., 2018; Pecorelli et al., 2019). It also induces inflammatory reactions, disrupting skin homeostasis and barrier function (Lee et al., 2016; Kim et al., 2016). Intracellular ROS are inevitable by-products of normal metabolism in oxygen-breathing organisms (Lushchark 2014), with the mitochondrial electron transport system and oxygen-dependent metabolic enzymes as the major intracellular sources of ROS (Sarniak et al., 2016). The impact of ROS on skin biology can be further increased by the Fenton reaction catalyzed by transition metals contained in PM or by the photochemical, photodynamic reactions mediated by UV in the skin (Mikrut et al., 2018; Dumax-Vorzet et al., 2015). PM can activate signaling pathways involving mitogen-activated protein (MAP) kinases such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAP kinase (Xiao

et al., 2016; Kim et al., 2019). PM also stimulates signaling pathways which lead to the activation of the redox-sensitive transcription factor, nuclear factor NF-κB (Donaldson et al., 2003; Ryu et al., 2019). PM has been shown to increase mRNA and protein levels of many inflammatory cytokines, including tumor necrosis factor (TNF), interleukin IL-1, IL-6, IL-8, and IL-18, in cells and in reconstructed human epidermis (Kim et al., 2019, Romani et al., 2018; Lecas et al., 2016). PM also induces the expression of chemokines, such as monocyte chemoattractant protein (MCP)-1, and cell adhesion molecules, such as intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 (Park et al.,2016; Montiel-Davalos et al., 2017). PM has been implicated in the activation of matrix metalloproteinase (MMP)-1, -2, and -9 that are involved in the degradation of extracellular matrix components, including collagen (Jin et al., 2018, Kim et al., 2016; Lecas et al., 2016, Morales-Barcenas et al., 2015).

PM has been shown to upregulate the production of prostaglandin (PG) E2, an eicosanoid mediator of inflammation (Lee et al., 2016; Fernando et al., 2018).

PM increased the expression of COX-2 by facilitating the activation of MAP kinases such as ERK, JNK, and p38 MAP kinase in keratinocytes (Lee et al., 2016; Zhang et al., 2017). Cellular mRNA levels of COX-1 and -2 and mPGES-1 and -2 have been shown to be upregulated by PM in keratinocytes (Ha et al., 2019).

Aryl hydrocarbon receptor (AhR) is known to regulate gene expression associated with air pollution toxicity (Mancebo and Wang, 2015, Hidaka et al., 2017). AhR mediates the PM effects ROS production, NOX activity, COX-2 expression, and PGE2 production and decreases the expression of filaggrin that physically aggregates keratin bundles and thereby enhances skin barrier function in human keratinocytes [Lee et al., 2016; Yokota et al., 2018). Cells also possess a system to detect and defend their oxidative status, which involves nuclear factor erythroid 2-related factor 2 (Nrf2) Nrf2 system mainly induces the expression of phase II metabolic enzymes, adding glutathione, acid, and other hydrophilic molecules to the foreign substances to enhance their clearance from the cells. The system can also induce antioxidant enzymes involved in the removal of ROS and repair of oxidative damage.

In a highly polluted atmosphere, impaired immune function can induce or exacerbate skin diseases such as atopic dermatitis (Ahn 2014, Song et al., 2011) and acne vulgaris (Krutmann et al., 2017; Liu et al., 2018). A continuous inflammatory response due to PM exposure has also been associated with premature skin aging (Vierkotter et al., 2010; Park et al., 2015) and hyperpigmentation (Roberts

2015). Simultaneous PM and ultraviolet radiation (UV) exposure exerts synergistic negative effects on the skin, associated with photo-aging and cancer (Soeur et al., 2017; Datzmann etal., 2018).
In healthy skin, exposure to urban PM has been associated with accelerated intrinsic and extrinsic aging (Vierkotter et al., 2010), its clinical symptoms being pigment spot formation, coarse wrinkle development, and elastosis (Yaar et al., 2002). Further evidence from *in vitro* studies epidemiological, clinical studies, and aging mechanisms have been reviewed (Krutmann et al., 2017, Vogeley et al., 2019; Parrado et al., 2019; Buke 2018), all indicating the generation of free radicals, the activation of aryl hydrocarbon receptor signaling, the induction of inflammatory cascade, and finally the impairment of the skin barrier.
In summary, air pollution and PM in particular have a major role in altering skin functions and challenging skin health by promoting acute oxidative stress and inflammatory conditions and by accelerating intrinsic and extrinsic aging-related skin disregulations.

4.3.1 Skin Aging

As a consequence of the direct contact between skin and environment, cutaneous aging is induced by both intrinsic and extrinsic factors, all leading to reduced structural integrity and loss of physiological function. There are, in fact, two clinically and biologically independent aging processes that occur simultaneously. The first is innate or intrinsic aging, a cumulative process which affects skin as it probably affects a variety of internal organs, by slow and irreversible degeneration of tissue. The second process is extrinsic aging or photoaging caused by environmental oxidative factors, primarily the exposure to UV irradiation and smoking, but also other pollution aggressors.

CHRONOAGING

Intrinsic aging, or chrono-aging, is a continuous and natural process that begins around 25 years of age: during this period the production of collagen, elastin and hyaluronic acid slows down, cell turnover becomes less rapid and the thickness of the superficial horny layer increases in spite of the "living" layers below. All this leads to decreased hydration, tone and elasticity, the appearance of fine wrinkles, expression lines and loss of volume (Glogau, 1996).
The speed with which chrono-aging becomes visible is related to one's genetic heritage. It is influenced by many factors, including, in particular, the race. The skin of African Americans, in fact, is more compact than that of Caucasians and has also a high content of intercellular lipids, which

can contribute to resistance to aging. It has been seen that wrinkles appear later and in a less severe way in Asians than in Caucasians (Farage et al., 2008).

Skin deterioration is not only an aesthetic factor. Without considering the problem of melanoma, in fact, dermatological disorders are omnipresent in older people, with a significant impact on quality of life. Although the number of cell layers remains stable throughout life, the skin will tend to thin in a progressive way.

The epidermis decreases in thickness (Harvell and Maibach, 1994), particularly in women, on the face, neck, upper chest and forearms (Boss and Seegmiller, 1981). Keratinocytes change shape, becoming shorter and thicker, while corneocytes become larger due to a decrease in epidermal turnover. Enzymatically active melanocytes decrease by 8-20% per decade, causing irregular pigmentation. Although the number of sweat glands does not change, sebum production decreases by up to 60%.

The aged epidermis is atrophic (Lavker et al., 1987); at a functional level it shows reduced barrier function and reduced repair capacity after damage (Choi et al., 2007).

Such changes in the epidermis increase sensitivity to a wide variety of skin problems, such as colonization by pathogenic bacteria and reduction of hydration and cohesion of the horny layer; this leads to itching and dryness of the skin, conditions prevalent in elderly individuals, and leading to a substantial negative impact on quality of life (Chang et al., 2013; Garibyan et al., 2013).

The thickness of the dermis decreases with age and thinning is accompanied by a decrease in vascularization and cell number. Inevitably the turnover of collagen and elastin decreases. During intrinsic aging, collagen and elastic fibers remain intact, but they lack to form a wider network.

The most consistent structural change is a flattening of the dermo-epidermal junction, which occurs as a result of dermal papilla loss and reduced interdigitation between layers. This flattening causes a lower resistance to shear forces and greater vulnerability to insults (Grove, 1989).

The reduced surface continuity between the layers creates a reduced cellular supply of nutrients and oxygen and an increased risk of dermo-epidermal separation, a process that can cause the formation of wrinkles. There is a change in subcutaneous fat distribution: it decreases in the face, hands and feet, while there is a relative increase in thighs, waist and abdomen. These changes probably occur to increase the thermoregulation function of the organs (Farage et al., 2013).

Also the skin glands, in particular sebaceae, progressively decrease their activity, with a consequent reduction in sebum production, increase in skin dryness and desquamation. This causes a lower protection of the skin from exogenous agents (Fisher et al., 2002).

PHOTOAGING

Extrinsic aging is driven by extrinsic factors of various kinds, such as solar radiation, cigarette smoking, or other polluting factors (Kammeyer and Luiten, 2015).

The factor with the greatest impact is chronic exposure to sunlight (Debacq-Chainiaux et al., 2012), so the term "photoaging" is referred to as a synonym for extrinsic aging (Klingman and Klingman, 1986).

Photodamaged skin is characterized by deep wrinkles, loss of elasticity, dryness, laxity, and pigmentation disorders (Kammeyer and Luiten, 2015). In recent decades, stratospheric ozone depletion has led to an increase in solar ultraviolet radiation at the Earth's surface, leading to an increased likelihood of the occurrence of photoaging in the population (Saewan and Jimtaisong, 2015).

The process of photoaging is mainly caused by chronic skin exposure to UV radiation.

The severity of photoaging also depends on the skin phototype: it is more prominent in fair-skinned individuals (phototypes I and II) and is less noticeable in people with phototype III or higher. Nonetheless, the severity of damage depends primarily on the cumulative dose of UV received and the pigmentation status of the skin (Kammeyer and Luiten, 2015).

Phototype	Skin reaction to sun exposure	Skin colour	Hair colour	Eye colour
I	Always burns, never tans	Pale, Fair	Blond	Blue
II	Usually burns, sometimes tans	Fair, Freckles	Blond, Red	Green
III	May burn, usually tans	Light Brown	Dark Blond, Light Brown	Hazel, Brown
IV	Rarely burns, always tans	Olive brown	Light Brown, Brown, Black	Dark brown
V	Seldom burns, always tans	Brown Moderate constitutional pigmentation	Dark Brown, Black	Dark brown
VI	Never burns	Black Marked constitutional pigmentation	Black	Dark brown

Figure 2: Skin type Fitzpatrick

It is now clear that UV radiation is the central driver of photoaging. Exposure of cells to UV irradiation, in particular keratinocytes and fibroblasts, induces activation of coordinate signal transduction pathways that leading to degrade structural proteins in the dermis that confer strength and resiliency to skin. In particular, UV-B radiation is characterized as a mutagenic and cancer-inducing agent because it causes direct damage to the DNA, while UV-A is mainly associated with oxidative stress (Fisher et al., 2002; Xu and Fisher, 2005; Weihermann et al., 2016).

Besides UV rays, also other components of the solar spectrum play an important role, even if secondary, towards photoaging: this is the case of visible light and infrared rays (IRA). The latter are absorbed by different chromophores than UV, as probably the enzyme cytochrome c oxidase, cause damage to cells and have a procarcinogenic potential without having direct effects on DNA (Schroeder et al., 2008).

Other factors such as an unbalanced diet and cigarette smoking also contribute to extrinsic aging, causing a buildup of free radicals that damage the skin (Vlassopoulos et al., 2014). In addition, air pollution from transportation has also been shown to be associated with the appearance of signs of extrinsic skin aging (Vierkotter et al., 2011).

The effects and damage due to photoexposure and free radicals are cumulative and add up in both the epidermis and the dermis since childhood: these are particularly evident in those parts of the body that are constantly exposed such as the face, neck and upper hands (Makrantonaki et al., 2012).

Other peculiar features of extrinsic aging are cellular hyperplasia, which determines a significant increase in the thickness of epidermis and dermis, and the so-called "solar elastosis", characterized by an accumulation of elastic material at dermal level and in particular of elastin, fibrillin, versican (large proteoglycan) and GAG (mainly hyaluronic acid). The pathogenesis of solar elastosis has not yet been fully elucidated, but it is thought to be caused by both a process of degradation and one of *ex novo* synthesis of these components (Sellheyer, 2003).

There is a high percentage of fragmented collagen at the dermal level and a significant reduction in total interstitial collagen (Fisher et al., 2002).

First, the cellular machinery that damages skin connective tissue is initiated by photochemical generation of reactive oxygen species (ROS), which induces mutations in mitochondrial DNA, and alters nuclear gene transcription, all of which contribute to both the clinical and histologic features of skin aging. This process is further exacerbated by the depletion of protective antioxidants (as glutathione, tocopherol and ubiquinone) in response to UV radiation (Fisher et al., 2002).

As a consequence of ROS generation, activation of cell surface growth factor is one of the earliest events, followed by activation of cytokine receptors on the surface of keratinocytes and fibroblasts. Activated receptors stimulate signal transduction cascades that induces transcription factor AP-1, wich stimulates transcription of matrix metalloproteinase (MMP) genes and inhibits, in fibroblasts, procollagen gene expression. Matrix metalloproteinases break down collagen and imperfect repair

of the dermal damage impairs the functional and structural integrity of the extracellular matrix, results in characteristc wrinkling of photodamaged skin. Ultraviolet irradiation also activates the transcription factor NF-κB that stimulates transcription of proinflammatory cytokine genes, including IL-1β, TNF-α, IL-6 and IL-8, that act through their cell surface receptors to activate AP-1 and NF-κB and thereby amplify the UV response (Fisher et al., 2002; Xu and Fisher, 2005).

Destruction of collagen is a hallmark of photoaging. Skin fibroblasts in response to UV-B irradiation produce MMP-1 (matrix metalloproteinase-1), the major enzyme responsible for collagen digestion. Ultraviolet-induced MMP-1 initiates cleavage of fibrillar collagen at a single site within its central triple helix. Once cleaved by MMP-1, collagen can be further degraded by elevated levels of MMP-3 and MMP-9. In addition to degrading mature collagen, UV irradiation inhibits production of type I and type III procollagen gene expression (Fisher et al., 2002; Xu and Fisher, 2005).

Another direct reflection of UV radiation is the loss of cutaneous elastic properties due by change in elastin production. Similar to other molecular components of the extracellular matrix such as collagen, elastin is a protein synthesized by fibroblasts and keratinocytes, that is present in several connective tissues and confers a unique physiological elasticity. Photoaged skin may be characterized by a reduction in functional elastic fiber production and an increase in non-functional fiber production, leading to an accumulation of dystrophic elastotic material in the dermis known as solar elastosis. A potential explanation for this phenomenon is an alternative splicing UV-induced of the elastin gene, wich leads to inadequate synthesis of elastic fibers. Additionally, the dermal elastin network is destroyed and consequently loss of elasticity causes greater predisposition for the appearance of wrinkles and cellulite (Weihermann et al., 2016).

4.3.2 Solar UV Radiation

One of the major risk factors in the onset and development of skin diseases is exposure to ultraviolet (UV) radiation, which, although representing only 5% of the entire solar spectrum, have a powerful impact on human skin (Dupont et al., 2013).

UV radiation is divided into UV-A, UV-B, and UV-C.

UV-C rays are the high-energy ones, whose wavelength (λ) is between 200 and 280 nm. They are filtered by atmospheric ozone and, since they do not reach the earth's surface, they have no effect on the skin.

UV-B rays (λ: 280-315 nm) are only partially filtered by the atmospheric ozone layer and are biologically active. They penetrate up to the innermost layer of the epidermis, where they interact

with endogenous chromophores such as melanin. These rays are responsible for the onset of sunburn and erythema and are also able to cause direct damage to DNA through the formation of cyclobutane-pyrimidine dimers (CPDs) and 6-4 photoproducts (PPs), which cause gene mutations (Gilchrest, 1996).

UV-A rays (λ: 315-400 nm) are less energetic than UV-B, but more penetrating, as they are able to reach the dermis and their energy is absorbed not only by melanin, but also by other chromophores such as riboflavin and urocanic acid (Bäumler et al., 2012). Because of their ability to penetrate all the way to the dermis, they are considered to be the major contributors to chronic skin damage associated with photoaging (Gilchrest, 1996). Recent genetic studies have confirmed the high sensitivity of dermal fibroblasts to UV-A radiation (Battie et al., 2014).

UV-A, in the range between 360 and 380 nm, are immunosuppressive and this contributes to the development of skin cancer triggered by UV rays themselves, which directly or indirectly cause mutations at the level of genetic material (Halliday et al., 2011). However, the main visible harmful effects of UVA radiation only appear after years of exposure (Battie et al., 2014).

The UV-B portion of the sunlight spectrum is potentially the most toxic to tissues and affects in particular the skin and eyes; potentially because only repeated and prolonged exposure can determine degenerative changes in the skin, characterized by aging and possible pre-malignant and malignant lesions. The effects due to prolonged UV exposure are inflammatory and ulcerative of epidermis and dermis, followed by fibrosis, elastosis and atrophy of the epidermis. However, the toxic reaction can continue and result in irreversible damage with tumor formation (Moehrle, 2008). Due to increased human outdoor activities and the depletion of the ozone layer that absorbs most UV radiation, the number of skin cancers has increased significantly in recent years (Moehrle, 2008).

4.3.3 Reactive Oxygen Species

Oxidative damage and free radicals

We can talk about a so-called 'theory of oxidative stress' as a phenomenon concomitant to biological aging. This theory provides that, under physiological conditions, there is a balance between the endogenous production of free radicals and antioxidant systems. When this balance is altered it creates a potentially harmful condition. The progressive accumulation of metabolites of oxidative damage would lead to the senescence of cells and tissues and an imbalance in the homeostasis of the body. The term free radical refers to any chemical species, whether atom or molecule, that, by

containing one or more unpaired electrons in its outer orbit, becomes extremely unstable and reactive, which leads it to react with molecules close to it (Borellini, 2015).

Free radicals important to living organisms include: hydroxyl (OH^-), superoxide ($-O_2^-$), nitric oxide (NO^-), and peroxynitrite (RO_2^-).

Peroxynitrite (ONOO-), hypochlorous acid (HClO), hydrogen peroxide (H_2O_2), singlet oxygen (O^2), and ozone (O_3) are not radicals, but can easily lead to radical reactions. The term ROS is also often used to refer to non-radicals.

There are two main sources of ROS: a mitochondrial one, with a role in aging, and a non-mitochondrial one. The mitochondrial source is represented by the electron transport chain and the nitric oxide synthase reaction (Murphy, 2009). The rate of mitochondrial respiration is responsible for the amount of ROS generated: the higher the metabolic rate, the shorter the cell lifespan, with some exceptions (Cutler, 1976).

The production of mitochondrial superoxide radicals, mainly produced by keratinocytes and fibroblasts (Kammayer and Luten, 2015), occurs primarily in two enzyme complexes in the electron transport chain: complex I (NADH dehydrogenase) and complex II via FADH2, then they are transferred to complex III (ubiquinone-cytochrome c reductase) and eventually to complex IV (Murphy, 2009). Under normal metabolic conditions, complex III is the main site of ROS production. The weakness of this system consists in the formation of anionic species of the radical semiquinone ion (-Q-). Once formed, it can readily transfer electrons to molecular oxygen resulting in the generation of superoxide radical.

ROS can also be responsible for activating substances that lead to ECM degradation. For example, they lead to the formation of transcription factor complexes such as AP-1 and NF-κB that regulate the transcription of MMPs and TIMPs (Kammeyer and Luiten, 2015).

Human cells possess many mechanisms to protect against ROS damage; antioxidants, for example, combat ROS by functioning as reducing agents (Sies, 1997).

The most important defense system against ROS is glutathione peroxidase (GPx). It can remove singlet oxygen, hydrogen peroxide, and organic peroxides through a reaction with the thiol group of glutathione (Tyrrel and Pidoux, 1986). Other antioxidant enzymes are superoxide dismutase (SOD) and catalase, which remove superoxide radicals and hydrogen peroxide, respectively (Kammeyer and Luiten, 2015).

Skin cells are constantly exposed to ROS and oxidative stress. ROS production increases with age and the ability of cells to repair damaged DNA steadily decreases over the years (Pons et al., 2010).

Functionally, reducing the production of free radicals is the more efficient strategy than trying to neutralize them after they have been produced.
It appears that long-term exposure of cells to ROS triggers a vicious cycle resulting in reduced stress responsiveness, reduced ATP synthesis, and a further increase in ROS production in affected cells (Ma et al., 2009).

Since reactive oxygen species have been considered involved in connective tissue damage, there have been to better define the role of distinct ROS in the up-regulation of matrix-metalloproteinases which are responsible for the connective tissue degradation in photoaging, tumor invasion and metastasis.
In young skin, fibroblasts adhere to the intact extracellular matrix (ECM) while in aged skin, fibroblast attachment is impaired due to progressive ECM degradation, resulting in fibroblast size reduction, decreased elongation and collapsed morphology. Reduced size is a key feature of senescent fibroblasts, and is correlated to increased mitochondrial ROS generation (Shin et al., 2019).
Therefore, Human Dermal Fibroblasts (HDFs) in monolayer culture are considered a useful model to study some aspects of intrinsic and extrinsic skin aging since these cells are easily cultivated and responsive to various age-inducing stimuli. Senescent cells secrete a collection of cytokines, chemokines, growth factors, matrix metalloproteases that are responsable of connective tissue degradation and inflammation.
DNA is the main intracellular chromophore for UV-B. DNA bases are able to absorb photons from UV-B and this interaction results in increased ROS production and structural rearrangement of nucleotides. As a result, cells respond by processes to prevent further DNA damage and increasing the expression of senescence-associated genes (Cavinato and Jansen-Dürr, 2017).
During photoaging, dermal collagen becomes sparser, degenerated and changed in composition and elastin accumulates as elastic material due to abnormalities in its structure. In fact, in contrast to those in young skin, collagen fibrils in aged skin are fragmented and coarsely distributed. This process leads to clinical changes, such as wrinkling and loss of elasticity, which are observed in both naturally and photoaged skin (Cavinato and Jansen-Dürr, 2017; Shin et al., 2019).
This phenomena are caused by increased expression and activity of matrix metalloproteinases (MMPs) and by inhibition or decrease in the activity of the tissue inhibitors of metalloproteinases (TIMPs). This imbalace accelerates progressive fragmentation in the dermis, and accelerates skin aging. ROS are a major driving forxe behind the increase in MMP levels in aged skin.

Moreover, increment in ROS production generated by UVB irradiation leads to activation of transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) as well as to increased expression of protein kinases such as mitogen-activated protein kinase (MAPK), comprised of extracellular signal-regulated kinase (ERK), p38, and c-Jun NH_2-terminal kinase (JNK). This activation increases MMP expression and inhibits transforming growth factor-β (TGF-β) signaling, which leads to collagen fragmentation and decreased collagen biosynthesis. This hinders the mechanical interaction between fibroblasts and the extracellular matrix (ECM), and consequently reduces the size of dermal fibroblasts. Aged fibroblasts produce a greater amount of ROS that increases the expression of MMPs and inhibits TGF-β signaling, creating a loop that accelerates dermal aging (Cavinato and Jansen-Dürr, 2017; Shin et al., 2019).

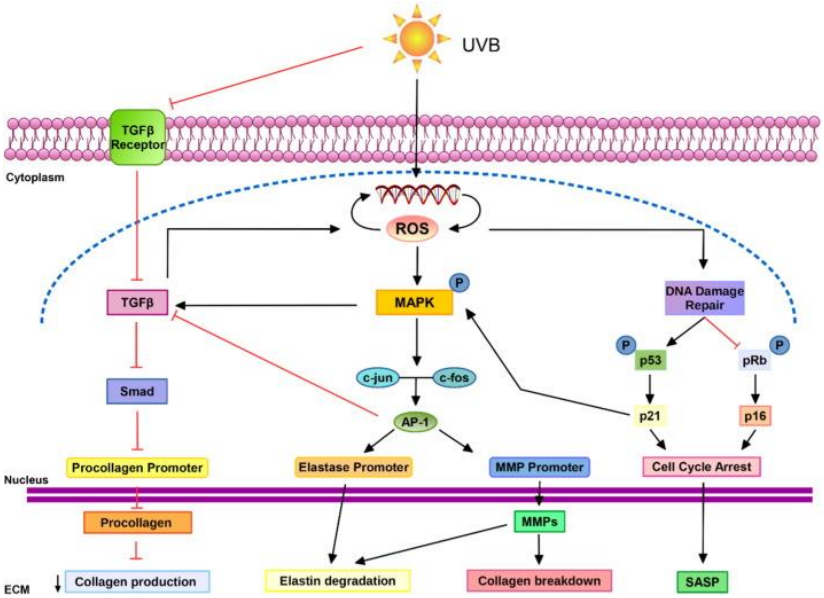


Figure 3: Schematic diagram showing the main signaling pathways involved in the process of UVB-induced photoaging. Adapted from:Cavinato and Jansen-Dürr, 2017

Understanding the molecular mechanisms of skin aging helps us to identify novel targets for antiaging treatments.

4.3.4 Inflammation

Inflammation is a response of the body to damage related to chemical or physical insults or to microorganisms and their toxins; it aims to inactivate or destroy the pathogen involved, remove irritants, and lay the foundation for tissue repair (Guzik et al., 2003).

The inflammatory process can be differentiated into acute or chronic. The acute form has a rapid onset (seconds or minutes), a short duration (up to a few days) and is marked by exudation (edema formation) and leukocyte migration.

Chronic inflammation has a longer duration and is associated with the presence of lymphocytes and macrophages, blood vessel proliferation, fibrosis, and tissue necrosis (Kumar et al., 2005).

The skin provides a barrier to protect the body from environmental insults including pathogens by maintaining temperature and controlling evaporation. Violations of this barrier are common occurrences and failure to restore this barrier function can cause health problems such as inflammatory skin conditions, defined as dermatitis, which are very common and have high morbidity. This group of diseases includes acute and chronic dermatitis, which have different etiology, pathological features, evolution, and cellular and molecular involvement, as described in the following paragraphs.

Dermatitis are inflammatory processes of the skin, with mono- or multifactorial etiology, with immune or irritative pathogenesis and with well-defined clinical pictures.

It is possible to distinguish dermatitis in acute forms, mostly consisting of irritative contact dermatitis (ICD), and chronic forms, represented by atopic dermatitis (AD) and allergic contact dermatitis (ACD). Among chronic dermatitis there are also xerosis and seborrheic dermatitis.

The above mentioned forms are the most frequent and the best recognized from a pathogenetic point of view, with different but consequential clinical aspects: the acute form is characterized by erythema, edema, vesicles or blisters, followed by serous exudation and formation of scabs, the detachment of which does not cause any outcome, except for transient dyschromic sequelae. The chronic form is characterized by a decrease of the previous symptoms and the onset of scaling, rhagades and lichenification. Both acute and chronic forms are accumulated by the scratching lesions due to the strong itching that characterizes the disease (Cainelli et al., 2000).

Dermatitis caused by air pollutants

Evidence shows a correlation between the level of environmental pollutants and inflammatory skin diseases, particularly evident in regions undergoing rapid industrialization processes. Epidemiological studies have shown an association between global warming, air pollution and allergic diseases. Several air pollutants, including volatile organic compounds, formaldehyde, toluene, nitrogen dioxide (NO_2), and particulate matter, act as risk factors for the development or exacerbation of dermatitis (Patella et al., 2020). In a recent paper, as a primary outcome, the authors evaluated the effect of weather and climate factors on the signs and symptoms of atopic dermatitis

using the Scoring Atopic Dermatitis (SCORAD) index. The results of the cited study demonstrated that climate change and air pollutants have a significant impact on skin reactivity and symptoms in patients with atopic dermatitis, increasing severity. Knowledge of the proportion of individual variables to symptoms and their severity could propose themselves as warning signs for exacerbations of the disease.

PM10 and volatile organic acid concentrations have been correlated with AD exacerbations. A study conducted in France, applying a complex dispersion model that combines traffic conditions, topography, meteorology and background traffic pollution, showed a significantly increased risk of developing asthma, rhinitis, eczema and pollen sensitization in children who had been living for at least three years in areas characterized by high concentrations of vehicular traffic pollutants, especially PM10 and benzene (Brunekreef et al., 2009).

4.3.5 Photocarcinogenesis

Skin cancer account for more than 40% of all cancers in the USA and continue to rise in incidence (Subhadarshani et al., 2020). Photocarcinogenesis is a multi-step model of cancer development in which UV-induced DNA damage leads to mutations resulting in activation of oncogenes or silencing of tumor-suppressor genes (Seebode, 2016).

A strong association exists between exposure to ROS and human skin cancer. Maybe UV-exposure and sunburns with subsequent influx of ROS generating inflammatory cells into the skin which play an important role in the etiology of cutaneous melanome. Exposure of fibroblasts to ROS generating systems reflects the situation that tumor cells may release high concentrations of ROS and thus stimulate synthesis of collagenase in the peritumoral fibroblasts. In fact, there is some evidence in the literature for this unique abuse of fibroblasts metabolism by tumor cell for their own advantage. Several genes have been studied that have important roles in skin carcinogenesis, including TP53. The basic function of p53 is to maintain a cell in normal status against various extracellular stress, or lead the cell into apoptosis when DNA is damaged in order to prevent carcinogenesis. A variety of cellular stresses, including UV-rays, active p53 by phosphorylation. The p53 gene is the most frequent target of genetic alteration identified so far in human cancers (Matsumura, 2002).

Botanical antioxidants have been traditionally used to prevent and treat skin cancer. Many botanical agents contain active principles of the polyphenol group with antioxidant and anti-inflammatory properties, cancer-preventive and anti-photoaging effects in the skin. Whereas photoaging and photocarcinogenic mechanisms are primarily induced by UV-radiation, the photoprotective

strategies include the blockage of UV radiation, DNA repair and removal of ROS with antioxidant agents. Many of these strategies involve active phytochemicals. This suggests the possibility that speific botanicals might be used in prevention and treatment of a variety of human skin disorders (Afaq and Mukhtar, 2006; Bosch et al., 2015).

5 CONVENTIONAL TREATMENT

As described above, prolonged exposure to environmental pollution causes premature aging and oxidative and inflammatory-related pathologies of the skin.

A good daily cosmetic practice is able to slow down and counteract the damage.

The cosmetic treatment of skin aging is mainly based on photoprotection, hydration, repair of the skin barrier, stimulation of cell turnover, collagen synthesis and anti-radical protection (Gao et al., 2008).

The modern cosmetic is a combination of active ingredients, preservatives, antioxidants and additives, linked by a formulation balance.

Among the best known active ingredients of anti-aging creams we find Hyaluronic Acid, which acts on hydration and thanks to its ability to swell and its small size can fit into the wrinkles filling them, and Retinol, a derivative of vitamin A that stimulates cellular turn over and also acts on the synthesis of collagen and elastin. These are often associated with a very important antioxidant, Vitamin C.

Antioxidants can regulate the transfer of electrons or quench free radical escaping from electron transport chain, therefore they are the best candidate to prevent damage inflicted by both UV and environmental pollution. UVR-induced skin damage is a rapid event, and antioxidants possibly prevent such damage only when present in relevant concentration at the site of action beginning and during oxidative stress (Poljšak and Dahmane, 2012).

Skin has a network of protective antioxidants. They include enzymatic antioxidants such as glutathione peroxidase (GSH), superoxide dismutase (SOD) and catalase, and nonenzymatic low-molecular-weight antioxidants such as vitamin E, vitamin C (ascorbic acid), uric acid and ubiquinol (Co-Q10). The levels of these antioxidants are reduced by intrinsic and extrinsic factors such as age and athmospheric aggressors. Restock these antioxidants either by topical application or by dietary supplementation can protect skin from ROS (McDaniel et al., 2018).

Vitamin C and Vitamin E slow the process of aging either by preventing free radicals from oxidizing or by reducing the formation of free radicals and quenching the already formed ROS (McDaniel et

al., 2018). In particular, vitamin C has long been used as an antiaging and hyperpigmentation topical agent for several decades. Ascorbic acid, in fact, eliminates most ROS due to the oxidation of ascorbate to monodehydroascorbate, and then to dehydroascorbate, and can maintain the normal physiological state of human skin. Moreover, it is a cofactor required for the synthesis of procollagen and elastin. Topical formulation containing ascorbic acid have clinical efficacy in antiaging treatments, however the poor skin penetration and chemical instability reduce the clinical efficacy of ascorbic acid and require additional research (Shin et al., 2019).

Vitamin E is the body's mayor lipid phase antioxidant and exists in eight molecular forms. It plays an important role in prevention lipid peroxidation. Vitamin E is found primarily in the stratum corneum and is the body's first line of defense against oxidative stress of sunlight and pollutants. Studies have shown that topically applied of vitamin E protects skin against UV-induced erythema, UV-induced lipid peroxidation, UV-induced photo-aging changes, Uv-induced immunosuppression, UV-induced photocarcinogenesis and inhibition of melanogenesis (Palmer and Kitchin, 2010).

Regarding Co-Q10, present in all human cells as part of the electron transportation chain, and its role in skin aging prevention, it may inhibit the production of IL-6, which stimulates the up-regulation of MMP. It was reported that Co-Q10 strongly inhibits oxidative stress in the skin induced by UVB via increasing SOD and glutathione peroxidase.

A number of experimental studies indicate also protective effects of β-carotene against acute and chronic manifestations of skin photodamage, revealing that β-carotene acts not only as an antioxidant, but also has unexpected prooxidant properties.

Uric acid should be important in quenching of singlet oxygen (Yamamoto, 2001).

In addition, antioxidants enzymes system such as GSH peroxidase and GSH reductase regenerate antioxidants, and other enzymes directly neutralize ROS, such as SOD, catalase and quinone reductases. Many of these enzymes are reduced in aging and photoaged skin (McDaniel et al., 2018).

The non-enzymatic antioxidant glutathione functions to maintain the exogenous antioxidants, such as vitamin C and E, in their reduced or active forms. Glutathione peroxidase (GPx) is an important intracellular enzyme that converts hydrogen peroxides to water and lipid peroxides to their corresponding alcohols mainly in the mitochondria and sometimes in the cytosol. This enzyme plays a crucial role of inhibiting lipid peroxidation process and therefore protects cells from oxidative stress.

The new great protagonists of cosmetics are biomedical peptides, amino acid sequences, as they are able to act on a well-defined biological target. There are many peptides, each of which has its own function and is able to act on different imperfections: wrinkles, dark circles, bags, skin spots, facial redness. Among the most used we find signal peptides, able to stimulate the synthesis of collagen and/or decrease its degradation; peptides that modulate neurotransmitters reduce or prevent the transmission of nerve impulses thus preventing the contraction of the muscle going to prevent or reduce wrinkles, and finally there are peptides that chelate metals: through the chelation of metal, useful to counteract air pollution (Lima and Pedriali Morales, 2018).

As for therapy in inflammatory skin conditions such as atopic dermatitis, we resort to the use of agents that intervene on the symptoms. The classic pharmacological treatment, almost always effective, generally refers to drugs such as cortisone or antihistamine. It is necessary to keep in mind, however, that, given the relapsing nature of skin sensitization stimulated by various factors, the treatment often involves prolonged periods of treatment that can cause the appearance of side effects. Obviously, the consideration just made assumes an even greater importance if we refer to the pediatric field.

Just because of the necessary limitations in the prolonged administration of synthetic drugs, it is interesting to consider the possibility of using remedies of natural origin that allow to face the problem of contact dermatitis in an effective and safe way, even for prolonged periods of time.

As we have just seen there are many active ingredients that can be used to slow or block the environmental impact on our skin. But these solutions are not without limitations and side effects.

It is for these reasons that recent trends in anti-aging research involve the use of natural products after scientific validation. Natural skin care products are rapidly absorbed by the surface layers of the skin and are usually hypoallergenic in nature. A large number of molecules from natural products can scavenge free radicals from skin cells, eliminate metabolic byproducts, prevent trans-epidermal water loss, include a sun protection factor (SPF) and help protect the skin from wrinkles, leading to a younger, healthier looking face. Today, herbal cosmetics are becoming increasingly popular due to their significant impact on aging skin. (Pandel et al., 2013; McDaniel et al., 2018).

5.1 Botanical Ingredients For Skin Protection

In order to be young longer, or at least *to look* younger, plant extract and herbal preparations have been used into topical skin care formulation since botanical ingredients have the power to reduce

the appearance of skin aging and enhance the beauty of the skin. There are some plants that can affect skin elasticity and tightness.

Medicinal plants have a lot of phytoconstituents such as polyphenols, alkaloids, tannins, saponins, carotenoids and terpenoids which possess antioxidant properties and can be used in treating the signs of aging. In particular, phenolic compounds have free-radical scavenging property. Numerous plants show capability of attenuate matrix skin degradation and inhibiting various enzymes such as hyaluronidase, elastase and MMPs, such as Chinese ginseng roots and cinnamon bark (Binic et al., 2013).

The list of botanical extracts with antiaging potential could be growing endlessly.

Several plant extracts exert a strong free radical scavengers capacity; indeed, botanical antioxidants have been shown to reduce the incidence of ROS-mediates photocarcinogenesis and photoaging and, since UV-B radiation plays an important role in cutaneous damage, they could be ideal photochemoprotective agents for skin aging and cancer. In general, these botanical antioxidants modulate cellular signaling pathways and protect the skin matrix through the inhibition of enzymatic degradation (Afaq and Muktar, 2010; Binic et al., 2013).

Plants produce many secondary metabolites to protect themselves from UV radiations and these molecules can be used as natural antioxidants able to protect the skin from photoaging. This has generated a great interest in using botanical supplements rich in antioxidants against photocarcinogenesis and prevent photoaging (Afaq and Mukhtar, 2010; Petruk et al., 2018).

Active compounds show not only antioxidant and antiaging properties but also anti-inflammatory, emollient, melanin-inhibiting and antimutagenic features. Several mechanisms are involved in order to protect the skin, among which are (i) absorbing UV radiations, (ii) inhibiting free radical reactions induced by UV in cells, and (iii) modulating endogenous antioxidant and inflammatory system (Petruk et al., 2018).

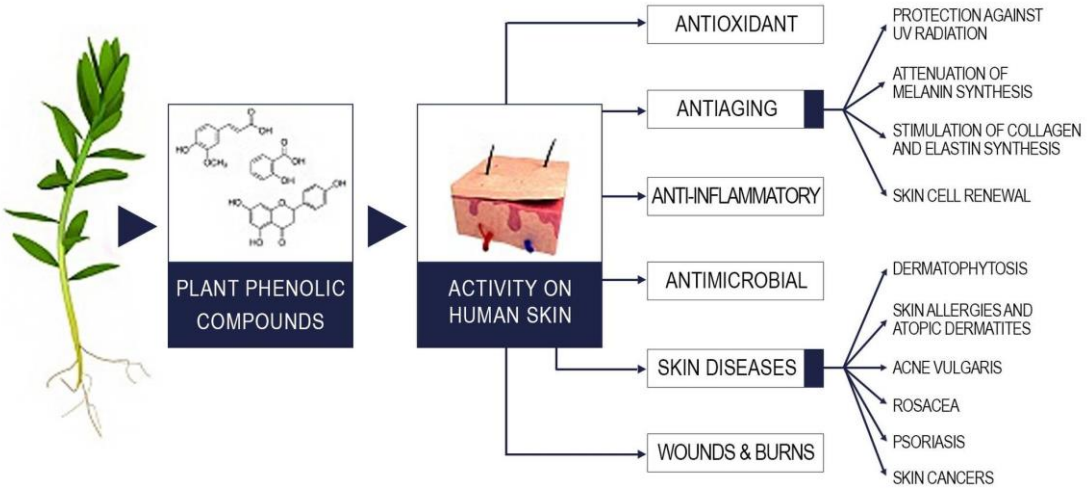


Figure 4: Potential role of plant phenolics in prevention and therapy of skin deseases. Adapted from: Działo et al., 2016

A great number of plant extracts are studied for their antioxidative action. Here some examples.

Fruit extract of *Coffea arabica* L. shows antioxidant activity due to the large amount of chlorogenic acid, a potent antioxidant polyphenol. The powerful antioxidant activity of the ethanol extract of licorice (*Glycyrrhiza glabra* L.) is attributed to the main constituent of *G. glabra*, glycyrrhizin and two molecules of glucoronic acid. Glycyrrhizin can be considered as a quenching agent of free radicals and blocking agent of lipid peroxidation chain reactions (Binic et al, 2013).

Green tea (*Camellia sinensis* (L.) Kuntze) is an abundant source of polyphenols: epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG) are the four major types. All of these act as potent antioxidants and ROS-scavengers, confering protective effects on the skin against UV irradiation (Afaq and Mukhtar, 2010; Roh et al., 2017).

Pomegranate (*Punica granatum* L.) extract possesses strong antioxidant and anti-inflammatory properties due to two types of polyphenolic compounds, anthocyanidins and hydrolyzable tannins (Afaq and Muktar, 2010; Binic et al., 2013).

The health-promoting effect of grape (*Vitis vinifera* L.) against aging have been demonstrated by several studies. This is due to the high content of resveratrol, a polyphenolic phytoalexin, which presents strong antioxidant, anti-inflammatory and antiproliferative activity (Afaq and Mukhtar, 2010; Petruk et al., 2018).

Extract of *Panax ginseng* C.A. Meyer have the ability to promote synthesis of collagen (Binic et al., 2013).

Polyphenol compounds	Natural sources
Resveratrol	Red grape skin, peanuts, mulberry, blueberry, and bilberry. Red wine contains significant amounts
Epigallocatechin-3-gallate (EGCG) and other epicatechins	Green tea, black tea
Rosmarinic, ursolic and carnosic acids and other pentacyclic triterpenes	Sage, rosemary, lavender, origan, thyme, peppermint, and other herbs
Ellagic acid	Pomegranate
Glycyrrhizic acid	Licorice

Table 1: Polyphenols and their natural sources commonly used in Skincare products to protect skin from photodamage. Adapted from: McDaniel et al., 2018

Phenolic compounds are a group of secondary metabolites, characterised by the presence of at least one phenolic ring and one or more hydroxyl substituents. The role of polyphenols in maintaining health and preventing disease is undisputed and has been attributed partly to their antioxidant properties and partly to the free radical scavenging capacity of these biomolecules (Bertelli et al., 2021).

Therefore, phenolic compounds, including flavonoids (catechins, isoflavones, proanthocyanidins and anthocyanins), phenolic acids (benzoic, gallic) and stilbenes, can prevent penetration of radiations into the skin, reduce inflammation and oxidative stress and, in addition, influence several signalling pathways through various mechanisms of action, such as inhibition of the ROS formation and neutralization of singlet oxygen; reducing the chelated metal ions, which catalyze reactions leading to the formation of ROS; blocking the cascade of free radical reactions in lipid peroxidation (Działo et al., 2016; Petruk et al., 2018).

Due to their natural origin, low toxicity and antioxidant properties, phenolic compounds are widely used as skin treatment to prevent skin disorders, both embarrassing minor problems (wrinkles, acne) or serious, as cancer. Dietary phenolic may influence the internal organ, however the most effective way of use it is surface and topical application (Działo et al., 2016).

In addition to antioxidants action, phenolic compound also have other important properties:

- *Anti-inflammatory properties*: phenols inhibit lipid peroxidation, pro-inflammatory mediators and neutralize the excess of free radicals produced during inflammation (Działo et al., 2016).

- *Antimicrobial action*: phenolic compounds possess potent antifungal, antiviral and antibacterial activity (Działo et al., 2016).
- *Stimulation of collagen and elastin*: phenolic compounds show suppressive activity against MMPs and elastases. However, despite the inhibitory activity against collagenases, the use of some polyphenols might be limited due to their molecular weight and lower permeability through the layers of the skin (Działo et al., 2016).
- *Decrease in melanin production*: melanin plays a crucial role in skin photoprotection, absorbing 50%-75% of UV radiations; an excessive irradiation may lead to dermal disorder, as hyperpigmentation. Several studies on skin cell cultures report that phenolic and flavonoidic compounds, thank to the anular ring, have similar structures to tyrosine, and therefore, they can act as false substrate inhibiting melanin synthesis. Resveratrol and oxyresveratrol are examples of melanin production inhibitor and they are supposed to act as tyrosinase inhibitor. However, these compounds are susceptible to photooxidation and their use in cosmetic formulation is limited (Działo et al., 2016).

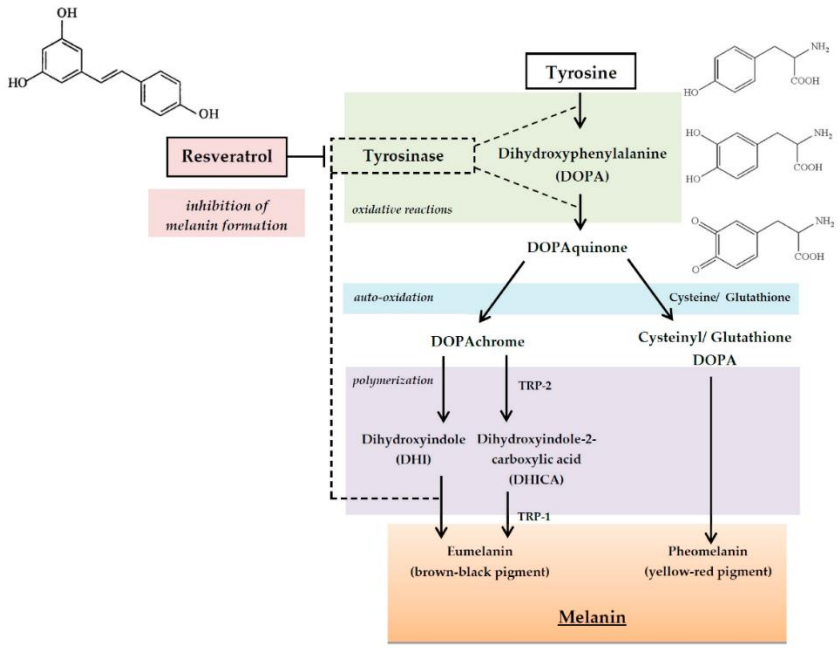


Figure 5: The involvement of the tyrosinase in the reaction of melanin synthesis and the inhibition of melanogenesis by the substrate analog inhibitor, i.e., resveratrol. Adapted from: Działo et al., 2016.

- *Anticancer properties*: phenolic compounds show anticancer action by inhibition of the proteasome, a multienzyme complex responsible for the degradation of numerous proteins involved in cell development. Catechin-3-gallate and epigallocatechin gallate, gallic acid, apigenin, curcumin and resveratrol are examples of natural compounds that inhibit

proteasome function leading to the inhibition of growth and spread of cancer cells (Działo et al., 2016).

In conclusion, phenolic compounds with free radical scavenging or antioxidant properties could be potentially effective in the treatment of various skin disorders. It is important to point out that because of their natural origin, plant extracts are often considered safe, but irritation, contact allergic dermatitis and other adverse reactions to natural product have been documented (Binic et al., 2013).

6 EXPERIMENTAL PART

6.1 Selection of the species of interest

6.1.1 Bibliographical research

The bibliographic research conducted in the first year of my Ph.D. program, mainly based on PubMed papers and supported by the starting of collaborations with other research groups, professionals and companies involved in the field of cosmetic and medical devices, allowed us to identify several Tuscan species with specific indication of skin protection.

We applied the following research criteria on PubMed: “ethnobotany + skin + (and or not + Tuscany)”; “herbal (or phytotherapy or extract) + skin (and or not + Tuscany)”; “herbal (or phytotherapy or extract) + skin + air pollution”; “herbal (or phytotherapy or extract) + skin aging”.

We identified more than 25 species of interest, typical of Tuscan territory or common in this zone.

As discussed extensively, among plant secondary metabolites, phenolic compounds play the most important role in the treatment of skin disorders due to their free radical scavenging or antioxidant properties, thus we applied a filter to screen species rich in polyphenols.

We added all valuable information obtained from Italian research groups involved in the sector of polyphenols, medicinal plants and skin protection: Institute on Research of Terrestrial Ecosystems, National Research Council, Florence Section, Sesto Fiorentino, Italy, Dr. Alessandra Bonetti and Cecilia Faraloni; University of Padua, Department of Pharmaceutical and Pharmacological Sciences, Prof. Monica Montopoli; University of Siena, Department of Biotechnology, Chemistry and Pharmacy, Prof. Stefania Lamponi; University of Milan, Department of Biomedical Sciences for Health, Prof. Alberto Bertelli.

According to the set screening program, then we rationally categorized all retrieved results in three subsets: species from the territory with established use in phytotherapy; species considered for their ethnobotanical traditional use in Tuscany; by-products obtained from food industry.

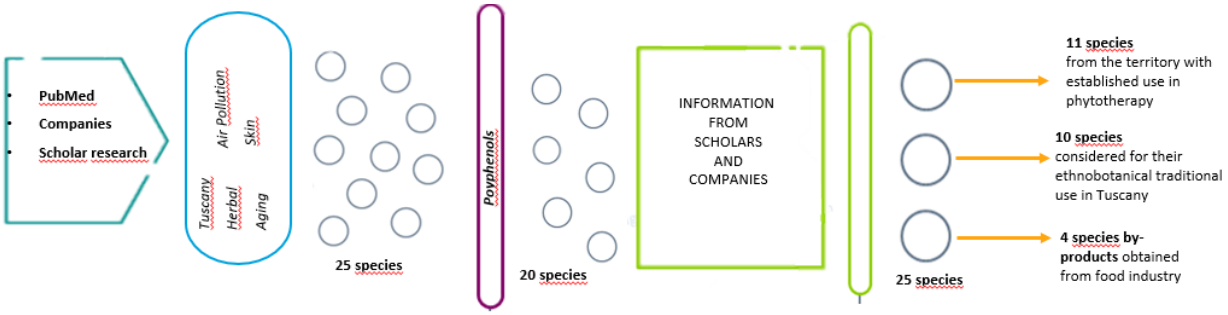


Figure 6: Screening program for species

We rated all bibliographical results according to some features in order to finalize the screening:

- Tuscan ethnobotany interest;
- Availability of herbal material;
- Scientific novelty;
- Papers on preliminary phytochemical and biological data;
- Marketing authorization.

One to five stars were assigned to each feature.

Species with 23 to 25 points were finally taken into account. Nine species resulted eligible.

As starting herbal products, the following drugs were considered:

- Species from the territory with established use in phytotherapy:
    - leaves and twigs of *Rosmarinus officinalis* L. (fam. Lamiaceae),
    - leaves of *Salvia officinalis* L. (fam. Lamiaceae),
    - flowering tops of *Hypericum perforatum* L. (fam. Hypericaceae),
- Species considered for their ethnobotanical use in Tuscany:
    - leaves of *Sedum telephium* L. (fam. Crassulacee),
    - leaves of *Balsamita major* Desf. (fam. Asteraceae),
    - flowering tops of *Calluna vulgaris* (L.) Hull. (fam. Ericaceae), mainly known as urinary disinfectant in Tuscany, but recently studied for skin photoprotection;
    - aerial parts of *Cistus creticus* L. subsp. *eriocephalus* (Viv.) Greuter & Burdet (fam. Cistaceae), mainly known for respiratory complaints, but recently studied for skin antioxidant protection;
- By-products obtained from Tuscan food industry:
    - leaves of *Castanea sativa* Mill. (fam. Fagaceae)
    - leaves of *Juglans regia* L. (fam. Juglandaceae)

6.1.2 Samples Collection And Preliminary Chemical Analyses

Collection

Samples were collected in south central Tuscany from healthy, pest-free wild plants or in private gardens.

The samples were collected during the full bloom stage.

- leaves and twigs of *Rosmarinus officinalis* L.: collected in Val d'Orcia, Siena in May;
- leaves of *Salvia officinalis* L.: collected in Val d'Orcia in April;
- flowering tops of *Hypericum perforatum* L.: collected in Siena, in June;
- leaves of *Sedum telephium* L.: collected from domesticated plants, private garden in Castelnuovo Berardenga, Siena, in July;
- leaves of *Balsamita major* Desf.: collected from domesticated plants, private garden in Fiesole, Florence, in May;
- flowering tops of *Calluna vulgaris* (L.) Hull.: collected in Maremma (Monteleoni, Grosseto), in August;
- aerial parts of *Cistus creticus* L. subsp. *eriocephalus* (Viv.) Greuter & Burdet: collected in Cala Violina, Grosseto, in August;
- leaves of *Castanea sativa* Mill.: collected in Amiata Mountain, Arcidosso, Grosseto, in October;
- leaves of *Juglans regia* L.: collected in Amiata Mountain, Arcidosso, Grosseto, in October.

The plant material was spread in a single layer and turned during drying. It was air-dried at room temperature until it reached a constant weight. After drying they were put in hermetically sealed containers in a dry place to avoid reabsorption of humidity from the air.
On the other hand, fresh leaves of *S. telephium* were pressed and juice obtained and analyzed.
Prof. Elisabetta Miraldi (University of Siena) identified the specimens and a voucher specimen was deposited in the Herbarium of the University of Siena, (SIENA), Italy.

Extraction

After collection, the sample were air dried and extracted in static conditions with ethanol 60% V/V for 72 hours. The drugs:extract ratio (DER) was 1:10.

Analysis of the total polyphenolis content

The amount of total phenolics in the extract were determined by the Folin-Ciocâlteu colorimetric method based on the reduction of a phosphowolframate-phosphomolybdate complex by phenolics to blue reaction products.

10 µL of each samples were diluted in the extraction solvent to a final volume of 3 mL into clean test tubes. 500 µL of Folin-Ciocâlteau reagent (Sigma-Aldrich, Milan), previously diluted 1:10 in distilled water, were pipetted to each tube and shaked; then were added 1 mL of sodium-carbonate solution (anhydrous sodium carbonate, Na_2CO_3, made up into a 30% w/v solution). Mixtures were kept in dark ambient conditions for 2 hours to stabilize the reaction. Distilled water were used as a blank.

The samples were scanned in UV/Vis spectrophotometer VICTOR® Nivo™ 3s, (Perkin-Elmer Italia, Milan) at 700 nm.

The measurements were performed in triplicate.

The total phenolic content were determined using a standard curve constructed with gallic acid (10000-78 mg/L, R^2>0.98) standard grade (Sigma-Aldrich, Milan). Results were expressed as % of total phenolics on the basis of dry weight of herbal material ± standard error mean (SEM) (Biagi et al., 2014).

The preliminary phytochemical analyses allowed us to verify that many of the considered species contain a high content of total polyphenols, more than 3% m/m dried weight (dw). Only *S. telephium* and *B. major* resulted less enriched.

The by-products from walnut and chestnut from Tuscany confirmed to be a valuable source of antioxidant metabolites; the same for *C. vulgaris* and *C. creticus*, species of ethnobotanical interest, but to date scarcely investigated for their potentiality in Italy.

Due to the non-significant inferiority of polyphenols content in the above species in comparison with well known and more investigated species such as *H. perforatum*, *R. officinalis* and *S. officinalis*, the experimental protocol was focused on four selected species: flowering tops of *C. vulgaris,* aerial parts of *C. creticus* subsp. *eriocephalus,* leaves of *C. sativa*, leaves of *J. regia*.

These species are described in 6.1.3-6.1.6.

6.1.3 *Calluna vulgaris* (L.) Hull

Domian: Eukaryota

Kingdom: Viridiplantae

Phylum: Streptophyta

DivisionClass: Magnoliopsida

Subclass: Asteridae

Order: Ericales

Family: Ericaceae

Genus: *Calluna*

Species: *Calluna vulgaris* (L.) Hull

Botanical description

The genus Calluna (fam. Ericaceae) is monotypic and therefore represented by a single species: *Calluna vulgaris* (L.) Hull.

The common name of the species "brugo" and the Italian term "brughiera" both derive from the Celtic language (Marcuzzi 1979). The English it is called heather.

Heather is a gregarious species which, depending on its development, can occur in compact and dense formations as bushes, shrubs or dwarf trees. It is an evergreen shrub of modest size (i.e. from 10 to 150 cm high), very branched and winding, with erect branches, or climbing or prostrate, and tough woody stems, covered with hairs only in the early stages of growth.

The leaves are very small, varying in colour depending on the season (e.g. dark green in summer, more reddish-brown in winter), simple, linear-lanceolate, persistent and scale-like, arranged in four very regular longitudinal lines along the stems, 1-5 mm long, about 1 mm wide, with entire margin and upper grooves.

The hermaphrodite flowers are whitish-pink in color and are gathered in unilateral racemes at the apex of the branches and generally have a 5 mm bell-shaped corolla with four separate petals, a more developed calyx enveloped on the outside by four ciliate bracts, eight stamens shorter than the corolla, the anthers with forward-facing filiform appendages and the style longer than the calyx.

The fruit is a 1.5 mm globular capsule, hairy, divided into four compartments and enveloped by the calyx when ripe. The capsule contains numerous brown, oval, spotted seeds.
The plant flowers in July-August, after leafing ("Scheda IPFI Acta Plantarum - *Calluna vulgaris* (L.) Hull" 2017; Australian Government 2003; Pignatti 1982).

Geographic distribution

C. vulgaris is widespread in Europe, North Africa, Asia and North America.

The brugo is present in areas of temperate-cold Europe characterized by high rainfall and short summer periods. In Italy, its distribution area includes the entire Alpine system and the northern Apennines areas up to 2,500 m altitude. As far as Tuscany is concerned, some populations can also be found in Maremma (Grosseto) and in the province of Siena. The species is very frequent towards Viareggio, descending almost to the sea, while towards the south it becomes rarer: the southernmost populations are found in Tuscany in the area of Follonica.
C. vulgaris is one of the dominant species of heathland and appears both in woodland and in open environments, often after fire. It has a great ecological range, as it is able to colonize lowland environments, hills, mountains up to 2000 m, maximum 2750 m high ("Scheda IPFI Acta Plantarum - *Calluna vulgaris* (L.) Hull" 2017; Australian Government 2003; Pignatti 1982).

The drug

The drug, the part of the medicinal plant which, in its dried state, is used for medicinal purposes, consist in the flowering tops, as in other Ericaceae.

Active ingredients

In 2010, Monschein and colleagues published a paper with a comprehensive overview of the findings on the phytochemical profile of the aerial parts of *C. vulgaris*.

From the data available to date, a complex pattern emerges with a predominance of the phenolic component, represented by flavonoids, chromones, procyanidins, phenolic acids and phenols, most of them in glycosidic form. Acetylated flavonol glycosides include galactosides and arabinosides,

which are specific to the Ericaceae family. The main phenolic compounds found in calluna are chlorogenic acid, callunin, hyperoside, isoquercitrin and keampferol-3-O-galactoside. Other compounds found are sterols, triterpenes, common fatty acids and ascorbic acid (Rodrigues et al., 2018; Mandim et al., 2019).

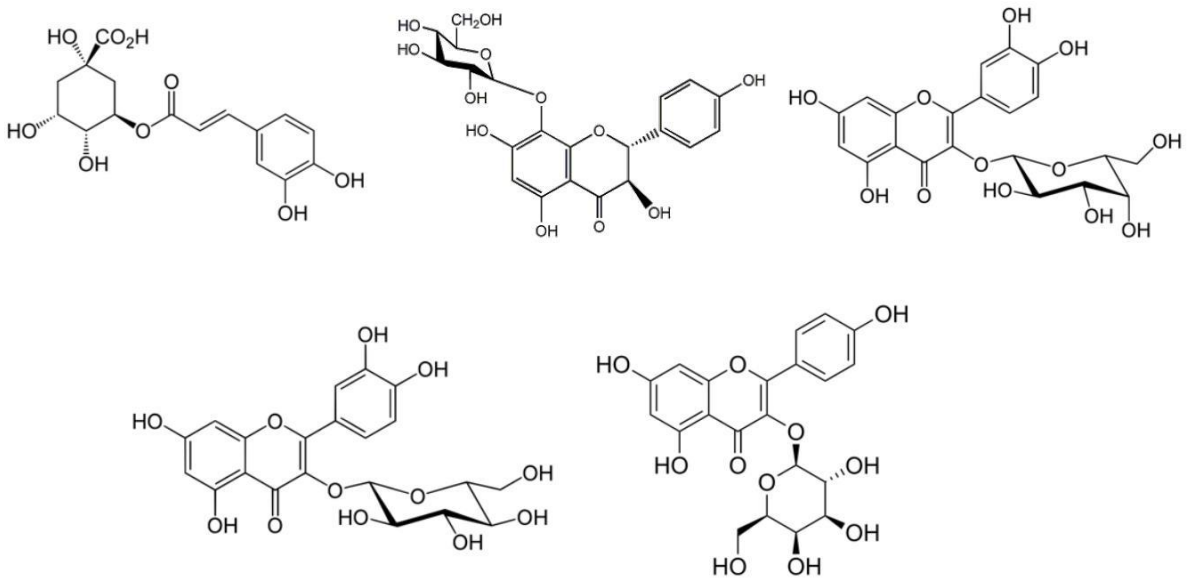


Figure 7: Chemical structures of the main phenolic compounds of C. vulgaris; from top left to bottom right chlorogenic acid, callunin, hyperoside, isoquercetin and campferol-3-O-galactoside

Traditional medicine uses

For centuries, the herbal teas obtained from the flowering tops and leaves of the brugo, have been known for their antiseptic and inflammatory action, used as a remedy for a variety of inflammatory disorders affecting the kidneys and urinary tract, such as cystitis with pyuria, colibacillosis, urethritis, cystopyelitis, chronic cystitis and prostatic cystitis (Monschein et al. 2010). This is the main traditional use of this species in Tuscany and all over Italy.

Preparations based on the aerial parts of *C. vulgaris* have been currently found with this indication also in the ethnobotany of Bosnia, Herzegovina and Sweden, and with that of antituberculars in Scotland and with that of antidepressants in Denmark (Jäger et al. 2013; Gordien et al. 2010; Redžić 2007; Tunon et al. 1995).

Pharmacology

Modern literature on *C. vulgaris* is rather poor and there are no clinical trials.

To date, the most important pharmacological activities ascribed to the aerial parts of *C. vulgaris* and obtained by means of *in vitro* tests are antibacterial (Vučić et al. 2014; Gordien et al. 2010; Braghiroli et al. 1996), antioxidant and anti-inflammatory (Dróżdż et al. 2017; Deliorman-Orhan et al. 2009; Pavlović et al. 2009; Calliste et al. 2001), which can be correlated with the high polyphenol content. Some of the aforementioned activities have recently led researchers to hypothize a cosmetic application for this species, encouraging them to perform *in vitro* (Olteanu et al. 2014; Perde-Schrepler et al. 2011) studies in order to assess its effectiveness in preventing photoaging (Filip et al. 2012; Filip et al. 2011a; Filip et al. 2011b).

The only *in vivo* study confirmed the chemo-preventive effect of a hydrogel containing a water *C. vulgaris* extract in UVB-exposed hairless mice (Olteanu et al. 2012).

6.1.4 *Cistus creticus* subsp. *eriocephalus* (Viv.) Greuter & Burdet

Domian: Eukaryota

Kingdom: Plantea

Division: Magnoliophyta

Class: Magnoliopsida

Order: Violales

Family: Cistaceae

Genus: Cistus

Species: *C. criticus*

Subspecie: *C. creticus* subsp. *eriocephalus* (Viv.) Greuter & Burdet

Other synonyms by which this species is commonly known are: *Cistus garganicus* Ten., *Cistus villosus* Auct. an L. and *Cistus incanus* L., the outdated scientific binomial by which it is universally known. For the sake of simplicity, from now on, in this work this entity will be referred as *Cistus incanus* (Pignatti, 1982).

Botanical description

C. creticus subsp. *eriocephalus* is an evergreen bush 30-100 cm high, not slimy, with an herbaceous smell, it has very branched stems, woody at the base, woolly and white-greyish at the apex, covered with simple hairs mixed with star-shaped hairs and reddish-brown bark. The leaves are opposite, with a short petiole (3-15 mm) dilated and tapering at the base, with an oval or elliptical lamina (1-2 x 2-4 cm), wrinkled and reticulate, with a flat or slightly wavy margin, edged with a white felt of star-shaped hairs; the lower page has a very evident pinnate-reticulate vein. The inflorescence is in terminal pauciflorous cymes with hermaphrodite flowers arranged on sturdy, hairy 5-15 mm peduncles at the axil of linear bra5eiform leaves; The flowers have a persistent calyx with 5 triangular, subequal, acuminate sepals with long, villous hairs that completely cover the stellate hairs and a dialipetal, soon to be deciduous, corolla with 5 large, pleated, 2-3 cm petals, deep pink or light purple (rarely white) with a yellow nail.

The stamens are numerous, orange-yellow in colour; the ovary is superficial with an elongated filiform style. The fruit is an ovate, hairy, brown capsule, dehiscent by 5 valves, containing numerous small polyhedral seeds.

The genus *Cistus* belongs to the Cistaceae family, to which three different genera belong: Helianthemum, Halimium and Cistus (Agnolucci and Meggiato, 2019).

Geographic distribution

C. creticus subsp. *eriocephalus* is a typical species of the Mediterranean area. It grows in the Lauretum as a plant in the undergrowth of the Mediterranean maquis or as a floristic component of degraded maquis and garrigue. It can be found on the islands, in Liguria and in all of peninsular Italy, but it can also be found in the north, even in Emilia-Romagna in the coastal area (backdunes of the Lidi Ferraresi) and in the hills of Romagna. It is also present in isolated stations on the Veneto coast. Each of these areas, in turn, is colonized by different species of cistus depending on climatic and soil conditions.

The drug

The drug of *C. creticus* subsp. *eriocephalus* consist in the aerial parts (Barrajón Catalán et al., 2011).

Active ingredients

Mediterranean bush species, such as *C. creticus* subsp. *eriocephalus* are naturally rich in polyphenols and could represent a source of bioactive compounds for the development of new drugs (Gori et al., 2016). Polyphenols can be divided into three categories, based on chemical structure. The first category includes gallic acid, ellagic acid and gallo/ellagitannins. The second category consists of flavonoids, while the third is composed of derivatives of phenolic acids (Barrajón-Catalán et al., 2011). In particular, the main polyphenolic constituents of cistus extract are myricetin glycosides (61.81 g/100 g of dry mass of the extract), including myricetin 3-ramnoside, which accounts for 47.3% of the mass of the extract. Other polyphenols present in the extract (although in much lower content) are quercetin glycosides (9.28 g/100 g), gallocatechins (6.27 g/100 g), epicatechins (3.07 g/100 g), kaempferol glycosides (1.97 g/100 g) and bis-hexahydroxydiphenol-glucose (1.22 g/100 g). Gallic acid, punicalagins, cornussin B, prodelfinidine and pendunculagin are present in very low quantities (<1 g / 100 g) (Moreira et al,2017).

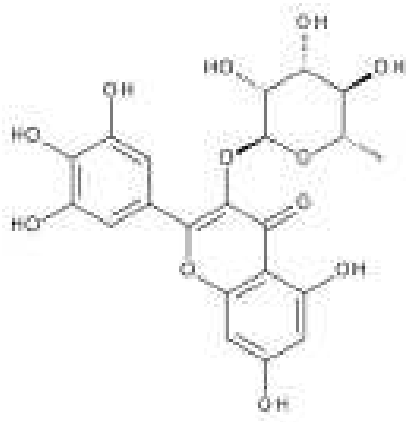


Figure 8: Chemical structure of myricetin 3-ramnoside

Traditional medicine uses

Traditionally, cistus is used to treat diarrhoea and as an anti-inflammatory. In the Mediterranean area, the Middle East and North Africa, herbal teas obtained from *C. creticus* subsp. *eriocephalus* aerial parts are used in situations of diarrhoea, peptic ulcer, skin problems, as an anti-inflammatory, and antispasmodic (Agnolucci and Meggiato, 2019); in Tuscany and Central part of Italy cistus tea are also used to treat symptoms of common cold.

On the Island of Giglio (Grosseto), the species C. creticus, has been used since time immemorial in medicine as an anti-inflammatory and healing agent. With the leaves

leaves are used to prepare an alcoholic macerate, known as "cistosina", which is applied to burns, sores, dermatitis. (D'Alonzo, 2002).

In the past, cistus was mainly used in cosmetics for its so-called labdanum, the gum-resin covering the leaves and stem, which has a very pleasant fragrance. Its sweet, vanilla and amber aroma was much loved by the ancient Egyptians, who used it in traditional blends such as Kyph. It is still used today as a fixative and/or fragrance component in lotions and perfumes. Labdanum is also known for its antiseptic effect against bacterial infections and is used in cosmetics for impure or inflamed skin. It was used internally in ancient times for catarrh and diarrhoea.

Pharmacology

Modern scientific research studied this medicinal plant and clinical trials and studies on animal models highlighted the potential antiviral effect of polyphenols in leaves and aerial parts. In addition, cistus polyphenols have demonstrated *in vitro* good antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus hirea* and *Pseudomonas aeruginosa*

(Agnolucci and Meggiato, 2019). More recent literature has highlighted the antioxidant and anti-inflammatory activity of cistus constituents. The high content of polyphenolic compounds in plant extracts is often correlated with significant antioxidant activity due to the proven reactive oxygen species (ROS) scavenging potential of several polyphenols (Agnolucci and Meggiato, 2019).

6.1.5 Castanea sativa Mill

Domain: Eukaryota

Kingdom: Plantea

Division: Magnoliophyta

Class: Dicotiledoni

Order: Fagales

Family: Fagaceae

Genus: *Castanea*

Species: *Castanea sativa* Mill.

Botanical description

Castanea sativa Mill. is a tree with a height of 10 to 30 meters; It has a rounded crown and a columnar stem with smooth, glossy, brown to grey bark.

The leaves are large and lance-shaped, with serrated margins, prominent veins and a sharp apex. The leaves are alternate with a small petiole, up to 22 cm long, 10 cm wide and leathery.

The chestnut tree produces both female and male flowers: the latter are whitish and grouped in glomerules, which in turn form erect catkins, while the former group together in a variable number of 1 to 3 in an involucre known as a dome, which later turns into a husk.

After fertilization, the fruits are formed and remain enveloped in the husk until they ripen in autumn (Roy, 1987).

The fruit is an achene, called chestnut, with a dark brown leathery pericarp, shiny on the outside and hairy on the inside. The shape is round, with one side flattened and one convex. Inside the spiny involucre one can find a single fruit or 2-3 ripe fruits.

Geographic distribution

C. sativa is a long-lived tree that is native to southern Europe, North Africa and western Asia, but is also widespread on the Atlantic coast of Morocco, on the shores of the Caspian Sea and in southern England.

The chestnut tree grows well in temperate and humid climates but can withstand cold temperatures of several degrees below zero. It prefers acidic, deep, fertile soil with a pH value of around 6.5 and cannot be grown in overly compact or calcareous substrates.

The area of italy richest in chestnut groves is Tuscany. There are three main cultivar, Bastarda rossa, Marrone and Cecio. Each cultivar differs, to a greater or lesser extent, in shape, size, colour of the skin, shape of the hilum, inner skin of the fruit and in its organoleptic characteristics.

The drug

The chestnut tree has food, nutraceutical and cosmetic functions provided by several components of the plant.
In the nutraceutical sector, the Italian Ministry of Health recognizes the possible use of the gems, leaves and fruits. Three health claims are associated with the leaves: "fluidity of bronchial secretions; regularity of intestinal transit; antioxidant.
The gems and leaves are used in cosmetics.

Active ingredients

The phytochemical composition of chestnut leaves is based on polyphenols; more specifically, there are high concentrations of tannins and flavonoids.

In a lesser extent we also find cinnamic derivatives with chlorogenic acid predominating (Almeida et al., 2010).

The tannin fraction is the most abundant, with a concentration varying between 6% and 8% of the dry weight of the leaf and is mainly made up of ellagic acid polymers such as pedunculagin, tellimagrandin I and II, castalagin, vesalagin (Basile et al., 2007; Almeida et al., 2010).

The flavonoid fraction, on the other hand, is represented by a class of secondary constituents that are widespread in the plant kingdom, usually in the form of glycosides, and well known for their

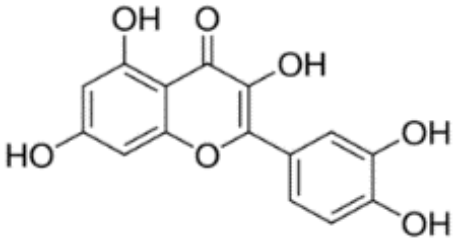
beneficial properties in human health, such as, for example, anti-inflammatory, antiviral, anti-allergic, anti-proliferative and anticarcinogenic activity.

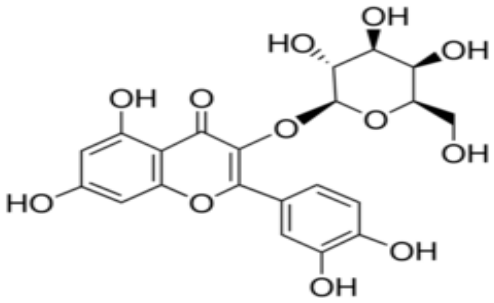
The wide variety of biological actions attributed to flavonoids depends mainly on their antioxidant activity in blocking free radical damage related to many chronic degenerative diseases (Morelli et al., 2005).

Flavonoids are present in percentages varying between 0.1% and 0.3% of the dry weight of the drug and among them are prevalent rutin, hesperidin, quercetin (and the derivatives quercetin 3-O-rutinoside and quercetin 3-O-glucoside), hyperoside (quercetin 3-O-galactoside), apigenin, morin, galangin, kaempferol (and kaempferol-3-O-rutinoside and kaempferol-3-O-glucoside derivatives), isoquercitrin, myricetin (and myricetin 3-O-glucoside derivative) (Basile et al., 2007; Almeida et al., 2010).

In the plant kingdom, phenolic compounds are synthesized by plants themselves in response to microbial infection (Cowan, 1999): in particular, by forming hydrogen bonds with the membrane proteins of microorganisms and causing changes in membrane permeability, they can lead to cell death.

Thus, phenols act as natural antimicrobials that inhibit the growth of pathogenic microorganisms (Rains and Jain, 2011). In humans, as outlined above, they have a strong antioxidant action.





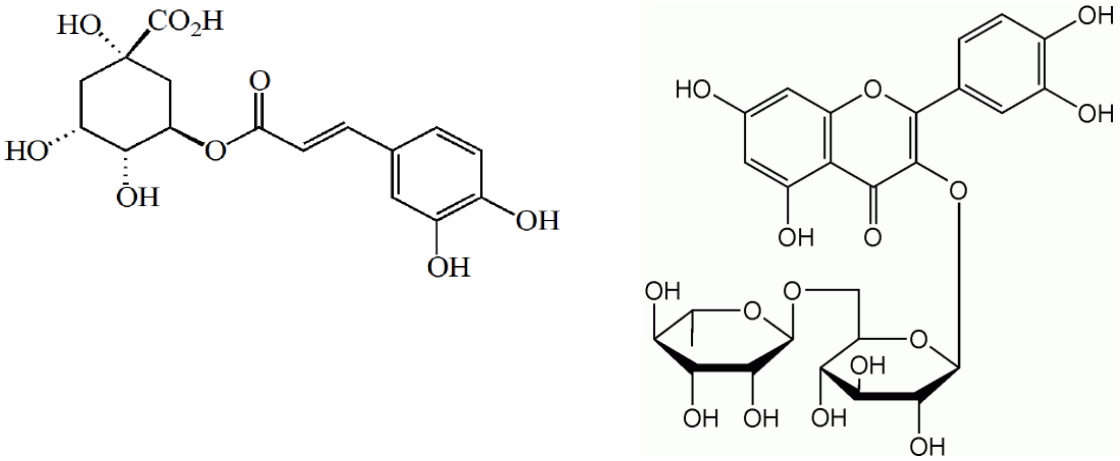


Figure 9:Structure formula of quercetin and hyperoside (above), chlorogenic acid (below left) and rutin (below right).

Traditional medicine uses

The development of the species was favored by the possible dual use of *C. sativa* by the population: the fruit as a starchy resource and the bark for making barrels, beams and poles for growing vines.

Thanks to its fruit rich in starch, vitamins and mineral salts, the chestnut tree was known as the 'bread tree'.

The use of *C. sativa* is also known in phytotherapy.

The fruit, being rich in minerals and vitamins, is particularly suitable for those suffering from anemia, chronic fatigue, psychophysical exhaustion and for those going through a period of convalescence following some illness, especially children and the elderly.

The bark, in the form of decoction, thanks to its high tannin content, has always been used as an astringent in cases of diarrhea and dysentery, and is an excellent skin astringent in cases of sebaceous hypersecretion. The tannins have a strong affinity for proteins and exert an anti-diarrheal action by forming a protective layer of coagulated proteins on the intestinal mucosa, which reduces the stimulus to intestinal peristalsis, limits the passage of pathogenic microorganisms and inhibits the action of inflammatory proteins. The same mechanism of action allows tannins to exert a gastroprotective action (Almeida et al., 2010).

Chestnut leaf infusions have long been used to treat diarrhea, rheumatism, whooping cough and, in general, all coughs of a convulsive nature (Dìaz Reinoso et al., 2012) as they have an effective astringent, calming and sedative action (Maugini et al., 2006).

Pharmacology

Flavonoids including quercetin, rutin, hyperoside, hesperidin, apigenin, morin, galangin and kaempferol have been identified in *C. sativa* leaf extracts. The composition of chestnut leaf extract also includes ellagic acid derivatives (Basile et al., 2000).

Skin-protective activity of *C. sativa* leaf extracts have been mostly investigated in *in vitro* tests: in 2008, a study carried out on an ethanol:water (7:3) extract of *C. sativa* Miller leaves from Mirandela (Portugal), demonstrated the *in vitro* efficacy of the extract against reactive oxygen and nitrogen species, closely associated with the high presence of polyphenols (Almeida et al., 2008). Some studies (Hseu et al., 2012) report that ellagic acid protects HaCaT cells from UVA-induced DNA damage and attribute this protective effect to increased nuclear translocation and Nrf-2 activation. In 2015, Almeida et al. tested another chestnut leaf but they found no evidence on Nrf-2 upregulation in HaCaT, probably due to a lesser amount of ellagic acid present in the extract (Almeida et al., 2015).

Recent papers also allow us to obtain information on the antioxidant activity of individual flavonoids present in chestnut leaves, such as quercetin, rutin and apigenin, and to assess its possible role in combating skin ageing and, in particular, in limiting photo-ageing. Antioxidant activity has also been demonstrated by non-flavonoid phenolic compounds such as chlorogenic acid: a study highlighted the ability of chlorogenic acid to suppress the expression of matrix metalloproteases and increase the synthesis of type I procollagen in UVB-stimulated mouse fibroblasts. In addition, this compound is also able to inhibit the intracellular production of reactive oxygen species (Cho et al., 2017).

The research group of Almeida and colleagues (Almeida et al., 2008; Almeida et al., 2015) published some preliminary clinical data and tested the skin tolerability of the hydroethanolic chestnut leaf extract by means of a validated patch test on 20 volunteers.

Preliminary findings on the biological effects of Portuguese chestnut leaf extracts at topical level prompted the investigation of Italian varieties as well.

6.1.6 *Juglans regia* L.

Kingdom: Plantae

Clade: Tracheophytes

Clade: Angiosperms

Clade: Eudicots

Clade: Rosids

Order: Fagales

Family: Juglandaceae

Genus: *Juglans*

Species: *Juglans regia* L.

Botanical description

The name *Juglans* originates from the Latin *Jupiter* and *glans* (acorn) thus meaning “Jupiter nuts”.

Juglans regia L., commonly known as walnut tree, belongs to family Juglandaceae and is the most widespread and cultivated nut tree worldwide, due to its edible kernel. It is a large, deciduous frost-tender tree growing to 20 m height.

Main bloom time is in June, while the seeds ripen in October. The species is monoecious, that is flowers are either male or female, but both can be found on the same plant. It is pollinated by wind (www.pfaf.org).

The leaves of the common walnut is imparipinnate with 7-9 single leaves. The leaflets are oblong ovate and up to 12 cm (4.7 in) long.

The leaves have a very strong, characteristic smell, aromatic and not unpleasant, but said to be injurious to sensitive people. They have three, sometimes four pairs of leaflets and a terminal one, the leaflets varying in size on the same leaf, being 2 1/4 to 4 inches in length and 1 to 1 1/2 inch wide, entire, smooth, shining, and paler below.

Geographic distribution

J. regia is a long-lived tree, may reach one thousand years old. Because of its long history of cultivation, the natural distribution range of this species is not clear. Probably it is native to the Mediterranean area, however it has been cultivated in the northern hemisphere and, apart from norther regions, it can be found in most of Europe (de Rigo et al., 2016). The common walnut requires special site conditions since is highly susceptible to winter. It, in fact needs a warm and sheltered site and it also prefers soils with pH values between 6 and 7,5. However, older trees are able to resist winter temperatures low as -30 °C.

The common walnut is susceptible to a number of parasites. The main fungal diseases affecting the tree are *Armillaria mellea*, *Phytophthora cinamomii* and *P. cambivora*, while antracnosis (*Gnomonia leptostyle*) causes summer leaf fall. Another serious disease, sometimes causing mortality in young tree, is walnut blight (de Rigo et al., 2016).

The drug

J. regia is a valuable species as its nut, walnut, is a worldwide consumed food. Also green walnuts, shell, husk, kernel, bark, root, and leaves have been extensively used in food, pharmaceutical and cosmetic industries. EMA consider comminuted walnut dried leaves for decoction as a traditional medicine for the relief of minor inflammatory conditions of the skin. https://www.ema.europa.eu/en/documents/herbal-report/final-assessment-report-juglans-regia-l-folium_en.pdf

Active ingredients

The phytochemical composition of walnut leaves is polyphenolic in nature; more specifically, there are high concentrations of tannins and flavonoids.

The leaves contain approximately 10% tannins of the ellagitannins type (Blumenthal 2000), and 3.4% of flavonoids (Wichtl 2004, Carnat et al. 1993), especially quercetin, hyperoside=quercetin 3-O-galactoside (0.6% according to Carnat et al. 1993), and 0.2-0.6% of quercitrin=quercetin 3-O-

rhamnoside (Wichtl 2004). Kaempferol and kaempferol 3-Oarabinofuranoside have also been isolated (Liu et al. 2004).

Beside phenolics compounds, the characteristic secondary metabolites is naphthoquinones; they are oxygen-derivatives of naphthalene. The most known naphthoquinone constituent is juglone (5-hydroxy-1,4-naphtoquinone) which occurs in fresh plant (leaf, stain) as glycoside of reduced form (2%in the stain, 0.6% in the leaves), but also in free state, particularly in the epicuticular leaf wax (to about 30%) (Wichtl 2004, Evans 2009, Hazra et al. 2004; Prasad and Gülz 1990).

This glucosidic form is decomposing by hydrojuglone-β-D-glucopyranoside-β-glucosidase to juglone (Morant 2008). Juglone is unstable and easily polymerized into yellow, brown and black pigments, so in the older leaves or dry leaves it is present only in trace amounts. When dried leaves were used for extract preparation, juglone was not detected, which means that this compound is not suitable for use in quality control of the dry plant (Amaral et al. 2004; Babula et al. 2009a, Gîrzu et al. 1998; Wichtl 2004; Wójcik 1984)

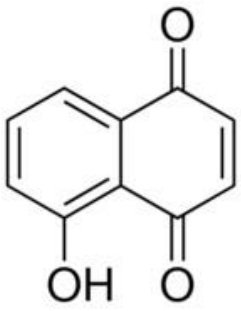


Figure 10: Chemical structure of Juglone.

Determination of the phenolic compounds demonstrate quite significant variations depending on seasonal growth and extractive methods (Fernández-Agulló et al., 2019; Vieira et al., 2019).

Traditional medicine uses

Walnut leaves have been intensively used in traditional medicine for their antioxidants and anti-inflammatory properties.

Many studies report the bioactivity of walnut leaves regarding their antidiabetic, antimicrobial, hepatoprotective, sedative, analgesic and neuroprotective properties. Moreover, other studies showed the anti-hemolytic and human renal cancer cell antiproliferative activities of a methanolic

extracts. Leaves are also internally used to treat constipation, coughs, diarrhoea and topically to treat skin diseases. *Juglans regia* leaf extract is widely used in topical skin care formulations since astringent, cleansing and abrasive functions it have been reported (Vieira et al., 2019).

Walnut leaves therapeutic effects have been related to its secondary metabolites, which present a radical scavenging activity. These compounds include phenolic acid, hydroxycinnamic acid, monomeric flavonoids (especially rutin, quercetin, hyperoside, quercitrin, myricetin, kaempferol), ellagic acid, galloyl derivatives and related oligomers, catechins, tocopherol and terpenes. Juglone is unstable and in dry leaves it is present only in traces (Vieira et al., 2019; Nour et al., 2013).

J. regia fruit include four parts: the kernel, the skin, the shell and the green husk. The kernel is the edible part of the fruit and is covered by the skin, a thin light brown layer. It has been reported that walnut skin is a rich source of antioxidants compounds acting against the deleterious effect of UV radiation, bacterial and other parasites.

J. regia seeds are widely used and they are associated with the reduction cardiovascular and neurodegenerative disorders. Topically they are used against dermatitis and eczema. The seed oil is also used in the treatment of menstrual problems and dry skin conditions (www.pfaf.org; Jahanban-Esfahlan et al., 2019).

Finally, the bark has anthelmintic, depurative and laxative activities (NirmlaDevi et al., 2011).

Pharmacology

J. regia leaves are recognized as a source of bioactive compounds with potential health effect, above all for cutaneous use, even if there are no data available from published clinical trials.

A *J. regia* leaf extract (ethanol:water) studied by Almeida and colleagues demonstrated a noticeable *in vitro* scavenger efficacy, due to its polyphenols, in particular in counteracting lipid peroxidation. Authors underlined the sounding data they obtained suggesting that walnut leaf extracts may have a high potentiality as treatment of oxidative stress- and inflammatory-relative skin diseases, as well as food preservation (Almeida et al., 2008).

A recent study confirmed the importance of green walnut leaves as a source of bioactive molecules, providing antioxidant, anti-inflammatory, anti-proliferative and antibacterial properties, with potential to be used in different industrial sectors (Vieira et al., 2019).

https://www.ema.europa.eu/en/documents/herbal-report/final-assessment-report-juglans-regia-l-folium_en.pdf

7 INVESTIGATION ON SELECTED SPECIES: *CALLUNA VULGARIS, CISTUS INCANUS, CASTANEA SATIVA, JUGLANS REGIA*

7.1 Optimization Of The Extractive Procedure Of Selected Species

Herbal material was newly collected in 2019, at the balsamic period of each species, according to the procedure described in xx.

- *Juglans regia* L. leaves collected in Foiano della Chiana, Arezzo, in October 2019.
- *Castanea sativa* Mill. leaves, var. Bastarda Rossa dell'Amiata collected in Arcidosso, Grosseto, in September 2019
- *Calluna vulgaris* (L.) Hull. aerial parts collected in the area of Maremma (Monteleoni, Grosseto), in September 2019
- *Cistus incanus* L. aerial part collected in Cala Violina, Grosseto, in June 2019

Different solvents were used for extraction of phenolic compounds and other secondary metabolites from selected species; in particular, various mixtures of ethanol and water in different ratios since these solvents have no toxicity and environmental problems and are usually considered to provide good extraction yields. In particular, used solvents were: water, ethanol 30% v/v, ethanol 60% v/v and ethanol 96% v/v.

In addition, extraction time was also examined. Two extraction methods were considered for hydroalcoholic extracts: a short maceration lasting 48 hours and a long maceration lasting 21 days at room temperature to avoid alteration of thermo-labile compounds. Water extracts were only obtained at 45 °C in ultrasound bath for 3 hours, followed by 24 hours of static maceration at 40 °C. According to what described in Farmacopea Ufficiale Italiana (Farmacopea Italiana XII ed., 2009), maceration was carried out by leaving the drug (20 g in this case) in a suitable quantity of solvent (10 times its weight in this case) for the necessary time, shaking from time to time and replenishing the solvent in case of evaporation. After the maceration period, all the extracts were filtered. Finally, the drug:extract ratio was standardized to 1:10 m/m.

Extracts from selected species were also obtained using an automated Extractor.

The technique of Estrattore Naviglio®, known in Italy since over 10 years, in the last years has earned a prominent role in university research (Biagi et al., 2014) and began to be used in industry thanks to machines with high capacity (up to 40 L) and high automation. Combining a high pressured and fully automated close system with a thermostatically controlled temperature, this innovative

technique adequately responds to the needs of extraction of polyphenols containing drugs and it is developed precisely to overcome issues related with the classic extraction techniques. Extraction procedure includes a static phase under high pressure conditions (7,5/8 bar) followed by solvent recirculation (dynamic phase) supplied by a hydraulic pump; by varying the parameters of static phase time, dynamic phase and number of complete cycles, it is possible to optimize extraction for each specific drug.

In this work a comparison between Estrattore Naviglio® and maceration technique was performed. A 500 cc Naviglio Estrattore® machine made by ATLAS Filtri, Padova, was used. The herbal material was weighed (45 g ca.) and transferred in a 50 µm extraction bag up to complete filling. The bag was placed in the extraction room that was filled with solvent (450 g ca. of EtOH 60% V/V) and then hermetically closed. Static phase time and dynamic phase laps for each cycle of extraction and number of cycles of extraction were set as follows: 80 cycles, 25 piston strokes per cycle, 5 minutes in static phase per cycle. At the end of extraction procedure, as for maceration, extracts were adjusted for a final drug:extract ratio 1:10.

7.2 Phytochemical Analysis

Chemical screening were carried out on the various extracts using standard procedure for qualitative and quantitative detection of phytoconstituents.

7.2.1 Determination of Total Phenolics

The amount of total phenolics in the extract were determined by the Folin-Ciocâlteu colorimetric method based on the reduction of a phosphowolframate-phosphomolybdate complex by phenolics to blue reaction products.

10 µL of each sample were diluted in the extraction solvent to a final volume of 3 mL into clean test tubes. 500 µL of Folin-Ciocâlteau reagent (Sigma-Aldrich, Milan), previously diluted 1:10 in distilled water, were pipetted to each tube and shacked; then were added 1 mL of sodium-carbonate solution (anhydrous sodium carbonate, Na_2CO_3, made up into a 30% w/v solution). Mixtures were kept in dark ambient conditions for 2 hours to stabilize the reaction. Distilled water were used as a blank.

The samples were scanned in UV/Vis spectrophotometer VICTOR® Nivo™ 3s, (Perkin-Elmer Italia, Milan) at 700 nm.

The measurements were performed in triplicate.

The total phenolic content was determined using a standard curve constructed with gallic acid (10000-78 mg/L, R2>0.98) standard grade (Sigma-Aldrich, Milan). Results were expressed as % of total phenolics on the basis of dry weight of herbal material ± standard error mean (SEM) (Biagi et al., 2014).

7.2.2 DPPH Scavenging Assay

The antiradicalic activity of all the extracts was tested by means of the DPPH (2,2'-diphenil-1-picrylhydrazyl) free radical scavenging method, one of the most frequently used test. It is based on the ability of antioxidant/antiradicalic compounds to reduce the odd electron of nitrogen atom in DPPH to the corresponding hydrazine. Due to the delocalisation of the spare electron over the whole molecule, DPPH is stable and do not dimerise despite the presence of the free radicals. A purple coloured DPPH$^{\bullet}$ change gradually to stable yellow DPPH-H, during the reaction. The resulting decolorizations were read at 515 nm and were proportional to the number of hydroxyl groups and thus, with the hydrogen-donor capacities of polyphenols for DPPH$^{\bullet}$.

Pure ascorbic acid was used as a reference substance.

Briefly, DPPH$^{\bullet}$ reagent (Sigma-Aldrich, Milan) was dissolved in 99.8% methanol for a solution concentration of $1x10^{-4}$ M. Samples were diluted in distilled water (1:10) and then aliquots were added at different concentrations (100-6.25 mg/mL) to the DPPH$^{\bullet}$ methanol solution (1:19). The control solution was prepared by mixing water and DPPH radical solution (1:19) while only water was used as a blank.

After 30 minutes of standing at room temperature in darkness, the absorbance of the resulting solution was measured.

Percentage of radical scavenging activity for each compound were calculated using the following reaction:

$$\%\, DPPH\bullet \;\; quenched: \frac{A_0 - A_1}{A_0 \times 100}$$

Where A_0 and A_1 stand for the absorbance of the control and antioxidants extract. The experiment was done in triplicate for each substance.

The DPPH$^{\bullet}$ method is interpreted on the basis of samples concentration necessary to quench 50% radicals in the reaction mixture, thus IC_{50} values were calculated and expressed as mg/mL of extracts (Biagi et al, 2009).

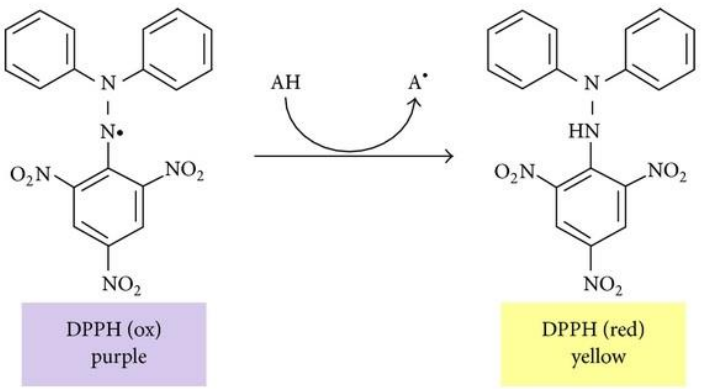


Figure 11 Principle of DPPH radical scavenging capacity assay.

7.2.3 Colorimetric Phytochemical Analysis of Optimized Extracts

Optimized extracts were further analyzed and phytochemical profile was better characterized.

Walnut Leaves Extract (WLE), *Castanea Leaves Extract* (CLE), *Calluna aerial parts Extract* (CVE) and *Cistus aerial part Extract* (CCE) were investigated for their flavan-3-ols.

The vanillin-HCl test, a colorimetric method, were used for the detection and quantitative determination of total flava-3-ols. A 0.5 mL of 1% m/V vanillin in ethanol and 1 mL of HCl (11.5 N) were added to 0.5 mL of the sample, previously diluted 1:50 in the extraction solvent. Positive results were indicated by the appearance of a light pink to deep cherry red coloration. Catechin (10000-78 mg/L, R^2>0.98) standard grade (Sigma-Aldrich, Milan) was used as standard.

The absorbance was measured at 500 nm and results were expressed as mg total flavan-3-ols per g of extract ± standard error mean (SEM). Thanks to this simple colorimetric reaction, monomeric catechins and oligomeric proanthocyanidins were easily detected (Biagi et al., 2014).

WLE was also analyzed for its triterpenes content and CVE for soluble sugars content.

Determination of total triterpenes was obtained by means of a colorimetric test too. 0.3 mL of vanillin-glacial acetic acid reagent (5% V/V) first and 1 mL of perchloric acid then, were added to 0.2 mL of the pre-diluted sample (1:20) in glacial acetic acid. After incubating at 60°C for 45 minutes, glacial acid acetic was poured until to the final volume of 5 mL. A blank was prepared consisting of all reagents and solvents without sample solution.

The content of total triterpenes was calculated from the calibration curve using β-sitosterol (10000-78 mg/L, R2>0.97) standard grade (Sigma-Aldrich, Milan).

The absorbance of the mixture was measured at 548 nm. Data analyses were expressed as mg total triterpenes per g of extract ± standard error mean (SEM) (Biagi et al., 2014).

All determinations were performed in triplicate.

7.2.4 Determination Of Total Polysaccharides

C. vulgaris water extract was tested for polysaccharides.

The basic procedure on the phenol-sulphuric method was derived from a method of total carbohydrate analysis by spectrophotometry. The amount of polysaccharides was calculated by means of the equation obtained from the calibration line, performed with standard solutions of D(+)-glucose, at concentrations between 0.01 and 0.1 mg/mL.

To 100 µl of sample, placed in a reaction tube, 200 µl of 6% m/V aqueous phenol solution was added. Subsequently, 0.5 ml of 98% m/m sulphuric acid was added and the reaction tube was quickly closed. After vigorous stirring, the reaction was allowed to develop for 30 minutes at 30 °C and finally absorbance readings were taken at a wavelength of 490 nm against a blank consisting of 200 µl of distilled H_2O.

7.2.5 HPLC-DAD Analysis

HPLC-DAD analysis was performed by using a Shimadzu Prominence LC 2030 3D instrument equipped with a Bondapak® C18 column, 10 mm, 125 Å, 3.9 mm x 300 mm column (Waters Corporation, USA).

Water solutions containing 0.1 % (v/v) formic acid (A) and acetonitrile with 0.1% (v/v) of formic acid (B) were used as mobile phase. The following program was applied: B from 10% at 0 min to 25% at min, then B 50% at 26 min. to 10% at 35 min; flow was set at 0.9 mL/min. Chromatograms were recorded at 280, 350 and 366 nm. Analyses were performed using 20 µL of extracts; gallic acid, chlorogenic acid, caffeic acid, catechin, rutin, hyperoside, isoquercitrin, quercetin, miricitrin and kaempferol (Sigma-Aldrich, Milan) were used as external standards. Calibration curves were established using reference standards ranging from 0.008 mg/mL to 0.500 mg/mL. The correlation coefficient (R^2) of each curve was > 0.99.

Phenolic compounds peaks were identified via comparison with the retention time of pure standard and UV spectra.

The absorbance was measured at 280 and 350 nm and the different constituents recognized and quantified, based on the retention time (RT) and UV-vis spectrum of the reference standards used (Sigma-Aldrich, Milan).

7.2.6 Electrochemistry

The voltammetry experiments were conducted in a three-electrode cell connected to the analyzer. The working electrode used is glassy carbon. The reported potentials are referred to the Ag/AgCl reference electrode. The counter electrode is a platinum wire.
The measurements were carried out at room temperature and under an inert atmosphere of ultra-pure nitrogen, in order to avoid both the reactivity of the compounds under examination with atmospheric oxygen and the reduction process of dissolved oxygen. In 5 ml of a 0.1 M NaCl aqueous solution, 1 ml of chestnut alcohol extract is added.

7.2.7 DPPH-HPLC-DAD Analysis

DPPH$^{\bullet}$ reagent (Sigma-Aldrich, Milan) was dissolved in 99.8% methanol for a solution concentration of 4 mg/mL.
The samples were diluted in DPPH (1:10), and the mixtures were vortexed prior to HPLC analysis in previously described conditions.

7.3 Biological analysis

7.3.1 Cell Lines

Human immortalized keratinocytes (HaCaT) and human foreskin fibroblasts (HFF) (kindly provided by University of Modena and Reggio-Emilia) cells were cultured in plastic flasks using 4500mg/L DMEM (Dulbecco's Modified Eagle's medium), a high glucose medium, supplemented with 10% V/V heat-inactivated fetal bovine serum (FBS), 1% m/V L-glutamine (Sigma-Aldrich, Milan), 1% m/V penicillin/streptomycin (Sigma-Aldrich, Milan). Plates were incubated at 37°C in a 5% CO_2 atmosphere. Once detached from the surface of the flasks (Euroclone, Milan) with a freshly prepared EDTA/trypsin solution, viable cells count was performed using a hemocytometer after staining with Trypan Blue dye.

7.3.2 Cell Viability Assay

5 x 10^3 cells/well were seeded in 96-multiwell plates in 0.1 mL of DMEM with 3% FBS and maintained at 37 °C in a 5% CO_2 atmosphere for 24h. After that, the medium was removed and replaced with fresh DMEM with 1% FBS and treatments: WLE and CLE 2, 1, 0.5, 0.25 mg/mL, CCE 2 mg/mL and CVE 4 mg/mL; plates were then incubated at 37 °C in a 5% CO_2 atmosphere. After 24 h, the medium was removed, and cells were incubated for 4 h with fresh medium in the presence of 10% v/v of CCK-8 (Cell Counting Kit, Sigma-Aldrich, Milan). The CCK-8 assay is a colorimetric method that provide reproducible results on cell viability. The assay is based on the reduction of a water soluble tetrazolium salt WST-8 operated by cell dehydrogenases that lead to formazan production, soluble and orange-colored. Formazan production and, thus, absorbance development is directly related to the cell viability in the tested sample. The absorbance of the formazan dye was measured at 450 nm with a microplate reader VICTOR® Nivo™3s, (Perkin-Elmer Italia, Milan). Cell viability in treated groups was reported in comparison with not treated cells (control), whose mean absorbance was normalized as 100%.

All samples were analyzed in replicate (n=6).

7.3.3 Human Pro-Collagen Type I Assay On Human Fibroblasts

5x10^3 HFF/well were seeded in 96-well plates as described above. After 24 h, the medium was removed and replaced with fresh DMEM with 1% FBS and treatment: WLE 1 mg/mL, CLE 1 mg/mL, CCE 2 mg/mL and CVE 4 mg/mL

Plates were then incubated at 37 °C in a 5% CO_2 atmosphere for 24 hours. Cell culture supernatants were then collected and quantified for the amount of procollagen I using the Human Pro-Collagen I α-1 Simple-Step ELISA kit (Abcam, kit ab210966), following the procedure in the data sheet.

Briefly, 50 µL of sample and 50 µL of Antibody Cocktail were added to each well in 96-well plates. The antibody cocktail was prepared by diluting the capture and detection antibodies in Antibody Diluent. The plates were then sealed and incubated for 1 hour at room temperature. Subsequently, cells were washed three times with 1X Wash Buffer and 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) solution was added to each well and incubated for 10 min in the dark. Diluted sulfuric acid was used as the stop solution. The absorbance was measured at 450 nm. A calibration curve was plotted against the concentration of a standard type 1 procollagen. All samples were analyzed in replicate (n=6).

7.3.4 Filaggrin And Loricrin And Occludin Dosage On Human Keratinocytes

Whole cell protein extracts were prepared by cell lysis into 1X SDS sample buffer (25 mM TrisHCl pH 6.8, 1.5 mM EDTA, 20% glycerol, 2% SDS, 5% β-mercaptoethanol,0.0025% Bromophenol blue). For immunoblotting equivalent amounts of cellular extracts were resolved by SDS-PAGE, electrotransferred to PVDF membrane (GE Healthcare) and immunoblotted with the following primary antibodies diluted 1:1000 in TBS 1X- BSA 1 µg/µl: anti-occludin (#GTX114949, Genetex), anti-filaggrin (#ENZ-ABS181-0100), anti-loricrin (#GTX127943, Genetex), mouse anti-Vinculin (#V4504, Merck KGaA). After washes with TBS 1X membranes were incubated with secondary antibodies HRP conjugated, goat anti-mouse (#A90116P, Bethyl Laboratories), goat anti-rabbit (#G21234, ThermoFisher Scientific) diluted 1:5000.

Membranes were blotted and scanned with Amersham Imager AI680 RGB (GE Healthcare), using chemiluminescent detection reagents Westar ηC and Supernova HRP substrates (Cyanagen).

7.3.5 Effectiveness Of Botanical Extracts In A Hyperosmotic Aging Model

A model of cellular dehydration was developed with the aim of assessing the hydration capacity by evaluating the protection against a hyperosmotic insult *in vitro*.

A cell line of immortalized human keratinocytes (HaCaT) was also used to perform this test. Cells were pretreated with WLE 1 mg/mL, CLE 1 mg/mL, CCE 2 mg/mL and CVE 4 mg/mL for 24 hours and left to incubate as already described. The medium was then replaced and NaCl was added until a concentration of 1.5% m/v was reached and left to incubate for 4 hours.

After this time frame, cell viability was assessed by means of CCK-8 assay. After two hours, a spectrophotometric reading was taken using a Perkin Elmer VICTOR® Nivo™3s plate reader at a wavelength of 450 nm.

The assay was performed in biological triplicate and technical duplicate (n=6).

7.3.6 Effectiveness Of Botanical Extracts In A Immunosuppressive Skin Aging Model

Cells were treated with a methylprednisolone 1 mg/mL for 24 hours, which minimizes the metabolic activity of keratinocytes and fibroblasts and simulating an aged epidermis condition; the aim of the test was to verify the possible effect of extracts in counteracting the effect of the corticosteroid.

Cell viability was assessed by using CCK-8.

The assay was performed in biological triplicate and technical duplicate (n=6).

7.3.7 UVA Irradiation Protection

In this work, a model of UV cellular damage was developed with the aim of evaluating the protective capacity of extracts against an *in vitro* UV insult.

An immortalized human keratinocyte (HaCaT) cell line was also used to perform this test. The cells were pretreated with the extracts WLE 1 mg/mL, CLE 1 mg/mL, CCE 2 mg/mL and CVE 4 mg/mL for 40 min. Subsequently, cells were subjected to UVA irradiation (36 W) at different timings 15, 30, 60 min.

After these times, CCK-8 was used to measure cell viability.

The assay was performed in biological triplicate and technical duplicate (n=6).

7.3.8 Effectiveness Of Botanical Extracts In Oxidative Stress Models

The intracellular ROS measurement were evaluated with H_2DCF-DA (Abcam ab133851), a chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate, which is a oxidative stress indicator. Inside the cells, it is first deacetylated by esterases and releases its chloromethyl group, which reacts with glutathione resulting in a fluorescent adduct, dichlorofluorescein (DCF). Therefore, the fluorescence signal intensity of DCF is proportional to the amount of ROS produced by the cells.

To verify the ability of CLE, WLE, CCE and CVE to reduce the intracellular level of ROS in keratinocytes stimulated with three different models (tert-butyl hydroperoxide (TBHP), hydrogen peroxide (H_2O_2), serum deprivation) HaCaT cells were seeded in 96-well plates at a cell density of $2.5x10^4$ cells/well and initially cultured prior to the experiment for 24 hours.

Cells were treated with WLE 1 mg/mL, CLE 1 mg/mL, CCE 2 mg/mL and CVE 4 mg/mL for 24h. In the serum deprivation model, cells were cultured in DMEM without FBS for 5 h. The medium was then removed and cells rapidly washed with assay buffer. Then, in order to stain cells, 100 µL of the diluted DCFDA solution was added to each well and plates were incubated for 45 min at 37 °C in the dark.

Subsequently, DCFDA solution was removed and 100 µL of buffer containing tested samples were added.

In this work, three models of oxidative stress were used:

- *TBHP stimulated Keratinocytes*
- *Hydrogen peroxide stimulated keratinocytes*
- *Serum deprivation stimulated keratinocytes*

Summarizing, the two prepared plates were organized as follows:

- Extracts used as pre-treatment:

1- Control (not treated cells)

2- Extracts (cells treated with WLE 1 mg/mL, CLE 1 mg/mL, CCE 2 mg/mL or CVE 4 mg/mL the day before experiment)

3- TBHP or H_2O_2 (cells stimulated with TBHP 200 µM or H_2O_2 1 mM)

4- Serum deprived medium

5- Extracts + TBHP or H_2O_2 (cells pre-treated with WLE, CLE, CCE or CVE and stimulated with TBHP 200 µM or H_2O_2 1 mM or in serum deprived medium)

Using the microplate reader VICTOR® Nivo™ 3s, (Perkin-Elmer Italia, Milan), the fluorescence was recorded at Ex/Em 488/535 nm, every 30 min for 3 hours.

ROS level produced by tested samples was reported as fold change in comparison with not treated cells (control), normalized as 1.

All samples were analyzed in replicate (n=6).

7.3.9 Inhibition Of ECM-Degrading Enzymes

In chrono- and photo-aged skin, there is an uncontrolled overproduction of collagenase, elastase and hyaluronidase leading to degradation of collagen, elastin and hyaluronic acid respectively. As result, wrinkle formation and sagging of skin occur. These dermal components are responsible to provide strength, elasticity and moisture to the skin, therefore inhibitory effects of the sample on collagenase, elastase and hyaluronidase were performed by using *in vitro* enzyme inhibitory assays based on spectrophotometric evaluation.

7.3.9.1 Anti-Collagenase Activity

Inhibitory effect was performed using Collagenase Activity colorimetric Assay kit (Sigma-Aldrich, Milan, MAK293). Assay kit measures proteolytic degradation when collagenase interact with synthetic FALGPA, a synthetic peptide that mimics collagen's structure; this leads to a reduction in the absorption of 340 nm in the presence of collagenase inhibitors.

Firstly, samples were diluted with ultrapure water to obtain different concentration ranging from 1000 to 40 mg/mL. All kit components were brought at room temperature before use. Reaction Mix (RM) enough for the number of wells were prepared with 40 µL of Collagenase Substrate (FALGPA) and 60 µL of Collagenase Assay Buffer. Then, in 96-well plate, 88 µL buffer, 2 µL test sample (WLE at different concentration) and 10 µL enzyme (collagenase provided in the kit) were added to each well. 100 µL of RM were added to adjust the volume to 200 µL. Inhibitor and positive control were prepared too. Absorbance was determined at 10 min intervals for 20 min.

Collagenase activity (U/mL) was calculated as follows:

$$\frac{\left(\frac{-\Delta A_{test}}{\Delta T} - \frac{-\Delta A_{Reagent\ background}}{\Delta T}\right)}{0{,}53 \times V} \times RV \times DF$$

Where ΔA was the difference between absorbance values A_2 and A_1 at two time points, and ΔT was the difference between the two time points T_2 and T_1.

RV is reaction volume (mL), DF is the dilution factor, 0.53 is the millimolar extinction coefficient of FALGPA and V is the enzyme volume (mL).

Percent inhibition activity was determined using the following equation*:*

$$\%\ Inhibition\ = \frac{Activity_{enzyme} - Activity_{Inhibitor}}{Activity_{enzyme}} \times 100$$

IC_{50} values were calculated and expressed as mg/mL of extracts.

All samples were analyzed in triplicate.

7.3.9.2 Anti-Elastase Activity

The Sigma-Aldrich kit Assay (Sigma-Aldrich, Milan, MAK213) was used to carry out anti-elastase potential test of samples. The principles of this assay are based on the interaction between elastase and a synthetic substrate. The proteolytically cleavage release a fluorophore which can be easily quantified by fluorescence.

As for collagenase inhibitory assay, all the components were brought at room temperature before use. Samples were diluted with assay buffer to obtain different concentration ranging from 250 to 10 mg/mL. Then, enough reagents for the number of assays were prepared as follow:

-for the Reaction Mix (RM), 48 µL of Assay Buffer and 2 µL of Neutrophil Elastase (enzyme) were mixed;

-for the Substrate Mix (SM), 23 µL of Assay buffer and 2 µL of Substrate were mixed.

Then, 50 µL of RM was mixed with the 25 µL of sample in the 96-well plates and incubated at 37°C for 5 min. After, 25 µL of SM were added to each reaction well.

Mixtures of enzyme and substrate solution without sample served as control. The fluorescence was measured at 505 nm in kinetic mode for 30 minutes at 37°C.

Fluorescences (FLU_1 and FLU_2, FLU/minute) at different two time points (T_1 e T_2) were determined to obtain the slope of the plot for each well. Anti-elastase activity was calculated as follows:

$$Slope = \frac{(FLU_2 - FLU_1)}{T_2 - T_1} = \Delta FLU/min$$

$$\%Relative\ Inhibition = \frac{(Slope_{EC} - Slope_{SM})}{Slope_{EC}} \times 100$$

Where $Slope_{SM}$ ans $Slope_{EC}$ were the slope of the Sample Inhibitor and the slope of the Enzyme Control, respectively.

IC_{50} values were calculated and expressed as mg/mL of extracts.

All samples were analyzed in triplicate.

7.3.9.3 Anti-Hyaluronidase Activity

Inhibition of hyaluronidase by samples were determined by slighting modifying and by optimizing the turbidimetric method (% Transmittance at 600 nm) described by Sigma-Aldrich in the method: Enzymatic Assay of HYALURONIDASE1 (EC 3.2.1.35).

All chemicals, substrates and hyaluronidase were purchased from Sigma-Aldrich, Milan.

A 0.3 M of sodium phosphate buffer (pH 5.35) was prepared. This buffer was used to prepare a hyaluronic acid solution 0.03% at pH 5.35.

The other used buffer was 0.02 M sodium phosphate buffer (pH 7.00) containing 0.01% (m/V) bovine serum albumin (BSA) and 77 mM of NaCl. This buffer was used as enzyme diluent.

The enzyme solution (hyaluronidase, 10 U/mL) was prepared immediately before the experiment using the cold enzyme diluent.

Finally, 0.24 M sodium acetate, 0.79 M acetic acid and 0.1% (w/v) BSA were mixed to prepare acidic albumin solution at pH of 3.75.

The assay reaction was performed as follows:

In test tubes, 0.75 mL of enzyme solution was diluted to 0.90 mL with enzyme diluent. 0.1 mL of tested samples at different concentrations ranging from 250 to 10 mg/mL were then added. Assay buffer was used as positive control and only enzyme diluent was used as a blank. Test tubes were

incubated a 37°C for 10 min. After this time, each sample was mixed with 1 mL of hyaluronic acid solution and after incubation at 37°C for 45 min, 0.5 mL of each sample and blank were transferred into a test tube containing 2.5 mL of acidic albumin and gently mixed.

After 10 minutes at room temperature, transmittance was measured. The activity of hyaluronidase for each sample was calculated according to the following formula:

$$\frac{Units}{mL} enzyme = \frac{(\%T_{test} - \%T_{blank}) \times DF}{14{,}84 \times V}$$

Where DF represented the dilution factor of enzyme and V was the volume (mL) of enzyme solution used in reaction.

The inhibitory activity of samples was calculated using the following formula:

$$\%\, Inhibition = \frac{Activity_{enzyme} - Activity_{Inhibitor}}{Activity_{enzyme}} \times 100$$

IC_{50} values were calculated and expressed as mg/mL of extracts.

All samples were analyzed in triplicate.

7.3.9.4 Tyrosinase Inhibitory Activity

Tyrosinase is an oxidoreductase that participates in the biosynthesis of melanin, a ubiquitous biological pigment found in hair, eyes, skin, etc. Inhibition of tyrosinase has been a long-time target in the skin health research, cosmetics, and agricultural industries. Skin whitening and bleaching products utilize natural or synthetic tyrosinase inhibitors in order to lighten the skin color. the natural compounds have been used as tyrosinase inhibitors.

Tyrosinase catalyzes the oxidation of tyrosine, producing a chromophore that can be detected at 510 nm. In the presence of kojic acid, a reversible inhibitor of tyrosinase, the rate of oxidation of the substrate is decreased.

Samples were diluted with assay buffer to obtain different concentration ranging from 250 to 10 mg/mL.

Then, enough reagents for the number of assays were prepared as follow:

-for the Tyrosinase Enzyme solution Mix (EM), 48 µL of Assay Buffer and 2 µL of Tyrosinase were mixed;

-for the Substrate Mix (SM), 23 µL of Assay buffer, 2 µL of Substrate, 5 µL Enhancer were mixed.

Then, 50 µL of EM was mixed with the 25 µL of sample in the 96-well plates and incubated at 25 °C for 10 min. After, 30 µL of SM were added to each reaction well.

Mixtures of enzyme and substrate solution without sample served as control. The absorbance was measured at 510 nm in kinetic mode for 30 minutes at 37 °C.

Calculate the slope for all sample, including Enzyme Activity Control (EC), by dividing the net ΔAbs (Abs_2- Abs_1) values by the time ΔT (T_2-T_1). Anti-Tyrosinase activity was calculated as follows:

$$\%Relative\ Inhibition = \frac{(Slope_{EC} - Slope_S)}{Slope_{EC}} \times 100$$

Where $Slope_S$ ans $Slope_{EC}$ were the slope of the Sample Inhibitor and the slope of the Enzyme Control, rispectively.

IC_{50} values were calculated and expressed as mg/mL of extracts.

All samples were analyzed in triplicate.

7.4 Effectiveness Of Selected Extracts Against Oxidative And Inflammatory Stress Induced By Urban Dust

7.4.1 Cell Viability On Keratinocytes Stimulated With Urban Dust

This test was performed according to 7.3.2, keratinocytes were stimulated with Urban Dust NIST®SRM®1648a (Sigma-Aldrich, Milan) $500\ \mu g/ml$ for 24 hours

7.4.2 Intracellular Ros Dosage in Urban Dust-stimulated human keratinocytes

This test was performed according to 7.3.8, keratinocytes were stimulated with Urban Dust NIST®SRM®1648a (Sigma-Aldrich, Milan) $500\ \mu g/ml$. for 2 h.

7.4.3 Proinflammatory Cytokines And Chemokines Dosage

Cytokine assay was performed after stimulation of cells with UD 500 μg/mL and treatment with WLE 1 mg/mL, CLE 1 mg/mL, CCE 2 mg/mL and CVE 4 mg/mL for 24 hours.

The inflammatory parameters assayed, using new generation ELISA kits, were the cytokines TNF-α, IL-6, IL-1β and IL-8.

ELISA kits for TNF-α assays were purchased from Afflimetrix Invitrogen. The plate reader used was the VICTOR® Nivo™3s.

The assays were conducted in duplicate on the independent triplicates of the cell groups (n=6). Non-competitive sandwich type ELISA assays were used for the assays.

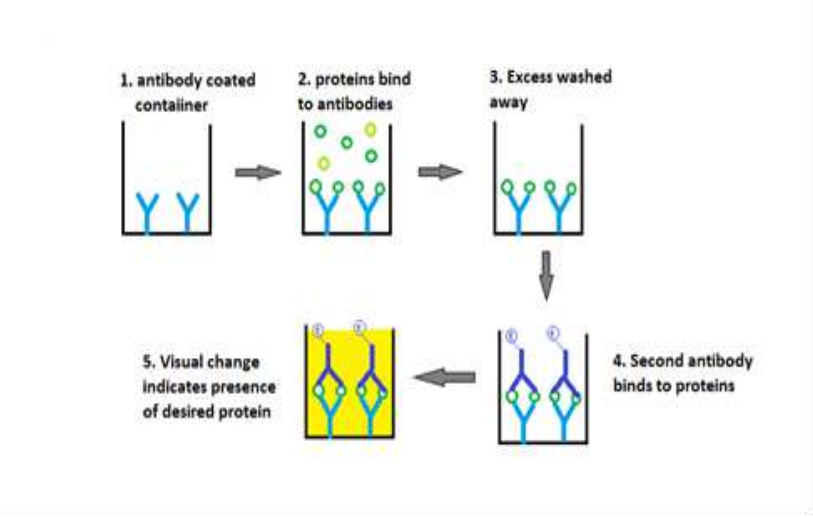


Figure 12: Sandwich ELISA assay procedure.

Samples coincubated with the test substances were subjected to cell lysis and frozen at -80 °C. Samples were analyzed by strictly following the data sheets provided by the kits:

- 100 µl of capture antibody was added to the coating buffer in each well and incubated overnight at 2-8 °C.
- Wash wells 4 times with wash buffer.
- Add 100 µL of assay diluent which is the blocking solution (200 µL for blank) to all wells.
- In two columns of wells (1 and 2) add 100 µL of solution containing the standard protein and make progressive dilutions (wells A to G) for the calibration curve; in well 1H and 2H insert the standards at concentration 0.
- In all other wells 100 µL of the different samples to be tested are added.
- Add 100 µL of biotinylated detection antibody in assay diluent to all wells and incubate at room temperature for two hours.
- Wash the wells 4 times with wash buffer.
- Add 100 µL of peroxidase conjugate in assay diluent to each well and incubate at room temperature for one hour.
- Wash the wells 5 times while soaking for 30 seconds to 1 minute per well.

- Add 100 µL of TMB (tetramethylbenzidine and hydrogen peroxide) substrate to all wells and incubate in the dark for 15 minutes.
- 100 µL of stop solution consisting of 2N sulfuric acid is added to each well.
- The plate is inserted into the plate reader and the absorbance read at 450 nm.

7.4.4 ERK 1/2, P38 And JNK Activation

ELISA kits for the assays of ERK 1/2, p38 and JNK were purchased from RayBiotech (PEL-MAPK-SK-1). The plate reader used is a VICTOR® Nivo™ 3s, (Perkin-Elmer Italia, Milan).
The protocol provided by the kit was followed for the assay of ERK, p38 and JNK, with assays performed in duplicate on the different replicates (n=6).

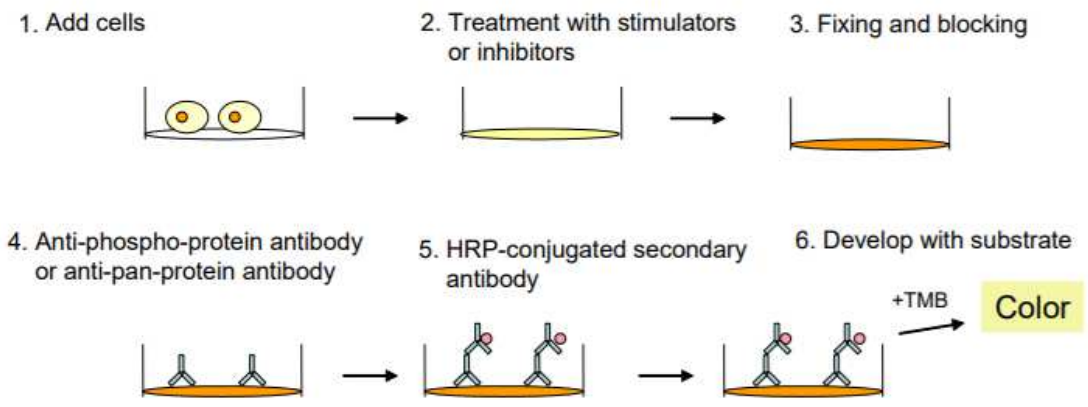


Figure 13: Sandwich ELISA assay procedure.

1. Seed 10,000-30,000 cells HaCaT into each well and incubate overnight.
2. Apply various treatments, WLE 1 mg/mL, CLE 1 mg/mL, CCE 2 mg/mL and CVE 4 mg/mL for 30, 60 and 120 minutes according to manufacture's instructions.
3. Add 100 µL of Fixing Solution into each well and incubate for 20 min at RT with shaking.
4. Add 200 µL of prepared 1X Quenching Buffer and incubate 20 min at RT.
5. Add 200 µL of Blocking Solution and incubate for 1 h at 37 °C.
6. Add 50 µL of 1X anti-phospho-protein specific antibody or anti-pan-protein specific antibody to each well and incubate for 2 h at RT.
7. Add 50 µL of prepared 1X HRP-Anti-Rabbit or Mouse IgG and incubate for 1 h at RT.

8. Add 100 µL of TMB One-Step Substrate Reagent to each well.
9. Incubate 30 min at RT.
10. Add 50 µL of Stop Solution to each well.
11. Read at 450 nm immediately.

7.4.5 Statistical Analysis

Analysys of variance (ANOVA) for the determination of significant differences was applied and followed by *t*-Test. Differences in dataset with $p<0.05$ were considered statistically significant. Graphs and results were obtained using GraphPrism® software and Microsoft Excel.

8 RESULTS and DISCUSSION

Phytochemical analyses confirmed that ethanol 60% v/v was the best extraction solvent to achieve a high yield in polyphenols; ethanol 30% v/v resulted comparable in terms of extraction capacity, with a mean decrease of polyphenols content ranging from 3% to 10% ca. Water extraction allowed to have the best yield in soluble sugar, particularly in *C. vulgaris*. Ethanol 96% v7V was the worst extraction solvent for all considered samples.

As regards extraction time, no significant variation was shown when short and long extraction procedures were compared.

The summary of obtained results is shown in tables 2 a-d.

C. vulgaris aerial parts	Water	Ethanol 30% v/v	Ethanol 60% v/v	Ethanol 96% v/v
	Polyphenols content (% dw)			
Extraction time: 3 days	++ *24h, ultrasound assisted	++	+++	+
Extraction time: 21 days	-	++	+++	+

C. creticus aerial parts	Water	Ethanol 30% v/v	Ethanol 60% v/v	Ethanol 96% v/v
	Polyphenols content (% dw)			
Extraction time: 3 days	++ *24h, ultrasound assisted	+++	+++	++
Extraction time: 21 days	-	+++	+++	++

C. sativa leaves	Water	Ethanol 30% v/v	Ethanol 60% v/v	Ethanol 96% v/v
	Polyphenols content (% dw)			
Extraction time: 3 days	+ *24h, ultrasound assisted	++	++	+/-
Extraction time: 21 days	-	++	++	+/-

J. regia leaves	Water	Ethanol 30% v/v	Ethanol 60% v/v	Ethanol 96% v/v
	Polyphenols content (% dw)			
Extraction time: 3 days	+	+	++	+/-

	*24h, ultrasound assisted			
Extraction time: 21 days	-	+	++	+/-

Table 2: Polyphenols content express as gallic acid in prepareds by maceration for 3 and 21 days. +++: > 5% ++: 3-5%: +: 2-3%: +/-: < 2%

These first results allowed us to choose ethanol 60% v/v as extraction solvent for *C. creticus*, *C. sativa* and *J. regia*, whereas water was chosen for *C. vulgaris* with the aim of maintaining the sugar fraction. The extraction procedure was then repeated for *C. creticus*, *C. sativa* and *J. regia* by using the automatic Naviglio® Estrattore, in the attempt of better standardize the procedure and shorten the extraction time.

The automatic procedure, lasted less than 15 hours, guaranteed the same polyphenols yield obtained with 3 or 21 days of static extraction.

Finally, optimized extracts were obtained, all as liquid extracts, drug:extract ratio 1:10 m/m: CVE, CCE, CLE and WLE.

8.1 Spectrophotometric And HPLC-DAD Analysis Of Optimized Extracts

Juglans regia leaves extract (WLE)

WLE was further analyzed first by means of colorimetric assays in order to obtain information on different classes of secondary metabolites present in the extract. In detail, colorimetric assays were performed to quantify total flavan-3-ols and total triterpenes. WLE showed a high content of total polyphenols, 4634 mg/L, corresponding to a yield of 46.3 mg per g of extracted dried raw herbal material (dry weight, dw). Total flavan-3-ols represented more than 20% of total polyphanols. As reported in literature, walnut leaves showed to contain a large amount of total triterpenes, 2970 mg/L in WLE.

HPLC-DAD analysis was subsequently applied to identify and quantify main single molecules in WLE. Flavonols were found as the main polyphenolic subclass; they were identified by means of their UV spectrum, with λ of maximum absorption at 270-280 nm and a second variable λ max at 345-355 nm (glycosides) or 360-370 nm (aglycones). The total content of flavonols in WLE, calculated as sum of peaks of all the compounds with characteristic UV spectrum was 1092 mg/L, expressed as hyperoside.

Compound	mg/g dw of herbal material
Total polyhenols	46.3±3.6
Total flavan-3-ols	10.3±1.9
Total hydroxycinnamic derivatives	4.3±0.5
Flavonols **Of which:** ***Hyperoside + isoquercitrin***	10.9±0.6 3.7 ±1.4
Total triterpenes	29.7±2.0

Table 3: Total phenolics, flavan-3-ols and triterpenes in WLE, expressed as mg/g extract. Phenolics are expressed as gallic acid, flavan-3-ols as catechin, triterpenes as b-sitosterol, flavonoid are quantified by means of peak area of related compounds in the chromatogram and expressed as hyperoside+ isoquercitin, respectively.

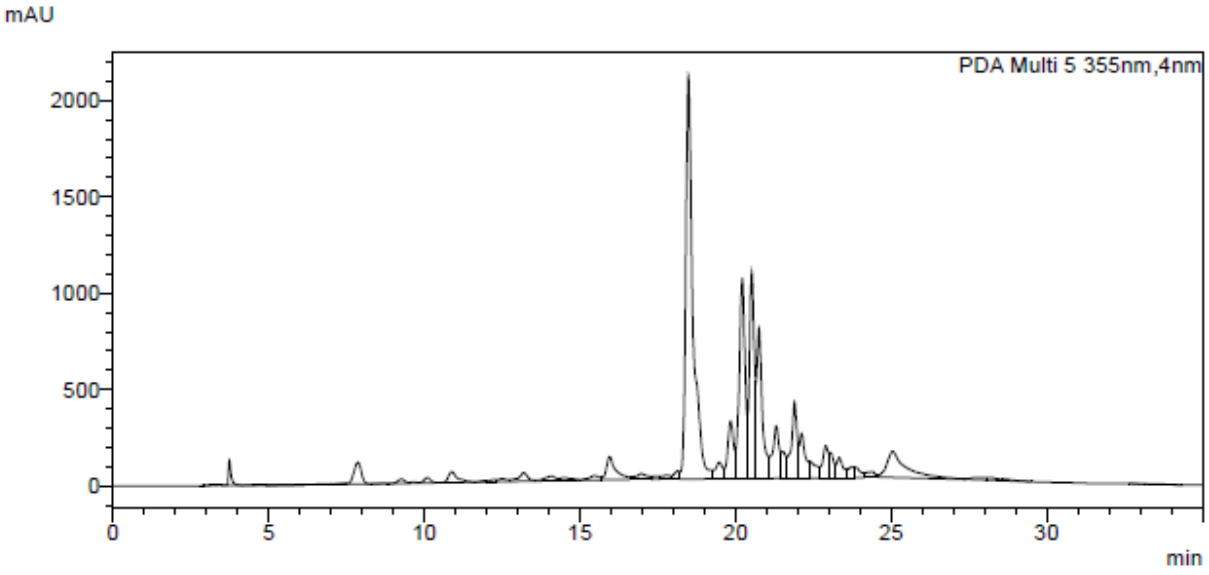


Figure 14: Chromatogram of walnut leaf extract recorded at 355 nm.

Four main flavonols were identified in WLE. By comparison with reference standards and their UV-vis spectra, all these flavonols were identified ad quercetin glycosides. Isoquercitrin and hyperoside, as usual not completely separated, were found at RT=18.8-19.0 minutes (371 mg/L as sum of the two flavonols) as the main flavonols. Rutin was also present in the extract, but in low concentration (RT= 18.1 minutes). Quercetin resulted the flavonoidic aglycone at highest content: 24 mg/L WLE (RT=25.1 minutes).

On the other hand, monomeric catechins were present only at very low content (<50 mg/L) suggesting that flavan-3-ols in WLE were mostly represented by condensed proanthocyanidins.

Six different hydroxycinnamic derivatives (HCD) were clearly separated at retention time (RT) 7.9-14.2 minutes (figure 14); among them chlorogenic acid (RT=10.9 min) and caffeic acid (RT=13.2)

were found. Peaks' area of different hydroxycinnamic derivatives were very similar and the content of single compounds of this class was found to be similar in WLE (total HCD in WLE, calculated as sum of peaks of all the compounds with characteristic UV spectrum: 433 mg/L).
A qualitative research of juglone was also performed comparing its published UV spectrum with those of single constituents separated and analyzed in HPLC: juglone was not detectable in WLE.

Castanea sativa extract (CLE)

Phytochemical analyses showed that CLE shared many features with WLE. Again, the total polyphenols content resulted very high, 3482 mg/L, and quercetin glycosides were the single compounds at highest content. The peak at Rt=18.8 minutes could be assigned to hyperoside or its isomer isoquercitrin or, more plausibly, could be considered as an overlapped peak. The main flavonol(s) represented more than 50% of the total of the subclass (191 mg/L, expressed as hyperoside).

Compound	mg/g dw of herbal material
Total polyphenols	34.8±3.7
Total flavan-3-ols	3.3±0.2
Flavonols **Of which:** *Hyperoside + isoquercitrin*	3.4±0.2 1.9±0.1

Table 4: Total phenolics and flavan-3-ols in CLE, expressed as mg/g extract. Phenolics are expressed as gallic acid, flavan-3-ols as catechin. Flavonoid are quantified by means of peak area of related compounds in the chromatogram and expressed as hyperoside.

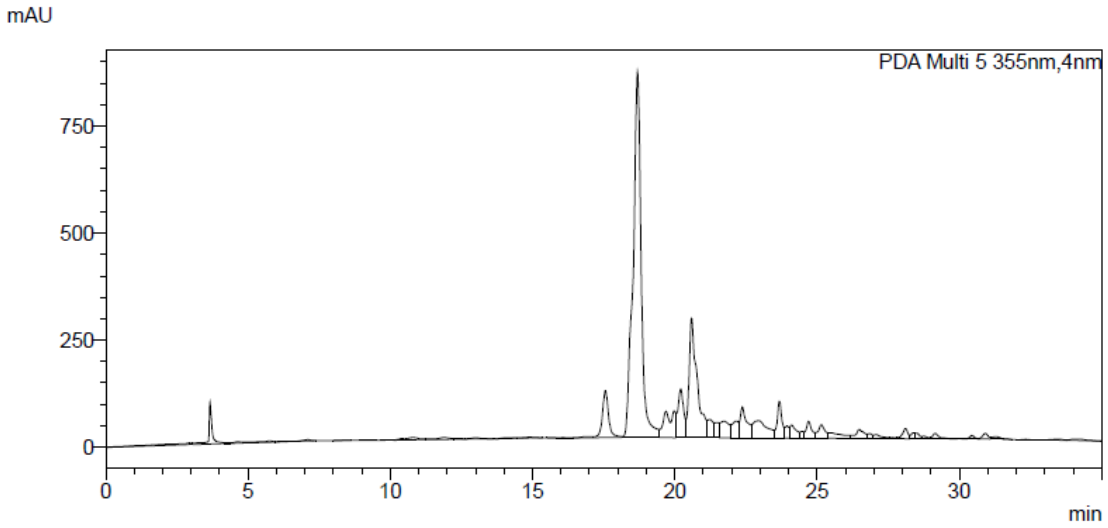


Figure 15: Chromatogram of chestnut leaf extract recorded at 355 nm.

The peak at 20.2 min could be assigned most likely to a quercetin glycoside; Rutin and quercetin were also present, but at low concentration (RT= 17.6 and 25.0 minutes, respectively).
Flavan-3-ols content was significantly lower than in WLE and the main non-flavonoid constituents of CLE could be assigned to the class of oligomeric galloyl derivatives, not currently identifiable.

Calluna vulgaris *aerial parts extract (CVE)*

Compound	mg/g dw of herbal material
Total polyphenols	33.3±4.0
Total flavan-3-ols	16.0±1.7
Chlorogenic acid	1.0±0.1
Flavonols	1.7±0.1
Hyperoside + isoquercitrin	0.2±0.1
Myricitrin	0.5±0.1
Callunin and derivatives	0.5±0,1
Soluble sugars	60.5±9.3

Table 5 :Total phenolics and flavan-3-ols in CVE, expressed as mg/g extract. Phenolics are expressed as gallic acid, flavan-3-ols as catechin. Flavonoid are quantified by means of peak area of related compounds in the chromatogram and expressed as hyperoside

The water extract of *C. vulgaris* resulted very rich in soluble sugars and polyphenols (6052 mg/L and 3332 mg/L, respectively).
Oligomeric flavan-3-ols counted for almost 50% of total polyphenols, consistently with their higher water solubility if compared with flavonols that resulted the main flavonoidic fraction (170 mg/L, expressed as hyperoside). Among these flavonoids, the main constituents recognized by comparison with the standards were hyperoside and isoquercitrin (RT=18.2 and 18.6 minutes, partially overlapped) and myricitrin (RT=17.8 minutes). Considering that the phytochemical data were very consistent with those reviewed by Monschein et al. (2010), and given the UV-vis spectrum, it was plausible to identify the main not identified flavonoid at RT=20.8 minutes as callunin ((2R,3R)-5,7,8,4'-tetrahydroxydihydroflavonol-8-O-β-D-glucopyranoside) or a related derivative, yet reported in literature.
Beside flavonoids, HPLC-DAD analysis revealed that the main polyphenolic constituent in CVE was chlorogenic acid (RT= 10.9), 100 mg/L. The chromatogram allowed us also to detect other HCD in CVE, not unequivocally identified.
The peak at RT=12.8 showed a UV profile which could be associated with arbutin, already reported in the literature as a minority constituent of *C. vulgaris*.

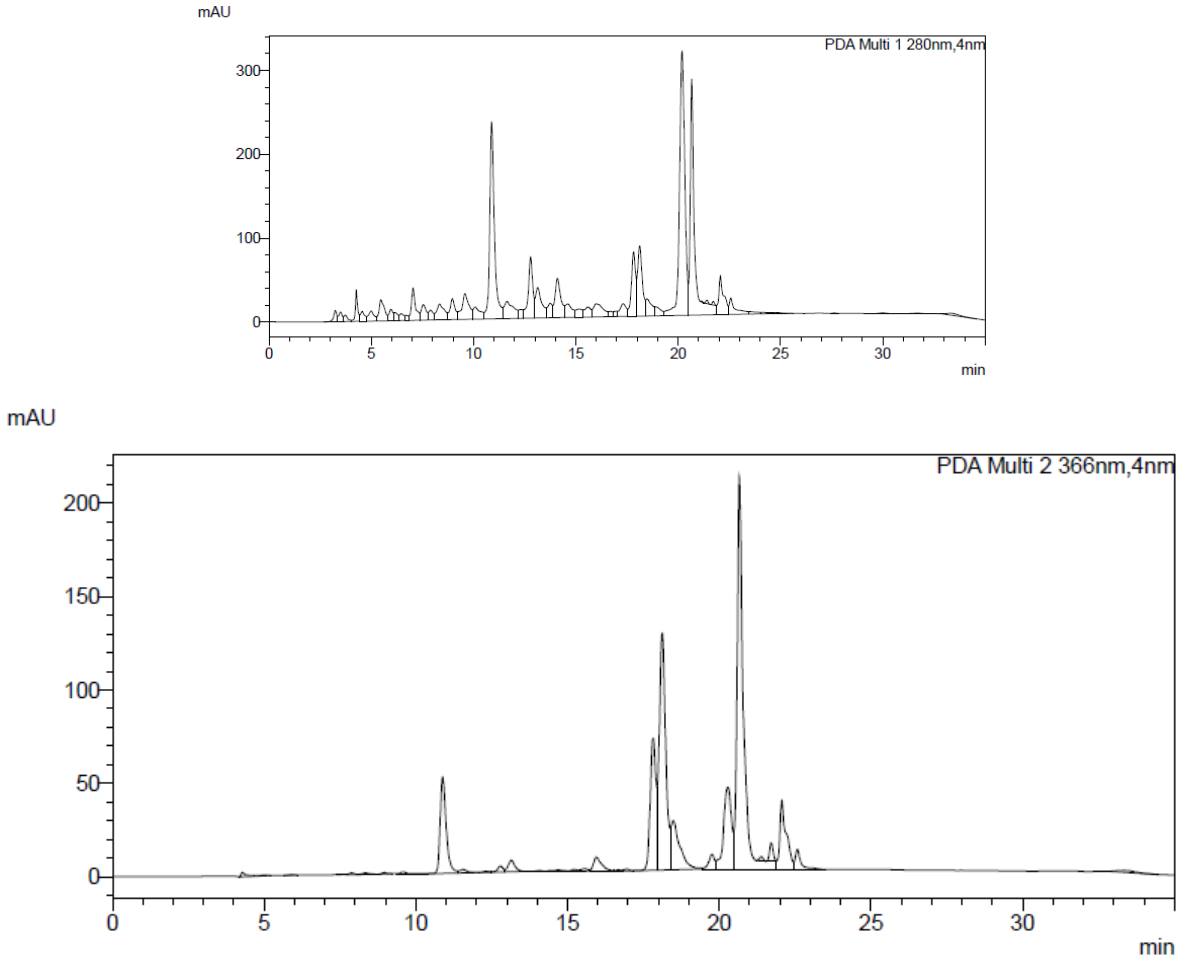


Figure 16 Chromatogram of C.vulgaris aerial part recorded at 280 and 366 nm.

Cistus creticus subsp. eriocephalus extract (CCE)

CCE resulted the extract with the highest content of total polyphenols (6541 mg/L), as yet shown in preliminary analyses. By observing the chromatogram, it was evident the presence of two main compounds at low RT; UV-vis spectra and published literature indicated a very plausible assignment referred to ellagitannins derivatives, such as punicalagins. These compounds have a λ_{max} at 270-280 nm and a low shoulder at 367-377 nm (Fyhrquist et al., 2020) (spectrum of compounds at RT=10.3 minutes in figure 17).

Quantification of these compounds were made by expressing data as gallic acid; ellagitannins represented 35% ca. of total polyphenols.

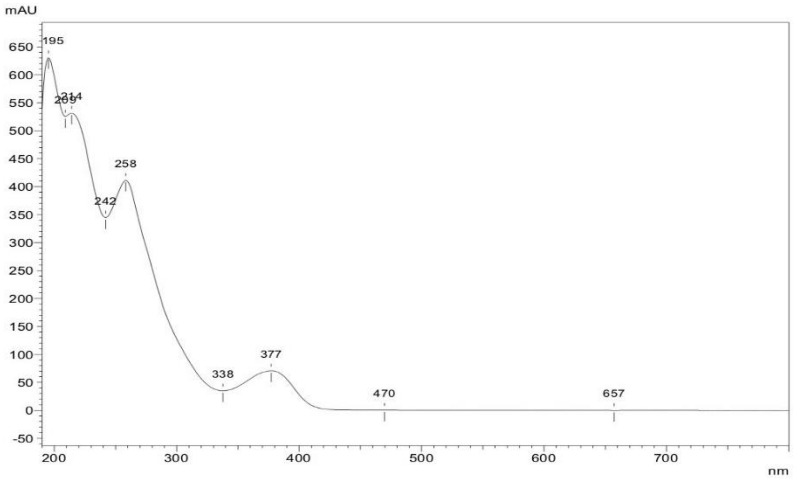


Figure 17: UV-visible spectrum of an ellagitaninus

CCE, as other studied extracts, was rich in flavonols, 550 mg/L. The flavonol fraction of CCE resulted similar to that of CVE; the three semi-overlapped myricitrin, isoquercitrin and hyperoside. It must be underlined that hyperoside, together with callunin, could be considered the chemical marker of *C. vulgaris* and myricitrin is often a secondary compound (Mandim et al.,2019), whereas the latter is the chemical marker of *C. creticus*, being isoquercitrin and hyperoside often in small amount (Aničić et al., 2021); in our samples we observed a peculiar inversion of the content of these flavonols in CVE and CCE.

Flavan-3-ols content in CCE was low: 344 mg/L.

Compound	mg/g dw of herbal material
Total polyphenols	65.4±2.5
Total flavan-3-ols	3.4±0.3
Flavonols	5.5±0.2
Hyperoside + isoquercitrin	1.3±0.1
Myricitrin	0.3±0.1
Ellagitannins	23.3±0.6

Table 6: Total phenolics and flavan-3-ols in CCE, expressed as mg/g extract. Phenolics are expressed as gallic acid, flavan-3-ols as catechin.

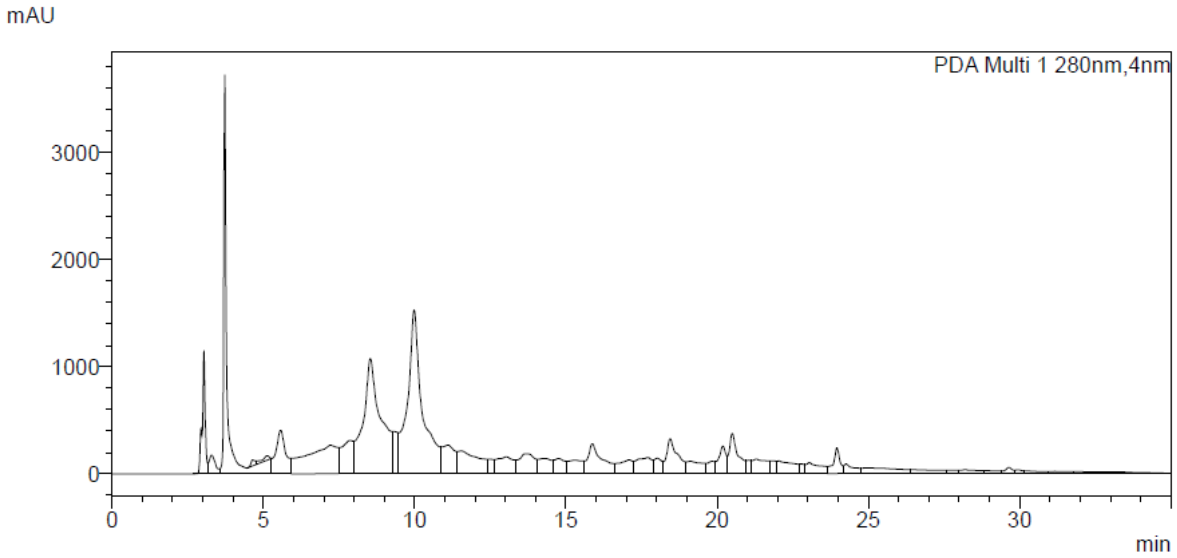


Figure 18: Chromatogram of cistus aerial part recorded at 280 nm.

8.1.1 DPPH Scavenging Assay

As antioxidant and antiradicalic activities could be considered pivotal in the context of skin protection from environmental pollution and aging-booster agents, the first activity tests carried out on selected extracts were focused on this direction.

In vitro cell-free tests, rapid and cheap, were considered first.

The DPPH scavenging activity of the samples were determined and expressed as IC_{50} values. The test showed positive results with high scavenging capacity exerted by all the extracts, as shown in table7.

Sample	IC_{50}
CLE	0.81±0.08 mg/ml
WLE	0.95±0.10 mg/ml
CCE	0.74±0.08 mg/ml
CVE	1.25±0.22 mg/ml

Table 7: IC_{50} values (mg/mL) resulting from DPPH test

All extracts showed comparable activity to other well-known botanical extracts or red wines, yet validated for their antiradical capacity (Biagi et al.,2009; Biagi et al., 2019).

The results were validated using ascorbic acid, which was confirmed to have an IC_{50} of less than 10 µg/ml (Biagi et al., 2009).

The data confirmed a good correlation between total phenolic content of extracts and antiradicalic activity, but not a clear correlation between activity and flavonols or flavan-3-ols.

In order to better investigate the different role of each extract in participating in the antiradicalic activity, DDPH test coupled with HPLC-DAD was repeated.

The figures below compare two chromatograms, the one above of samples before the DPPH reaction and below recording sample after DPPH reaction. Since high DPPH concentration was used and reaction immediately analyzed, the DPPH-HPLC-DAD allowed us to understand which compounds in each extract exerted the highest and more rapid DPPH quenching activity by monitoring their oxidation. As reported in Governa and co. (2021), all compounds that underwent DPPH-dependent oxidation were recorded with lower peaks' area.

Obtained results were very interesting.

WLE and CLE, extracts from by-products, showed a very similar behavior, showing a reduction of the entire flavonoidic fraction after DPPH reaction, suggesting that all flavonols participated to the antiradicalic activity in the same way (Figure 19-20)

Even in CVE, myricetrin, hyperoside, isoquercitrin and the not unambiguously identified callunin derivative displayed a strong and rapid antiradicalic activity; also chlorogenic acid almost completely disappeared. On the other hand, other flavonoids such as that at RT=20.3 and the secondary constituents at RT=21.2-22.4 exerted a lower antiradicalic activity against DPPH.

In CCE, the ellagitannins fraction was completely reduced, confirming the high antiradicalic capacity reported in the literature for this class of polyphenols. A strong reduction was also observed for myricitrin, whereas hyperoside was only partially active and other flavonoids at RT>20 minutes did not exert a rapid and clear antiradicalic effectiveness.

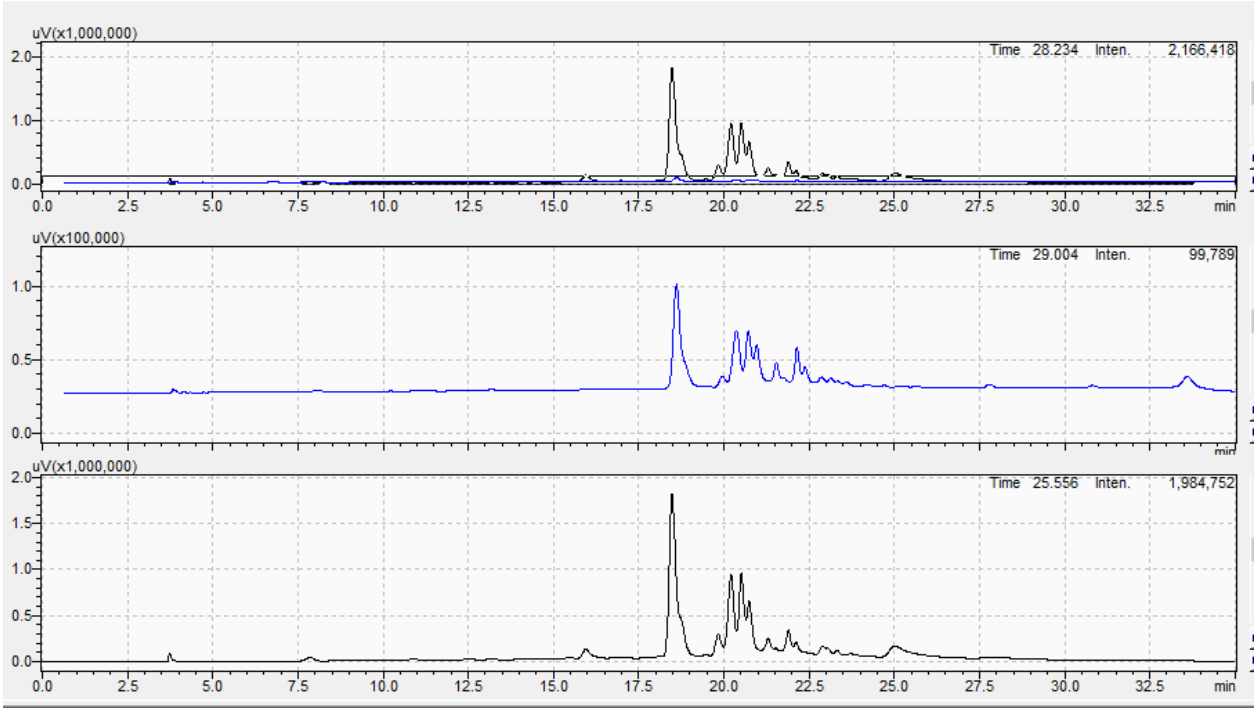


Figure 19: HPLC-DAD chromatograms of WLE, before (black) and after (blue) DPPH addition,

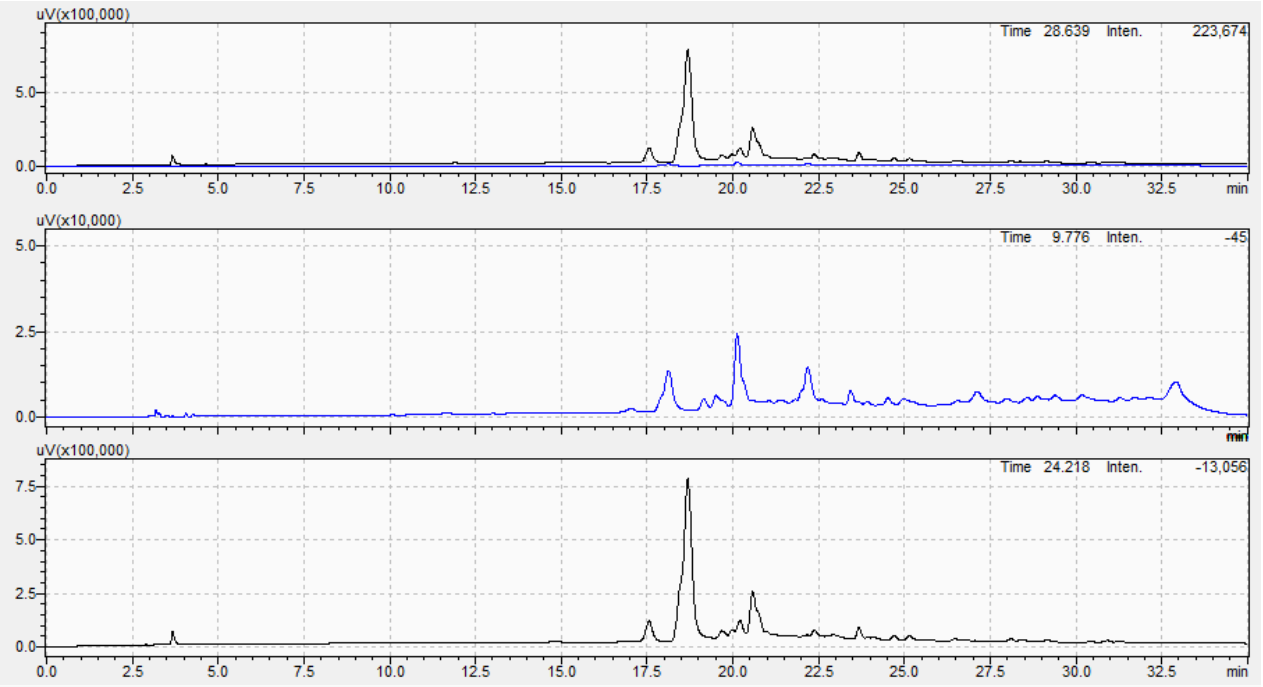


Figure 20: HPLC-DAD chromatograms of CLE, before (black) and after (blue) DPPH addition,

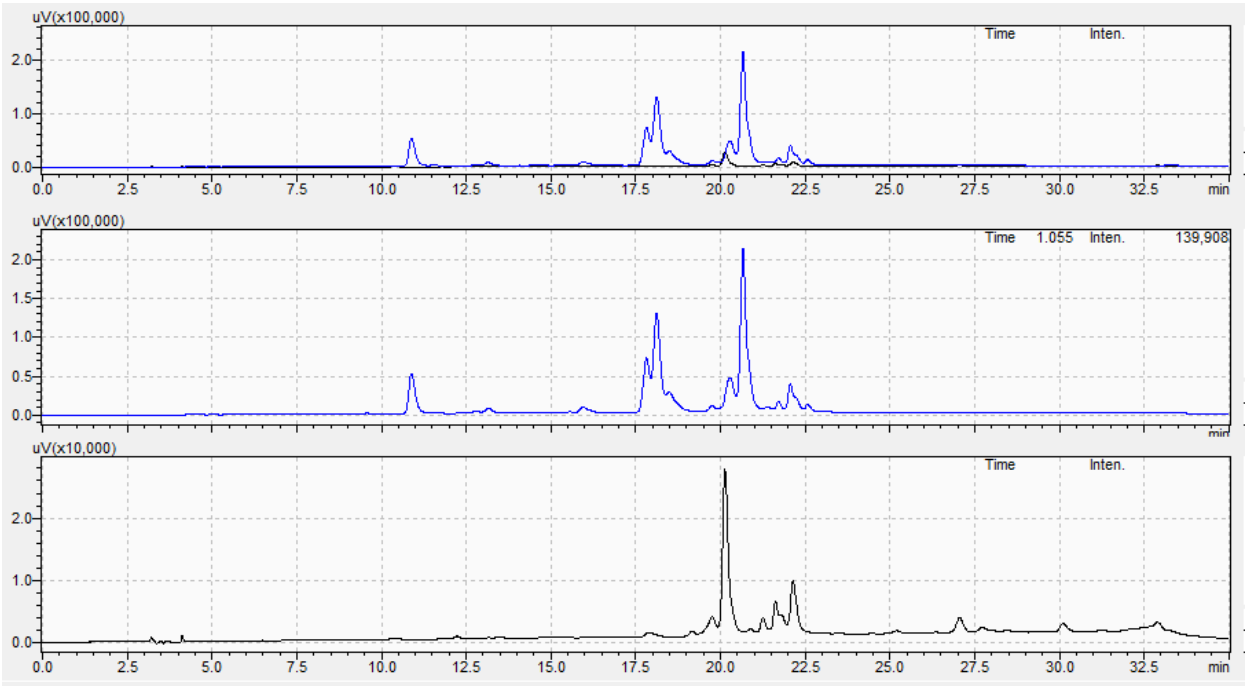


Figure 21: HPLC-DAD chromatograms of CVE, before (blue) and after (black) DPPH addition.

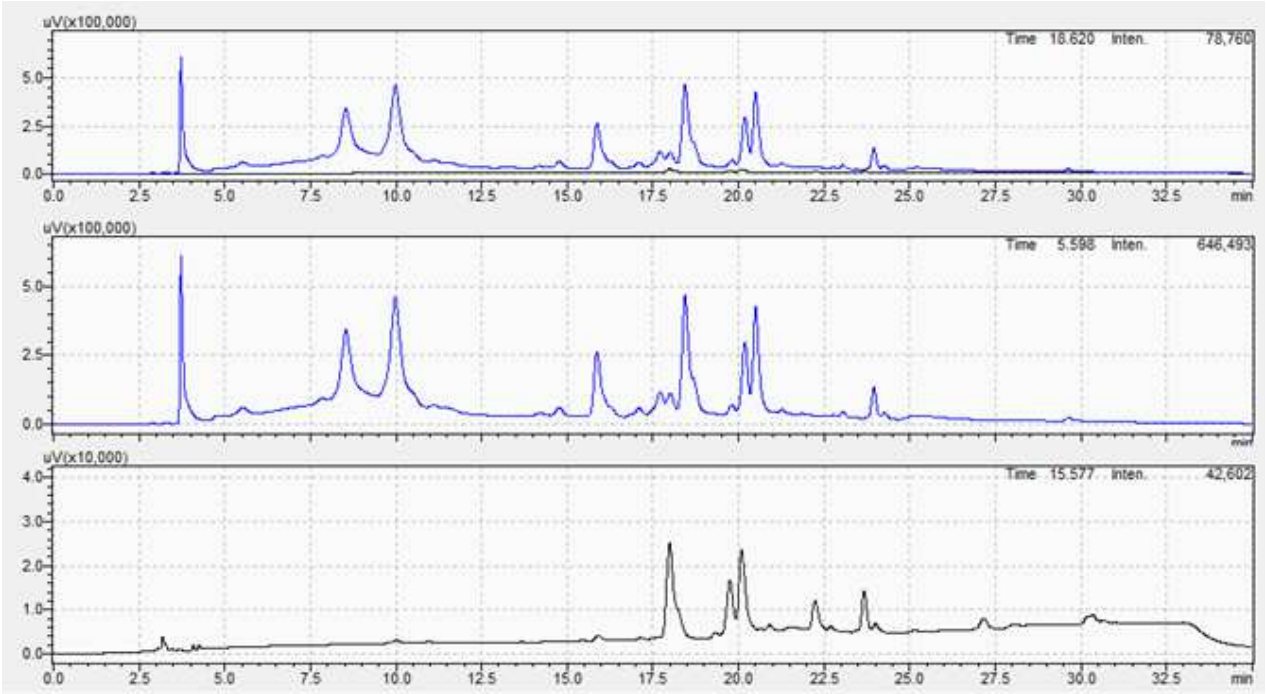


Figure 22: HPLC-DAD chromatograms of CCE, before (blue) and after (black) DPPH addition.

8.1.2 Electrochemistry

Electrochemical experiments (cyclic voltammetry and differential pulse voltammetry (DPV)) were carried out to obtain information on the redox properties of the extracts and also of its main flavonoids.

Cyclic voltammograms recorded in the solution containing quercetin showed a quasireversible oxidation process with an anodic peak at +0.33 V. The same profile was recorded in the solution containing hyperoside, but the anodic peak potential was reached at +0.44 V (data not shown in figures). More complicated redox behaviour was recorded in the extracts solution.

Indeed, the cyclic voltammogram of the extracts solution showed clear irreversible oxidative processes at low potentials for all considered samples.

CCE showed two main oxidation processes at low and medium potential: +0.36 and 0.46 V, respectively. Only CCE recorded an oxidation process at potential < 0.40 V and this strong red/ox activity could be linked to the peculiar presence of ellagitannins, yet main actors due to their antiradicalic capacity in DPPH test. The second oxidation process at 0.46 V of CCE could be related to the red/ox activity of flavonols, as confirmed by similar reactivity recorded for WLE (one main oxidation processes at +0.47 V and CVE, at +0.46 V). Nevertheless, CLE, also rich in flavonols, showed one main oxidation process with a peak potential of +0.55 V suggesting that in WLE and CVE other synergistic compounds were present, such as HCD, even this hypothesis could be not confirmed currently. More plausibly, both WLE and CVE received a very positive contribution from flavan-3-ols, as both these extracts that contained the highest amount of these compounds, displayed a similar oxidation process at +0.41 and +0.40, respectively.

CVE displayed a third distinct processes at +0.79 V, not easily understandable.

The current intensity measured at the peak potential in all recorded processes, both in cyclic voltammetry and pulse voltammetry, was in the μA range and reported a marked process associated with a good oxidation capacity exerted by the extracts. Electrochemical measurements, on the other hand, showed no reductive process, suggesting a pure, irreversible capacity of the extract to undergo oxidative processes and thus to serve as protective agents in presence of oxidant species.

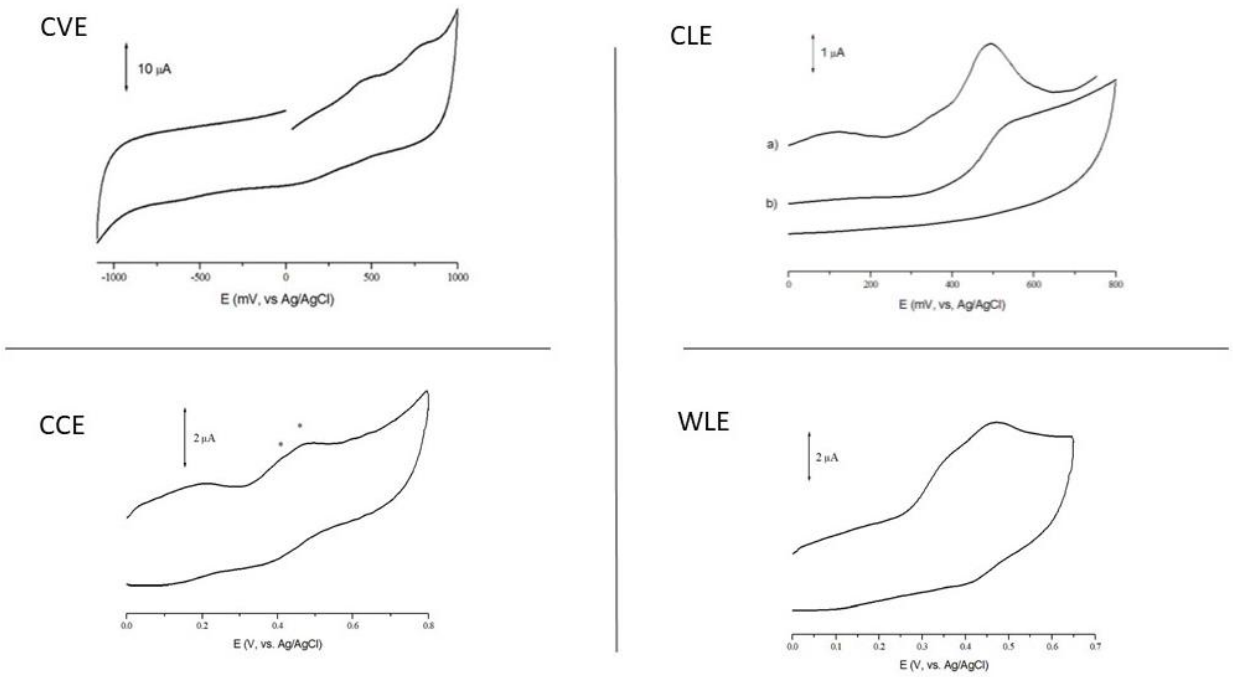


Figure 23: Cyclic differential voltammogram recorded on glass-carbon electrode. Scan rate: 20 mV/s.

Taken together, DPPH test and electrochemical measurements showed that the abundance of polyphenols in selected extracts actually played a marked antiradicalic and antioxidant activities, basilar in the context of the research for skin anti aging agents. Not only: these tests depicted the distinct role of polyphenols subclasses and provided a clearer correlation between chemical composition of extracts and their biological potential in counteracting cell oxidative stress.

8.2 *In vitro* tests on human skin cells

8.2.1 Cell Viability Assay

With the aim of defining the samples concentration dependent response on cell viability, the CCK-8 method was applied both on human keratinocytes and fibroblasts, treated with CCE, CVE, CLE and WLE for 24 hours.

As shown in figure 24 and 25, the samples did not significantly influence keratinocytes viability. Only at 2 mg/mL in HaCaT cells, CLE and WLE produced a slight decrease in cell viability.

HFF viability in treated cells increased slightly in samples tested at various concentrations but the differences compared to untreated cells were statistically significant only for CCE 2 mg/mL, CLE 1 mg/mL and WLE 0.25 mg/mL.

Based on these initial cell viability experiments and after some preliminary tests, it was decided to use WLE 1 mg/mL, CLE 1 mg/mL, CCE 2 mg/mL and CVE 4 mg/mL in all subsequent biological tests.

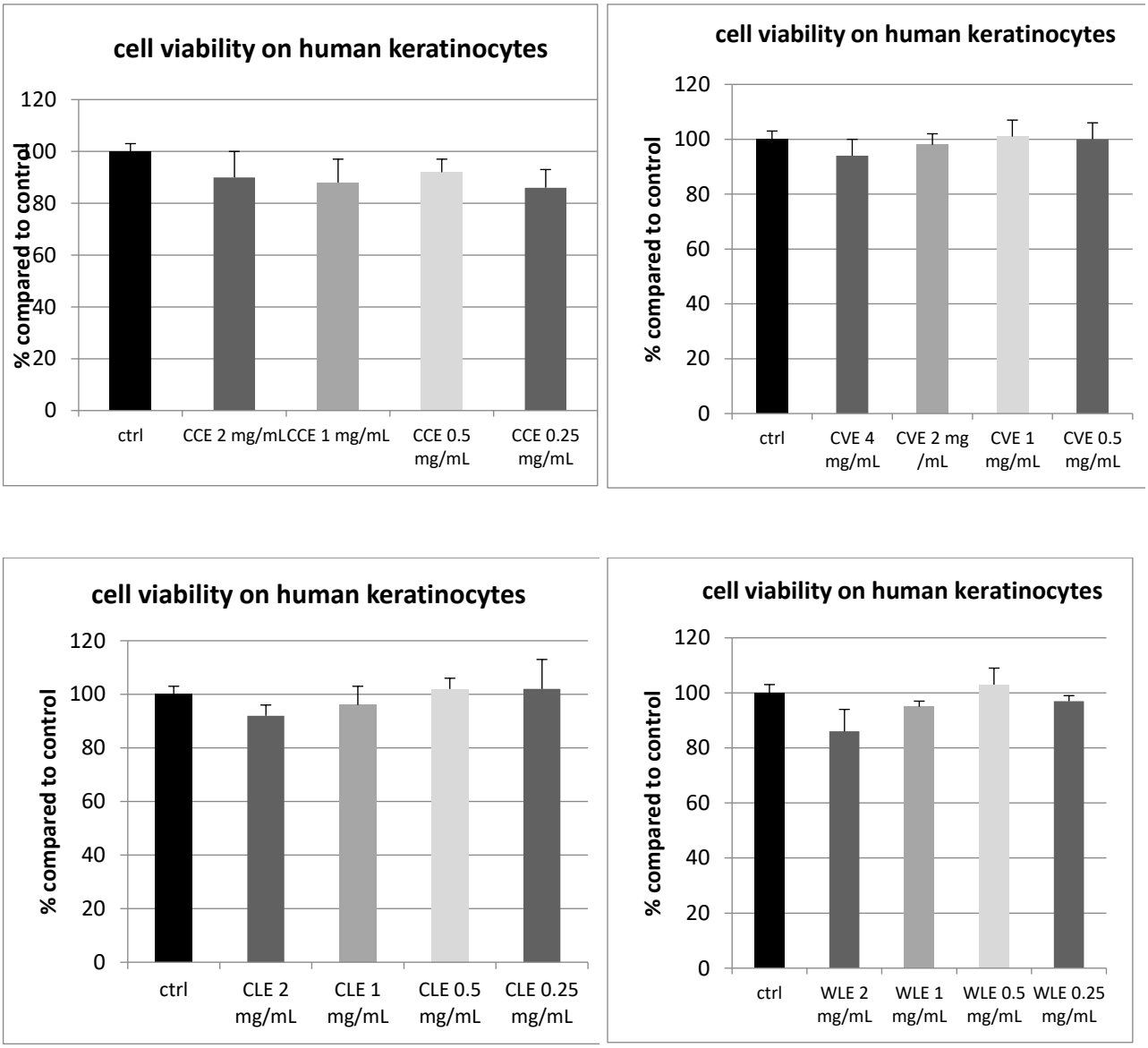


Figure 24: HaCaT cell viability

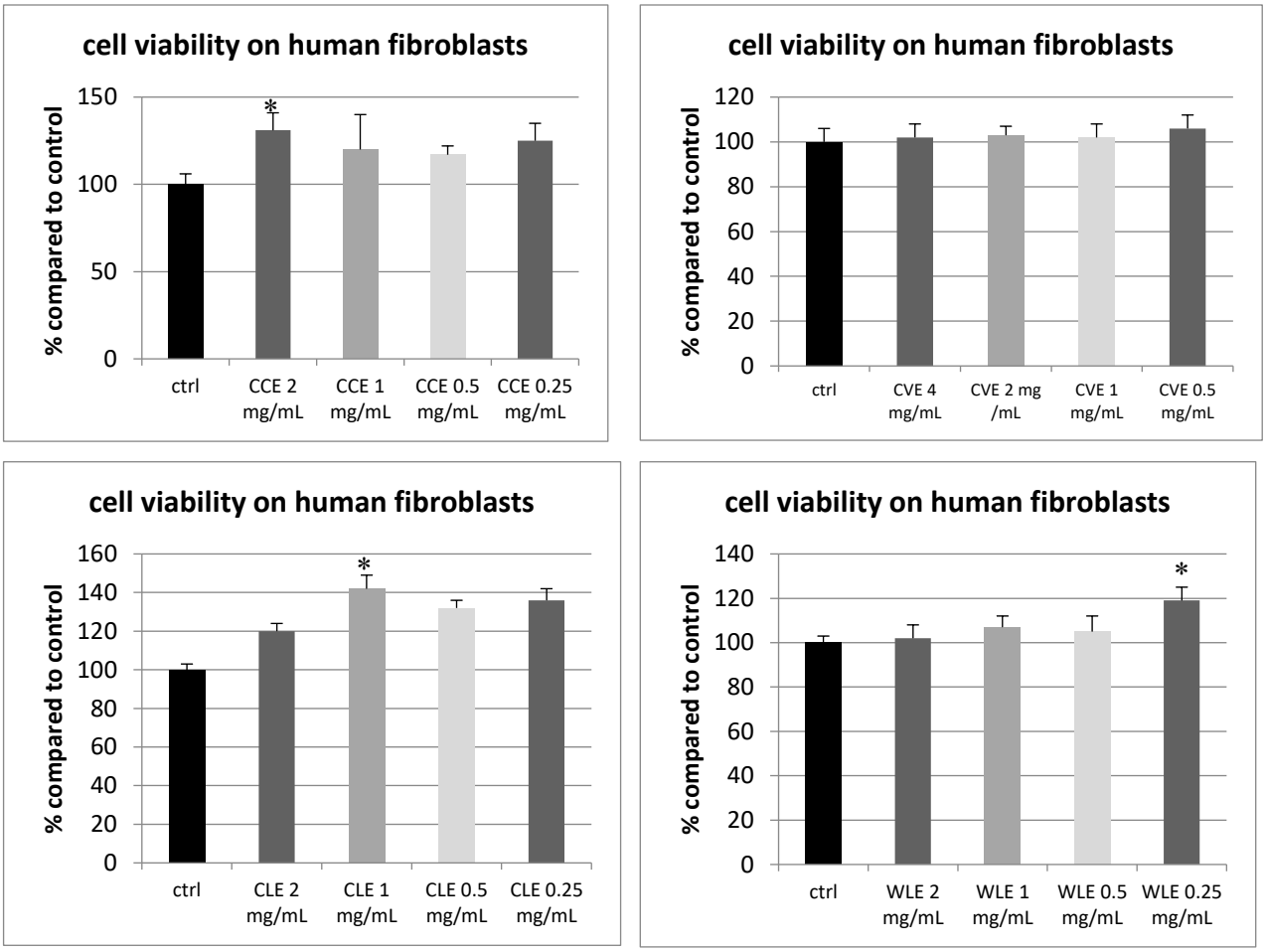


*Figure 25: HFF cell viability. *: p<0.05 vs ctrl*

8.2.2 Cell viability in an immunosuppressive model

It is well established that skin aging is related to epidermal and dermal immune impairment; indeed, decreased immune response may alter normal cell metabolism, viability and turnover, dysregulate barrier proteins synthesis in keratinocytes, such as filaggrin and loricrin, tight junctions, such as occludin and in fibroblasts mainly impairs ECM synthesis, namely collagen and elastin (Evrard et al., 2021).

In this work human keratinocytes and fibroblasts were treated with a corticosteroid drug, methylprednisolone (CORT) 1 and 2 mg/mL, respectively, which minimized the metabolic activity miming a skin aging condition.

In CORT-stimulated HaCaT and HFF the cell viability of was reduced by 20% ca., confirming the experimental model used was ideal to produce a marked cell stress.

Any tested sample was able to increase keratinocytes viability under conditions of slowed vitality. On the other hand, CVE 4 mg/mL and WLE 1 mg/mL, were effective in counteracting CORT-induced

lower cell viability in human fibroblasts ($p<0.05$ *vs* CORT). CCE 2 mg/mL and CLE also produced an increase in CORT-stimulated HFF viability, but differences between treated and only CORT-stimulated cells were not significant (Fig. 26).

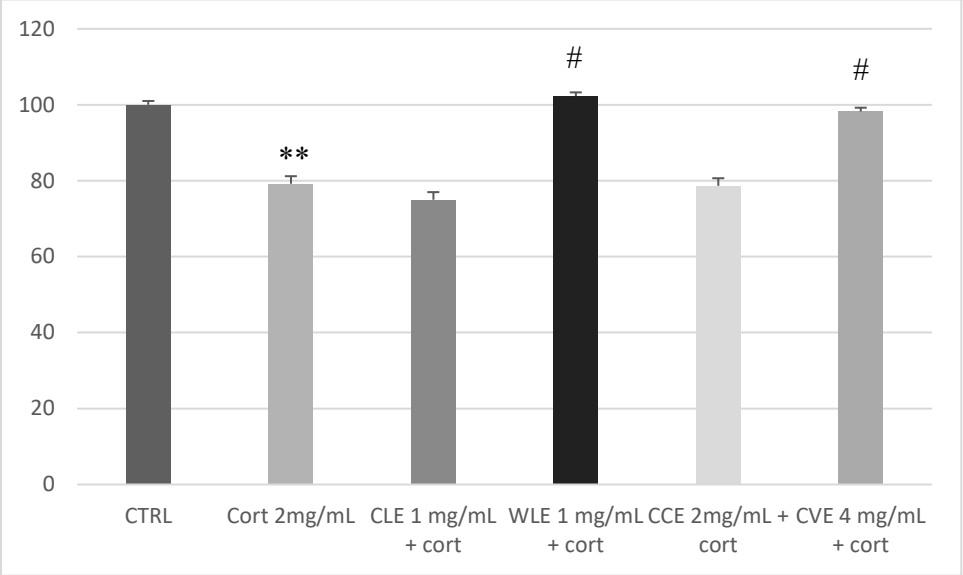


*Figure 26:HFF Cell viability in an immunosuppressive model. **: $p<0.01$ vs ctrl; #:$p<0.05$ vs CORT*

8.2.3 Filaggrin, loricrin and occludin dosage in human keratinocytes and pro-collagen I and elastin

After having evaluated the impact of selected extracts in skin cells viability in normal and aging-mimed conditions, we studied the effect of samples in modulating the synthesis of the most important proteins characteristic of both keratinocytes and fibroblasts that in aged human skin naturally slowed down. The expression of filaggrin and loricrin, two of the main proteins involved in the maintenance of the skin barrier in the epidermis, was analyzed after treating keratinocytes with the test substances, whereas pro-collagen I and elastin were dosed in human fibroblasts.

The WB analysis showed a slight increase in the expression of filaggrin and loricrin induced by CLE 1 mg/mL, even if the difference with control was not statistically significant. Conversely, CCE 2 mg/mL and CVE 4 mg/mL slightly lowered proteins' expression, again not in a significant manner.

With regards to the tight junctions protein occludin, WB analysis showed a significant increase in protein levels following WLE 1 mg/mL and CVE 4 mg/mL treatment of keratinocytes ($p<0.05$ *vs* ctrl). Again, CCE slightly lowered occluding expression compared to control.

Summarizing, selected extracts did not produce a marked effect in modulating cell viability in human keratinocytes, in normal and in immune-impaired conditions, nor in promote the expression of skin

barriers proteins, such as filaggrin and loricrin; despite this, a noticeable effect of WLE and CVE in upregulating occluding expression was shown.

As regards human fibroblasts, selected extracts resulted ineffective in modulating pro-collagen I and elastin expression.

After having studied the studied samples in a preliminary way both on keratinocytes and fibroblasts, subsequent stress models were focused only on HaCaT cells, considering the dramatic importance of aging-related impairment at epidermal level.

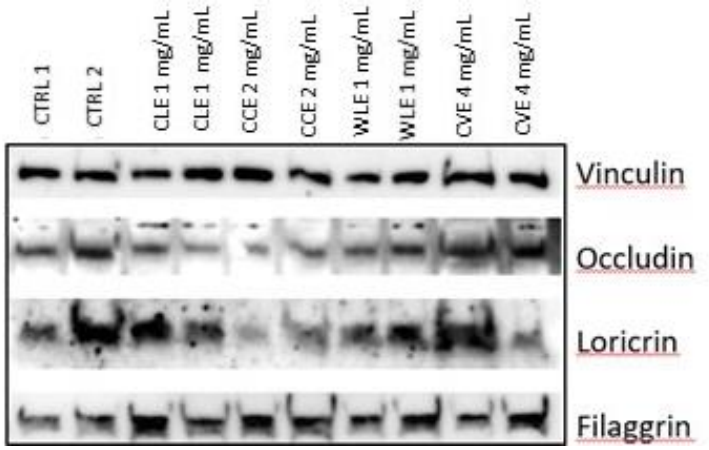


Figure 27: *Western Blot analysis*

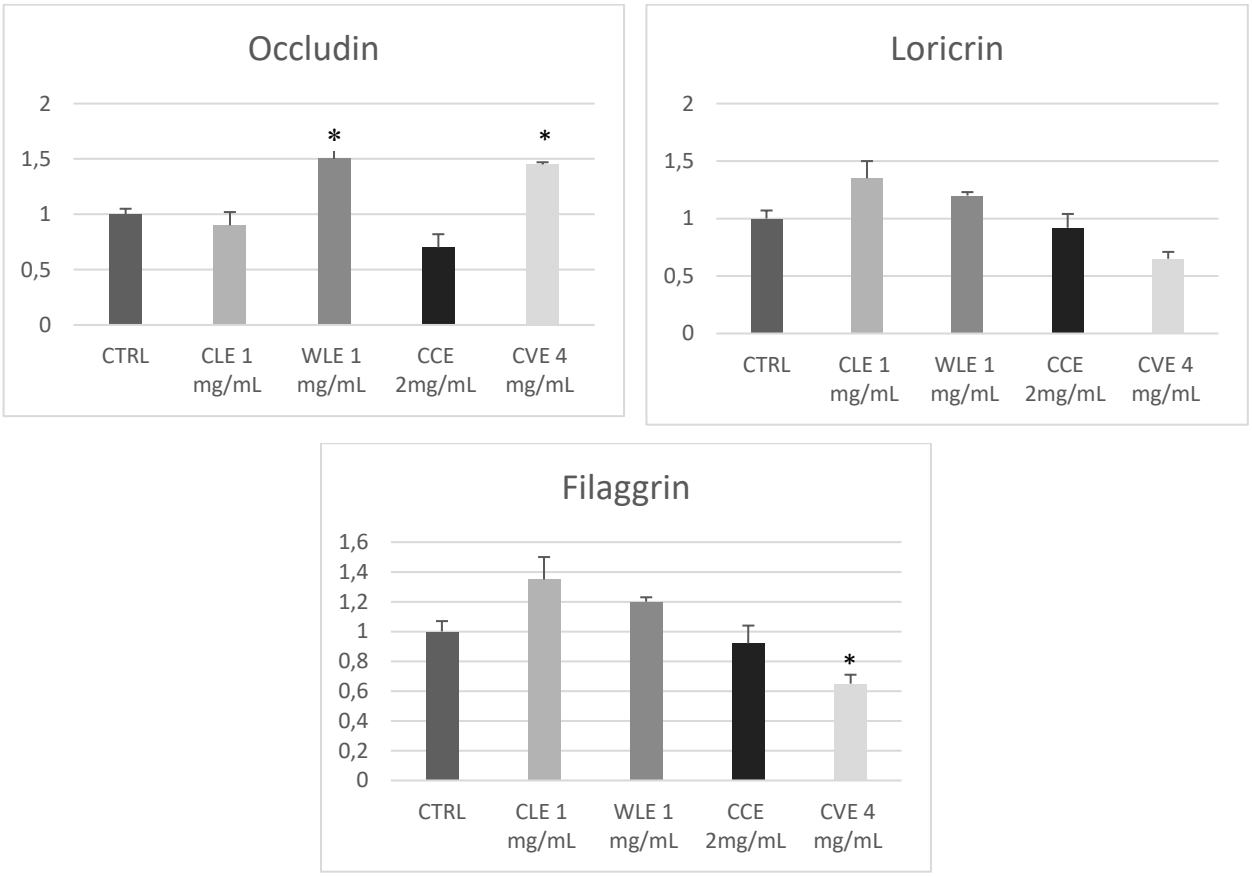


Figure 28: *Expression of occludin (A), loricrin (B), and filaggrin (C) in cultured keratinocytes after treatment with CLE, WLE, CCE and CVE, respectively. *: p<0.05 vs ctrl.*

8.2.4 Effectiveness of botanical extracts in a Hyperosmotic aging model

Moisturizing agents are fundamental to preserve skin barrier integrity and epidermis and dermis homeostasis when, such as in aged skin, hydration is compromised. Skin hydration depends on the presence of hydrophilic polymers such as hyaluronic acid, that bonds a massive quantity of water and regulate water movement towards and from cells, through different types of aquaporins; in addition, epidermal barrier limits the transepithelial water loss (Verdie-Sevrain and Bonte, 2007). A key element in maintaining skin hydration is, thus, preserving keratinocytes barrier function in case of osmotic dysregulation.

The stress model set up in this work took into account this osmotic impairment.

In a hyperosmotic environment, obtained with high NaCl concentration, keratinocytes viability dramatically decreased by 60% ca.

Interestingly, WLE 1 mg/mL and CVE 4 mg/mL partially counteracted the hyperosmotic damage and the differences between treated and untreated cells were statistically significant (figure 29).

In contrast, CCE, CLE were not able to counteract the damage induced.

The effectiveness of WLE and CVE in hydrating keratinocytes in a hyperosmotic stress model and in modulating occludin expression suggested the fascinating hypothesis of a link between these activities, since the role of tight junctions in controlling transmucosal permeability to solutes, water, and electrolytes was clearly elucidated (Musch et al., 2006).

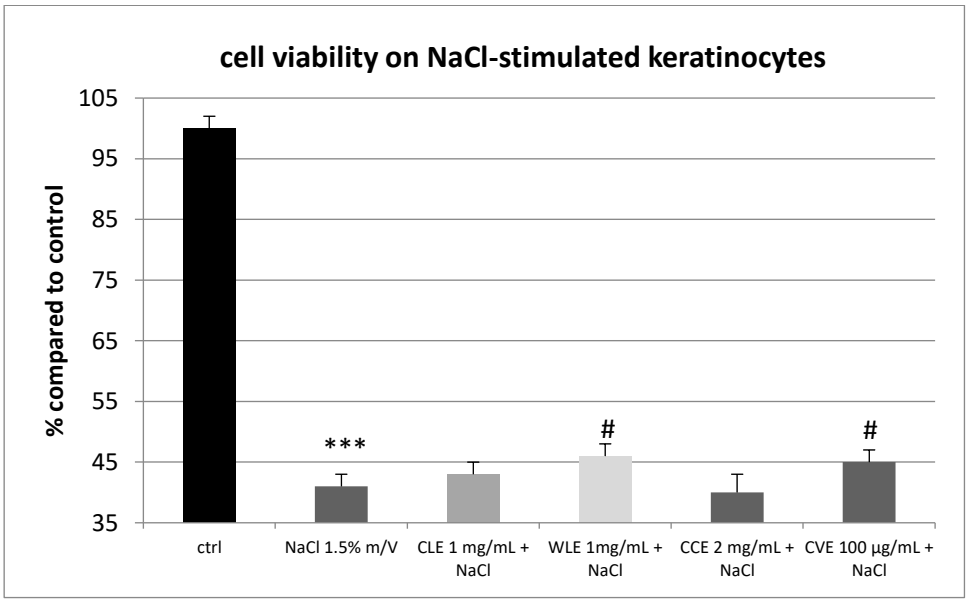


*Figure 29: cell viability on NaCl-stimulated keratinocytes. ***: $p<0.001$ vs NaCl; #: $p<0.05$ vs NaCl.*

8.2.5 UVA irradiation protection

The assessment of the high antiradicalic and antioxidant activities exerted by selected extracts in cell-free tests, leaded us to plan a large *in vitro* protocol consisting in cell-based evaluations. The first test considered the photo-protective action of the extracts against the effect of UVA in cell viability.

The HaCaT cell line is considered a good model for toxicity and photogenotoxicity studies involving human skin (Burlando et al, 2008; Horinouchi and Arimoto-Kobayashi, 2011).

The test was carried out using the following experimental model: HaCaT cells were pre-treated with WLE 1 mg/mL, CLE 1 mg/mL, CCE 2 mg/mL and CVE 4 mg/mL for 4 hours and then subjected to UV irradiation at different times 15, 30 min and 1 hour. Values were expressed as a percentage of viability compared to the negative control with 100% viability. Exposure of the HaCaT cell line to UVA resulted in a reduction of the viability of cells

Induction of UVA in 15 minutes showed a statistically significant reduction in keratinocyte viability ($p<0.05$ *vs* ctrl); after 1 hour of UVA exposure, the damage became particularly significant, reaching a 50% decrease in viability ($p<0.001$ *vs* ctrl).

As shown in figure 30, CVE and CCE were particularly effective in counteracting cytotoxicity after 60 of UVA exposure (cell viability UVA-stimulated cells: 54.0% vs 61.6% in CVE+UVA and 61.3% in CCE+UVA, $p<0.01$ *vs* UVA-ctrl) and also after 30 minutes of UVA exposure (cell viability UVA-stimulated cells: 61.6% vs 68.5% in CCE+UVA and 66.1% in CVE+UVA, $p<0.05$ *vs* UVA-ctrl); on the other hand, they were not effective after short UVA exposure. WLE and CLE, used at lower concentrations, did not exhibit efficacy in this test.

The different kinetic of activity of CVE and CCE, more effective at longer times, suggested that these extracts had a detoxification capacity related to a direct scavenger activity, adequately confirmed in our previous experiments but also plausibly related to intracellular antioxidant systems modulation. The effect of many polyphenols in improving keratinocytes survival after photo-toxicity by targeting Nrf-2/SOD signaling has been confirmed for curcumin and grape seed extracts (Brand et al., 2018), as well as their effects in enhancing DNA repair response, as demonstrated for *Perilla frutescens* (L.) Britt. extracts (Lee and Park, 2021).

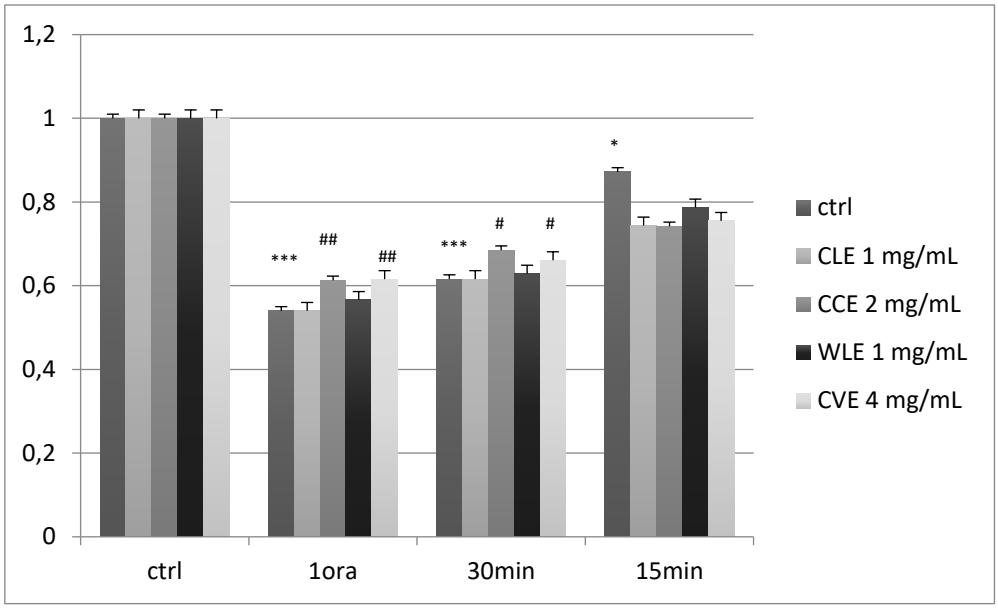


*Figure 30: Vitality of keratinocytes after UVA exposure treated with CLE, WLE, CCE and CVE. *: p<0.05 vs ctrl; ***: p<0.001 vs ctrl; #: p<0.05 vs UVA; ##: p<0.01 vs UVA.*

8.2.6 Effectiveness of botanical extracts in oxidative stress models

Three different *in vitro* oxidative-stress models were set up to mimic the upregulation of ROS level in human keratinocytes related to skin aging.

Two models, classical and validated, consisted in HaCaT stimulation with TBHP and H_20_2; the third could be considered a physiological model of oxidative stress caused by cellular suffering due to serum deprivation.

In this work, the conditions for doubling ROS production in keratinocytes after stimulation were optimized.

The aim of the tests was to evaluate the capacity of samples of reducing harmful high ROS levels in stimulated epidermis cells. The tested samples were, again, WLE 1 mg/mL, CLE 1 mg/mL, CCE 2 mg/mL and CVE 4 mg/mL.

In the first tests HaCaT cells were pre-treatment with samples for 24 hours in order to better investigate their capacity to activate intracellular antioxidant and antiradicalic defenses.

As shown in figure 31, stimulation with TBHP after 3 hours doubled production of ROS in stimulated cells compared to unstimulated cells.

CVE, and in a lesser extent CLE, were able to preserve the cell from the release of high ROS levels in a statistically significant manner (CVE -22.55% *vs* TBHP, CLE -15.20% *vs* TBHP, p<0.05 *vs* TBHP); WLE and CCE were not effective in this model.

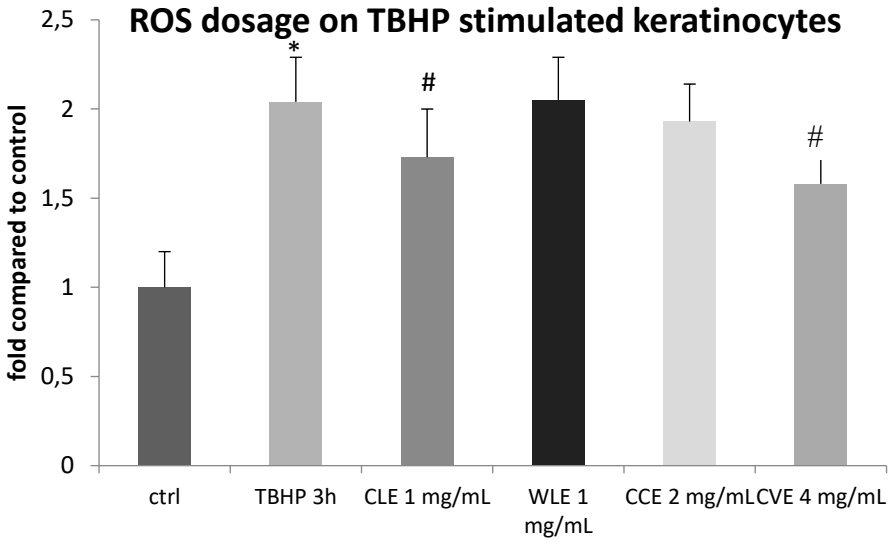


*Figure 31: ROS dosage on TBHP stimulated keratinocytes. *: p<0.05 vs ctrl; #: p<0.05 vs TBHP.*

The second test evaluated the effectiveness of the extracts to reduce oxidative stress was carried out by stimulating the cells with H_2O_2.

This experiment showed that, similarly that in the photo-protective test, treating HaCaT with CCE 2 mg/mL and CVE 4 mg/mL for 24 hours and H_2O_2 1mM for 45 minutes resulted in a statistically significant decrease in the level of intracellular ROS (CVE -31.97% *vs* H_2O_2, CCE -24.66% *vs* H_2O_2, $p<0.05$ *vs* H_2O_2).

CLE and WLE did not prove efficacy in protecting cells from induced oxidative stress.

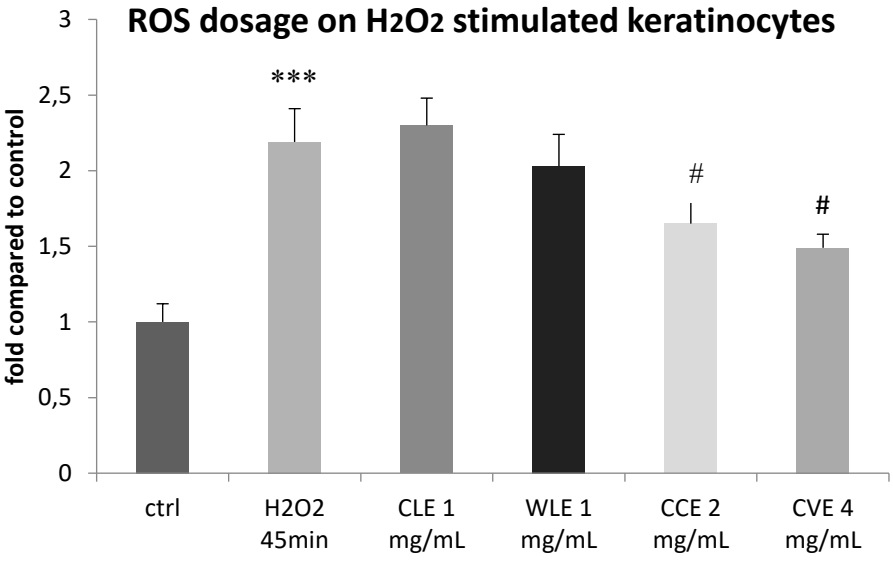


*Figure 32: ROS dosage on H_2O_2 stimulated keratinocytes. ***: p<0.001 vs ctrl; #: p<0.05 vs H_2O_2.*

In summary, in the *in vitro* models of ROS excess production in human keratinocytes induced by classical stimuli such as TBHP and H_2O_2, as well as in UVA-induced cell oxidative model, it was highlighted that CVE 4 mg/mL was markedly effective in reducing intracellular reactive species and preserving cell viability, providing good evidence that Italian *C. vulgaris* may actually represent a new skin protective agent.

In the serum deprivation model, that mimed a sub-chronic oxidative stress, interestingly, it was noted that in 24 h, ROS almost doubled (+94% compared to normally-cultured cells) All tested samples exhibited efficacy in reducing ROS production, the samples that stood out for their effectiveness were CCE and CVE that completely preserved ROS production. CLE treated cells had -21.10% ROS production compared to positive control, but difference was not statistically significant; WLE -27.32% ($p<0.01$ *vs* pos. ctrl), CCE and CVE > 99% reduction ($p<0.001$ *vs* pos. ctrl).

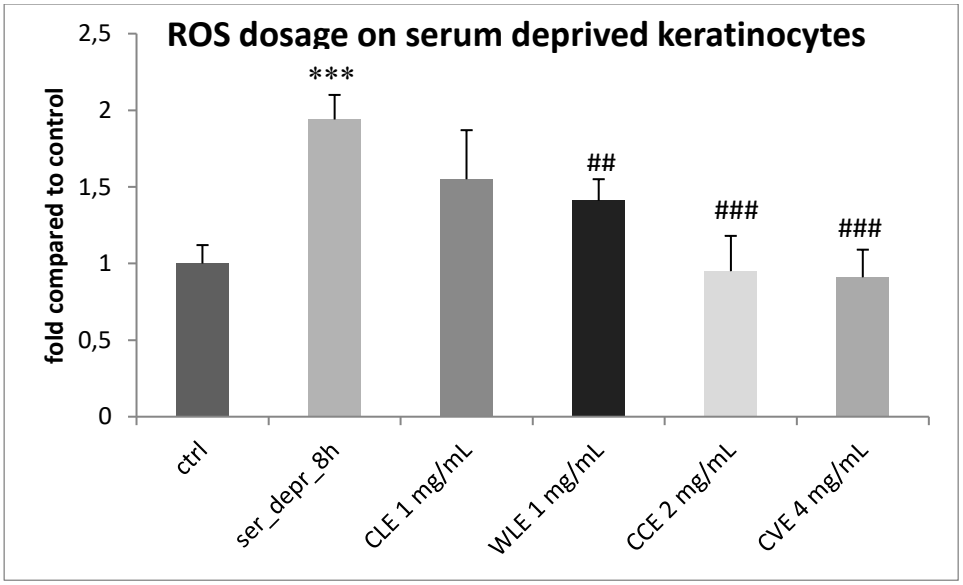


*Figure 33: ROS dosage on serum deprived keratinocytes. ***: $p<0.001$ vs ctrl; ##: $p<0.01$ vs ser_depr.; ###: $p<0.001$ vs ser_depr.*

UVA exposure aimed to represent the skin photo-damage, TBHP- and H_2O_2-induced ROS production wanted to simulate an acute oxidative stress, while serum deprivation was chosen to simulate a slower, more physiological condition of ROS excess in skin cells. Each condition is related to skin aging and, in many cases, they act as sum of events as in the case of constant exposure to environmental pollution. Noteworthy, all selected extracts, even if with different extent, demonstrated effective antioxidant skin protective activity.

8.2.7 Inhibition of ECM-degrading Enzymes

UV rays and high ROS levels, as well as inflammatory factors, both in epidermis and dermis, in aged skin unbalance the ratio between synthesis and catabolism of skin structures, in particular connective ECM; indeed, skin aging is related to the upregulation of the activity of collagenases, elastases, hyaluronidases and tyrosinase.

By means of validated cell free *in vitro* enzymatic assays, CLE, WLE, CCE, CVE, were tested as inhibitor of these degrading enzymes.

It was important to note the selectivity of the extracts in the enzymatic inhibition of extracellular matrix degradation targets such as elastase, hyaluronidase and collagenase, not observed on tyrosinase, which is known to primarily act on skin with a depigmenting effect.

CCE proved to be the most active extract on all targets. CCE showed a very strong activity in inhibiting elastase activity (IC_{50} < 1 mg/mL), whereas it had a comparable activity on collagenase and hyaluronidase; CVE also produced a very good inhibitory activity on hyaluronidase but, interestingly, it resulted ineffective on other targets. WLE exerted a good effect on hyaluronidase activity, and a fair efficacy on elastase and collagenase; CLE did not exerted inhibitory effect on collagenase, but it provided to be fairly effective on elastase and hyaluronidase. Results on the inhibitory effect on ECM degrading enzymes activity are summarized in table 8.

Noteworthy, overall data suggested a different role of each specific extracts' phytocomplex in enzyme activity inhibition: the plausible role of CCE characteristic ellagitannin should be better investigated, but also flavan-3-ols of CVE and WLE in hyaluronidase.

Sample	Tyrosinase	Elastase	Collagenase	Hyaluronidase
		IC_{50} (mg/mL)		
CLE	>10	8.1±1.0	>10	5.1±1.0
WLE	>10	9.4±0.8	4.7±1.0	2.5±0.6
CCE	>10	0.7±0.1	2.1±0.4	1.6±0.4
CVE	>10	>10	>10	2.1±0.2

Table 8: Inhibition of the activity of tyrosinase, elastase, collagenase and hyaluronidase exerted by extracts.

These tests concluded the third and largest part of this work, after the screening phase and chemical analyses and cell-free antiradicalic/antioxidant essays that were included in the second phase.

8.3 Activity Of Selected Extracts On Human Keratinocytes Stimulated With Urban Dust

As a specific and very important part of this research work, we evaluated the cell impact of urban dust (UD) on human keratinocytes. We immediately confirmed that UD was impactful at the level of epidermal cells: indeed, it altered vitality, oxidative stress through the production of ROS and inflammatory response. In order to better investigate this latter, we evaluated pro-inflammatory cytokines induced by NF-κB activation at downstream level and upstream, the activation of transduction factors such as MAPKs.

8.3.1 Cell Viability On Keratinocytes Stimulated With Urban Dust

The viability of UD-stimulated cells decreased by 20% ca. The samples tested, WLE 1 mg/mL, CLE 1 mg/mL, CCE 2 mg/mL and CVE 4 mg/mL showed no modulation of cell viability of UD-stimulated keratinocytes after 24 hours of treatment.

8.3.2 Oxidative Stress Models With Urban Dust

This work investigated the capacity of substances to reduce the production of ROS caused by UD. UD, after 2 h of exposure, caused the strongest release of ROS in HaCaT cells, 2.96 folds respect to control.

CVE 4 mg/mL and WLE 1 mg/mL were only able to slightly modulate ROS production UD-induced and CLE was devoid of effect; on the other hand, CCE reduced by 20.3% ($p<0.05$ *vs* UD) ROS production and confirmed to exert a high antioxidant/antiradicalic activity.

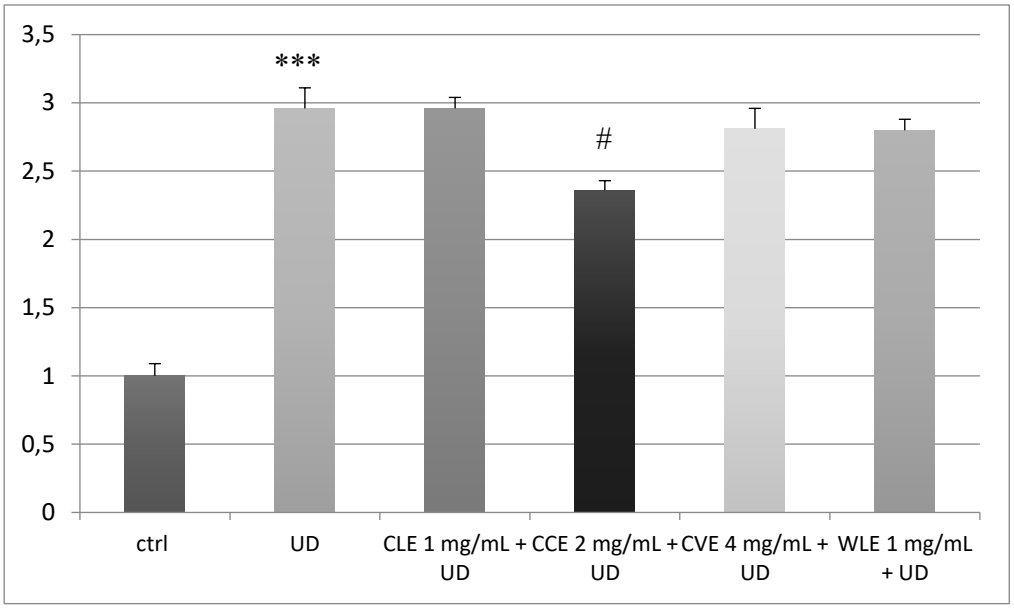


*Figure 34 ROS dosage on UD stimulated keratinocytes. ***: $p<0.001$ vs ctrl; #: $p<0.05$ vs UD.*

8.3.3 Anti-Inflammatory Activity Of Samples On Keratinocytes Stimulated With Urban Dust

The developed experimental model showed that dosed inflammatory cytokines were over-regulated when keratinocytes were stimulated with UD.

In animal models with a damaged skin barrier, topical application of particulate resulted in upregulating cytokines expression, dermal cell infiltration and increased epidermal thickness (Jin et al., 2018). This indicates that particulate matter could exacerbate symptoms of skin diseases with an already damaged skin barrier as in AD, supporting the epidemiological evidence that particulate matter in the air can exacerbate AD symptoms.

Even in our *in vitro* model, UD stimulation on HaCaT significantly stimulated IL 6 and 1β production, TNF-α, in a lesser but significant extent, while only slightly modulated IL 8 level (+20% ca. compared to control).

CCE 2 mg/mL showed a marked effect in inhibiting cytokines release UD-induced; this extract completely reverted the over-regulation of IL-6 and TNF-α and reduced IL-1β over-regulation UD-induced (folds to control: 1.45 *vs* 1.61 UD, $p<0.05$). The slight IL-8 over-regulation was not reverted by CCE.

CLE, WLE and CVE were not able to lower TNF-α, IL-1β and IL-8, but they had a weak, although non-significant effect on IL-6. On the other hand, even if in different extent, CLE, WLE and CVE showed a synergistic effect with UD in promoting TNF-α, IL-1β and IL-8 expression; the understanding of this effect exerted by CLE, WLE and CVE currently is not complete and it is worthy to be investigated,

but it could be taken into account an UD complementary mechanism, such as the modulation of transduction and/or transcription factors different from NF-κB, classically triggered by the mixed pro-oxidant/inflammatory stimulus provided by UD.

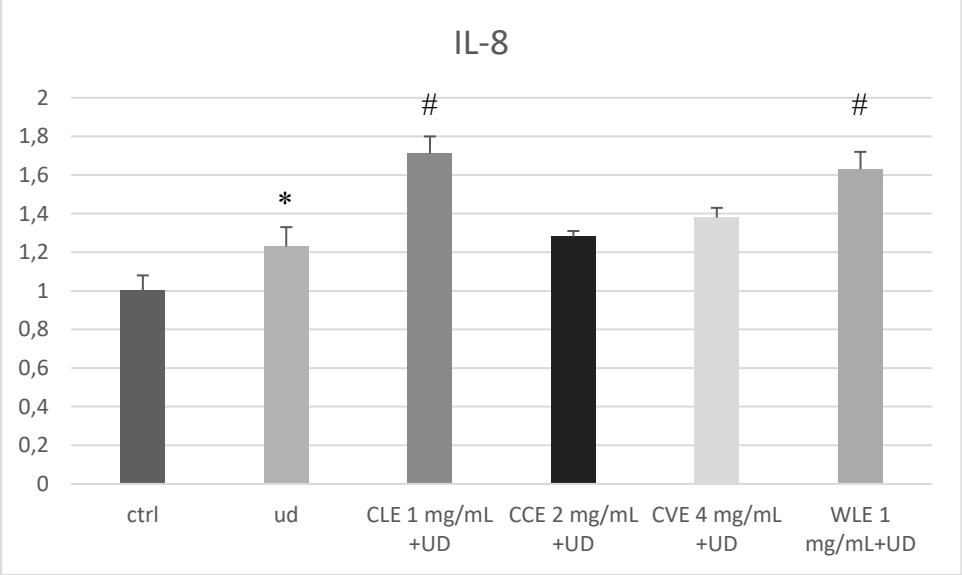


*Figure 35: Inhibition of IL-8 secretion in UD-stimulated HaCaT cells by extracts. *: p<0.05 vs ctrl; #: p<0.05 vs UD.*

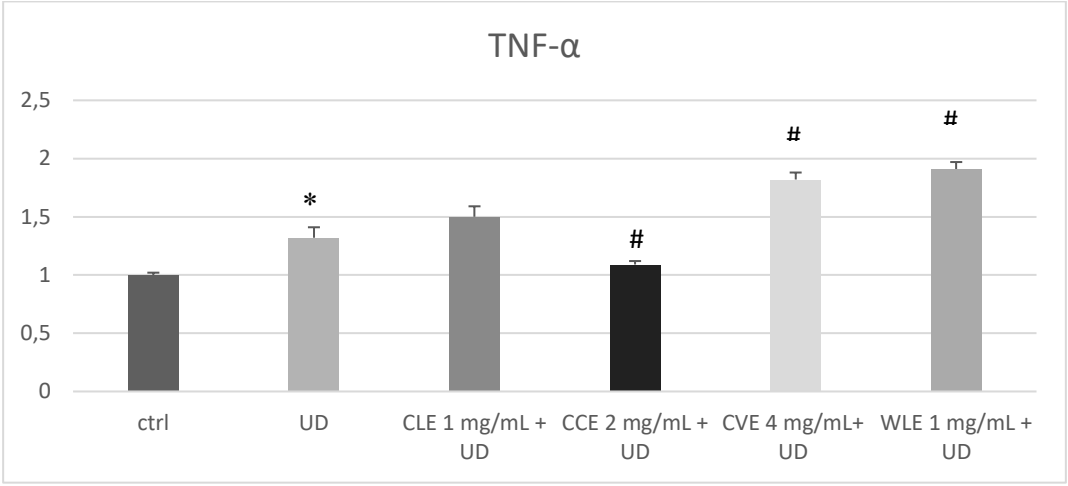


*Figure 36: Inhibition of TNF-α secretion in UD-stimulated HaCaT cells by extracts. *: p<0.05 vs ctrl; #: p<0.05 vs UD.*

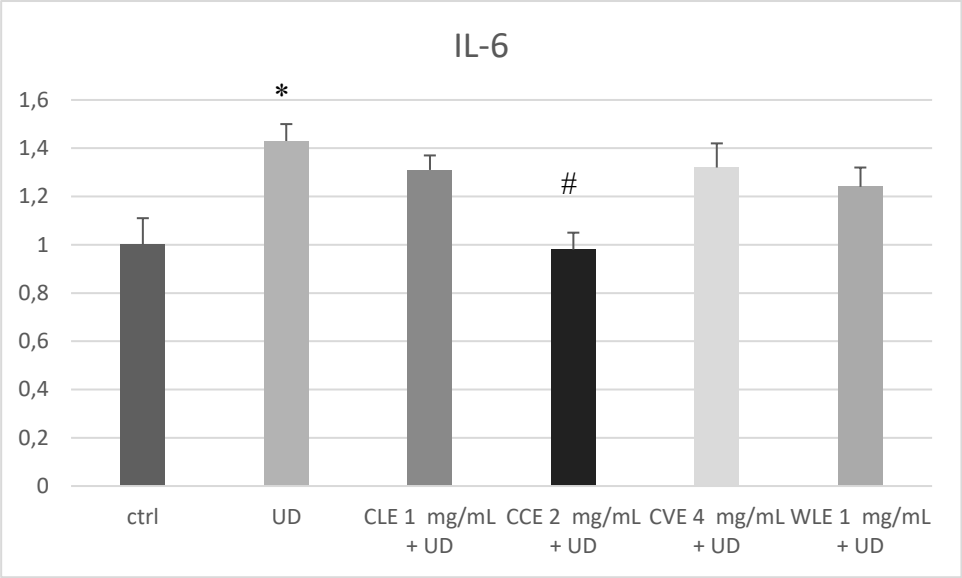


*Figure 37: Inhibition of IL-6 secretion in UD-stimulated HaCaT cells by extracts. *: p<0.05 vs ctrl; #: p<0.05 vs UD.*

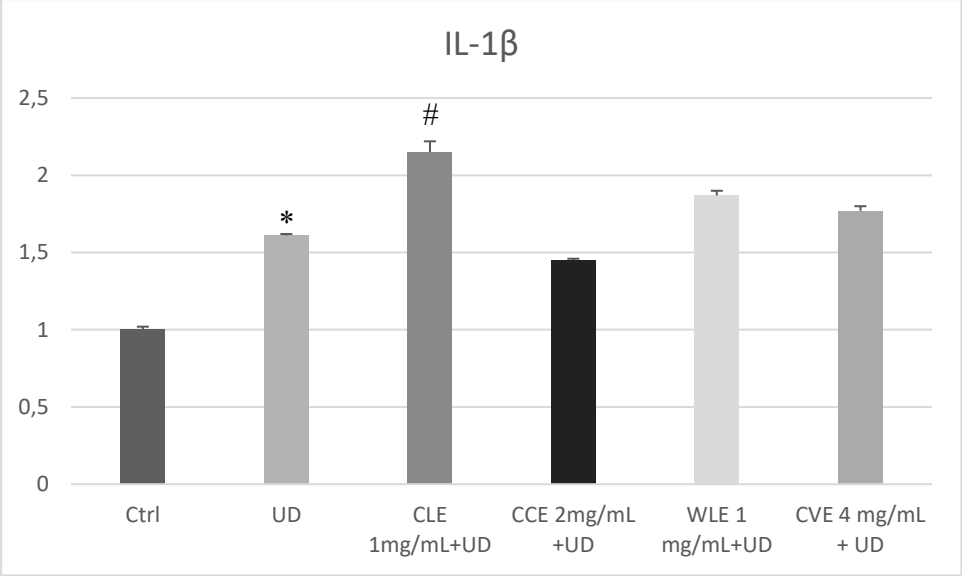


*Figure 38: Inhibition of IL-1β secretion in UD-stimulated HaCaT cells by extracts *: p<0.05 vs ctrl., #: p<0.05 vs UD.*

8.3.4 Evaluation of MAPK activation (JNK, ERK1/2, p38)

Given the rapid cytokine release produced by HaCaT cells treated with samples and UD, an analysis of activation pathways was carried out on this same line, studying the phosphorylation of the main MAPKs, *i.e.* p38, ERK1/2 and JNK, at different timings (30, 60 and 120 minutes). The phosphorylated protein (p-p38, p-ERK, p-JNK): total protein ratio allowed as to study the factors' activation.

p-38 and p-JNK level always resulted increased in UD-stimulated cells compared to control after 30 and 60 minutes; despite this, only p38 and JNK at 60 minutes resulted significantly modulated by UD ($p<0.05$ *vs* ctrl). Results of the ERK1/2 analysis were also very interesting; indeed, at 30 minutes UD produced p-ERK upregulation, which remained fairly constant even at 60 and 120 minutes (at 30 and 60 minutes ERK1/2 activation was significant compared to unstimulated cells, $p<0.05$ *vs* ctrl). Interestingly, despite it was not able to modulate cytokines release, CLE 1 mg/mL reverted almost completely the slight ERK1/2 activation at 120 minutes and the more marked p38 and JNK activation at 60 minutes, providing evidence of a clear modulation of MAPKs signaling. CCE 2 mg/mL showed a strong effect in reverting JNK activation, a mechanism very plausibly linked to the effect of the extract in modulating cytokines release.

Overall results may suggest that UD in HaCaT cells primarily promoted cytokines release through a cell oxidative stress that triggers transcription factors such as AP-1 and NF-κB, as suggested by low cytokines release compared to ROS production and, more important, the null downstream effect of CLE, even its modulation of MAPKs activation.

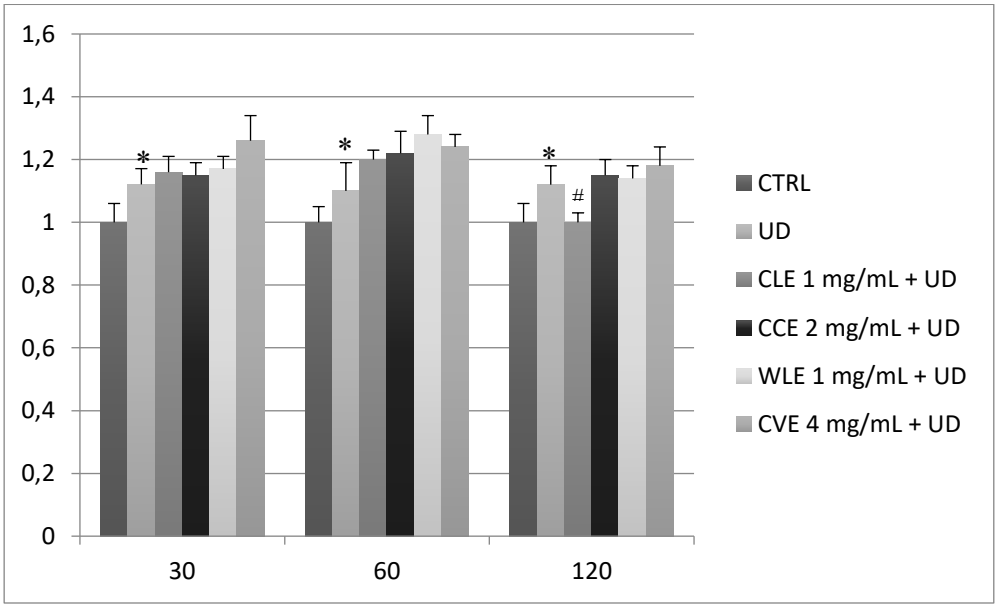


Figure 39: ERK1/2 activation. Results are expressed as p-ERK1/2:totERK1/2 ratio and results are normalized to the control ratio
**: $p<0.05$ vs ctrl; $^{\#}$: $p<0.05$ vs UD*

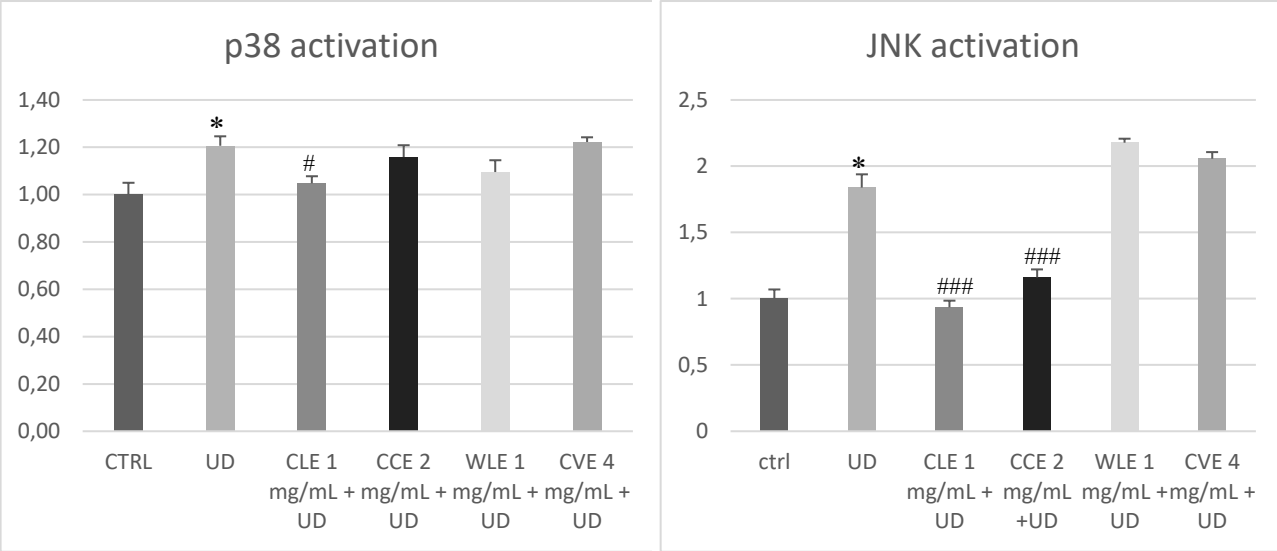


*Figure 40: Activation of p38 and JNK at 60 minutes. *: p<0.05 vs ctrl; #: p<0.05 vs UD, ##: p<0.01 vs UD; ###: p<0.01 vs UD.*

At the end of the experimental protocol, it was possible to draw an overall discussion on the activity of selected extracts from by-products and species of ethnobotanical interest.

The table 9 summarizes all results of different performed tests.

	CLE	**WLE**	**CCE**	**CVE**
Antiradicalic (DPPH)/antioxidant (voltammetry) activity	+	+	++	+
Most active compounds	FLA	FLA	ELL	FLA/HCD
Membrane proteins expression (filaggrin, loricrin)	-	-	-	-
Tight junctions expression (occludin)	-	++	-	++
Collagen/Elastin neo-synthesis	-	-	-	-
Immunosuppressive skin aging model				
keratinocytes	-	-	-	-
fibroblasts	+/-	+	+/-	+
Hyperosmotic aging model	-	+	-	+
oxidative stress models				
photodamage: UVA	-	-	+	+
acute damage: TBHP/H_2O_2	+/-	-	+/-	++
Low grade prolonged stress: serum deprivation	+/-	+	+++	+++
Inhibition of ECM-degrading enzymes activity				
Tyrosinase	-	-	-	-
Elastase	+/-	+/-	+++	-
Collagenase	-	+	++	-

Hyaluronidase	+	++	++	++
Oxidative stress models UD	-	-	+	-
Pro-inflammatory cytokines over-expression	-	-	++	-
MAPK activation				
ERK1/2	++	-	-	-
p38	+	-	-	-
JNK	+++	-	++	-

Table 9: Summary all results of different performed tests.

As it could be observed, all the selected extracts proved to be interesting candidates for a development in the field of dermatology for skin protection.

CLE, WLE and CVE showed more likely specific skin protective activities, whereas CCE resulted more or less effective in all performed tests.

In detail, CLE, rich in flavonols, exerted a good antiradicalic activity, linked to a fair efficacy in acute stimuli- and serum deprivation-induced ROS excess lowering; furthermore, CLE proved to be effective in inhibiting elastase and hyaluronidase and it resulted the most effective extract for its activity in counteracting UD-induced MAPK activation. Given these results, CLE could be seen as a good anti-inflammatory agent and a fair antioxidant. CLE biological features suggested its potential development in the context of skin aging-related inflammatory conditions.

The second extracts obtained by by-products, WLE, rich in flavonols and oligomeric catechins, exerted a good antiradicalic capacity and positive red/ox characteristics; it proved to be effective in protecting fibroblasts from immunosuppressive lowered viability and keratinocytes from dehydrative stress, at least in part probably related to the promotion of occluding expression. In acute and UD stimulation tests on human keratinocytes, WLE did not exerted a clear effectiveness, but it was able to counteract ROS release in serum deprived model and it exerted a good anti-collagenase and hyaluronidase activity. These results clearly indicated a very interesting plausible application of WLE in the context of the management of physiological chrono-aging, as general epidermis protective and active moisturizer agent.

CVE, rich in polysaccharides, oligomeric catechins, flavonols and HCD, in particular chlorogenic acid, shared the most important biological characteristic with WLE, being the most important of these keratinocytes dehydrative protection with occludin expression promotion, and fibroblast protection from immunosuppressive stimulus. CVE was only active in inhibiting hyaluronidase activity, but it exerted a markedly higher protective activity in UVA-induced keratinocytes damage and in acute and slow oxidative stress. It must be underlined that CVE was the extract studied at the highest

concentration, 4 mg/mL against 1 mg/mL of by-products extracts and 2 mg/mL of CCE, but this could not scale back the importance of obtained results. For these reasons, CVE resulted a very interesting extract with a versatile plausible application to preserve most important aging-related skin functions impairment. Italian *C. vulgaris*, species of ethnobotanical interest, is actually worthy to be considered for further studies in the context of functional cosmetic and in the broader field of dermatology.

Tuscan *C. creticus*, taken into account mostly being a species of ethnobotanical interest very scarcely investigated in the field of skin protection, provided the most unexpected and positive results. Indeed, CCE, rich in ellagitannins, flavonols and, in a lesser extent, flavan-3-ols, exerted the best cell-free antiradicalic/antioxidant profile, mostly given by the ellagitannins fraction. The most interesting results obtained with CCE were related to its strong activity against UD-induced keratinocytes stress, both oxidative and inflammatory; these accompanied by a clear photoprotective effect and the most important impact in the inhibitory effect on elastase, collagenase and hyaluronidase activity.

For these reasons, CCE could be considered the best prototype of a botanical extract capable of protect skin from air pollution-induced dysregulations.

8.4 Laboratory Development And Prototyping Of New Topical Formulations

During this Ph.D. project, we have tried to give concreteness to the data obtained by developing prototypes of formulations for topical use, capable of delivering the active components to the targets of the skin layer, the dermis and epidermis, where their biological action could be exerted.
One of the main aims of this project was the valorization of waste and by-products of natural products. For this reason, prototypes of cosmetic formulations based on walnut and chestnut leaves were created, which could be introduced into the cosmetic market.

Nutrient face cream with walnut leaves extract

WLE was incorporated into a commercially available cream base purchased from Galeno (Comeana, Prato) at different concentrations: 1%-5% m/m.
The cream resulted stable after 1, 7 and 15 days. It had a glossy appearance, a characteristic fragrance, and a hazelnut color. It was also stable after centrifugation at 3000g for 15 minutes and 30 minutes.
The skin tolerability of the cream (3% WLE m/m) was assessed by a patch test.
For the patch test, 7 healthy volunteers were recruited, and sterile gauze pads were used.
After application on the forearm for 24 hours, the formulation proved to be well tolerated by all subjects and showed no hypersensitivity reactions.
Excellent stability and good tolerability were fundamental requirements for the realization of a preliminary study in human, that we assessed.
The primary objective of this human trial was to evaluate whether the cosmetic product containing WLE had an effect in improving skin moisturization, skin elasticity an in reducing skin roughness. The trial also had the following secondary objectives: evaluate the action of the product on sebum, pH and skin temperature.
The tested product (base cream containing 3% WLE m/m) was given to 7 enrolled subjects. They used the product on the face, twice a day for 21 consecutive days. Specific end-point variables were analyzed at baseline time (before the use of the product), and after 21 days of treatment.
The results obtained indicated, at a preliminary level, the effect of the product in improving skin moisturization, skin elasticity and in reducing skin roughness, primary objective of the study. Besides this phenomenon, a non-variation of pH, sebum, skin temperature was also observed.
These tests were carried out by Bio Basic Europe S.r.l.

As shown in figure 41, compared to the baseline (T0), it can be observed a increase in variable moisturization of 11% (A) an increase of the variable elasticity of 9% (B) a decrease of the variable skin roughness of 3% (C) after 21 days of treatment.

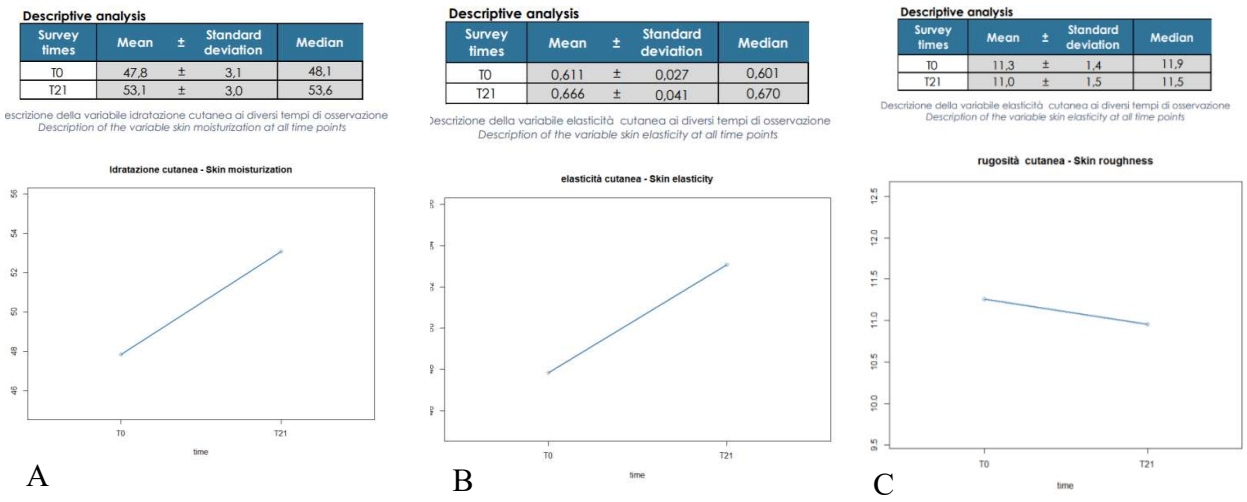


Figure 41: Description of the variable skin moisturization (A), elasticity (B) roughness(C) at all time point.

With concerning the secondary objectives of the study, the variables ph (D), sebum (E) and temperature (F) remained approximately equal to the baseline value at all observation times.

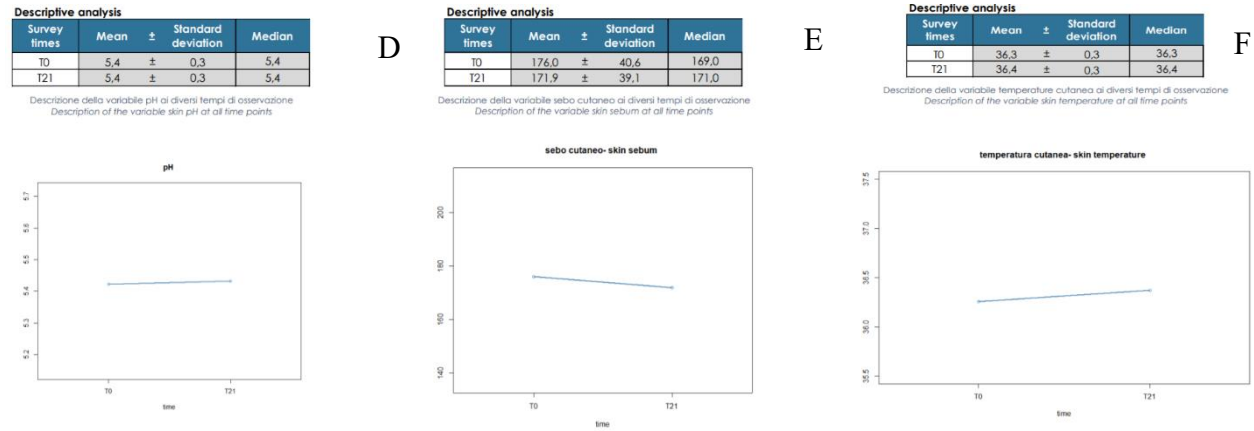


Figure 42: Description of the variable skin ph(D), sebum (E) temperature(C) at all time point.

<u>Formulative development and evaluation of sun protection factor (SPF) of chestnut leaf extract</u>

CLE was included in a basic cream to assess its filtering capacity by determining SPF *in vitro*.

SPF assessments were conducted under the guidance of Prof. Marisanna Centini in her research laboratories at the Department of Biotechnology, Chemistry and Pharmacy of the University of Siena.

Chestnut extract was included in the base cream at three different concentrations: 1%, 3% and 5% m/m.

The cream was stable after 1, 7 and 15 days. It had a shiny appearance, a characteristic odour and a white colour.

The results showed that the chestnut leaf extract did not show any filtering power: the SPF value did not change compared to that of the base cream alone, despite the increase in the concentration of the extract from 1% to 5%. For this reason, a second test was carried out, in which two UV filters, Parsol MCX (UVB filter) and Parsol 1789 (UVA filter) were added to the same formulation, to test the SPF booster capacity of the CLE 1% m/m.

Tables 10 and 11 show the results of SPF measurements of the base emulsion with filters with and without the addition of the extract.

	SPF	STD	COV %	STAR CATEGORY	UVA/UVB RATIO	λ
1-5	7.60-9.25	1.51	2.37	Good	0.61-0.66	375
Mean SPF: 7.27 ± 1.51						

Table 10: Basic emulsion with filters.

	SPF	STD	COV %	STAR CATEGORY	UVA/UVB RATIO	λ
1-5	7.71-10.53	1.06	1.72	Good	0,63	375
Mean SPF: 8.94 ± 1.06						

Table 11: Basic emulsion with filters and CLE 1% m/m.

In this case, with the addition of CLE 1% m/m, the average SPF value was higher compared with cream without the extract and the increase was statistically significant ($p<0.05$ *vs* cream without CLE).

CLE therefore had an interesting booster effect of sun filters which, combined with the good intrinsic antioxidant and antiradical properties already demonstrated, proposes this product as an outstanding protector against sun radiation.

Cosmetic application of Italian Calluna vulgaris *Hull. extract*

The promising results obtained, proposed CVE as a good candidate for the cosmetic sector.

Thanks to the collaboration with Qualiterbe (Pitigliano, Grosseto), a Tuscan company involved in the research and development, production and marketing of herbal products, again operating between tradition and modernity, it was possible a line of natural biocosmetics called Biokalluna, based on CVE. Biokalluna is now market available and first feedbacks retrieved from customers are very positive.

Linea BioKalluna

NUOVISSIMA LINEA DI COSMESI NATURALE BIOLOGICA BIOKALLUNA, NATURALLY AGELESS, ANTIPOLLUTION SKIN CARE

Una straordinaria e scientificamente moderna linea di cosmetici naturali Bio, realizzati artigianalmente con processi qualitativi tradizionali ed esclusivi del nostro laboratorio di ricerca e sviluppo, supportati dalla compentente collaborazione scientifica dell'Università degli Studi di Siena.
La miscela di ingredienti attivi la rende un ideale supporto per contrastare i segni del tempo e i danni causati dall'inquinamento, donando idratazione e luminosità alla pelle del viso.

Prodotti dermatologicamente testati, senza Parabeni e Peg, Vegan OK

- Cosmetici fatti a mano
- Certificati Bio
- Materie prime a km 0
- Packaging Ecosostenibile
- Aumento produzione di Collagene
- Attività Antipollution
- Azione scravenger diretta
- Rinnovamento cutaneo
- Biofilm protettivo
- Effetto superidratante
- Protezione dal danno ossidativo

9 CONCLUSIONS

Cutaneous aging is a complex biologic process affecting various layers of the skin, but the major changes are seen in dermis. There are two clinically and biologically independent aging processes that occur simultaneously. The first is innate or intrinsic aging, which affects skin and a variety of internal organs by slow, irreversible degeneration of tissue. The second is the result of chronic exposure to UV irradiation which causes photoaging: it can be viewed as an environmental damage superimposed on chronological aging. DNA photo-damage and UV-generated reactive oxygen species (ROS) are the initial molecular events that lead to most of the typical histological and clinical manifestations of chronic photodamage of the skin. Wrinkling and pigmentary changes are directly associated with premature photo-aging and are considered its most important cutaneous manifestations. In addition, other atmospheric factors such as air pollution (such as smog, ozone, particulate matter) have been implicated in premature skin aging (Rittie and Fisher, 2015; Zouboulis et al., 2019).

Skin aging mirrors age-related deficiencies for the entire human body and correlates well with certain systemic diseases. Both *chrono* and *photo* aging, are closely connected with the development of a wide range of degenerative and non-degenerative diseases (Zouboulis et al., 2019).

Despite natural aging is genetically determined, extrinsic aging mediated by high ROS levels and inflammatory factors, could be prevented or, at least, managed. For this reason, several approaches using topically applied ingredients, particularly antioxidants that penetrate and protect the skin, have been developed. To limit the degradation of the skin primary structural constituents, such as collagen and elastin, is another key point of the scientific research on skin aging.

The use of plants as a source of human therapeutic medicine is an old-recorded history. The importance of plants as agents of therapeutic components is increasingly being recognized in line with current technology advances.

In recent years, naturally occurring herbal compounds such as phenolic acids, flavonoids, and high molecular weight polyphenols have gained considerable attention as beneficial protective agents.

Indeed, phenolic antioxidants play the very important role in protecting against harmful effects of UV-induced skin damage (Binic et al. 2013). The plant phenolics may be efficient in the treatment of both serious dermal disease and minor skin problems (Afaq and Mukhtar, 2010; Dzialo et al., 2016).

Many endemics or typically Italian species are reported in literature for their potential for human health and, in this scenario, also some skin care and anti aging extracts were investigated; nevertheless, many ethnobotanical knowledges and traditional uses of Italian species for skin protection remain unexplored. This Ph.D. project aimed to fill the gap: living in a Department where environmental sciences and ecotoxicology deeply contaminated my way of working, I was able to concentrate my research on species of the territory and their role in challenging air pollution-induced skin impairments.

A comprehensive bibliography research, supported by collaborations with scholars and companies, made possible to identify the most interesting species, from territorial ethnobotany and established use in phytotherapy, such as *Rosmarinus officinalis* L., *Salvia officinalis* L., *Hypericum perforatum* L., to recently investigated species such as *Calluna vulgaris* (L.) Hull, *Sedum telephium* L., *Balsamita major* Desf, *Cistus creticus* L. subsp. *eriocephalus* (Viv.) Greuter & Burdet (= *Cistus incanus* L.).

In this work I wanted to include by-products or waste products of the Tuscan flora species, which are an important resource of active ingredients. The by-products investigated here were leaves of *Castanea sativa* Mill and leaves of *Juglans regia* L.

Preliminary chemical and biological analyses carried out and the will of privileging by-products and species of ethnobotanical interest drove us to focus the attention on *C. sativa* and *J. regia* leaves and *C. vulgaris* and *C. criticus* aerial parts, respectively. These latter are species described for their use as skin protective by people that carried with them direct ethnobotanical experiences, that I later also found in some bibliography.

In the first part of this work, I focused on the optimization of extraction of herbal material, a parameter too often underestimated, but always of primary interest, since the best yield of active principle is still the basis of the rational use of plant products.

I have therefore tried to find the best time of harvest and the best extraction, implementing traditional extractions, but also extractions exploitable in modern industrial processes.

All species were collected in Tuscany and extractive procedure optimized in order to define the best method to produce phenolic enriched extracts.

Chestnut and walnut extracts were optimized considering the collecting time in October, the best time to consider the leaves an actual by-product, as edible fruits are yet harvested at this time.

It was observed that 60% v/v of ethanol was the most efficient solvent in order to have the highest yield in phenolics aerial parts of *C. creticus*, leaves of *C. sativa* and leaves of *J. regia*.

For flowering tops *C. vulgaris*, the best extraction solvent was water, due to the important presence of the polysaccharide component in addition to the phenolic component.

Ethanolic extracts were produced by using the Naviglio® Automatic Extractor, which combines efficiency, speed and a standardized method working with a dynamic pressure variation on the surface of the liquid phase containing the solid material to be extracted.

Subsequently, various *in vitro* and innovative models were developed, and they allowed me to gain scientific competence, enabling me to formulate concrete hypotheses of effectiveness.

All selected extracts showed to be a very interesting candidates in the context of skin aging, worthy to be further investigated and developed. In fact, if skin aging is in part genetically determined and could be only slowed down, it is true that extrinsic factors that trigger different skin cells structures, such as sun UV rays and air pollution *in primis*, should be limited and contrasted by using effective products.

The results obtained from these models revealed an activity of these extracts that is particularly interesting in the context of skin aging. Indeed, it is precisely on oxidative and inflammatory protection that we obtained positive results.

These results prompted me to go into more detail, developing a specific model of environmental pollution. The results were in line with those obtained in previous tests. This gave me positive answers about the effectiveness of the extracts tested.

With a view to the applicability of the acquisitions of the expertise I created, I wanted to gain experience adding a small clinical trial part and a formulation phase.

Some of the species that I did not focus on, became the object of scientific collaborations and in 2020 and 2021 two peer-reviewed papers and a proceeding abstract were published in international journals, on *Rosmarinus officinalis* L. and *Balsamita major* Desf., and *Sedum telephium* L., respectively.

10 BIBLIOGRAPHY and SITOGRAPHY

- Afaq F, Mukhtar H. Botanical Antioxidants for Skin Protection: An Overview. Nutrition for Healthy Skin, 2010; 51–63.
- Afaq F, Mukhtar H. Botanical antioxidants in the prevention of photocarcinogenesis and photoaging". Experimental Dermatology, 2006; 15(9), 678–684.
- Agnolucci A, Meggiato C. Cistus Incanus L., una pianta mediterranea particolarmente efficace contro i disturbi influenzali. 2019.
- Ahn K. The role of air pollutants in atopic dermatitis. J. Allergy Clin. Immunol. 2014, 134, 993–999.
- Almeida IF, Costa PC, Bahia MF. Evaluation of functional stability and batch-to-batch reproducibility of a *Castanea sativa* leaf extract with antioxidant activity. AAPS PharmSciTech., 2010; 11(1):120-125.
- Amaral JS. Seabra RM, Andrade PB, Valentao P, Pereira JA, Ferreres F. Phenolic profile in the quality control of walnut (Juglans regia L.) leaves. Food Chemistry, 2004; 88:373–379.
- Amirlak B, Shahabi L, Javaheri S, Talavera F and Stadelmann W.: Skin Anatomy. Medscape. 2013.
- Aničić N, Patelou E, Papanikolaou A, et al. Comparative Metabolite and Gene Expression Analyses in Combination With Gene Characterization Revealed the Patterns of Flavonoid Accumulation During *Cistus creticus* subsp. *Creticus* Fruit Development. *Front Plant Sci*. 2021; 12:619634.
- Araviiskaia E, Berardesca E, Bieber T, Gontijo G, Sanchez Viera M, Marrot L, et al. The impact of airborne pollution on skin. J Eur Acad Dermatology Venereol. 2019; 33:1496–505.
- Arda O, Göksügür N, Tüzün Y. Basic histological structure and functions of facial skin. Clin. Dermatol. 2014; 32, 3–13.
- Australian Government. Weed management Guide, Heather (*Calluna vulgaris*). CRC Weed Management. 2003.
- Babula P, Adam V, Havel L, Sladky Z, Kizek R. Naphthoquinones as allelochemical triggers of programmed cell death. Environ Exp Botany, 2009; 65:330–337.
- Bakke JV, Wieslander G, Norback D, Moen BE. Eczema increases susceptibility to PM10 in once indoor environments. Arch. Environ. Occup. Health 2012; 67, 15–21.
- Barrajón-Catalán E, Fernández-Arroyo S, Roldán C, Guillén E, Saura D, Segura Carretero A, Micol V (2011). A systematic study of the polyphenolic composition of aqueous extracts deriving from several Cistus genus species: evolutionary relationship. Phytochemical analysis, 2011; *PCA*, *22*(4).
- Basile A., Sorbo S., Giordano S., Ricciardi L., Ferrara S., Montesano D., et al.: Antibacterial and allelopathic activity of extract from *Castanea sativa* leaves. Fitoterapia, 2000.
- Battie C, Jitsukawa S, Bernerd F, Del Bino S, Marionnet C, Verschoore M. New insights in photoaging, UVA induced damage and skin types. Exp. Dermatol., 2014; 23 (s1): 7-12.
- Bäumler W, Regensburger J, Knak A, Felgenträger A, Maisch T. UVA and endogenous photosensitizers - the detection of singlet oxygen by its luminescence. Photochem. Photobiol. Sci., 2012; 11 (1): 107–117.
- Beelen R, Hoek G, van den Brandt PA, Goldbohm RA, Fischer P, Schouten LJ, Armstrong B, Brunekreef B. Long-term exposure to traffic-related air pollution and lung cancer risk - Epidemiology, 2008; 19: 702-710.

- Bertelli A, Biagi M, Corsini M, Baini G, Cappellucci G, Miraldi E. Polyphenols: From Theory to Practice. *Foods*. 2021; 10(11):2595.
- Biagi M, Manca D, Barlozzini B, Miraldi E, Giachetti D. Optimization of extraction: of drug containing polyphenols using an innovative technique. Agro food Ind Hi-Tech, 2014; 25, 60-65.
- Biagi M, Miraldi E, Figura N, Giachetti D. Antiradical activity and in vitro inhibition of Helicobacter Pylori by Italian red wine. Nat. Prod. Commun, 2009; 4(2), 255-260.
- Binic I, Lazarevic V, Ljubenovic M, Mojsa J, and Sokolovic D. Skin Ageing: Natural Weapons and Strategies, Hindawi Publishing Corporation -Evidence-Based Complementary and Alternative Medicine Volume 2013; 1-10.
- Blumenthal M, (ed). Expanded German Commission E Monographs Walnut Leaf Integrative Medicine Communications, 2000; 401-403.
- Blume-Peytavi U, Kottner J, Sterry W, Hodin MW, Griffiths TW, Watson Hay RJ, Griffiths CEM. Age-associated skin conditions and diseases: current perspectives and future options. Gerontologist 2016; 56, S230–S242.
- Borellini U, La divina cosmesi, I edizione, Mondadori, Milano, 2015.
- Bosch R, Philips N, Suárez-Pérez J, Juarranz A, Devmurari A, Chalensouk-Khaosaat J, González S. Mechanisms of Photoaging and Cutaneous Photocarcinogenesis, and Photoprotective Strategies with Phytochemicals. Antioxidants, 2015; 4(2), 248–268.
- Boss GR, Seegmiller JE. Age-related physiological changes and their clinical significance. Western Journal of Medicine, 1981; 135(6), 434-440.
- Braghiroli L, Mazzanti G, Manganaro M, Mascellino MT, Vespertilli T. Antimicrobial activity of *Calluna vulgaris*. Phytotherapy Research.1996; 10 (1):S86-S88.
- Brand RM, Wipf P, Durham A, Epperly MW, Greenberger JS, Falo LD Jr. Targeting Mitochondrial Oxidative Stress to Mitigate UV-Induced Skin Damage. Front Pharmacol. 2018, 20;9:920.
- Briggaman RA, Wheeler CE. The epidermal dermal junction. J. Invest. Dermatol. 1975; 65, 71–84.
- Brunekreef B, Annesi-Maesano I, Ayres JG, Forastiere F, Forsberg B, Künzli N, Pekkanen J, Sigsgaard T. Ten principles for clean air. Eur Respir J 2012; 39:525-8.
- Burke KE. Mechanisms of aging and development—A new understanding of environmental damage to the skin and prevention with topical antioxidants. Mech Ageing Dev. 2018; 172:123–30.
- Burlando B, Parodi A, Volante A, Bassi AM. Comparison of the irritation potentials of Boswellia serrata gum resin and of acetyl-11-keto-beta-boswellic acid by in vitro cytotoxicity tests on human skin-derived cell lines. Toxicol Lett. 2008; 15;177(2):144-9.
- Cainelli T, Giannetti A, Rebora A. Manuale di Dermatologia Medica e Chirurgica. 2000. II edizione, McGraw-Hill. Passim.
- Cainelli T, Giannetti A, Rebora A. Manuale di dermatologia medica e chirurgica. McGraw-Hill, 2012.
- Calliste CA, Trouillas P, Allais DP, Simon A, Duroux JL. Free radical scavenging activities measured by electron spin resonance spectroscopy and B16 cell antiproliferative behaviors of seven plants. Journal of Agricultural and Food Chemistry. 2001; 49 (7): 3321–3327.
- Campbell KL, Lichtensteiger CA. STRUCTURE AND FUNCTION OF THE SKIN. Hanley & Belfus. L.B.T.-S.A.D.S. Campbell, Philadelphia, 2004; 1–9.

- Carnat A, Petitjean Freyytet C, Muller D, Lamaison L. Teneurs en principaux constituants de la feuille de noyer Juglans regia L. Plantes Med Phytother. 1993; 26:332-339
- Castano P, Cocco L, De Barbieri A, D'Este L, Floriani F, Gheri G, Mondello MR, Papa S, Petriglieri P, Pizzini G, Ridola C, Rossi S, Sacchi G, Sirigu P, Spinella S. Anatomia umana. Edi-ermes, 2006.
- Castano-Vinyals G, Cantor KP, Malats N,Tardon A, Garcia-Closas R, Serra C, Carrato A, Rothman N, Vermeulen R, Silverman D, Dosemeci M, Kogevinas M. Air pollution and risk of urinary bladder cancer in a case-control study in Spain - Occup Environ Med, 2008; 65,56-60.
- Cavinato M, Jansen-Dürr P. Molecular mechanisms of UVB-induced senescence of dermal fibroblasts and its relevance for photoaging of the human skin. Experimental Gerontology, 2017; 94, 78–82.
- Chang AL, Wong JW, Endo JO, Norman RA. Geriatric dermatology review: Major changes in skin function in older patients and their contribution to common clinical challenges. Journal of the American Medical Directors Association,2013; 14(10), 724-730.
- Cho YH, Bahuguna A, Kim HH, Kim DI, Kim HJ, Yu JM, Jung HG, Jang JY, Kwak JH, Park GH, Kwon OJ, Cho YJ, An JY, Jo C, Kang SC, An BJ. Potential effect of compounds isolated from Coffea arabica against UV-B induced skin damage by protecting fibroblast cells. J Photochem Photobiol B., 2017; 174: 323-332.
- Choi EH, Man MQ, Xu P, Xin S, Liu Z, Crumrine DA, Jiang YJ, Fluhr JW, Feingold KR, Elias PM, Mauro TM. Stratum corneum acidification is impaired in moderately aged human and murine skin. Journal of Investigative Dermatology,2007; 127(12), 2847-2856.
- Cowan MM.: Plant products as antimicrobial agents. Clin Microbiol Rev., 1999; 12(4): 564-582.
- Cutler RG. Cellular ageing, concepts and mechanisms. Karger, 1976
- D'Alonzo R. L'uso delle erbe nella tradizione rurale della Toscana, EFFEEMME LITO srl, ARSIA • Regione Toscana, 2002, vol 1.
- Daly CH. Biomechanical properties of dermis. J. Invest. Dermatol., 1982; 79 (1): s17-s20,
- Datzmann T, Markevych I, Trautmann F, Heinrich J, Schmitt J, Tesch F. Outdoor air pollution, green space, and cancer incidence in Saxony: A semi-individual cohort study. BMC Public Health 2018, 18, 715.
- De Rigo D, Enescu CM, Houston Durrant T, Tinner W, Caudullo G, *Juglans* regia in Europe: distribution, habitat, usage and threats. (Eds.), European Atlas of Forest Tree Species, 2016; 103.
- Debacq-Chainiaux F, Leduc C, Verbeke A, Toussaint O. UV, stress and aging. 2012; 1;4(3):236-40.
- Deliorman-Orhan D, Şenol S, Kartal M Orhan I. Assessment of antiradical potential of *Calluna vulgaris* (L.) Hull and its major flavonoid. Journal of the Science of Food and Agriculture 2009; 89 (5): 809–814.
- Dìaz Reinoso B, Couto D, Moure A, Fernandes E, Domìnguez H, Parajò JC. Optimization of antioxidants-extraction from *Castanea sativa* leaves. Chem Eng J. 2012; 203:101-109.
- Donaldson K, Stone V, Borm PJA, Jimenez LA, Gilmour PS, Schins RPF, Knaapen AM, Rahman I, Faux SP, Brown DM; MacNee W. Oxidative stress and calcium signaling in the adverse e_ects of environmental particles (PM10). Free Radic. Biol. Med. 2003, 34, 1369–1382.
- Donaldson K, Tran L, Jimenez LA. Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure - Part Fibre Toxicol, 2005; 2,10.

- Dróżdż P, Sentkowska A, Pyrzyńska K. Biophenols and antioxidant activity in wild and cultivated heather. Natural Product Research, 2017; 31 (10): 1181–1184.
- Dumax-Vorzet AF, Tate M, Walmsley R, Elder RH, Povey AC. Cytotoxicity and genotoxicity of urban particulate matter in mammalian cells. Mutagenesis 2015, 30, 621–633.
- Dupont E, Gomez J, Bilodeau D. Beyond UV radiation: a skin under challenge. International journal of cosmetic science, 2013; 35(3), 224-232.
- Działo M, Mierziak J, Korzun U, Preisner M, Szopa J, Kulma A. The Potential of Plant Phenolics in Prevention and Therapy of Skin Disorders. International journal of molecular sciences, 2016; 17, 160.
- Eckhart L, Zeeuwen PLJM. The skin barrier: Epidermis vs environment. Exp Dermatol. 2018; 27(8):805-806.
- Evans WC. Trease and Evans Pharmacognosy. Sixteenth Edition. Saunders Elsevier, Edinburgh - London 2009.
- Evrard C, Lambert de Rouvroit C, Poumay Y. Epidermal Hyaluronan in Barrier Alteration-Related Disease. *Cells*. 2021;10(11):3096.
- Farage MA, Miller KW, Elsner P, Maibach HI. Characteristics of the aging skin. Advances in wound care, 2013; 2 (1), 5-10.
- Farage MA., Miller KW., Elsner P., Maibach HI., Intrinsic and extrinsic factors in skin ageing: a Review, International Journal of Cosmetic Science, 2008, 30, 87–95.
- Fernández-Agulló A, Castro-Iglesias A, Freire MS, González-Álvarez J, *Op*timization of the Extraction of Bioactive Compounds from Walnut (Juglans major 209 x Juglans regia) Leaves: Antioxidant Capacity and Phenolic Profile. Antioxidants, 2019; 9(1), 18.
- Fernando IPS, Jayawardena TU, Sanjeewa KKA, Wang L, Jeon YJ, Lee WW. Anti-inflammatory potential of alginic acid from Sargassum horneri against urban aerosol-induced inflammatory responses in keratinocytes and macrophages. Ecotoxicol Environ Saf. 2018; 30, 160:24-31.
- Filip GA, Clichici S, Daicoviciu D, Catoi C, Bolfa P, Postescu ID, Gal A, Baldea I, Gherman C, Muresan A. Chemopreventive effects of *Calluna vulgaris* and *Vitis vinifera* extracts on UVB-induced skin damage in SKH-1 hairless mice. Journal of Physiology and Pharmacology, 2011a; 62 (3): 385.
- Filip GA, Daicoviciu D, Clichici S, Mocan T, Muresan A, Postescul D. Photoprotective Effects of Two Natural Products on Ultraviolet B–Induced Oxidative Stress and Apoptosis in SKH-1 Mouse Skin. Journal of Medicinal Food, 2011b; 14 (7–8): 761–766.
- Filip GA, Postescu ID, Tatomir C, Muresan A, Clichici S. *Calluna vulgaris* extract modulates NF-κB/ERK signaling pathway and matrix metalloproteinase expression in SKH-1 hairless mice skin exposed to ultraviolet B irradiation. Journal of Physiology and Pharmacology. 2012; 63 (4): 423–432.
- Fischer F, Achterberg V, März A, Puschmann S, Rahn CD, Lutz V, Krüger A, Schwengler H, Jaspers S, Koop U, Blatt T, Wenck H, Gallinat S. Folic acid and creatine improve the firmness of human skin in vivo, J. Cosmet. Dermatol.2011; 10(1):15-23.
- Fisher GJ, Kang S, Varani J, Bata-Csorgo Z, Wan Y, Datta S, Voorhees JJ, Mechanisms of Photoaging and Chronological Skin Aging. Archives of dermatology,2002; 138(11),1462–1470.
- Fuchs E. Finding one's niche in the skin. Cell Stem Cell, 2009; 4 (6): 499-502.

- Fyhrquist P, Salih EYA, Helenius S, Laakso I, Julkunen-Tiitto R. HPLC-DAD and UHPLC/QTOF-MS Analysis of Polyphenols in Extracts of the African Species *Combretumpadoides*, *C. zeyheri* and *C. psidioides* Related to Their Antimycobacterial Activity. *Antibiotics (Basel)*. 2020;9(8):459. **2020**.
- Gao X, Wei ZH, Chen H, Efficacy and safety of innovative cosmeceuticals, Clinics in Dermatology, **2008**; 19, 371-374.
- Gilchrest BA. A review of skin aging and its medical therapy. Br. J. Dermatol. **1996**; 135: 867-875.
- Gîrzu M, Carnat A, Privat AM, Fialip J, Carnat AP, Lamaison JL. Sedative effect of walnut leaf extract and juglone, an isolated constituent. Pharm Biol. **1998**; 36:280–286.
- Glogau RG. Physiological and structural changes associated with aging skin, Dermatol. Clin., **1997**, 15 (4), 555-559.
- Gordien AY, Gray AI, Ingleby K, Franzblau SG, Seidel V. Activity of Scottish plant, lichen and fungal endophyte extracts against *Mycobacterium aurum* and *Mycobacterium tuberculosis*. Phytotherapy Research. **2010**; 24 (5): 692–698.
- Gori A, Ferrini F, Marzano MC, Tattini M, Centritto M, Baratto MC, Pogni R, Brunetti C. Characterisa4on and Antioxidant Activity of Crude Extract and Polyphenolic Rich Frac4ons from *C. incanus* Leaves. International journal of molecular sciences, **2016**; 17(8), 1344.
- Governa P, Manetti F, Miraldi E., Biagi M. Effects of in vitro simulated digestion on the antioxidant activity of different *Camellia sinensis* (L.) Kuntze leaves extracts. *Eur Food Res Technol* **2021**.
- Grove GL. Physiologic changes in older skin. Clinics in geriatric medicine, **1989**; 5(1), 115-25.
- Guzik TJ, Korbut R, Adamek-Guzik T. Nitric oxide and superoxide in inflammation and immune regulation. Journal of physiology and pharmacology. **2003**; 54(4): 469-487.
- Ha JW, Song H, Hong SS, Boo YC. Marine Alga Ecklonia cava Extract and Dieckol Attenuate Prostaglandin E2 Production in HaCaT Keratinocytes Exposed to Airborne Particulate Matter. Antioxidants (Basel). **2019**; 21;8(6):190.
- Halliday GM, Byrne SN, Damian DL.: Ultraviolet A radiation: its role in immunosuppression and carcinogenesis. Semin. Cutan. Med. Surg., **2011**; 30 (4): 214-221.
- Harvell JD, Maibach HI. Percutaneous absorption and inflammation in aged skin: a review. Journal of the American Academy of Dermatology, **1994**; 31(6), 1015-1021.
- Hazra B, Das Sarma M, Sanyal U. Separation methods of quinonoid constituents of plants used in Oriental traditional medicines. J Chromatogr B Analyt Technol Biomed Life Sci. **2004**; 812:259-275.
- Hidaka T, Ogawa E, Kobayashi EH, Suzuki T, Funayama R, Nagashima T, Fujimura T, Aiba S, Nakayama K, Okuyama R, Yamamoto M. The aryl hydrocarbon receptor AhR links atopic dermatitis and air pollution via induction of the neurotrophic factor artemin. Nat Immunol. **2017**; 18(1):64-73.
- Horinouchi M, Arimoto-Kobayashi S. Photomicronucleus assay of phototoxic and pseudophotoclastogenic chemicals in human keratinocyte NCTC2544 cells. Mutat Res. **2011**; 14;723(1):43-50.
- Hseu YC., Chou CW., Senthil Kumar KJ., Fu KT., Wang HM., Hsu LS., Kuo YH., Wu CR., Chen SC., Yang HL.: Ellagic acid protects human keratinocytes (HaCaT) cells against UVA-induced oxidative stress and apoptosis through the upregulation of the HO-1 and Nrf-2 antioxidant genes. Food Chem. Toxicol. **2012**; 50 1245-1255.

- https://www.pfaf.org
- Jabłońska-Trypuć A, Krętowski R, Kalinowska M, Świderski G, Cechowska-Pasko M, Lewandowski W. Possible Mechanisms of the Prevention of Doxorubicin Toxicity by Cichoric Acid-Antioxidant Nutrient. Nutrients. 2018; 5;10(1):44.
- Jäger AK., Gauguin B, Andersen J, Adsersen A, Gudiksen L. Screening of plants used in Danish folk medicine to treat depression and anxiety for affinity to the serotonin transporter and inhibition of MAO-A. Journal of Ethnopharmacology. 2013; 145 (3): 822-825.
- Jahanban-Esfahlan A, Ostadrahimi A, Tabibiazar M, Amarowicz R, A Comprehensive Review on the Chemical Constituents and Functional Uses of Walnut (Juglans spp.) Husk. International Journal of Molecular Sciences, 2019; 20(16), 3920.
- Jin SP, Li Z, Choi EK, Lee S, Kim YK, Seo EY, Chung JH, Cho S. Urban particulate matter in air pollution penetrates into the barrier-disrupted skin and produces ROS-dependent cutaneous inflammatory response in vivo. J Dermatol Sci. 2018; 30:S0923-1811(18)30202-0.
- Kammeyer A, Luiten RM, Oxidation events and skin aging. Ageing Research Reviews, 2015; 21, 16–29.
- Kanitakis J.: Anatomy, histology and immunochemistry of normal human skin. Eur. J. Dermatol., 2002; 12 (4): 390-399.
- Kim KE, Cho D, Park HJ. Air pollution and skin diseases: Adverse effects of airborne particulate matter on various skin diseases. Life Sci. 2016; 152:126–34.
- Kim M, Kim JH, Jeong GJ, Park KY, Lee MK, Seo SJ. Particulate matter induces pro-inflammatory cytokines via phosphorylation of p38 MAPK possibly leading to dermal inflammaging. Exp. Dermatol. 2019, 28, 809–815.
- Kligman LH. and Kligman AM. The nature of photoaging: its prevention and repair. Photodermatol., 1986; 3 (4): 215-27,.
- Kolarsick PAJ. Anatomy and Physiology of the skin. J. Dermatol. Nurses Assoc., 2011; 3 (4): 203-213.
- Krutmann J, Bouloc A, Sore G, Bernard BA, Passeron T. The skin aging exposome. J Dermatol Sci. 2017; 85:152–61.
- Krutmann J, Liu W, Li L, Pan X, Crawford M, Sore G, Seite S. Pollution and skin: from epidemiological and mechanistic studies to clinical implications. J Dermatol Sci. 2014;76(3):163-8.
- Krutmann J, Moyal D, LiuW, Kandahari S, Lee GS, Nopadon N, Xiang LF, Seite S. Pollution and acne: Is there a link? Clin. Cosmet. Investig. Dermatol. 2017, 10, 199–204.
- Kumar V, Abbas AK, Fausto N, 2005. Le basi patologiche delle malattie. Elsevier Italia, Milano. DH, Oh JH, Chung JH. Glycosaminoglycan and proteoglycan in skin aging. J. Dermatol. Sci., 2016; 83 (3): 174-181.
- Lavker RM, Zheng PS, Dong G, Aged skin: a study by light, transmission electron, and scanning electron microscopy. Journal of investigative dermatology, 1987; 88(3), s44-s51.
- Lecas S, Boursier E, Fitoussi R, Vie K, Momas I, Seta N, Achard S. In vitro model adapted to the study of skin ageing induced by air pollution. Toxicol. Lett. 2016, 259, 60–68.
- Lee CW, Lin ZC, Hu SC, Chiang YC, Hsu LF, Lin YC, Lee IT, Tsai MH, Fang JY. Urban particulate matter down-regulates filaggrin via COX2 expression/PGE2 production leading to skin barrier dysfunction. Sci. Rep. 2016, 6, 27995.

- Lee H, Park E. *Perilla frutescens* Extracts Enhance DNA Repair Response in UVB Damaged HaCaT Cells. Nutrients. 2021;13(4):1263.
- Lima TN, Pedriali Moraes CA. Bioactive peptides: applications and relevance for cosmeceuticals. Cosmetics, 2018; 5(1): 21.
- Liu W, Pan X, Vierkotter A, Guo Q, Wang X, Wang Q, Seite S, Moyal D, Schikowski T, Krutmann JA. Time-Series Study of the Effect of Air Pollution on Outpatient Visits for Acne Vulgaris in Beijing. Skin Pharmacol. Physiol. 2018, 31, 107–113.
- Liu YM, Xu YP, Gao JM, Yang XP, Liu YS. Analysis of volatile components from leaves of Juglans regia by GC/MS. Acta Bot. Boreali-occidentalia Sin. 2004; 24:1100–1102.
- Lushchak VI. Free radicals, reactive oxygen species, oxidative stress and its classification. Chem. Biol. Interact. 2014, 224, 164–175.
- Ma YS, Wu SB, Lee WY, Cheng JS, Wei YH. Response to the increase of oxidative stress and mutation of mitochondrial DNA in aging. Biochim. Biophys. Acts, 2009; 1790 (10): 1021-1029.
- Makrantonaki E, Bekou V, Zouboulis CC. Genetics and skin aging. Dermatoendocrinol., 2012; 4 (3): 280-284.
- Mancebo SE, Wang SQ. Recognizing the impact of ambient air pollution on skin health. J. Eur. Acad. Dermatol. Venereol. 2015, 29, 2326–2332.
- Mandim F, Barros L, Calhelha RC, Abreu RMV, Pinela J, Alves MJ, Heleno S, Santos PF, Ferreira ICFR. *Calluna vulgaris* (L.) Hull: chemical characterization, evaluation of its bioactive properties and effect on the vaginal microbiota. Food Funct. 2019; 10(1):78-89.
- Marcuzzi G. The Heaths in European Ecosystems". Springer. 1979;343–378
- Matsumura Y. Molecular mechanisms of photocarcinogenesis. Frontiers in Bioscience, 2002; 7(1-3), 765.
- Maugini E, Bini LM, Lippi MM. Manuale di botanica farmaceutica. Piccin-Nuova Libraria, 2006.
- McDaniel D, Farris P, Valacchi G, Atmospheric skin aging—Contributors and inhibitors. J. Cosmet. Dermatol. 2018;17(2) ,124–137.
- McGlone F, Reilly D. The cutaneous sensory system. Neurosci. Biobehav. Rev. 2010; 34, 148–159.
- Micali G, Potenza MC, Fabbroncini G, Monfercola G, Tosti A, Veraldi S. Le basi della dermatologia. Springer Science & Business Media, 2014.
- Mikrut M, Regiel-Futyra A, Samek L, Macyk W, Stochel G, van Eldik R. Generation of hydroxyl radicals and singlet oxygen by particulate matter and its inorganic components. Environ. Pollut. 2018, 238, 638–646.
- Moehrle M. Outdoor sports and skin cancer. Clin dermatol, 2008;. 26 (1) : 12-15.
- Monschein M, Neira JI, Kunert O, Bucar F. Phytochemistry of heather (*Calluna vulgaris* (L.) Hull) and its altitudinal alteration. Phytochemistry Reviews. 2010; 9 (2): 205–215.
- Montagna W, Celleno L.: Biologia comparativa della pelle. in Serri F. Trattato di dermatologia. Piccin, 1986.
- Montagna W, Kligman AM, Carlisle KS. Atlas of normal human skin. Springer Science & Business Media, 2012.
- Montiel-Davalos A, Silva Sanchez GJ, Huerta-Garcia E, Rueda-Romero C, Soca Chafre G, Mitre-Aguilar IB, Alfaro-Moreno E, Pedraza-Chaverri J, Lopez-Marure R. Curcumin inhibits activation induced by urban particulate material or titanium dioxide nanoparticles in primary human endothelial cells. PLoS ONE 2017, 12, e0188169.

- Morales-Barcenas R, Chirino YI, Sanchez-Perez Y, Osornio-Vargas AR, Melendez-Zajgla J, Rosas I, Garcia-Cuellar CM. Particulate matter (PM10) induces metalloprotease activity and invasion in airway epithelial cells. Toxicol. Lett. 2015, 237, 167–173.
- Morant AV, Jørgensen K, Jørgensen Ch, Paquette SM, Sanchez-Perez R, Møller BL, Bak S. β-Glucosidases as detonators of plant chemical defense. Phytochemistry 2008; 69:1795-1813.
- Moreira H, Slezak A, Szyjka A, Oszmianski J, Gasiorowski K, An4oxidant and cancer chemopreven4ve ac4vi4es of cistus and pomegranate polyphenols. Acta poloniae pharmaceuVca, 2017; *74*(2), 688–698. 81
- Morelli I., Flamini G., Pistelli L. Manuale dell'erborista, biosintesi, estrazione e identificazione delle sostanze di origine vegetale. Milano, Tecniche Nuove, 2005.
- Murphy MP. How mitochondria produce reactive oxygen species. Biochem. J., 2009; 417 (1): 1-13.
- Musch MW, Walsh-Reitz MM, Chang EB. Roles of ZO-1, occludin, and actin in oxidant-induced barrier disruption. Am J Physiol Gastrointest Liver Physiol. 2006; 290(2):G222-31.
- NirmlaDevi T, Apraj V, Bhagwat A, Mallya R, Sawant L, Pandita N. Pharmacognostic and Phytochemical Investigation of *Juglans regia* Linn. Bark. Pharmacognosy Journal, 2011; 3(25), 39–43.
- Nour V, Trandafir I, Cosmulescu S, HPLC determination oh phenolic acids, flavonoids and juglone in walnut leaves. Journal of Chromatographic Science, 2013; 51(9), 883-90.
- Olteanu ED, Baldea I, Clichici S, Bolfa P, Cenariu M, Perde-Schrepler M, Alupei MC, Muresan A, Filip A. *In vitro* studies on the mechanisms involved in chemoprevention using *Calluna vulgaris* on vascular endothelial cells exposed to UVB. Journal of Photochemistry and Photobiology B: Biology. 2014; 136 (C): 54–61.
- Olteanu ED, Filip A, Clichici S, Daicoviciu D, Achim M, Postescu ID, Bolfa P, Bolojan L, Vlase L, Muresan A. Photochemoprotective effect of *Calluna vulgaris* extract on skin exposed to multiple doses of ultraviolet B in SKH-1 hairless mice. Journal of Environmental Pathology, Toxicology and Oncology. 2012; 31 (3): 233–243.
- Palmer DM, Kitchin JS, Oxidative damage, skin aging, antioxidants and a novel antioxidant rating system. Journal of Drugs in Dermatology, 2010; 9(1), 11-5.
- Pan TL, Wang PW, Aljuffali IA, Huang CT, Lee CW, Fang JY. The impact of urban particulate pollution on skin barrier function and the subsequent drug absorption. J. Dermatol. Sci. 2015, 78, 51–60.
- Pandel R, Poljšak B, Godic A, Dahmane R. Skin Photoaging and the Role of Antioxidants in Its Prevention. International Scholarly Research Notices,2013; 930164.
- Park S. Seok JK, Kwak JY, Suh HJ, Kim YM, Boo YC. Anti-Inflammatory Effects of Pomegranate Peel Extract in THP-1 Cells Exposed to Particulate Matter PM10. Evid. Based Complement. Altern. Med. 2016, 2016, 6836080.
- Park SY, Byun EJ, Lee JD, Kim S, Kim HS. Air Pollution, Autophagy, and Skin Aging: Impact of Particulate Matter (PM10) on Human Dermal Fibroblasts. Int. J. Mol. Sci. 2018; 19, 2727.
- Parrado C, Mercado-Saenz S, Perez-Davo A, Gilaberte Y, Gonzalez S, Juarranz A. Environmental stressors on skin aging. Mechanistic insights. Front Pharmacol. 2019; 10:759.
- Patella V, Florio G, Palmieri M, Bousquet J, Tonacci A, Giuliano A, Gangemi S. Atopic dermatitis severity during exposure to air pollutants and weather changes with an Artificial Neural Network (ANN) analysis. Pediatr Allergy Immunol. 2020; 31(8):938-945.

- Pavlović RD, Lakušić B, Došlov-Kokoruš Z, Kovačević N. Arbutin content and antioxidant activity of some Ericaceae species. Die Pharmazie-An International Journal of Pharmaceutical Sciences.2009; 64 (10): 656–659.
- Pecorelli A, Woodby B, Prieux R, Valacchi G. Involvement of 4-hydroxy-2-nonenal in pollution-induced skin damage. Biofactors. 2019;45(4):536-547.
- Perde-Schrepler M, Chereches G, Virag P, Barbos O, Soritau O, Tatomir C, Fischer-Fodor E, Filip A, Vlase L, Postescu ID. Photoprotective effect of *Calluna vulgaris* extract against UVB-induced phototoxicity in human immortalized keratinocytes. Journal of Environmental Pathology, Toxicology and Oncology 2011; 30 (4): 323–331.
- Petruk G, Del Giudice R, Rigano MM, Monti DM. Antioxidants from Plants Protect against Skin Photoaging. Oxid Med Cell Longev. 2018; 1454936.
- Piao MJ, Ahn MJ, Kang KA, Ryu YS, Hyun YJ, Shilnikova K, Zhen AX, Jeong JW, Choi YH, Kang HK, Koh YS, Hyun JW. Particulate matter 2.5 damages skin cells by inducing oxidative stress, subcellular organelle dysfunction, and apoptosis. Arch Toxicol. 2018;92(6):2077-2091.
- Pignatti S. *Calluna vulgaris* (L.) Hull. in Flora d'Italia. Edagricole. 1982; (2): 259.
- Poljšak B, Dahmane R. Free Radicals and Extrinsic Skin Aging. Dermatology Research and Practice, 2012;135206.
- Pons B, Belmont AS, Masson-Genteuil G, Chapuis V, Oddos T, Sauviago S. Age-associated modifications of base excision repair activities in human skin fibroblast extracts. Mech. Ageing Dev., 2010;131 (11): 661-665.
- Prasad RBN, Gülz PG. Surface waxes from leaves and fruits of walnut. Phytochemistry 1990; 29:2097- 2099.
- Puizina-Ivić N. Skin aging. Acta Dermatovenerol. Alp. Pannonica Adriat., 2008; 17 (2): 47-54.
- Rains JL. and Jain SK. Oxidative stress, insulin signaling and diabetes. Free Radic Biol Med. 2011; 50(5): 567-575.
- Rawlings V, Harding CR. Moisturization and skin barrier function. Dermatol. Ther., 2004; 17 (s1): 43-48.
- Rinnerthaler M, Bischof J, Streubel MK, Trost A, Richter K. Oxidative stress in aging human skin. Biomolecules, 2015; 5 (2): 545-589.
- Rittié L, Fisher GJ. Natural and sun-induced aging of human skin. *Cold Spring Harb Perspect Med*. 2015;5(1):a015370.
- Ritz-Timme S, Laumeier I, Collins MJ. Aspartic acid racemization: Evidence for marked longevity of elastin in human skin. BR J. Dermatol., 2003; 149 (5): 951-959.
- Roberts WE. Pollution as a risk factor for the development of melasma and other skin disorders of facial hyperpigmentation is there a case to be made? J. Drugs Dermatol. 2015; 14, 337–341.
- Rodrigues F, Moreira T, Pinto D, Pimentel FB, Costa ASG, Nunes MA, Gonçalves Albuquerque T, S Costaa H, Palmeira-de-Oliveira A, Oliveira AI, Sut S, Dall'Acqua S, Oliveira MBPP. The phytochemical and bioactivity profiles of wild Calluna vulgaris L. flowers. Food Res Int. 2018; 111:724-731.
- Roh E, Kim J E, Kwon J Y, Park J S, Bode A M, Dong Z, Lee K W, Molecular mechanisms of green tea polyphenols with protective effects against skin photoaging, Critical Reviews. Food Science and Nutrition, 2017; 57(8), 1631-1637.
- Romani A, Cervellati C, Muresan XM, Belmonte G, Pecorelli A, Cervellati F, Benedusi M, Evelson P, Valacchi G. Keratinocytes oxidative damage mechanisms related to airbone particle matter exposure. Mech Ageing Dev. 2018; 172:86-95.

- Roy H. Reader's digest encyclopaedia of garden plants and flowers. Selezione dal Reader's Digest S.p.A., Milano, 1987.
- Ryu YS, Kang KA, Piao MJ, et al. Particulate matter induces inflammatory cytokine production via activation of NFκB by TLR5-NOX4-ROS signaling in human skin keratinocyte and mouse skin. Redox Biology. 2019; 21:101080.
- Saewan N, Jimtaisong A. Natural products such as photoprotection. J. Cosmet. Dermatol., 2015; 14 (1): 47-63.
- Sandilands A, Sutherland C, Irvine AD, McLean WI. Filaggrin in the frontline: role in skin barrier function and disease. J. Cell. Sci., 2009; 122 (9): 1285-1294.
- Sarniak A, Lipinska J, Tytman K, Lipinska S. Endogenous mechanisms of reactive oxygen species (ROS) generation. Postepy Hig. Med. Doswiadczalnej 2016, 70, 1150–1165.
- Schröder P, Schieke SM, Morita A, Premature skin aging by infrared radiation, tobacco smoke and ozone - B.A. Gilchrest, J.Krutmann(Eds.),Skin Aging, Springer-Verlag, New York, 2006, 45-55.
- Schroeder P, Gremmel T, Berneburg M, Krutmann J. Partial depletion of mitochondrial DNA from human skin fibroblasts induces a gene expression profile reminiscent of photoaged skin. J. Invest. Dermatol., 2008;128 (9): 2297-2303.
- Seebode C, *Lehmann J, Emmert*, S. Photocarcinogenesis and skin cancer prevention strategies. Anticancer research, 2016; 36(3), 1371-1378.
- Seeley RD, Stephens TD, Tate P. Anatomia e cenni di istologia e fisiologia. Idelson-Gnocchi, 2005.
- Sellheyer K. Pathogenesis of solar elastosis: synthesis or degradation? J Cutan Pathol, 2003. 30(2): 123–127
- Shapiro SD, Endicott SK, Province MA, Pierce JA, Campbell EJ. Marked longevity of human lung parenchymal elastic fibers deduced from prevalence of D-aspartate and nuclear weaponsrelated radiocarbon. J. Clin. Invest., 1991; 87 (5): 1828-1834.
- Shimizu H. Structure and function of the skin. Shimizu's Dermatology, 2016; 1–42.
- Shin JW, Kwon SH, Choi JY, Na JI, Huh CH, Choi HR, Park KC. Molecular Mechanisms of Dermal Aging and Antiaging Approaches. International Journal of Molecular Sciences,2019; 20(9), 2126.
- Sies H.: "Oxidative stress:oxidants and antioxidants". Exp. Physiol., 82 (2): 291-295, 1997
- Sivamani RK, Jagdeo JR, Elsner P, Maibach HI.: Cosmeceuticals and active cosmetics. CRC Press, 2016.
- Soeur J, Belaïdi JP, Chollet C, Denat L, Dimitrov A, Jones C, Perez P, Zanini M, Zobiri O, Mezzache S, Erdmann D, Lereaux G, Eilstein J, Marrot L. Photo-pollution stress in skin: Traces of pollutants (PAH and particulate matter) impair redox homeostasis in keratinocytes exposed to UVA1. J Dermatol Sci. 2017;86(2):162-169.
- Song S, Lee K, Lee YM, Lee JH, Lee SI, Yu SD, Paek D. Acute health effects of urban fine and ultrafine particles on children with atopic dermatitis. Environ Res. 2011; 111(3):394-9.
- Subhadarshani S, Athar M, Elmets CA. Photocarcinogenesis. Current Dermatology Reports, 2020;9, 189–199.
- Theocharis AD, Skandalis SS, Gialeli C, Karamanos NK. Extracellular matrix structure. Adv. Drug. Deliv. Rev.. 2016; 97: 4-27,
- Tunon H, Olavsdotter C. Bohlin L. Evaluation of anti-inflammatory activity of some Swedish medicinal plants. Inhibition of prostaglandin biosynthesis and PAF-induced exocytosis. Journal of Ethnopharmacology. 1995; 48 (2): 61–76.

- Tyrrel RM, Pidoux M. Endogenous glutathione protects human skin fibroblasts against the cytotoxic action of UVB, UVA and near-visible radiations. Photochem.Photobiol., 1986; 44 (5): 561- 564.
- Valacchi G, Sticozzi C, Pecorelli A, Cervellati F, Cervellati C, Maioli E. Cutaneous responses to environmental stressors. Ann N Y Acad Sci. 2012; 1271:75–81.
- Vieira V, Pereira C, Pires TCSP, Calhelha RC, Alves MJ, Ferreira O, Ferreira IC FR.Phenolic profile, antioxidant and antibacterial properties of Juglans regia L. (walnut) leaves from the Northeast of Portugal. Industrial Crops and Products, 2019; 134, 347–355.
- Vierkötter A, Krütmann J. Environmental influences on skin aging and ethnic-specific manifestations. Dermato-Endocrinology Landes Bioscience, 2012; 4 (3): 227-231,
- Vierkotter A, Schikowski T, Ranft U, Sugiri D, Matsui M, Kramer U, Krutmann J. Airborne particle exposure and extrinsic skin aging. J. Investig. Dermatol. 2010, 130, 2719–2726.
- Vlassopoulos A, Lean ME, Combet E. Oxidative stress, protein glycation and nutrition-interactions relevant to health and disease throughout the lifecycle. Proc Nutr Soc., 2014; 73 (3): 430-438.
- Vogeley C, Esser C, Tüting T, Krutmann J, Haarmann-Stemmann T. Role of the aryl hydrocarbon receptor in environmentally induced skin aging and skin Carcinogenesis. Int J Mol Sci. 2019; 20:6005.
- Vučić Dragana M., Miroslav R. Petković, Branka B. Rodić-Grabovac, Olgica D. Stefanović, Sava M. Vasić e Ljiljana R. Čomić. *In vitro* activity of heather [*Calluna vulgaris* (L.) Hull] extracts on selected urinary tract pathogens». Bosnian Journal of Basic Medical Sciences 2014; 14 (4): 234.
- Weihermann AC, Lorencini M, Brohem CA, de Carvalho CM. Elastin structure and its involvement in skin photoageing. International Journal of Cosmetic Science,2016; 39(3), 241–247.
- Wichtl M. Juglandis folium. Walnut leaf In: Herbal Drugs and Phytopharmaceuticals. Medpharm GmbH Scientific Publisher, Stuttgart. 2004; 281-282.
- Wójcik E. Spektrofotometryczne oznaczanie zawartości juglonu w Folium Juglandis. .Spectrophotometric estimation of juglone contents in Juglans leaves. Polish Farm Pol. 1984; 40:523- 525.
- Xiao X, Wang R, Cao L, Shen ZX, Cao YX. The Role of MAPK Pathways in Airborne Fine Particulate Matter-Induced Upregulation of Endothelin Receptors in Rat Basilar Arteries. Toxicol. Sci. 2016, 149, 213–226.
- Xu Y, Fisher G J, Ultraviolet (UV) light irradiation induced signal transduction in skin photoaging. Journal of Dermatological Science Supplement,2005; 1(2).
- Yaar M, Eller MS, Gilchrest BA. Fifty years of skin aging. J Investig Dermatology Symp Proc. 2002; 51–8.
- Yamamoto Y. Role of active oxygen species and antioxidants in photoaging. Journal of Dermatological Science, 2001; 27, 1–4.
- Yokota M, Yahagi S, Masaki H. Ethyl 2,4-dicarboethoxy pantothenate, a derivative of pantothenic acid, prevents cellular damage initiated by environmental pollutants through Nrf2 activation. J. Dermatol. Sci. 2018, 92, 162–171.
- Zhang H, Chen MK, Li K, Hu C, Lu MH, Jie ST. Eupafolin nanoparticle improves acute renal injury induced by LPS through inhibiting ROS and inflammation. Biomed. Pharmacother. 2017, 85, 704–711.
- Zouboulis CC, Ganceviciene R, Liakou AI, Theodoridis A, Elewa R, Makrantonaki E. Aesthetic aspects of skin aging, prevention, and local treatment. Clin Dermatol. 2019; 37(4):365-372.