Valorization of natural sources and by-products for pharmaceutical and cosmetic applications

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Summary

Chapter 1

Introduction

1. Natural ingredients in cosmetic products

For thousands of years, cosmetic products have been used to improve the appearance for cultural or religious purposes. Initially, all the cosmetic products were made with natural ingredients, derived from plants, animals or minerals (1).

For example, in ancient Egypt, cosmetics were a part of daily life, and men and woman used vegetable and animal oils, butters and waxes for treat their skin, herbs and pigments for makeup, and essential oils for fragrance. Egyptians were sophisticated chemist formulators! They used animal far or vegetable oils like cleansing creams, red ochre ground with water for lips and cheeks, and henna for fingernails and palms of the hands. It is famous the use of kohl: kohl was a significant cosmetic product used by ancient Egyptians for both cosmetic and medicinal purposes, for cosmetic appeal or for treating conditions, diseases and preventing infections of the eyes. It is a dark cosmetic in powder or paste in a stick, applied on the eyebrows, eyelashes and perimeter of the eyes. It was typically made from a mixture of soot, galena, and other ingredients, and it was applied around the eyes using a stick or a small spoon-like implement. Egyptians believed that kohl not only enhanced the appearance of the eyes by darkening the eyelids and eyelashes, but also provided protection against the harsh sun and evil spirits. Today, it is still used in parts of Northern Africa, but in the U.S., Food and Drug Administration (FDA) does not approve the use of original khol for the presence of lead, that is toxic: the products called “kohl” today, is only a marketing term to describe extremely dark eyeliners. Another important part of the Egyptian cosmetic is perfume, composed by animal or vegetable facts, that can absorbs fragrance molecules, and perfume like cardamon, cassia, cinnamon, frankincense, myrrh, thyme, marjoram, chamomile, lavender, lily, peppermint, rosemary, cedar, rose, aloe, olive oil, sesame oil, and almond oil (1) (2).

Traditional systems of medicine such as Traditional Chinese Medicine in China and Ayurveda in India incorporated natural ingredients into skincare and beauty rituals, using herbs, plant extracts, and minerals to promote health and well-being. In the Traditional Chinese Medicine, practiced in China for thousands of years, medicinal herbs are used to restore balance and harmony in the body, between the internal organs and the external body. Together with acupressure, exercising and other practices like moxibustion (burning herbal leaves near the body) and cupping (hot cups on the body to increase blood flow), herbal extracts were used like remedies: plant and animal extracts were mixed to treat lots of health problems, but also skin problems like dermatitis, eczema, and psoriasis. The most used herbal extracts, taking different parts of the plants like leaves, roots and barks, were *Chartamus tinctorius*, *Artemisia stelleriana*, *Semen Benincasae*, *Semen Persicae*, *Oryza sativa* (rice), *Semen Aermeniaceae amarum*, mica and *Gingko Biloba*. Also nail polish was introduce in China around 3000 BC, made of beeswax, egg whites, and gelatine, with orchids and roses, and red and black tones were preferred, while silver and gold dust were chosen for higher social ranking (1) (3). Also Ayurveda, originated in India over 5000 years ago and practiced for more than 3000 years, is an herbal medicine system, based on the harmony between mind, body and spirit thanks to the diet, yoga and meditation. The aim of Ayurved is the balance between physiological and psychological being. The skincare is an important part of Ayurveda, focused on skin beauty: water and water are believed to play an important role for the health of the skin. Ayurvedic treatments focus on skin beauty and skin disorders as acne vulgaris, aging, eczema, psoriasis, and vitiligo. A lots of botanical ingredient were used in Ayurvedic treatment like sesame oil like base for cosmetic preparations, aloe vera, *Calendula officinalis*, *Curcuma longa*, *Centella asiatica*, but also buttermilk and goat milk for face masks (1).

Also India, has a very important history in the use of natural ingredients for cosmetic formulations, like kajal, with the same composition of kohl, and used for protecting the skin against UV radiation and for religious reasons, and henna or *Lawsonia inermis*, for hair and body painting (1).

Similarly, ancient civilizations like the Greeks and Romans also valued cosmetics made from natural ingredients such as olive oil, honey, and herbs. These early cosmetic formulations were often used for skincare, haircare, and perfumery purposes, with natural fragrances and botanical extracts prized for their aromatic and therapeutic properties. In particular, in ancient Rome, we read in the writings of Pliny the Elder and Cornelius Celsus and other literature and poetry (like Ovid) (4). They used natural ingredients, like *Alcyonium,* the soft coral (sponge type) from the Mediterranean Sea, Narcissus, Iris, Honey and Myrrh for treating acne, wound, vitiligo, skin-aging and skin-whitening, like lead-based creams used by Greeks (1).

During the Reinassance, between the Middle Age and Modern era, cosmetics and especially make-up, fell out of favor in Europe due to religious and cultural shifts, under the influence of Catholic Church on society: make up was viewed like a defiance of God's natural creation (1), but they continued to be used in other parts of the world, including Asia and the Middle East. The most famous and well-known cosmetics used were Venetian Ceruse, famous for being used by Queen Elizabeth I, also known for the use of lipstick containing cinnabar, a mercury sulfide mineral, used also on the cheeks (1).

2. The transition from natural ingredients to those of synthetic origin

During the twentieth century, cosmetic industries began to use synthetic ingredients. Natural cosmetic ingredients have been replaced by synthetic ones for several reasons. First of all, synthetic ingredients are often cheaper to produce compared to the natural ones. Chemical synthesis, in fact, allows for the production of large quantities of ingredients at lower costs than harvesting or extracting natural ingredients from plant or animal sources. For the same reasons, synthetic ingredients are often more readily available in consistent quantities: natural ingredients, in fact, can be subject to seasonal, climatic, or geographical variations. This ensures greater continuity in the production of cosmetic products and reduces the risk of supply chain disruptions.

Another important reason is the control that can be had during the production of synthetic raw materials. Synthetic ingredients, in fact, offer greater control over quality and purity compared to natural ones. Chemical synthesis enables the creation of compounds with precise chemical compositions and greater uniformity, reducing the risk of contamination from pesticides, environmental pollutants, or allergens present in natural ingredients. Furthermore, synthetic ingredients, after the production, have a greater stability compared to natural ones. This means that cosmetic products containing synthetic ingredients may have a longer shelf life and a lower likelihood of deterioration or bacterial contamination compared to those containing natural ingredients. They are often preferred also for the consistency and texture: during the production they can be engineered to provide a pleasing consistency and texture, which is a fundamental quality for the finished cosmetic product. The final consumer, in fact, is attracted by the fell and the appearance of the product. An example are silicones, inserted into cosmetic formulations because they can modify the texture and rheology of cosmetic products, giving desired characteristics such as smoothness. They can also act like viscosity modifiers, thickeners, and suspending and enhancing agents, improving product spreadability and providing a luxurious sensory experience. At the same time, silicones act like active ingredients thanks to emollient properties, stability and optical effect, especially for hair providing volume and body, heat protection, anti-frizz and silky effect, and smoothness and shine thanks to the

filming property. The problem of silicones is their environmental impact: they are not biodegradable and they also have ecotoxicological effects. Certainly, the development of cosmetic ingredients of synthetic origin has also led to an innovation that the use of ingredients of natural origin would never have achieved. Research and development in the field of cosmetic chemistry allow for the creation of new synthetic ingredients with unique properties and specific benefits for skin or hair (5).

3. The recent return to ingredients of natural origin

The modern era saw a resurgence of interest in natural cosmetic products, fuelled by growing awareness of environmental sustainability, health concerns, and a desire for more holistic approaches to beauty and skincare.

In recent years there has been a growing demand, especially in the European countries, for more natural and sustainable cosmetic products, to provide benefits to consumers, called “green consumers”. Green products have the same characteristics and functions of synthetic products, but without harming the environment. Consequently, many companies are exploring the use of natural and biodegradable ingredients in their formulations to meet this demand and reduce the environmental impact of their products, replacing their synthetic ingredients with naturals (1) (6).

From the late 1990s to the early 2000s, the market began to change: there was a proliferation of natural and organic cosmetic brand offering products made, in a large percentage, from plant-based ingredients, essential oils, and botanical extracts. These products were marketed as safer and healthier alternatives to conventional cosmetics, free from synthetic chemicals, artificial fragrances, and harsh preservatives. Companies started to reintroduce natural ingredients into formulations, following consumer demands, maybe due to the influence of social media and internet, and driven by a growing attention to the environment. The aim is to reduce the use of hazardous chemicals, the energy consumption, and also encourage the use of wastes and renewable sources.

Today, natural cosmetic products continue to enjoy widespread popularity, with consumers increasingly seeking out products that are environmentally friendly, ethically sourced, and free from harmful ingredients. From skincare and haircare to makeup and fragrances, the market for natural cosmetics continues to expand, reflecting a growing demand for products that promote both personal well-being and planetary health.

The size of the market of natural cosmetic products was valued at USD 35.2 billion in 2021, USD 36.5 billion in 2022 and USD 37.9 billion in 2023, with a constant annual growth. The forecast for the period 2024-2032 is a CAGR (Compound Annual Growth Rate) of 5.1%. The focus on natural cosmetic products is above all aimed at Skin Care and Sun Care with the largest industry 20.1%, USD 15.5 billion in 2023. The second fragment is Body care (18.7%) followed by hair care, make up, fragrances and men’s grooming. Europe is the leader of natural cosmetics market (7).

3.1 The role of packaging for natural cosmetic products

Packaging plays a crucial role in the natural cosmetics industry, not only for containing and protecting products, but also because it communicates brand identity, values, and sustainability commitments to consumers. In the natural cosmetics consumers, there is a growing demand for sustainable packaging: in fact, a lot of brands are choosing biodegradable, compostable, refillable packaging, or recyclable materials such as glass, paperboard, aluminium, and bio-based plastics. Reducing packaging waste and incorporating recycled materials into packaging are becoming standard practices. Many natural cosmetic brands use minimalistic packaging to reflect simplicity, purity, and transparency in line with

the trend of a minimalist lifestyles. Not only the simplicity, but also transparency is used by brands to provide consumers with clear and accurate information about product ingredients, sourcing, and manufacturing processes. Clear labelling, ingredient lists, and certifications such as organic, cruelty-free, and vegan are essential for building trust and credibility with consumers who prioritise transparency and authenticity. The packaging can also acts as a vehicle for brand storytelling, allowing natural cosmetic brands to communicate their values, mission, and commitment to sustainability. In this way, the brand can create an emotional connection with consumers. Packaging for natural cosmetic products should be functional and user-friendly, ensuring ease of use and maintaining product integrity, but also avoiding waste through minimizing product contamination and oxidation, preserving the efficacy and freshness of natural ingredients through the use of airless containers, droppers bottles and pump dispenser.

3.2 The use of solid formulations

To avoid packaging and minimize waste, the use of solid products is being developed by many companies. This kind of products plays a significant role in promoting sustainability within the beauty and personal care industry. First, they can be packaged in minimal or plastic-free packaging such as cardboard boxes or reusable container avoiding plastic bottles or tubes, like traditional liquid formulations. But avoiding the packaging is not the only reason that reduces the environmental impact of solid cosmetic products: in fact, they are made without water and consequently without preservatives, have a longer shelf life because they remain stable for longer periods, reducing purchase frequency and waste. Solid cosmetics often require less energy and resources for the production, package, and transport compared to liquid formulations. Their compact size results in lower transportation costs and reduced greenhouse gas emissions associated with logistics and distribution. By minimising energy consumption and carbon emissions throughout the product lifecycle, solid cosmetics help mitigate climate change and promote environmental sustainability. They are also versatile and portable for consumers, making them convenient choices for consumers that want sustainable beauty solutions: their compact size makes them ideal for travel, gym bags, and on-the-go lifestyles, encouraging consumers to use multi-functional and travel-friendly products, reducing consumption of single-use packaging. Furthermore, most of the solid cosmetic brands prioritize natural, plant-based ingredients and transparent formulations free from synthetic chemicals, preservatives, and artificial fragrances. By using high-quality, ethically sourced ingredients, solid cosmetics offer consumers a more sustainable and environmentally friendly alternative to conventional beauty products. As consumer awareness of environmental issues continues to grow, the demand for solid cosmetics is expected to increase, driving further innovation and adoption of sustainable beauty practices.

3.3 Plant-based ingredients for skin-care

With the high quantity of raw materials of vegetal origin, it is possible to act on a high quantity of skin problems. For example, to act on skin hydration it is possible to use emollient, occlusive and humectant natural ingredients. With emollient properties it is possible to use butters and oils like shea butter, cocoa, avocado oil, sunflower oil, coconut oil, almond oil, and a lot of other natural ingredients. Oils like olive oil, jojoba oil and coconut oil, and waxes of candelilla and bees can be used like occlusive ingredients for avoiding the Trans Epidermal Water Loss (TEWL) (8) (9). Natural humectant ingredients, moisturizing agents that bind water, are honey, hyaluronic acid, glycerine, panthenol and sugar like sorbitol (8) (10). Another important aspect of skincare is the maintenance of the lipid barrier: agents that repair the lipid barrier are present into cosmetic formulations. Skin barrier act as a defence, but it can be damaged by external agents, age, UV radiation and an aggressive skincare, such as the use of unsuitable cleansing. Barrier repair agents are fatty acids, antioxidants like phenolic compounds and

tocopherols, phospholipids, cholesterol and ceramides (8). Fatty acids play a critical role in the maintenance of the skin barrier, and oil like flaxseed, olive, chia, jojoba, walnut, grapeseed, coconut, argan, sesame, castor and sunflower oils, rich in fatty acids, are widely used in natural cosmetic formulations (11) (8) (12). Natural ingredients in cosmetic formulations are also used like skin whitening and lightening, acting as inhibitors of tyrosinase (a key enzyme in the production of melanin) and/or in the transfer of melanosome, accelerating the epidermal *turn-over*, or acting with antioxidant and anti-inflammatory activities. This natural ingredients are extracts like liquorice, citrus, white mulberry and bearberry extracts and some molecules like kojic acid, arbutin, allantoin, vitamin C, hydroquinone, retinoids, resveratrol, and alpha and beta hydroxy acids (8) (13). Liquorice root, chamomile, calendula, oats, turmeric and nuts are for example plants with anti-inflammatory activity against sensitive skin or acne condition, while aloe vera, green tea, coconut oil, grape seeds and ginger have phytochemicals that can protect against UV radiations and the damages that they cause, like photoaging (dark spot and wrinkles) sunburns and skin cancer (8) (10) (14).

Phytochemicals are molecules produced by secondary metabolism by plants with the aim to protect plants from external agents, like atmospheric agents or sun and microbes. Some of them have antioxidant activity and can react with free radicals, like phenolic acids, polyphenols, glutathione, coenzyme Q10 and carotenoids that can be used in cosmetic formulation to defend the skin. In general, phytochemicals have a wide range of effects like photoprotection, anti-inflammatory, anti-bacterial and anti-viral, and anti-age activities The most used and famous phytochemicals are probably vitamins that have antioxidant properties, but also beneficial effects for the skin: vitamin C, for example, can increase collagen production as well as neutralize free radical; vitamin E in an emollient and antioxidant active, and Vitamin A can increase collagen production and renew the skin (8).

Another class of natural cosmetic ingredients are gums derived from plant sources and they are used like stabilizing agents, enhancing product viscosity, texture and improving the Spreadability. The most common gums are xanthan, agar, guar, acacia or arabic gums, and carrageenan. They are also used like emulsifiers, film-forming agents, moisturizers, and emollients.

Also essential oils are typical natural ingredients, used like perfumes and like actives. In fact, they are rich in volatile compounds which give the fragrance, but also properties such as anti-inflammatory and antiseptic activities. There is a huge variety of essential oils and some of the most famous are lavender, peppermint, tea tree, rose, immortelle, eucalyptus, chamomile, neroli and rosemary essential oils (12).

In general, some of the most used botanical species are *Vitis vinifera*, *Butyrospermum parkii*, *Glycine soja*, *Simmondsia chinensis*, *Helianthus annus*, *Theobroma cacao*, *Calendula officinalis*, *Glycyrrhiza glabra*, green tea (*Camellia sinensis*), *Coffea arabica*, *Punica granatum* , *Aloe vera*, *Citrus limon*, *Prunus dulcis*, Cocos nucifera (15) (8).

One of the well-known examples is *Vitis vinifera*. It is possible to use extracts from different parts of the plant and for this reason it is possible to find it in International Nomenclature Cosmetic Ingredients (INCI) with different names like for example *vitis vinifera* (grape) seed oil, *vitis vinifera* (grape) fruit extract, palmitoyl grape seed oil, palmitoyl grapevine shoot extract, palmitoyl grape seed extract, *vitis vinifera* extract and *vitis vinifera* seed extract. Each part of the plant contains different molecules: the stems and the aerial parts of the plant are rich in resveratrol (16) (17), seed oil contains an high concentration of linoleic acid and it is used like emollient (18), while the seed extract is rich in catechin, epicatechin and epicatechin gallate and it is used like anti-pollution ingredient (19) (20) (21). The extract of the fruit is rich in antioxidants like vitamin C, E, polyphenols and carotenoids (22) (15).

Instead, the most used botanical is *Butyrospermum parkii*, which can be found in INCI like *Butyrospermum parkii* (shea) butter or *Butyrospermum parkii* (shea) Butter Extract. The butter is extract from the mature shea fruit and it contains 90% of triglycerides and 10% is represented by the unsaponifiable fraction. The main fatty acids are stearic, oleic, palmitic, linoleic and arachidic acid, that provide to the skin moisturizing and a barrier protective action (15) (23) (24). The second most used botanical is *Glycine soja* (soy), and also in this case, many parts of the plant are used. For example, in INCI it is possible to find *Glycine soja* (soybean) oil, rich in triglycerides of linoleic (54%), oleic (24%) and linoleic (7%), and saturated fatty acids. It is generally used like anti-aging ingredient, like *Glycine soja* (soybean) germ extract, rich in isoflavones, that we can also find in INCI like Soy isoflavones. Soy peptides are present in INCI like "Hydrolized soy protein", which provides to the skin small peptides and isolated aminoacids (15) (25). Another botanical widely used is *Simmondsia chinesis* (jojoba), represented like *Simmondsia chinesis* (jojoba) seed oil, *Simmonsia chinensis* oil and jojoba esters. It is composition is full of fatty acids and triglycerides with a composition similar to skin sebum, and it is also full of polyphenols that provide an antioxidant activity (15) (26) (27). We can continue with *Helianthus annuus* (sunflower), rich in oleic and linoleic acids, and represented as *Helianthus annuus* seed oil and *Helianthus annuus* seed wax (15) (28). *Theobroma cacao* (cocoa), is represented like *Theobroma cacao* seed butter and *Theobroma cacao* (cocoa) butter extract, used like emollient and antioxidant thanks to the grant content of triglycerides, polyphenols like flavonoids, xanthine derivatives, caffeine and theobromine (15) (26) (29) (30) (31). For the *Calendula officinalis*, the flower is used and represented as *Calendula officinalis* flower extract, rich in terpenoids, carotenoid, flavonoids and volatile oils. It shows antioxidant and antimicrobial activities, providing skin elasticity, hydration, reduction of TEWL and anti-inflammatory and anti-pollution activities (15) (32) (33) (34) (35) (36) (37) (38). Also *Green tea*, *Coffea arabica* and pomegranate extracts are used for the high antioxidant properties that can scavenge free radicals, while aloe vera is mainly used like moisturizer and anti-inflammatory activity. Licorice (Glycyrrhiza glabra) is used especially for the depigmenting ability while almonds oil and coconut oil are used to obtain a soft skin (8). These are only the most well-known and used examples, but the list of ingredients and natural extracts used in cosmetics is very long.

4. The role of sustainability in natural cosmetics

Products with natural and sustainable connotation are the 25% of total cosmetic consumption in 2023, equal to over €2,890 million (39).

Consumers demand a return to natural ingredients, driven by awareness of the environmental changes in recent years. The natural ingredients that consumers want in cosmetic formulations are indeed those that do not harm the environment: a specific example is the use of sunscreens that have no harmful effects on the marine ecosystem (40) (41) (42) (43) (44) (45) (46).

The sustainability of natural cosmetic ingredients and final products concern different aspects of the production. First, the ingredient source has to be renewable, organic, and ethically harvested. This often supports local farmers and communities, promoting biodiversity, and avoiding ingredients associated with deforestation or environmental degradation. About the environment, industries have to minimize their environmental footprint by reducing water usage, energy consumption, and greenhouse gas emissions, during manufacturing and transportation. They may also employ eco-friendly practices such as using renewable energy, implementing efficient production processes, and reducing waste generation (47).

Many natural cosmetic products give security to the consumers with the certifications of organic such as COSMOS Organic and Ecocert, verify their adherence to specific sustainability standards and ethical

practices. These certifications provide transparency and assurance to consumers regarding the environmental and ethical sustainability of the products. These certifications of organic cosmetics, in Europe, are carried out by independent certification bodies that assess cosmetic products through specific organic standards to determine their compliance with organic principles. They conduct audits, inspections, and product testing during the entire supply chain to verify compliance with organic standards. Products that meet the requirements of organic certification may display the certification logo or seal on their packaging. The label is another important concern for the certification: for example, when the product is EcoCert certified and has the certification logo on the label (Figure 1), it assures the consumer of at least 10% organic ingredients and at least 95% ingredients of plant origin. Labelling requirements allow consumers to choose and distinguish between genuine organic products and those that make misleading claims.

Figure 1. The Ecocert certification logo.

We also discuss about packaging, but it is another important aspect for the sustainability of cosmetic products: it has to be recyclable, biodegradable or reusable, to minimize waste and pollution. In recent years, packs suitable for a second life are available, which can be refilled once finished. These packs are made of glass, hard plastic or with a second smaller container inside which can be replaced one the product is finished, by a full one, reducing the amount of waste. Another alternative is to avoid the use of a packaging: the trend of the solid cosmetic products.

For most consumers, another aspect regarding sustainability is the respect for animals. Usually, cosmetic industries avoid the use of cosmetic raw materials of animal origin, which, although natural, imply animal exploitation. Among these there are collagen and elastin, keratin, lanolin, snail slime, milk and by-products of various animal species such as cows or donkeys, the red dye E120 extracted from cochineal as a colorant in make-up, and bee products such as beeswax, propolis and honey. In fact, even if animal testing of raw materials and finished cosmetic products is prohibited in Europe by the Regulation (EC) N° 1223/2009 (48) on cosmetic products, this does not limit the use of animal substances within the products. Cosmetic industries that pay attention to the sustainability use plant-based or synthetic alternatives to animal-derived ingredients to ensure ethical sourcing and production.

In general, we can say that sustainability of natural cosmetic products pay attention to ethics and environment, prioritizing the well-being of people, animals, and the planet. By choosing sustainable natural cosmetics, consumers can support companies that align with their values and contribute to a more sustainable and eco-conscious beauty industry.

5. Natural extract in cosmetic products

In 2011, one third of the ingredients in the INCI (International Nomenclature of Cosmetic Ingredients) system at the Personal Care Products Council were classified as “botanical extracts” (15).

These ingredients were obtained from different extraction procedures, solvents and different parts of the plants.

5.1 Green extraction methods

Extraction of bioactive compounds like antioxidants, such as polyphenols, requires the use of extraction method based on the use of mechanical, chemical, thermal or electrical methods, and on the use of sustainable organic solvents.

These techniques affect cell membranes, with permeabilization or physical rupture which allow a rapid and high diffusion of bioactive molecules into the extracellular environment. In fact, bioactive and antioxidant compounds such as polyphenols are located in the vacuoles or chloroplasts of plant cells, and separated from the outside environment by the cell membrane, which hinders their release (49) (50) (51).

Conventional extraction methods require the use of organic solvents, long processing time, high temperature (that can also influence the properties of the bioactive compounds), large amounts of energy, and can also have a negative impact on human health and environment.

Conventional extraction methods are replaced by green ones that have been chosen for several reasons, mainly related to environmental sustainability and the reduction of negative impacts on human health and the planet. In fact, green extraction methods reduce the use of aggressive and toxic chemical solvents, which can contaminate air, water, and soil during the extraction process. By using safer and biodegradable solvents, the impact on the ecosystem is reduced, and the production of hazardous waste is limited. The use of less toxic and less flammable solvents in extraction processes reduces also the risk of exposure and accidents for operators and production staff, improving workplace safety.

For many green extraction methods, renewable resources are used, such as plant-based solvents, such as ethanol or terpenese, and water. These solvents can be obtained from sustainable and biodegradable sources, reducing dependence on non-renewable resources like petroleum.

Most of the green extraction methods require lower energy inputs compared to conventional methods, contributing to overall energy efficiency and a reduction in greenhouse gas emissions.

Reducing time, energy and temperature, green extraction methods may better preserve the quality and integrity of extracted compounds, maintaining the nutritional, sensory, and functional properties of the final products. This can lead to higher quality products and increased perceived value for consumers.

It is also necessary to consider that the use of sustainable extraction methods responds to market needs: with the increasing awareness of environmental issues and the demand for sustainable products from consumers, companies are increasingly searching green extraction methods to meet this demand and demonstrate their commitment to sustainability and social responsibility.

All this can be summarized with the definition of green extraction of natural products: *“Green Extraction is based on the discovery and design of extraction processes which will reduce energy*

consumption, allows use of alternative solvents and renewable natural products, and ensure a safe and high quality extract/product" (52).

Overall, green extraction methods offer numerous advantages over traditional methods, contributing to a more circular, sustainable, and environmentally respectful economy. Their adoption represents an important step towards creating a greener and more responsible cosmetic industry.

5.1.1 Ultrasound-Assisted Extraction (UAE)

Ultrasound-assisted extraction (UAE) is an innovative technique increasingly recognized for its efficiency and sustainability in extracting bioactive compounds from various natural sources. This method utilizes high-frequency sound waves (ultrasound) to disrupt cell walls thanks to the acoustic cavitation process, and enhance extraction of target compounds from plant materials, microorganisms, and other matrices. The high pressure and temperature involved in this process destroy the cell walls, increasing the surface area exposed to the solvent, and increasing accordingly the release of intracellular compounds (49). Ultrasound extraction can be done using an ultrasonic bath, in which a stainless-steel tank is connected to a transducer, where the matrix is dispersed into the solvent. This system is easy and affordable, but reproducibility and scale-up are limited. The alternative is the use of an ultrasonic probe system where a probe is connected to the transducer and immersed in the solvent with the matrix, minimizing the energy loss (Figure 2). This second method is usually preferred for bioactive extractions, thanks to the greater intensity that can be generated: the concentration of the energy in a specific area of the matrix makes the cavitation more efficient, with a single limit which is the volume (49) (53) (54). The parameters influencing UAE efficiency are power, frequency, temperature, and time with a similar influence on the yield: increasing them, also the efficacy of the extraction increases. This is possible up to a limit beyond which by increasing the parameters, the yield decreases (55). The power during UAE can be expressed as amplitude percentage in the range 0 to 100%, where 100% amplitude indicates the rated power of the equipment and power density (W·mL−1) which is calculated as power dissipated per unit volume of the extraction medium. The power applied for the extraction of bioactives from fruit and vegetables by-products depends upon the compound to be extracted and the types of plant matrix selected for the extraction, and falls in the range from 20 to 700 W. The yield of the UAE increases with increase in power and then decreases after reaching a peak. The frequency is usually between 20 kHz and 120 kHz, and a great variety of solvents can be used like ethanol, water and other alcohols (54). UAE offers several advantages over conventional extraction methods, including reduced extraction time and consequently rapidity, safety, versatility, simplicity, eco-friendliness and cost-effectiveness, due to the low expensive solvent consumption and reduced consumption of time and energy (56) (57) (49). UAE preserve the integrity and bioactivity of thermosensitive phenolic compounds, alkaloids, vitamins, essential oils and for antioxidant compounds. In general, the extraction is efficient for thermolabile compounds thanks to the mild conditions of the extraction, like temperature, that can be controlled. It is a fast and cheap method, with high extraction yields (58).

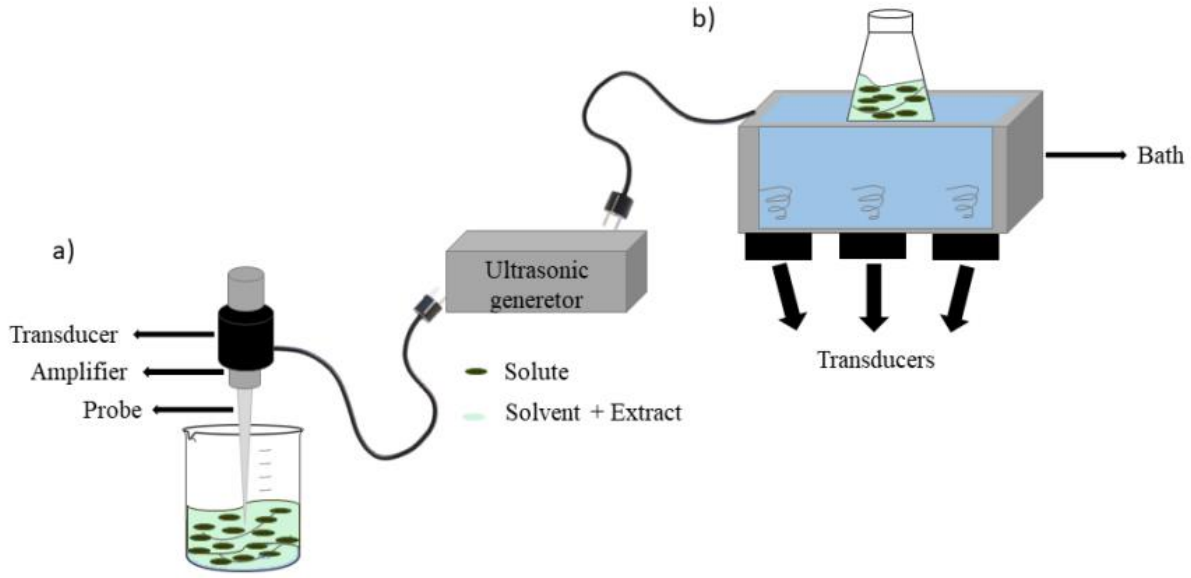

Figure 2. Schematic representation of an ultrasonic probe system (a) and an ultrasonic bath system (b) *(49)*.

UAE is a versatile technique that can be scaled up for industrial applications and represents a promising approach for obtaining high-quality bioactive compounds from natural sources, with potential applications in the food, pharmaceutical, and cosmetic industries. This method can contribute to sustainable and eco-friendly extraction practices, eliminating the need for high temperatures and harsh chemicals, making it a greener and more environmentally friendly extraction method.

5.1.2 Microwave-Assisted Extraction (MAE)

Microwave-assisted extraction (MAE) is an innovative and efficient green extraction technique that utilizes microwave radiation to facilitate the extraction of bioactive compounds from various natural sources. This method involves the application of electromagnetic waves which generate heat inside the matrix after penetrating it, causing the breakdown of cell walls accelerating the extraction process by promoting the release of target compounds from the matrix (59). The rise in temperature and pressure of water vapor inside the cell, due to the generation of heat during the microwave exposure, lead to the swelling of the cell wall until its rupture with resulting in release of intracellular compounds in the outside environment (49). For the extraction of natural products there are two types of MAE systems. In the closed-vessel system, the extraction is carried out under controlled conditions of temperature and pressure, and it is usually used for extraction with very high temperature. Microwaves interact with the plant sample, placed in extraction vessels in a closed-vessel chamber, from all directions. In the open-vessel system, the extraction vessel is only partially exposed to microwaves: a waveguide directs the microwaves towards the extraction vessel inside the microwave cavity (Figure 3) (55). MAE offers several advantages including reduced extraction time, higher extraction yields and good efficiency, low solvent consumption and the possibility to develop a solvent-free process, low power consumption and good reproducibility (49). Additionally, MAE operates under mild conditions, preserving the integrity of sensitive compounds, such as vitamins, enzymes, and polyphenols. It is a good extraction method for polyphenols, polysaccharides and carotenoids (58). Factors that improve the efficiency of MAE are temperature, time, microwave power, and the choice of solvent and matrix. The temperature can be higher the boiling point of the solvent, but, also if higher temperatures can improve extraction efficiency, it is necessary to pay attention to thermolabile

components. How to concern power and time (usually MAE does not exceed 30 min. at 30 W) they are mutually dependent factors: the efficiency of the extraction is higher increasing the microwave power, but at the same time the time is greatly reduced (1 o 2 min. at 150 W). For the extraction of phenolic compounds, a high power can result in a poor yield due to the degradation of thermolabile components. The same thing can happen with an exposure for a long time, for the disruption of chemically active compounds, like polyphenols. To avoid the thermal degradation of phenolic compounds, extraction time can be manipulated (55). Another important aspect of microwave extraction is the choice of solvent: it is necessary to take into consideration the microwave adsorption by the solvent, the interaction of the matrix with the solvent and the solubility of the analyte into the solvent. For the efficiency and the selectivity of the MAE, the solvent must have a high dielectric constant and absorb a high amount of microwave energy. Green solvents like water and ethanol are sufficiently polarized and heated by microwaves (49). Other two features that increase the effectiveness of the extraction are the characteristics of the matrix: the content of water and the size. The use of matrices containing water, by absorbing the energy of the microwaves, increase the internal temperature until cellular rupture, increasing the extraction of intracellular compounds. By avoiding the step of drying the matrix it's also possible to save energy (60). Hot to concern the size, to improve extraction yield is possible to reduce the matrix into fine particles (100 µm-2mm) to increase the interaction between the matrix and the solvent (increasing the surface area) so that the microwaves penetrate deeper in the matrix (61).

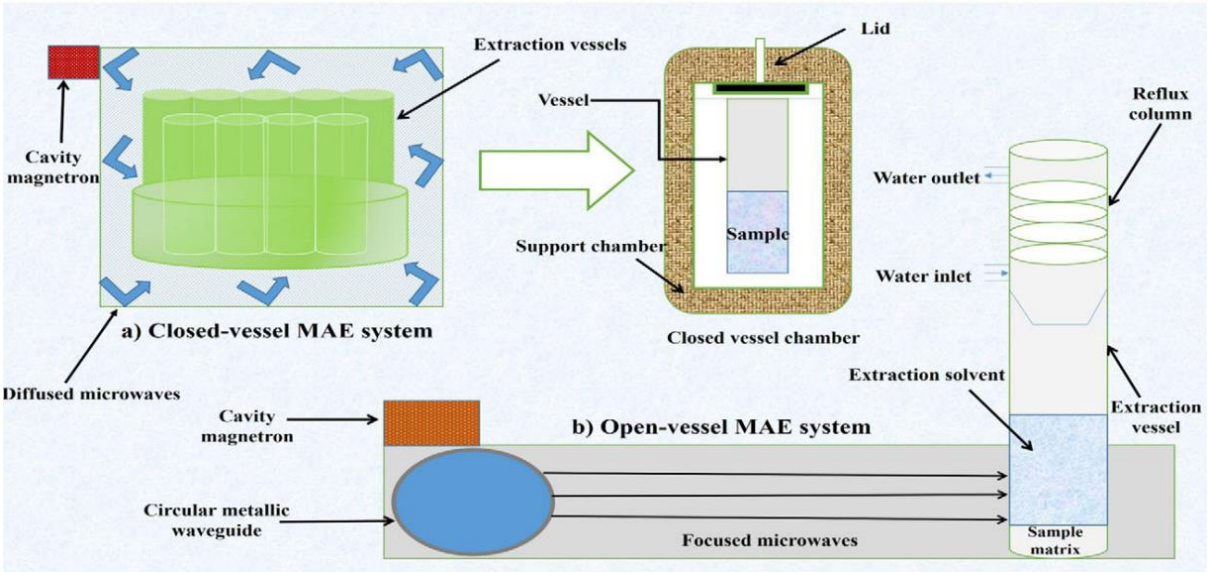

Figure 3. Schematic representation of the mechanism of Microwave-Assisted Extraction (MAE) system: closed-vessel MAE system (a) and open-vessel MAE system *(55)*.

In general, MAE is a green extraction technique that requires minimal solvent consumption and can be easily scaled up for industrial applications. By reducing energy consumption and solvent waste, MAE contributes to sustainable and eco-friendly extraction practices, making it a promising approach for a rapid and efficient extraction of bioactive compounds from natural sources, with potential applications in various fields such as food, pharmaceutical and cosmetic.

5.1.3 Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction (SFE) is a cutting-edge extraction technique widely explored in research and industry for its efficiency and environmentally friendly characteristics. This method employs supercritical fluids, typically carbon dioxide (CO_2) as extraction solvent, due to its low critical

temperature and pressure. Supercritical fluids are fluids existing in a phase with characteristics of both liquids and gases above their critical temperatures and pressures. The critical temperature is the temperature at which the gas does not become liquid until the application of an extra pressure. The critical pressure is the minimum quantity of pressure required to liquefy a gas at its critical temperature (55). Controlling temperature and pressure, the supercritical fluid is brought in the supercritical region, above which it possesses the physicochemical properties of both, the liquid and the gas (62). In fact, if the liquid phase has an higher density and due to this has more power to dissolve analytes, the gas phase is more diffusible thanks to its lower viscosity, and the simultaneous presence of these properties in supercritical fluids leads to an increase in the extraction of the compounds from the matrix to the supercritical fluid (63). The first step of this method is the extraction, where the fluid is compressed to the required pressure and brought to the required temperature, and then spreads in the sample. In this way, the material is dissolved and transferred out of the matrix. In the second step, the separation part, pressure and temperature are then adjusted to reduce the supercritical fluid solubility leading to the precipitation of the compounds (Figure 4) (62). One of the major advantages of SFE is its tunability, as the solvent properties of supercritical fluid can be adjusted by varying pressure and temperature, allowing for selective extraction of specific compounds. Additionally, SFE operates under mild conditions, preserving the integrity of bioactive sensitive compounds, such as essential oils, terpenes, carotenoids, polyunsaturated fatty acids and cannabinoids, and in general thermolabile compounds. It is a very selective extraction method (58).

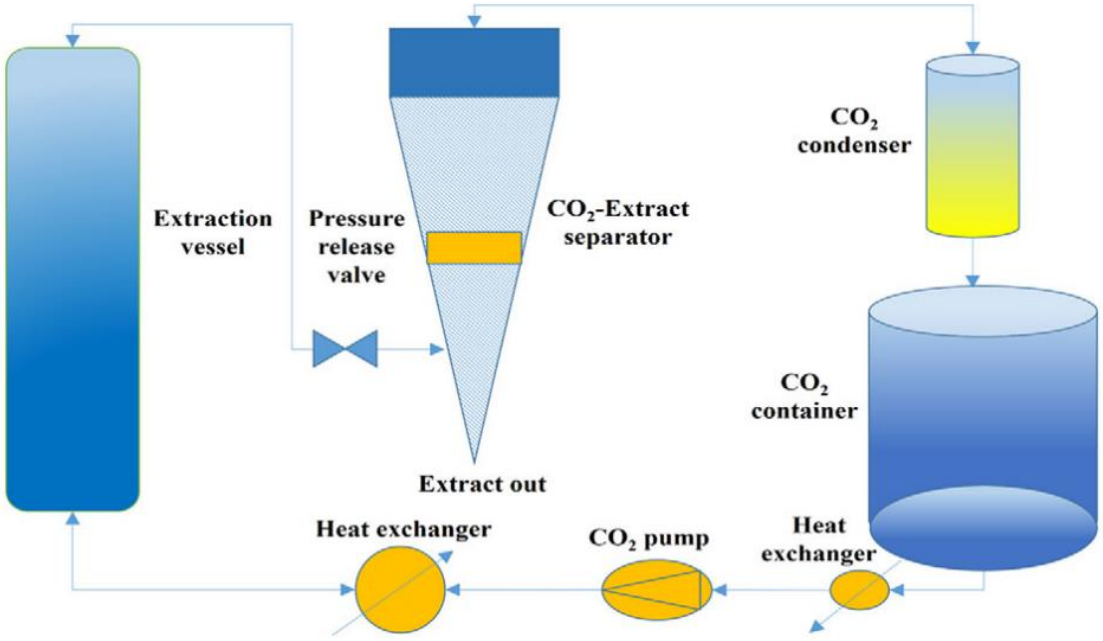

Figure 4. Schematic representation of the mechanism of a supercritical fluid extraction (SFE) system (55).

The most common supercritical fluid used like solvent is SFE, is CO_2, generally recognised as safe (GRAS) by European Food Safety Authority and United Stated Food and Drug Administration and it is also inexpensive (55). The supercritical region, for the CO_2 is above 31.1°C and 73.8 bar, with a low critical temperature and pressure, making it suitable for thermos-sensitive molecules. CO_2 can penetrate plant materials or other matrices and dissolving target compounds through a combination of diffusion and solubility (63). The SFE with CO_2 is not so good for polyphenols extraction because they have a low degree of solubility in supercritical CO_2. Sometimes some modifiers are added to CO_2 to improve the solubility and the recovery of phenolic compounds, with acetonitrile, acetone, methanol, ethyl ether,

ethanol and water, where ethanol is the best choice thanks to lower degree of selectivity but also in terms of green choice, thanks to the low toxicity (55). Moreover, supercritical CO_2 is non-toxic, non-flammable, non-corrosive, inexpensive, readily available and with a low viscosity and high diffusivity. These properties make it a safe and sustainable alternative to conventional organic solvents. It can also be reused (62).

SFE is a fast extraction method with an high extraction yield thanks to the simultaneous properties of liquids and gases, that eliminates the use of organic or toxic solvents avoiding purification steps, and making this method economically acceptable at industrial scale (49).

SFE has found applications in various industries, including food, pharmaceuticals, nutraceuticals, and cosmetics, for extracting bioactive compounds from natural sources like herbs, spices, botanicals, and biomass. SFE represents a promising green extraction method that offers high selectivity, efficiency, and environmental sustainability for the extraction of valuable bioactive compounds.

5.1.4 Pressured-Liquid Extraction (PLE)

Pressured-liquid extraction (PLE), also known as pressurized solvent extraction or accelerated solvent extraction, is a modern extraction technique used to efficiently extract target compounds from solid or semi-solid samples. In PLE, elevated pressure and temperature are applied to a solvent, typically water or organic solvents such as ethanol, to increase its solvating power and enhance extraction efficiency. The sample is placed in a stainless-steel extraction cell, and the pressurized solvent is pumped through the cell, lead to a rapid dissolution of target compounds from the matrix. The sample is placed into an extractor where the solvent is pumped into the extraction vessel with and HPLC pump. The sample is maintained at the desired temperature and pressure. The extraction process begins when the combination of desired temperature and pressure is reached. Due to the back pressure, after the end of the extraction cycles, blocking valves are opened in order to maintain the flow rate. At the end, when the extraction cycle is completed, the heating system and the HPLC pump are shut down. Eventually, the residual solvent is removed from the extractor, using inert gases such as nitrogen (Figure 5) (55). The increased pressure helps to penetrate the sample more effectively, while elevated temperatures accelerate the extraction kinetics, reducing extraction times compared to traditional solvent extraction methods. Increasing the temperature of the solvent above its boiling point allows increasing in solubility and extraction rates between the matrix and the solvent. The increase of temperature decreased also the extractant viscosity leading to high solubility of the molecules. It also breaks the bonding forces like dipole-dipole, van der Waals and hydrogen bonding, facilitating the diffusion of phenolic compounds out of the solid matrix (55).

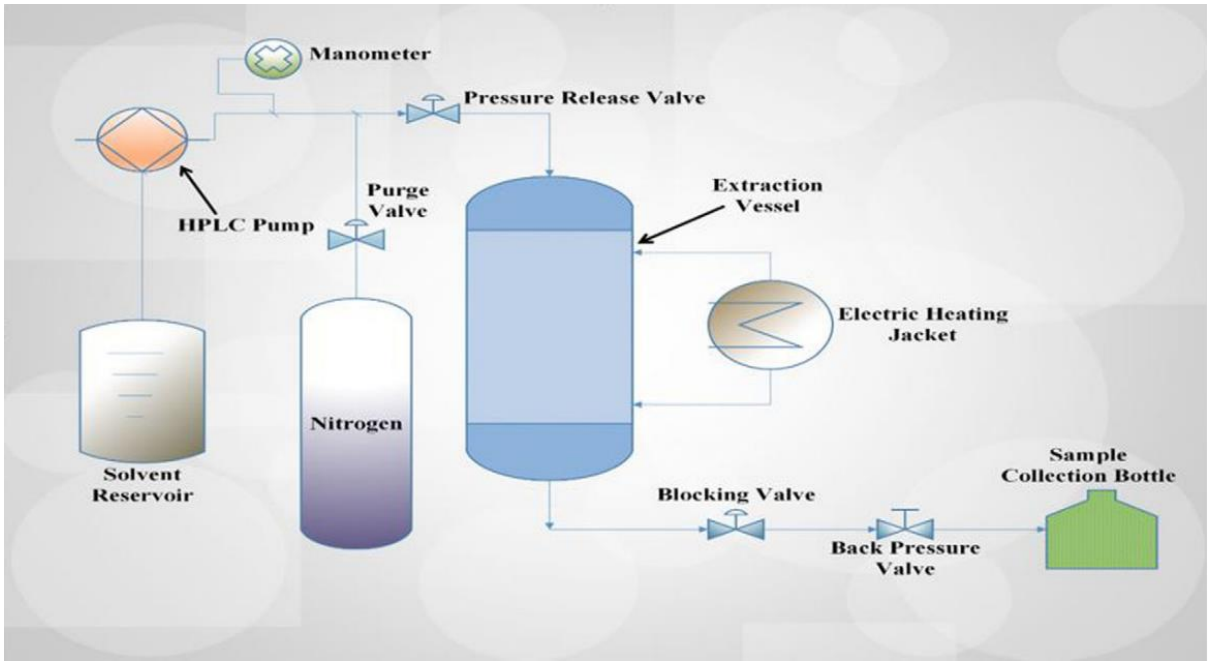

Figure 5. Schematic mechanism of pressurized liquid extraction (PLE) system *(55)*.

One of the main advantages of PLE is its ability to extract a wide range of compounds, including polar and non-polar analytes, such as for example polyphenols and carbohydrates. In fact, in recent years, PLE is recognized as green method for polyphenols extraction from herbal preparations, extracting in shorter time with a minimum solvent, but also with a minimum matrix pretreatment (55). Furthermore, PLE requires low volumes of solvents, but for a high total extraction yield, making it a good choice in terms of environmental problems (58). The choice of an appropriate solvent is necessary to obtain a high extraction yield of extraction, but it is also necessary to choose a safe and non-toxic solvent, looking also to the economic and environmental aspects, with a low toxicity and ease to remove is good. For the extraction of phenolic compounds, usually binary solvents are used, like ethanol-water mixture, more effective and environment friendly than pure organic solvents. Moreover, PLE minimizes the need for multiple extraction steps, making it a more environmentally friendly extraction technique compared to traditional methods. PLE is also highly automated, allowing for control over extraction conditions and reproducibility.

Overall, pressure liquid extraction offers a rapid, efficient, and environmentally friendly approach to extract target compounds from solid samples.

5.1.5 Pressured Hot-Water Extraction (PHWE)

Pressurized hot water extraction (PHWE), also known as subcritical water extraction, is an advanced extraction technique that utilizes hot water under elevated pressure to extract target compounds from solid or semi-solid samples, like plants and botanicals. In PHWE, water is heated above its boiling point at atmospheric pressure, typically between 100 to 374 °C, while maintaining pressure to prevent boiling. This creates a solvent with properties intermediate between liquid and gas, enhancing its solvating power and facilitating the extraction process.

One of the key advantages of PHWE is its ability to extract, in a rapid way, a wide range of polar and semi-polar compounds from different matrices. The use of water as solvent eliminates the need for organic solvents, reducing environmental impact and potential health hazards associated with other solvents. The use of water makes this process cheap, non-toxic, non-flammable and eco-friendly.

Moreover, PHWE operates under relatively mild conditions, preserving the integrity of sensitive compounds, such as vitamins, antioxidants, and essential oils. How to concern polyphenol extraction depends on the solubility of polyphenols in water, and on pressure, temperature, extraction time, flow rate and additives, that can be changed to enhance the solubility in water. For example, increasing the temperature, the solubility rates increase, but it's necessary to pay attention to the thermal degradation of thermolabile phenolic compounds. Also a high flow rate, reducing the retention time of the compounds at elevated temperatures, enhances the recovery of polyphenols.

The extraction process takes place in an extraction vessel, where the matrix ground or powdered is placed, in an extractor chamber, where heating and pressure are controlled by a control system. Pressurized water, increased to above atmospheric pressure, typically ranging from 100 to 300 bar, is pumped into the matrix. This high pressure increases the boiling point of water, allowing it to reach temperatures above its normal boiling point of 100 °C without evaporating. The temperature is increased above 100 °C to create superheated water and is controlled to optimize the extraction process. In this way, the hot pressurized water into contact with the matrix, it penetrates the cell walls and solubilizes the target bioactive compounds. The high pressure and temperature enhance the solubility and diffusion of the compounds into the water, promoting efficient extraction. The extraction process typically requires a certain retention time to allow sufficient contact between the water and the source material for optimal extraction. The duration of the extraction process can depend on factors such as the type of matrix and the desired compounds. After the extraction process is complete, the extract-containing water, is collected from the extraction vessel and may undergo further processing, such as filtration or concentration, to obtain the desired concentration of bioactive compounds. The used water, now containing extracted compounds, can be recycled or treated for reuse in subsequent extraction cycles, making PHWE a more sustainable extraction method compared to traditional solvent-based methods (Figure 6).

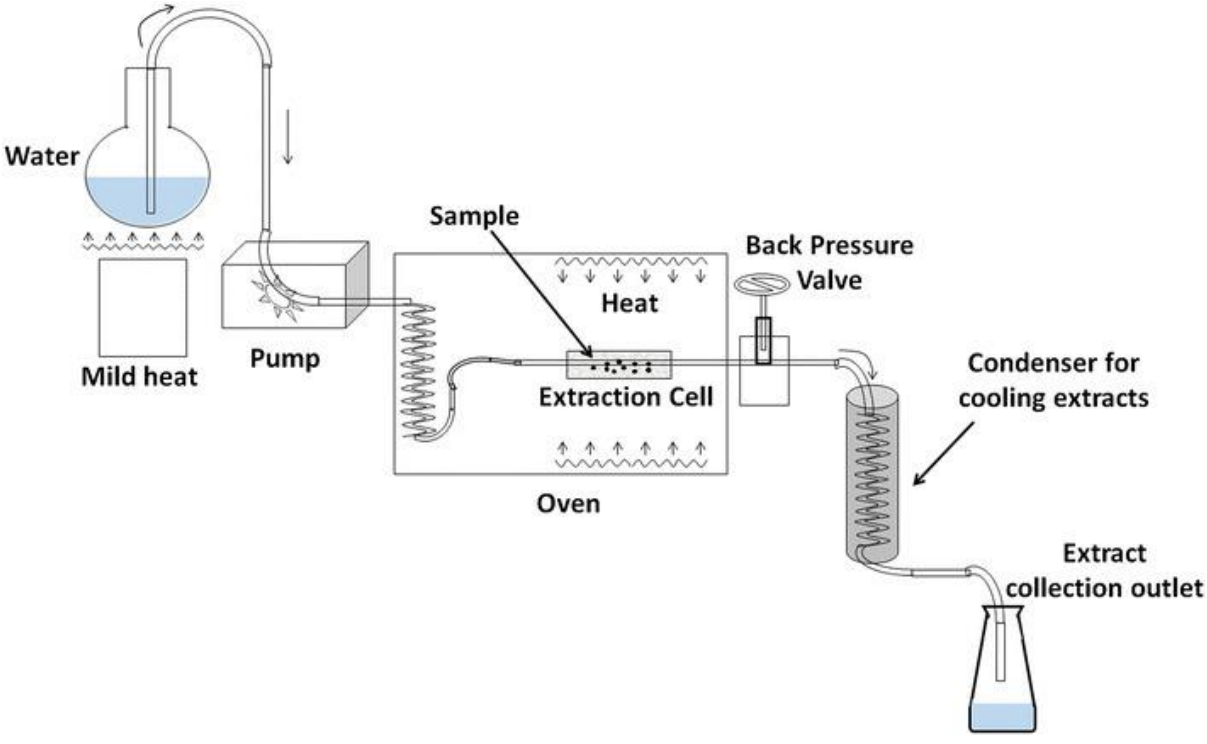

Figure 6. Schematic representation of a pressurized hot-water extraction system *(64)*.

PHWE is an efficient, environmentally friendly, and cost-effective method for extracting bioactive compounds from natural sources. It is a versatile extraction technique that permit to adjust parameters such as temperature, pressure and extraction time, for the extraction of different actives. PHWE can also be coupled with analytical techniques such as chromatography or mass spectrometry for the analysis and quantification of extracted compounds. These properties make PHWE a popular extraction method in pharmaceuticals, cosmetics, food, and nutraceuticals industries (55).

5.1.6 Pulsed Electric Field (PEF)

Pulsed Electric Field (PEF) extraction is an innovative green extraction method based on electrical pulses to extract bioactive compounds from natural sources. PEF extraction involves the application of short, high-voltage electrical pulses to the raw material, like plant materials, in a wet form. These electrical pulses create temporary pores or openings in the cell membranes of the plant cells, allowing for the release of intracellular compounds into the solvent. The creation of this pores on the cell membrane, can be reversible or irreversible and depends on the treatment intensity, size and morphological characteristics of the biological cells. This method improves the efficiency of the extraction of compounds from plant tissues of fruit and vegetable origin, as compared to conventional extraction method, facilitating the penetration of the solvent into the cells and the consequent release into the medium (65) (66). The efficacy of PEF depends on several parameters like electric field (usually repetitive and very short) strength, total energy input (relatively low) and the temperature of the treatment (Figure 7) (67).

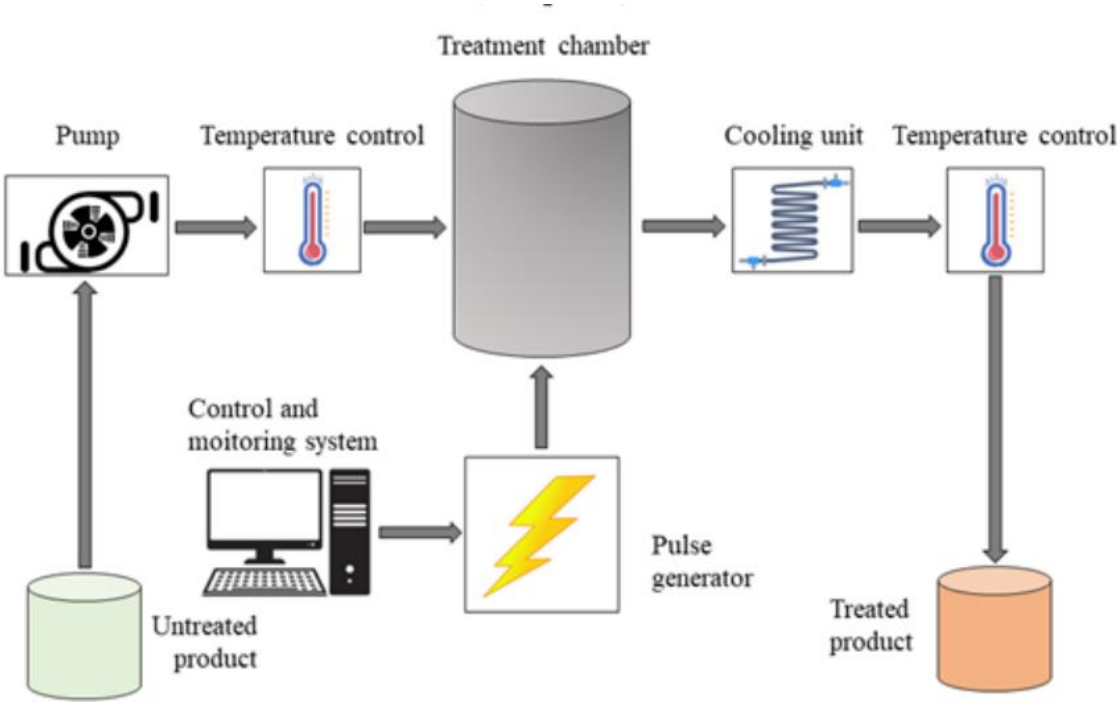

Figure 7. Schematic representation of a Pulsed Electric Fields system *(49)*.

PEF extraction has some key advantages. One of them is that it is a non-thermal process. Unlike traditional extraction methods that use heat or solvents to disrupt cell membranes, PEF extraction operates at room or slightly elevated temperatures, minimizing the risk of thermal degradation of sensitive compounds, like polyphenols, maintaining their properties. It is in fact gentle on the cellular structure of the plant material, preserving bioactive compounds. Furthermore, thanks to the electrical pulses that increase the permeability of the cell membranes, this extraction method often requires

less solvent to obtain an efficient extraction, compared to conventional extraction methods. This solvent reduction leads to a lower environmental impact. In terms of yield of extraction, the electrical pulses disrupt cell walls and membrane enhancing the release of intracellular compounds obtaining an high extraction yield in a shorter extraction time, compared to traditional methods. There properties improve the efficiency and the productivity, with a low energy consumption, making PEF easy to scale up (49) (58).

PEF extraction can be applied to a wide range of natural sources, including fruits, vegetables, herbs, and botanicals. It can extract various bioactive compounds, including polyphenols, antioxidants, vitamins, and essential oils, but also sterols, polysaccharides and carotenoids, making it suitable for diverse applications in industries such as food, pharmaceuticals, nutraceuticals, and cosmetics (58).

FEF extraction offers a sustainable and environmentally friendly alternative to traditional extraction methods, with advantages such as reduced solvent use, enhanced extraction efficiency and preservation of bioactivity, for obtaining high quality extract.

5.1.7 High Voltage Electrical Discharge (HVED)

High voltage electrical discharge (HVED), also known as pulsed high voltage electric field (PHVEF) extraction, is an emerging green extraction method that utilizes electrical discharges for the extraction of bioactive compounds from natural sources. HVED involves the application of high-voltage electrical discharges to the raw wet material, typically plant matrix or aqueous solutions containing target compounds. The high current/high voltage electrical discharges, usually of short duration, generate a series of plasma channels within the extraction medium, leading to the formation of shockwaves, cavitation bubbles, and microjets. The intense physical forces generated by the electrical discharges cause disruption of cell structures, including cell walls and membranes, within the plant material. This disruption facilitates the release of intracellular compounds, such as bioactive molecules, enzymes, and nutrients, into the surrounding extraction medium (Figure 8) (49) (68).

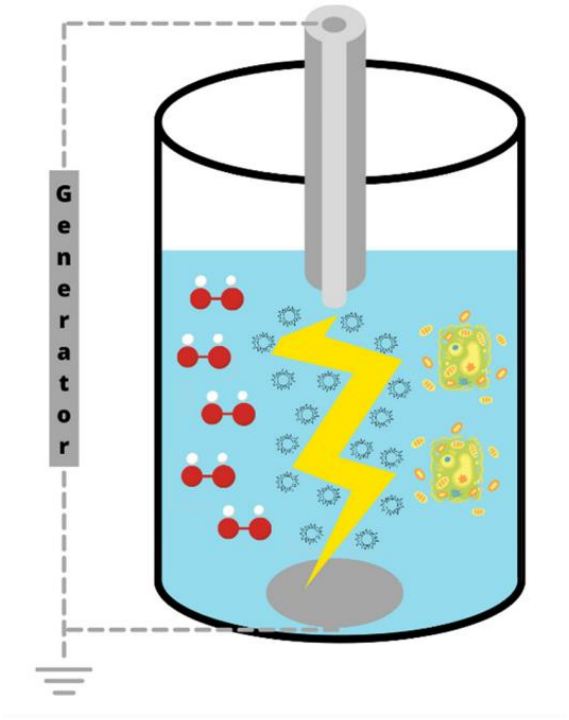

Figure 8. Representation of the mechanism of High Voltage Electrical Discharge (HVED) system, leading to cell disintegration with processes such as cavitation bubbles and shockwaves *(49)*.

HVED is a non-thermal extraction method, meaning it operates at room or slightly elevated temperatures without the need for heating. Unlike traditional extraction methods that use heat or solvents, HVED minimizes the risk of thermal degradation or denaturation of thermolabile compounds, preserving their bioactivity value. It also has high extraction yields and low energy consumption, if compared to conventional extraction methods: the electrical discharges in fact, lead to a higher extraction efficiency in a shorter extraction times, with a low solvent consumption.

A problem of this extraction technique is that, the non-selective extraction and the release of all cellular material may increase the operational problems and costs of the purification (49).

HVED can be applied to a wide range of natural sources, including fruits, vegetables, herbs, and botanicals, as well as aqueous solutions containing bioactive compounds. It can extract various types of molecules, including phenolics, flavonoids, alkaloids, essential oils, and proteins, making it suitable for diverse applications in industries such as food, pharmaceuticals, nutraceuticals, and cosmetics.

HVED, like green and sustainable extraction method, offers several environmental benefits, including reduced solvent and energy usage, minimal waste generation, and lower environmental impact compared to traditional extraction methods. By eliminating the need for organic solvents and operating at ambient temperatures, HVED contributes to sustainability and also to the obtaining of high quality extracts.

5.1.8. High-Pressure Homogenization (HPH)

High-pressure homogenization (HPH) is a green extraction method gaining attention for its ability to efficiently extract bioactive compounds from natural sources while minimizing the use of solvents and energy. HPH subject the sample to high pressures, typically ranging from 100 to 2000 bar, followed by rapid decompression. This process creates intense shear forces and turbulence within the sample,

leading to the disruption of cell walls and the release of intracellular compounds into the surrounding solvent. The mechanical forces generated during homogenization, lead to mechanical stress of the fluid and a temperature increase of 2-3 °C for every 10MPa of homogenization pressure (69). These forces cause the full cell disruption, associated to the release of intracellular material. This process facilitates the extraction of bioactive compounds using only water or other environmentally friendly solvents, reducing solvent consumption and minimizing environmental impact (70) (71) (49). If the pressure is between 0 and 50 Mpa is a standard homogenization while with a high-pressure homogenization (HPH) the pressure is between 50 and 300 Mpa. The homogenization at very high pressure (UHPH) acts with a pressure equal or greater than 400 Mpa. The application of HPH leads to the disruption of plant tissues, cell walls, membranes and organelles, improving the transfer of the solvent into the matrix, recovering high added value compounds. The intensity of the pressure and the characteristics of the matrix, affect the degree of cell disruption (Figure 9) (72) (73).

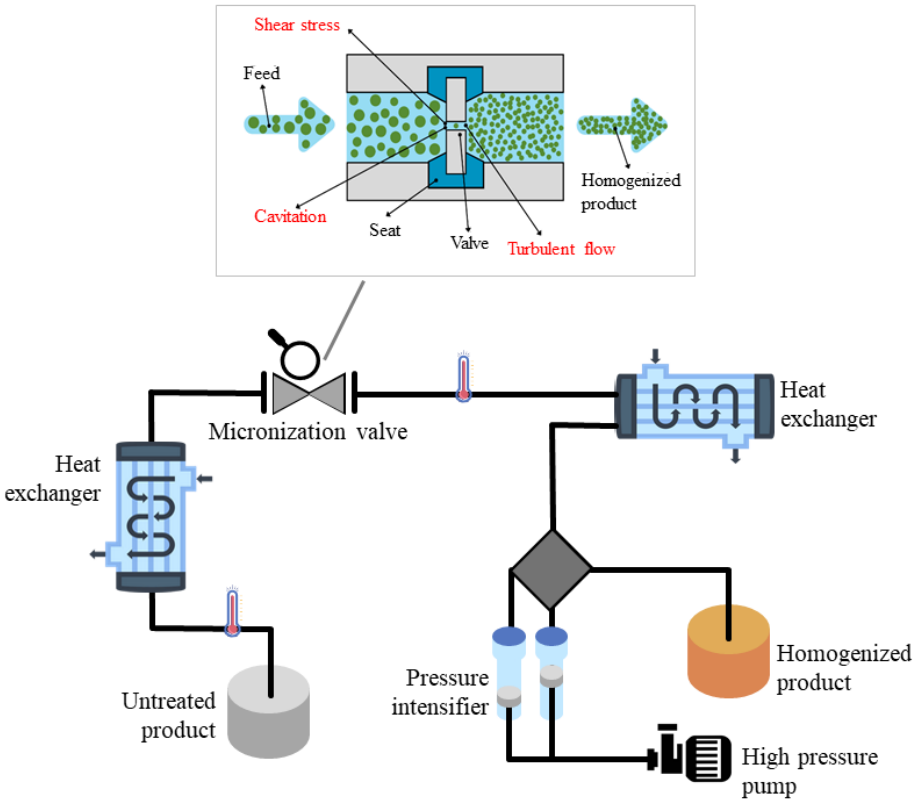

Figure 9. Schematic representation of the principle of a typical HPH system with the homogenization valve *(49)*.

The advantages of HPH are that it requires minimal or no solvent, and it is an energy-efficient extraction method, if compared to conventional techniques such as maceration or Soxhlet extraction. In fact, the high-pressure homogenizer requires relatively low energy inputs to achieve the disruption of cells and the extraction of target compounds, resulting in lower energy consumption and reduced greenhouse gas emissions, but with an high extraction yield.

HPH is also a technique that preserves the bioactivity of extracted compounds by minimizing exposure to heat and oxidative degradation. The short processing time and absence of high temperatures prevent the degradation of thermally sensitive compounds, that maintain their properties. That's why HPH can be used to extract a wide range of bioactive compounds, including antioxidants, vitamins, phenolics, flavonoids, and essential oils, from various natural sources such as fruits, vegetables, herbs,

and microorganisms. The versatility of HPH makes it a good extraction process for industries, including food, pharmaceuticals, nutraceuticals, and cosmetics, also because it is a scalable extraction technique suitable for both laboratory and industrial-scale applications. The modular design of high-pressure homogenizers, in fact, allows for easy and efficient scale-up of the extraction process to meet production demands, but maintaining the quality of the products.

In summary, HPH offers a green extraction method with minimal environmental impact that combines efficiency, sustainability, and preservation of bioactivity, reducing solvent consumption, energy usage, and processing time, and without the generation of CO_2 emissions and pollutants.

5.1.9 Soxhlet-extraction, a traditional extraction technique that can be made more sustainable

Soxhlet extraction is a classical extraction technique commonly used in laboratories to extract target compounds from solid samples. It takes its name from the inventor Franz von Soxhlet and it consist in a continuous extraction process using an extractor, consisting of a glass thimble, extraction flask, condenser, and reflux system. In the process, the solid sample, often ground or powdered, is placed inside a cellulose or glass thimble and loaded into the Soxhlet extractor. A solvent, which is chosen depending on the matrix and on the compounds that must be extracted, typically an organic solvent like hexane, ethanol, or methanol, is added to the extraction flask. The solvent is heated to reflux, causing it to evaporate and rise into the Soxhlet extractor. When the hot solvent vapor condenses in the condenser, it drips down onto the solid sample in the thimble, extracting the target compounds. Once the solvent level in the extractor reaches a certain height, it falls back into the flask, leaving the extracted compounds behind. The process continues cyclically, with fresh solvent continuously circulated through the solid sample, maximizing extraction efficiency (Figure 10). Soxhlet extraction is particularly suitable for the extraction of non-volatile or semi-volatile compounds from solid samples and it offers several advantages, including simplicity, repeatability, and the ability to extract large quantities of sample and different compounds. However, Soxhlet extraction requires time and a significant amount of solvent, leading to environmental concerns. Additionally, the high temperatures used in the process may cause degradation of thermally sensitive compounds.

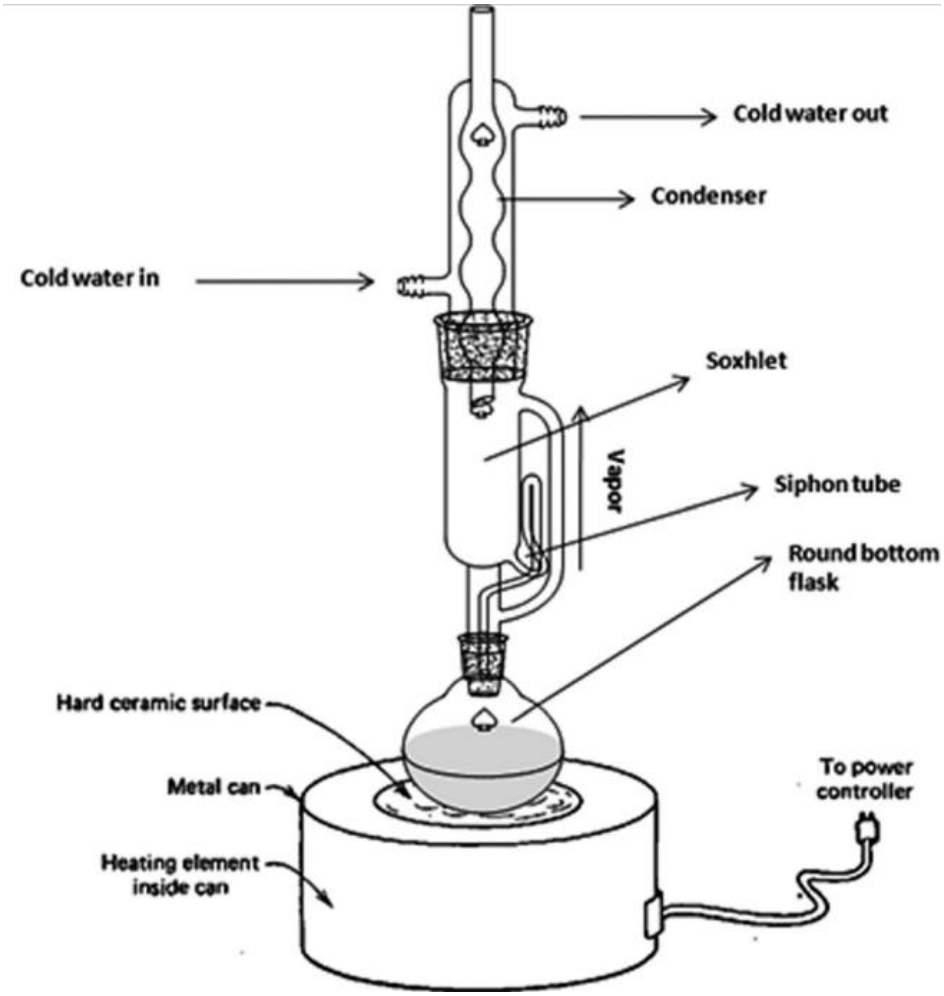

Figure 10. Schematic representation of a Soxhlet apparatus *(74)*.

Soxhlet extraction, it is not traditionally considered a green extraction method for the high solvent consumption and long extraction times, but it can be adapted to be more environmentally friendly through certain modifications and considerations. First, solvent can be recovered: Soxhlet extraction allows for the continuous recycling of solvent, which can reduce solvent consumption. Using solvent recovery systems, such as rotary evaporators or distillation setups, the solvent used in Soxhlet extraction can be recovered and reused, minimizing waste and environmental impact. Furthermore, the choice of solvent in Soxhlet extraction can influence its environmental impact: using green solvents such as ethanol or water, Soxhlet extraction can become more environmentally friendly and it can reduce health and safety risks for laboratory personnel. Other green practices that can be integrated with Soxhlet extraction are the use of energy-efficient heating methods, the optimization of extraction parameters to minimize extraction times, and the implementation of sustainable laboratory practices. By incorporating these green principles, Soxhlet extraction can be conducted in a more environmentally responsible manner.

Therefore, also if Soxhlet extraction is not considered a green extraction method, it can be modified and optimized to reduce its environmental footprint and can contribute to sustainability.

5.2 Green extraction solvents appropriate for cosmetic applications

Choosing the right solvent is a fundamental step in the extraction processes of bioactive substances from natural sources. This is because the choice of solvent influences the extract yield and quality but

is also fundamental for determining the cost of the extraction and its environmental sustainability. The use of green extraction methods requires the use of green and sustainable solvents, avoiding the petrochemically derived solvents.

Petrochemical solvents are solvents derived from petroleum or natural gas. These solvents are commonly used in various industrial processes, including manufacturing, cleaning, and extraction. Some examples of petrochemical solvents include hydrocarbons, aliphatic and aromatic hydrocarbons, and chlorinated solvents. Petrochemical solvents are widely used due to their effectiveness, versatility, and low cost. However, they are also associated with environmental and health risks, including air and water pollution, toxicity, and flammability. As a result, there is increasing interest in developing alternative solvents derived from renewable resources or utilizing greener extraction techniques to reduce reliance on petrochemical solvents and minimize their environmental impact.

Green extraction methods require the use of environmentally friendly solvents, or in any case the possibility of being able to recover the solvent and be able to reuse it. Green solvents are preferred for the low toxicity, the environmental sustainability (they can be biodegradable or recyclable) and because they are sourced from renewable materials or have low synthesis cost. Some examples of green solvents, compatible with cosmetic use, are supercritical carbon dioxide (CO_2), ethanol, water, deep eutectic solvents (DES), terpenes such as limonene and glycerol.

5.2.1 Bio-Based Solvents

5.2.1.1 Water

Water is often regarded as the greener solvent due to its abundance, low cost, and environmental impact. Its use as a solvent in various industrial processes, including cosmetics production, offering several advantages from a sustainability perspective. Water is one of the most abundant substances on Earth, making it readily available, inexpensive and high accessible. Unlike many organic solvents, which may rely on fossil fuel extraction or synthesis, water can be sourced sustainably from natural reservoirs, rivers, and groundwater: its ubiquity ensures a stable supply chain and reduces the environmental impact associated with sourcing and transportation. Water is non-toxic, non-flammable, and generally regarded as safe (GRAS) for human health and the environment. Its use as a solvent eliminates concerns about chemical residues or harmful by-products that may be associated with other solvents. Additionally, water-based formulations are often less irritating to the skin and respiratory system, making them suitable for sensitive individuals, and in general water-based extraction methods safer for both operators and consumers. How to concern the environment, water is readily biodegradable, meaning it can be broken down into harmless substances by natural processes, such as microbial degradation. This reduces the risk of water pollution and environmental contamination associated with solvent use, particularly in the case of accidental spills or releases. Water does not contribute to air pollution or greenhouse gas emissions (49) (75).

How to concern water-based extraction methods they require lower energy inputs compared to solvent-based techniques, further reducing their environmental footprint. Water in fact, has a high specific heat capacity and can transfer heat efficiently. This can result in reduced energy consumption and lower greenhouse gas emissions, contributing to overall process sustainability.

Furthermore, this solvent is selective for the extraction of specific compounds based on their polarity. By adjusting extraction conditions such as temperature, pH, and pressure, researchers can improve water-based extraction processes to target desired compounds. Water is compatible with a wide range of natural products, including botanicals and herbs. Its versatility allows for the extraction of various

bioactive compounds, such as polyphenols and flavonoids, without compromising their chemical integrity or biological activity.

In cosmetic field, water is not only a versatile solvent, but it can also be used within a wide range of cosmetic formulations, including emulsions, suspensions, gels, and solutions. Its polarity allows for the dissolution of polar and ionic compounds, while its ability to form hydrogen bonds enhance the dispersion of particles and the stabilization of emulsions.

The use of water as green solvent in cosmetic formulations offers a sustainable and environmentally friendly alternative to conventional solvents: thanks to the properties of water, cosmetic companies can create products that are effective, safe, and environmentally responsible, meeting the growing demand for sustainable beauty solutions.

Water like solvent can be used in some emerging extraction techniques to improve the extraction yield, like PEF, HVED, UAE and HPH. These techniques can be combined with water according to the cell rupture mechanisms, the plant matrix and tissue physical and chemical propertied of the target compounds like solubility and polarity (49). Furthermore, water-based extraction methods align with regulatory requirements for green and sustainable practices. As consumer demand for environmentally friendly products grows, the use of water as a solvent for extraction can enhance product transparency and compliance with eco-labelling standards.

Also the water present in plant tissues can be seen like a solvent. For example, growing interest has recently focused on microwave irradiation based on the hydro diffusion phenomenon that exploits in situ the water present in the plant tissue: microwave irradiation induced the heating of the internal water within the plant tissue of by-products, allowing the destruction of the cell tissue and the release of the intracellular bioactive compounds (76). An example of integrated strategy is the extraction of essential oil and polyphenols from orange peel using microwave and ultrasound technology together, using the water inside the matrix like solvent. Microwaves cause the evaporation of interstitial water and induce the release of the essential oil. Essential oil–vapor mixture is then condensed, the essential oil collected, while water can be recycled and used for the polyphenol's extraction from the MAE residues, performed by UAE. This extraction process enabled the obtainment of high-added value compounds in a shorter time than conventional extraction, through a closed cycle using only water provided by the plant (77).

In summary, the use of water as a solvent for green extraction offers numerous benefits, including safety, sustainability, and regulatory compliance. By harnessing the inherent properties of water, researchers and industries can develop environmentally friendly extraction processes that promote both human and environmental health.

5.2.1.2 Ethanol

Ethanol is a green solvent especially when sourced from renewable biomass: bioethanol, produced through the anaerobic fermentation of sugar, is the most used green solvent (78). Ethanol is frequently recognized as a green solvent due to several environmentally friendly attributes. Derived primarily from renewable biomass sources such as corn, sugarcane, or cellulosic materials, ethanol offers a sustainable alternative to fossil fuel-derived solvents. Additionally, ethanol is non-toxic, non-carcinogenic and non-irritating and exhibits low volatility, reducing emissions and environmental impact during production and use. Is generally regarded as safe (GRAS) for human health and is widely used in food, pharmaceutical, and cosmetic applications, and is considered safe for both operators and consumers. How to concern the attention for the environment, ethanol is also biodegradable and breaks down into harmless by-products through natural processes, reducing environmental impact

and minimizing pollution compared to synthetic solvents. Ethanol can be easily recovered and recycled using distillation or other separation techniques. This allows for solvent reuse, minimizing waste generation and reducing environmental footprint. Ethanol exhibits good solubility for a wide range of compounds, including polar and non-polar substances. It can effectively extract various bioactive compounds such as phenolics, flavonoids, terpenes, and cannabinoids from natural sources like plants, herbs, and botanicals.

Ethanol-based extraction processes typically operate under mild conditions of temperature and pressure, preserving the integrity of bioactive compounds, preserving their properties. In order to obtain an high percentage of polyphenol during the extraction, ethanol is usually combined with water and the final composition of the mixture is defined by the nature of the compound that has to be extracted. In general, the mixture of ethanol and water shows an high efficiency in the extraction of antioxidant compounds than pure water or pure ethanol, because the change in the polarity, reaches the polarity of different compounds (49) (79) (80).

In general, ethanol is a versatile, safe, and sustainable solvent for green extraction processes, and it is widely accepted for use in green extraction processes and complies with regulatory requirements for sustainability and environmental stewardship. The use of ethanol is in line with green chemistry principles and eco-labelling standards, meeting the consumer demand: its use contributes to the development of environmentally friendly products while promoting resource efficiency and minimizing environmental impact. Despite these advantages, it's important to consider potential drawbacks such as high energy consumption during purification. Nonetheless, with proper management and optimization, ethanol remains a valuable green solvent option for numerous applications.

5.2.1.3 Glycerol

Glycerol, also known as glycerine, is recognized as a green solvent due to its versatile properties and environmentally friendly characteristics. It is a natural byproduct of biodiesel production and is also derived from renewable sources such as vegetable oils and animal fats, through the hydrolysis of triglycerides, then purified to be used like solvent (75). Glycerol is biodegradable, non-toxic, non-flammable, and exhibits low volatility, making it a safer and more environmentally sustainable alternative to many traditional organic solvents. It has a high boiling point and excellent solvent power for polar and non-polar compounds, making it suitable for a wide range of applications, including extraction, purification, and synthesis processes. Glycerol is a versatile solvent that can dissolve a wide range of compounds, especially polyphenols, including polar and non-polar substances. Its ability to solubilize various types of molecules makes glycerol suitable for extracting bioactive compounds from natural sources such as plants, herbs, and botanicals. Several studies show glycerol like bio-solvent for the extraction of polyphenols, increasing the extraction yield, especially if in combination with emerging extraction technologies like for example UAE, exploiting the disruption of vegetable cell tissue made by ultrasounds (81) (82) (83). Glycerol-based extraction processes operate under mild conditions, minimizing the degradation of heat-sensitive compounds and preserving the bioactivity of extracted molecules. This preservation of bioactivity ensures that the extracted products maintain their nutritional value, flavor, aroma, and functional properties, making them suitable for use in food, pharmaceutical, nutraceutical, and cosmetic applications, without any colour or odour and with a sweet taste. Is generally recognized as safe (GRAS) by regulatory authorities such as the Food and Drug Administration (FDA) in the United States and the European Food Safety Authority (EFSA) in the European Union. Its regulatory acceptance and low toxicity make glycerol a preferred solvent for extraction processes in industries requiring compliance with strict safety and quality standards. Additionally, glycerol is widely available and relatively inexpensive (cheaper than ethanol), and

chemically stable during storage, further enhancing its attractiveness as a green solvent. Furthermore, glycerol can be easily recycled and reused, contributing to overall process sustainability.

Despite its many advantages, challenges such as its high viscosity and limited solubility for certain compounds may need to be addressed through process optimization and formulation adjustments (49). The advantage is that it can be used together with solvents or co-solvents to make its viscosity lower, like DESs, water and ethanol, also to enhance extraction efficiency and selectivity (84). Overall, glycerol represents a promising green solvent option that aligns with the principles of green chemistry and sustainable development.

Overall, glycerol offers a sustainable and environmentally friendly solution for extraction processes, aligning with the principles of green chemistry and sustainability. It is an attractive green solvent option for industries seeking to reduce their environmental footprint and promote sustainable practices.

5.2.1.4 Terpenes and D-Limonene

Terpenes are naturally occurring organic compounds found in a wide variety of plants, including fruits, vegetables, herbs, and flowers. In fact, they are bio-based solvents, extracted from fats and oils, particularly interesting to substitute low polarity organic solvents, such as hexane thanks to their physio-chemical properties (85). They are known for their distinctive aromas and taste and have gained attention in recent years as potential green solvents due to their eco-friendly properties. They are easy to recovery and reuse, biodegradable and non-flammable: terpenes are derived from renewable plant sources and are biodegradable, meaning they can break down into harmless substances through natural processes. Unlike petroleum-based solvents, terpenes are not derived from fossil fuels and do not contribute to carbon emissions or environmental pollution.

Terpenes are generally considered safe for human health and the environment. They are non-toxic and do not expose to the same health risks of some synthetic solvents, such as volatile organic compounds (VOCs) or chlorinated solvents. The also have excellent solvent properties and can dissolve a wide range of compounds, including fats, oils, resins, and pigments with an high solvent power. Terpenes are versatile solvents that can be used in various applications, including cosmetics, personal care products, cleaning products, and pharmaceuticals. They can also be mixed to achieve desired solvency, volatility, and aroma characteristics. In addition to the solvent properties, terpenes may also act like antimicrobial, antioxidant, and anti-inflammatory actives, enhancing the performance and efficacy of products and minimizing the need for additives or preservatives.

Among the terpenes, D-limonene is emerging in recent years. It is a naturally constituent of some essential oils from citrus, and it is considered GRAS by Food and Drug Administration (FDA). It is used like green alternative to *n*-hexane for the extraction of bioactive natural products like for example carotenoids (49).

In general, terpenes offer a promising alternative to traditional solvents, providing a green and sustainable option for green extraction methods.

5.2.2 Supercritical fluid (SF)

Supercritical fluid are substances with pressure and temperature higher than the critical values, that combine physical characteristics intermediate between those of a liquid and those of a gas (49). This kind of solvents have high density, similar to liquids, and low viscosity, like gases. The properties can be adjusted controlling temperature and pressure, making these solvents versatile for various applications (86) (87). The characteristics of SFs like the density, the low viscosity, the diffusivity, near-

zero surface tension, pressure and temperature make them able to penetrate a microporous matrix to extract intracellular compounds (88).

The most used SFs are supercritical CO_2 and supercritical water, thanks to the particularly evident health and safety benefits. However, the use of these solvents are controversial due to the high energy consumption, the risk of hydrolysis and degradation reaction, and for the high CO_2 emissions and high costs (89).

5.2.2.1 Carbon dioxide like Supercritical Fluid

The use of carbon dioxide (CO_2) as a solvent for green extraction has gained significant attention due to its unique properties and environmental benefits. It is inexpensive, renewable, easily recyclable minimizing waste generation and it doesn't have any odour. CO_2 is non-toxic, non-flammable, inert, and generally recognized as safe (GRAS) for use in food, pharmaceutical, and cosmetic applications, without risks associated with the use of organic solvents. It does not leave behind harmful residues or contaminants in extracted products, ensuring consumer safety and regulatory compliance.

CO_2 is abundant in the atmosphere and does not contribute to greenhouse gas emissions when used as a solvent. Its use in extraction processes minimizes environmental impact compared to traditional organic solvents, which may be derived from fossil fuels and emit volatile organic compounds (VOCs).

CO_2 is a versatile solvent, with properties that can be modified by changing pressure and temperature, allowing for selective extraction of target compounds: adjusting extraction parameters, researchers can extract specific bioactive compounds. CO_2 can be used to extract a wide range of compounds, including lipids like fatty acids, essential oils, antioxidants, vitamins, cannabinoids, flavonoids, carotenoids, sterols, tocopherols and natural pigments, from various natural sources such as plants, herbs, and botanicals. It is mainly used for the extraction of poorly polar compounds thanks to the value of the dielectric constants that make it a non-polar substance, but some co-solvents can be added for the extraction of more polar compounds (49). Ethanol it seems to be the best co-solvent for example for food applications, but also a mixture of water/ethanol can be used (90) (91).

With CO_2 it's possible to operate under relatively mild conditions of temperature and pressure, minimizing the degradation of sensitive compounds and preserving the chemical integrity and bioactivity of extracted compounds: it is in fact widely used for the extraction of thermosensitive compounds.

The limits of this solvent are the high processing costs and the complexity of the industrial equipment, in comparison with conventional processes. When combined with other extraction methods like for example ultrasound, this problem can be limited and the extraction yield and the antioxidant activity of the extract can be enhanced. This is a perfect example of the synergistic effect of methods and solvents (49) (92).

The use of CO_2 as a solvent for green extraction offers numerous advantages, including safety, sustainability, selectivity, and versatility. Exploiting the properties of CO_2, researchers and industries can develop environmentally friendly extraction processes that promote both human health and environmental stewardship.

5.2.2.1 Water like Supercritical Fluid

Water is a solvent that can be used at supercritical or subcritical conditions. As a supercritical fluid (SCW) has attracted interest in various industries due to its unique properties and potential applications: it's considered the cleanest solvent, and it can be used in many reactions. The critical

point of water occurs at a temperature above 374 °C and a pressure above 22.1 MPa. At these conditions, water transitions into a supercritical state where it exhibits properties of both a liquid and a gas. It can be considered a non-polar solvent thanks to the low dielectric constant and few hydrogen bonds, with less persistence than liquid water (93). Supercritical water (SCW) has several properties that make it a versatile solvent: it has high density comparable like liquids, allowing it to dissolve a wide range of polar and non-polar compounds, and low viscosity similar to that of gases, enabling it to penetrate materials efficiently.

The solvation power of SCW can be adjusted by controlling temperature and pressure, making it suitable for various applications. SCW, in fact, is used for the extraction of bioactive compounds from natural sources such as plants, algae, and food products. It offers advantages such as high selectivity, rapid extraction kinetics, and minimal solvent residue. Subcritical water shows high extraction yields and antioxidant activities if compared with other solvents (49). In particular, if combined with 30% of NADESs lead to a significant increment in the extraction of phenolic compounds for example for winery by-products if compared with pure subcritical water (94).

Despite its potential, the use of SCW can include equipment corrosion, high operating costs, and limited scalability for industrial applications. These problems are challenges that require advancements in materials science, process engineering, and reactor design (49).

Also if the use of water as a supercritical fluid solvent promises a wide range of applications in industries such as pharmaceuticals, food and cosmetic, continue research and development efforts are essential to unlock the potential of SCW and realize its benefits for sustainable and efficient processes.

5.2.3 Neoteric Solvents

DESs and NADESs

Deep eutectic solvents (DESs) are gaining attention as green solvents due to their unique properties and environmentally friendly characteristics. DES are typically formed by mixing a hydrogen bond acceptor, such as a quaternary ammonium salt, with a hydrogen bond donor, such as a carboxylic acid, amide, or alcohol, mixed at a suitable temperature at specific ratios to create a eutectic mixture with a lower melting point than the individual components (49) (95) (96). One of the key advantages of DESs is their ability to be easily synthesized from inexpensive, renewable, and biodegradable starting materials, making them sustainable alternatives to conventional organic solvents derived from fossil fuels. DESs exhibit low toxicity, high thermal stability, and are often non-flammable, reducing health and safety risks in industrial applications. Moreover, DESs can be tailored to meet specific extraction or separation requirements by adjusting the composition and properties of the solvent system. These characteristics make DESs promising candidates for a wide range of applications, contributing to the green extraction methods. They have a great solubilization capacity of different compounds with low water solubility, and an adjustable viscosity, sometimes very high.

DESs are often recyclable and reusable: after use, DES can be regenerated by simple methods such as evaporation or extraction, allowing for solvent recovery and minimization of waste generation. This property reduces the environmental footprint of DES-based processes. They are gaining acceptance by regulatory authorities as green alternatives to traditional solvents. Their low environmental impact align with the principles of sustainability, making them a good option for industries to reduce the hazardous chemicals.

DESs have an high potential as solvents when combined with innovative extraction techniques like HVED, MAE and UAE. The combination of these technologies with these solvents, improves the

extraction yield which is attributed to the heat generated by ultrasound and microwaves, that reduce the viscosity of DESs, enhancing the penetration into the matrix and the dissolution also of phenolic compounds (49) (97) (98).

NADES (Natural Deep Eutectic Solvents) are a particular class of DES, composed of plant metabolites. If DES are usually synthesized from organic salts and hydrogen bond donors, NADES are made by naturally substances like sugars, amino acids, organic acids and polyols (75). DESs and NADESs show the same characteristics but NADESs are more environmentally friendly thanks to their natural origin.

DESs offer a promising green solvent alternative with numerous advantages in terms of sustainability, efficiency, and versatility. As research and development in this field continue, DESs and NADEs are expected to play an increasingly important role in promoting environmentally friendly practices in various industrial sectors.

6. Overexploitation of the plants and the use of the wastes

The increasing demand for natural ingredients and their extracts is leading to over-exploitation of plant sources: some plants have become extinct, and the large-scale uncontrolled harvesting are making some species at risk of extinction, like “Devil’s claw” used by pharmaceutical laboratories to treat rheumatism and sandalwood and rosewood. It’s necessary to preserve biodiversity for future generations. To solve this problem of overexploitation, some precautions have been taken such as the use of cultivated plant instead of plant take from their natural habits. Another alternative to preserve plants is the natural selection of varieties with much higher concentrations of active ingredients: an example is the extraction of artemisinin from *Artemisia annual L.* normally present in the plant in very low concentrations, until the production, with a lot of experimental work, of varieties of this plant with higher concentrations of the active principle. A new technology is the “plant-milking”, developed for the extraction of substances without destroying the plant: plants are grown in a greenhouse, in a liquid medium, and the secretion and exuding of substances from the roots are collected in the liquid medium with physical, chemical and biological stimulation (52).

The use of industrial waste materials in cosmetic production represents a promising avenue for sustainability and resource efficiency in the cosmetics industry. With growing concerns about environmental situation and resource depletion, many cosmetic companies are exploring innovative ways to exploit waste materials from other industries to create value-added products.

One common approach is the utilization of by-products or waste materials from the food and agriculture industries. For example, fruit and vegetable peels, seeds, and pulp that are typically discarded as waste can be transformed into natural cosmetic ingredients such as extracts, oils, and exfoliants. These ingredients often contain beneficial compounds such as antioxidants, vitamins, and essential fatty acids, which can offer skincare benefits such as hydration, rejuvenation, and protection against environmental damage. A well-known and studied example is the use of by-products from wine production, discarded by winery from the wine industry: stems, peels and seeds, known as pomace, are a rich source of polyphenols and precious antioxidant compounds, that can be reused by cosmetic industries. Similarly, waste materials from the textile industries such as sericin from silk production, and forestry industries, such as cellulose fibres, can be repurposed for use in cosmetics. These materials can serve as natural exfoliants, thickeners, or emollients in cosmetic formulations, providing texture, viscosity, and skin conditioning properties.

Also for the packaging, another emerging trend is the use of upcycled or recycled ingredients: industries transform post-consumer waste materials, such as plastics, glass, or metals, into cosmetic packaging or containers. By incorporating recycled materials into packaging designs, cosmetic companies can reduce the use of virgin resources, minimize waste generation, and contribute to a circular economy.

The use of industrial waste materials in cosmetic production offers several potential benefits, including resource conservation, waste reduction, and the development of innovative, sustainable product formulations. By leveraging waste streams from other industries, cosmetic companies can create products that are not only effective and safe but also environmentally friendly and socially responsible, meeting the growing demand for sustainable beauty solutions.

7. Natural do not mean safe and ethical

While natural products are often perceived as safer alternatives, it is important to understand that the term "natural" does not mean “safe” or “ethical”. Many natural substances can be toxic or allergenic, and their effects can vary depending on factors such as concentration, purity, and individual sensitivity. In fact, plants contain a lot of compounds that can be harmful if applied to the skin in large quantities or pure, like for example essential oils. Additionally, natural products can interact with medications or exacerbate certain health conditions. Therefore, it is essential for consumers to educate themselves, read labels carefully, and consult healthcare professionals before using natural products, just as they would with synthetic ones, and don't just believe in trends or claims.

Is also important to underline that several cosmetic ingredients may pose sustainability challenges due to various factors such as environmental impact, resource depletion, social implications, and ethical concerns. An example is the use of palm oil and its derivatives if they are not certified organic: they are widely used in cosmetics for their emollient and surfactant properties, but palm oil cultivation is unsustainable contributing to deforestation, habitat loss, biodiversity depletion, and social conflicts, particularly in tropical regions. The expansion of palm oil plantations is also a problem for some species in danger of extinction such as orangutans, tigers, and elephants.

From the ethical point of view, it is important to underline that some cosmetic ingredients are also derived from animals or animal by-products, such as lanolin, beeswax, collagen and carmine. The use of animal-derived ingredients raises ethical concerns about animal welfare, exploitation, and cruelty. Not all products formulated with ingredients of natural origin can be defined as vegan and cruelty free.

8. Why some consumers do not switch to natural?

If some consumers are fooled by the "natural" product, for others it is difficult to rely on green products, even if certified. *Sadiq M. et al.* studied why consumers do not purchase eco-friendly products. They divided the reasons in functional barriers, like usage, value and risk of innovations, and psychological barriers like tradition and image. In fact, a lot of consumers are resistant to use new, innovative but unfamiliar products, and they also do not trust to certification and manufacturing process, because they are not confident of certification agencies, and they have a low eco-label knowledge. Many of them also perceive a risk linked to the use of green cosmetics.

How to concern the psychological barriers, they are related to tradition and image. Consumers think that innovations would change their habits and lifestyle and they think that organic products have a

shorter shelf life and will give low satisfaction to them, compared with conventional products. They also don't trust in the efficacy of green products, and the image barrier is given by scepticism and some doubt about the originality of claims.

The value barrier is one of the strongest to eradicate, for the perceived performance and monetary value of a new product: consumers do not believe that the higher costs of the finished eco-friendly product are linked to greater quality and effectiveness, and that they are not right for the value of the products.

Certainly, what drives consumers to choose eco-friendly cosmetic products is attention to the environment and health, which reduce the negative influence of barriers like tradition, image, risk and all the other barriers. Consumers with a higher level of sensitive about environmental well-being are more oriented to choose green products instead of conventional products. Also people that have an important health concern choose organic products, because they feel that organic products are healthier than conventional products (99).

8.1 The higher costs of eco-friendly products in comparison with conventional

Usually, green cosmetic products have a higher cost that can be attributed to several factors due to the high cost of their sources, production and formulation. In fact, the production of natural and organic ingredients using sustainable and environmentally friendly practices, such as cultivation, harvesting, and extraction, usually have high labour costs, strict quality control, and certification requirements.

How to concern industries, they have to invest in eco-friendly equipment and processes to minimize environmental impact and promote sustainability. They have to invest for the implement of energy-efficient technologies, waste reduction measures, and water conservation practices with high costs.

In addition, to develop green cosmetic formulations is necessary to invest also in extensive research and development (R&D) activities, testing, and innovation to ensure product efficacy, safety, and sustainability. In fact, green cosmetics require safe, novel, natural and eco-friendly ingredients and botanical extracts.

Costs are also increased by certification standards like COSMOS Organic or Fair Trade that involve rigorous testing, documentation, and auditing processes, and by eco-friendly packaging materials and labelling practices. Sustainable packaging, used for minimising environmental footprint and promote recycling, such as glass, aluminium, biodegradable plastics, and recycled materials, are usually more costly than conventional plastic packaging. Additionally, eco-friendly inks, adhesives, and labels can increase the production costs.

Finally, the last step of the investments of green cosmetic brands are marketing strategies for the communication of values, ethics, and sustainability. It's important, in fact, for the brand to educate consumers about green beauty, and promote transparency in ingredient sourcing and manufacturing.

9. Conclusions

The demand for cosmetics with ingredients of natural origin has grown significantly in recent years, thanks to the planet concern and the vision according to which this type of product is better for the health. These kinds of products, require to manufacturing industries to adapt to green extraction methods, both in terms of the methodologies and the solvents used. Extraction methods must save energy, not degrade bioactive compounds, reduce production times and costs, and use solvents

reusable or without any environmental impact. These products require certifications, higher costs and attention to safety. Despite the increase in the use of green cosmetic products, a high percentage of consumers still do not use them due to the lack of trust in certifications, the higher costs, and the difficulty in abandoning their habits. This is why we still need to work on convincing consumers about these products, in order to preserve the planet and animals, which are currently in danger.

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Chapter 2

Evaluation of Physicochemical and Microbial Properties of Extracts from Wine Lees Waste of Matelica's Verdicchio and Their Applications in Novel Cosmetic Products

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Abstract

Wine lees are sediments deposited on the walls and bottom of barrels resulting from wine fermentation and mainly consist of yeasts. Saccharomyces cerevisiae extracts, rich in beneficial components for the skin, have already been used in cosmesis, while wine lees have not been well exploited by the cosmetics industry yet. The aim of this work was the full characterization of the wine lees from Verdicchio's wine, with the aim to exploit it as a beneficial ingredient in new cosmetic products. After mapping the microbial composition of the sample waste, the parameters for the sonication extraction process were optimized and the physicochemical properties of the extract were analyzed. The efficiency of the aqueous extraction—and in particular the yeast cell lysis necessary for the release of proteins from the cell—was assessed by evaluating cell shape and size, and protein release, under scanning electron microscopy (SEM), dynamic light scattering (DLS) and Bradford's protein assays. Thus, the total phenol content and antioxidant capacity of the supernatant recovered from native and sonicated lees were determined by Folin–Ciocalteu's and spectrophotometric assays, respectively. To quantify the heavy metals and highlight the presence of microelements beneficial for the skin, inductively coupled plasma-mass spectrometry (ICP-MS) was applied. In vitro metabolic activity and cytotoxicity were tested on both HaCat keratinocytes and human gingival fibroblasts, showing that wine lees are safe for skin's cells. The results show that sonicated lees appear to be more interesting than native ones as a consequence of the release of the active ingredients from the cells. Due to the high antioxidant capacity, content of beneficial elements for skin and an appropriate microbiologic profile, wine lees were included in five new solid cosmetic products and tested for challenge test, compatibility with human skin, sensory analysis, trans epidermal water loss (TEWL) and sebometry.

1. Introduction

During the winemaking process, thousands of tons of winery waste is generated. Among the various sub-products of the wine industry—such as grape pomace, stalks and dewatered sludge—wine lees represent around 12% of the total waste, and it is defined as the residue formed at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following filtration or centrifugation of this product. Wine lees are among the most underexploited residues in the oenological industry [1,2,3].

There are two types of classifications for lees; on the one hand, they are classified depending on the stage of vinification: first and second-fermentation lees, which are formed during the alcoholic and malolactic fermentations, respectively, and aged wine lees formed during wine aging in wood barrels. On the other hand, they can also be classified depending on particle size: heavy lees (between 100 µm and 2 mm) and light lees (<100 µm).

Wine lees can be found as semi-solid residue constituted by liquid and solid lees. The solid fraction is a combination of yeasts, organic acids (mainly tartaric acid), insoluble carbohydrates (such as cellulosic or hemi-cellulosic materials), inorganic salts, lignin, proteins, phenolic compounds, pulp and other parts of the grape. The liquid fraction is mainly composed of ethanol and organic acids, such as lactic and acetic acids.

Quantitative and qualitative characteristics of winery wastes vary widely according to the type of wine produced, the winemaking technologies employed, the size of winemaking facilities and the season [4,5].

Yeasts are the main component of the lees, in fact they are the main responsible for the process of alcoholic fermentation in the production of wine, together with many microorganisms including other fungi and bacteria.

Yeasts have already been exploited by the cosmetics industry: in particular, some studies report that they have positive effects on the skin, demonstrated by evaluating transepidermal water loss (TEWL), skin moisture (SM) and skin microrelief (SMR) [6].

Saccharomyces cerevisiae extract in particular has a prominent role among biotechnological raw materials in cosmetic products, because it is rich in amino acids and peptides that can have moisturizing properties, as well as proteins and polysaccharides, which are beneficial for cell renewal effects. These extracts are also rich in components that provide benefits such as the prevention of photoaging and oxidative stress diseases; there are also molecules such as vitamins B6 and B12; minerals (enzyme co-factors) such as zinc, copper and manganese; phytosterols and phenolics, including catechins and trans-resveratrol with antioxidant activity, as they are produced by Saccharomyces cerevisiae in the adaptive response to oxidative stress. As such, these components can improve photoaged skin [7].

By-products derived from wine-making, like lees, pomace and stalks, are subject to a defined method of management, which includes the obligation of total or partial delivery to the distillery or disposal under control for alternative uses [8]. Usually, wineries send wine lees to distilleries, which then use the material to recover alcohol; part of this waste is used by the winery itself to separate spent yeast and reuse it in the subsequent wine production process, as these fungi act as fermentation activators. Alternatively, wine lees are sent to companies that use them for the recovery of organic acids as tartaric acid.

The increasing attention to the circular economy represents an opportunity to both reduce disposal costs and improve the environmental and economical sustainability of the wine supply chain. In this scenario, the exploitation of grape pomace products such as spirits and grape seed oil—and for extracting additives like polyphenols—is already well established [9,10,11], while wine lees, the second largest by-product of winemaking, have so far received only a modest attention regarding their possible valorization.

The treatments obtained in the lees mainly concern the recovery and transformation of compounds with high added value. Wine lees are traditionally fundamental raw materials for the production of ethanol and tartaric acid [12], and they are used for the production of methane-rich biogas through anaerobic digestion [13]. Proteins, lipids and polysaccharides are found mainly inside yeast cells and on the cell wall. At the end of the alcoholic fermentation process, yeasts die and a process of cell lysis begins. This process allows the release of cytoplasmic material (peptides, fatty acids, nucleotides, amino acids) and cell wall compounds (mannoproteins) [14,15]. Autolysis is a very long and slow process, but in industrial processes it can be induced by physical inductors (rise in temperature, alternate freezing and thawing and osmotic pressure), chemical inductors (pH, detergents and antibiotics) or biological inductors (aeration and starvation) [16].

Some studies report the extraction of bioactive molecules of cosmetic interest, such as polyphenols (flavonols and phenolic acids), squalene or for the recovery of mannoproteins and beta-glucans [5,17,18,19,20,21]. Usually, the recovery of these substances is favored by pre-treatments that allow their release.

Even though the beneficial properties of lees for the skin have already been described [22,23,24], there are few cosmetic industries that have used this agro-industrial waste in cosmetic formulations, and in many cases wine lees have been used as is, without transformation.

The aim of this study is the optimization of extraction and the full characterization of a sonicated extract obtained from Matelica's Verdicchio wine lees. A preliminary microbiological analysis is intended to exclude harmful effects of wine lees and highlight the benefit of obtaining raw material by yeast cells extract, favoring the breakdown of cells with ultrasound. This works is also intended to verify if there are differences between the native and sonicated lees, and to understand how to better exploit the beneficial properties of wine lees.

Finally, an attempt is made to formulate a set of cosmetic products containing this waste, with a low environmental impact. Final original solid cosmetic formulations are thus proposed, with the intention of verifying positive cosmetic effects on the skin, in a context where solid waste reduction and recycling are becoming increasingly important for consumers who care for the environment.

2. Materials and Methods

2.1. Wine Lees

The native wine lees (NWLs) used in this study were kindly supplied by Belisario's winery (Matelica, Italy) and are derived from the vinification process of 2020 Matelica's Verdicchio. This wine is produced starting from the first phase of the harvesting of Verdicchio's grapes, which are then pressed. Briefly, grape pomaces obtained by pressing are removed from the must, which, at this point, undergoes the process of alcoholic fermentation. After the fermentation process, the wine is poured in other casks to separate the components which gradually decant. Lees obtained from the first racking are usually richer in yeasts, whereas the ones obtained from the second racking have a lower concentration of

yeasts, in detriment of the higher quantity of tartaric acid, which crystallizes due to the acid pH after salification with potassium ion. NWLs provided for this study comes from the second racking of the wine (Figure 1) to highlight the potential of exploiting a waste considered of lower quality.

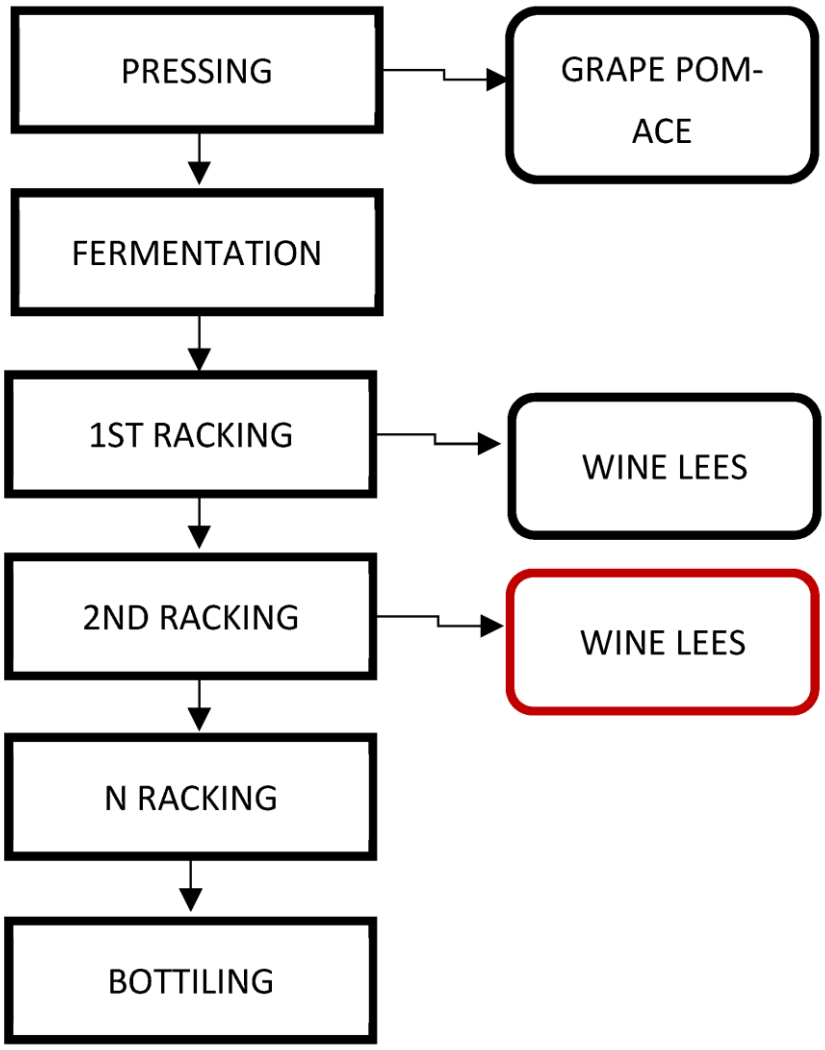

Figure 2. Vinification process from which wastes are produced, Inside the red box, the type of less received from Belisario Winery.

2.2. Reagents and Materials Other Than Wine Lees

Deionized water was produced with a G3 RO CUBIC-S2 demineralizer (Gamma3, Castelverde, Italy). Hexane, acetonitrile and ethanol were purchased from Carlo Erba Reagents (Cornaredo, Mi, Italy). Reagents used for the antioxidant assays, such as 2,2- diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2&39;-azino-bis(3-ethylbenzothiazolin-6-sulfonic acid) (ABTS), manga-nese dioxide, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), HCl 37%, sodium acetate, iron tri-chloride, Folin reagent, sodium carbonate and gallic acid were purchased from Sigma-Aldrich (Stenheim, Germany). A soil DNA isolation plus kit was purchased from the Norgen Biotek Corporation (Thorold, ON, Canada). The human keratinocyte cell line (HaCaT) was purchased from AddexBio, catalog numberT0020001, (AddexBio, San Diego, CA, USA). Reagents for metabolic activity assays, such as dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were purchased from Merck Life Science (Milan, Italy); medium and sera for cell cultures, such as Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS) and penicil-

lin/streptomycin were purchased from Euroclone (Milan, Italy); tissue culture-treated plates were purchased from Falcon®, Corning Incorporated, New York, NY, USA. CytoTox 96® non- radioactive assay (Promega Corporation, Fitchburg, WI, USA) was used to determine Lactate dehydrogenase (LDH) release (cytotoxicity assay). Ex Taq® DNA Polymerase, dNTPs (10 mM) and 10X Buffer (Mg 2 mM) were purchased from Takara Bio Inc. (San Jose, CA, USA). Primer F (pro 341 F) and Primer R (pro 805 R) were from Sigma-Aldrich (Stenheim, Germany). Klebsiella pneumonie preparation, strain ATCC13883, was made in a laboratory in 2019. De Man, Rogosa and Sharpe agar (MRS), Tryptic Soy Agar (TSA) and Sabouraud Dextrose Agar (SDA), MacConkey Agar (MCA), Pseudomonas Cetrimide Agar (PCA) and Mannitol Salt Agar (MSA) media were from Thermo Fisher Scientific (Waltham, MA, USA). Chloramphenicol solution (20 mg/mL) was pre-pared using chloramphenicol from Sigma-Aldrich (Stenheim, Germany) and 95% etha-nol. GenElute bacterial genomic DNA Kit was purchased from Sigma-Aldrich (Stenheim, Germany). Squalene analytical standard was purchased from Merck KGaA, (Darmstadt, Germany).

2.3. Microbiological Analysis of Native Wine Lees

2.3.1. Extraction of Total DNA

To determinate the bacterial and fungal species total content in NWLs, the total DNA was extracted using a Soil DNA isolation plus kit (Nalgene, Waltham, MA, USA), following the manufacturer's instructions and protocol. Starting material volume of NWLs was 400 µL. The final volume of the eluate from the column 50 µL. Four replica samples were prepared, following the same procedure described above, and they were stored at −20°C.

2.3.2. Quality Check by PCR and Agarose Gel Electrophoresis

A PCR protocol was developed to make the quality check by amplification of a portion of the 16S rRNA gene. The protocol developed is standard and very much like the one suggested by the biotech company committed in the NGS workflow analysis (BMR Genomics, Padova, IT). A Bio-rad iCycler PCR system was used. A Klebsiella pneumoniae total chromosomal DNA preparation already available in the lab was used as the positive control.

Reaction mixtures for amplification contained 1X TaKaRa Buffer (TaKaRa Bio, Kusatsu, Japan), 2 mM MgSO4, deoxynucleotides—0.2 mM each, 0.5 Units Ex Taq Polymerase (TaKaRa Bio, Kusatsu, Japan) and 5 µL of the genomic DNA prep. Oligonucleotide primers (Pro341F: 5′-CCTACGGGNBGCASCAG-3′ and Pro805R: 5′-GACTACNVGGGTATCTAATCC-3′) were added at a final concentration of 0.4 µM each [25]. The final reaction volume was 25 µL. The negative control containing no DNA was subjected to the same procedure to exclude or detect any possible contamination. Thermal cycling condition were as follows: 1 cycle at 94 °C for 1 min, then 25 cycles (94 °C for 30 s, 55 °C for 30 s; 68 °C for 45 s); 1 cycle at 68 °C for 7 min.

After amplification, 5 µL of loading dyes were added to each PCR tube. Aliquots of each sample of 20 µL and 6 µL of 1 kb standard were loaded on a 1.2% agarose gel, prepared in 0.5X buffer of TBE, embedding ethidium bromide (0.5 µg/mL) into the gel matrix during gel casting. Amplicons were visualized on a TFX-20.M UV transilluminator.

2.3.3. Preparation and Shipment of Samples

Aliquots of 45 µL of sample obtained in "2.3.1" were transferred into 4 PCR tubes and were sent to the biotech company BMR Genomics (Padua, Italy) for analysis; two duplicates (named GDA and GDB) were sent for 16S rDNA-based NGS, and the other two (named GDC and GDD) for 18S rDNA ITS NGS sequencing.

2.3.4. Preparation and Inoculation of Microbial Growth Media

To verify the presence of living microbial cells in NWLs that may pose a potential health concern, a 1:10 (F1:10) and a 1:100 (F1:100) dilution of lees in saline were prepared for inoculum on different enrichment solid media (Tryptic Soy Agar—TSA; De Man, Rogosa and Sharpe agar—MRS and Sabouraud Dextrose Agar—SDA). Aliquots of 100 µL of F1:10 solutions were inoculated onto 2 TSA plates, 2 MRS plates, 1 SDA plates and 1 SDA plate with chloramphenicol (20 mg/L final concentration). The latter was added to inhibit bacterial growth. A total of 100 µL aliquots of F1:100 were inoculated following the same scheme. All plates were checked after 7 days under the incubation conditions summarized in Table 1.

Table 1. Incubation conditions of the plates inoculated with wine lees samples (NWLs).

Medium	Sample	Incubation Temperature (°C)	Atmospheric Conditions
TSA	F1:10	37	Aerobiosis
TSA	F1:100	37	Aerobiosis
TSA	F1:10	Room temperature	Aerobiosis
TSA	F1:100	Room temperature	Aerobiosis
SDA	F1:10	37	Aerobiosis
SDA	F1:100	37	Aerobiosis
SDA + chloramphenicol	F1:10	37	Aerobiosis
SDA+ chloramphenicol	F1:100	37	Aerobiosis
MRS	F1:10	Room temperature	Aerobiosis
MRS	F1:100	Room temperature	Aerobiosis
MRS	F1:10	Room temperature	Anaerobiosis
MRS	F1:100	Room temperature	Anaerobiosis

2.3.5. Bacterial Population Enrichment

Single colonies from the enrichment media plates (2.3.4) were transferred to 4 TSA plates, each divided into 16 quadrants, to grow each single isolate. Plates were incubated for 24 h at 37 °C.

2.3.6. Microbial Growth on Selective and Differential Media

The bacterial colonies grown in the above sectorial plates (2.3.5) were replica plated on-to selective and differential media MacConkey Agar (MCA), Plate Count Agar (PCA) and Mannitol Salt Agar (MSA), for the isolation and identification of specific microorganisms [26], respectively of Enterobacteriaceae (e.g., E. coli), Pseudomonas aeruginosa and Staphylococcus aureus, [27], the same addressed for in the microbiological quality of the cosmetic products (as per ISO 17516:2014) [27]. Specifically, 4 plates were prepared for each type of media, and microbial growth was evaluated after 48-h incubation at 37 °C.

2.3.7. DNA Extraction by GenElute Bacterial Genomic DNA Kit

For the preparation of the chromosomal bacterial DNA from isolates (2.3.5), GenElute bacterial genomic DNA Kit (Merck KGaA, Darmstadt, Germany) was used following the protocol for gram-positive bacteria, which is more efficient in cell disruption and DNA release. Single colonies of each isolate grown in all TSA plates (2.3.5) were pooled together into 500 µLl of TSB, obtaining a polymicrobial culture. After 1.5 h of incubation at 37 °C, total DNA was extracted. At the last step of the procedure of purification the final elution volume was 200 µL.

2.3.8. Quality Check by PCR and Agarose Gel Electrophoresis

Extracted DNAs (2.3.7) were subjected to 16S ribosomal RNA gene region PCR amplification using the same protocol applied for quality check (2.3.2. and 4). Additionally, a genomic DNA prep from a reference strain of Klebsiella pneumoniae was used as positive control. A reaction with no DNA was added as a negative control. After amplification, 7–8 µL from each sample were loaded on the agarose gel. After electrophoresis (6–7 Volts/cm), the banding patterns were visualized by UV irradiation.

2.3.9. Preparation and Shipment of Samples for NGS Analysis

A total of 30 µL of Genomic DNA 1 and Genomic DNA 2 were transferred into PCR tubes and sent to BMR Genomics (Padua, Italy) for 16S rDNA NGS analysis.

2.3.10. NGS Bioinformatic Analysis Using DADA2

Analysis of generated sequences by 16S and 18S ITS NGS at step 2.3.1 or 2.3.7 was performed using the DADA2 pipeline [28]. Taxonomic assignment of sequences was done using the SILVA reference database for 16S rDNA sequences, compiled for use in the DADA2 pipeline and downloaded from https://doi.org/10.5281/zenodo.4587955 (accessed on 13 October 2022) [29], while for 18S ITS, the training set was from https://doi.org/10.5281/zenodo.4310151 (accessed on 13 October 2022). The DADA2 pipeline was run using Galaxy with default settings [30].

2.4. Ultrasound-Assisted Yeast Lysis of NWLs and Physicochemical Characterization of the Aqueous Extract

2.4.1. Ultrasound-Assisted Yeast Lysis of NWLs

To break up yeast cells of NWLs for the release of cytoplasmatic and wall-associated material, an ultrasonic processor (US, Sonicator Q500, QSonica, Newton, CT, USA), with a 19-mm probe made of titanium, was used. Several preliminary conditions were tested in order to optimise a fast and effective cell break, easily scalable for further industrial purposes, and finally, the following conditions were retained. NWL was diluted in 1:10 (g/g) of water as solvents. Samples were sonicated at a constant frequency of 20 kHz for 5 min. The energy input was controlled by setting the amplitude at 99%, by considering that the total energy of the instrument is 500 W. The probe was submerged in a 250-mL beaker containing 100 g of diluted lees, and the beaker was placed in an ice bath and carefully fixed during sonication.

The cell lysis was confirmed by examining small aliquots under a Dialux 22 microscope (Ernst Leitz, Wetzlar, Germany) at a magnification of 100×.

All the obtained materials were characterized. This product will be identified as sonicated wine lees (SWL).

2.4.2. Scanning Electron Microscopy (SEM)

NWL and SWL were analyzed under scanning electron microscopy to determine the particle shape and size of the cells. A drop of the suspensions (from both NWL and SWL) was deposited on a stub leaving for spontaneous solvent evaporation necessary for the analysis. Samples were fixed on the stub for the FE-SEM using a double-sided carbon pad and metallized by depositing a 10 nm chromium film with a metallizer (Quorum Technologies' Q150T, Laughton, UK). For the SEM analysis, a ZEISS microscope (FE-SEM SIGMA 300) was used (Oberkochen, Germany).

2.4.3. Dynamic Light Scattering (DLS)

The particle size and the PDI of NWL and SWL were characterized by dynamic light scattering (DLS) (Zetasizer Nano-S90, Malvern instruments, Malvern Panalytical, Malvern, UK) at a fixed 90° scattering angle at 25 °C. The samples were suspended in ultrapure water and measurements were performed in triplicate.

2.4.4. Bio-Rad Protein Assay

The determination of the total soluble proteins content in NWL and SWL was carried out by Bradford's method using the BioRad Protein Assay reagent [31]. Bovine serum albumin (BSA) was used as standard. To prepare the BSA standard solution, 2.8 mg of BSA were weighed and dissolved in 1 mL of deionized water. To obtain a concentration of 0.1 mg/mL, 20 µL of solution were diluted with 540 µL of water. The calibration curve was obtained using 6 dilutions of BSA. The equation relative to the calibration curve obtained from the absorbance read by the spectrophotometer and related to the BSA standard solution concentration is y = 0.0481x + 0.075 (R2 = 0.998). The absorbances (595 nm) were measured at 15 min and one hour after sample preparation. For the measurements, a SHIMADZU UV-1700 PharmaSpec spectrophotometer (Kyoto, Japan) was used. This analysis was performed on the sample supernatants after centrifugation at room temperature at 4000 rpm for 15 min in a Heraeus Megafuge 1.0R (Kendro Laboratory Products GmbH, Hamburg, Germany). The lyophilized supernatants were suspended in water, and the difference between the supernatant of the NWL and SWL was evaluated.

2.4.5. Quantitative Element Analysis

The samples (NWL and SWL) were prepared by homogenizing the lees with the T 25 digital ULTRA-TURRAX homogenizer (Ika, Leiden, The Netherlands) and then centrifuged at room temperature for 15 min at 4000 rpm. The lyophilized supernatants of NWL and SWL were first dispersed in Grade 1 water (Millipore Milli-Q Advantage A10 system, resistivity 18.2 MΩ cm) and the optimized digestion of the samples was carried out by placing the sample in a Teflon digestion vessel, followed by 1 mL of HNO3 (65%) and 4 mL of H2O2 (20%) Suprapure grade. A total of 50 µL of Be, Ru and Au solution (2 mg/L) was added as the recovery standard. The vessel was immediately sealed and placed in a microwave closed vessel system (Berghof Speedwave four, Berghof, Eningen, Germany) for digestion. The microwave digestor program is indicated in Table 2. Digested solutions were transferred to a 10 mL volumetric flask and diluted with ultrapure water, then diluted again 10 times to obtain a solution with the correct acid concentration.

Table 2. Microwave digestor program.

Step	**1**	**2**	**3**
T (°C)	150	190	50
P (Bar)	36	36	0
POWER (%)	70	90	0
RAMP (Min)	5	5	1
Step (Min)	10	20	20

The concentrations of elements in the processed samples from the first and the second preparation were measured by an Agilent ICP-MS (7500cx series) with the following operating conditions: power 1550 W, carrier gas 1.03 L/min, make-up gas 0.00 L/min, sample depth 7.5 mm, nebulizer pump 0.1 r.p.s. and spray chamber temperature 2 °C. The 7500cx series instrumentation can be operated in NoGas/He mode, in order to overcome most of the polyatomic interference by the collision cell. A

typical performance test in He mode was performed with a 1 ppb tune solution and gave the following results: He flux 3.0 mL/min, m/z 9 (1000 cps), m/z 45 (1600 cps), m/z 115 (1500 cps), m/z 140 (2000 cps) and m/z 209 (1200 cps). A solution containing Sc, In, Ce and Bi (10 mg/L) was used as the internal standard for ICP-MS measurements. Calibration curves for investigated elements were obtained using aqueous (1.0% nitric acid) standard solutions prepared with appropriate dilution of stock standards (Fluka Analytical, Aldrich, Milan, Italy). The calibration straight was made with the following solutions for microelements (Li, Be, B, Al, Ti, V, Cr, Mn, Fe, Co, Cu, Zn, Ga, As, Se, Rb, Sr, Mo, Ru, Pd, Ag, Cd, Sn, Sb, Cs, Ba, Au, Tl, Pb, U): 0.01 ppb; 0.10 ppb; 1.00 ppb; 5.00 ppb; 10.0 ppb; 50.0 ppb; 100.0 ppb; 500.0 ppb. For the calibrations straight of macroelements (Na, Mg, P, S, K, Ca) the solutions used were: 0.50 ppm; 1.00 ppm; 2.50 ppm; 5.00 ppm; 10.0 ppm; 25.0 ppm; 50.0 ppm. Solutions for the calibration straight of Hg element was as followed: 0.1 ppb; 0.5 ppb; 1.0 ppb; 5.0 ppb; 10.0 ppb.

2.4.6. Total Phenol Content (Folin-Ciocalteu Assay)

The total phenol content (TPC) of NWL and SWL was determined according to the Folin–Ciocalteu spectrophotometric method [32]. The reagent was prepared adding 6 mL of Folin's reagent in 24 mL of distilled water. For the samples, the lyophilized supernatants of NWL and SWL were used. A total of 100 µL of each sample was added in the first row of a 96-well microplate, including the gallic acid control solution made with 1 mg of gallic acid in 1 mL of distilled water, which is used like a standard. Serial dilutions of 1:2 (v/v) were made. In the last well of the 96-plate, a control with deionized water was prepared. Subsequently, 150 µL of the reagent and 50 µL of a saturated Na2CO3 solution, previously prepared, were added to each well. The plate was incubated at 37 °C for 30 min, and then it was analyzed in a microplate reader (FLUOstar Omega, BMG Labtech GmbH, Ortenberg, Germany). The absorbance of each well was determined at 765 nm, and the measurements were compared to the calibration standard solution of gallic acid (GA). The final results of the assay have been calculated using Graph Pad Prism 9 and they were expressed in mg of gallic acid equivalent/g of sample (mg GAE/g). All the measurements were performed in triplicate.

2.4.7. Antioxidant Assays

The antioxidant activity was determined on the lyophilized supernatant of NWL and SWL. It was evaluated by measuring 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging activity, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS•) radical scavenging capacity and Ferric reducing antioxidant capacity (FRAP). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX) was used in every assay as standard for the calibration curve. The results of the assays were calculated using Graph Pad Prims 9, and the values were expressed in IC50, which represents the concentration of the samples required for a decrease of 50% of the initial DPPH, ABTS or iron concentrations, and in µmol or mg of Trolox equivalent/g of sample [32].

DPPH Radical Scavenging Method

DPPH (1.2 mg) was dissolved in 30 mL of ethanol, obtaining a purple solution. For the calibration standard, a solution of Trolox in ethanol (1 mg/mL) was prepared, and the diluted 1:10 (v/v) in ethanol. 100 µL of each sample were added in the first row of a 96-well microplate, including the Trolox. Serial dilutions 1:2 (v/v) were made. Deionized water was used as control. A total of 150 µL of DPPH solution, previously prepared, was added in every well, and the plate was incubated in the dark at 37 °C. After 30 min of incubation, the absorbance of each well was measured at 517 nm with the microplate reader (FLUOstar Omega, BMG Labtech GmbH, Ortenberg, Germany). All the measurements were performed in triplicate.

ABTS Radical Scavenging Method

ABTS (9.8 mg) and 0.6 g of MnO_2 were added in 3.6 mL of H_2O, obtaining a green solution, which was incubated in a dark place for 20 min. After this period, the solution was filtered and 1 mL of this was added in 30 mL of water. The Trolox solution was prepared following the same method used for DPPH assay. A total of 100 µL of each sample was added in the first row of the 96-well microplate, including the Trolox solution. Serial dilutions 1:2 (v/v) were made, and in the last well of the plate a control was made with deionized water. A total of 150 µL of the ABTS solution was added in every well, and the plate was incubated in the dark for 30 min, at 37 °C. After this period, the absorbances of each well were measured at 734 nm, with the same microplate reader as the DPPH assay. All the measurements were performed in triplicate.

Ferric Reducing Antioxidant Power (FRAP)

An acetate buffer pH 3.6 was prepared with 2.46 g of sodium acetate added in 80 mL of distilled water. This solution was acidified with acetic acid until pH 3.6 and made up to 100 mL with water. A solution of 15.6 mg of 2,4,6-tripyridil-s-triazine (TPTZ) in 5 mL of HCl 40 mM and another solution of 16.2 mg of $FeCl_3$ in 5 mL of HCl 40 mM, were added to 50 mL of the acetate buffer to obtain the reagent FRAP. A Trolox solution for the calibration curve was prepared with the same procedure for DPPH and ABTS assays. A total of 50 µL of each sample was added in the first row of the 96-well plate microplate, including the standard Trolox. Serial dilutions 1:2 (v/v) were made, and in the last well a control was prepared with deionized water. A total of 175 µL of the FRAP solution was added to each well, and the plate was incubated for 60 min at 37 °C. After this period, the absorbances were measured at 593 nm, with the same microplate reader as the other assays. All measurements were performed in triplicate.

2.4.8. Quantitation of Squalene by High-Performance Liquid Chromatography Coupled with Diode Array Detection

Squalene was obtained by a lipid extraction with a sonication process. For the extraction, 1 g of NWL was sonicated in 100 mL of n-hexane used like solvent, and the extraction was made in a water bath to avoid increasing the temperature. The sonication was made with a duty cycle with an active interval of 8 sec, using the 19 mm titanium probe, for 29 min, with an amplitude of 97%. After the sonication, the sample was centrifuged at 4000 rpm for 15 min and the solvent was removed under vacuum (40 °C).

The identification and quantitation of squalene were carried out using an HPLC Agilent (1200 Series chromatograph), coupled with a diode array detector (DAD). Chromatographic separation was performed using a ALLTIMA (C18, 150 mm × 4.6 mm) with a particle diameter of 5µm. The column temperature was set at 30 °C. The analysis was performed in presence of an elution gradient with a mobile phase of acetonitrile at a constant flow rate of 1.5 mL/min [33]. Squalene standard was added in 2 mL of mobile phase and then sonicated for 10 min to allow complete solubilization. Before injection, samples were filtered through a 0.45 µm membrane filter to remove undissolved particles. Five µL of squalene standard and 5 µL of each sample were injected into the HPLC. Analytes were monitored with a UV detector at a wavelength of 195 nm. Quantitative analyses in UV/Vis-based detection systems were performed using a linear calibration curve generated with the squalene standard.

2.5. Determination of Cell Metabolic Activity and Cytotoxicity

2.5.1. Cell Culture and Treatments

HaCaT cell line was cultured in DMEM high glucose supplemented with 10% of fetal bovine serum (FBS) and 1% of penicillin/streptomycin. Human gingival fibroblasts (HGFs) were extracted as previously reported [34], after having received the approval of the Local Ethical Committee of the University of Chieti (Chieti, Italy; approval number. 1173, approved on 31 March 2016). Both the cell cultures were kept at 37 °C in a humid atmosphere with CO2 5%.

HaCat and HGFs were seeded at density of 8000 and 6700, respectively, in a 96-well tissue culture-treated plate, and then allowed to adhere for 24 h. NWL and SWL were previously dispersed in sterile water (starting solution 3 mg/mL) and then administered to Ha-Cat and HGFs at 1, 5, 10 and 20% in culture medium, for 24, 48 and 72 h.

2.5.2. Cell Metabolic Activity Test (MTT)

HaCaT cell line was cultured in DMEM high glucose supplemented with 10% of fetal bovine serum (FBS) and 1% of penicillin/streptomycin. Human gingival fibroblasts (HGFs) were extracted as previously reported [33], after having received the approval of the Local Ethical Committee of the University of Chieti (Chieti, Italy; approval number. 1173, approved on 31 March 2016). Both cell cultures were kept at 37 °C in a humidified atmosphere with CO2 at 5%.

HaCat and HGFs were seeded at density of 8000 and 6700, respectively, in a 96-well tissue culture-treated plate and then let adhere for 24 h. NWL and SWL were previously dispersed in sterile water (starting solution 3 mg/mL) and then administered to Ha-Cat and HGFs at 1, 5, 10 and 20% in culture medium, for 24, 48 and 72 h.

2.5.3. Cytotoxicity Assay

To assess cytotoxicity occurrence, the release of LDH into cell supernatants was quantified by the CytoTox 96® non- radioactive assay, which uses a 30-min coupled enzymatic assay, after 24 and 48 h of treatment. The optical density was measured at 490 nm with a correction at 690 nm, by means of a spectrophotometer (Multiskan GO, Thermo Scientific, Waltham, MA, USA). The percentage of released LDH was therefore normalized on the optical density values obtained from the metabolic activity assay.

2.6. Statistics

Statistical analysis was performed using the GraphPad 7 software (GraphPad Software, San Diego, CA, USA) by means of ordinary one-way ANOVA, followed by post−hoc Tukey's multiple comparisons test. Values of $p < 0,05$ were considered statistically significant.

2.7. Formulation of Cosmetic Products

Five cosmetic formulations containing the SWL were developed. For the formulations, the whole ingredient recovered after the sonication was used composed of both the insoluble particles. According to the International Nomenclature Cosmetic Ingredients (INCI), the wine lees will be identified as Saccharomyces/Grape Lees Ferment extract, and it is obtained under the lyophilization of the extract recovered after sonication. The others ingredients used in the formulations are listen in Table 3. Formulations were selected to improve product consistency, strength, texture and functionality.

Table 3. Ingredients used in the formulation of cosmetic products, their function and the supplier.

Commercial name	Ingredients (INCI)	Supplier
TEGO® Betain CK D MB	Cocamidopropyl BetaineSodium Chloride, Aqua	Evonik, Essen, Germany
	Stearic acid	Acef, Fiorenzuola d'Arda (PC), Italy
	Cetearyl alcol	
	Theobroma Cacao Seed Butter	
	Butyrospermum Parkii Butter	
	Citric acid	
	Glycerin	
	Kaolin	
	Zinc oxide	
	Helianthus Annuus Seed Oil	
	Cera Alba	
	Hydrogenated castor oil	
	Oryza Sativa Starch	
	Argania Spinosa Kernel Oil	
	Oryza Sativa Bran Oil	
ACNIBIO PE 9010	Phenoxyethanol, ethylhexylglycerin	
GLDA Chelating	Aqua, Tetrasodium glutamate diacetate, Sodium hydroxide	
	Butylene Glycol	
	Oryza Sativa Bran Oil	
	Sodium Stearate	
	Thocopheryl Acetate	Basf, Ludwigshafen, Germany
	Sodium Coco-Sulfate	
	Glyceryl Stearate	
	Betaine	
	Erylite	Eurotrading, Civitanova Marche (MC), Italy
	Cocos nucifera oil	Esperis, Milan, Italy
	Copernicia Cerifera Cera	Natura Tec, Louis Lépine Fréjus, France
Turkey Red Oil	Castor Oil Sulfated, Sodium Salt, Aqua	Zschimmer & Schwarz, Lahnstein, Germany
PROTELAN ENS	Stearic acid, Sodium lauroyl glutamate, Cetearyl alcohol, Glyceryl stearate	
	Hydrogenated Coco-glycerides	Farmalabor, Canosa di Puglia (BT), Italy
	Polyglyceryl-10 Caprylate	Bregaglio, Biassono (MB), Italy
SORBOSIL™ AC 36	Hydrated silica, Aqua	
	Cetyl Alcohol	
	Sodium Cocoyl Glutamate	Prodotti Gianni, Milan, Italy
	Hydroxyethylcellulose	Vevy Europe, Genova, Italy

Table 4 indicates the list of ingredients (INCI names) and procedures used for the preparation of the cosmetic products. When homogenization was necessary, a T25 Ultra-Turrax® (IKA™, Staufen, Germany) was used (the speed is indicated time to time). The stirring was performed by an IKA™ EUROSTAR 20 High Speed Digital Overhead Stirrer.

Table 4. List of ingredients and preparation of cosmetic formulations.

Cosmetic Formulation/ Consistency	List of Ingredients According to the International Nomenclature Cosmetic Ingredients (INCI)	Method of Preparation
Facial cleanser Solid	Sodium Coco-sulfate, Oryza Sativa Starch, Sodium Cocoyl Glutamate, Cetearyl Alcohol, Glyceryl Stearate, Hydrogenated Coco-glycerides, Saccharomyces/Grape Lees Ferment Extract, Butyrospermum Parkii Butter, Betaine, Hydrogenated Coco—glycerides, Aqua, Theobroma Cacao Seed Butter, Tocopheryl Acetate, Tocopherol, Parfum, Citric Acid	The lipidic phase A was weighed and heated in a water bath up to 85 °C until complete melting. Vitamin E was added to the Phase A once the latter was removed from the water bath and allowed to cool under stirring. Sodium coco-sulfate, oryza sativa starch and the Saccharomyces/Grape Lees Ferment were weighed and homogenized to the lipidic phase at 3500 rpm to obtain a pasty texture. Citric acid was weighed and stirred until complete solubilization in water and added to the paste to reach the desired pH. Perfume was added as the last step.
Facial cleansing Powder	Oryza Sativa Starch, Saccharomyces/Grape Lees Ferment Extract, Oryza Sativa Bran Oil, Sodium Cocoyl Glutamate, Erylite, Silica, Betaine, Cocamidopropyl Betaine, Sodium Chloride, Parfum, Aqua	The oil was added drop by drop to be adsorbed into the weighed starch by using a pestle and a mortar. Thus, first Saccharomyces/Grape Lees Ferment, and then betaine, silica and erylite were added and mixed with a mortar to obtain a homogeneous powder. Perfume was added as the last step.
Make-up remover Solid	Helianthus Annuus Seed Oil, Hydrogenated Coco-glycerides, Cera Alba, Polyglyceryl-10 Caprylate, Saccharomyces/Grape Lees FermentExtract, Theobroma Cacao Seed Butter, Castor Oil Sulfated, Cocos Nucifera Oil, Butyrospermum Parkii Butter, Hydrogenated Castor Oil, Sodium Salt, Tocopheryl Acetate, Parfum, Aqua.	The lipidic phase was weighed and heated in a water bath up to 90 °C until complete melting. Vitamin E was added after the removal from the water bath of the mixture and allowed to cool under stirring. Saccharomyces/Grape Lees Ferment was dispersed in water and added to sulfated castor oil s, heated up to 40 °C under stirring to promote the formation of the mixture and added to the lipidic phase. Perfume was added as the last step.
Melting face mask Solid	Cocos Nucifera Oil, Kaolin, Saccharomyces/Grape Lees FermentExtract, Butyrospermum Parkii Butter, Copernicia Cerifera Cera, Castor Oil Sulfated, Sodium Salt, Zinc Oxide, Tocopheryl acetate, Parfum, Aqua.	Cocos nucifera oil, butyrospermum parkii butter and cera carnauba were weighed and heated up to 80 °C for 30 min under stirring with kaolin and zinc oxide. After removing the ingredients from the water bath, vitamin E was added. Saccharomyces/Grape Lees Ferment was dispersed in water and added to sulfated castor oil, heated up to 40 °C under stirring to promote the formation of the mixture and added to the other ingredients. Perfume was added as the last step.
Purifying face mask Solid	Aqua, Kaolin, Glycerin, Saccharomyces/Grape Lees FermentExtract, Butyrospermum Parkii Butter, Sodium Stearate, Butylene Glycol, Glyceryl Stearate, Cetearyl alcohol, Cera Alba, Stearic Acid, Sodium Lauroyl Glutamate, Tocopheryl Acetate, Phenoxyethanol, Hydroxyethylcellulose, Parfum, Ethylhexylglycerin, Tetrasodium Glutamate Diacetate, Sodium Hydroxide.	Phase A, composed of water, chelating and wetting agents, kaolin, sodium stearate and Saccharomyces/Grape Lees Ferment, was weighed and heated up to 75 °C under stirring. Hydroxyethylcellulose was then slowly added to the phase A under stirring. Phase B, composed of the lipidic ingredients, was heated up to 75 °C and added to the phase A under homogenization (3500 rpm) until the temperature reached 40 °C. Vitamin E, preservative and parfum were added as the last step.

2.8. Physicochemical Characterization of Formulations

The formulations were analyzed for pH with a pH 60 Violab bench pHmeter equipped with the electrode XS Sensor Flow S7 Ag/AgCl, with an internal solution (electrolyte) of KCl 3M. The density of the cleansing powder was determined by measuring the volume in a graduated cylinder of the accurately weighted powder. The evaluation of the stability under accelerated conditions was performed placing the formulations at both 4 and 40 °C alternatively, under triplicate cycles. All the

formulations were also tested for a long-term stability at room conditions for 1 year. The formulations were considered to have passed the test if no changes in organoleptic characteristics occurred (color, odor).

2.9. Challenge Test

In spite of the fact that all formulations are solid cosmetic formulations where a minimum amount of water has been used and only remains in small amount in the final product, the evaluation of possible microbial contamination of the products under use is necessary, which is why a challenge test was performed according to Regulation CE 1223/2009. The challenge test was performed according to the method ISO 11930:2012 Cosmetics–Microbiology–Evaluation of antimicrobial protection of a cosmetic product [35]. The antimicrobial activity was screened against Pseudomonas aeruginosa ATCC 9027, Staphylococcus aureus ATCC 6538, Candida albicans ATCC 10231, Aspergillus brasiliensis ATCC 16404 and Escherichia coli ATCC 87394. Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli were incubated for 48–72 h at 30 °C on Tryptic Soy Agar (TSA); Candida albicans was incubated for 48–72 h at 30 °C on Sabouraud Dextrose Agar (SDA); and Aspergillus brasiliensis was incubated for 72–120 h at 22.5°C on Potato Dextrose Agar (PDA). The media TSA, SDA and PSA were from Sigma Aldrich, Stenheim, Germany. Initial inoculi were 300,000, 480,000, 700,000, 41,000 and 9000 UFC/g for Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Candida albicans and Aspergillus brasiliensis, respectively. The test was followed for 28 days.

2.10. Local Compatibility Test with Human Skin (Irritant Potential)

The test for the evaluation of the compatibility (irritant potential) of the cosmetic formulations with human skin was performed under normal conditions according to the Helsinki Declaration (64th WMA General Assembly, Fortaleza, Brazil, October 2013) and to the COLIPA guidelines [36]. A panel of 10 volunteers (age 28 ± 3 years old) of female and male gender was used. The products were left in contact with human skin for 48 h (model Curatest® F, adhesive strips for patch test, Lohmann and Rauscher International, Rengsdorf, Germany) in a sufficient amount to fill a 1 cm2 test disk. The assessment was made with the comparison with a negative control. The reactions of the skin were evaluated 15 min after patch removal, and again after 24 h, according to defined parameters (erythema, desquamation, oedema and vesicles). The tests were performed in single blind mode, under the directions of a medical doctor certified in dermatology.

2.11. Measurement of the Trans Epidermal Water Loss (TEWL)

The measurements of trans epidermal water loss (TEWL) for the evaluation of the efficiency of the skin barrier were conducted with a VapoMeter® (Delfin Technologies, Kuopio, Finland) that evaluates humidity with a closed chamber unaffected by ambient airflows. The increase of relative humidity (RH) was measured by the sensor and the evaporation rate value (g/m2h) was automatically calculated from the RH increase. The measurements were conducted after the application of the formulations on the forearm divided in six parties of the 20 volunteers, in a room with controlled humidity and temperature (57.2% and 23.4 °C). Five measurements for each product were taken: the first before the application of the products, and 60, 120, 180 and 240 min after rinsing the products. The two masks were left 15 min on the skin after the application. The measuring time was around 10 s.

2.12. Measurement of the Sebometry

The amount of sebum (µg/cm2) on the skin was measured using SebumScale (Delfin Technologies, Kuopio, Finland) in a room with controlled temperature and humidity of 23 °C and 54%. A disposable quartz crystal sensor was placed on the skin for some seconds to absorb the sebum. The equipment

measures the mass of the collected sebum analyzing the changes on the quartz crystal resonance frequency. Measurements were made after the application on the forehead of the 20 volunteers of the solid facial cleanser, cleansing powder and two masks, avoiding the make-up remover, which represents a preliminary step during skin care. Products were left on the skin for 15 min. Five measurements for each product were taken: the first before the application of the products and after 30, 60 and 120 min after rinsing the products. The measuring time was around 15 s.

3. Results and Discussions

3.1. Microbiological Analysis

3.1.1. Analysis of the Microbiome in the Lees

Since the microbiological variability of the wine lees under study is unknown and potentially high, a Next Generation Sequencing approach was used to obtain information on the microbiome of the sample. This is an inexpensive and fast approach that can guide subsequent steps to characterize the viable fraction of microbes with a focus on potential pathogens. The 16S rDNA NGS analysis was used to evaluate the bacterial diversity in the lees, while the 18S ITS NGS analysis was used for the eukaryotic cells, which are expected to be mainly represented by yeasts.

The results are shown in Figure 2. As expected, the Saccharomyces genus is the most represented (95–99%), and the associated ITS sequences found a match at the level of species to the Saccharomyces cerevisiae, which is essential and well-known for driving fermentation processes in the winery. In turn, this evidence is supported by DLS and SEM analysis, which revealed cellular bodies having shape and size compatible with yeast cells. This evidence is quite important in the context of the present work, as the main claim here is the major contribution of the cells and cellular components of the Saccharomyces cerevisiae contained in lees for the development of cosmetic products.

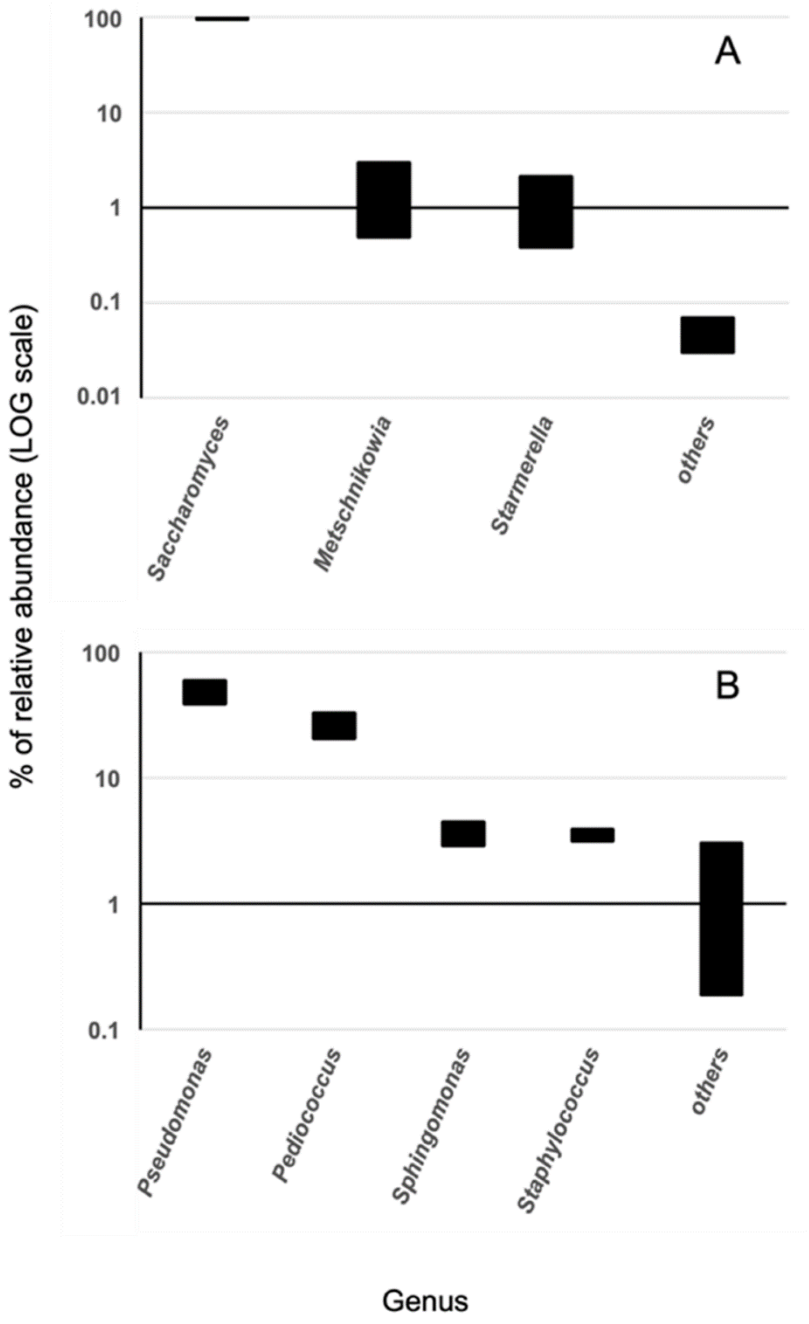

Figure 3. Relative abundance of genus represented in the lees sample. (A): Fungal diversity by 18S ITS NGS analysis. (B): bacterial diversity by 16S rDNA NGS analysis. The group "others" comprises low represented genus (<3% relative abundance). Bars represent ranges of abundance obtained by two measurements.

It is worth noting the presence of other non-Saccharomyces yeasts in the lees, which may be significant from an oenological point of view, namely the genera Starmerella and Metschnikowia (Figure 2). The former has been resolved at the species level as Starmerella bacillaris (synonym Candida zemplinina) [37], and is often used in combination with Saccharomyces in alcoholic fermentation to increase the quality of the process [38]. The latter corresponded to the species M. pulcherrima, which has a strong biocontrol activity against other undesirable yeast species possibly present in winemaking [38].

The bacterial diversity was featured by the prevalence of species belonging to the genus Pseudomonas (49.1%) and Pediococcus (26.6%), and to a lesser extent Sphingomonas (3.7%) and Staphylococcus (3.5%). The Pseudomonas genus is ubiquitous in the environment and includes more than one hundred

species. Few species are human opportunistic pathogens such as P. aeruginosa. However, NGS analysis did not assign it in the lees sample [39]. Various species belonging to the genus Pediococcus are utilized in industrial fermentations of foods and silage, are considered a probiotic and may be present during the production of wines. Their presence may be desirable both for biocontrol and to add a special taste to the final product [40]. Some species have been rarely associated with human infections, but only in immunocompromised patients [41]. The genus Staphylococcus is mainly associated with animals, and it includes many different species, some of which are responsible for diseases in humans [42]. Sphingomonas is notoriously very resistant to various stress conditions and can be found in various environments [41]. Different genera were among the less prevalent (<3%). Faecalibacterium (e.g., F. prausnitzii) is a mesophilic bacterium usually isolated from human feces [43]. It is considered a marker of healthy intestinal microbiota and non-pathogenic. The genus Tatumella spp., typical of fruit and soil, has been associated with wine production [41]. Species belonging to the genus Acinetobacter and Gluconacetobacter are important soil bacteria [44]. Acinetobacter spp. are environmental, and only a few species are associated with nosocomial infectious diseases in immunocompromised patients. Genus Variovorax spp. has been isolated in a diverse range of environments [45]. Enhydrobacter genera have not been associated with any type of disease. The name genus Burkholderia refers to a group of Gram-negative bacteria characterized by species isolated from humans, animals and plants [46]. Only a few species are potentially pathogenic. Being ubiquitous in nature and a common airborne organism, Methylobacterium spp. is found in a wide variety of environmental, industrial and clinical environments [27,47]. Additionally, Micrococcus occurs in a wide range of environments, including water, dust and soil; this genus is generally thought to be saprotrophic or commensal, though it can be an opportunistic pathogen, particularly in hosts with compromised immune systems, such as HIV patients [48]. Natronobacillus is a bacterial genus that can be found in soils [49], while Alistipes is primarily isolated from the intestines of patients with appendicitis. Bacteria of the genus Bacteroides are commensals of the intestinal and urogenital tracts of humans and other animals; they are found in the oral cavity and are the most numerous bacteria in human feces [50].

Based on this NGS analysis, we planned the analysis of the viable bacteria content of the lees to exclude the presence of undesired mesophilic species according to the ISO 17516 standard [27].

3.1.2. Count of Mesophilic Bacteria, Anaerobes, and Fungi

Like all products on the market, cosmetic products must meet certain requirements to ensure product safety and protect the health of consumers. The microbiological quality of cosmetic products is regulated by the ISO 17516 standard that involves total aerobic mesophile counting and the search for specific microorganisms, including Enterobacteriaceae, Pseudomonas spp., S. aureus and C. albicans [51]. Microbial contamination may derive from one or more sources, such as raw material [52].

The total count of viable mesophiles was 5 × 102 CFU/mL of lees. This value is below the maximum limit indicated by requirements for a finished cosmetic product, which is set to 103 CFU/mL [51]. Considering that the quantity of lees used for formulation would certainly be a fraction of the total amount of the final product, the results are promising in terms of microbiological safety related to the source material. Moreover, the final product usually contains actives to control microbial growth, which could evenly decrease microbial viability in the final product. Interestingly, no viable fungi nor anaerobes were found. The first result would indicate that the fraction of yeast cells composing the lees, which is predominant, is not viable.

3.1.3. Growth of Isolates on Selective Media

Sixty-four bacterial isolates from the plates used to make the total count of viable mesophiles were randomly selected and subcultured onto MCA, PCA and MSA, which are selective and differential. MSA allows the growth of halotolerant bacteria, while the color change from purple to yellow is visible only when bacteria can ferment mannitol. MCA medium is used to favor the growth of lactose fermenting Enterobacteriaceae and is selective, thanks to the presence of crystal violet and bile salts. PCA facilitates the growth of bacteria resistant to cetrimide, such as Pseudomonas spp. No bacterial growth occurred on PCA and MCA. This result indicated that the sample is most probably devoid of any viable Enterobacteriaceae and Pseudomonas. On the contrary, a clear growth was visible on MSA plates. Eighty-three per cent of them were able to ferment mannitol. This would be an indication of the putative presence of Staphylococcus aureus. Although growth with a color change is also possible for some other bacterial species—and the lees sample cannot be directly associated with a human sample, so that contamination with S. aureus is unlikely—the results suggest further microbiological analysis to confirm or exclude the presence of pathogens considered by ISO 17516 [27].

3.1.4. Identification of Mesophiles Isolates Grown onto MSA by 16S rDNA Analysis

Isolates from plates were pooled together. Total DNA from the pooled cells was extracted and the identification was determined through 16S rDNA analysis by NGS. Bacterial genera found in the sample shown in Figure 3 belong to the phylum Firmicutes and are environmental [53,54,55,56,57]. They are Gram-positive, halotolerant, aerobic, or facultative anaerobic and spore-forming. Their general growth characteristics are summarized in Table 5 and correlate with those used to isolate and differentially cultivate them in this study.

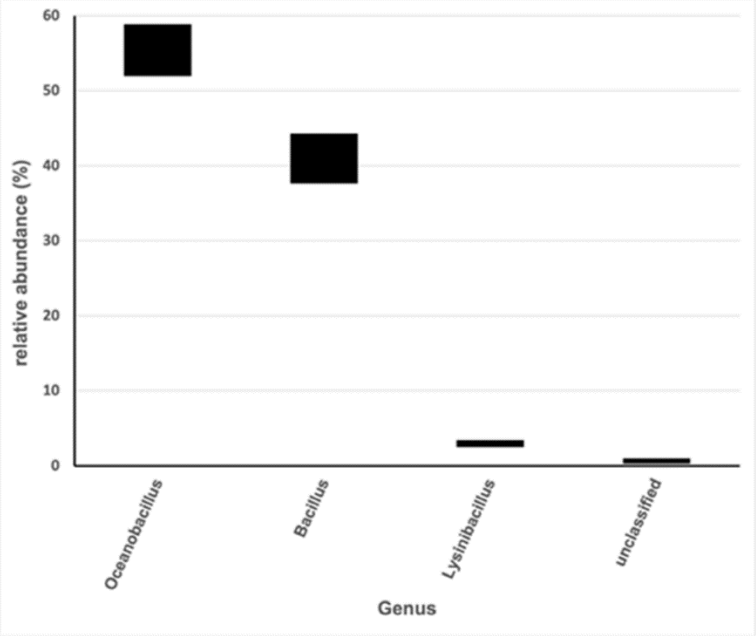

Figure 4. Relative abundance of genus represented in the pool of isolates from viable cells counting experiments. Bacterial diversity was assessed by 16S rDNA NGS analysis. The group “others” comprises low represented genus (<1% relative abundance). Bars represent ranges of abundance obtained by determinations made on two independent pooled samples.

Table 5. Optimum growth conditions for the bacteria found in the samples (GDA-GDD).

Bacterial Genus	Temperature Range(°C)	pH Range	Salt Range (%)	Oxygen	Mannitol Fermentation	References
Bacillus	4–49	6–9	0–10	aerobic	+	[58]
Lysinibacillus	10–45	5.5–9.5	5–7	aerobic	-	[59]
Oceanobacillus	10–40	6.5–10	3–10	aerobic	+/–	[60]

The colour change onto MSA is explained by the fact that most of the identified bacterial species have the enzymatic pathway enabling them to ferment mannitol. Overall, the results allow us to exclude, under these experimental conditions, a measurable contamination by potentially pathogenic viable bacterial cell. However, it is important to consider that this analysis pertained to one single batch of lees. A more extensive analysis considering a significant number of batches would give a reliable evaluation of the general microbial diversity of the Belisario lees (and lees from other wines), which, in turn, would help in guiding the safe inclusion of this important and valuable byproduct in cosmetics.

3.2. Optimization of Ultrasound-Assisted Extraction Method for the Yeast Cells Breakdown in Water and Evaluation of the Properties of the Aqueous Extract

During the present study, a systematic optimization of the sonication conditions, such as, for example, the dilution, the percentage of amplitude or the sonication time, has been performed in order to reduce both energy and time necessary to break up the cells.

The optimization of the extraction method has been performed by comparing the samples produced under different sonication conditions under both optical (prevalently used routinely) and scanning electron microscopy, and DLS analysis, and verifying the capacity of each specific treatment to favor the yeast breakdown. The pictures obtained with SEM were compared with images of yeast cells found in the literature [61]. Figure 4 shows the aspect of NWL (Figure 4A,B) and the SWL (Figure 4C,D), obtained under the optimized conditions, observed under SEM. The cells of NWL appear to be separated from each other, even if they do not exhibit a plump and oval-shaped morphology with a smooth envelope, thus suggesting they are not vital and lysed (Figure 4A,B). In contrast to the NWL, after sonication, the shape of the cells showed various degrees of deformation, and some cells totally collapsed (Figure 4C,D and Figure 5). The sonicated sample exhibits a clear cells breakdown (Figure 5A), which leads to cellular aggregation (Figure 5B). As described in the Methods section, the optimized sonication occurred for 5 min of sonication of a 1:10 water diluted suspension, with a constant sonication frequency of 20 kHz, an amplitude of 99% and a 19-mm probe with a continuous sonication mode. Lower sonication amplitude or a pulsated sonication mode or less diluted samples requested longer time for cell breaking up. The percentage yield of dry weight was calculated for both NWL and SWL. A yield of 4.9% and 5.6% were obtained for NWL and SWL, respectively. The weights of the supernatant of both NWL (3.5 g) and SWL (2.8 g) from the total 10 g of starting material were calculated. Considering the amount of supernatant calculated in the starting material and the water added for dilution (90 g per sample), a dry weight concentration of 2.1 mg/ and 1.5 mg/mL was obtained for NWL and SWL, respectively.

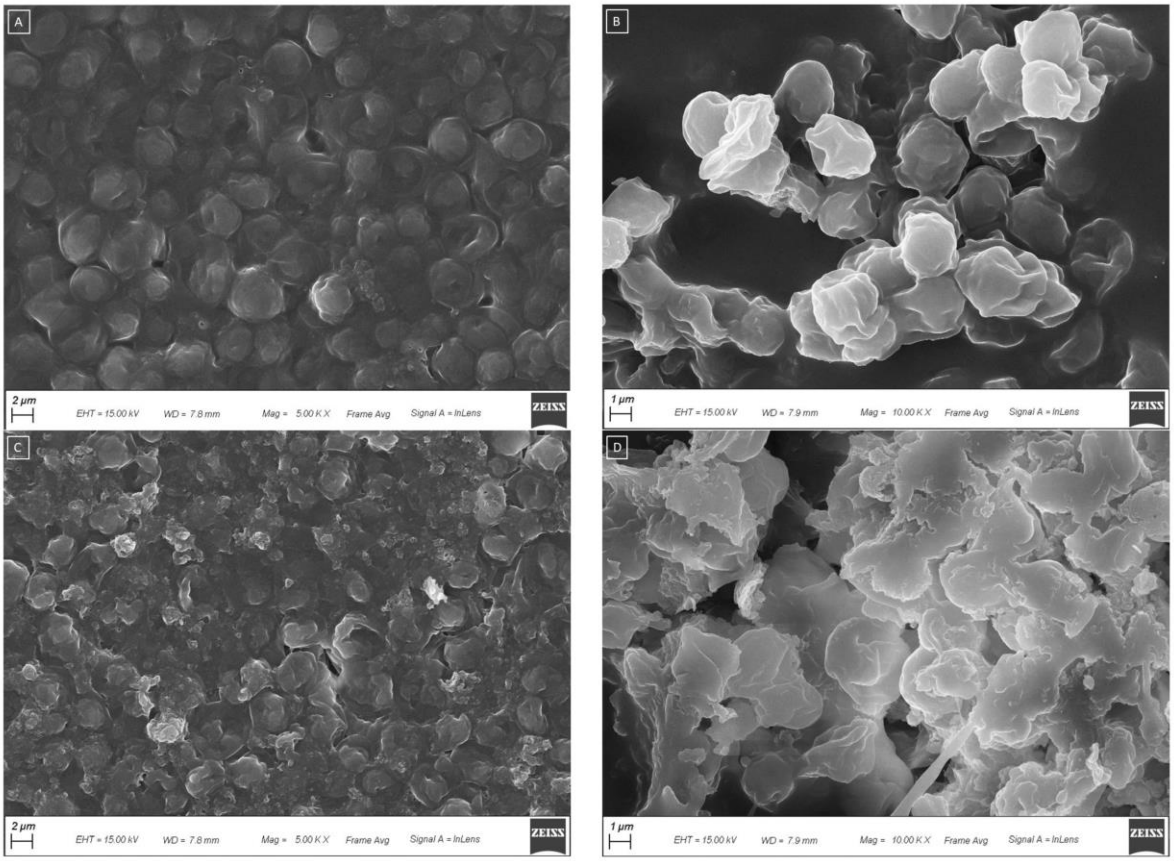

Figure 5. SEM images of (A) NWL (5.00K X); (B) lyophilized NWL sample (10.00K X); (C) SWL (5.00K X); (D) and lyophilized SWL (10.00K X).

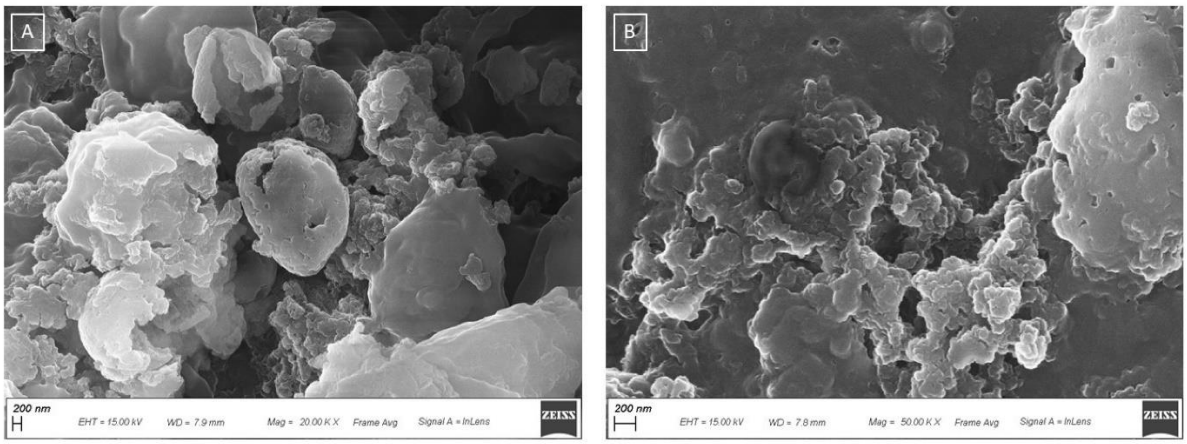

Figure 6. SEM images of lyophilized SWL showing yeast cells breakdown and cellular aggregation. SEM of (A) SWL (20.00K X) and (B) SWL (50.00K X).

The diameter of some cells was measured (Figure 6), which fell in the range of 2848–3000 nm, in agreement with the 1000–10,000 nm range reported in the literature for yeast cells [62].

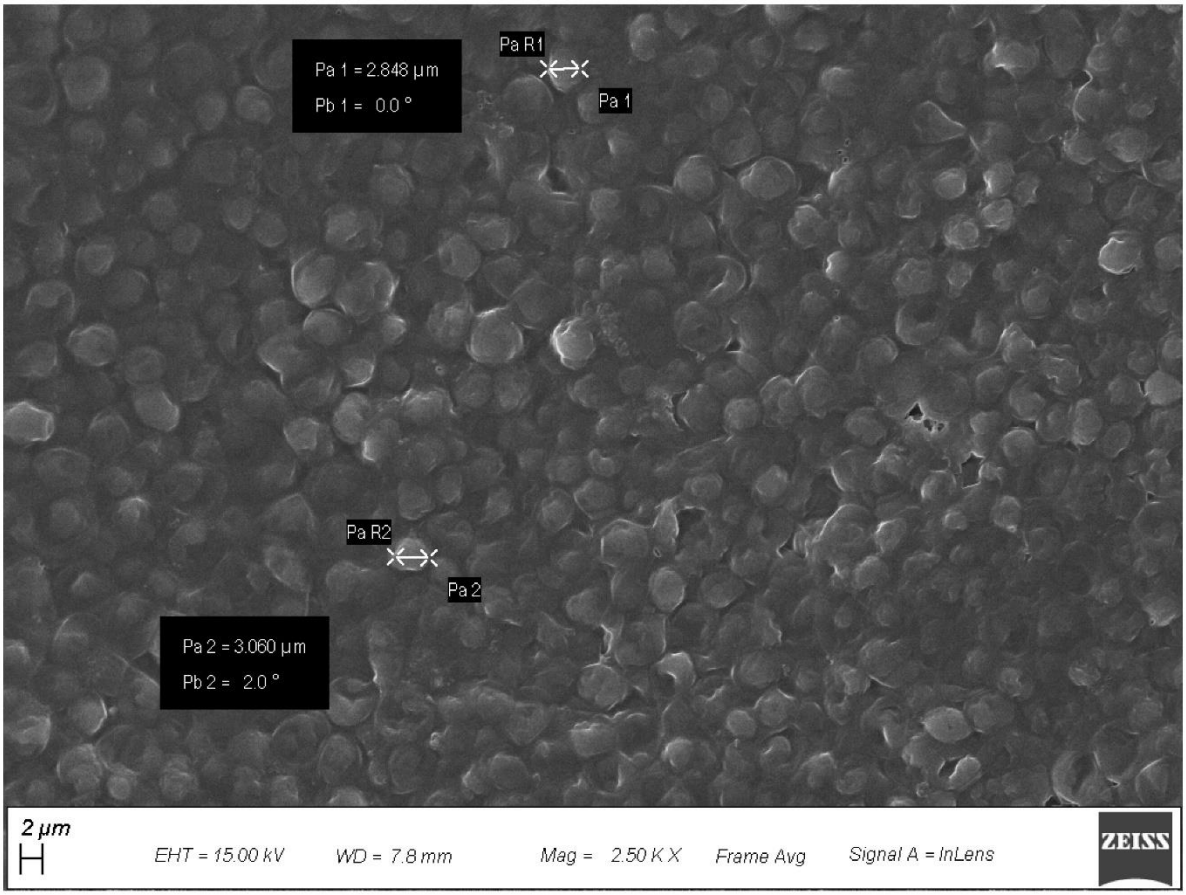

Figure 7. Yeast cells size measurement by SEM analysis of NWLs.

The breakdown and perforation of the cell wall has been described for several different cells. In a recent study, the effects of ultrasound on yeast cells are related to the breakdown of the cell walls, disruption and thinning of cell membranes [59]. The mechanism of action by which ultrasound disrupts cells is called cavitation: when sound waves travel through the liquid system, they generate cycles of particle compression and expansion, and many cavities are formed thereof; these cavities are filled with air or vapor [61], producing strong implosions and explosions in the medium with a major increase in temperature and pressure, which could be responsible for cell wall perforation with subsequent rupture.

To further confirm the efficiency of sonication in breaking down of the yeast cells, the yeast size was determined by DLS analysis, and the results are shown in Table 6. The largest yeast size was observed for the sample (NWL0) diluted 1:10 in water, not subjected to any treatment (neither lyophilization of sonication). Once lyophilized (NWL1), the cell size decreased because of the water loss, and cells appeared with a wrinkled cell wall (Figure 4B). Sonication strongly affects the cell size for the samples (SWL0 and SWL1), which is the lowest among all the samples, and the lees' samples subjected to sonication, but not lyophilized has a smaller value. Therefore, disruption of the yeast cells through sonication could lead to an average reduction in yeast cell size. The SWL1, resuspended in water after lyophilization to allow the DLS analysis, exhibited a higher size than the sample SWL0, which was not lyophilized. This is probably due to the fact that the lyophilized sample has a strong affinity for water, and tends to absorb a large amount of water that allows for a swelling. This same phenomenon cannot be described for the NWL, because the sample redispersed in water after the lyophilization NWL1 is smaller than the NWL0. Independently of the treatment, the diameter of the yeast cells fell within the

1000–10,000 nm range found in the literature [62], which confirms the results obtained by SEM analysis.

Table 6. Different size measured at the DLS between yeast cells of native, sonicated and lyophilized lees samples.

SAMPLES	FREEZE-DRYING	SONICATION	SIZE	PDI
NWL_0	-	-	2945 ± 26	0.084
SWL_0	-	+	1476 ± 64	0.442
NWL_1	+	-	2447 ± 28	0.676
SWL_1	+	+	1723 ± 35	0.291

Protein analysis was performed to confirm the efficiency of sonication in allowing the release of both the cytoplasmic proteins, and the one associated with the yeast cell wall. The total protein content for the supernatant of the NWL was 0.117 ± 0.001 mg/mL, while the supernatant of the SWL was 0.284 ± 0.009 mg/mL. This result indicates the substantial disruption of the yeast cell that causes the release of cell proteins. Prolonging sonication can cause significant protein denaturation resulting from the final temperature increase driven by insufficient cooling [63]. In this case, an increase in protein content occurred in the supernatant of the lees subjected to the sonication process, indicating that sonication time and power are appropriate.

The element analysis was performed to evaluate the safety of wine lees regarding to the heavy metals content, while valorizing the wine lees regarding the benefits for the skin due to the presence of macro and microelements. The ICP-MS results of the total element content are reported in Table 7, and a certain difference in concentration for most of the elements between the supernatant of NWL and SWL can be highlighted. For some elements, the concentration is higher in the supernatant of SWL (Li, B, Na, K, Ca, Mn, Zn, Rb, Sr, Ba), and this could be due to the escape of the elements in saline form from the yeast cells after breaking. Contrarily, Mg, P, Cu and Fe are found in higher concentration in the supernatant of the NWL with respect to the sonicated ones, and it could depend on the binding with the biological tissues, after being released from the yeast cells.

Table 7. Different concentration of element in the samples of wine lees before and after treatments of sonication and centrifugation.

	ELEMENT ANALYSIS		
	NWLs (ppm)	**Supernatant of the NWLs (ppm)**	**Supernatant of the SWLs (ppm)**
Li	0.06 ± 20.29	0.20 ± 6.41	47.26 ± 3.12
B	58.18 ± 1.11	168.94 ± 1.35	236.22 ± 1.90
Na	68.98 ± 2.91	315.89 ± 1.18	729.07 ± 4.23
Mg	577.88 ± 2.07	1735.23 ± 1.55	2125.98 ± 2.94
P	10,888.45 ± 1.56	9146.64 ± 1.27	7862.77 ± 2.56
S	3593.28 ± 3.11	4928.72 ± 1.78	5383.26 ± 5.85
K	13,514.31 ± 1.74	19,195.52 1.68±	65,338.26 ± 3.23
Ca	2687.06 ± 1.45	1130.35 ± 1.34	7927.05 ± 2.49
Cr	0.43 ± 2.15	1.10 ± 1.12	1.39 ± 3.41
Mn	10.72 ± 1.13	8.48 ± 1.40	20.63 ± 2.80
Fe	11.48 ± 1.92	8.28 ± 2.03	4.49 ± 6.68
Cu	227.34 ± 1.74	588.19 ± 0.91	262.41 ± 2.67
Zn	5.16 ± 4.94	14.46 ± 2.47	45.54 ± 2.25
Rb	12.21 ± 1.14	35.03 ± 1.72	62.21 ± 2.80
Sr	9.11 ± 1.81	11.93 ± 0.70	25.66 ± 1.62
Ba	2.16 ± 1.25	3.25 ± 1.12	10.53 ± 1.85
Hg	0.00 ± 6.49	0.02 ± 5.91	0.00 ± 14.57

Concerning the limits in heavy metals, the Regulation (EC) 1223/2009 indicates that heavy metals are technically unavoidable in traces, and the absence of limit values, requires a case-by-case assessment [55,64]. The absence of quantitative limits has led some Member State authorities, such as the 'Ministry of Health', to establish guide values to be respected. In Italy, the "Istituto Superiore di Sanità" (ISS) established maximum limits as "technically unavoidable traces" [64]. The concentration of heavy metals in lees can be assessed after optimizing the method for the element's quantification. Table 7 shows the content in chromium that can be considered safe. Other heavy metals were not detected.

The antioxidant capacity of NWL and SWL was evaluated. The results, reported in Table 8, show that all the assays share the same trend. NWL showed the lowest antioxidant capacity, while the sonication of the lees favored the release of molecules with an antioxidant capacity. In fact, all the cells of Saccharomyces Cerevisiae, present in the lees, have a natural antioxidant defense system composed of small molecules like glutathione (GSH) and ascorbic acid, and enzymes like superoxide dismutases (SODs), catalases, glutathione peroxidases (GPXs) and peroxiredoxins (Prxs) [65] that can be released after the breakup of the cells wall [66].

Table 8. Results of the antioxidant assays.

	ABTS		**DPPH**		**FOLIN**	**FRAP**
	IC50 (mg/mL)	**μmol TE/g**	**IC50 (mg/mL)**	**μmol TE/g**	**mg GAE/g**	**mg TEA/g**
NWL	0.107 ± 0.003	204.525 ± 4.756	0.457 ± 0.009	870.692 ± 17.961	81.333 ± 9.905	55.048 ± 6.419
SWL	0.068 ± 0.001	182.489 ± 2.643	0.228 ± 0.020	607.195 ± 64.368	136.533± 8.942	82.133 ± 8.987
TROLOX	0.004 ± 0.000	7.991 ± 0.190	0.003± 0.000	120.577 ± 14.872		

Wine lees is known for its high content in lipids, which include the presence of squalene [17]. In the lipidic extract of Matelica's Verdicchio obtained with ultrasound, the squalene content detected by using HPLC-DAD is 2.272 mg/g. It can be considered an important vegetable and ethical source of squalene comparable to olive oils, with the advantage f being recovered from waste [67]. The use of

wine lees as a rich source of this bioactive compound also represents a promising approach for a cost-effective, environmentally friendly investment by the winery industry and an alternative and sustainable source of ingredients for the cosmetic sector.

3.3. In Vitro Metabolic Activity and Cytotoxicity Assays

First, SWLs were administered to HaCat keratinocytes cell line in percentages ranging from 1 to 20% for 24, 48 and 72 h. The metabolic activity was read by means of MTT test. After 24 h of treatment, a statistically significant increase in cell viability was recorded in the presence of 1% SWLs respect to untreated cells (Ctrl); after 48 h the metabolic activity was significantly augmented with 10 and 20% SWLs compared to the control, while, after 72 h of treatment, the viability of HaCat exposed to all the SWLs tested percentages (1, 5, 10 and 20%) appeared significantly increased with respect to the control (Figure 7A). Then, in order to evaluate the cytotoxicity level within the culture medium, a LDH assay, after 24 and 48 h of treatment, was carried out. After 24 h of SWLs exposure, a statistically significant reduction of released LDH was recorded in HaCat treated with 1% SWLs with respect to untreated cells, whereas, after 48 h, all the administered SWLs per-centages led to a significant decrease in LDH spread within the culture medium with respect to the control cells (Figure 7B).

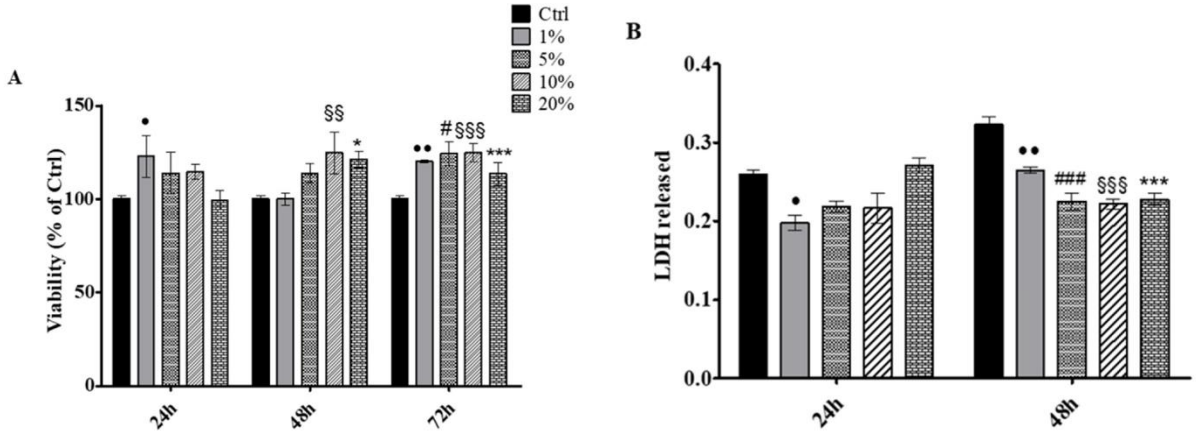

Figure 8. (A) MTT assay performed on Hacat keratinocytes treated for 24 h, 48 h and 72 h with SWLs in percentages ranging from 1 to 20%. Metabolic activity was normalized to control cells treated with DMEM culture medium. The most representative of three separate experiments is shown. Data are presented as the mean ± standard deviation. • vs. Ctrl 24 h $p < 0.01$, * vs. Ctrl 48 h $p < 0.01$, §§ vs. Ctrl 48 h $p < 0.001$, §§§, # vs. Ctrl 72 h $p < 0.01$, •• vs. Ctrl 72 h $p < 0.001$, §§§, *** vs. Ctrl 72 h $p < 0.0001$. (B) LDH release from HaCat keratinocytes treated for 24 and 48 h with SWLs in percentages ranging from 1 to 20%. The most representative of three separate experiments is shown. Data are presented as the mean ± standard deviation. • vs. Ctrl 24 h $p < 0.01$, •• vs. Ctrl 48 h $p < 0.001$, ###, §§§, *** vs. Ctrl 48 h $p < 0.0001$.

Taken together, these data lead us to assume that SWLs definitely show a safe and beneficial profile on keratinocytes by promoting, on the one hand, an appreciable increase in cell viability, and, on the other hand, by significantly reducing the cytotoxicity level.

Second, SWLs were tested on connective tissue cells represented by primary HGFs. The metabolic activity and cytotoxicity level were measured. An MTT test was carried out after 24, 48 and 72 h administering grape lees at 1, 5, 10 and 20 % to HGFs. After 24 h of SWLs exposure, a statistically significant reduction of cell viability was evidenced in HGFs treated with 10 and 20% SWLs with respect to untreated cells. After 48 h, the cell viability level appeared significantly reduced with respect to the control, when 20% SWLs was administered, while, after 72 h, HGFs exposed to 5, 10 and 20% SWLs disclose a statistically significant reduction of cell viability with respect to untreated cells (Figure 8A). The obtained results underline the fact that very high SWL percentages (10 and 20%) could lightly

affect HGF viability, thus suggesting, as already evidenced elsewhere [34], on the importance of continuing SWL biological evaluations with lower concentrations (1 and 5%), in order to totally preserve HGF viability. To conclude, LDH release within the culture medium was evaluated, finding, after 24 h of treatment, a significant augmentation of released LDH% in 20%-treated HGFs with respect to untreated cells, while, after 72 h, a statistically significant reduction of released LDH% was disclosed in HGFs exposed to 1% SWls (Figure 8B).

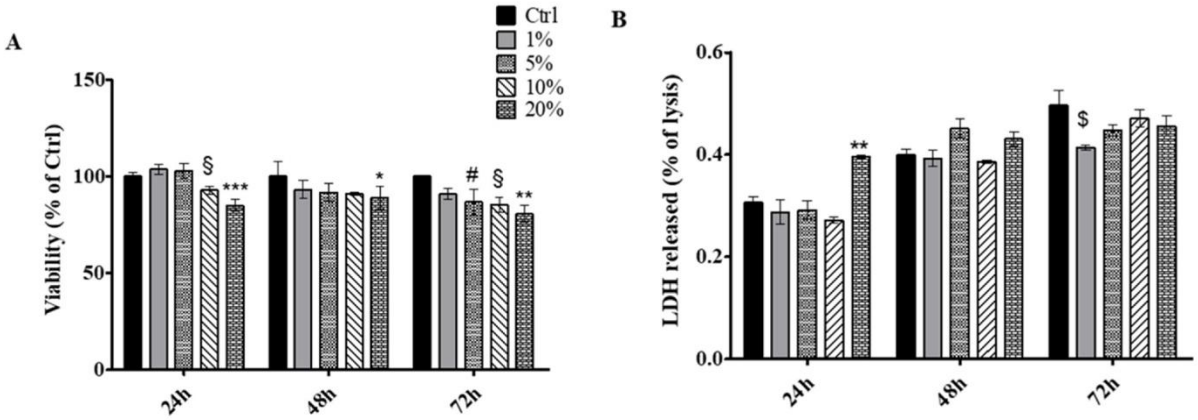

Figure 9. (A) MTT assay performed on HGFs treated for 24 h, 48 h and 72 h with SWLs in per-centages ranging from 1 to 20%. Metabolic activity was normalized to control cells treated with DMEM culture medium. The most representative of three separate experiments is shown. Data are presented as the mean ± standard deviation. § vs. Ctrl 24 h $p < 0.01$, *** vs. Ctrl 24 h $p < 0.0001$, * vs. Ctrl 48 h $p < 0.001$, #, § vs. Ctrl 72 h $p < 0.01$, ** vs. Ctrl 72 h $p < 0.001$. (B) LDH release from HGFs treated for 24 and 48 h with SWLs in percentages ranging from 1 to 20% 24 h and 48 h after treatment with wine lees. The most representative of three separate experiments is shown. Data are presented as the mean ± standard deviation. ** vs Ctrl 24 h $p < 0.001$, $ vs. Ctrl 48 h $p < 0.01$.

3.4. Cosmetic Products

Due to the properties of the SWLs, five new solid cosmetic products were formulated. All products were formulated to create an attractive and innovative beauty routine with the least amount of water and using natural ingredients. The use of minimal amounts of water supports product preservation (solid cosmetics last longer than liquid) and plays within the respect of water waste. Solid products can be easily transported, and are suitable for travelling. They do not require plastic packaging, as they can be easily contained in paper packaging. Most of the products are formulated in a convenient size to ensure easy application. The general idea is to follow the current trend of the world cosmetics market, with the concept of sustainability.

The addition of Saccharomyces/Grape Lees Ferment into the formulations, thanks to its antioxidant properties and cell viability, corresponds with the skin protective action of the cosmetic products; the content of polyphenols, proteins, squalene and microminerals that can be found in an appreciable quantity in the SWL offer a precious cosmetic ingredient with potential hydrating properties. Finally, the complexity in the microbial composition, within its safe profile, which could correspond with benefits for the skin microbiota, completes the cosmetic interest for SWL. The Saccharomyces/Grape Lees Ferment used in all the formulations was obtained after sonication and the lyophilization of the whole extract, which is composed of both the liquid and the solid fractions.

3.4.1. General Characteristics of the Cosmetic Products

The general characteristics of the five cosmetic formulations are reported in Table 9. All the formulations were in a pH range between 5.8 and 6.8, allowing for care of the skin's pH. The density of the cleansing powder is 380 g/L, and is the only product not formulated for being monoportion.

Table 9. Developed products with relative properties.

Cosmetic Formulation	pH	Physicochemical Stability		Challenge Test	Mean Irritation Index	
		Accelerated Stability	Long Term Stability		15 min	24 h
Solid facial cleanser	5.85	Stable	Stable	Passed	0	0
Facial cleansing powder	5.84	Stable	Stable	Passed	0	0
Make-up remover	6.08	Stable	Stable	Passed	0	0
Melting mask	6.05	Stable	Stable	Passed	0	0
Purifying mask	5.80	Stable	Stable	Passed	0	0

All the formulations were stable under both accelerated and long-term conditions. No physicochemical instability phenomena were observed, such as changes in smell or color. All the formulations passed the challenge test, and they were also well tolerated by normal human skin, with a mean irritation index of 0, observed 15 min and 24 h after application, for all the tested volunteers.

3.4.2. Evaluation of Clinical Efficacy

Trans epidermal water loss (TEWL) is an indication for an alteration in the capacity of the skin to contrast a water loss from the more external skin layers, and in general it increases as a consequence of a damaged skin. Apart from pathological conditions, the use of aggressive cleansers can lead to a significant increase in the TEWL. None of the five formulations showed a change in TEWL with respect to the starting values. Small changes visible on the graph (Figure 9) are not statistically significant (significance $p < 0.01$).

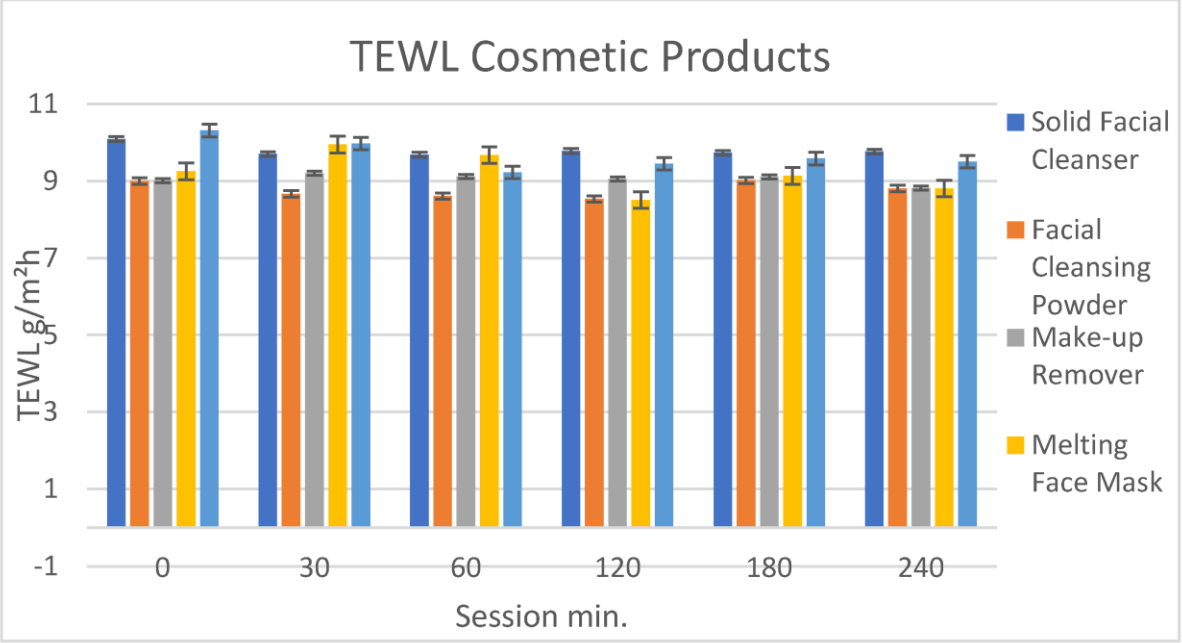

Figure 10.TEWL measurement before the application of the cosmetic products and 30, 60, 120, 180 and 240 min after the removal.

3.4.3. Measurements of the Sebometry

Sebometry is a test that evaluates the capacity of a cosmetic cleanser product to remove the sebum layer during a cleansing procedure. Sebum removal can occur mechanically or most frequently thanks to the presence in the formulation of surfactants or absorbing agents.

The results are reported in Figure 10. To better interpret the results, it is important to highlight that the differences in the time 0 (before the application of the cosmetic products) depend on the different application points of the forehead (Figure 11). An immediate statistically significant ($p < 0.01$) decrease in the sebum level was observed after 30 min of the treatment for all the products. This means that the application of these products allowed for the removal of the sebum from the skin surface, which is appropriate for such a product, thanks to the presence of ingredients such as light surfactants. The sebum content tends to be restored 120 min after treatment for the solid facial cleanser, the facial cleansing powder and the melting mask. The purifying face mask that contains higher amounts of Saccharomyces/Grape Lees Ferment did not restore the initial sebum level during the testing time, making it possible to support a higher purifying capacity of this ingredient.

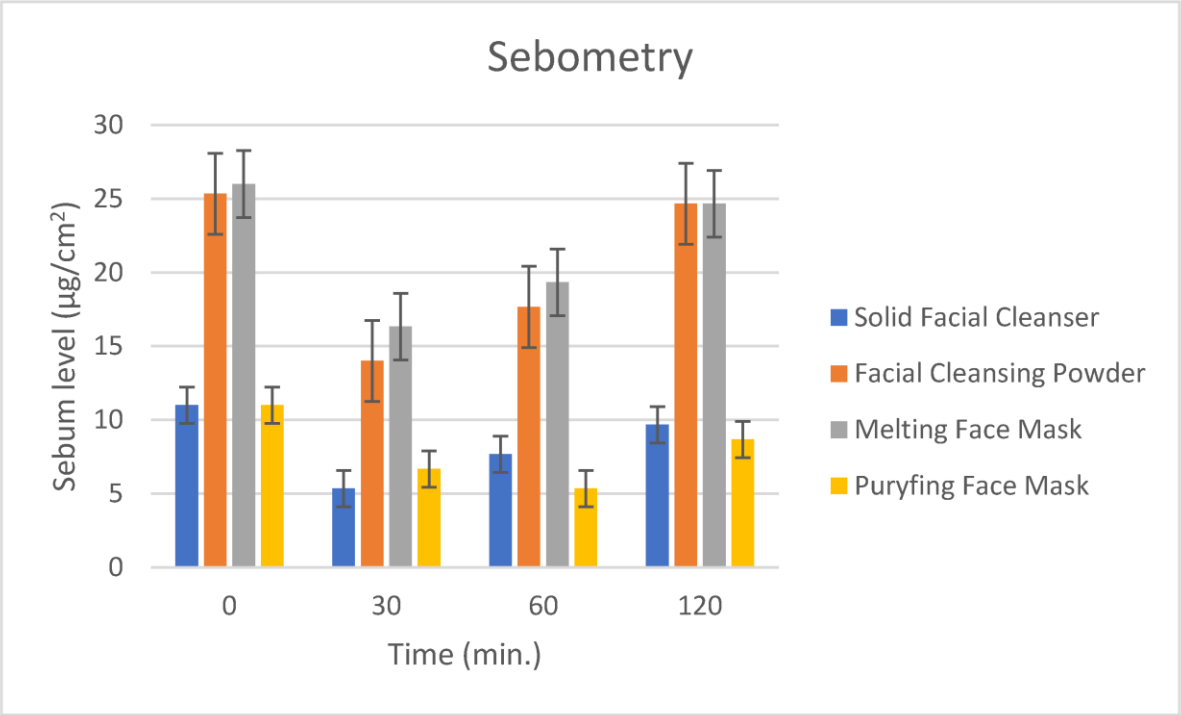

Figure 11. Skin sebum measurement before the application of the cosmetic products, and after 30, 60 and 120 min after the removal of that.

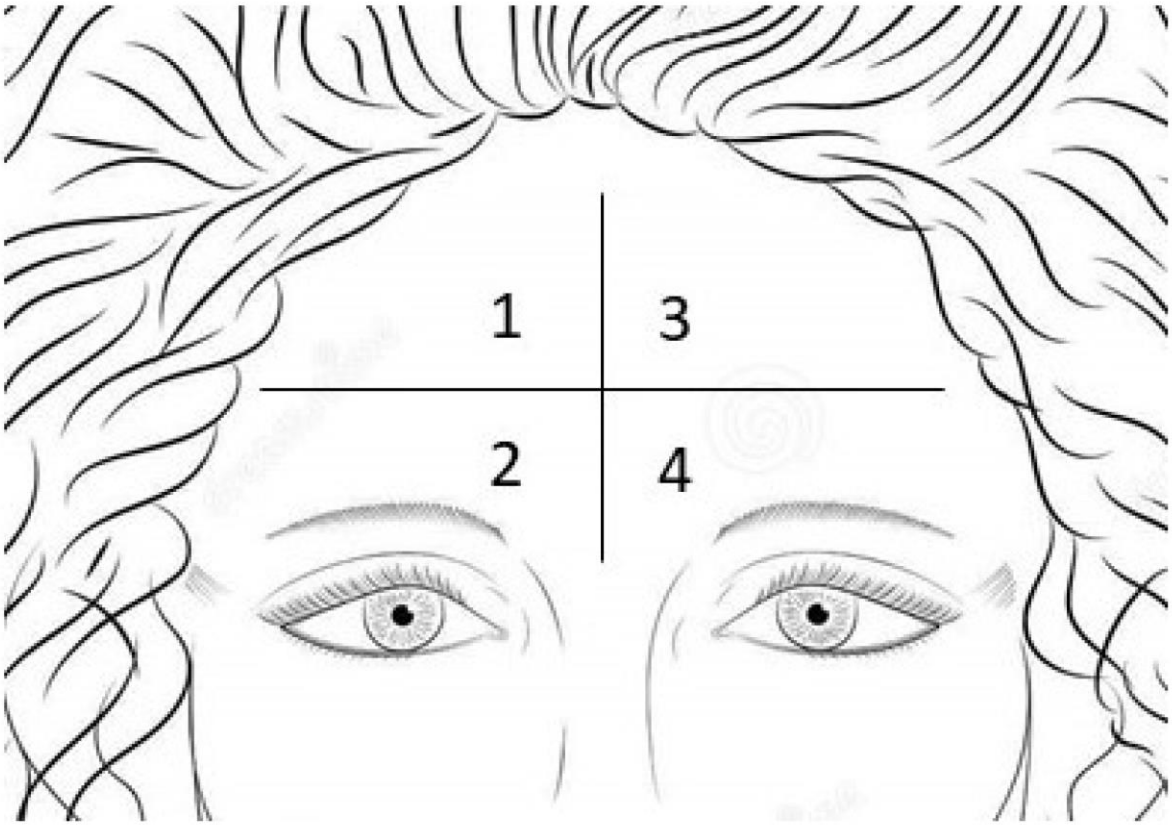

Figure 12. Forehead division for measurements of sebometry. Each product was applied in one of the four areas of the forehead.

4. Conclusions

This study is intended to encourage the exploitation of sonicated wine lees, a wine waste that is not yet exploited as a beneficial ingredient in the cosmetic industry. The results of the present study represent of a good example of the exploitation of waste from food industry from an upcycling perspective.

The results obtained in this study for this specific batch cannot be directly extended to other batches or types of lees, and thus a batch-to-batch characterization will be necessary for product marketing.

Author Contributions

Conceptualization, P.D.M. and M.R.G.; methodology, L.D.N., M.C., L.A.V., R.G., S.F., S.Z., V.D.V., A.C. and R.C.; software, L.D.N.; validation, M.Z., L.A.V., S.F. and S.Z.; formal analysis, M.F., L.D.N., M.C., D.V.P., M.Z., S.I.C. and S.Z.; investigation, L.A.V., R.G., S.F., S.Z., A.C., M.R.G., R.C. and P.D.M.; resources, L.A.V., S.F., A.C. and P.D.M.; data curation, L.D.N., L.A.V., S.F., R.G., S.Z., M.R.G., R.C. and P.D.M.; writing—original draft preparation, L.D.N.; writing—review and editing, P.D.M., L.A.V. and S.Z.; supervision, L.A.V., S.F., A.C. and P.D.M.; funding acquisition, P.D.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki. Recusol s.r.l has performed clinical studies., that is a private company. According to the Italian low, clinical studies performed by private companies are not subjected to the approval of an Ethical Committee. In vitro studies have been performed after the approval of the Local Ethical Committee of the University of Chieti (Chieti, Italy; approval number 1173, approved on 31 March 2016).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the subjects involved in this study to publish this paper.

Data Availability Statement

Data is unavailable due to privacy or ethical restrictions.

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Conflicts of Interest

The authors declare no conflict of interest. Recusol s.r.l. proceeded with the formulations of the cosmetic products and with the clinical tests.

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Chapter 3

Upcycling squalene as penetration enhancer in dermatological and cosmetic formulations

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Abstract

Wine lees are a waste of the wine production rich in *Saccharomyces cerevisiae* cells, that are producers of squalene, a molecule naturally present in human sebum and widely used in cosmetic formulations. For a lot of years, squalene was extract from shark liver with a cruel practice, and currently it is extracted from vegetable oils, but with a low productivity caused by the low amount in the oils and by the long-life cycle of the plants. This may explain the interest in microorganisms like *S. Cerevisiae* as valid alternative for squalene extraction. Wine less, being a waste full of yeast cells, can be a perfect choice for the recovery of squalene from a green point of view. The aim of this work was the extraction of squalene from wine lees, a waste from winery industry, with a green point of view. After finding an effective extraction method using ultrasound, squalene was purified using a Thin Layer Chromatography (TLC). A green palladium-mediated catalytic hydrogenation of squalene was performed to obtain the stable saturated derivative squalane. For a green and cheap catalytic hydrogenation, three clays were used: montmorillonite, sepiolite and palygorskite, obtaining a high yield of squalane, confirmed by ^{1}H-NMR. Both squalene and squalane were tested like penetration enhancers using quercetin like model molecules, because it is an active molecule but with a very low ability to permeate though the skin. The *in vitro* permeability test, for the evaluation of penetration enhancing, was made using the artificial biomimetic membrane PermeaPlain®, a semi-permeable and cell-free cellulose membrane, and dissolving quercetin in water, glycerin, squalene and squalane. The permeability test showed that squalene, but above all squalane, allowed quercetin to pass the membrane surface, acting like a penetration enhancer and making squalane obtained from wine lees a good ingredient for cosmetic applications.

1. Introduction

Squalene is a ubiquitous triterpene, a polyunsaturated hydrocarbon consisting in 6 isoprene units, with formula C30H50. Squalene is in animals, plants and microorganisms like an intermediary and precursor of sterols, like cholesterol (1). In humans, squalene is present in a high amount on the skin, and it is one of the main component of skin surface lipids, around 13%. It is also present in low quantity in human organs and tissues.

The serum squalene originates from endogenous cholesterol synthesis and from dietary sources. Together with wax esters, cholesterol triglycerides, free fatty acids, diglycerides and other small components, it is one of the main components of sebum (around 13 %) (2). It is secreted by sebaceous glands in hair follicles to lubricate the skin and the hair of animals and humans, where it is present especially in face and scalp (3).

The role of squalene on the skin surface is the reduction of the free radical oxidative damage, exposed to stress like sunlight and pollutants. It protects the human skin surface from the lipid peroxidation, acting like a quencher of singlet oxygen. This is why, together with food and pharmaceutical industries, approximately 60-65% of the squalene market is for cosmetic industry, where it is used in cosmetic formulations and efficiently absorbed into the skin without any oily residues (4). It is widely used as emollient, antioxidant and like moisturizer thanks to its occlusive property for the reduction of water loss, and because it can maintain moisture in the stratum corneum (5). Squalene has also antitumor activity and it can prevent skin tumorigenesis (6) and act as a chemopreventive and therapeutic agent (7) (8) (9).

The richest source of squalene is shark (Squaluss spp.) liver, that represents around 20-25% of the weight of the animal and from which it takes its name. Squalene has attracted the attention after the discovery by Dr. Tsujimoto in 1903, and it was considered for a long time the main source of squalene, where it represents 40-70% of the liver mass (1). But this is a very cruel practice for the recovery of squalene because for economic reasons, fishermen discard the rest of the body of the shark after the use of the liver. The overexploitation of the sharks is making them vulnerable to extinction and it is a very big problem for the balance of the marine ecosystem, where sharks are top predators.

To avoid this brutal practice, squalene can be recovered from other sources, like plants and microorganisms. Olive oil is the main vegetable source of squalene, containing an amount between 200-700 mg/100 g of biomass (486 mg/100 g oil (10)) depending on the fruit maturation and the period. Another rich source of squalene is amaranth oil (9.87 g/100 g oil (11)) but in general, squalene is present in all vegetable oils, such as soybean, grape seed, palm, wheat germ, peanut, rice bran, sunflower seeds, sesame, pumpkin and flaxseed oils. The quantity of squalene in vegetable sources is low and plants take also one year to complete their life cycle before being harvested. This is why the productivity in terms of mg/g/day of squalene from plants is very low and the quantity cannot satisfy the demand (12). Squalene is also found in some microorganism sources, like microalgae, fungi and yeast like Saccharomyces cerevisiae. Microorganisms are a valid alternative to shark liver for the recovery of squalene. In fact, although they do not accumulate an amount of squalene as sharks and plants, their rapid growth makes them an attractive choice for squalene recovery (12) (13).

Squalene is highly susceptible to the oxidation for the presence of six carbon double bonds (C=C) and for its chemical instability; this is why it is hydrogenated in squalane (C30H62). Squalane is the saturated derivative of squalene, and it is also identified in human sebum and thanks to the inert properties, it is widely and highly preferable used, especially for cosmetic and nutraceutical applications, with the same emollient, moisturizing and antioxidant properties of squalene. Squalane

can be obtained by catalytic hydrogenation of squalene derived from natural sources. Usually the hydrogenation is performed using high temperature and high pressure, with a significant energy consumption an high greenhouse emission, but also using catalysts environmentally harmful such as platinum or nickel, and harmful solvents (1) (14) (15).

The skin is the protection of our body and for its nature it has a barrier function, thanks to the presence of the external layer of the skin, the stratum corneum. Especially for the presence of lipids, the skin is protected against the external environment, and it has the impermeability. The penetration of active compounds through the skin can be promoted, without disrupt the skin barrier and respecting the nature of the skin, by physical methods like electroporation, microneedles or laser, and chemical methods. For the chemical methods, over 360 molecules have been shown to enhance the permeation of active compounds across the stratum corneum. These substances are called penetration enhancers or penetration promoters, and they can act with different mechanism of action: for example, the increase of fluidity or the modification of the lipid bilayers of the stratum corneum, the increase of the hydration of the stratum corneum, the protein denaturation on the desmosome or on the keratin. Penetration enhancers allow active compounds that hardly pass through the stratum corneum to reach the epidermis. According to the REGULATION (EC) No 1223/2009 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 30 November 2009 on cosmetic products, "'cosmetic product' means any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours". This means that, even with the help of penetration enhancers, cosmetic ingredients must not go beyond the epidermis.

Chemical enhancers can be divided for the chemical structure: alcohols (like glycerol, propylene glycols and ethanol), fatty acids (like oleic acid and linoleic acid), amines (like triethanolamine), esters (like isopropyl palmitate and isopropyl myristate), amides (like urea), surfactants (like sodium laureate), terpenes (like D-limonene), sulfoxides (like dimethyl sulfoxides), phospholipids (like lecithin) and hydrocarbons (like alkanes and squalene) (16) (17) (18).

In facts, squalene and squalane are also used in cosmetic, pharmaceutical and nutraceutical applications for the capacity to penetrate the skin and enhance the permeation of active compounds. Squalene and squalane are considered safe penetration enhancers, improving the permeability of compounds, like polyphenols (19) (20) (21).

The aim of this work is the optimization of an extraction method for the recovery of squalene, from wine lees, that is rich in S. Cerevisiae (22) (23). This molecule, highly susceptible to oxidation, is then hydrogenated with a green catalytic hydrogenation, in squalane, that is more stable. Finally, a skin permeability test is proposed using Franz cell, to verify and confirm the ability of both squalene and squalane like penetration enhancers, using quercetin like model. Quercetin in fact is a polyphenol with a very low ability to permeate though the skin but with antioxidant and anti-inflammatory properties that can improve health skin (24).

2. Materials and methods

2.1 Materials and Reagents

Wine lees used for squalene extraction were kindly supplied by Belisario's winery (Matelica, Italy). They are derived from the vinification process of 2022 Matelica's Verdicchio. Quercetin hydrate (≥95%) (No. CAS: 849061-97-8) was purchased from Sigma-Aldrich (Stenheim, Germany). Deionized water was produced with a G3 RO CUBIC-S2 demineralizer (Gamma 3, Castelverde, Italy). Squalene analytical standard was purchased from Merck KGaA (Darmstadt, Germany). Cyclohexane > 99.5% was purchased by Honeywell Riedel-de Haën™(Charlotte, Noth Carolina, US), ethyl acetate > 99.5% was purchased by Sigma-Aldrich. *n*-Hexane and acetonitrile were purchased from Carlo Erba Reagents (Cornaredo, Mi, Italy). For the catalytic hydrogenation, palladium (II) nitrate hydrate was purchased by Alfa Aesar (Haverhill, Massachusetts, US; the clays Montmorillonite, sepiolite and palygorkite were purchased by Sigma Aldrich. For the NMR analysis, dymethyl sulfoxide (DMSO) was purchased by Sigma Aldrich. For the purification of squalene, Silica Gel 60A (35-70 μm) was purchased by Carlo Erba.

2.2 Ultrasound-assisted lipid extraction from wine lees

For squalene extraction from wine lees, were used the parameters of the sonicator used in the study by *Di Nicolantonio et al.,* changing the sonication time and the quantity of wine lees and solvent (23). Wine lees were subjected to a sonication process with 25 mm titanium probe (Sonicator Q500, QSonica, Newton, CT, USA). To optimize a fast and cheap method for the extraction of the squalene, different timing were tested: 10, 15, 20 and 29 minutes. 100 g of fresh wine lees were diluted in 1L of hexane and sonicated at a constant frequency of 20 kHz with and energy input controlled by setting the amplitude at 97 % considering the total energy of the instrument (500 W). The sonication was made with a duty cycle with an active interval of 8 sec. The Becher was placed in an ice bath in order to avoid the overheating of the sample. After the extraction, the samples were centrifuged at 5000 rpm for 10 min and the solvent was removed under vacuum (40°C). After testing that the best extraction yield was obtained with 29 minutes of extraction, fresh wine lees were lyophilized before the extraction: the ultimate sonication was made using dried lees and for 29 minutes, that resulted the best extraction time. The lyophilized lees were used in order to avoid the interference of water with the non-polar solvent hexane, allowing a better contact between the lipidic material of the yeast cells of the lees and hexane. The cellular breakdown that occurs thanks to the action of ultrasounds and which allows the release of squalene into the solvent, is confirmed by using an optical microscope.

2.3. Quantification of squalene by High-Performance Liquid Chromatography Coupled with Diode Array Detection (HPLC-DAD)

The quantification of squalene was carried out using an HPLC Agilent (1200 Series chromatograph), coupled with a diode array detector (DAD). The HPLC method was based on the one described by *Di Nicolantonio et al*. (23). Chromatographic separation was performed using the column ALLTIMA (C18, 150 mm x 4.6 mm) with a particle diameter of 5 μm. The temperature of the column was set at 30 °C. The analysis was performed with an elution gradient with a mobile phase of acetonitrile at a constant flow rate of 1.5 mL/min. Squalene standard was added in 2 mL of mobile phase and sonicated for 10 min for the complete solubilization. Samples were filtered through a 0.45 μm membrane filter to remove undissolved particles. 5 μL of each sample were injected into the HPLC. Analytes were monitored with a UV detector at a wavelength of 195 nm. A linear calibration curve, generated with the squalene standard, was used for the quantitative analyses in UV/Vis-based detection systems.

2.4 Purification of squalene with column chromatography and separation with Thin Layer Chromatography (TLC)

Purification of squalene was made with a column chromatography. The system was composed of silica (SiO_2) gel (100 g) as stationary phase and a mixture of cyclohexane and ethyl acetate (95:5 v/v) as mobile phase. After the column packing, 2 g of squalene standard were added in the column. The fractions recovered from the column chromatography were evaluated with Thin Layer Chromatography (TLC), using a mixture of cyclohexane and ethyl acetate (98:2 v/v) like mobile phase. The interesting fractions were recovered.

2.5 Palladium-mediated catalytic hydrogenation of squalene in squalene

For the catalytic hydrogenation of squalene in squalene, the 3 clays montmorillonite, palygorskite and sepiolite were clean with deionized water and dried at 120° C. For the catalyst, Palladium (II) nitrate hydrate was solubilized in 15 ml of 3N HNO3 solution, and 1 g of clay was added. The mixture was dried and hydrogenated for 2 hours (25).

15 mg of each catalyst and 50 mg of squalene were put in a pressurized glass reactor with 25 mL of ethanol anhydrous, and hydrogenated under 23 psi for 1.5 h. After the reaction, the solutions were filtered with filter paper and dried. A colorless oil was obtained, and it was analyzed with 1H-NMR, using 30 mg of squalene and 20 mg of the obtained oils in 750 mL of DMSO.

2.6 Evaluation of squalene and squalane like penetration enhancers

2.6.1 Evaluation of solubility of quercetin in squalene and squalane

The equilibrium solubility of quercetin was determined by adding an excess of powder to 50 ml of water, glycerin, squalene or squalane and maintaining the system at room temperature (20 ± 0.5°C) under continuous stirring in an incubator (Velp Scientifica, FTC 90E, Usmate, Italy). The equilibrium solubility was assumed to have been reached when the standard deviation of three subsequent measurements was smaller than 1%. Upon reaching the equilibrium, generally after 24 h, aliquots were taken, filtered through a regenerated cellulose filter syringe of 0.45 µm pore size (Filalbet, Rossello, Barcelona, Spain) and the concentration of the filtrate was determined by HPLC. Assays were performed in triplicate.

2.6.2 *In vitro* skin permeation test

The synthetic biomimetic cellulose hydrate membrane PermeaPlain® barrier (PermeGear, Bechenheim, Germany) of 1.7 cm in diameter was used as model for the *in vitro* permeation test. The membrane, that simulates the passive transport throught human's membrane, was mounted on vertical Franz's diffusion cells (PermeGear, Bechenheim, Germany) taking care of the correct exposure of the membrane facing the receptor compartment of the system. An appropriate amount of quercetin (0.5 mg/ml) able to leave an excess of undissolved quercetin, was added in four different liquids: water, glycerin, squalane and squalene. Suspensions (200 µl) were placed in the donor compartment. The diffusion area of the Franz's cell was 0.64 cm^2 and the cell volume was 5 mL. The membrane was placed between the donor compartment and the receptor compartment. The receptor compartment was filled with 5 ml of 0.1 M phosphate buffer (pH 7.2) and placed in contact with the membrane, taking care to avoid the presence of air. During the experiments, the solution in the receptor compartment was stirred continuously and the temperature was set at 32.0 ± 1.0 °C.
At specific time intervals, 200 µL of receptor medium was collected and replaced with "fresh" medium thermostated at the same conditions. At predetermined time points (from 5 to 480 min.), the skin was removed from the diffusion cell, cleaned with methanol-soaked cotton, and homogenized in methanol. The solvent was evaporated under reduced pressure, and the dry residue was dissolved in the mobile

phase for the HPLC determination. The results corresponded to the amount of quercetin retained in the skin.
Aliquots were analyzed by HPLC and each measurement represents the amount of quercetin passing through the membrane over the time. The membrane was left under stirring in 3 ml of ethanol 70% for 24 hours and the solution was then analyzed by HPLC to determine the concentration of quercetin retained in the membrane, using the calibration curve previously prepared.
HPLC analysis was performed by a HPLC 1090 Hewlett Packard Series I (Ramsey, USA), equipped with a Hewlett Packard HP1100 MSD Chemstation Rev. A.08.03. Separation was performed on a reversed-phase column (Mightysil RP-18 GP, 4.6 mm, i.d. 150 mm, Kanto Corporation, Portland, USA) using a mobile phase consisting of methanol, water and phosphoric acid at a ratio of 100:100:1 in weight. The detection wavelength was 360 nm, and trans-ferulic acid was used as internal standard. Samples (1 mL) were injected manually. Standard quercetin solutions showed linearity over the concentration range of 0.1–200 µg/mL with a correlation coefficient (r) of 0.999. The quantification limit in the HPLC assay was 0.03 µg/mL.

2.6.3 Evaluation of results and statistical analysis

The amount of permeated quercetin was analyzed by HPLC. The permeated quercetin as a function of diffusion time was fitted to Higuchi's equation (1):

$$\frac{Q_t}{Q_0} = k \times t^{1/2} \qquad \text{Equation (1)}$$

where, Qt/Q0 is the fraction of permeation of drug, and the constant k expresses drug diffusion rate, also related to the interaction between drug and vehicle. The linear regression in the initial linear range of the curve was then determined. Data were analyzed by one-way analysis of variance, using a Bonferoni test. The statistical analysis was conducted using an Origin software (version 8.5). Results are shown as mean ± S.D. (standard deviation), and considered significantly different when $P < 0.05$.

3. Results and discussion

3.1 Ultrasound-assisted squalene extraction

Different extraction conditions were tested, based on the extraction protocol made by Naziri et al. (22) and optimizing the method, in order to increase the amount of recovered squalene. The protocol that gave the best result in terms of squalene amount was the UAE extraction lasted 29 minutes with lyophilized wine lees. This method showed a substantial difference in the detected content of squalene giving 25.93 mg/g dry lees (Naziri et al. showed a maximum content of 5.90 mg/g dry lees). This important result can be explained by the high total energy of the sonicator (500 W). The pictures obtained with the optical microscope confirmed the lysis of the yeast cells, which allowed the release of squalene into the extracellular environment. Squalene in fact, is an intracellular accumulated compound, and the cell lysis is essential for the release of this molecules (Figure 1).

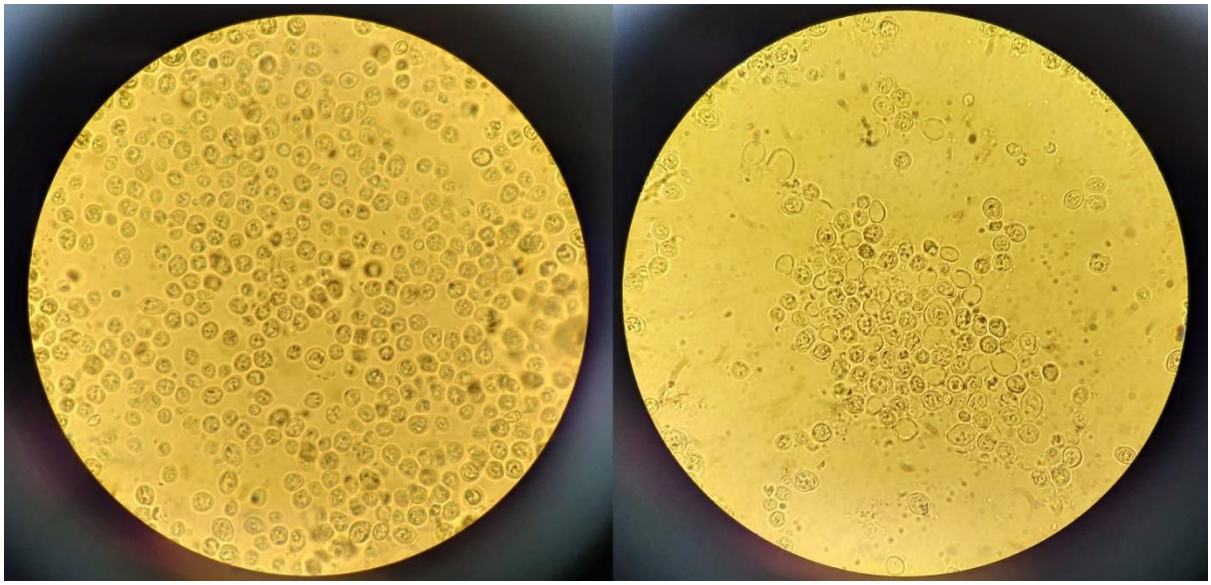

Figure 1. Optical microscope images (100X) of yeast cells before and after the sonication for the squalene extraction. The cell lysis, confirmed by the breaking of the cell wall and by the cellular aggregation *(Di Nicolantonio L., 2023)* allows the release of squalene into the extracellular environment.

3.2 Catalytic hydrogenation of squalene in squalane

After the extraction, squalene was purified and recovered from the lipidic extract using a column chromatography, and then evaluated with TLC. After the purification, a green palladium mediated hydrogenation was made. Squalene in fact, is a highly oxidable molecule, thanks to the presence of six carbon double bonds (C=C) (14). Squalane is more stable and usually is the industrial product used in cosmeceutical and nutraceutical formulations. Squalane is usually obtained by hydrogenation of squalene extracted from natural sources (1). For the green hydrogenation, clays were used thanks to the low cost and easy availability, changing the clay based on the catalysis (15). In this study, 3 different clays were tested: montmorillonite, sepiolite and paygorskite. After the hydrogenation, the collected solution resulted in colourless oil with a yield of squalane of 70% for montmorillonte clay and 60 and 90% respectively for palygorskite-metal and sepiolite-metal. The 1H-NMR confirmed the hydrogenation of squalene in squalane (Figure 2 and 3): in fact, in the spectra of the hydrogenated samples there are no peaks at 5 ppm, that are typical of the vinylic protons of the squalene. On the contrary, there are signals between 0.5 and 2 ppm, that are typical peaks of the aliphatic structure of the squalane. These spectra confirmed the synthesis of the squalane. For a green catalytic hydrogenation of squalene in squalane, clay-based materials are a perfect choice for the easy availability and the cost effectiveness of the clays. Clay-based material catalysts have the possibility to replace the interlamellar cations with other cations or small molecules. Natural clays can be modified by physical and chemical modifications, changing properties like acidity, pore size and surface area, with the aim to improve catalytic performance (15).

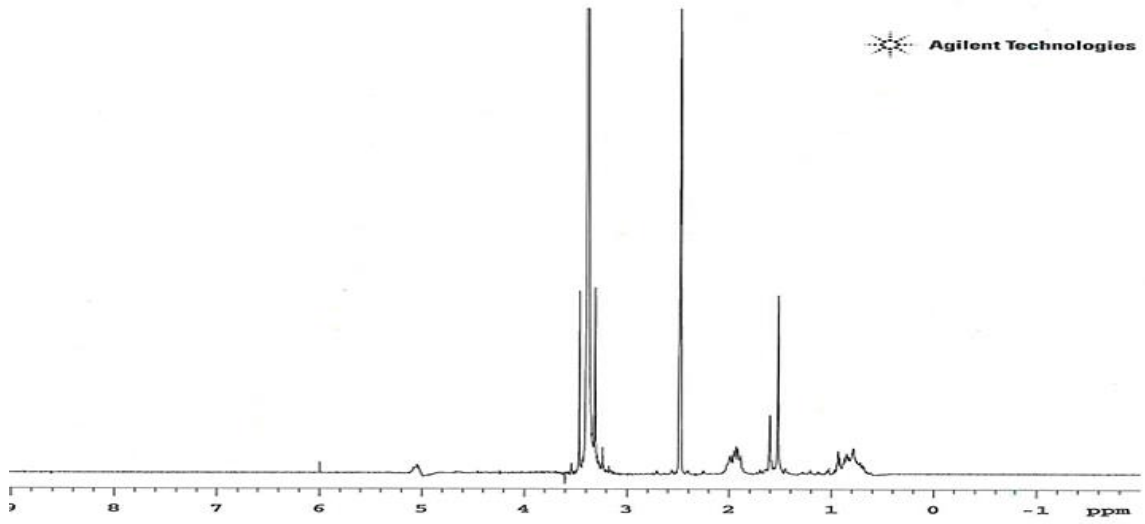

Figure 2. ^{1}H-NMR of squalene after purification.

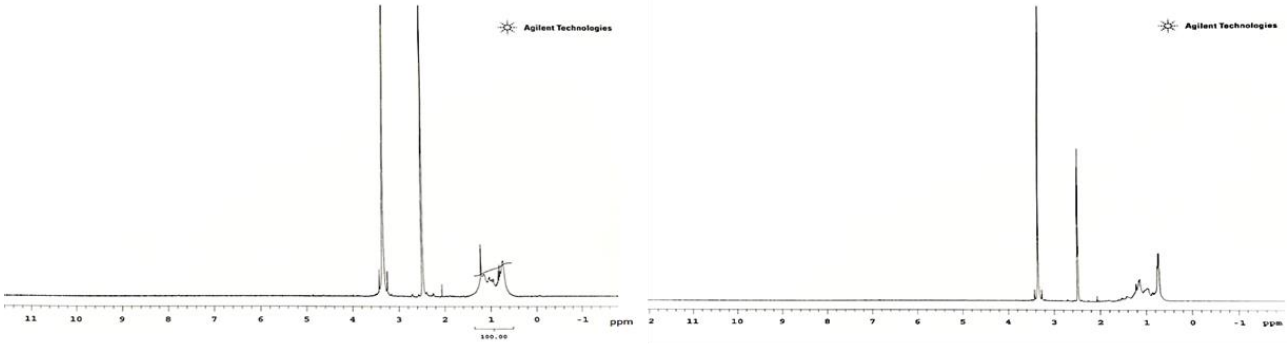

Figure 3. ^{1}H-NMR of hydrogenated squalene using montmorillonite-metal and palygorskite-metal. The hydrogenation in squalane is confirmed by the disappearance of the signals at 5 ppm, characteristic of the vinyl protons of squalene, also by the signals between 0.5 and 2 ppm, characteristic of the aliphatic structure of squalane. Signals at 2.5 and 3.2 ppm are respectively DMSO and water.

3.3 *In vitro* permeability test

In contrast to the very low water solubility of quercetin (0.003 ± 0.001 mg mL^{-1} at 20 °C) determined during the present study and in agreement with the literature (26) (27), and the low solubility of quercetin in glycerin (0.050 ± 0.007 mg mL^{-1} at 20 °C) the solubility of quercetin varied from 3.650 ± 0.011 and 4.012 ± 0.213 mg mL-^{1} at 20 °C, respectively for squalene and squalane (Table 1).

Table 1. Quercetin hydrate solubility in different liquids

Liquid	Solubility / mg mL^{-1} at 20 °C
Water	0.003±0.001
Glycerin	0.050±0.007
Squalene	3.650±0.011
Squalane	4.012±0.213

For the evaluation of the permeation through a membrane, it was decided to select a biomimetic artificial membrane PermeaPlain®, a semi-permeable and cell-free cellulose membrane, that is characterized by the absence of lipids on the surface. The choice felt in prefer the use of an artificial membrane instead of an *ex vivo* tissue to simplify avoiding multiple factors effects. In the last few years, the replacement of *ex vivo* models with artificial membranes is gaining success. Cell-free membranes are easier to implement, have a low cost and easy application (28). The distribution of quercetin in the three compartments is shown in Figure 4. When the quercetin is dissolved in squalene and squalane, respectively the 68% and the 72% is retained within the membrane, while most of the quercetin dissolved in water and glycerin remains on the membrane surface without penetrating, respectively the 85 % and 76 %. When quercetin is dissolved in penetration enhancers, in this case squalene and squalane, it can penetrate in abundance within the skin, mimicked in this study by the membrane. In particular, these results in which the molecule is retained into the membrane, show how thanks to penetration enhancers, molecules are transported and retained into the epidermis, where cosmetic ingredients work. This indicates how, thanks to the use of penetration enhancers, molecule like antioxidants can penetrate deeper, into the epidermis, without remaining on the surface, going beyond the stratum corneum.

The permeated quercetin as a function of diffusion time (Figure 5) was fitted to the Higuchi's equation, calculating the drug diffusion rates of the molecule, reported in Table 2. Quercetin in squalane has the highest drug diffusion rate of 0.164 ± 0.023 (mg cm^{-2} min^{-2}), confirming how squalane is an excellent cosmetic ingredient, thanks to the emollient, moisturizing and antioxidant properties, the high stability and penetration enhancing.

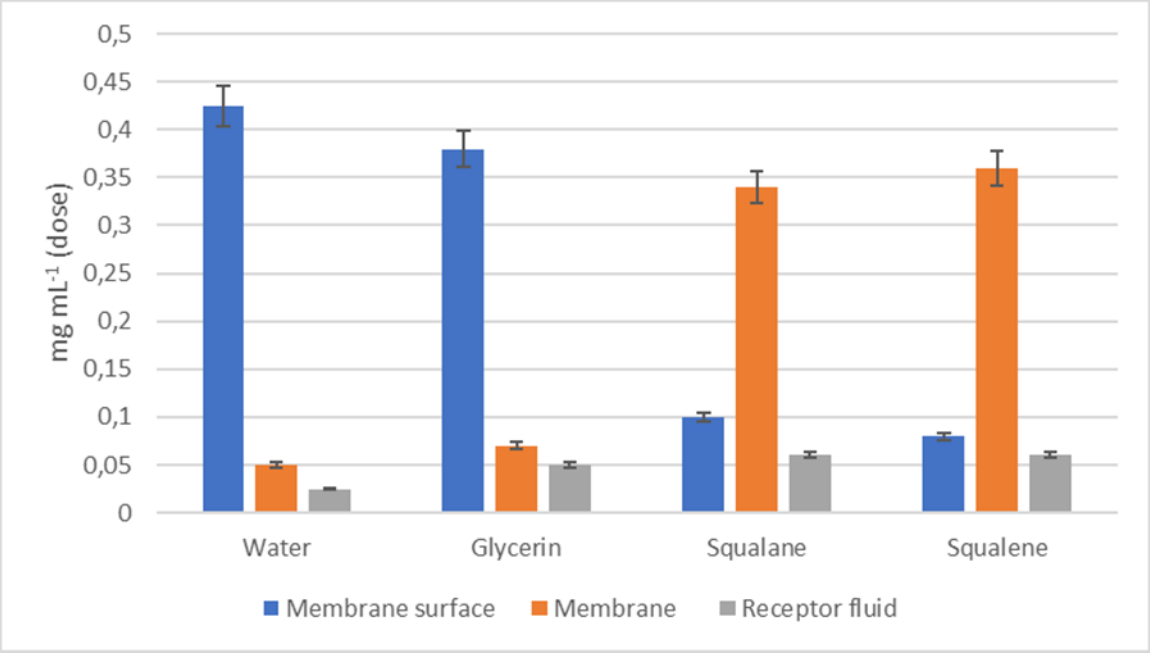

Figure 4: Distribution of quercetin in the 3 compartments of Franz's cells according to the dissolution medium.

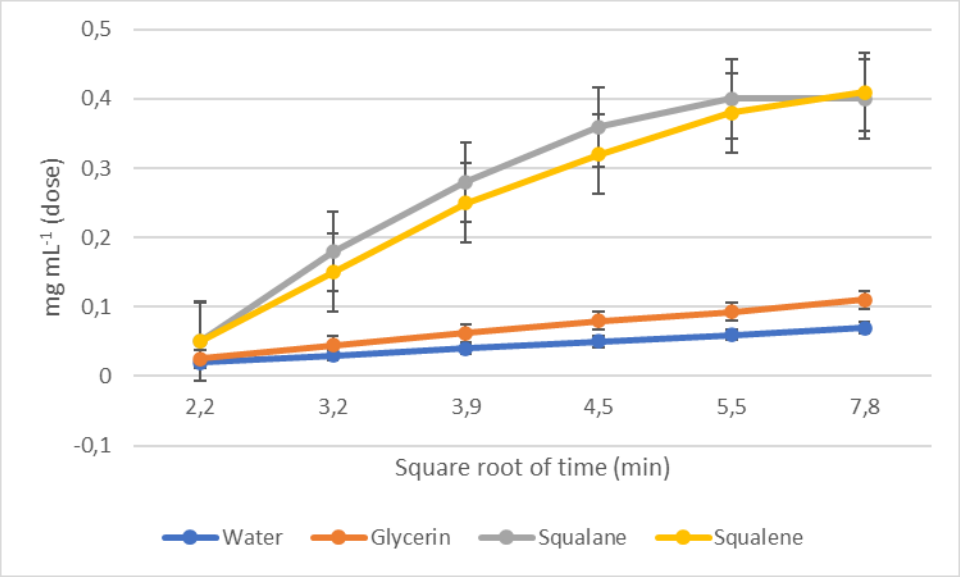

Figure 5. Permeated quercetin as a function of the diffusion time fitted to the Higuchi's equation.

Table 2. Drug diffusion rates of quercetin derived from the Higuchi's equation.

Solutions	Drug diffusion rate (mg cm^{-2} min^{-2})
Quercetin in water	0.028 ± 0.015
Quercetin in glycerol	0.065 ± 0.013
Quercetin in squalene	0.159 ± 0.020
Quercetin in squalane	0.164 ± 0.023

It is reported that the epidermis has a higher amount of antioxidants than the dermis: this is due to the higher exposure of the outermost layer of the skin to harmful effects of UV rays. The skin has a good network of antioxidants, but the ones in the external layers, at the interface between the body and the environment, are the target for UV radiations and other harmful effects like pollutants, and they are rapidly consumed by oxidative damage. A good antioxidant for the topical application on the skin is a molecule that shows a good permeability into the stratum corneum and that can act as reservoir penetrating in the deeper layers of the skin. When this is not possible because of the nature of the molecule, it's possible to enhance the penetration of these molecule thanks to lipophilic molecules, such as squalene and squalane, similar to the lipophilic nature of the stratum corneum (Thiele J. J., 2001) (Abla M. J., 2013) (Marti-Mestres G., 2007).

4. Conclusions

Wine lees are an excellent alternative and ethical source of squalene thanks to the high presence of yeast cells, which are squalene producers. For the catalytic hydrogenation of squalene in squalane, clays can be used to obtain an easy and cheap green approach. The results of this study showed how both squalene and squalane can be also used as penetration enhancers, to improve the absorption of molecules with a low permeability capacity.
This study showed how wine less, that is a wine waste, can be exploited by the cosmetic industries for the recovery of squalene and squalane.

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Chapter 4

Anti-aging cosmetic formulations starting from *H. Crenulata* (thanaka) extracts: in vitro and in vivo evaluation

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Abstract

Thanaka is a Burmese plant typical of the traditional medicine used for skin care and topical application. Coumarins give to this plant beneficial properties for the skin like antioxidant and anti-inflammatory capacity, and anti-acne and anti-age activity. Marmesin is a coumarin, present in thanaka's bark that gives to this plant UV protection, contributing to the anti-age properties. The aim of this work was the evaluation of thanaka's extracts, made by Soxhlet extraction ultrasound-assisted extraction, using different solvents. All the extracts were evaluated in terms of antioxidant capacity using spectrophotometric assays and Folin Ciocalteu's assay. The extracts with the higher antioxidant capacity were then evaluated for the anti-collagenase activity and for the quantification of Marmesin using a High-Performance Liquid Chromatography Coupled with Diode Array Detection (HPLC). The two extracts with the highest antioxidant capacity were then tested with Human Gingival Fibroblasts (HGFs) for a biological evaluation of Cell Viability (MTT Assay), Morphological Analysis, Gene Expression of MMP9 and Col1α1, Antioxidant Capacity and PGEF2 Secretion. Finally, sample extracted with ethanol 70° and Soxhlet extraction method was used for the formulation of a face cream, that was then characterized in terms of density, pH, stability and microbial stability. The formulation cosmetic efficacy and safety were evaluated on 20 volunteers after 30 days of daily use of the cream; specifically, the local compatibility test, Trans Epidermal Water Loss (TEWL), deep and surface hydration, and skin elasticity were assessed. After 30 days, image skin analyzes were also performed for the evaluation of wrinkles and fine lines, redness (hemoglobin), color (melanin) and pores.

1. Introduction

Hesperethusa Crenulata Roem., syn. Naringi crenulata and Limonia acidissima L., with common name of “thanaka”, is an original tropical plant of the Indian subcontinent and Southeast Asia, especially of Myanmar. The Hesperethusa Crenulata or thanaka tree represents, for Burmese people, the source of a yellowish powder that has been used as a traditional skin care product for more than 2,000 years. In Myanmar, people use thanaka grounding the bark, on a circular flat stone, called kyauk pyin, to form a powder wet with water. The formed paste, with a floral sandalwood-like fragrance, is applied on the skin with fingers or with a brush giving a colling sensation, due to the presence of menthol and alcohol (1). Burmese people attribute their beautiful skin to this paste. In fact, thanaka’s paste can prevent acne, smooth the skin, and protect it against sun. It is also used like mosquito repellent and like make up, making drawings on the face, also applied by men. After the application of thanaka’s paste, the water evaporates, leaving the powder on the face (2) (3). In general, the dermatologic uses of thanaka are photoprotection, acne treatment and prevention, skin whitening, skin cooling, make up, anti-wrinkle activity, pruritus relief, scar reduction, and odor prevention (1). The antioxidant capacity of thanaka bark’s extract was confirmed by different studied and has been attributed to the presence of polyphenols, which have high antioxidant power (4) (5) (6) (7) (8). Wangthong et al. (2010) also reported the anti-inflammatory activity of the thanaka bark’s extract, probably given by polyphenols especially coumarins (4) (9). Anti-oxidant and anti-inflammatory activities, together with the demonstrated antimicrobial activity, make this plant an excellent remedy for treating acne (4) (6) (10) (8). Thanaka’s extracts are known for the anti-aging properties. In fact, in addition to the antioxidant properties that protect against premature aging caused by free radicals, it has a skin whitening effect thanks to a mild tyrosinase inhibition activity (4) (11). Tyrosinase is the enzyme responsible for melanin synthesis, so thanaka’s extract, thanks to the presence of arbutin, can prevent pigmentation on the skin, one of the sign of ageing and photoaging (1). The anti-age activity is also given by the collagenase inhibitory activity of thanaka, reported by Ito J. et al (2018) (11). The antioxidant and anti-inflammatory activities have been attributed to the presence of phenolic compounds such as coumarins (12) (13) (14) (9). Coumarins absorb UV-light, and have some pharmacological activities like anti-inflammatory, anti-oxidant, anti-microbial and anti-cancer (15). Among all the coumarins, the most representative is marmesin, a dihydrofuranocumarin, with a chemical structure characterized by chromophore group, the aromatic rings, which allows the absorption of solar energy (16).

Interestingly, although there is a very limited amount of data on the chemical constituents and biological activities of H. crenulata bark, this powder has started to receive increasing attention from many Asian cosmetic companies that have incorporated thanaka bark powder as an ingredient in many of their cosmetic products (4) (8).

The aim of this work is to obtain an extract able to preserve all the beneficial properties of the powder used in the traditional practice and being usable as cosmetic ingredient in anti-aging formulations.

2. Materials and methods

2.1 Thanaka bark

A bark of H. Crenulata from the city of Thandwe, located in western Myammar, was used for this study.

The bark was ground into a powder with a grater and then dried with a ventilated oven (VEC2103/8, Everest, Rimini, Italy) at 40 °C for 24h.

2.2 Reagents and Materials other than thanaka bark, ingredients for the cosmetic formulations and biological assays

To study antioxidant properties and polyphenol content, the reagents used for the tests were: the chemicals 2,2-diphenyl-1-picrylhydrazyl (DPPH); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid(Trolox); 2,2'-Azino-bis(3- ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS); Folin & Ciocalteu's phenol reagent; gallic acid; 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ); hydrochloric acid from which a 40mM solution was prepared were purchased from LB, Sigma-Aldrich® (St. Louis, MO - USA). Manganese oxide (MnO_2) was purchased from Fluka Chemika (Ronkonkoma, N.Y., Buchs, Switzerland); sodium acetate from the company J.T. BAKER (Deventer, Holland); ferric chloride from B.H. Schilling (Bologna, Italy); sodium carbonate for the preparation of a saturated solution and acetic acid were purchased from the company Carlo Erba Reagents (Val de Reuil Cedex). Collagenase enzyme, FALPGA (N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala) collagen mimicking substrate and buffer, all from LB, Sigma-Aldrich® (St. Louis, MO - USA). HPLC analysis was performed using acetonitrile purchased from Carlo Erba Reagents (Val de Reuil Cedex) and methanol with purity >=99.9% from Fisher chemical company (Loughboorough, UK).

Dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) were purchased from Merck Life Science (Milan, Italy); medium and sera for cell cultures, such as Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Euroclone (Milan, Italy); tissue culture-treated plates and flasks were purchased from Falcon®, Corning Incorporated, NY, USA. RNA extraction, DNAse block and RNA quantification were carried out by RNA PureLink® RNA Mini Kit, On-column PureLink® Dnase Treatment and Qubit® RNA BR Assay Kit, respectively (all from Life Technologies, Carlsbad, CA, USA). Reverse transcription was carried out by cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA), quantitative PCR through PowerUpTM SYBRTM Green Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA); Col1α1, MMP9 and GAPDH primers genes were all purchased from Merck Life Science (Milan, Italy). ELISA kits to read Col1 and PGE2 secretion were purchased from Cosmo-Bio Co., (Tokyo, Japan) and Enzo Life Sciences (Farmingdale, NY, USA), respectively. The cell antioxidant activity was measured through Total Antioxidant Capacity Assay Kit (Merck Life Science, Milan, Italy).

Ultrapure water was obtained through a Milli-Q ultrafiltration system (Millipore, Rome, Italy); ethanol was purchased by Carbo Erba Reagents (Val De Reuil Cedex, France) and ethyl acetate was purchased by Sigma-Aldrich (Stenheim, Germany).

Marmesin analytical standard was purchased by Phytolab GmbH & Co, (Vestenbergsgreuth, Germany).

2.3 Ultrasound-assisted extraction (UAE)

For the ultrasound-assisted extraction (UAE), an ultrasonic processor (US, Sonicator Q500, Qsonica, Newton, CT, USA), with 19-mm probe made of titanium, was used. An appropriate amount of dried thanaka powder (1 g) was weighted and added to 100 ml of the solvent (water, ethanol 30, 50 or 70°, or mixtures of ethanol and ethyl acetate 50:50) in a 250 mL beaker, and then placed in an ice bath. Samples were sonicated at a frequency of 20 kHz for 5 minutes, controlling the energy input by setting the amplitude at 95%, considering the total energy of the instrument (500 W). Samples are named and listed in Table 1.

Each extract was filtered with filter paper, and the solvent (ethanol and/or ethyl acetate) removed under reduced pressure at 40 ° C (IKA RV8, RM-Solutions, Napoli, Italy); the residual humidity was removed under lyophilization at -53 °C for 24 h at 0.03 millibar (FreeZone, LABCONCO, Kansas City, MO, USA). The yield percentage of the extracts was calculated.

Table 1. Summarization of the samples obtained under Ultrasound-assisted or Soxhlet extractions.

Solvent Mixtures	Samples		Parameters	
			Heat level	Extraction cycles
Ethanol 30°	1UAE	1SE	19	5
Ethanol 70°	2UAE	2SE	18	5
H_2O	3UAE	3SE	20	5
Ethanol 50°	4UAE	4SE	19	5
EtOH: EtOAc 50:50	5UAE	5SE	15	10

2.4 Soxhlet extraction (SE)

The Universal Extractor E-800 Extractor (Büchi, Flawil, Switzerland) was used to perform the Soxhlet extraction. On the basis of preliminary essays, three extraction parameters were kept constant for all the extractions: maximum valve opening rinsing time (5 minutes), and drying time (10 minutes). The other parameters, such as the solvent mixture, the heat level, and the extraction cycles, were adapted to the solvent mixture (Table 1). Dried thanaka (3 G) was placed in the filter paper chamber and 250 ml of solvent were used; once the extraction completed, each extract was filtered with filter paper, and the solvent removed under the same conditions previously described for the UAE. The yield percentage of the extracts was calculated.

2.5 Antioxidant capacity (AC)

The antioxidant capacity of the samples was evaluated by measuring 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging activity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS•+) radical scavenging capacity and Ferric reducing antioxidant capacity (FRAP). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used for the calibration curve, like standard for each assays. Results were expressed in IC50, that is the concentration of the samples required for the decrease of 50% the initial concentration of DPPH, ABTS and iron, and in mg/g Trolox equivalent antioxidant capacity (TEAC) of samples.

2.5.1 Scavenging of diphenyl-picrylhydrazyl (DPPH) radical

In a 96-well plate, 100 µL of each sample (1 mg/ml) per well were added and a positive control was made with a solution of Trolox in ethanol at a concentration of 0.1 mg/ml. A serial dilution (1:2) of 50 µL with ethanol was made. Finally, DPPH reagent, (150 µL) previously prepared dissolving 1.2 mg of DPPH in 30 ml of ethanol, was added. The reaction takes place in the dark at 37° and after 30 minutes of incubation, the absorbance is read on the spectrophotometer (FLUOstar Omega, BMG Labtech GmbH, Ortenberg, Germany) at a wavelength of 517 nm. All measurements were performed in triplicate.

2.5.2 Scavenging of (2,2'-azinobis(3-etilbenzotiazolin-6-solfonato) (ABTS) radical

ABTS reagent was prepared incubating for 20 minutes 9.8 mg of ABTS and 0.6 g of MnO2 in water. The solution was filtered and then 1 ml was added in 30 ml of water. In a 96-well plate, 100 µL of each sample (0.25mg/ml) were added per well and a positive control was made with a solution of trolox (0.1 mg/ml). A serial dilution (1:2) of 50 µL with ethanol was made. Finally, ABTS reagent (150 µL) was

added. The reaction takes place in the dark at 37° for 30 minutes, after which the absorbance is read on the FLUOstar Omega spectrophotometer at a wavelength of 734 nm. All measurements were performed in triplicate.

2.5.3 Scavenging of (2,4,6-tris(2-pyridyl)-s-triazine) (FRAP) radical

2.46 mg of sodium acetate were added in 80 ml of distilled water, for the preparation of an acetate buffer. The solution has been acidified with acetic acid until pH 3.6 and made up with water, up to 100 ml. 15.6 mg of TPTZ were added in 5 ml of HCl 40 mM and 16.2 mg of FeCl3 were added in 5 ml of HCl 40 mM. These two solutions were added to 50 ml of buffer acetate to obtain FRAP reagent. In a 96-well plate, 50 µL of each sample (0.75 mg/ml) per well were added and a positive control was made with a solution of trolox (0.1 mg/ml). A serial dilution (1:2) of 25 µL with ethanol was made. Finally, FRAP reagent (175 µL) was added. The reaction took place in the dark at 37° for 60 min, after which the absorbance was read on the FLUOstar Omega spectrophotometer at a wavelength of 593 nm. All measurements were performed in triplicate.

2.6 Total phenolic content (TPC)

The amount of total phenolic compounds in each extract was determined with the Folin–Ciocalteu assay. In a 96-well plate, 100 µL of sample (0.5 mg/ml) were added per well and a positive control was made with a solution in water of gallic acid at a concentration of 0.125 mg/ml. A serial dilution (1:2) of 50 µL with water was made. 150 µl of a solution, previously prepared adding 6 ml of Folin's reagent in 24 ml of water and 50 µL of saturated Na_2CO_3 solution were added to each well. The reaction takes place in the dark at 37° for 30 min, after which the absorbance is read on the FLUOstar Omega spectrophotometer at a wavelength of 734 nm. The results were expressed in mg of gallic acid equivalents per g of dried matrix (mg GAE/g). All measurements were performed in triplicate.

2.7 Determination of anti-collagenase activity

The activity of collagenase enzyme against FALPGA (N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala) substrate, which mimics collagen, was compared in presence or absence of H. Crenulata's extract. In detail, we focused on the samples 4UAE and 2SE. In one well, 5 µL of collagenase and 95 µL of buffer were plated. In the other wells, 5 µL of collagenase, 5 µL of the sample (1 mg/ml) and 90 µL of buffer were added. 100 µL of reaction mix (60 µL buffer and 40 µL FLPGA) were added to each well. The samples were read with the FLUOstar Omega spectrophotometer at a wavelength of 345 nm at a temperature of 37 °C and at different time intervals (2, 5, 15, 30 and 60 min). Finally, the percentage inhibition of collagenase enzyme was calculated.

2.8 Quantification of marmesin by High-Performance Liquid Chromatography Coupled with Diode Array Detection

The extracts were analyzed for marmesin content, the substance which allows the UV rays protection (16). The identification and quantification of marmesin were carried out using an HPLC Agilent (1200 Series chromatograph), coupled with a diode array detector (DAD). Chromatographic separation was made using a reverse phase column Gemini C18 column (250 × 3.0 mm, 5 µm), (Phenomenex, Torrance, CA, USA). The column temperature was set at 40 °C. The analysis was performed in presence of an elution gradient, with a mobile phase A of water and a mobile phase B of acetonitrile in a ratio of 60:40 at a constant flow rate of 0.6 mL/min. The volume of a single injection was 0.1 µl for the standard, and 1 µl for the samples. Sample preparation involves solubilization of 1 mg of matrix in 1 ml of methanol, 5 minutes of sonication through an ultrasonic bath for the complete solubilization, centrifugation for 10 minutes at 13000 rpm, and finally filtration with a 0.45 um filter (Sartorius

Biotech, Gottingen Germany) to remove undissolved particles. The first injection is that of the reference standard, in our case marmesin, after which, having found the retention time, the samples are passed. The quantitative analysis was performed using a linear calibration curve prepared with the marmesin standard.

2.9 Biological in vitro evaluation

2.9.1 HGF culture and cell treatment

HGFs were obtained from gingival biopsies derived from third molar extractions (17). The Local Ethical Committee of the University of Chieti (Chieti, Italy; approval number 1173) approved the project on 31/03/2016. The cell culture was maintained at 37° C in a humidified atmosphere with CO2 5%. Cells were cultured in DMEM high glucose with 10% of fetal bovine serum (FBS) and 1% of antibiotics (penicillin/streptomycin) and used at passages 5-12 for the following experimental procedures.

HGFs were seeded in a 96-well tissue culture-treated plate at density of 6700 per well. The day after seeding 2S and 4UAE extracts, previously dissolved in culture medium (solutions 1mg/ml) were administered at 0.5, 1, 2 and 4% dilutions up to 72 h. Cells receiving basal medium without extracts were established as control sample (Ctrl). After 24 h of treatment with extracts 2S and 4UAE at 2% and 4% phase contrast images were acquired with an inverted light microscope Leica DMI1 (Leica Cambridge Ltd., Cambridge, UK Leica) equipped with a camera Leica MC120 HD (Leica Cambridge Ltd., Cambridge, UK).

2.9.2 Metabolic activity assay (MTT test)

After 24 and 72 h of treatment, the metabolic activity of cells exposed to 2s and 4UAE extracts at 0.5, 1, 2 and 4% dilutions, was evaluated by means of MTT test (Merck Life Science, Milan, Italy). HGFs were probed in the presence of 100 µl/well of MTT (0.5 mg/ml), diluted 1:10 within DMEM, for 5 h within an incubator. Then, the medium containing MTT was discarded and replaced by an equal volume of DMSO to solubilize formazan crystals.

The final solution absorbance was spectrophotometrically read at 540 nm wavelength by a spectrophotometer (Multiskan GO, Thermo Scientific, Waltham, MA, USA). The experiment was repeated three times and each time in six biological replicates. The metabolically active cells were expressed as percentage by normalizing with values of Ctrl cells, set as 100%.

2.9.3 RNA extraction

RNA extraction was assessed by RNA PureLink® RNA Mini Kit (Life Technologies, Carlsbad, California, USA) following the manufacturer's instructions. Briefly, after 24 of culture in the presence of 2S and 4UAE extracts at 2% and 4% dilutions within the culture medium, cells were detached and centrifuged. 300 µL of supplied lysis buffer, added with 1% of 2-mercaptoethanol, were added to each pellet, vortexed and passed 10 times through an 18-gauge needle. After that, for each experimental sample, an equal volume of ethanol 70% was added to the previous solution and the whole volume was then transferred into the Spin Cartridge for RNA extraction and purification. In order to remove contaminating DNA, samples were incubated in the presence of DNAse mixture (On-column PureLink® Dnase Treatment, Life Technologies) for 15 min at room temperature. RNA was finally eluted in 30 µL of Nuclease-Free Water, RNA concentration, expressed as ng/µL, was obtained by Qubit® RNA BR Assay Kit (Life Technologies, Carlsbad, CA, USA).

2.9.4 Reverse transcription (RT) and real-time RT-polymerase chain reaction (real-time RT-PCR)

The high-capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) was applied to reverse transcribe 300 ng of RNA. Reactions were performed in volumes of 20 µL and then incubated in a thermal cycler at 25 °C for 10 min, 37 °C for 2 h, 85 °C for 5 min, concluding at 4° C. Quantitative PCR was applied to determine gene expression by means of PowerUpTM SYBRTM Green Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA). Each reaction of amplification was performed in a MicroAmp® Optical 96-weel Reaction Plate (Life Technologies, Carlsbad, CA, USA); 20 µL was established as final volume consisting of 10 µL of SYBR Green, 1 µM of each primer (stock solution 10 µM), 10 ng of cDNA and Nuclease-Free Water. The sequences of forward and reverse primers are reported in Table 2.

The run method consisted of the following steps: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min in QuantStudio 3 (Thermo Fisher Scientific, Waltham, MA, USA). Obtained data were elaborated by QuantStudio™ Design & Analysis Software v1.5.1 (Thermo Fisher Scientific, Waltham, MA, USA). The analysis of the melt curve confirmed authenticity of PCR products. The investigated genes (Col1α1 and MMP9) fold changes were measured in relation to GAPDH gene expression, used as housekeeping. The comparative ΔΔCT method (relative quantification) was applied to calculate the fold change of Col1α1 and MMP9 genes and expressed in relation to Ctrl.

Table 10. Primers sequences for quantitative RT-PCR

Primer	Sequence (5'-3')	Reference
GAPDH-FW	GGGTGTGAACCATGAGAAGTA	Primer blast
GAPDH-RW	ACTGTGGTCATGAGTCCTTC	Primer blast
Col1α1-FW	CAGGCTGGTGTGATGGGATT	Primer blast
Col1α1-RW	GGGCCTTGTTCACCTCTCTC	Primer blast
MMP9-FW	CGACGTCTTCCAGTACCGAG	Primer blast
MMP9-RW	GTTGGTCCCAGTGGGGATTT	Primer blast

2.9.5 Collagen1 (Col1) and Prostaglandin E2 (PGE2) secretion

A human Col1 (Cosmo Bio Co., Ltd., Tokyo, Japan) and a human PGE2 (Enzo Life Sciences Farmingdale, NY, USA) ELISA kits were used to determine the amount of Col1 and PGE2 secreted in the culture medium after treatment of HGFs with 2S and 4UAE extracts at 2 and 4% dilutions, after 48 h for Col1 and after 24 and 48 h for PGE2, following the manufacturers' instructions. The absorbance of the final solutions was spectrophotometrically read at 450 nm and at 405 nm wavelengths (for Col1 and PGE2, respectively) by a microplate reader (Multiskan GO, Thermo Scientific, Inc., Waltham, MA, USA). The concentration of Col1 (µg/mL) and of PGE2 (pg/ml) was calculated after having generated for both the two kits a standard curve with provided standards and then normalized with the OD values of each sample obtained from MTT test.

2.9.6 Cell antioxidant capability measurement

The total antioxidant capability of HGFs exposed to 2SE and 4UAE extracts at 2 and 4% dilutions after 24 and 48 h of treatment was evaluated through Total Antioxidant Capacity Assay Kit (Merck Life Science, Milan, Italy) following the manufacturer's instructions. Briefly, 100 µL of supernatant, collected from each experimental sample, were added into wells, then 100 µL of Cu2+ Working Solution were added to each well. The reaction was incubated for 90 minutes at room temperature in the dark and the absorbance of the final solution read at 570 nm though a microplate reader

(Multiskan GO, Thermo Scientific, Inc., Waltham, MA, USA). The values obtained from the provided Trolox Standards were used to plot a standard curve. The total antioxidant capability was expressed in nmole of Trolox per µl.

2.9.7 Statistical analysis

Statistical analysis was performed by one-way ANOVA, through Prism 5.0 software (GraphPad) followed by Dunnett's post-hoc test. Statistically significant values were established for $p < 0.05$. Each experiment has been performed in triplicate.

2.10 Formulation of a face cream with thanaka's extract

A cosmetic emulsion for face containing thanaka's extract was formulated. For the formulation, the lyophilized extract (INCI: limonia acidissima extract) was used together with the other ingredients listed in Table 3. The ingredients were weighted, and five different phases were prepared. The phase A (aqueous phase) and the phase B (oily phase) were heated at 70 °C, and then the oily phase was added to the aqueous phase under stirring. For the homogenization, the stirring was performed by an high speed stirrer (IKA™ EUROSTAR 20 High Speed Digital Overhead Stirred, Staufen Germany), with a speed of 3000 rpm. When the emulsion reached the temperature of 40 °C, the phases C and then D were added. The phase E was added when the formulation was completely cold (room temperature).

Table 3. List of the ingredients used for the formulation of the face cream containing thanaka's extract.

Phase	Commercial Name	INCI	Supplier
A	Water	Aqua	Deionized water was internally produced (G3 RO CUBIC-S2 demineralizer, Gamma3, Castelverde, Italy).
	Glycerol	Glycerin	Acef, Fiorenzuola d'Arda (PC), Italy
	KELCO-CARE™ Diutan Gum	Sphingomonas Ferment Extract	Biochim s.r.l., Casarile (MI), Italy
	Dermofeel® PA-12	Sodium phytate	Evonik, Essen, Germany
B	TEGO® Care PS MB	Methyl Glucose Sesquistearate	Evonik, Essen, Germany
	Cutina® GMS V	Glyceryl stearate	BASF, Ludwigshafen, Germany
	Lanette® O	Cetearyl alcohol	BASF, Ludwigshafen, Germany
	CETIOL® C5	Coco-caprylate	BASF, Ludwigshafen, Germany
	Grapeseed oil	Vitis vinifera (grape) seed oil	Acef, Fiorenzuola d'Arda (PC), Italy
	Cetiol® CC	Dicaprylyl carbonate	BASF, Ludwigshafen, Germany
C	Vitamin E acetate	Tocopheryl acetate	BASF, Ludwigshafen, Germany
	E-leen 8	Caprylyl glycol, glycerin, water	Active up, Milano, Italy
D	Thanaka extract	Limonia acidissima extract	
E	Perfume	Parfum	Farotti s.r.l., Rimini, Italy

2.11 Physiochemical Characterization of the formulation

The formulation was analyzed for pH with a pHmeter 60 Violab (pHmeter Violab, Bormac, Carpi, Italy), equipped with an electrode specific for cosmetic applications (XS Sensor Flow S7 Ag/AgCl, Bormac, Carpi, Italy). The density of the emulsion was analyzed by measuring its volume in a glass cylinder and weighing the formulation.

The stability of the formulation was determined in terms of both accelerated, and long term stability. For the accelerated physiochemical stability, a sample of the emulsion was centrifuged at 4000 rpm at 20 °C for 30 min (Micro-centrifuge Scilogex D3024R, Rocky Hill, CT, USA). Another amount of the formulation was also placed alternatively at 4 and 40 °C for three times, subjected to a temperature shock. For the long term stability, a reference sample was left at room conditions for 1 year. The emulsion was considered stable when no changes in organoleptic characteristics or phase separation occurred.

2.12 Challenge Test

The evaluation of microbial contamination of the products was performed with a challenge test, according with the method ISO 11930:2012, "Cosmetics, Microbiology, Evaluation of antimicrobial protection of a cosmetic product". The test was followed for 28 days and the antimicrobial activity was screened against Pseudomonas aeruginosa ATCC 9027, Staphylococcus aureus ATCC 6538, Candida albicans ATCC 10231, Aspergillus brasiliensis ATCC 16404 and Escherichia coli ATCC 87394, according to the Regulation CE 1223/2009. E. coli, S. aureus, and P. aeruginosa were incubated for 48–72 h at 30 °C on Tryptic Soy Agar (TSA) (Sigma Aldrich, Stenheim, Germany), C. albicans was incubated for 48–72 h at 30 °C on a Sabouraud Dextrose Agar (SDA) (Sigma Aldrich, Stenheim, Germany), and A. brasiliensis was incubated for 72–120 h at 22.5 °C on a Potato Dextrose Agar (PDA) (Sigma Aldrich, Stenheim, Germany). Initial inoculi were respectively 700,000, 480,000, 300,000, 41,000, and 9000 UFC/g for E. coli, S. aureus, P. aeruginosa, C. albicans, A. brasiliensis.

2.13 Local Compatibility test with Human Skin (Irritant Potential)

The irritant potential of the cosmetic formulation was evaluated, with a Local Compatibility Test, under normal conditions of use, according to the Helsinki Declaration (64th WMA General Assembly, Fortaleza, Brazil, October, 2013) and to the COLIPA guidelines. 20 volunteers, male and female (age 27 ± 4 years old), were used like a panel. The formulation was left in contact with the skin of the volunteers for 48 h (model Curatest® F, adhesive strips for patch test, Lohmann and Rauscher International, Rengsdorf, Germany) in a sufficient amount to fill a test disk of 1 cm2. The skin reactions were evaluated 15 min and 24 h after patch removal, evaluating the appearance of erythema, desquamation, oedema and vesicles. The test, made under the direction of a medical doctor certified in dermatology, was performed in single blind mode.

2.14 Measurement of the Trans Epidermal Water Loss (TEWL)

The efficiency of the skin barrier was evaluated with the measurement of the Trans Epidermal Water Loss (TEWL), before and after 15 and 30 days of daily use of the face cream, morning and night. The analysis was conducted using a VapoMeter® (Delfin Technologies, Kuopio, Finland), that evaluates the humidity through a closed chamber unaffected by ambient airflows. The evaporation rate (g/m2h) was calculated from the increase of the relative humidity (RH), measured by a sensor. The analysis was conducted on 20 volunteers, male and female (age 25-55), in a room with controlled humidity and temperature (56.0±5.0 % and 21.0±1.0 °C). Six measurements for each time point were taken: one on

the forehead, one on the chin, and two for each cheek. The first measurement was taken before the application of the cream (t0), and the other measurements after 15 days (t1) of daily use and 30 (t2) days of daily use. The measuring time was around 10 s.

2.15 Measurement of the skin hydration

The skin hydration was evaluated in terms of deep skin hydration and skin surface hydration. The instrument MoistureMeterEpid® (Delfin Technologies, Kuopio, Finland) was used for the evaluation of the deep skin hydration, considering the water content in the epidermal layer. The skin surface hydration, related to the content of water in the stratum corneum, was evaluated using the instrument MoistureMeterSC® (Delfin Technologies, Kuopio, Finland).

For the measurement of the deep hydration, the MoistureMeterEpid® was placed on the area and the instrument measures the local percentage water content in the epidermis, showing the percentage water content on the display, ranging from 0 to 100%. The instrument generates a low electromagnetic wave with high frequency of 300 MHz on the tissue and the obtained dielectric constant is proportional to the water content on the area of the tissue, converted to the Percentage Water Content (PWC 0-100%).

For the measurement of the skin surface hydration, the MoistureMeterSC® was used, measuring the hydration in the stratum corneum (SC). The instrument uses an electromagnetic field of 1.25 MHz, penetrating through the skin, to measure the dielectric constant of the skin, revealed by a sensor. The dielectric constant is given by the moisture held in the stratum corneum and the thickness of the stratum corneum's dry layer. The skin surface hydration is in fact a function of this two variables.

The measurement principle of the instrument is based on the layered capacitive structure of the skin. The capacitor plates are formed from the probe and the highly conductive epidermal layer, while the dry later of the stratum corneum acts as an insulator. When measuring with an electromagnetic field penetrating through the skin, upper-most layer is predominantly capacitive, while the deeper layers are conductive. Since the dielectric constant of water is much higher than that of solid compartment of the SC, the dielectric constant of the SC is mainly dependent on its water content. The measurement depth of the instrument is determined by the thickness of the stratum corneum's dry layer, and the measurement is equal to the thickness of the dry layer of the skin. The measuring time was around 4 s. The value is expressed in Skin Hydration (SH). Both the analyses were conducted on 20 volunteers, male and female (age 25-55). Six measurements for each time point were taken: one on the forehead, one on the chin, and two for each cheek. The first measurement was taken before the application of the cream (t0), and the other measurements after 15 days (t1) and 30 days (t2) of daily use, morning and night (2 applications per day).

2.16 Measurement of the skin elasticity

The analysis of the skin elasticity was conducted with the instrument ElastiMeter® (Delfin Technologies, Kuopio, Finland), before and after 15 and 30 days of daily treat with the cream. The instrument evaluates the elasticity of the skin, analysing the deformation of the skin surface thanks to a 0.3 mm indenter placed in a plate that is pressed against the skin, without altering the structure of the skin. The instant skin elasticity is determined by a sensor, that measures the force required to produce the deformation on the skin by the indenter, expressed in stiffness (N/m). The analysis was conducted on 20 volunteers, male and female (age 25-55). Six measurements for each time point were taken with a skin contact of 0.5 seconds: one on the forehead, one on the chin, and two for each cheek. The first measurement was taken before the application of the cream, and the other measurements after 15 days (t1) of daily use and 30 (t2) days of daily use. The measuring time was around 10 s.

2.17 Image skin analysis

3D photos for the evaluation of pores, wrinkle, fine lines, redness (hemoglobin) and color (melanin) were carried out by Antera 3D® Camera (Miravex, Dublin, Ireland), a device with a camera for pictures acquisition for the analysis of the skin. The measuring area of the camera is 5.6 X 5.6 cm and the camera opening is placed directly onto the skin, without excessive pressure, so the acquisition of the images are not affected by the surrounding lighting conditions. The acquired images were converted into data for the evaluation of wrinkles, fine lines, and pores. With the multispectral analysis, the instrument measures the content of the 2 pigments, hemoglobin and melanin, related to the redness and to the pigmentation of the skin. Data were analyzed by the Antera software.

The analyzes were conducted on 20 volunteers, male and female gender (age 25-55). Acquisitions were performed on the forehead, on the cheeks and on the periorbital areas, before the application of the cream (t0) and after 15 (t1) and 30 days (t2). Triplicate measurements were performed.

3. Results and discussions

3.1 Characterization of the extracts

The yields of the lyophilized extracts for the different thanaka's batches are summarized in Table 4. Comparing the ultrasound and soxhlet extraction techniques, the sample 4UAE shows a better performance in terms of yield (7.74 %); however, the soxhlet yield values of the sample 2SE is very similar (6.90 %). Both techniques are efficient, these, in fact, are the most widely used even at the industrial level. Focusing on solvent mixtures, ethanol 70° and 50° are the most effective, for both UAE and SE in terms of yield.

Table 411. Summary of the characteristics of the thanaka's extracts.

	Yield (%)	DPPH TEAC (mg/g)	ABTS TEAC (mg/g)	FRAP TEAC (mg/g)	Total Phenol Content (TPC) (mgGAE/g)	Anticollagenase assays (inhibition %)	Marmesin content (μg/g DW)
ULTRASOUND ASSISTED EXTRACTION (1g/100 ml)							
1UAE	5.86 ± 0.98	1.52 ± 0.15	38.67 ± 9.89	1.48 ± 0.44	5.11 ± 0.21		
2UAE	7.43 ± 1.35	3.70 ± 0.78	82.39 ± 11.34	3.29 ± 1.22	7.71 ± 0.31		268.39
3UAE	4.12 ± 0.78	0.39 ± 0.06	13.17 ± 5.44	0.96 ± 0.09	3.30 ± 0.15		
4UAE	7.74 ± 0.99	3.28 ± 0.95	98.53 ± 9.89	3.96 ± 0.76	9.83 ± 0.66	16.08 ± 2.33	176.43
5UAE	4.25 ± 0.65	2.61 ± 0.56	44.28 ± 12.77	2.40 ± 0.74	1.42 ± 0.78		
SOXHLET EXTRACTION (3g/250 ml)							
1SE	3.95 ± 0.21	3.27 ± 0.70	45.05 ± 9.87	2.50 ± 0.77	3.48 ± 0.80		
2SE	6.90 ± 0.54	4.12 ± 0.44	101.94 ± 18.93	4.97 ± 0.23	6.14 ± 1.29	16.12 ± 0.76	480.77
3SE	1.20 ± 0.09	0.15 ± 0.07	2.62 ± 0.99	0.28 ± 0.02	1.04 ± 0.12		
4SE	5.90 ± 0.01	3.57 ± 0.43	57.82 ± 2.67	3.95 ± 0.09	4.71 ± 0.89		360.58
5SE	4.48 ± 0.12	1.68 ± 0.33	15.31 ± 3.35	1.96 ± 0.25	0.52 ± 0.05		

3.2 Antioxidant activity

The antioxidant capacity of thanaka extracts was evaluated with three different assays, namely DPPH, ABTS and FRAP. The results obtained are expressed in TEAC (mg equivalent of the reference substance, the trolox, per g of dried matrix). Results are summarized in Table 5.

The extract 2SE, obtained by the Soxhlet's method with ethanol 70° shows excellent values for all the three assays. In detail, in DPPH assay, TEAC value is 4.12 mg/g for the extract 2SE and 3.70 mg/g for the extract 2UAE. Also with ABTS assay, the extract 2SE gives the best result, with a TEAC of 101.94 mg/g, while as regards the extracts with ultrasound the best value is obtained with the 4UAE sample, 98.53 mg/g. The last assay performed was FRAP where the highest TEAC value is again from the extract 2SE with 4.97 mg/g of trolox equivalents and again with of the extract 4UAE with 3.96 mg/g of Trolox equivalents.

3.3 Total phenolic content (TPC)

The results of the TPC are summarized in Table 5. Also in the case of polyphenol content, the best results are obtained for the samples 4UAE and 2SE, respectively with 9.83 mg GAE/g and 6.14 mgGAE/g.

3.4 Anti-collagenase activity

The anti-collagenase activity was evaluated only for the two samples exhibiting the best antioxidant activity (4UAE and 2SE). The samples 4UAE and 2SE showed, respectively 16.08% and 16.12% of collagenase inhibition. Results show that the samples have anti-collagenase activity confirming the anti-collagenase activity reported in literature (11).

Collagenases are metalloproteinases capable to degrade other molecules such as collagen, which is the responsible for the tensile strength of the skin, and it is produced by fibroblasts. Photoaging affects fibroblasts and induces the expression of collagenases, resulting in wrinkles and change in thickness. Thanaka's extract can inhibit collagenases, protecting the skin from collagen degradation, and this property, together with the antioxidant activity, acts against the aging caused by ROS (18).

3.5 Quantification of marmesin in thanaka's extracts with HPLC-DAD

Marmesin is an important molecule for the properties of thanaka. In fact, it is a bioactive compound extracted for the first time by Joo and colleagues in 2004 (16) which determined the molecular structure and found that marmesin has UV-absorbing chromophores that absorb UVA radiations. Our results confirm the sun-protective property of thanaka, which also contributes to the anti-aging action of the extracts in cosmetic applications.

Quantification of marmesin was performed in the best extracts in terms of yield and antioxidant activity: 2UAE, 4 UAE, 2SE and 4SE. Results are reported in Table 5. The higher marmesin content was observed for the extract 2SE, with a value of 480.77 µg/g Dry Weight (DW).

3.6 Biological evaluation on HGFs

Based on the above reported results, the two extracts with better performances, 2SE and 4UAE, were selected to be included in an in vitro model evaluation represented by primary HGFs exposed up to 72 h to the mentioned extracts. Firstly, a metabolic activity test, after 24 and 72 h of treatment, was assessed in order to select the better concentrations for each extract to be applied in further and more specific experiments. MTT test was carried out administering extracts 2SE and 4UAE at percentages ranging from 0.5 to 4% in the culture medium starting from an initial solution of 1mg/ml. For 2SE extract MTT results disclosed that, after 24 h of treatment, all tested percentages of the abstract are able to guarantee a percentage of metabolically active cells comparable to untreated cells, assumed as control of the experimental model. Similarly, for extract 4UAE percentages 1, 2 and 4% do not affect cell viability whereas a slight decrease, compared to control, is detectable in HGFs exposed to 4UAE at 0.5% (Figure 1A). After 72 h of extracts administration, all tested percentages, for both 2SE and 4UAE, keep levels of viable cells higher than 91%, thus comparable to values recorded for Ctrl (Figure 1B). These preliminary results clearly revealed that all tested percentages are totally tolerated by HGFs without affecting cell viability. Thus, based on these evidences, the highest concentrations (2% and 4%) for both the two extracts, were selected for further experiments.

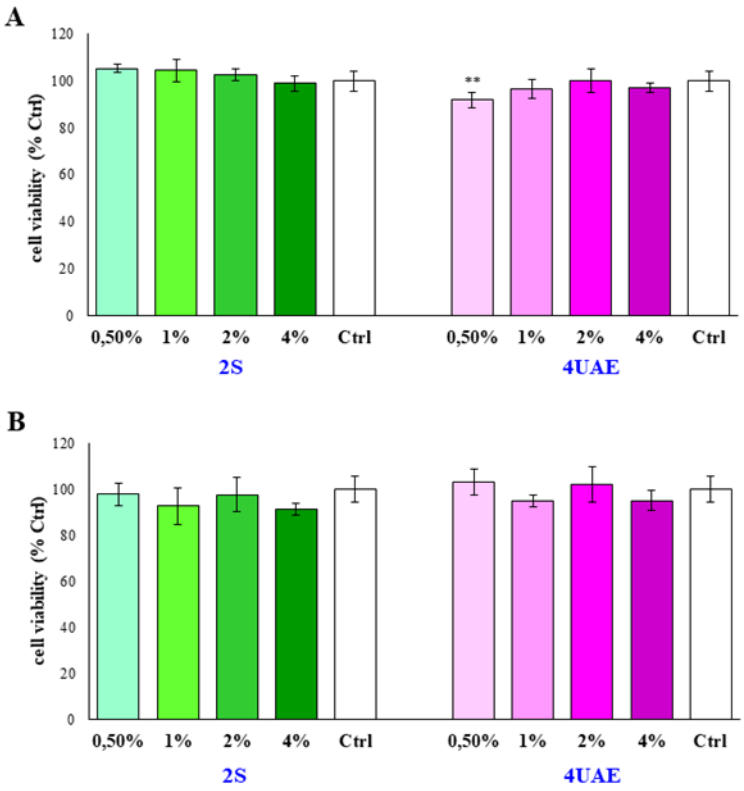


Figure 1. MTT test performed on HGFs exposed to 2SE and 4UAE extracts at 0.5, 1, 2 and 4% for 24 h (A) and 72 h (B). Cells treated with basal culture medium were assumed as control (Ctrl). Each bar represents the cell viability ± standard deviation, expressed as % Ctrl. The most representative of three independent experiment is shown. ** vs Ctrl p=0,005.

Then, a morphological analysis of HGFs exposed to 2SE and 4UAE at 2% and 4%, after 24 h of treatment, was assessed by means of a phase contrast observation. The morphological analysis accurately reflects the MTT results disclosing, for all experimental samples, viable cells perfectly adherent to the plate surface, the morphology is elongated with very long cytoplasmic extensions. Moreover, in the Ctrl sample some floating dead cells are detectable (black arrows) which are not identified in the extracts-treated samples thus underlining again the beneficial effect of tested extracts on cell metabolism and, as consequence, on cell viability (Figure 2).

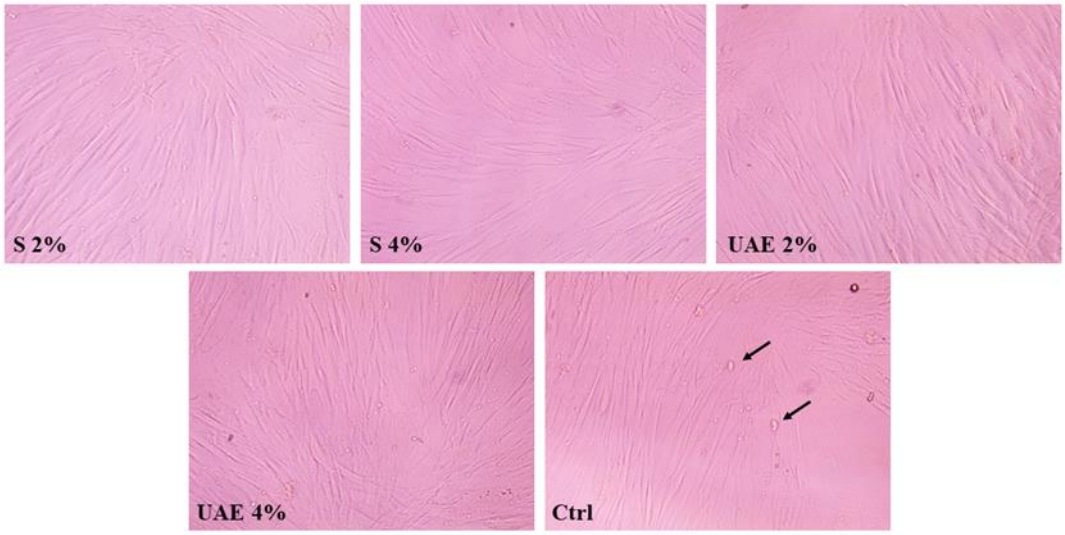


Figure 2. Phase contrast observation of HGFs exposed to 2SE and 4UAE extracts, both at 2% and 4%, after 24 h of treatment. Ctrl: untreated cells. Black arrows indicate dead cells detached from the plate surface; magnification 20x.

Fibroblasts are highly and continuously involved in the synthesis and release of Col1, the most abundant protein secreted by them, thus allowing a correct adhesion process and, at the same time, the renewal of collagen fibers belonging to the extracellular matrix (ECM) (19). In turn, collagen fibrils start to be fragmented due to increasing activity of zinc dependent endopeptidases, namely matrix metalloproteinases (MMPs), synthetized by fibroblasts. After an initial cleavage of Collagen fibrils by MMP-1, several isoforms of the MMP family (such as MMP-3 and MMP-9) are involved in a further degradation of Col1 (20). Based on this knowledge, a gene expression analysis of both Co1α1 and MMP9, after 24 of treatment in the presence of thanaka's extracts, was assessed by means of a Real Time RT-PCR. The obtained results showed that in all tested experimental points Co1α1 gene expression was higher than in Ctrl sample, in particular, in HGFs exposed to 2SE and 4UAE extracts at 2% this increase appears statistically significant (Figure 3B). In parallel MMP9 mRNA appears detectable only in Ctrl sample while a suppression of its expression is remarkable in all cell samples exposed to 2SE and 4UAE extracts (Figure 3A). This result is further supported by Col1 secretion within the culture medium measured after 48 h of treatment through an ELISA assay: our results clearly evidence a marked increase of Col1 secretion with respect to Ctrl in the presence of extracts, statistically significant when 2SE at 2%, 4UAE at both at 2% and at 4%, are administered (Figure 3C). Taken together these results positively characterize thanaka's extracts because they, on one hand, enforce the hypothesis before described (11) of an anti-collagenase activity and, on the other hand, demonstrate a consistent capability to stimulate synthesis and release of Col1 thus promoting the deposition of ECM fibrils, even when administered at lower percentages.

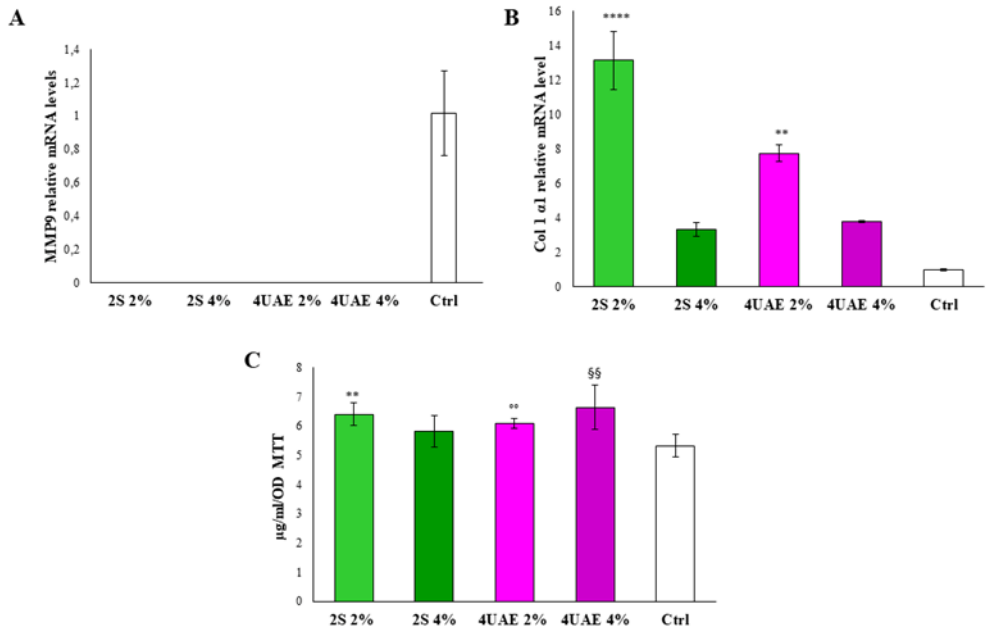


Figure 3 A and B. Relative gene expression of MMP9 and Col1α1, respectively, in HGFs cultured for 24 h in the presence of 2SE and 4UAE extracts, both at 2% and 4% concentrations. Ctrl: untreated cells. Data are expressed as relative to Ctrl (calibrator sample defined as 1). The bar graph displays densitometric values expressed as mean ± standard deviation, Y-axis, fold change. **** vs Ctrl p<0,0001, ** vs ctrl p=0,0012. C ELISA assay for Col1 secretion in HGFs cultured for 24 h in the presence of 2S and 4UAE extracts, both at 2% and 4% concentrations for 48 h. Ctrl: untreated cells. Secretion levels are reported as µg/mL/MTT OD MTT. The most representative of three separate experiments is shown. The bar graph displays densitometric values expressed as mean ± standard deviation. ** vs Ctrl p= 0,0013, °° vs Ctrl p=0,0012, §§ vs Ctrl p= 0,0045.

As previously reported, thanaka's extracts demonstrated an appreciable antioxidant ability, this aspect was checked and positively confirmed also in the biological model by measuring the total antioxidant capability of the cells, after 24 and 48 h of treatment, and expressing it in nmole of Trolox/µl. No significant differences between extracts-treated cells and Ctrl are revealed after 24 h of treatment while, after 48 h, our results underline that 2S extract, at 2% and 4%, and 4UAE extract, at 4% compared to Ctrl, significantly increase the release of Trolox within the culture medium, thus strengthening the supposed antioxidant activity for thanaka's extracts (Figure 4).

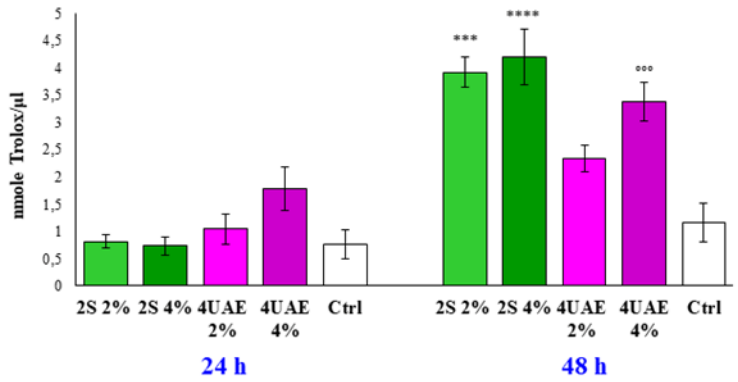


Figure 4. Total antioxidant capability of HGFs exposed to 2SE and 4UAE extracts at 2 and 4% dilutions for 24 and 48 h. The bar graphs represent the mean of Trolox nmole/ µl of medium ± standard deviation. The most representative of three separate experiments is shown. **** vs Ctrl p<0,0001, *** vs Ctrl p=0,0001, °°° vs Ctrl p=0,0009.

Prostaglandins are mediators obtained, constitutively or after inflammatory stimuli, from arachidonic acid thanks to the activity of cyclooxygenase.

Inhibition of PGE2, the most abundant prostaglandin involved in several inflammatory pathways, has represented a valuable anti-inflammatory strategy in the last century (21). For this reason, PGE2 secretion by HGFs within the culture medium, after 24 and 48 h of exposure to 2SE and 4UAE thanaka's extracts, was measured and expressed as pg/ml. After 24 h of treatment a statistically significant decrease in PGE2 secretion level in sample exposed to 2SE extract at 2% is recorded compared to Ctrl sample, whereas no significant differences between extracts-exposed samples and Ctrl can be identified after 48 h of Ctreatment (Figure 5).

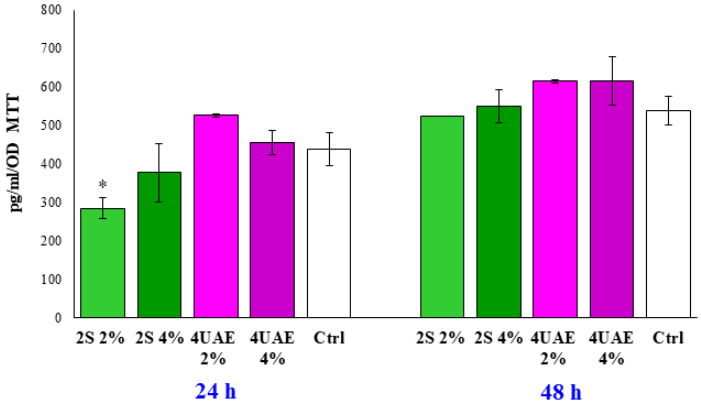


Figure 13. ELISA assay for PGE2 secretion of HGFs exposed to 2SE and 4UAE extracts at 2 and 4% dilutions for 24 and 48 h. Secretion levels are reported as mean pg/ml/OD MTT values ± standard deviation of three separate experiments. * vs Ctrl p=0,0442.

This result underlines the capability for 2SE extract to exert an appreciable anti-inflammatory effect by reducing the release of the most important cytokine, able to trigger a flogosis response. However, this beneficial effect is totally lost after 48 h of treatment, probably due to chemical modifications of bioactive components, thus leading to suggest that a daily refresh of thanaka's extracts in the culture medium could help to control the release of pro-inflammatory cytokines.

3.7 Formulation of a Cosmetic Face Cream

In agreement with the chemical results concerning the thanaka's extracts, the 2SE was selected for the formulation of a cosmetic cream and used as a powder. Usually, thanaka's bark is used rubbing the bark on a stone, creating a paste with water that is put directly on the face. Using the lyophilized extract as ingredient in a cosmetic formulation is possible to obtain more benefits than the paste thanks to the greater concentration on the bioactive compounds responsible for antioxidant, anti-age, and anti-UVA properties. Furthermore, using the freeze-dried ingredient, allows to maintain the physiochemical characteristics of the extract.

3.8 Characteristic of the cosmetic formulation

The pH of the formulation was 5.6, that respects the skin pH, and the density was 1.03 g/mL. The cream resulted physico-chemically stable under accelerated and long-term condition, without any instability phenomena, such as separation phase or changes in color or odor during the time interval considered for the observation. The formulation also passed the challenge test, confirming the efficacy of the preservative system to avoid microbial contamination.

The formulation was also well tolerated by human skin, showing a mean irritation index of 0 for all the volunteers, after both 15 min and 24 h of the application.

3.9 Evaluation of efficacy of the cosmetic product

The formulation was tested on the volunteers for the evaluation of the Trans Epidermal Water Loss (TEWL). This value is an evaluation of the capacity of the skin to retain water, contrasting the water loss from the external skin layers. TEWL is an indication of the health of the skin's barrier function which can be affected by external and environmental factors such as UV rays, cold, pollution, incorrect use of cosmetic products, and skin aging, but also endogenous factors such as age or disease, like dermatitis (22).

After the daily use of the face cream enriched with thanaka's extract, we assisted to a decrease of the evaporation rate value. Before the daily use of the face cream (t0) the value was 24.40 g/m2h, that decreased to 17.38 after 15 days (t2) and to 16.02 after 30 days (t3). This trend indicated that the cream has the capacity to restore the skin barrier integrity and repair the damages. This can be explained by the antioxidant capacity of the thanaka's extract and the presence of coumarins, especially marmesin, that both help to protect the skin against pollution, inflammation, and UV rays.

The skin hydration is an evaluation of the water content in the skin, and in this study it is evaluated in terms of Deep Skin Hydration, related to the water content in the epidermal layer, and in terms of Skin Surface Hydration, referred to the water content in the stratum corneum. In general, the water content is strictly related to the TEWL, and, in fact, in our study the water content both in the epidermal layer and in the stratum corneum improved after 30 days of constant use of the cream. The water content in the epidermal layer, progressively increased from 27% at t0, to 31.33% at t1, and to 33% at t2, showing an improvement in the Deep Skin Hydration. The Stratum Corneum Hydration, indicating the water content in the SC, is expressed as Skin Hydration (SH) and there is an increase from 12.00 (t0) to 12.86 after 15 days (t1) and to 18.26 after 30 days (t2).

The loss of elasticity can be associated to the skin aging process, which can be contrasted by the use of specific cosmetic products able to increase the quantity of collagen and elastin, for example by stimulating fibroblasts to produce them, or by inhibiting proteases and thus decreasing their degradation. In our study, the use of the cosmetic face cream favoured an increase in the skin elasticity from 63.4 N/m to 69.8 N/m at t1, and to 80.3 N/m at t2. The increase in skin elasticity can be explained by the capacity of thanaka's extract to increase the expression of the genes Col1α1 and to decrease the expression of the gene MMP9, as proven by the in vitro tests. Also the antioxidant activity of the extract is important for the skin elasticity: in fact, the molecules of the extracellular matrix like collagen, that give elasticity to the skin, are targets for free radicals.

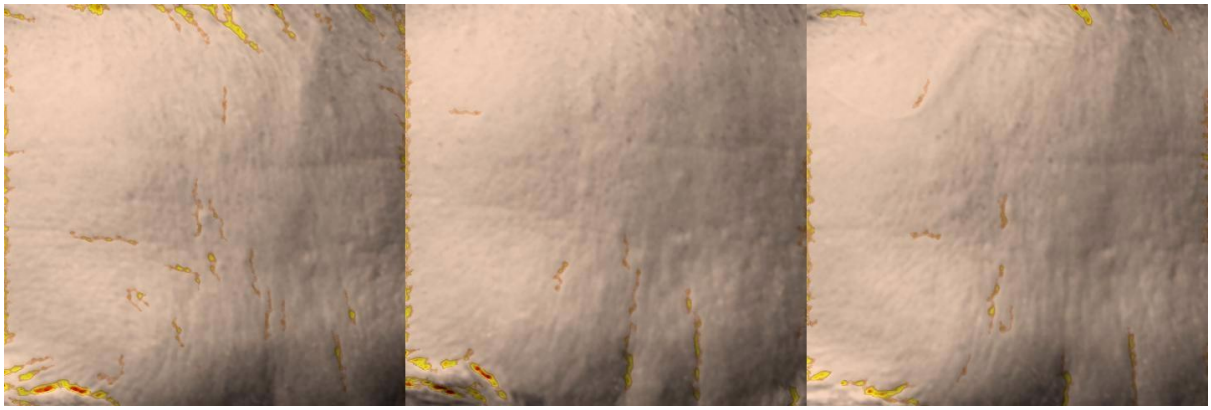
3.10 Skin imaging analysis by Antera 3D and data analysis

Antera 3D is a device that can analyses wrinkles and fine lines, redness, pigmentation and pores, relating the skin imaging to data analysis. With this instrument it is possible to compare the health of the skin, before and after the use of a cosmetic product. of the daily application of our face cream promoted a significant improvement in the skin condition.

Considering the amount of data obtained from the volunteers, we have decided here to report in detail the case of a single volunteer as an example. For other volunteers, the results are very similar. From our evaluation, we have mapped out the extreme situations, i.e. those that presented the greatest improvement and those that did not show variation over time.

Values are the average for measurements of specific areas of the face on a same volunteer (forehead, cheeks and periorbital areas).

Concerning the wrinkles (Figure 6), there was an important reduction in the wrinkles length from 71.06 ± 28.33 mm (t0) to 42.21 ± 17.35 mm (t1), and to 38.09 ± 8.47 mm after 30 days; in the width from 0.73 ± 0.47 mm at t0, to 0.64 ± 0.35 mm at t2 and to 0.58 ± 0.88 mm at t3. There was also a reduction in the average depth of the wrinkles (t0=0.048 ± 0.003 mm; t1=0.043 ± 0.020 mm; t2=0.039 ± 0.077 mm) and in the maximum depth (t0=0.094 ± 0.003 mm; t1=0.075 ± 0.021 mm; t2=0.057 ± 0.097). The number of the wrinkles was reduced from 14.2 ± 8.97 (t0) to 10.4 ± 6.30 (t1) to 7.5 ± 5.43 (t3).



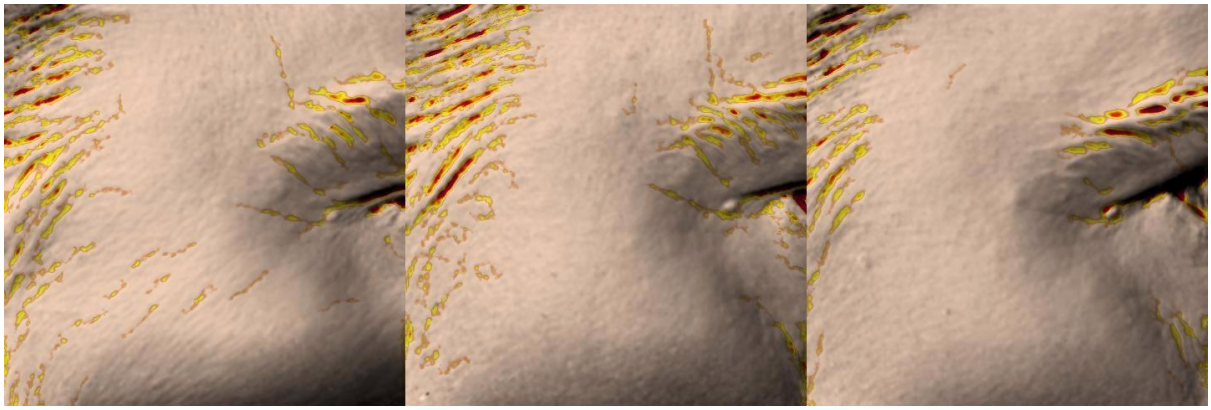


Figure 6. Picture of the forehead and right periorbital area obtained by Antera 3D, showing a decrease in length, width, depth and number of wrinkles.

For the forehead there is an important decrease of the length from 41.6 ± 8.90 mm (t_0) to 13.9 ± 4.44 (t_1) to 3.56 ± 3.32 (t_2), of the width from 0.765 ± 0.344 (t_0) to 0.569 ± 0.190 (t_1) to 0.455 ± 0.115 (t_2) and of the number of wrinkles from 8 ± 3 (t_0) to 4 ± 2 (t_1) to 1 ± 2 (t_2). The average depth is reduced from 0.0302 ± 0.0470 (t_0 and t_1) to 0.0264 ± 0.0111 (t_2) and the maximum depth from 0.0641 ± 0.0223 (t_0) to 0.0504 ± 0.0379 (t_1) to 0.0363 ± 0.0112 (t_2). For the right periorbital area, the length decreases from 34.60 ± 12.12 (t_0) to 26.76 ± 11.89 (t_1) to 7.54 ± 9.43 (t_2) and the width from 0.641 ± 0.286 (t_0) o 0.622 ± 0.214 (t_1) to 0.603 ± 0.165 (t_2). The number of wrinkles is reduced from 10 ± 5 (t_0) to 8 ± 4 (t_1) to 4 ± 3 (t_2), and for the depth there is an average reduction from 0.0659 ± 0.0433 (t_0) to 0.0437 ± 0.0222 (t_1) to 0.0389 ± 0.0221 (t_2). The maximum depth is reduced from 0.0789 ± 0.0351 (t_0) to 0.0557 ± 0.0111 (t_1) to 0.0487 ± 0.0090 (t_2).

For the fine lines (Figure 7), the situation was similar, and the data followed the same trend. There was an important reduction in the length from 304 mm ± 54.00 (t0) to 243 ± 131.88 mm (t1) and to 216.25 ± 87.88 mm after 30 days; in the width from 0.65 ± 0.93 mm at t0, to 0.64 ± 1.03 at t2 and to 0.62 ± 1.35 at t3. There was also a reduction in the average depth of the fine lines (t0=0.017 ± 0.006 mm; t1=0.015 ± 0.030 mm; t2=0.014 ± 0.120 mm) and an important reduction in the maximum depth (t0=0.071 ± 0.110 mm; t1=0.066 ± 1.243 mm; t2=0.052 ± 0.355 mm). The number of the fine lines was reduced from 52.40 ± 11.32 (t0) to 35.00 ± 23.34 (t1), that remained constant also after 30 days, 35 ± 11.24 (t3).

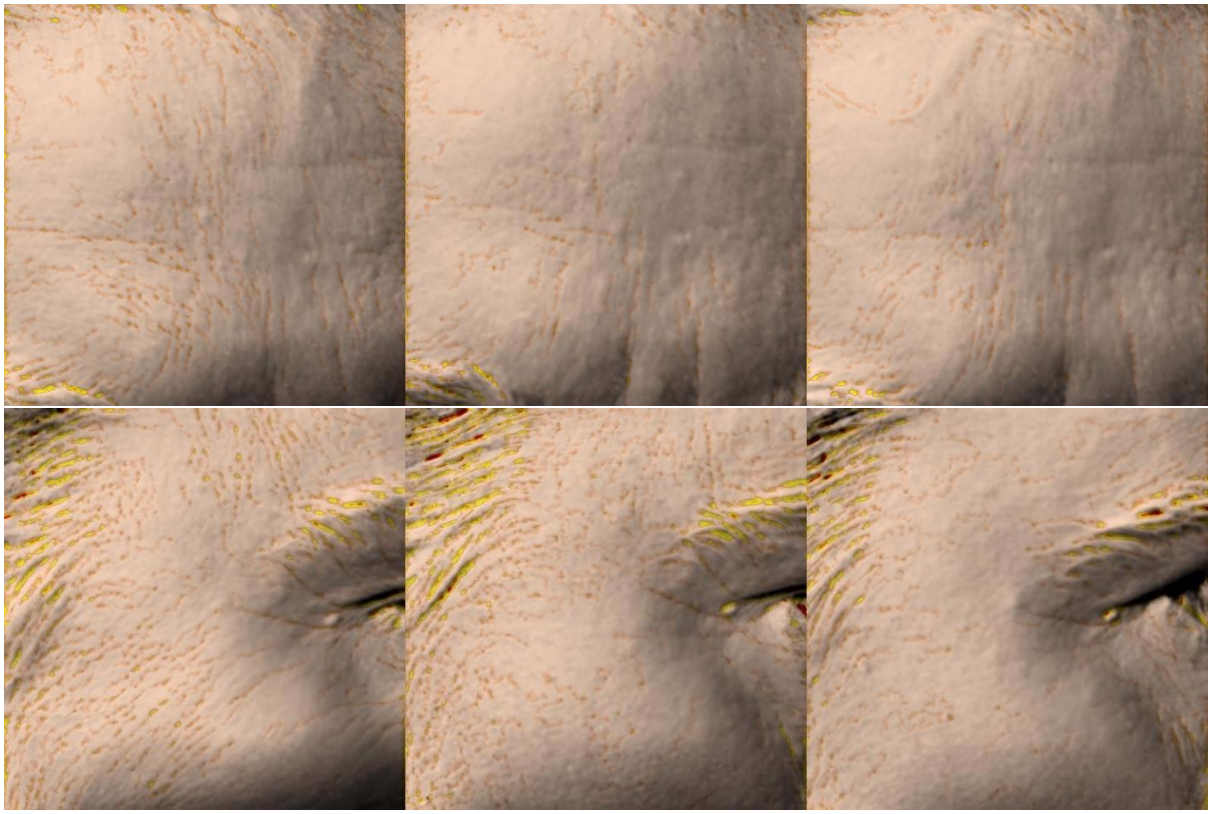


Figure 7. Picture of the forehead and right periorbital area obtained by Antera 3D, showing a decrease in length, width, depth and number of fine lines.

For the forehead there is an important decrease of the length from 234 ± 112 mm (t0) to 111 ± 87 (t1) to 98.3 ± 76 (t2), of the width from 0.62 ± 0.23 (t0) to 0.59 ± 0.26 (t1) to 0.62 ± 0.29 (t2) and of the number of fine lines from 47 ± 21 (t0) to 35 ± 19 (t1) and stable at t2, 35 ± 9. The average depth is reduced from 0.017 ± 0.009 (t0) to 0.015 ± 0.009 (t1) to 0.014 ± 0.007 (t2) and the maximum depth from 0.06 ± 0.06 (t0) to 0.05 ± 0.06 (t1) to 0.04 ± 0.05 (t2). For the right periorbital area, the length decreases from 203 ± 85 (t0) to 162 ± 90 (t1) to 76 ± 33 (t2) and the width from 0.630 ± 0.220 (t0) o 0.624 ± 0.200 (t1) to 0.621 ± 0.190 (t2). The number of fine lines is reduced from 36 ± 40 (t0) to 35 ± 23 (t1) to 17 ± 11 (t2), and for the depth there is an average reduction from 0.017 ± 0.009 (t0) to 0.016 ± 0.007 (t1) to 0.014 ± 0.009 (t2). The maximum depth is reduced from 0.054 ± 0.040 (t0) to 0.049 ± 0.029 (t1) to 0.048 ± 0.023 (t2).

Redness is a condition of inflammation and irritation. The redness of the skin is due to the dilation of the blood vessels, resulting in an influx of hemoglobin into the area. Pictures, showed an evident decrease in redness (Figure 8), confirmed by the data given from the software, expressed in redness average concentration, that decreases from 33.42 ± 12.22 (t0) to 29.26 ± 11.30 after 15 days (t1), and 26.92 ± 8.99 after 30 days (t2). The software also allowed us to obtain the maximum redness value in the selected area, that was 48.74 ± 45.45 at t0, 43.82 ± 33.24 at t2 and 41.32 ± 56.33 at t2. The data of the redness, related to the inflammation and irritation condition, confirmed the anti-inflammatory activity of thanaka reported in literature (4) (9), also highlighted in this study by the inhibition of the release from HGFs of the cytokine PGE2.

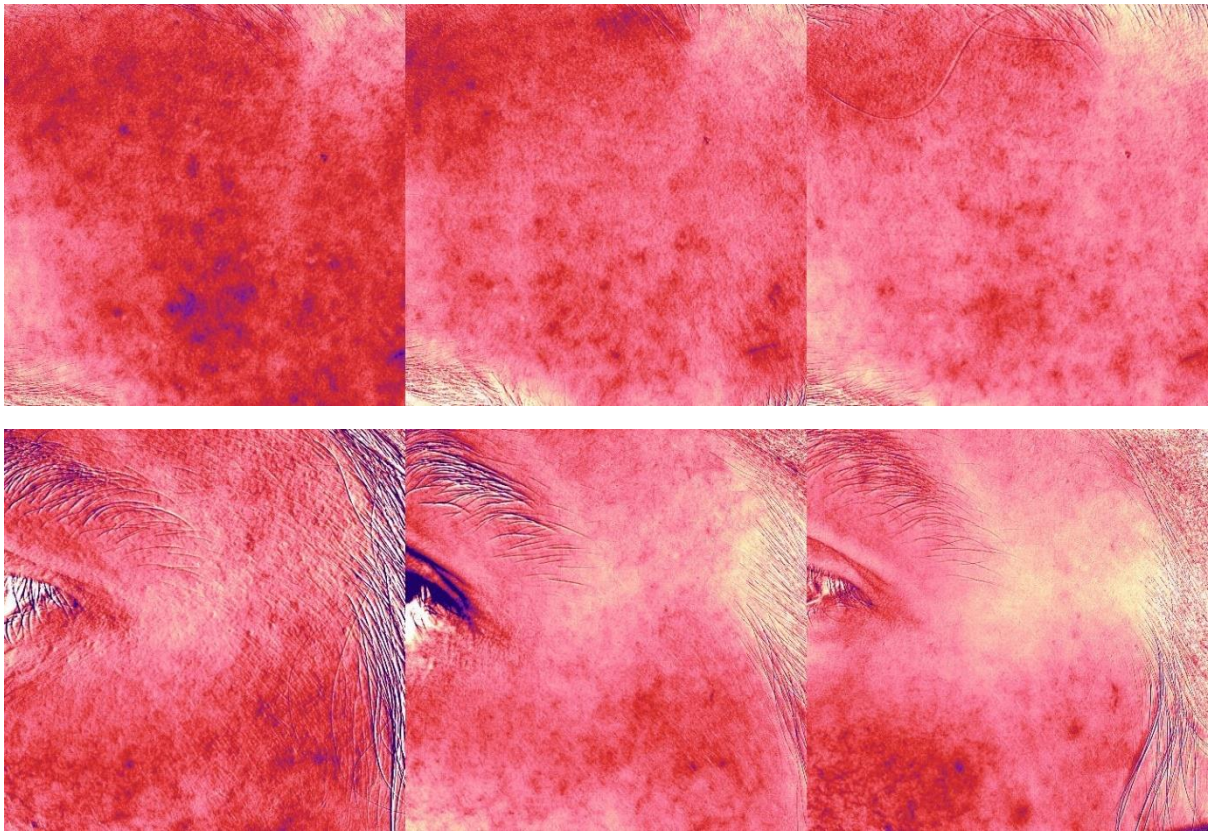


Figure 8. Picture of the forehead and left periorbital area obtained by Antera 3D, showing a decrease in redness. The Redness average concentrations for the forehead are 33.8 ± 28.8 (t_0), 29.5 ± 27.3 (t_1) and 26.8 ± 11.3 (t_2), while the maximum redness values are 47.5 ± 12.2 (t_0), 43 ± 8.9 (t_1) and 38 ± 14.3 (t_2). The redness average concentrations for the left periorbital area are 28.3 ± 8.8 (t_0), 26.4 ± 8.7 (t_1) and 22.5 ± 11.2 (t_2), and the maximum redness values are 43.2 ± 13.6 (t_0), 39.1 ± 13.4 (t_1) and 38.4 ± 8.8 (t_2).

Pigmentation of the skin is given by the quantity of melanin, produced and accumulated on the skin. Melanin is a pigment produced by the melanocytes with a reaction catalysed by the enzyme tyrosinase. It is proved that thanaka has a light anti-tyrosinase activity (4) (11). Our work confirmed this action, thanks to a reduction of the pigmentation of the skin, after the application of the cream containing thanaka's extract. The value was expressed in terms of pigmentation index, related to the concentration of melanin. The index decreased from 30.0 ± 12.12 (t0) to 26.20 ± 4.43 after 15 days (t1) and to 22.4 ± 12.45 (t2), indicating a lower concentration of melanin in the skin. The instrument also permitted to quantify the minimum and maximum concentration of melanin in the area of the surface of the area selected for the measurement. The minimum value of pigmentation decreased from 38.22 ± 31.22 (t0) to 37.02 ± 23.89 (t1) to 36.34 ± 19.78 (t2), while the maximum value decreases from 59.66 ± 46.55 (t0) to 57.86 ± 34.67 (t1) and to 54.44 ± 33.52 (t1). The medium concentration of the pigmentation related to the surface of the area selected for the measurement decreases from 44.60± 39.70 (t0) to 43.74 ± 38.99 (t1) to 43.12 ± 34.80 (t2). These values indicated the reduction of pigmentation, related to the decrease of melanin in the skin. This can confirm the anti-tyrosinase activity of the thanaka's bark. The images are not shown since the reduction of pigmentation cannot be visually assessed.

Measurements of skin pores (Figure 9) are expressed in terms of total volume of the pores (mm3) in the selected area, medium volume of a single pore (mm3), total area of the pores in the area of the measurement and the medium area (mm2) of a single pore (mm2), maximum depth of the pores (mm), density expressed in number of the pores per cm2, number of the pores in the selected area

and the pore index, that considers the medium depth, the total area and the density of the pores. The sum of the volume of all the pores in the selected area decreased from 2.54 ± 0.99 mm3 (t0) to 1.66 ± 1.12 mm3 (t1) to 1.25 ± 1.24 mm3 (t2), and the medium volume decreased from 0.0046 ± 0.0034 mm3 (t0) to 0.0030 ± 0.0027 (t1) mm3 to 0.0025 ± 0.0014 (t2) mm3. The total area of all the pores was reduced from 172.25 ± 78.55 mm2 (t0) to 130.33 ± 80. 22 mm2 (t1) to 132.00 ± 111.90 mm2 (t2) and the medium area of a single pore decreased from 0.281 ± 0.113 mm2 (t0) to 0.199 ± 0.099 mm2 (t1) to 0.171 ± 0.146 mm2 (t2). The maximum depth of the pores decreased from 0.048 ± 0.019 mm to 0.043 ± 0.022 mm to 0.036 ± 0.019 mm; the density was reduced from 67.28 ± 24.78 cm-2 (t0) to 58.58 ± 34.66 cm-2 (t1) to 54.52 ± 13.80 cm-2 (t2); and the number of the pores decreased from 590.4 ± 224.1 (t0) to 514 ± 360.8 (t1) to 471.4 ± 180.3 (t2). In general, pore index decreased from 2.96 ± 0.77 (t0) to 1.93 ± 0.97 (t1) to 1.39 ± 1.04 (t2).

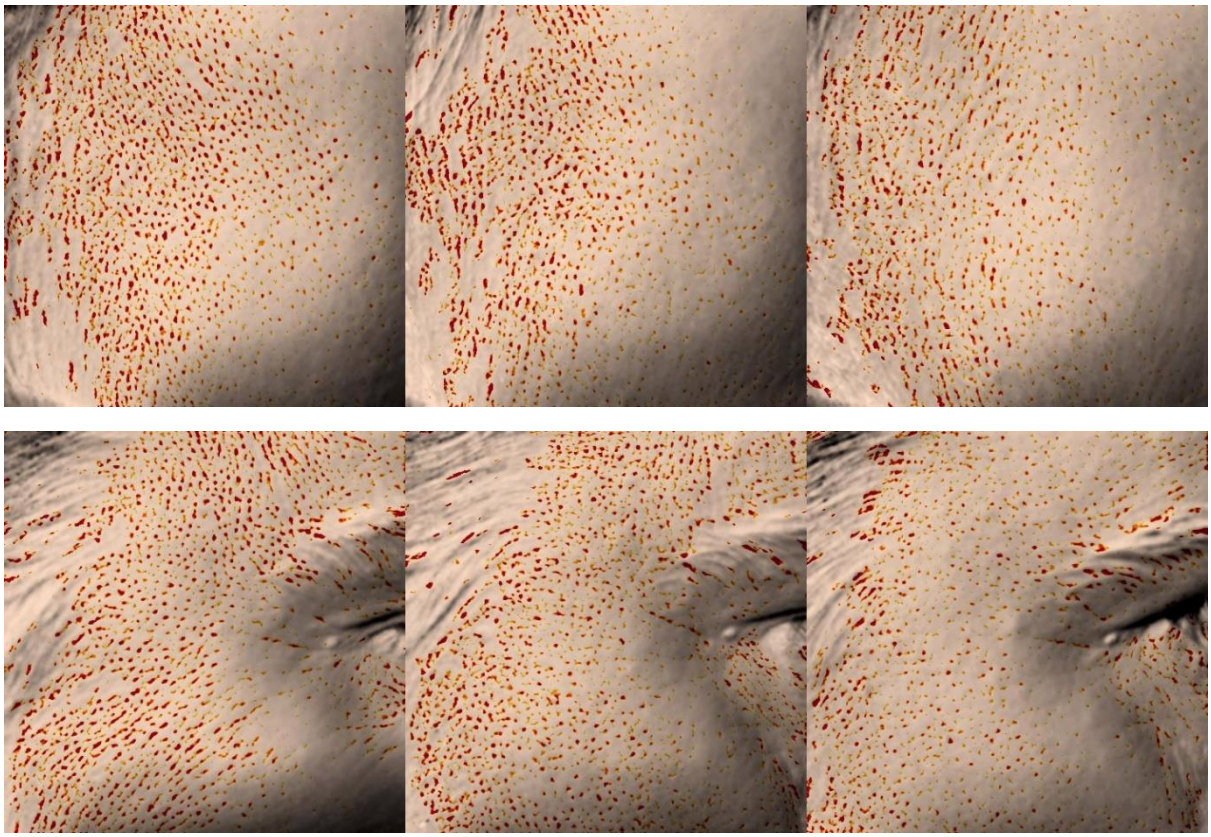


Figure 9. Picture of the right cheek and right periorbital area obtained by Antera 3D, showing an improvement in the visibility of the pores. For the cheek the total volume of the pores decrease from 3.56 ± 1.22 mm^3 (t_0) to 2.84 ± 0.96 mm^3 (t_1) to 1.74 ± 66 mm^3 (t_2) and the medium volume from 0.005 ± 0.003 mm^3 (t_0) to 0.004 ± 0.003 mm^3 (t_1) to 0.003 ± 0.001 mm^3 (t_2). The total area decrease from 215 ± 198 mm^2 (t_0) to 177 ± 166 mm^2 (t_1) to 118 ± 85 mm^2 (t_2), and the medium area is reduced from 0.304 ± 0.250 mm^2 (t_0) to 0.259 ± 0.178 mm^2 (t_1) to 0.195 ± 0.111 mm^2 (t_2). The maximum depth is decrease from 0.051 ± 0.031 mm (t_0) to 0.048 ± 0.029 (t_1) to 0.036 ± 0.018 (t_2) while the density of the pores decreases from 64.1 ± 31.3 cm^2 (t_0) to 61.8 ± 25.5 cm^2 (t_1) to 54.8 ± 24.6 cm^2 (t_2). The number of the pores decreases from 708 ± 376 (t_0) to 681 ± 303 (t_1) to 604 ± 247 (t_2) and the pore index decrease from 3.23 ± 1.89 (t_0) to 2.58 ± 2.10 (t_1) to 1.58 ± 1.13 (t_2). For the right periorbital area the total volume of the pores decrease from 1.59 ± 1.12 mm^3 (t_0) to 1.30 ± 1.17 mm^3 (t_1) to 0.71 ± 1.08 mm^3 (t_2) and the medium volume from 0.005 ± 0.001 mm^3 (t_0) to 0.003 ± 0.001 mm^3 (t_1) to 0.002 ± 0.002 mm^3 (t_2). The total area decrease from 96.3 ± 23.2 mm^2 (t_0) to 84.6 ± 23.3 mm^2 (t_1) to 48.9 ± 17.2 mm^2 (t_2), and the medium area is reduced from 0.372 ± 0.115 mm^2 (t_0) to 0.232 ± 0.124 mm^2 (t_1) to 0.166 ± 0.87 mm^2 (t_2). The maximum depth is decrease from 0.037 ± 0.011 mm (t_0) to 0.034 ± 0.012 (t_1) to 0.035 ± 0.019 (t_2) while the density of the pores decreases from 74.1 ± 31.3 cm^2 (t_0) to 61.4 ± 34.5 cm^2 (t_1) to 59.8 ± 31.1 cm^2

(t_2). The number of the pores decreases from 365 ± 257 (t_0) to 303 ± 233 (t_1) to 295 ± 231 (t_2) and the pore index decrease from 3.22 ± 1.23 (t_0) to 2.63 ± 1.45 (t_1) to 1.45 ± 1.11 (t_2).

4. Conclusion

This study aims to fully evaluate the properties of an extract recovered from thanaka bark, widely used in the Burmese popular tradition, and obtained from sustainable approaches. The extract was proven to be safe in cells, where potent antioxidant and anti-collagenase activities could be highlighted, confirming the predictions revealed during the spectrophotometric analyses. The inclusion of the extract in a cosmetic formulation allowed to confirm the beneficial properties in 20 volunteers, where an unquestionable antiaging activity emerged.

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Chapter 5

Chemical, Antioxidant, and Antimicrobial Properties of the Peel and Male Flower By-Products of Four Varieties of *Punica granatum* L. Cultivated in the Marche Region for Their Use in Cosmetic Products

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Abstract

We are now seeing an increase in the production of agri-food waste, which is an essential resource for the recovery of bioactive compounds that may be employed as innovative natural ingredients in cosmetics. To date, the approach to cosmetics preservation has seen a significant shift in the search for biological components that give healthier alternatives for customers and help businesses operate in an environmentally friendly manner. To achieve this goal, we studied pomegranate extracts using the peel and, for the first time, extracts from the male flowers of a wide pomegranate variety cultivated in the Marche region, specifically, the Wonderful, Mollar de Elche, Parfianka, and less-studied G1 varieties. We studied the phenol compounds profile, antioxidant capacity, antimicrobial activity, and cell viability of the obtained pomegranate extracts. The identification and quantification of phenol compounds belonging to different classes, such as hydrolysable tannins, hydroxybenzoic acid, hydroxycinnamic acid, dihydroflavonol, gallocatechin, and anthocyanins, were performed using UPLC-ESI-MS/MS. Punicalagin isomers and punicalin resulted in the most abundant polyphenols found in the peel and male flower extracts. Mollar de Elche 2020 peel extract revealed a high concentration of punicalagin A and B (7206.4 mg/kg and 5812.9), while the content of gallic acid revealed high results in the G1 and Parfianka varieties. All extracts were spectrophotometrically analysed to determine their total phenol content (TPC) using the Folin-Ciocalteu method and their antioxidant capacity (AC). In terms of the total phenol obtained by the Folin-Ciocalteu colorimetric method, Mollar de Elche 2020 extracts reported the highest TPC content of 12.341 µmol GAE/g. Results revealed that the Mollar de Elche and Wonderful 2020 peel extracts demonstrated the highest TPC and AC. Furthermore, AC results indicated that the peel extracts displayed higher AC than the male flower extract due to the high punicalagin content detected by UPLC analysis. The antimicrobial activity testing revealed that the Wonderful and G1 2020 peel extracts resulted active against Escherichia coli, while all extracts exhibited promising anticandidal activity. Additionally, the cytocompatibility was evaluated in keratinocytes HaCaT cells by testing concentrations of pomegranate extracts ranging from 0.15 to 5.00 mg/mL. Extracts were non-toxic for the cells in the tested concentration range. The acquired results may help exploit pomegranate agri-food waste products provided by the Marche region's short supply chain for their use as an antimicrobial and antioxidant booster in the formulation of cosmetic products.

1. Introduction

In recent years, the cosmetics sector's new focus has been on two crucial challenges: ecology and sustainability. Given this sustainable approach, companies are currently using natural ingredients to enhance the quality of the cosmetics, keep them free of microorganisms contamination, enhance their shelf-life, obtain safer products, and avoid the use of synthetic preservatives.

Furthermore, the typical approach to cosmetic preservation is to employ the most significant number of conventional preservatives allowed (refer to Annex V of the European Commission's Regulation (EC) No 1223/2009 for a list of authorized preservatives [1]). Conventional preservatives have shown many secondary effects and risks on human health. Recent studies have also reported side effects from preservatives on the skin microbiota [2,3]. The use of cosmetic products has recently been shown to change the equilibrium of the skin microbiota, which can cause it to lose its healthy status. This effect can be linked to various causes, including the preservatives' residual impact on the skin.

Against this background, the approach to cosmetics preservation can be managed with several strategies to prevent microbial contamination. Various methods can be used to achieve these goals. One solution is based on the concept of "Hurdle Technology", which encompasses all intelligent ways of preventing and reducing microbe proliferation throughout the formulation production process. As a result of this approach, several steps must be taken to avoid microbial contamination, ranging from strict adherence to GMP, raw material microbial control, and water treatment using a different strategy to reduce water activity, as water is one of the most abundant ingredients in many cosmetic formulations. In adherence to GMP, qualified employees can manage cross-contamination, disinfection of equipment, and proper packaging. (e.g., airless dispensers, pumps) are essential [4]. Any of these measures, when combined, can help lower any microbial contamination. On the other hand, using novel raw materials obtained from agri-food waste is one way to avoid using conventional preservatives and it depends more and more on innovative ingredients. This strategy provides healthier options for customers and assists businesses in working in an ecologically sustainable manner. This method is also feasible since it is widely recognised that some natural ingredients may play many roles in cosmetic formulation, such as absorbed water making a formulation alkaline or acid. They can also be applied as a natural antibacterial. Herbal extracts and essential oils have shown evidence of microbial activity despite not being preservatives but also exhibiting antimicrobial activity with double safety rules: safety of the formula, where we do not want microorganisms, and protection of the skin, where we do not want side effects [5]. Currently, a feasible strategy for reintroducing agri-food waste into natural cosmetics manufacturing is the circular economy [6–8]. Natural compounds obtained from agri-food waste products can be used successfully as a natural preservative in cosmetic compositions [9,10]. One of the most interesting by-products that may be utilised for this purpose is pomegranate by-products, which are becoming recognised as having numerous bioactive compounds with considerable antioxidant activity and antibacterial properties [11].

Pomegranate, or Punica Granatum L., one of the most ancient fruits, is a fruit that belongs to the family Punica and has many benefits to human health. It is grown worldwide with approximately 1500 ha of cultivars in Italy, mostly in Sicily, Puglia, Calabria, Campania, and Lazio [12]. Still, many of these cultivations are also present in the Marche region. In the last decade, the interest in the cultivation of Punica Granatum L. also highlights the varieties that have encouraged the revival of pomegranates in Italy as an income crop. Therefore, many entrepreneurs, driven by the need to diversify and expand their business, have identified a viable alternative to traditional orchards. Consequently, this situation has led to a significant increase in waste. Usually, pomegranates generate 669 kg of waste materials for every 1 ton of fresh fruit, with 78% composed of peel and 22% seed [13,14]. Different studies report

pomegranate peel as an interesting by-product [12,15,16], but we also identified another part of the pomegranate tree that provides an abundant waste material. During the flowering period, from May to July, the pomegranate tree develops two principal types of flowers: male flowers and hermaphrodite flowers. The first one has a poorly designed or no pistil and atrophied ovaries with few ovules. It is infertile and drops down without fruit set, representing considerable waste material. In contrast, the hermaphrodite flowers are fertile, with an ovary producing fruit. The primary by-products of the pomegranate flower, the male ones, have not been investigated singularly as potential sources of bioactive polyphenols. To the best of our knowledge, no data are available.

Pomegranate peel and flowers represent an enormous waste of raw materials. As reported by the literature, pomegranates contain a high number of phenol ingredients in their pulp, seed, and juice [17–20]. Polyphenols represent the predominant phytochemicals of the pomegranate peel and are mainly composed of hydrolysable tannins (pedunculagin, punicalin, punicalagin, and ellagic acids). Among them, the predominant hydrolysable tannin of pomegranates is known as the punicalagin [21]. In addition, there are also other flavonoids such as quercetin, kaempferol, luteolin glycosides, catechins, anthocyanins, and complex flavonoids [20,22]. These bioactive compounds are responsible for many pharmacological properties, notably their significant antioxidant activity and antibacterial characteristics [18,23]. Total phenolic content, antioxidant capacity, and antibacterial activity against various microbes have been highly correlated [24]. Pomegranate peel extracts and other fruit by-products, such as juice or seeds, have exhibited remarkable antioxidant and antibacterial properties, with a high scavenging capacity, reducing the microbiological proliferation of several bacteria, among other therapeutic benefits to the human body [25].

Due to their high content of ellagitannins, such as punicalagin, Punica Granatum L. peel extracts show a remarkable antimicrobial capacity influenced by the different types of cultivars, which has been demonstrated against pathogenic such as Staphylococcus aureus [26]. The antimicrobial activity was associated with a high concentration of punicalagin and an ellagic acid concentration in the pomegranate peel extract, demonstrating that the ellagic acid content substantially impacts the antimicrobial activity [27–29].

Based on the potential benefits of pomegranate extract and the need for research exploring natural ingredients that may be used as alternative preservatives in cosmetic formulations, our study aims to evaluate the antioxidant capacity and anti-microbial activities of extracts of the peel and male flower by-products of four pomegranate varieties cultivated in the Marche region. The US green extraction processes were applied to efficiently recover the polyphenols from the pomegranate peel and male flower extract. The main polyphenol compounds present in the peel and male flower extracts were identified and quantified by UPLC-ESI-MS/MS. The majority of the data on pomegranate extracts in the literature came from one of the most common varieties. As a result, our research presents a chemical and antibacterial analysis of pomegranate varieties that have received less attention in the past, such as G1 and Parfianka. To our knowledge, the characterisation of polyphenols and antibacterial activity for the combination of pomegranate and other fruits, such as the G1 cultivar, has never been studied. As a result, this study will provide a scientific foundation for supporting the valorisation of pomegranate peels and male flower by-products received through a short supply chain as a substantial source of natural preservatives used in the production of cosmetics with a reduced rate of conventional ones. To achieve this goal, pomegranate extracts might be used as a preservative booster with antimicrobial properties, decreasing the need for synthetic preservatives in cosmetics and as a booster fulfilling other specific purposes, such as moisturiser or antioxidants.

2. Materials and Methods

2.1. Chemicals

The chemicals 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), (±)6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX), 2,20 - Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt (98%TLC), ABTS, gallic acid, sodium carbonate monohydrate ACS reagents, and ethanol (final ethanol grade) were purchased from Sigma-Aldrich (Steinheim, Germany); Manganese (IV) oxidise activated (>90%), and Folin–Ciocalteu's phenol reagents were purchased from Fluka (Buchs, Switzerland). Analytical grade solvents and reagents were used for all of the work. Ellagic acid, gallic acid, punicalagin A and B, punicalin, cyanidin 3-glucoside, cyanidin 3,5-diglucoside, pelargonidin 3,5-diglucoside, and pelargonidin 3-glucoside were obtained from Sigma-Aldrich (Stenheim, Germany). Formic acid and acetonitrile for LC/MS were purchased from Carlo Erba Reagents (Cornaredo, MI, Italy). Sigma Aldrich provided all the other chemicals (Steinheim, Germany). The ultrapure water was produced from the Millipore system (Millipore Sigma, Darmstadt, Germany), and filtered with a 0.20 µm Sartolon polyamide filter (Sartorius Stedim Biotech, Göttingen, Germany).

1.2. Pomegranate Samples

In this study, Wonderful, Mollar de Elche, Parfianka, and G1 pomegranate cultivars, grown in the Marche region, were harvested in October 2019 and 2020 at commercial maturity at the agricultural enterprise "Onori Maria Rosaria", Sant'Elpidio a Mare (Fermo, Marche). Immediately after the fruits were collected, the pomegranate peels were manually removed to accurately separate the exocarp (rind) used for the extraction process from the mesocarp (white spongy tissue). The exocarp was dried at 37 ◦C in a ventilated oven (VEC2103/8, Everest, Rimini, Italy). The male flowers, and more specifically sepals and stamen, of Wonderful and Mollar de Elche were picked during the flowering period, in June 2020, from the same agricultural enterprise from which we obtained all of the materials for our investigation. The waste male flowers were promptly dried in a ventilated oven at 37 ◦C until they reached a consistent weight and were then utilised for extraction. For each sample, three extractions were performed.

1.3. Extraction of the Phenol Compounds from Pomegranate Peel and Male Flowers

Extractions from the pomegranate peel and male flowers were carried out in an ultrasonic bath (FALC-LABSONIC LBS2, Treviglio, BG, Italy), with a maximum capacity of 10 L. Extractions were performed using a water and ethanol at 70:30 (v/v) mixture as extraction solvent. The plants (1 g) were dispersed in 100 mL of solvent and then immersed in an ultrasonic bath (50 ◦C, 59.2 W/cm2 for 15 min). The extracts were lyophilised at −53 ◦C for 24 h at 0.03 millibar (FreeZone, 1 L, LABCONCO, Kansas City, MO, USA). Each sample was kept at −20 ◦C in 50 mL screw-top cap polyethylene vials (BD Falcon TMBD Biosciences, Bedford, MA, USA). The lyophilised solids were reconstituted with appropriate solvents for subsequent investigations.

1.4. Ultra-Performance Liquid Chromatography Mass Spectrometry Analysis

UPLC (Agilent 1290 Infinity Technologies UPLC, Santa Clara, CA, USA) with a diode array detector and a triple quadrupole mass spectrometer (MS-QQQ), combined with an electrospray ionization source (ESI), was used to identify and quantify polyphenols in pomegranate extracts. The UPLC had a binary pump and an auto-sampler. We used a Luna (C18 1504.6 mm) column (Phenomenex, Castel Maggiore, BO, Italy) at 25 ◦C for chromatographic separation. The gradient elution used water/0.1% formic acid (solvent A) and acetonitrile/0.1% formic acid (solvent B) at a 1 mL/min flow rate. The optimised

gradient was 0–3 min, 7% B, 3–30 min, 27% B, 30–60 min, and 100% B, and 5 µL was injected. The mass spectrometer detection in both negative and positive ionisation modes was utilised to identify the different signals. However, in the negative mode, a significant group of compounds matching deprotonate molecular ions of diverse chemicals were identified.

In contrast, in the positive mode, the characteristics of anthocyanins of pomegranate were detected. The peak characterisation was performed based on their retention time and accurate molecular mass in the MS and MS2 experiment. The ESI source worked at 350 ◦C, 12 L/min, and a nebulizer pressure of 55 psi. Samples were diluted 1:2 with mobile phase, sonicated for 5 min, then centrifuged at 12,000 rpm for 10 min (Scilogex D3024R High-Speed Refrigerated Micro-Centrifuge, Rocky Hill, CT, USA). Next, a 0.20 µm filter (Captiva Econofilter, PTFE) was used to filter the samples. A full scan mass spectrometer analysis was performed in m/z 150–1500 (negative) and 50–1500 (positive). Following that, several production experiments were conducted on the corresponding precursor ion. The quantification of polyphenols was carried out for individual phenol compounds using a calibration curve of the respective reference external standards. To this end, the mixed standard was prepared at 1000 mg/L. The linearity, sensitivity, accuracy, and precision of the developed method were verified according to the Food and Drug Administration Guidelines (FDA), as already mentioned in our previous studies [30]. The linearity was explored in the concentration range of 1–50 mg/L for ellagic acid, gallic acid, punicalagin A and B, and punicalin. External anthocyanins standards as cyanidin 3-glucoside, cyanidin 3,5-glucoside, pelargonidin 3,5-glucoside, and pelargonidin 3-glucoside were used with a concentration range of 1–20 mg/L, obtaining the corresponding calibration curve for further calculations (Table 1). The performances of this method were also tested for accuracy, precision, and sensitivity (Table 1). The sensitivity was evaluated in terms of the limit of detection (LOD) and limit of quantification (LOQ) by triple injections of a standard mixture at concentrations responsible for a signal-to-noise ratio equal to 3 and 10 for LOD and LOQ, respectively. To evaluate the method's accuracy, we chose a concentration of 10 mg/L for all standards. They were injected in triplicate among the unknown samples in the daily sequence. The accuracy was expressed as standard deviation percentage and reported in the Supplementary Materials. The overall method precision was evaluated by analysing nine extracts of the same sample. Each section was then injected individually as an unknown sample. The accuracy was calculated for each compound and expressed as a relative standard deviation percentage.

Table 1. Linearity, sensitivity, accuracy, and precision of the developed UPLC-ESI-MS/MS method for pomegranate peel and male flower by-products extracts.

Analytes	**RT (min.)**	**Linearity[a]**		**Sensibility**		**Accuracy[d]**	**Precision[e]**
		Regression curves	r^2	LOD[b]	LOQ[c]		
Gallic acid	3.83	y=109036x	0.973	0.07	0.24	14.7	0.4-16.8
Punicalin	3.75	y=115291x	0.992	0.04	0.14	11.9	0.5-18.8
Punicalagin A	9.25	y=139898x	0.997	0.04	0.14	6.8	0.4-6.7
Punicalagin B	11.71	y=149562x	0.998	0.04	0.14	4.7	0.4-3.8
Ellagic Acid	21.84	y=233729x+84858	0.994	0.03	0.11	7.8	0.2-19.7
Cyanidin 3,5-diglucoside	6.2	y=491074x	0.998	0.08	0.24	3.0	3.6-14.4
Pelargonidin 3,5-diglucoside	8.4	y=557447x	0.999	0.05	0.14	1.6	0.5-13.2
Cyanidin 3-glucoside	10.3	y=1073535.261	0.998	0.03	0.11	2.8	9.4-15.7
Pelargonidin 3-glucoside	12.0	y=1095405x	0.993	0.01	0.03	1.7	0.2-18.6

[a] explored in the concentration range of 1–50 mg/L for ellagic acid, gallic acid, punicalagin A and B, and punicalin; and in a concentration range of 1–20 mg/L in the case of cyanidin 3-glucoside, cyanidin 3,5-diglucoside, pelargonidin 3,5-diglucoside, and pelargonidin 3-glucoside, obtaining the corresponding calibration curve for further calculations. [b] Limit of detection. [c] Limit of quantification. [d] Accuracy was expressed as standard deviation %. [e] The precision was calculated for each compound, and it was expressed as relative standard deviation percentage. A range of SD% was reported in the table.

The matrix effect (ME) for the matrices used in this study was also evaluated. The Matrix Effect percentage (ME%) was calculated using this Equation (1):

$$\mathrm{ME}\ \% = \frac{\mathrm{B}}{\mathrm{A}}\ \mathrm{X}\ 100 \qquad (1)$$

where B is the difference between the areas of the fortified sample and non-fortified one, and A is the area of the standard solution in the pure solvent. Thus, it was possible to evaluate if the matrix effect induced an ion suppression (values < 100%) or an ion enhancement (values > 100%). A ME value equal to 100% means that no matrix effect occurs; deals over 100% reveal a signal enhancement and matters less than 100% reveal a signal suppression [31].

2.5. Determination of the Total Phenol Content

Total Phenol Content (TPC) was determined by using the Folin–Ciocalteu spectrophotometric method according to Gigliobianco et al. [30,32,33]. The results were expressed as milligrams of gallic acid equivalents (GAE) per grams of by-product (mg GAE/g).

2.6. Antioxidant Capacity (AC) Evaluation

Three methods were adopted in our approach to measure antioxidant activity: DPPH• radical scavenging activity, 2,2'-and-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS•+) radical cation scavenging capacity, and Ferric Reducing Antioxidant capacity (FRAP) [22,23]. Trolox, which is 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, was used as a standard. Results were shown in terms of IC50, which is the concentration of the test sample that would reduce the concentration of DPPH, ABTS by 50%, as well as the amount of Trolox equivalent (TE)g-1 in the sample.

The DPPH free radical scavenging activity was assessed using previously published methods with slight modifications [34,35]. In a 96-well microliter plate, 100 µL of the sample (10 mg mL−1) and standard were added to 150 µL of DPPH in 100 ethanol. At 517 nm, the absorbance of each well was measured after 20 min of incubation at 37 °C.

The ABTS analysis was carried out as described in [36] using a 96-well microliter plate [35]. We made the ABTS•+ solution by oxidizing it with MnO2 in water for 30 min. The ABTS•+ solution (150 µL) was added to a 50 µL aliquot of the different concentrations of the sample (concentration 10 mg mL−1) and standard (Trolox). After 10 min of incubation at room temperature, the absorbance of each well was measured at 734 nm.

The FRAP values of the extracts were calculated using a previously described technique [37], with minimal modifications [38]. The FRAP reagent was produced by dissolving the following three solutions: 50 mL 0.3 M acetate buffer pH 3.6 (1.23 g sodium acetate in 50 mL water acidified with acetic acid); 5 mL stock solution of 5 mM TPTZ (2,4,6-tripyridyl-s-triazine) (15.6 mg) in 40 mM HCl; 5 mL stock solution of 5 mM FeCl3 6H2O (16.2 mg) in 40 mM HCl. The FRAP solution was warmed to 37 °C. Aliquots of 50 µL sample solution (10 mg mL−1) were applied in triplicate to the 96-well plate (BD FalconTM). Each well was initiated with 175 µL of FRAP reagent. The plate was immediately shaken for 30 s in a FLUOstar Omega plate reader, and the reaction was allowed to run for 10 min before being read on a plate reader (593 nm). Meanwhile, a reference solution of Trolox was run to create the calibration curve by linear regression with the linearity of R2 = 0.9996. The data were presented in µM Trolox equivalent (TE) g-1 sample.

2.7. In Vitro Antimicrobial Activity Assay

Extracts were tested against the Gram-negative bacterial species Pseudomonas aeruginosa (ATCC 9027) and Escherichia coli (ATCC 8739), the Gram-positive Staphylococcus aureus (ATCC 6538), and the yeast Candida albicans (ATCC 10231). These microorganisms were from the culture collections maintained by the In Vitro Testing Laboratory of Abich s.r.l. (Verbania, VB, Italy).

The raw materials of the pomegranate peel and male flower extracts were initially evaluated for their TVC (total viable counts) according to ISO 21149 for bacteria and ISO 16212 for yeast and moulds. The bacterial TVC were < 10 UFC/g for all analysed pomegranate cultivars. Only male flowers had a bacterial TVC > 10 UFC/g, probably because the flowers were collected directly from the field after they had fallen from the tree. No contamination was detected in the yeast and moulds except for the Mollar de Elche peel (2019 and 2020). Furthermore, we also found no microbiological contamination in pomegranate extracts. The antimicrobial activity of extracts was evaluated by following the ISO 20645

based on an agar-disk diffusion method with some modifications for C. albicans and the filter paper size used. Petri dishes were prepared with a bottom layer of about 10 mL of a non-inoculated agar medium, which was Tryptone Soy Agar (TSA) (VWR, Milano, Italia) for bacteria and Sabouraud Dextrose Agar (SDA) for Candida. Then a top agar inoculated with 106 CFU/mL of cells was poured onto the bottom layer. After solidification, a sterile filter paper disc (about 6 mm in diameter) containing the test compound was placed on the surface of the inoculated top agar. The disks were prepared by soaking up 100 µL of extract and then placing them still wet on top of the agar layer. Negative control disks were soaked in the solvent used for the extraction. Plates were incubated at 37 °C for 24 h. To assess the antimicrobial activity of the extracts, bacterial and yeast growth inhibition under and around the disk was evaluated, and the diameter of the inhibition zone was measured and given in millimetres. The growth inhibition was calculated on a scale of 0% to 100%, with 0% indicating no inhibition and 100% indicating the complete absence of growth below the disk. Each experiment was repeated three times.

Effect of Pomegranate Extracts on Bacterial Growth

The susceptibility of the four microorganisms to different concentrations of pomegranate extracts was determined by the broth microdilution method, and the inhibition of growth was assessed by a turbidimetric assay. A serial dilution of the extracts (highest concentration: 50 µg/µL) was prepared in a growth medium and inoculated with bacteria at 37 °C for 24 h. A calibration curve correlated the turbidimetric values with bacterial cell counts. Fertility and sterility tests were run in parallel with ranging concentrations of Tryptone soy broth (TSB) (VWR, Milano, Italia) and extracts, respectively. After the incubation at 37 °C for 24 h, the optical density at A600nm was determined, and the percentage of inhibition was obtained by the following Equation (2):

$$Inibition\ (\%) = 100 - \frac{O.D./mL_{extract}}{O.D./\ mL_{no\ extract}}\ \text{X}\ 100 \qquad (2)$$

2.8. Cell Viability Evaluation of Pomegranate Extracts in Keratinocyte Cells

The human keratinocyte cell line HaCaT was cultured at 37 °C in a humidified incubator with 5% CO2 in Dulbecco's Medium (DMEM) supplemented with 10% foetal bovine serum, two mM L-glutamine, 50 U/mL penicillin, and 50 g/mL streptomycin. To assess the intracellular antioxidant activity of HaCaT cells, they were seeded at a density of 3104 cells per well in 96-well plates. The studies were conducted 24 h after incubation at 37 °C in 5% CO2.

Cell viability was determined by decreasing MTT as described in our previous work [30]. At 37 °C in 5% CO2, HaCaT cells were treated for 24 h with various concentrations of extracts (0.160–5 mg/mL). The treatment medium was changed to MTT in HBSS (0.5 mg/mL) for two hours at 37 °C in 5% CO2. Formazan crystals were dissolved in isopropanol after being washed with HBSS. The formazan concentration was determined (570 nm, reference filter 690 nm) using the VICTORTMX3 multilabel plate reader (PerkinElmer, Waltham, MA, USA). The viability of the cells was expressed as a percentage of total vitality.

2.9. Statistical Analysis

As appropriate, we used one-way ANOVA together with Dunnett or Bonferroni post hoc tests to conduct our statistical study. On a Windows platform, analyses and Pearson correlation analysis were performed using GraphPad PRISM software (version 5.0; GraphPad Software, La Jolla, CA, USA).

3. Results and Discussion

3.1. Qualitative Polyphenols Identification

We obtained the mass data in the full-scan analysis, and product ion mass data were acquired by the information-dependent acquisition method. Thirty-one phenol compounds, including hydrolysable tannins such as ellagitannins and gallotannins, and phenol acids from the peel extract of pomegranate, were identified based on previous studies [39,40,41,42,43]. Compounds were observed by their [M − H]− ions in negative ESI mode and their [M]+ ions in positive mode, which was helpful to detect their precise mass measurement. To organize the description of peak assignment and further characterization of individual compounds, all the phenol compounds were divided according to their compound classes in hydrolysable tannins, hydroxybenzoic acids, hydroxycinnamic acids, and dihydroflavonol for the portion of the negative ion. While for positive ions, four anthocyanins were identified. To obtain the most valuable chemical information and achieve better attribution of every peak of specific substances, G1 peel extract was selected out of all the extract samples for the investigation.

A comprehensive description of the method adopted for identifying all the substances detected by UPLC-MS/MS can be found in the Supplementary Materials (Tables S1 and S2). Accurate mass measurements, retention time, and main MS/MS product ions for all phenol compounds were reported in the Supplementary Materials.

3.2. Quantitative Analysis Using UPLC-ESI-MS/MS

The methodology established in this research may be used for the quantitative measurement of secondary metabolites contained in the peel and male flower extracts of four different pomegranate varieties. Quantitative data were expressed as mg/kg dry weight matter for all extract samples. Selected polyphenol compounds were quantified and compared among pomegranate peel and male flower extracts. The contents of the nine chosen compounds (gallic acid, punicalin, punicalagin A and B, ellagic acid, cyanidin 3,5-diglucoside, cyanidin 3-glucoside, pelargonidin 3,5 diglucoside, pelargonidin 3-glucoside) in the peel and male flower extracts of pomegranate from four different varieties of pomegranate are shown in Table 2 and Figure 1.

Table 2. Quantification of polyphenols by using UPLC-ESI-MS/MS. Each sample was isolated in triplicate and analysed separately. Three repetitions were used to determine the standard deviation.

Analyte	Wonderful peel 2019		Mollar de Elche peel 2019		G1 peel 2019		Wonderful male flowers 2020		Mollar de Elche male flowers 2020		Wonderful peel 2020		Mollar de Elche peel 2020		G1 peel 2020		Parfianka Peel 2020	
	Conc.[1]	DS%	Conc.[1]	DS%	Conc.[1]	DS%	Conc.[1]	DS%	Conc.[1]	DS%	Conc.[1]	DS%	Conc.[1]	DS%	Conc.[1]	DS%	Conc.[1]	DS%
Gallic acid	9.7	12.6	33.6	2.2	17.2	1.7	925.2	5.2	789.5	1.4	28.5	1.9	47.8	13.1	53.3	16.8	58.5	0.4
Punicalin	7.7	0.5	34.1	18.8	<LOQ	<LOQ	5948.2	1.0	2143.8	3.3	638.7	1.2	946.4	3.7	670.1	8.1	67.6	3.1
Punicalagin A	478.9	6.5	2176.7	4.9	325.3	9.5	3562.2	0.4	430.4	1.32	2754.8	7.9	7206.4	4.6	3622.3	4.2	3767.3	6.7
Punicalagin B	947.8	1.9	3343.6	0.6	540.7	3.8	4757.8	0.5	667.5	3.3	3320.1	1.3	5812.9	1.3	2805.7	3.1	5367.8	0.4
Ellagic acid	48.9	12.8	231.2	0.4	19.7	6.6	42.4	2.4	87.1	3.5	418.9	0.2	289.7	2.7	337.3	1.5	123.2	3.0
Cyanidin 3,5-diglucoside	25.2	3.6	<LOQ	<LOQ	3.4	14.1	6.1	14.4	<LOQ	<LOQ	4.7	9.7	<LOQ	<LOQ	<LOQ	<LOQ	5.7	4.2
Cyanidin 3-glucoside	23.9	13.2	8.3	32.5	0.5	9.2	<LOQ	<LOQ	<LOQ	<LOQ	7.1	14.1	<LOQ	<LOQ	<LOQ	<LOQ	8.3	13.7
Pelargonidin 3,5-diglucoside	8.4	9.8	9.4	9.4	20.6	13.4	2.4	14.5	8.1	14.9	3.5	15.7	<LOQ	<LOQ	<LOQ	<LOQ	3.9	12.1
Pelargonidin 3-glucoside	13.1	18.6	7.2	18.6	1.8	0.2	1.1	14.4	<LOQ	<LOQ	7.0	10.1	<LOQ	<LOQ	<LOQ	<LOQ	8.1	13.2

[1] *The mean value is expressed as mg/Kg of DM (dry matter).*

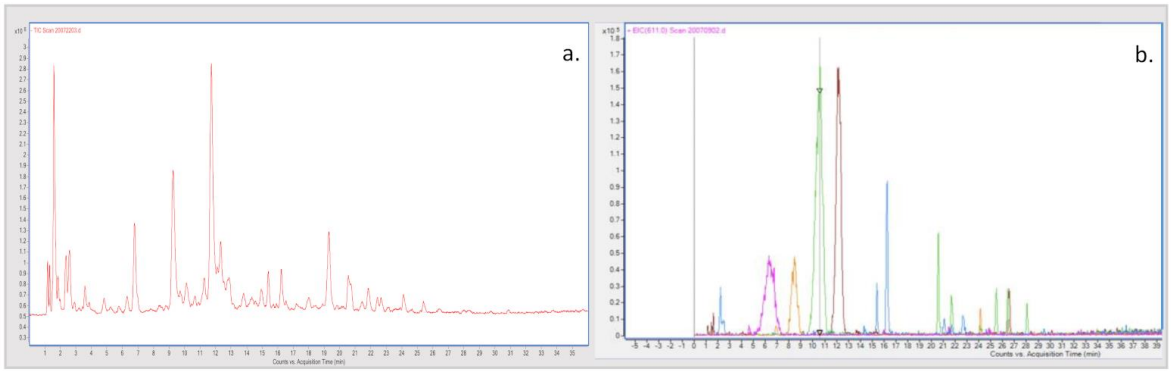

Figure 1. HPLC chromatographic profile of the phenol compounds (a), and quantified anthocyanins (b) (A4 in brown, A2 in green, A3 in orange, A1 in fuchsia), present in pomegranate peel extracts (variety G1). For peaks identification see Tables S1 and S2.

Among phenol compounds, ellagitannins represent the predominant class of phenol compounds present in pomegranate peel and flowers [15,40]. The ellagitannins, punicalagin A and B, punicalin, and ellagic acid were quantified in our work. High levels of punicalagin A and B (7206.4 mg/kg and 5812.9 mg/kg, respectively) were detected in the Mollar de Elche 2020 peel extract. The content of punicalagin A was also high in the Parfianka (3767.3 mg/kg) and G1 (3622.3 mg/kg) 2020 peel extracts. The Mollar de Elche 2019 peel extract (2176.7 mg/kg) and the Wonderful 2020 peel extract

(2754.8 mg/kg) revealed a content of punicalagin A higher than the other pomegranate varieties taken into account in this study. The Parfianka peel extract reported a high content in punicalagin B (5367.8 mg/kg), followed by the peel extracts of Mollar de Elche 2019 and Wonderful 2020. Our results are consistent with previous research that found extremely high levels of punicalagin (10,543.4 mg/g) in pomegranate peels. [13,15,44]. The punicalagin in pomegranate peel extract represented the most dominant component in the extract compared with ellagic acid, gallic acid, catechin, and epicatechin [45].

A high concentration of punicalin was detected in the Mollar de Elche 2020 peel extract, followed by the Wonderful and G1 2020 peel extracts. The Wonderful peel extract demonstrated a higher ellagic acid content than the other pomegranate varieties. The ellagic acid in the Wonderful 2020 peel extract was 428.9 mg/kg, while in the Mollar de Elche and G1 2020 peel extracts it was 289.7 and 337.3 mg/kg, respectively. The peel extract of Mollar de Elche 2019 resulted in an ellagic acid concentration of 231.2 mg/kg, while in the Wonderful and G1 peel extracts, the concentration of ellagic acid was lower.

Among the male flower extract, Wonderful 2020 showed a high level of punicalin followed by punicalagin B and A. In Mollar de Elche, the content of punicalin was higher compared to the concentration of punicalagin A and B. In the male flower extract, the ellagic acid content was low for Wonderful (42.4 mg/kg) and Mollar de Elche (87.1 mg/kg). Our work investigated the content of gallic acid, which belongs to the hydroxybenzoic acid class. Among the peel extracts, the samples with a high gallic acid content were the G1 and Parfianka 2020 peel extracts, followed by the Mollar de Elche 2020 and 2019 peel extracts. The concentration of gallic acid in male flower extract of Wonderful showed a high concentration of 925.2 mg/kg compared to the concentration obtained for the Mollar de Elche male flower extract (789.5 mg/kg). The concentrations of phenols detected in our study matched those observed in previous studies. The range of punicalagin in the peel extract was higher than the range of ellagitannins and hydroxybenzoic acids [46,47]. We also investigated the content of anthocyanins which revealed differences in their contents in different cultivars. In detail, the amount of cyanidin 3,5-diglucoside was 25.2 mg/kg in the Wonderful 2019 peel extract, while in the 2020 variety, the concentration was much lower (4.7 mg/kg). This compound was also detected in the Wonderful male flower extract, where the concentration was 6.1 mg/kg, while in Mollar de Elche peel and male flower extract, the concentration of cyanidin 3,5-diglucoside and cyanidin 3-glucoside was under the limit of quantification. Pelargonidin 3,5-diglucoside was detected in high concentration in G1 2019 peel extract (20.6 mg/kg), although, in the G1 2020 peel extract the concentration was under the detection limit. In the Wonderful and Mollar de Elche male flower extract, the pelargonidin 3,5-diglucoside was 2.4 mg/kg and 8.1 mg/kg, respectively. The mono glycoside pelargonidin was 13.1 mg/kg in the Wonderful 2019 peel extract, followed by Parfianka 2020 peel extract and Mollar de Elche 2020 and 2019 peel extracts. In the male flower extract, the content of pelargonidin 3-glucoside was 1.1 mg/kg for the Wonderful variety, while it was under the limit of detection in Mollar de Elche.

Our results confirmed that the phenol composition of pomegranate is strongly influenced by the fruit part (such as peel, mesocarp, and arils), cultivar, environmental conditions, solvent, and methods used for the extraction, as also reported in other studies [21,48,49]. Additionally, our work used a green extraction technique based on US technology, one of the most advantageous greenways for bioactive component extraction. This technology employs cavitation to collapse membrane cells, enabling the extraction of many molecules. The US technology provides various advantages, including low prices and the capacity to reduce extraction time, solvent use, and energy

consumption [16,21,50,51,52]. Many studies highlight how US technology is used mainly on natural products, including pomegranate phenol extraction [12,16,21,48,51,53].

3.3. The Total Phenol Content (TPC) and Antioxidant Capacity (AC) of Pomegranate Extracts

Table 3 reports the TPC and AC results obtained for the four pomegranate cultivars and their peel and male flower extracts. The Folin–Ciocalteu colorimetric method determined total phenol content and demonstrated impacts that ranged between 0.50 to 12.34 µmol GAE/g for peel extracts, and 0.46 and 0.778 µmol GAE/g for male flower extracts. Among the analysed samples, the peel extract from Mollar de Elche 2020 reported content of 12.341 µmol GAE/g, which represented the highest TPC, while Wonderful 2019 showed the lowest TPC. For the male flower extract samples, the TPC results were 0.778 and 0.746 µmol GAE/g for Wonderful and Mollar de Eche, respectively. The results show that pomegranate peel extracts have a phenol content 20 times higher than the corresponding extracts obtained from the male flowers of the same cultivars of Wonderful and Mollar de Elche. Our results are in line with the TPC of pomegranate peel extract reported in other works [54,55]. Based on the obtained results, we might highlight the impact of the type of cultivars and seasonal variation genotype of TPC in the biosynthetic pathway, as reported in previous studies [26,56,57,58]. The antioxidant capacity was obtained by two methods based on the evaluation of the free-radical scavenging capacity of the peel and male flower extract (DPPH and ABTS) and one method based on the reducing power of the extract samples (FRAP).

Table 3. Total phenol content (TPC) and antioxidant capacity (AC) of pomegranate extracts.

Samples	Folin Ciocalteau	ABTS		FRAP	DPPH	
	(µmol GAE/g)	(µmol TEA/g)	$IC_{50\ (mg/mL)}$	(µmol TEA/g)	(µmol TEA/g)	$IC_{50\ (mg/mL)}$
Wonderful Peel 2019	0.500 ± 0.004	0.076 ± 0.002	0.016 ± 0.001	2.170 ± 0.003	0.242 ± 0.056	0.065 ± 0.056
Mollar de Elche Peel 2019	2.304 ± 0.006	3.290 ± 0.001	0.001 ± 0.001	3.299 ± 0.028	0.455 ± 0.007	0.035 ± 0.007
G1 Peel 2019	1.872 ± 0.002	2.121 ± 0.001	0.001 ± 0.001	3.730 ± 0.001	1.524 ± 0.012	0.011 ± 0.012
Wonderful male flowers 2020	0.778 ± 0.003	6.808 ± 0.002	0.002 ± 0.001	0.615 ± 0.022	1.149 ± 0.014	0.014 ± 0.013
Mollar de Elche male flowers 2020	0.746 ± 0.003	3.168 ± 0.002	0.001 ± 0.001	0.458 ± 0.013	0.444 ± 0.020	0.036 ± 0.023
Wonderful Peel 2020	6.346 ± 0.001	29.301 ± 0.001	0.001 ± 0.002	7.015 ± 0.024	34.361 ± 0.001	0.001 ± 0.001
Mollar de Elche Peel 2020	12.341 ± 0.002	18.862 ± 0.004	0.001 ± 0.001	12.435 ± 0.801	3.230 ± 0.003	0.003 ± 0.001
G1 Peel 2020	9.283 ± 0.015	21.754 ± 0.001	0.002 ± 0.001	12.407 ± 0.739	5.029 ± 0.010	0.002 ± 0.003
Parfianka Peel 2020	6.098 ± 0.001	15.875 ± 0.001	0.001 ± 0.001	4.860 ± 0.237	4.393 ± 0.002	0.003 ± 0.001

To determine whether the pomegranate extracts produced had bio-active antioxidant properties due to their high content of phenol compound, which is widely known as being responsible for antioxidant activity, the in vitro antioxidant activity of all extracts was evaluated using the DPPH, ABTS, and FRAP assays [59]. Our results show that pomegranate peel and male flower extracts display variability in inhibitory activity against DPPH radicals ranging between 0.242–34.361 µmolTEA/g. Among the tested pomegranate peel extract, the highest radical scavenging activity was detected for the Wonderful 2020 peel extract, followed by the G1 2020 peel extract and the Parfianka 2020 peel extract. Meanwhile, male flower extracts show their highest inhibitory activity against DPPH radicals in the following order: Wonderful > Mollar de Elche.

Our work also evaluated the antioxidant activity by ABTS radical cationic decolorization assay, showing that the highest radical activity was detected for the Wonderful 2020 peel extract (29.301 µmol TEA/g) the lowest one in the Wonderful 2019 peel extract. Among male flowers, it was found that the antioxidant activity was 6.808 and 3.168 µmolTEA/g for the Wonderful and Mollar de Elche male flower extracts, respectively.

The ferric reducing and antioxidant power assay was employed. The FRAP results of pomegranate peel and male flower extract were determined to compare the four varieties of pomegranate in our investigation. The Mollar de Elche 2020 and G1 2020 peel extract reported the highest FRAP value

among all peel extracts tested, followed by Wonderful 2020, Parfianka 2020, G1 2019, Mollar de Elche 2019, and Wonderful 2019.

We found a positive and high Pearson correlation with a significant p value (* $p < 0.05$; **** $p < 0.0001$) between antioxidant activity measured with FRAP and ABTS and total phenolic contents, suggesting that the antioxidant activity is dependent on the number of phenolic compounds present in the extracts.

Results show that the peel extracts displayed higher AC than the male flower extracts. The AC found in pomegranate peel extracts was connected with the punicalagin concentration determined by our quantitative study, and it was also consistent with other studies [15,55].

3.4. Antimicrobial Activity Evaluation

To explore the possibilities of using pomegranate by-products as a natural preservative in cosmetic formulations alone or as a booster to reintroduce agri-food waste into the cosmetics formulation as a natural bioactive component, we investigated the antimicrobial activities of peel and male flower extracts derived from Wonderful, Mollar de Elche, Parfianka, and G1 cultivars. The evaluation of our extracts' activity against the Gram-positive and Gram-negative was obtained by the disk-diffusion testing [60] (Table 4). The extracts activities were tested against Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Candida albicans which are microbial species referenced in the challenge test for cosmetic products. The Wonderful 2020 peel extract was the most efficient against Staphylococcus aureus, with a 70% growth inhibition in microdilution tests and an inhibition zone diameter of 3 mm by disk diffusion. The highest antimicrobial activity against the Escherichia coli was demonstrated by the Wonderful 2020 and G1 2020 peel extracts, while all cultivars were active against Candida albicans. In particular, a good activity was detected for the G1 2019 and 2020 peel extracts, the Wonderful 2020 peel extract, and the Mollar de Elche 2020 peel extract. The antimicrobial activity obtained for peel extracts was in line with a previous work by Khan et al. [61] reporting high antimicrobial activity for pomegranate peel extract against Gram-negative bacteria. Besides, Kupnik et al. [62] found that E. coli and P. aeruginosa were more susceptible to pomegranate peel extract than to other parts of the fruit. In accordance with other studies, peel and male flower extract were effective also against Gram-positives [5,26,62,63,64].

Table 4. Evaluation of the antimicrobial activity by using S. aureus, E. coli, and C. albicans.

Samples	Diameter of inhibition (mm)	Evaluation [b]
	S. aureus	
Peel Wonderful 2019	8	++
Peel Wonderful 2020	12	+++
Peel Mollar de Elche 2019	<8	++
Peel Mollar de Elche 2020	8	++
Peel G1 2019	8	++
Peel G1 2020	8	+
Peel Parfianka 2020	8	+
Male flower Mollar	-	+
Male flower Wonderful	-	+
	E. coli	
Peel Wonderful 2019	10	
Peel Wonderful 2020	10	+
Peel Mollar de Elche 2019	10	
Peel Mollar de Elche 2020	10	+
Peel G1 2019	8	++
Peel G1 2020	10	+
	C. albicans	
Peel Wonderful 2019	10	++
Peel Wonderful 2020	10	++
Peel Mollar de Elche 2019	10	++
Peel Mollar de Elche 2020	12	++
Peel G1 2019	12	+++
Peel G1 2020	14	+++

The peel G1 extract was chosen as the most promising among the tested extracts for further antibacterial investigation using a turbidimetric test. Table 5 shows the IC50 obtained against S. aureus and P. aeruginosa in G1 2020 peel extract. It was detected that a 1:2 dilution of the extract was effective in reducing 97% of the bacteria.

Table 5. Determination of IC50 against S. aureus and P. aeruginosa in G1 2020 peel extract.

Sample %[a]	OD/mL	Rid. %
	S. aureus	
0	0.4467	-
5	0.2733	23
10	0.2232	34
18	0.2932	20
20	0.2398	30
25	0.2318	32
30	0.1520	52
50	0.0052	97
	P. aeruginosa	
0	0.7013	-
35	0.4600	34
40	0.3837	45
45	0.4630	34
50	0.4500	35

[a]Tested sample dilution expressed in (%).

Our pomegranate extract demonstrated a variable antimicrobial activity against the four microbial species considered. This might be attributed to the pH values ranging from 3.9 ± 0.2 to 4.6 ± 0.3 that were found in all of the extracts and the high concentration of polyphenols that can inhibit microbial growth. Data herein indicated that peel extract, mainly from the G1 and Wonderful varieties, had an effective antimicrobial activity, due to its inhibitory effect on bacterial growth. At a concentration of 10 mg/disc, the G1 extract induced the formation of a clear inhibition zone ranging from 1 to 4 mm against all microorganisms tested. Another extract with a measurable antimicrobial activity was the Mollar de Elche 2020 peel extract, with an ability to inhibit the growth of the Aspergillus brasiliensis (data not shown). Furthermore, this conclusion is supported by the TVC of the yeasts and moulds, which were found lower than 10 UFC/g in raw materials from the Mollar de Elche 2019 and 2020 peel extract.

Our results concur with several studies on the antimicrobial activity of pomegranate extracts [5,26,64].

Accordingly, we can correlate the antimicrobial activity of pomegranate peel extract with the high concentration of polyphenol compounds such as punicalagin and punicalin as evidenced by the UPLC-ESI-MS/MS analysis. The pomegranate peel extract obtained from the G1 cultivars demonstrated interesting inhibitory activity and had among the highest concentrations of punicalagin (3622.3 and 2805.7 mg/kg for two isomers A and B, respectively). Punicalagins use their hydrophilic part to interact with the polar regions of the cell membrane compromising its ability to transport substances inside the cell [5,63]. Additionally, phenols can render substrates unavailable to microorganisms or interfere with protein secretion.

The results against the Gram-negative for the G1 and Wonderful pomegranate extracts pave the way for their further application as booster antimicrobial agents to be used in combination with common synthetic antimicrobials, which might be added in lower amounts thereby increasing skin tolerance and product safety. Furthermore, since these extracts are obtained from the peel and male flowers of pomegranate, they provide a concrete example of natural products made from agri-food waste that have been reintroduced into the cosmetics' circular economy concept.

3.5. Cytocompatibility of Pomegranate Extract in Human Keratinocytes

The cytocompatibility was evaluated in the pomegranate peel and male flower extracts 2019 and 2020 in keratinocytes HaCaT cells to find the nontoxic concentration of pomegranate extracts and further exploitation of these extracts as antimicrobial agents in cosmetics formulations. The use of keratinocytes was based on the concept that these cells play a significant role in creating skin structure and the sustenance of homeostasis, including the production of the skin barrier and the extracellular matrix (ECM). The cells were treated with extract concentrations ranging from 0.15 to 5.00 mg/mL for 24 h. The vitality of the cells was then determined by the MTT test. The results showed no significant difference in cell viability between the different pomegranate extracts ($p > 0.05$) varieties at any extract concentration. Figure 2 shows the cytotoxicity results obtained for the extracts selected with the most interesting antimicrobial properties, such as the G1 2020 and 2019 peel extracts and the Wonderful 2020 and 2019 peel extracts. Additionally, no change in the morphology was observed after treatment of keratinocytes with the extracts (data not shown).

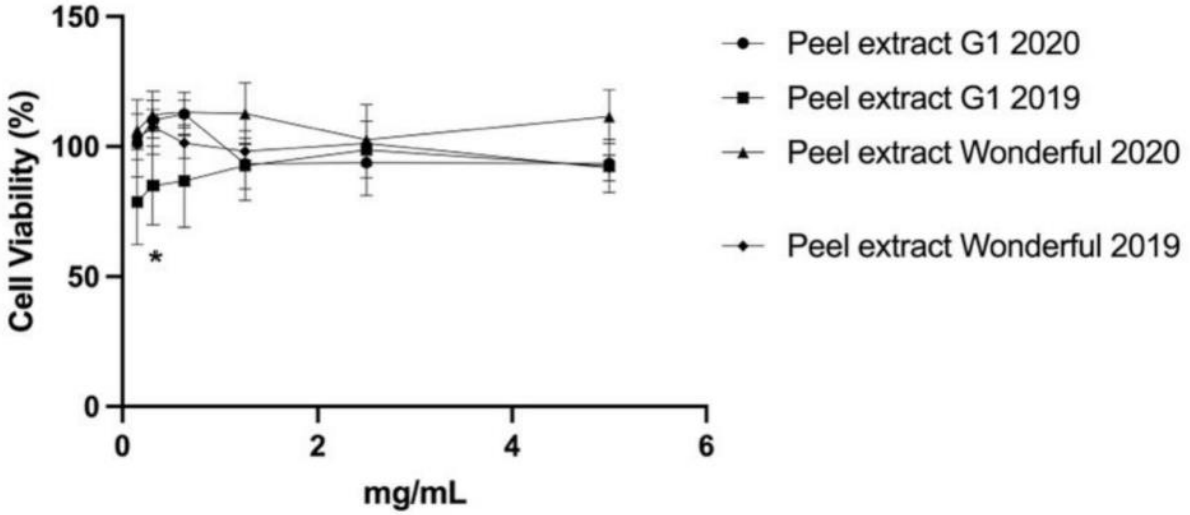

Figure 2. Cytotoxicity of the pomegranate peel extracts G1 and Wonderful 2019 and 2020 in HaCaT cells evaluated by MTT assay. For 24 h, cells were treated with an extract at different concentrations (0.15–5.00 mg/mL). The data are shown as a percentage of control cells and as the mean ± SEM of four separate experiments. (* $p < 0.01$ vs. untreated cells; one-way ANOVA with Dunnett post hoc test).

Our results agreed with a previous study that reported null cytotoxicity of pomegranate extract against HaCaT cells [65]. Liu et al. [66] also demonstrated that the pomegranate extract resulted in no cytotoxicity in a concentration range from 6.25 to 100.00 µg/mL. These promising results obtained in the cytotoxicity tests confirm the suitability of the extracts as a natural booster ingredient for cosmetic formulations.

4. Conclusions

The main phenol components profile, antioxidant capacity, antibacterial activity, and cell viability of four pomegranate types cultivated in Marche were investigated. The UPLC-ESI-MS/MS study revealed that Mollar de Elche had a high concentration of punicalagin A and B, whereas G1 and Parfianka had high concentrations of gallic acid. Moreover, antibacterial activity testing revealed that all extracts were promising. Finally, this work shows the value of studying bioactive chemicals in agri-food waste products to generate innovative natural antibacterial and antioxidant components for cosmetic

formulation. Further research will focus on the antibacterial activity of suitable pomegranate extract mixes and pomegranate extracts combined with conventional preservatives to minimise their concentration in cosmetic products.

Supplementary Materials

The following are available online at https://www.mdpi.com/article/10.3390/antiox11040768/s1, Table S1: Identification of polyphenols in pomegranate by-product extracts by UPLC-ESI-MS/MS analysis, Table S2: Identification of anthocyanins in pomegranate by-products extracts by UPLC-ESI-MS/MS analysis.

Author Contributions

Conceptualization, P.D.M. and M.R.G.; methodology, P.D.M., M.R.G., M.C., G.L. and E.B.; software, M.R.G., M.C. and E.B.; validation, M.C., E.B., P.D.M. and G.L.; formal analysis M.C., M.R.G., L.D.N. and S.N.; investigation, M.R.G., M.C., L.D.N. and S.N.; resources, P.D.M. and R.C.; data curation, M.R.G., P.D.M., M.C., L.D.N. and S.N.; writing—original draft preparation, M.R.G. and P.D.M.; writing—review and editing, P.D.M., M.R.G., L.A.V., E.B. and S.N.; visualization, P.D.M., M.R.G., D.V.P. and L.A.V.; supervision, R.C., M.R.G., L.D.N. and L.A.V.; project administration, P.D.M. and R.C.; funding acquisition, P.D.M. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Data is contained within the article and Supplementary Materials.

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Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 6

Future perspectives and Conclusions

Future perspectives

Many other by-products of natural origin can be exploited and used in the cosmetic industry thanks to their properties.

Physiochemical characterization and pre-formulation studies of sericin of different molecular weights for cosmetic and dermatologic formulations

Sericin is a protein from silkworm cocoons (*Bombyx mori*), usually discarded by the textile industry. It is the 20-30% of the cocoon and it has a molecule weight (MW) ranging from 10 to 400 kDa, depending on the extraction methods. The process of separation of sericin from the silk is called *degumming* (1). In cosmetic industry sericin is used for the similarity with filaggrin and for the strong affinity with hydrophobic proteins, such as keratin. It shows moisturizing properties, increase the skin elasticity, anti-wrinkle and anti-aging effect, antioxidant properties and photoprotection against UVB-light.

Sericin with MW < 20 kDa is used for skincare in cosmetic applications thanks to the capacity to penetrate up to the stratum corneum, while sericin with MW > 100 kDa is used for its filming properties. Therefore, sericin with MW > 20 kDa is generally used for the production of drug delivery system and hydrogels.

The aim of our work is the optimization of extraction methods of sericin with different MW for different cosmetic and dermatological application, and its characterization.

A Design of Experiment (DoE) was prepared for the optimization of the degumming of sericin from silk waste (Table 1).

The independent variables were temperature (X1=60-100°C), extraction time (X2=30-120 min.) and solvent (X3=water, urea 8M and Na_2CO_3 0.5% w/w), while the dependent variables were the yield (Y1) and the MW (Y2).

Table 1. Factors and the corresponding levels investigated by the 1-Optimal design.

Independent Variables	**Type**	**Coded factors**	**Coded levels**	
			-1	**+1**
Temperature (°C)	Numeric	X_1	60	100
Time (min)	Numeric	X_2	30	120
Additive	Categoric	X_3	H_2O Na_2CO_3 Urea	
Dependent Variable		**Coded factor**		
Yield (%)		Y_1		
MW (kDa)		Y_2		

For the extraction, silkworm cocoons were cut into small pieces. The extraction was made in water bath with controlled temperature, and using the three different solvents: water, Urea 8M or Na_2CO_3 0.5%. Bases are used to extract sericin from silk thanks to the capacity to hydrolize the protein by breaking peptide bonds of the amino acids. The protein is released into the alkaline solution, where it is highly soluble (2).

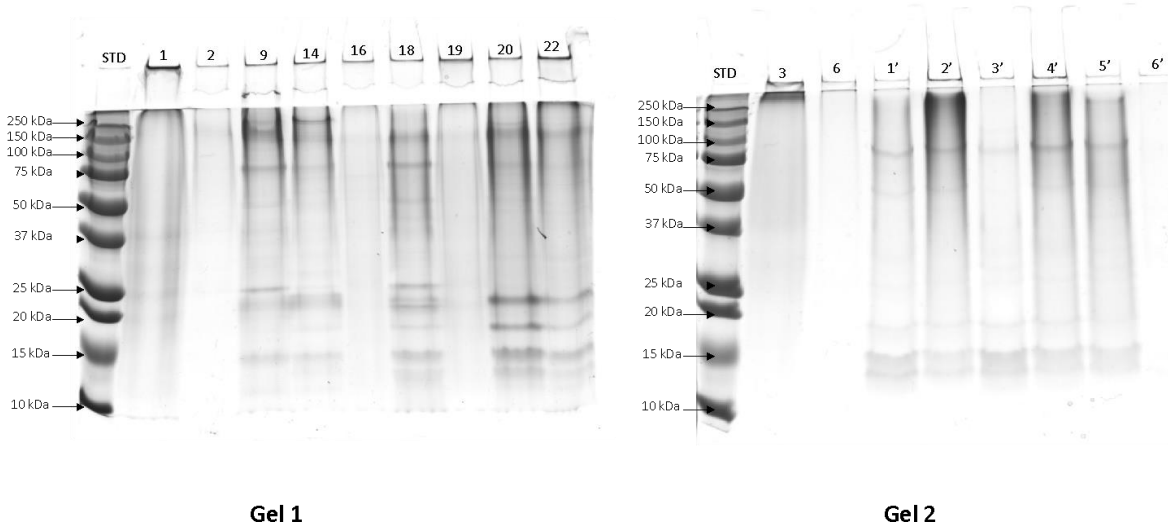
After the extraction, Urea and Na_2CO_3 were removed with dyalisis, and the solutions lyophilized in order to obtain a powder. All the experiments are listed in table 2.

Table 12. Listed experiments for sericin extraction.

Run	Temperature (°C)	Time (min)	Solvent
1	80	120	Na_2CO_3
2	91.4	48.45	Urea
3	100	75	Na_2CO_3
4	100	120	Urea
5	86.4	59.25	Acqua
6	80	30	Na_2CO_3
7	73.2	90.3	Acqua
8	68.6	100.65	Urea
9	73.4	60.15	Urea
10	91.4	99.75	Acqua
11	60	120	Acqua
12	68.4	48.9	Acqua
13	91.4	99.75	Acqua
14	68.6	100.65	Urea
15	100	30	Acqua
16	100	30	Na_2CO_3
17	68.4	48.9	Acqua
18	60	30	Urea
19	80	120	Na_2CO_3
20	60	30	Na_2CO_3
21	91.4	48.45	Urea
22	60	75	Na_2CO_3

The extracts were then characterized. The yield (Y1 response) for each extraction was calculated weighted sericin powder obtained after dyalisis and lyophilization. The molecular weight (Y2 response) was determined by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2).

FTIR spectra (Figure 3) showed the characteristic energy absorption in line with literature (3, 4). No major difference is observed in the IR spectra of tested samples extracted from different methods and prepared using different additives.



Gel 1 **Gel 2**

Figure 2. Electrophoretic separation of sericin.

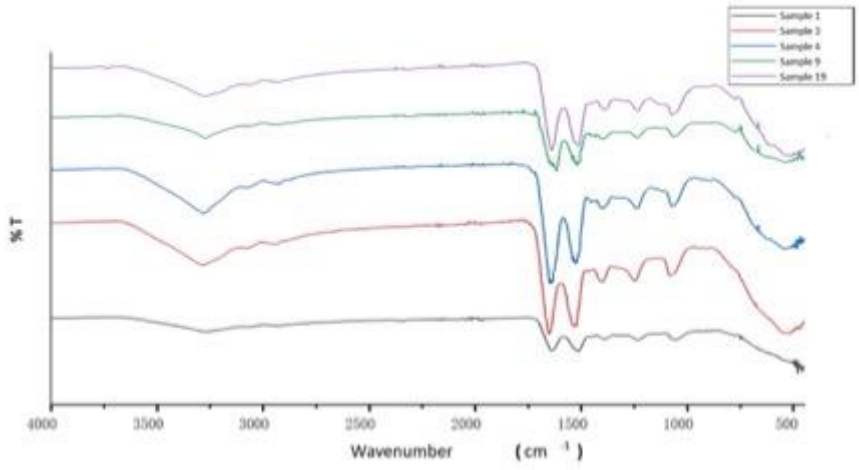


Figure 3. FTIR spectra of sericin powder.

The DoE (Table 3) was used to determine how the parameters influence the results of the experiments.

Table 3. Yield percentage and fractions of the MW for each extract obtained.

Run	Yield	Normalized volume (Optical Density)		
	(%)	12-20 kDa	21-99 kDa	100-250 kDa
1	17,76	1334,68	538.164,22	
2	19, 83			4684368,7
3	12,28			
4	21,46		111788,8	7546353
5	18,85	2.207.991,07	278591,7	4529032
6	1,62			
7	18,48	686339,4		1895817
8	20,06	1.563.610,5	1.303.364,8	24.426.487
9	12,72	5.703.468,743	13.665.649,213	36.420.950,4
10	22,73	9.620.879,8	353016,6	50.529.468
11	4,56	30.942.800		4419770
12	4,15	4.435.129,5	499691	5859168
13	25,34	791.283,969	368951,698	42.562.940,2
14	15,25	5.102.568,99	16.108.363,4	35.089.779
15	13,24			7.973.892,97
16	34,18			1365325,62
17	2,93	1050835,14		
18	1,2	11.373.197,19	20.647.829	7285836,2
19	21,74			1402733,55
20	0,78	19.616.843,23	15.427.603,16	5.519.761,694
21	22,70		407.253,2074	37764254
22	1,93	4443857,886	5.561.783,801	4062880,56

The relationship between the parameters and the yield of the extracts (Y_1 response) was calculated using a reduced linear model (Figure 4).

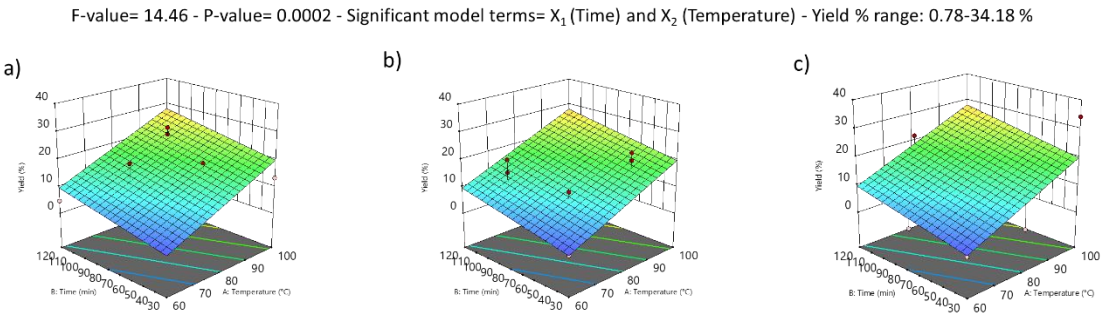


Figure 4. 3D Surfaces of the effect of Time (min.) versus Temperature (°C) on sericine extraction Yield (%) using H_2O (a), Urea8M (b) and Na_2CO_3 0.5 % as solvents.

In the case of Y_2 response, sericin fractions with different MW were obtained and were grouped in 3 main intervals respectively (12-20; 21-99; 100-250 kDa).

In this case, the use of solvent X_3 (H_2O, Na_2CO_3 or Urea) was found to be a significant model term influencing the MW of sericin extracted with the procedure used (data not reported).

The DoE allows to select the most convenient method for the recovery of sericin of three fractions of different MW: < 20 kDa, 20-99 kDa and 100-250 kDa. The next step will be the preparation of the gels using sericin with high MW (> 100 kDa) and to find which extraction method is convenient for obtaining this kind of sericin. The gelation will be then examined using a rheometer, to evaluate the strength of the gels. The gelation of sericin dissolved in aqueous solution occurred by the formation of hydrogen bonding between sericin molecules. It has been proved that the network formation is dependent on the temperature, on the MW and on the ethanol content.

Exploitation of Pistachio Hull (*pistacia vera. l,* variety bronte) for the recovery of anacardic acids, and the synthesis of surfactants

Different Ultrasound-Assisted extraction methods were tested for the recovery of extracts from Pistachio Hull. An ultrasonic bath and a sonicator with a probe were used, changing the frequency and the amplitude, the solvents and the extraction times. Extraction parameters are reported in Table 4.

Table 4. Parameters of the extractions.

Sonicator	Labsonic LBS2-10, Falc	Sonicator Q500, QSonica
Operative Conditions	Frequency 59 kHz at 50% amplitude (Total energy 300W) Room temperature	Frequency 20 kHz at 20% amplitude (Total energy 500W) Room temperature
Extraction time points	10 min 20 min 30 min	10 min 20 min 30 min
Solvents	EtOH 70° H_2O Hexane Ethyl Acetate MeOH/H_2O/HCl (80:19.9:0.1)	EtOH 70° H_2O Hexane Ethyl Acetate MeOH/H_2O/HCl (80:19.9:0.1)

The extracts were analysed in terms of HPLC-DAD-EMI/MSn, antioxidant assays (DPPH, ABTS, FRAP), and Total Phenol Content. Among the 37 compounds found in the extracts, the attention was for a particular class of compounds named “anacardic acids”, for their use in cosmetic products. The total amount of anacardic acids was higher in the sample extract with hexane like solvent and the sonicator with the probe, for 30 minutes. Results are reported in Table 5.

Table 5. Quantification of anacardic acids by HPLC-DAD-ESI/MSn.

	Hexane		**Ethyl acetate**		**MeOH/H_2O/HCl (80:19.9:0.1)**	
Sonicator	S1	S2	S1	S2	S1	S2
	Compound (g/kg)					
Total Anacardic Acid	80.16	87.49	53.46	81.00	65.04	38.66

After the optimization of the extraction method, these molecules were purified using a standard with a Thin Layer Chromatography. The idea is to synthetize a surfactant using triethanolamine:

triethanolamine anacardate (5). An ^{1H}NMR analysis will be made to compare the surfactant obtained, with the one found in literature.

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Conclusions

In recent years the attention to natural cosmetics has become increasingly greater. This has led companies and brands to conform to this trend, through technological innovations such as the use of extraction methods with reduced environmental impact and the use of eco-friendly solvents. The strategies that companies use are the use of raw materials of natural origin, biodegradable, plastic-free, reusable or recyclable packaging, and certifications that guarantee the final consumer the naturalness of the product and respect for the environment.

The research presented in this thesis show some green extraction methods of bioactive molecules from natural raw materials, for cosmetic applications. These studies also show the applicability and the efficacy of the cosmetic formulations with these extracts or bioactive compounds, through some *in vivo* studies.

Despite the effort of many companies and the efficacy of these products, there are still many problems to be solved, such as making the extraction methods completely eco-friendly and convincing the consumer of the truthfulness of the information on the label.

Curriculum vitae

Lucrezia Di Nicolantonio was born in Ascoli Piceno, Italy, on February 25, 1993. She attended the secondary school Liceo Scientifico B. Rosetti in San Benedetto del Tronto (AP), where she graduated in 2012. In the same year she started her studies in Biology Sciences at Marche Polytechnic University, in Ancona, Italy. During her academic studies, she completed the internship at EcoReach s.r.l., Ancona, studying the effect of sunscreens on the marine ecosystem. She graduated with a bachelor's degree in 2016, with a compilative thesis titled ""Impact of diet in shaping gut microbiota revealed by comparative study in children from Europe and Rural Africa." In the same year she started her master degree in "Molecular and Applied Biology" at Marche Polytechnic University and also in this case she completed the internship at Ecoreach s.r.l. She graduated in 2019 with a research thesis titled "Layered double hydroxide encapsulating resveratrol: preparation, characterization and in vitro study of antioxidant activity", under the supervision of Prof Giovanna Mobbili. In 2020 she started her Master in University of Camerino (UNICAM), in Science of Cosmetic and Dermatological Products, completed in 2021 with a research thesis titled "A comparative study of volatile compounds and antioxidant activity of two essential oils of *Helichrysum Italicum* growing in Tuscany and Marche (Italy)", under the supervision of prof. Dr. Roberta Censi and Dr. Dolores Vargas Peregrina. In 2020 she also started her Industrial PhD in Chemical and pharmaceutical sciences and biotechnology, at the School of Pharmacy at UNICAM, working at the same time in Recusol s.r.l., in Camerino. The PhD was under the supervision of Prof. Dr. Piera di Martino, Prof. Dr. Roberta Censi, Dr. Maria Rosa Gigliobianco and Dr. Dolores Vargas Peregrina, and it was completed in 2024. The thesis, titled "Valorization of natural sources and by-products for pharmaceutical and cosmetic applications", studying the recovery of natural sources and wastes though the qualitative and quantitative characterization of green extracts from these sources, with the final formulation of cosmetic products, followed by *in vitro* and clinical efficacy tests.

List of publications from this thesis

Di Nicolantonio, L.; Ferrati, M.; Cristino, M.; Peregrina, D.V.; Zannotti, M.; Vitali, L.A.; Ciancia, S.I.; Giovannetti, R.; Ferraro, S.; Zara, S.; et al. Evaluation of Physicochemical and Microbial Properties of Extracts from Wine Lees Waste of Matelica's Verdicchio and Their Applications in Novel Cosmetic Products. Antioxidants 2023, 12, 816.

Gigliobianco, M. R., Cortese, M., Nannini, S., **Di Nicolantonio, L**., Peregrina, D. V., Lupidi, G., Vitali, L. A., Bocchietto, E., Di Martino, P., & Censi, R. (2022). Chemical, Antioxidant, and Antimicrobial Properties of the Peel and Male Flower By-Products of Four Varieties of *Punica granatum* L. Cultivated in the Marche Region for Their Use in Cosmetic Products. *Antioxidants (Basel, Switzerland)*, *11*(4), 768.

Other publications

Di Nicolantonio, L.; Gigliobianco, M.R.; Peregrina, D.V.; Angeloni, S.; Ilorini, L.; Martino, P.D.; Censi, R. Impact of the Interactions between Fragrances and Cosmetic Bases on the Fragrance Olfactory Performance: A Tentative to Correlate SPME-GC/MS Analysis with That of an Experienced Perfumer. Cosmetics 2022, 9, 70. https://doi.org/10.3390/cosmetics9040070