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PhD Project

Sustainable use of plants adapted to the Mediterranean climate as sources of bioactive compounds with health and environmental applications

PhD candidate: Miriam Bazzicalupo

Tutor: Prof.ssa Laura Cornara

Coordinator of the PhD course: Prof. Marco Firpo

Coordinator of the PhD school: Prof. Mauro Mariotti

Summary

Abstract

This PhD project was focused on the identification of some species of Mediterranean climate to be used according to eco-sustainability criteria for the obtainment of bioactive compounds with health properties or with applications in the environmental field.

Himantoglossum robertianum flowers and *Carpobrotus edulis* leaves were micromorphologically characterized and used to obtain an hydroalcoholic and an aqueous extract, respectively. These extracts were then analysed from the phytochemical point of view. In addition, antioxidant and anti-inflammatory properties, inhibitory activities against enzymes involved in the extracellular matrix degradation and in inflammations, and their cicatrizing abilities were evaluated. Tests were performed both by *cell-free* enzymatic techniques and using *in vitro* cell cultures.

H. robertianum is a terrestrial epiphytic orchid that can reach up to 80 cm in height, commonly known as Giant orchid due to its showy inflorescence and its considerable dimensions. According to previous data, this species resulted rich orchicyanin I and II and serapianin, anthocyanins already used in cosmetic formulations. With this in mind and considering that the species is in progressive expansion along Mediterranean coasts, we used its flowers to obtain a hydro-alcoholic extract to be tested for biological properties. Since *H. robertianum* is a protected species, we also planned and optimized techniques for *in vitro* micropropagation of the plant.

Concerning *C. edulis*, the Hottentot fig, it is highly cited in ethnobotanical surveys for the treatment of various ailments and skin affections. This plant is an alien invasive species now naturalized in the Mediterranean area to the detriment of indigenous flora, thus representing a serious environmental problem. The species is therefore subject to several and repeated eradication campaigns. In a perspective of circular economy and sustainable use of natural resources we decided of using the leaves material with the aim to evaluate the biological activity of an aqueous extract obtained from the leaves. Data collected support the use of *C. edulis* as a natural resource for skin care and confirm the advisability of using material removed from the natural environment for this purpose.

Finally, again concerning the sustainable use of resources, peels from three different species of *Citrus* spp, widely cultivated in Southern Italy, were employed for the obtainment of their essential oils. Peels nowadays represent a consistent by-product of the citrus industry. Sites of production of bioactive compounds were morphologically characterised and essential oils obtained were evaluated from the phytochemical point of view. Essential oils were then tested for their phytotoxic activity against weeds and for their potential use as bioherbicides.

1. Introduction

The use of ingredients of natural origin is as old as mankind. Nowadays, a significant portion of world's population depends on plant resources such as forests, wetlands, and cultivated fields.
It has been calculated that up to 80 % of people from developing countries have a total dependence on herbal drugs for their primary healthcare, with a 25 % of prescribed medicines obtained from wild plant species (Chen et al., 2016).
The natural resources obtained from plants can be exploited for various uses: mainly for medicinal purposes, food, animal feeding, buildings, and others. The traditional knowledge of natural resources uses has still a pivotal role in the lives of poor people in rural areas, but also in the modern society is receiving increasing attention, principally due to the widespread idea that everything which is natural is safe. Moreover, more recently, for ethical reasons, the desire not to take products of animal origin is also increasing. Therefore, the use of natural products increments constantly, with more and more plant bioactive compounds and extracts studied to produce pharmaceuticals, nutraceuticals, cosmeceuticals, dietary supplements or bio-herbicides.

These plant bioactive compounds are secondary metabolites with no known direct function for the organism, but playing ecological roles in the interaction with other species and showing a wide spectrum of biological and pharmacological properties (Wink, 2015; Wink, 2020).

1.1 Brief overview on secondary metabolites

The main representative plant secondary metabolites are: phenols (flavonoids, tannins, lignin, salycilic acid), terpenoids (aromatic oils, resins, waxes, steroids, rubber, carotenoids), alkaloids (often showing toxicity, for example nicotine, caffeine, strychnine, capsaicine, cocaine). These compounds play an ecological and evolutive role in herbivory avoidance, protection against microbial attacks, attraction of pollinator or seed-dispersing animal vectors by means of pigments and scents, buffering activity for water regulation, allelopathic activity in competition phenomena among different plant species. The production of secondary compounds, which represent an adaptation of a species to a changing environment, have been indeed inherited and specialized in

million years of evolution. These substances are nowadays a very useful tool for taxonomists to comprise species radiations, mechanisms of co-evolution and to distinguish among entities in phylogenetic analyses (Wink, 2015; Wink, 2020). Given the great diversity of compounds and their functions, the way in which secondary metabolites act on biological targets are various. As medicines, they can be administered as isolated compounds or as mixture, they can be very effective and less dangerous than synthetic drugs or even exert a synergistic action with other components in the curative preparation (Seca & Pinto, 2019). Neuroreceptors, enzyme cleaving neurotransmitters, ion pumps and ion channels or cytoskeleton elements are typical targets of secondary metabolites (Roberts & Wink, 1998; Wink, 2000; Wink & van Wyk, 2010). Other examples concern the action on DNA topoisomerases, inhibition of phosphodiesterases and adenosine receptors, DNA intercalation (Wink, 2007; Wink, 2015 and references therein, Wink, 2020). The allelopathic activity exerted by secondary compounds on competing plants involves different mechanisms, like: change of membrane structure and interference with the signal transduction pathway; alteration of cell morphology and cell cycle by acting on replication, protein synthesis and mitosis; interference with normal phytohormone activities; perturbations of energy metabolism (respiration and photosynthesis); change of water balance and stomata overture/closure mechanisms; inhibition of pigment synthesis and turn-over; block of enzymatic activities (Lotina-Hennsen et al., 2006).

There is also a lot of interest in understanding the mechanisms of action of secondary compounds with key roles in plant life and in the relationship with the environment. It is first useful to have a basic understanding of evolutionary processes: the occurrence of specific secondary metabolites in unrelated taxonomic groups could be due to convergent evolution (Ober, 2005). However, genes encoding secondary compounds biosynthesis were found to have a wider distribution than that of the secondary metabolites. This aspect raised the hypothesis that the activation/expression of a certain pathway in a taxonomic group could depend on differential gene regulation (Wink, 2008). Since the majority of plant organisms has close relationships with mycorrhizal fungi (Brundrett & Tedersoo, 2018), which are also able to synthesize secondary compounds, it remain to be established who, (plant host - fungal partner), is the real responsible of compound

production, or if the fungus could have transferred the gene encoding the secondary metabolite to its host (Wink, 2008, Teoh, 2016 and references therein). Whatever their origin, the role of secondary metabolites in plant-pathogens, plant-herbivores and plant-pollinators interactions is object of several studies. Schoonhoven, Loon & Dicke (2005) stated that the defence against herbivores is the primary function; later, this view has been partially revisited (Wink, 1988; Agrawal & Weber, 2015 and references therein). Proposed mechanisms of action for secondary compounds in herbivory avoidance rely principally on their toxicity and carcinogenic activity (Bennett & Wallsgrove, 1994; Iason, 2005 and references therein). In fact, many secondary metabolites act as agonists or antagonists of neuronal signal transduction, being many of them alkaloids with structures very similar to those of some neurotransmitters (Wink, 2018). Some mechanisms are also involved in pollinators/seed dispersers attraction and/or in their food reward. Generally, pollinators or seed-dispersers are attracted by the smell and colour of flowers and fruits. These mechanisms are linked to the presence of volatile compounds, flavonoids, anthocyanins, carotenoids, terpenoids, amines and phenylpropanoids (Wink, 2018). Toxins and antimicrobic metabolites could interfere with the physiology, metabolic activity, and reproductive traits of potential enemies (bacteria and viruses and other pathogens included).

Stated all these biological activities, an astonishing number of plants could therefore be potentially employed in agriculture, or in medicine for the treatment of infections and health conditions (Wink, 2020).

Secondary metabolites accumulate in different organs of the plants: they can be concentrated in the roots, stems, leaves, flowers, fruits or seeds. Their presence and levels can vary from individual to individual of the same species, depending upon the variety, methods of obtainment, processing, and conditions of growth (Saxena et al., 2013).

Phenolic compounds are highly bioactive secondary metabolites widely distributed in plants and important components of the human diet. They are a large class of plant secondary metabolites, and include flavonoid (anthocyanins, flavonols, flavones, etc.) and other non-flavonoid compounds (phenolic acids, lignins, stilbenes) (Ribeiro et al., 2015), whose antioxidant activity varies depending on their molecular structure (Maqsood & Benjakul, 2010a). The molecular

structure-activity relationship indicates the number of hydroxyl groups as the most important factor in determining the antioxidant activity of phenolic compounds (Zhang et al., 2014). Plants rich in phenolic compounds can be used for example to prevent the dangerous effects of UV radiation on the skin. Daily diet, medicines, food supplements and cosmetics are the different options with which phenolic compounds can be supplied to the body.

1.2 The benefit of plant extracts for human health

1.2.1. Use of plants in the skin-care sector

The ingredients of natural origin have been widely used over the centuries also as cosmetic ingredients. Nowadays, the use of natural products in cosmetics is constantly increasing, and more and more compounds and molecules of plant origin are tested to evaluate their biological properties. The choice of formulations based on plant substances responds to consumer concerns regarding the use of synthetic substances or extracts of animal origin.

The application of plants in cosmetics mainly concerns hair care and skin care. The skin provides physical protection against the external environment and defends against microbial attacks; participates in the processes of thermoregulation, excretion, and tactile perception. It consists of two overlapping tissues, one facing outwards (the epidermis) and the other consisting of the dermis and hypodermis, in a relationship of contiguity with the rest of the body. The epidermis, organized in turn in several layers, transmits the effects of the external environment, aging and the use of topical agents to the underlying tissues, regulates cellular homeostasis, and provides mechanical protection through keratinocytes. It also produces melanin, the pigment that determines skin color. The dermis is made up of fibroblasts, collagen, and elastic fibers, which impart tensional strength and flexibility, and is crossed by a network of capillaries and nerve endings. The hypodermis, on the other hand, houses sweat glands and is rich in adipocytes, which function as an energy reserve and as tissue deposit for the body. These layers can undergo more or less serious alterations, ranging from simple dryness of the skin to more complex ailments, such as photodermatitis, dermatitis caused by pathogens (bacteria and viruses) and pathologies of tumor origin (Amerio et al., 2003; Gambardella et al., 2014).

The term "cosmeceutical" is commonly used to define as cosmetic certain products with ingredients promoting effects similar to drugs, since their composition includes elements with a beneficial topical action, and able to provide protection against degenerative skin conditions (Ribeiro et al., 2015). Such products can also improve skin appearance, conveying the nutrients necessary for its health and improving its tone, texture and shine, reducing wrinkles.

Many pharmaceuticals simulate the activity of natural constituents of the dermis: in their composition there is indeed a high content of phospholipids (useful for hydration and protection from UV rays), emollient mucilages (which prevent dryness and skin exfoliation), astringent tannins and anti-inflammatory flavonoids (Aburjai & Natsheh, 2003).

Products of plant origin could contain vitamins, antioxidants, essential oils, hydrocolloids, proteins, terpenoids and other bioactive compounds (Dubey et al., 2004; Dureja et al., 2005; Ribeiro et al., 2015). Safety and efficacy of these products are generally evaluated with *in vitro* tests. Furthermore, in the case of plant extracts, careful controls of the extraction method, the plant-solvent proportion and the titration of the active ingredients is required (Fowler et al., 2010; Aburjai & Natsheh, 2003; Ribeiro et al., 2015). The study of new ingredients involves their chemical characterization and *in vitro* evaluation on human cell lines to identify the correct concentration to use, avoiding any cytotoxic effects. Then follows a screening of the irritating potential of the cosmetic formulation with *in vivo* tests on human individuals (Ribeiro et al., 2015). Depending on their composition, plant extracts may have different properties, among which the antioxidant activity and the influence on proteases are some of the most important for skin care applications.

1.2.2. Tissue damage and enzymes involved in extracellular matrix degradation

Several studies highlight how cells initially exposed to a stressful agent could reply through mechanisms of adaptation and compensation, such as atrophy, hyperplasia, or metaplasia (Miller, 2017 and references therein). However, if the pathological stimulus lasts over time, or exceeds the adaptive capacity of the cells, it can cause the onset of tissue damage. In this case, the tissue

can lose its integrity and continuity triggering inflammation processes, which can lead to more or less effective repair of the damage, usually involving production of connective tissue.

Physical traumas, exposure to chemicals and other toxic substances or events linked to some pathological conditions (i.e. diabetes secondary ulcers) commonly cause damage to the skin. In response to the injurious event, an inflammatory response occurs, with different consequences for the tissue involved (Eming et al., 2007).

Firstly, ROS (reactive oxygen species) will be produced by leukocytes involved in the response against pathogens (by NADPH oxidase action). However, a greater presence of free radicals aimed at eliminating microbial agents could also be harmful to the tissue. Another event that occurs during the inflammatory process is the activation of the main group of enzymes responsible for the collagen and other protein degradation in extracellular matrix (ECM) , such as metalloproteinases (MMPs) (i.e. collagenases) (Nagase, 1997), hyaluronidase and elastase (Lee et al., 2020). The role of these enzymes consists in the extracellular matrix remodeling to facilitate the arrival of macrophages at the site of the damage. In addition, MMPs induce the release of growth factors to favor the repair process. There are, however, pathological conditions in which there is an increase in the level of free radicals (e.g. ROS concentration) and/or in the activity of proteases. Similar conditions can be due, for example, to the presence of a chronic inflammation status, exposure to UV rays and other oxidant factors or the aging process. It may therefore be useful to counteract the action of free radicals and the action of the matrix proteases, to safeguard the integrity of the skin and its well-being (Lee et al., 2020).

Skin aging, loss of tissue elasticity and other tissue damages are mainly due to oxidative stress mechanisms.

The production of free radicals, however, can be contrasted by various compounds known as antioxidants, radical scavengers that provide protection by inhibiting various oxidation chain reactions. In particular, the topical application of antioxidants is an effective strategy to decrease oxidative damage caused by UV radiation and preventing diseases strictly related to oxidative stress (Cooke et al., 2003).

1.3. Use of bioactive compounds for weed control

The spread of pests and weeds is an increasingly serious problem that causes reduction of biodiversity and conspicuous economic losses. It has been calculated that the management of these species takes away about one-third of the total production of field crops (Gnanavel, 2015). To control weed germination and development, substances like glyphosate, dicamba and 2,4-dichlorophenoxyacetic acid (2,4-D) have been widely used.

Mechanisms of action of commercial herbicides are various: for examples, many of them deal on enzymes involved in amino-acids biosynthesis, or on quinone-binding protein D-1 causing photosynthesis inhibition, or determining the formation of free radicals (Duke, 1990). Although the employment of herbicides in the fight against weed species constitutes a rapid solution for farmers to satisfy the crop demands, the continuative misuse of such chemical substances leads to other problems both environmental and related to human and animal health. Herbicides determine ecological imbalances in the environment, exert toxicity to animals (pollinators included) and cause pollution (Berger et al., 2013; Gnanavel, 2015; Pileggi et al., 2020). In addition, common herbicides negatively affect belowground interactions between soil organisms, causing changes in fungal and microbial communities, functions, and structures (Trappe et al., 2003; Zaller et al., 2014; Swarts & Dixon, 2017). Moreover, the prolonged utilization of chemical herbicides favors the development of resistant weed germplasm (Green & Owen, 2011). All these factors are increasingly arousing the interest of the scientific community and highlight the need to find eco-sustainable alternative methods for weed control and prevention (Cordeau et al., 2016; Radhakrishnan et al., 2018). In this context, specific biological activities useful against weed seed germination and growth have been recorded in bacteria, fungi and even plant products (Radhakrishnan et al., 2018 and references therein).

In a given environment, several plant compounds are secreted and are able to inhibit weed germination and growth: organic acids soluble in water, aldehydes, alcohols, ketones, fatty acids, lactones, polyacetylenes, quinones, phenolics, coumarins, cinnamic acids, tannins, flavonoids, terpenoids and steroids are some examples (Soltys-Kalina et al., 2013). There are cases of specificity of a certain plant extract, which may act on weeds without damaging surrounding crops

(Mendes et al., 2014): this could be due to the presence of specific receptor in weeds (Hosni et al., 2013; Radhakrishnan et al., 2018). Many plant-derived compounds, such as extracts or essential oils, are studied for their selective inhibition potential of the germination and seedling growth of weeds that severely damage crops. For example, essential oils rich in monoterpenes or sesquiterpenes have shown selective cytotoxicity against common weeds (Verdeguer et al., 2020).

1.4 Plant-derived compounds: from traditional uses to scientific validation

Many species, belonging to different plant families in different countries bordering the Mediterranean, have been used since ancient times in traditional medicine for the treatment of various health conditions. Such a great richness in floristic species, characteristic of the Mediterranean mountains and coasts, is in fact translated in a considerable number of herbal folk remedies (Tsioutsiou et al., 2017, Leonti & Verpoorte, 2017). Several ethnobotanical surveys dedicated to traditional medicine still investigate plant species to find other principles and molecules of medicinal interest. Since many understudied plant species can be a source of useful bioactive molecules, research aimed at amplifying knowledge about botanical features, phytochemistry and biological properties is indeed still ongoing (Oh et al., 2011; Pintus et al., 2015), because of the possible applications in medicinal fields or for the formulation of new products to be used for biological control. As stated by Tsioutsou et al. (2017), this field is still largely unexplored. However, as reported by Chen et al. (2016) in their review, at the global level an estimate of 15.000 medicinal species is at risk of extinction due to several factors like habitat destruction and overcollection. Huang (2011) observed that some plant families (i.e. Orchidaceae or Magnoliaceae in China) not only include more medicinal species than others, but also a major number of endangered entities. Therefore, accurate conservation actions by *ex* and/or *in-situ* strategies must be put in place to ensure the sustainable use of a certain medicinal threatened species. *Ex situ* conservation not only is necessary to cultivate and naturalize threatened species reducing their risk of extinction in the wild, but also could be useful to obtain additional plant material for pharmaceutical and medicinal purposes (Pant, 2013; Chen et al., 2016; Calevo et al., 2020).

On the other hand, there is a growing interest in finding bioactive molecules from invasive species for turning these "enemies" into value. In fact, many invasive species have potential chemical and pharmaceutical properties, and this fact could help mitigate the costs of their management. Ethnobotanical literature reports medicinal and therapeutic uses of many invasive taxa: such plants can prove to be a valuable abundant source of bioactive metabolites that can become the basis for new and effective medicines or cosmetic products (Arfin Khan et al. 2011). For example, in China *Ailanthus altissima* (Miller) Swingle, is used in the folk medicine against colds and gastrointestinal diseases: Luìs et al. (2012) showed that the extract from the leaves is particularly rich in phenolic compounds, flavonoids and alkaloids, which make it a valuable source of antioxidants.

In addition, the phytochemical composition and the allelopathic activity showed by several invasive alien plants, or by native entities and their byproducts, could be exploited for environmental eco-sustainable applications, pests and weed control included (Soltys-Kalina et al., 2013). Several species belonging to Mediterranean region have been already successfully tested for this purpose by Araniti et al. (2012).

Since globalization creates substantial changes and puts new unprecedented challenges for waste management and recycling, it is essential to exploit the resources that nature makes available. In this context, it is interesting to study also invasive plant species and plant industrial wastes, which can be used in a circular economy system. The results of these studies and the identification of useful bioactive plant compounds could lead to economic benefits, especially in those countries where invasive plants are widespread or where the production of waste from the agri-food sector is very high.

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2. Aim of the thesis

My PhD thesis has been focused on biological properties of extracts and essential oils from species of Mediterranean climate, with health (skin-care) and environmental (bioherbicides) applications. The first step concerned the study of two species showing different, diametrically opposite, conservation status. The extracts of these two species have been studied for their possible medicinal properties, in particular for dermatological/cosmetic applications.

One case concerns a terrestrial orchid, *Himantoglossum robertianum* (Loisel.) P. Delforge, largely diffused in Liguria and known as Giant Orchid for its great size and its showy inflorescence. The species has been previously included by Strack et al. (1989) in a comparative study dedicated to the determination of the anthocyanin patterns in flowers of terrestrial European orchids. These authors found a considerable content in orchicyanin I and II serapianin, interesting compounds already used in the cosmetic field. Therefore, we carried out a study on the hydroalcoholic extract from the inflorescence of the giant orchid, aimed at evaluating its bioactivities and cicatrizing properties.

Moreover, to avoid the possible exploitation of this orchid with damage to the natural populations, we contributed to the optimization of *ex-situ* propagation protocols for terrestrial and epiphytic orchids, *H. robertianum* included, formulating a new substrate for *in vitro* culture.

The second case is represented by the invasive alien halophyte *Carpobrotus edulis* (L.) N.E. Br., naturalized in the Mediterranean area and largely utilized by folk healers in South Africa. We validated the traditional use of this species for the treatment of skin affections and pathological conditions, also providing new phytochemical data from plant material harvested in the Ligurian coasts.

Finally, again in the context of a sustainable use of resources, in the third step we studied the essential oils extracted from peels of three different citrus entities (*Citrus* spl.), widely cultivated on the Mediterranean coasts. Citrus peels often represent a consistent by-product of the citrus

industry. The essential oils obtained have been phytochemically characterized and tested for their phytotoxic activity on selected seeds, for a possible re-use as natural herbicides.

2.1 Results obtained during the PhD thesis

The researches briefly described above have been the subject of my PhD thesis, and have led to the publication of the following articles:

- **Bazzicalupo M**., Burlando B., Denaro M., Barreca D., Trombetta D., Smeriglio A., Cornara, L. (2019). Polyphenol Characterization and Skin-Preserving Properties of Hydroalcoholic Flower Extract from *Himantoglossum robertianum* (Orchidaceae). Plants, 8(11), 502. https://doi.org/10.3390/plants8110502.

- Calevo J., Copetta A., Marchioni I., **Bazzicalupo M**., Pianta M., Shirmohammadi N., Cornara L., Giovannini A. (2020). The use of a new culture medium and organic supplement to improve *in vitro* early stage development of five orchid species, Plant Biosystems. DOI: 10.1080/11263504.2020.1840454

- Caputo L., Cornara L., **Bazzicalupo M.**, De Francesco C., De Feo V., Trombetta D., Smeriglio A (2020). Chemical Composition and Biological Activities of Essential Oils from Peels of Three *Citrus* Species. Molecules. doi: 10.3390/molecules25081890. PMID: 32325864; PMCID: PMC7221518.

- **Bazzicalupo M**., Burlando B., Cascini A., Trombetta D., Denaro M., Smeriglio A., Cornara L. (in press) *Carpobrotus edulis* (L.) N.E.Br. extract as skin preserving agent: from traditional medicine to scientific validation. Accepted by Journal of Integrative Medicine.

3. Materials and methods

3.1 Material investigated, reagents & cells

In this thesis, we used:

- Hydroalcoholic flower extract from the inflorescence of *Himantoglossum robertianum* (Loisel) P. Delforge;
- EOs extracted from the peel of *Citrus* x *bergamia* Risso & Poit., *C.* x *myrtifolia* Raf. and *C. limon* (L.) Osbeck.
- Aqueous extract from leaves of *Carpobrotus edulis* (L.) N. E. Br.

Cell cultures and chemicals were from Sigma-Aldrich (St. Louis, MO, USA), unless differently specified. For our *in vitro* cell-based tests, we used HaCaT keratinocytes and L929 murine fibroblasts cell lines. HaCat were purchased from the Biological Bank of the Azienda Ospedaliera Universitaria San Martino-IST and from DKFZ, Deutsches Krebsforschungszentrum, Heidelberg, Germany, while L929 cells were from the Genoa Tissue Bank (San Martino Hospital) Genova, Italy. Cells were maintained at 37°C, in a 5% CO_2, humidified atmosphere, with Dulbecco's Modified Eagle Medium (DMEM, EuroClone, Milan, Italy) enriched with 10% (*v/v*) FBS, 1% glutamine, and 1% antibiotic.

For micropropagation of orchids, new culture media were obtained by using almond milk, in different concentrations (0 mL/L, 50 mL/L, 100 mL/L) as low-cost organic supplement, was purchased from Condorelli® (Belpasso, CT, Italy). The newly proposed media (CG, see article attached) were then compared with common substrates used for orchid micropropagation such as 1/4 Murashige & Skoog (1962) including vitamins (Duchefa) and enriched with coconut water (Knudson C by Duchefa; Malmgren modified medium M551 by Phytotechnology).

3.2 Plant material: *H. robertianum* flowers, *Citrus* spp peels, *C. edulis* leaves

-H. robertianum

Flowers of *H. robertianum* were sampled from wild populations growing at Taggia (43°52'05.2" N, 7°50'14.6" E), and Carpasio (43°57'24.8" N, 7°50'38.7" E) (Imperia, Liguria, Italy) in 2018. Sampling of the inflorescences of the species, protected under law, was allowed by the Ligurian Region Government with act n. 363/29-01-2018. A voucher specimen was deposited at the Herbarium of DISTAV, University of Genova, Genova, Italy (number: GE 1038).

- *Citrus spp*

Ripe fruits of *C. limon* (Limone Costa d'Amalfi IGP) were collected in November 2019, from biological orchards of the Amalfi coast (Salerno, Italy), while fruits of *C.* × *bergamia* (Fantastico variety) and *C.* × *myrtifolia* were harvested in November 2019 from biological orchards of the Ionian coast (Reggio Calabria, Italy) by local farmers, and immediately sent to the laboratory.

-C. edulis

C. edulis leaves were harvested in autumn from plants growing in Ospedaletti (Italy, 396963,7; mE/4850464,9 mN), to obtain samples with highest antioxidant power following Chokoe et al. (2008, reference in the article attached).Specimen identification was conducted according Pignatti (2017, reference in the article attached). A voucher herbarium specimen was deposited at the Herbarium of DISTAV, University of Genova, Italy (number: GE 2292).

3.3 Micromorphological analyses (LM and SEM)

We performed micromorphological analyses on plant material by transmission light and/or by epifluorescence using a Leica DM 2000 microscope coupled with a DFC 320 camera (Leica Microsystems,Wetzlar, Germany On the basis of the different samples to be examined, various dyes were used, such as metachromatic staining with Toluidine Blue (TBO) to highlight

polyphenols and carboxylated polysaccharides, Phloroglucine - HCl for lignin, or clarification with hypochlorite to better highlight stomata and shape of the epidermal cells, etc.

To perform SEM analyses, samples were placed in FineFIX working solution (Milestone s.r.l., Bergamo, Italy) with 70% ethanol and left overnight at 4 °C, according to Chieco et al. (2013). Dehydration by graded ethanol series from 70 to 100%, for 60 min each, was carried out, then samples were subjected to critical point drying (K850CPD 2M Strumenti S.r.l., Roma, Italy). Small pieces were placed on aluminum stubs and covered with a 10 nm layer of gold particles. Observations were carried out with a VEGA3-Tescan-type LMU microscope (Tescan Orsay Holding, a.s., Brno, Czech Republic), at an accelerating voltage of 20 kV.

3.4 Extraction protocols

3.4.1 *H. robertianum*

Flowers were gently grounded by a blade mill (IKA® A11 basic analytical mill) under liquid nitrogen. 100 ml of 70% ethanol were added to 10 g of powdered sample mixing for 3 min. The extraction was carried out three times by sonication in an ice-cold bath for 5 min using a 3 mm titanium probe, set to 200W power and 30% amplitude signal (Vibra CellTM Sonics Materials inc., Danbury, CT, USA). Thereafter, the sample was centrifuged at 3000 rpm for 15 min (NEYA 10R, REMI, Carpi, Italy) and the supernatant was collected and evaporated by rotary evaporator (BUCHI R-205, Cornaredo, Italy).

3.4.2 *Citrus* spp

Fruits were peeled off manually by using a potato peeler and hydro-distilled by a Clevenger device, until no significant increase in the EOs volume was recorded (~3 h). EOs were dehydrated on Na_2SO_4 and stored in the dark with nitrogen headspace until use.

3.4.3 *C. edulis*

Ten grams of *C. edulis* leaves were powdered by a blade mill with liquid nitrogen (IKA ® A11 basic analytical mill), thereby obtaining an aqueous extract. The powdered sample was placed in a flask with 200 mL of distilled water and stirred on a magnetic plate for 3 h at 90°C. Sample was centrifuged at 3000 × g for 10 minutes, the supernatant filtered on Whatman filter paper no. 4, and then evaporated by rotary evaporator (≤ 40°C). After calculating the yield, the dried CAE was stored at -20°C until subsequent analyses.

3.5 Phytochemical screening and characterization

3.5.1 Carbohydrates

The carbohydrate content was determined in the case of *C. edulis* aqueous extract. 10 mg of extract were placed in a glass test tube with screw cap, then were added with 0.5 mL of 2.5 N HCl and placed in a water bath at 100 °C for 3 h. After cooling, sample was neutralized with sodium carbonate brought to 10 mL with distilled water, and centrifuged at 3500 × g for 5 min. Twenty microliters of sample, blank (distilled water) or reference standard (glucose 5-100 µg/mL), brought to 200 µL with distilled water, were added with 200 µL of 5% phenolic solution and 1 mL of 96% H_2SO_4. The reaction mixture was stirred for 10 minutes, incubated in a water bath at 30°C for 20 minutes and then the absorbance recorded at 490 nm. The carbohydrate content was calculated using the following equation:

$$\text{Total carbohydrates (\%)} = \frac{x}{0.02} \times 100$$

where *x* corresponds to sample concentration (mg/mL) extrapolated from the glucose calibration curve, and 0.02 is the sample volume (mL) in the reaction mixture.

3.5.2 Total phenols

50 µL of extract solution (150-1200 µg/mL) was added to Folin-Ciocalteu reagent (1:10 *v/v*) and brought to 1 mL with deionized water. After 3 min, 10% sodium carbonate (500 mL) was added, and sample left in the dark at room temperature for 1 h mixing every 10 min. Sample was read at

785 nm by an UV–Vis spectrophotometer (Shimadzu UV-1601, Kyoto, Japan). Results were expressed as g of gallic acid equivalents (GAE)/100 g DE, by using gallic acid as reference compound (75–600 μg/mL).

3.5.3 Flavonoids

200 μL of extract solution (150-1200 μg/mL) was added to 2 mg/mL $AlCl_3$ (1:1, *v/v*), and brought to 1.6 mL with 50 mg/mL sodium acetate. After 2.5 h, the absorbance was read at 440 nm by an UV–Vis spectrophotometer (Shimadzu UV-1601, Kyoto, Japan). Results were expressed as g of rutin equivalents (RE)/100 g DE, by using rutin as reference compound (125–1000 μg/mL).

3.5.4 Vanillin Index

The proanthocyanidin and anthocyanin contents were determined in the case of *H. robertianum* flower extract, according to the method described by Monforte et al. (2018, reference in the attached article), by recording the absorbance of sample with an UV–Vis spectrophotometer (Shimadzu UV-1601, Kyoto, Japan) at 500 nm against a blank. Catechin was used as reference compound (0–500 μg/mL) and results were expressed as mg of catechin equivalent (CatE)/100 mg of sample FW.

3.5.5 Proanthocyanindin and Anthocyanin Content

The proanthocyanidin content was determined in the case of *H. robertianum* flower extract, by an UV–Vis spectrophotometer (Shimadzu UV-1601, Kyoto, Japan), following the methodology described by Monforte et al. (2018) and by Rapisarda et al. (2000) (references in the attached article). Results were expressed as mg of Cyanidin chloride equivalents (CyE)/100 g of sample FW and Chrysanthemin (Cyanidin-3-*O*-glucoside) chloride equivalents (ChE)/100 g of sample FW, respectively.

3.5.6. Antioxidant activity and free-radical scavenging assays

FRAP test

The FRAP-test (Ferric Reducing Antioxidant Power) is a method used for the evaluation of the antioxidant activity, based on the ability of the test extracts to reduce, by electron transfer, iron ions from Fe 3+ to Fe 2+, that in the presence of TPTZ (2,4,6-tris (2-pyridyl) -s-triazine) form a blue complex (Fe 2 + - TPTZ), which has an absorption maximum of 593nm. The FRAP reagent contains a solution of 10 mM TPTZ in HCl 40 mM, $FeCl_3 \cdot 6H_2O$ 20 mM and acetate buffer (300 mM, pH 3.6). The reactive was maintained at a temperature of 37 ° C and its initial absorbance was measured; later, for every cuvette containing 1.5 ml of the FRAP reagent, 50 µl of sample (7.5-60 µg / mL) were added, and after 4 minutes of incubation (at room temperature in dark) absorbance was measured at 593 nm using an UV-VIS spectrophotometer (Shimadzu UV-1601). Trolox was used as the reference standard (0.312-5 µg / mL). The reducing ability of the sample, obtained as the average value of three independent experiments carried out in triplicate, was expressed as IC 50, that is, a concentration able to inhibit radical activity by 50%, bringing the respective confidence limits back to 95%.

ORAC test

The ORAC Test is a sensitive and versatile method based on the ability of an antioxidant substance to inhibit the oxidative degradation of a fluorescent molecule caused by peroxyl radicals (ROO •); this test uses fluorescein as reference compound and 2-2 azobis- (2- amidinopropane) - dichlorohydrate (AAPH) as a source of peroxides. 120 µl of a freshly prepared 117 nM fluorescein solution were added of 20 µl of the sample (0.625-5 µg / mL). A first incubation was carried out for 15 minutes at 37 ° C; thereafter, samples were rapidly added of 60 µl of fresh AAPH solution (40 mM). Fluorescence was recorded at 30 seconds intervals for 90 minutes (λex 485; λem 520), by means of a fluorescence plate reader (FLUOstar Omega, BMG LABTECH), monitoring the decrease. Trolox was used as the reference standard (0.25-2.5 µg / mL). The antioxidant ability of the sample, obtained as the average value of three independent experiments carried out in

triplicate, was expressed as IC 50, that is, as concentration able to inhibit radical activity by 50% by restoring the respective confidence limits to 95%.

TEAC test

The TEAC test allows to compare the antioxidant activity of the samples with that of Trolox, compound with known antioxidant activity used as standard. The method is based on the capacity of the test sample to reduce the radical ABTS • + (acronym for 2,2'-azino-bis- (3-ethylbenzothiazolin-6 sulfonic), a blue-green chromophore with an absorption maximum at 734 nm. The cationic radical ABTS • + is metastable and must be generated just before the experiment. The reaction mixture was prepared by mixing an aqueous solution of ABTS (1.7 mM) with potassium persulfate (4.3 mM) in the ratio 1: 5 (v/v), by incubating for 12-16 hours in the dark, at room temperature. Before use, the reaction mixture was diluted with phosphate buffer (pH 7.4), to obtain an absorbance of 0.7 ± 0.02 at 734 nm. Subsequently, 50 µl of the test sample (7.5-60 µg/mL) were added to 1 mL of the reaction mixture and incubated in the dark for 6 minutes at room temperature. The absorbance was later recorded at 734 nm by means of a UV-Vis spectrophotometer (Shimadzu UV-1601). Trolox was used as the reference standard (0.312-5 µg/mL). The scavenging ability of the sample, obtained as the average value of three independent experiments carried out in triplicate, was expressed as IC 50, that is, as concentration able to inhibit the oxidizing activity by 50%, restoring the respective confidence limits to 95%.

DPPH assay

The method allows to determine the free-radical scavenging power of a given substance or an extract under examination using a solution of the stable 2,2-diphenyl-1-picrilhydrazilic radical (DPPH), purple-colored; this reaction leads to a discoloration of the solution, due to the disappearance of the radical, with consequent decrease in absorbance at 517 nm, maximum peak of absorbance of the radical.

1.5 ml of a methanolic solution of DPPH (10 -4 M) prepared extemporaneously, were added to 37.5 µl of the test sample (30-240 µg / mL), shaking for 10 seconds at room temperature. The

absorbance was recorded at 517 nm after 20 minutes of incubation in the dark at room temperature, by comparing the blank (distilled water), using a UV-Vis spectrophotometer (Shimadzu UV-1601). Trolox was used as the reference standard (0.315-5 μg / mL). The radical activity was expressed as IC 50, or as a concentration able of inhibiting the radical activity of 50%, restoring the respective confidence limits to 95%.

3.5.7 Iron-Chelating Activity

The iron-chelating activity extract has been evaluated on *H.robertianum* flower extract. 50 μL of $FeCl_2 \cdot 4H_2O$ solution (2.0 mM) was added to 100 μL of extract (62.5–100 μg/mL), and mix was incubated at RT for 5 min. After that, 100 μL of ferrozine solution (5 mM) was added to the reaction mixture and the sample solution diluted to 3 mL with deionized water, mixed and incubated for 10 min at RT. The absorbance was read at 562 nm using an UV-VIS Spectrophotometer (Shimadzu UV-1601, Kyoto, Japan). Results were expressed as inhibition (%) of the Fe^{2+} chelating capacity calculating the half-maximal inhibitory concentration (IC_{50}) with the respective C.L. at 95%.

3.5.8 β-Carotene Bleaching Assay

This test was performed by aliquoting the fresh β-carotene emulsion (8.0 mL) in 320μL of *H-robertianum* flower extract solutions (12.50–200 μg/mL). An emulsion without β-carotene was employed as negative control. The absorbance was recorded at 470 nm at the starting time (t = 0), and then incubated at 50 °C in a water bath for 120 min, recording the absorbance every 20 min. Butylated-hydroxytoluene (BHT) 1 mg/mL was used as positive control. The antioxidant activity was expressed as inhibition (%) of the β-carotene bleaching calculating the half-maximal inhibitory concentration (IC_{50}) with the respective C.L. at 95%.

3.5.9 Characterization of *H. robertianum* hydroalcoholic extract

The qualitative and quantitative determination of polyphenols of flower extract was carried out by Reversed Phase Liquid Chromatography with Diode Array Detection (RP-LC-DAD). An Agilent high performance liquid chromatography system (1100 series, Santa Clara, CA, USA), equipped with a photodiode-array detector (DAD, G1315), was used. Briefly, the chromatographic separation was carried out by a 5 µm, 250 mm × 4.6 mm ODS3 Prodigy column (Phenomenex, Torrance, CA, USA), maintained at 25 °C, with solvent A (water/acetic acid, 97:3, *v/v*) and solvent B (methanol). The gradient elution was: 0–3 min, 0%B; 3–9 min, 3% B; 9–24 min, 12% B; 24–30 min, 20% B; 30–33 min, 20% B; 33–43 min, 30% B; 43–63 min, 50% B; 63–66 min, 50% B; 66–76 min, 60% B; 76–81 min, 60% B; 81–86 min, 0% B and equilibrated 4 min for a total run time of 90 min. Flow rate and injection volume was 1.0 mL/min and 50 µL, respectively. UV-Vis spectra of polyphenols were recorded from 190 to 400 nm. Peak identity was confirmed by comparing the retention time and the absorption spectra with those of pure (≥ 99%) commercially available standards (Extrasynthese, Genay, France). Chromatograms were acquired at 260 and 292 nm for phenolic acids and flavan-3-ols, and at 330 nm for flavones and coumarins. Quantitative analysis was carried out using external calibration curves of reference compounds (concentration range 0.1–20 µg/mL).

Table 1. Reference compounds used for quantitative analysis.

Peak n. 1	**Compound**	**Rt (min)**	**λmax (nm)**	**Regression Coefficient (R2)**
1	Protocatecuic acid	15.055	260; 294	0.9999
2	Hydroxybenzoic acid	25.545	255	0.9997
3	Catechin	29.453	234; 279	0.9997
4	Chlorogenic acid	31.401	294; 326	0.9999
5	Caffeic acid	33.617	232; 323	0.9998
6	Vanillic acid	35.254	260; 292	0.9996
7	Epicatechin	42.065	232; 280	0.9999
8	Coumaric acid	43.813	233; 310	0.9999
9	Scopoletin	47.375	296; 344	0.9999
10	Isovitexin	55.302	270; 337	0.9997

11	Naringenin-7-*O*-glucoside	55.881	284; 340	0.9998
12	Vitexin	57.145	268; 338	0.9996
13	Rutin	59.282	256; 356	0.9997
14	Kaempferol-3-*O*-rutinoside	61.977	266; 348	0.9998
15	Roifolin	64.620	266; 338	0.9999
16	Luteolin	74.695	254; 350	0.9999
17	Apigenin	76.418	236; 338	0.9998

*Peak numbers refer to Figure 4 of Chapter 4.5

3.5.10 Characterization of *Citrus* spp essential oils

Analytical gas chromatography (GC) was performed by an Agilent gas chromatograph (7890A), equipped with a flame ionization detector (FID) (Agilent Technologies Santa Clara, CA, USA) coupled with a data handling processor. The separation was carried out using a HP-5MS capillary column (30 mm, 0.25 mm coated with 5% phenyl methyl silicone, 95% dimethyl polysiloxane, 0.25 μm film thickness) and helium as carrier gas (1 mL/min). One microliter of 10% EO/CH_2Cl_2 *v/v* solution was injected in split mode (50:1), setting the injector and detector temperature at 250 °C and 280 °C, respectively. Elution was carried out according to the following program: 60 °C for 6 min, increased to 270 °C at 3 °C/min, and held at 270 °C for 4 min.

Gas chromatography-mass spectrometry (GC-MS) analyses were carried out on the same instrument, coupled with a mass detector (5975C), with the same column and operative conditions used for the analytical GC but setting the ionization voltage to 70 eV, the electron multiplier to 900 V, and the ion source temperature to 230 °C. EO components were identified by comparison of GC retention index (relative to C7-C40 n-alkanes), literature data, matching of mass spectral data with MS library NIST 08, comparison of MS fragmentation patterns with those reported in literature, and co-injection with commercially available terpene standards. Quantification was carried out by extrapolation of the compound's peak areas from GC-FID profiles.

3.5.11 Characterization of *C. edulis* aqueous extract

The phytochemical characterization was carried out using a reversed-phase liquid chromatography coupled with diode array detection and electrospray ion trap mass spectrometry (RP-LC-DAD-ESI-MS) analysis. The chromatographic separation was obtained using a Luna Omega PS C18 column (150 x 2.1 mm, 5 µm; Phenomenex, Torrance, CA, USA) at RT and with a flow rate of 0.4 mL/min. The mobile phase consisted of solvent A (0.1% formic acid) and solvent B (acetonitrile), according to the following elution program: 0–3 min, 0% B; 3–9 min, 3% B; 9–24 min, 12% B; 24–30 min, 20% B; 30–33 min, 20% B; 33–43 min, 30% B; 43–63 min, 50% B; 63–66 min, 50% B; 66–76 min, 60% B; 76–81 min, 60% B; 81–86 min, 0% B; 86–90 min, 0% B. The injection volume was 5 µL. The UV-Vis spectra were recorded in the 190-600 nm range and

the acquisition wavelength, chosen to show the polyphenol profile at which all the identified peaks are visible, was 260 nm.

3.6 Cell cultures (HaCat)

MTT tests with HaCat keratinocytes were carried out to assess cytotoxicity of extracts and the ability of *H. robertianum* flower extract of improving cell viability after treatments with increasing concentrations of H_2O_2 (500, 750, 1000 µM). Wound healing activity was evaluated with HaCat keratinocytes, while *C. edulis* extract potential to enhance collagen production by skin cells was assessed on mouse fibroblast L929 following an ELISA technique.

3.6.1 MTT test

The MTT colorimetric assay is utilized to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. The principle of the test is based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells.

20.000 cells were seeded in 96-well plates, left overnight in the culture medium, and then incubated with the test substances for 48 hours. Subsequently, the solutions were removed from

wells and cells were exposed for 3 hours to PBS added with 10 µL of a 5 mg/mL solution of MTT. Then, wells were emptied, and cells exposed to an acidified solution of 2-propanol with which formazan crystals present in viable cells are solubilized to give a blue/violet coloration of the medium. A darker solution indicates a major number of metabolically active living cells. Plates were read at 570 nm using a Varian-Cary Spectrophotometer.

3.6.2 Scratch-wound assay

Through the scratch wound assay, it is possible to evaluate *in vitro* the capacity of a substance to promote cell growth and proliferation by decreasing the healing time between two distinct cell monolayer flaps. Cells were cultured in 12-well plates with an initial cell density of 20,000 cells/well. Cells were incubated in DMEM culture medium at 37 ° C up to confluence. Two cuts per well were then made using a 100 µL sterile tip. A wash with PBS was then carried out to remove any residue. We then proceeded immediately by fixing the samples corresponding to T0 with sterile FineFix solution and staining the cells with 0.1% TBO. Controls and negative controls were set up by incubating the cells with the complete culture medium and in the second case with medium without FBS. In the remaining wells, cells were exposed to different concentrations of tested extracts and to allantoin as positive control. Medium was removed after 24h incubation, then followed a washing in PBS. Cells were then fixed with sterile FineFix solution and stained with TBO 0.1%.

The healing of the cuts was followed and analysed through images of the samples acquired with a Leica stereomicroscope M205 C associated with a Leica EZ 2.1.5 camera. The distance between the two margins of the cuts was measured with ImageJ software (https://imagej.nih.gov/ij/). Cell migration was then expressed as a percentage of healing of the cut according to Muniandy et al. (2018 reference in the attached article).

3.6.3 Collagen production

An ELISA assay was used to quantify the production of type I collagen by fibroblasts exposed to *C. edulis* extract for 48 h. Cells were washed once with PBS (100 µL/well), fixed with FineFIX

for 10 min, washed 3 times with washing buffer (0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% Triton in PBS, 100 μL per well), incubated for 30 min with blocking buffer (3% bovine serum albumin (BSA) in washing buffer), washed, and incubated under agitation for 2 h with mouse anti-human collagen type I primary antibody (ab6308, Abcam, Cambridge, UK) diluted 1:300 in washing buffer containing 1% BSA (50 μL per well). Cells were then washed 3 times with washing buffer, incubated for 60 min under agitation with HRP-conjugated rabbit anti-mouse IgG secondary antibody (ab97046, Abcam) diluted 1:1000 in washing buffer containing 1% BSA (50 μL per well), washed 3 times as above, incubated for 15 min with 50 μL per well of the Pierce 1StepTM Ultra TMB ELISA Substrate Solution (Thermo Fisher Scientific, Waltham, MA), and blocked with 2 M sulfuric acid. Plates were read at 450 nm in the Varian spectrophotometer. Eight replicates for each condition were run. This technique allows sample collagen content to be measured reliably in a range between 0 and 2000 pg/mL.

3.7 Enzymatic tests

3.7.1 Elastase inhibition assay

Elastase (EC 3.4.21.36) inhibition was evaluated in a reaction mixture containing 200 mM tris(hydroxymethyl)aminomethane (TRIS) pH 8.0, 10 mM N-succinyl-Ala-Ala-Ala-p-nitroanilide (Sigma-Aldrich, cat. S4760), 4 units/mL of elastase from porcine pancreas (Sigma-Aldrich, E1250), and serial water dilutions of extract stock solutions in 30% ethanol (final concentrations: 5, 50, 500 μg/mL). After incubation at RT for 10 min, plates were read at 410 nm on a Varian Cary-50 Bio spectrophotometer (Agilent, Milan, Italy).

3.7.2 Collagenase inhibition assay

Collagenase (EC 3.4.24.3) inhibition was assayed in a mix containing 0.16 units/mL of collagenase from *Clostridium histolyticum* (Sigma-Aldrich, cat. MAK293B), 20% N-[3-(2-furyl)acryloyl]-L-leucyl-glycyl-L-prolyl-L-alanine (FALGPA, substrate, MAK293C), 74% collagenase assay buffer (MAK293A), and water dilutions of extract stock solution in 60%

ethanol, to reach the indicated concentrations. Plates were read at 345 nm, at 37 °C, for 15 min in kinetic mode. A dose of 1mM 1,10-phenanthroline (MAK293D) was used as positive control.

3.7.3 Hyaluronidase inhibition assay

Hyaluronidase (EC 3.2.1.35) inhibition was evaluated in the case of *C. edulis* aqueous extract by using hyaluronidase type I from bovine testes, bovine hyaluronic acid as substrate, and epigallocatechin gallate (EGCG; 70935, Cayman Chemical Company, USA) as positive control. The enzyme solution was composed of about 5 units of hyaluronidase (Sigma-Aldrich, H3506) dissolved in enzyme diluent (20 mM NaH_2PO_4, 77 mM NaCl, 0.01% BSA (w/v), pH 7.0, at 37 °C). 8x sub-stocks of each extract final concentration to be tested (5, 50, 500, 1000 µg/mL), and of EGCG (0.2 mg/mL), were prepared by dissolving extract and EGCG in enzyme diluent. Mixtures of enzyme and extract or EGCG solutions were pre-incubated in a 3:1 ratio at 37 °C for 10 min. A blank composed of enzyme diluent, enzyme-only samples containing enzyme solution and enzyme diluent, extract-only samples containing extract at the different concentrations, and EGCG-only samples, were prepared. After pre-incubation, samples were diluted 1:1 (*v/v*) with the hyaluronic acid substrate, consisting of 0.015% (*w/v*) bovine hyaluronic acid sodium salt (Sigma-Aldrich, H7630) dissolved in phosphate buffer (300 mM NaH_2PO_4, pH 5.35, at 37°C). Subsequently, samples were incubated at 37°C for 45 min, diluted 2.5 times with acidic albumin (24 mM sodium acetate, 79 mM acetic acid, 0.1% BSA (*w/v*), pH 3.75, at 25 °C), incubated 10 min at room temperature, and read at 595 nm. Hyaluronidase inhibition was calculated by the following formula:

$$I\% = 1 - \frac{(T_{\mathrm{en/ex}} - T_{ex})}{(T_{\mathrm{en}} - T_{bk})} \times 100$$

(Transmittances: $T_{en/ex}$ = enzyme and extract, or EGCG, mixture; T_{ex} = extract or EGCG without enzyme; T_{en} = enzyme without extract or EGCG; T_{bk} = blank).

3.8 Toxicity assessment of Citrus essential oils

3.8.1 Phytoxicity test

The phytotoxic activity was evaluated on germination and radical elongation of several species: *Raphanus sativus* L., *Lactuca sativa* L., *Lepidium sativum* L., *Solanum lycopersicum* L., *Lolium multiflorum* Lam., and *Portulaca oleracea* L. These seeds are often used for their easy and well-known germinability.

Seeds were sterilized in 95% ethanol for 15 s and sown in Petri dishes (Ø = 90 mm), on three layers of Whatman filter paper. They were then impregnated with 7 mL of distilled water used as first control to verify the germinability of the seeds, 7 mL of water–acetone mixture (99.5:0.5, *v*/*v*) as second control because EOs were dissolved in this mixture for their apolarity, or 7 mL of the tested solution at different doses (100, 10, 1 and 0.1 µg/mL). The germination conditions were 20 ± 1 °C, with a natural photoperiod. Seed germination was observed in Petri dishes every 24 h. A seed was considered germinated when the protrusion of the root became evident. On the fifth day (after 120 h), the effects on radicle elongation were measured in cm. Each determination was repeated three times, using Petri dishes containing 10 seeds each. Data are expressed as the mean ± standard deviation for both germination and radicle elongation.

3.8.2 Toxicological evaluation of essential oils

Dried cysts were placed in a hatcher chamber containing artificial seawater and incubated for 24-48 h at room temperature (RT). Natural ventilation and continuous illumination favoured the larvae (nauplii) migration towards the opened centre of the chamber, where they hatched. Stock solutions 1000–0.01 mg/mL of each Citrus EO (*C. limon*, *C.* × *bergamia*, and *C.* × *myrtifolia*) in DMSO were prepared. Each concentration was seeded in a 24 well plate in triplicate and diluted 1:1000 *v/v* with artificial seawater (DMSO 0.1%). After that, 10 nauplii per well were added and incubated at RT for 48 h. Surviving nauplii without abnormal swimming behavior in each well were counted by a stereomicroscope (SMZ-171 Series, Motic) after 24 and 48 h. Three independent experiments in triplicates (n = 3) were carried out for each Citrus EO concentration.

Two negative control groups (10 nauplii and artificial seawater added with DMSO 0.1%, and 10 nauplii and artificial seawater, respectively) as well as a positive control ($K_2Cr_2O_7$ 50 μg/mL) were also evaluated.

3.9 *In vitro* micropropagation of orchids

After sterilization in 1% NaClO for 20 min and three 5 minutes washing cycles in sterilized dH_2O, seeds were sown in a laminar flow hood in Petri dishes (four for each medium) on the six different media (see section 4.6) added with 2g/L of activated charcoal, an enhancer and supporter of plant health (Roy et al., 2011; reference in the attached article). Sown seeds were kept at dark at 21 ± 2°C. Production of rhizoids from ruptured testas was used as an indicator for germination. Observations were carried out every 7 days for 90 days after sowing by dividing Petri dishes into four quadrants, each quadrant containing around 100 seeds with embryos. The average germination percentage of each Petri dish was calculated as the mean of the four quadrants, while the total percentage per medium was evaluated as the average of germination percentages of each Petri dish ± SE. After germination, seedlings were transferred to a growth chamber under a 16/8 h light/dark photoperiod, 200 mMm-2s-1-white fluorescent light (Philipps Master TL-D36W/ 840 lamp), at 21 ± 2°C for 5 months.

Complete methodology has been described in Chapter 4.6.

3.10 Statistical analyses

To evaluate the wound healing rates, data were analyzed with GraphPad Prism v.8.0. The statistical analysis used both in the case of H. robertianum and C. edulis studies was based on one-way analysis of variance (ANOVA) and Bonferroni post-hoc test for multiple pairwise mean comparisons.

Collagen production and antioxidant activity data were analyzed by one-way ANOVA with Dunnett's and t-Student post-hoc test, respectively. Results were considered statistically significant for $p < 0.01$ or $p < 0.05$, as indicated.

Data relative to phytotoxicity and lethality *Citrus* essential oils were analysed by one-way ANOVA, using Dunnett's multiple comparisons test for phytotoxicity and brine shrimp lethality assay, and Tukey's test for phytochemical characterization, by GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA). Results were considered significant for $p < 0.05$.

4. Orchidaceae: traditional uses, scientific studies, and conservation actions

Orchids can be a source of interesting bioactive compounds and their possible utilization opens new perspectives. However, several factors for a correct and sustainable employment of these endangered species must be considered. In this chapter we aim to present orchids with a special look on European Mediterranean species, and their traditional usage together with notes on available scientific information on their phytochemistry. This part of my study led to a first article published in *Plants* (2019) regarding the use of an ethanol extract from inflorescences of *Himantoglossum robertianum* (Loisel) P. Delforge for therapeutic applications. A second article published in *Plant Biosystems* (2020) was focused on the optimization of *in vitro* protocols for a sustainable *in vitro* propagation of this orchid and other terrestrial and epiphytic species.

4.1. Generalities on orchids and orchid biology

Family Orchidaceae, with about 28.000 species distributed all over the globe except for poles and deserts, is one of the most fascinating and varied group of plants (Swarts & Dixon, 2017; Christenhusz & Bying, 2016).

These herbs show an astonishing complexity of forms, colors, smells, and adaptive strategies; they could reach considerable dimensions (e.g. *Grammatophyllum speciosum*) or being tiny entities with flowers not larger than a millimeter. Their growth could be sympodial or monopodial; they often show pseudobulbs or, in the case of terrestrial species, below-ground tubers; flowers are constituted by two whorls, with the outer showing three sepals and the inner arranged with three petals (Pant & Raskoti, 2013). For their peculiar characters, orchids have attracted scientists and many passionate people since antiquity, being one of the most interesting family of plants for studying evolution and co-evolution processes, ethnobotanical usages, secondary metabolites and, especially nowadays, for horticultural aims.

About 30% of species are terrestrial, while the rest is characterized as litophytes or epiphytes (Gravendeel et al., 2004). Such a great number of species alongside the lack of abundant fossil material have been reflected in several difficulties in the establishment of taxonomical divisions, with various classification ambiguities: in general, the APG system now classify the family in order Asparagales and recognizes five subfamilies (Apostasioideae, Cypripedioideae, Orchidoideae, Epidendroideae, Vanilloideae).

Despite their evolutionary success, orchids are among the most endangered entities in the world (Swarts & Dixon, 2017), as a recent estimate said that 40% of plant species are at risk of extinction, mainly due to climate changes, use of pesticide and pollinators disturbance by anthropogenic pressure, human harvesting, etc. The reasons behind this worrying prevision are various. Orchids put in place significant below and above-ground interactions with symbiont organisms (in the case of epiphytic species, host trees: Fay, 2016), which persistence in nature is susceptible to various stimuli of abiotic and/or biotic origin. In the below-ground portion, actors like fungi and bacteria are fundamental hosts which enable seeds to germinate and acquire nutrients and/or immunity (Yeung, 2017); in the above ground, plants depend on the action of pollinators (birds, bees, wasps, moths, butterflies) for their reproductive success and diffusion. According to recent estimates (Wraith & Pickering, 2018; Wraith et al., 2020) hundreds of species are threatened, with terrestrial orchids particularly represented in the IUCN Red list (Fay, 2018). All members of Orchidaceae have been therefore included in Appendix II or higher of the Convention on International Trade in Endangered Species, CITES.

Anthropogenic pressure is, as overmentioned, a key element to explaining orchid distribution and the actual decreasing trend: however, although in particular illegal harvesting is still at the base of several medicinal terrestrial orchids rarity (Wraith & Pickering, 2018), steps have been taken to ensure that the harvest is regulated, or that the plant portions come from *in vitro* propagation processes. This practice can offer, at the same time, both individuals to be put back into the field to restore populations (Fay, 1994), and plant material to be exploited for therapeutic purposes (Pant, 2013; Pant & Raskoti, 2013; Pant, 2014; Teoh, 2016).

The high floral diversification and the wide spectrum of fragrances that orchid can reproduce represent a highly advanced terminal line of floral evolution in monocotyledons (Hsiao et al., 2011). This is reflected in the considerable popularity of these plants in the global market, but also indicates a high specialization in plant-pollinators interactions (Schiestl & Schlüter, 2009).

Orchid pollinators are usually bees, wasps and flies, moths, butterflies, gnats, or birds. The ways orchids attract pollinators are various (Cozzolino & Widmer, 2005): e.g. they can produce nectar to entice their visitors (nectar-reward), or deceit animals by advertising general floral signals typical of rewarding plants (Jersáková et al., 2006), beginning their flowering earlier than that of nectariferous plants (Kindlmann and Jersáková, 2006; Pellissier et al., 2010; Ostrowiecka et al., 2019). Another interesting deception mechanism is the sexual deception strategy (typical of *Ophrys* species concerning the Mediterranean Area). In this case plants mimic shape and sexual pheromones of insect females to attract males which, while copulating with the flower, receive the pollen to be transferred to another plant (Schiestl et al., 2003; Schiestl et al., 2009).

After pollination has been achieved and fertilization successfully occurs, capsules develop seeds, which are tiny (for the majority of species, smaller than 1 mm, the so called “dust seeds”), produced in high quantity and lacking endosperm and other storage reserves (Arditti & Ghani, 2000). Their germination and subsequent seedling development under natural conditions require the intervention of compatible symbiotic fungi and the establishment of mycorrhizal associations (McKendrick et al., 2000; Smith & Read, 2008; Rasmussen & Rasmussen, 2014). The identification of the fungal partner(s) is necessary not only to understand phylogenetical features (Rasmussen & Rasmussen, 2014; Jacquemyn et al., 2011), ecology, distribution (Calevo et al., 2020a), plant phytochemistry and physiology, but also to obtain successfully acclimatized plants and pursue *ex situ* conservation actions. However, good results in seed germination and seedling development are also achievable thanks to the optimization of several culture media and techniques for asymbiotic *in vitro* propagation (Calevo et al., 2020b).

Figure 1. A) *Ophrys bertolonii*; B) *Orchis patens;* C) *Serapias vomeracea;* D) orchid seeds; E) fungal iphae observed in orchid root.

4.2. Brief overview on orchid propagation

Orchid propagation is gaining more and more importance for horticultural aims, for the exploitation of medicinal principles and for conservation purposes (Pant, 2013; Pant, 2014).

Indeed, the growing number of threats to populations and the decline of habitats contribute to complicating the survival of adult plants and the emergence of new seedlings. (Swarts & Dixon, 2017). In addition, orchids need long periods for seedling development, and they require the finding of the best conditions for mycorrhizal associations establishment. The germination niche of orchid seeds, which must be considered as a coalition of the species and its fungal counterpart, is indeed often altered by biotic and abiotic factors (Rasmussen et al., 2015).

Orchid production for commercial (horticultural and medicinal) aims is principally practiced through vegetative propagation via division of plants, tubers, and *in vitro* cloning (callus induction

included) (Morel, 1965), to avoid variations in plants characters due to the high heterozygosity of the family (Pant, 2013). Concerning conservation purposes, seed sowing trials are preferred because they ensure that a genetic reinforcement would be applied to populations, and allow a phytosanitary control of diseases (Pant, 2013; Swarts & Dixon, 2017).

If the appropriate fungal partners are available, they can be used for symbiotic growth. However, in-depth studies calibrated on each species individually are required to elucidate orchid-fungus interactions, fungal metabolism, inoculation conditions and evaluation of the increment in germination and seedling development deriving from the application of the appropriate fungi (Swarts & Dixon, 2017; Dulić & Ljubojević, 2020).

Concerning asymbiotic methods, a significant contribution to orchid propagation had been firstly provided by Knudson, who optimized a medium (with subsequent modifications: Knudson, 1922; Knudson, 1946) suitable for seed germination. Common substrates for orchid culture are for example Knudson C, Murashige & Skoog, FAST, or Malmgren 1996 (and its modifications). Depending on the species to be propagated, different formulations can be used which vary from simple three-salt solutions to complexes made of 20 or more macro and micro elements (Chang and Chang 2000a; Pant, 2013; Calevo et al., 2020b). In the culture media, auxins and cytokinins are often added (Pant & Gurung, 2005; Stewart & Kane, 2006; Johnson et al., 2007; Pant et al., 2011; Pant, 2013). In addition, also organic complex supplements as banana pulp, coconut water, tomato juice, honey and other low-cost materials have shown their importance in orchid propagation media (Thepsitar et al.,2010; Gonçalves et al., 2012).

Epiphytic orchids are relatively easy to germinate and acclimatize (as seen in the case of the commercial success for several species like *Phalaenopsis, Dendrobium, Cymbidium, Vanda*), and they have a lower requirement of mycorrhizal association during *in vitro* development. Conversely, terrestrial species need more attention in all the passages of *in vitro* production. Techniques for improving the seedling progression to adult plants and their transfer from axenic conditions to native soil are constantly under study (Batty et al., 2006), considering also aspects linked to seed maturity, balanced seed sterilization times in NaOCl (that is also needed to favour

dormancy breaking and *testa* disruption), the presence of organic nutrients in specific moments of plant growth for rooting and shooting enhancement, temperature conditions and the correct establishment of *in vitro* symbiotic associations, etc. (Yamazaki & Miyoshi, 2006; Kitsaki et al., 2004; Pokorny et al., 2013; Zeng et al., 2012; Pant, 2013; Calevo et al., 2020b; Calevo & Bazzicalupo, 2020).

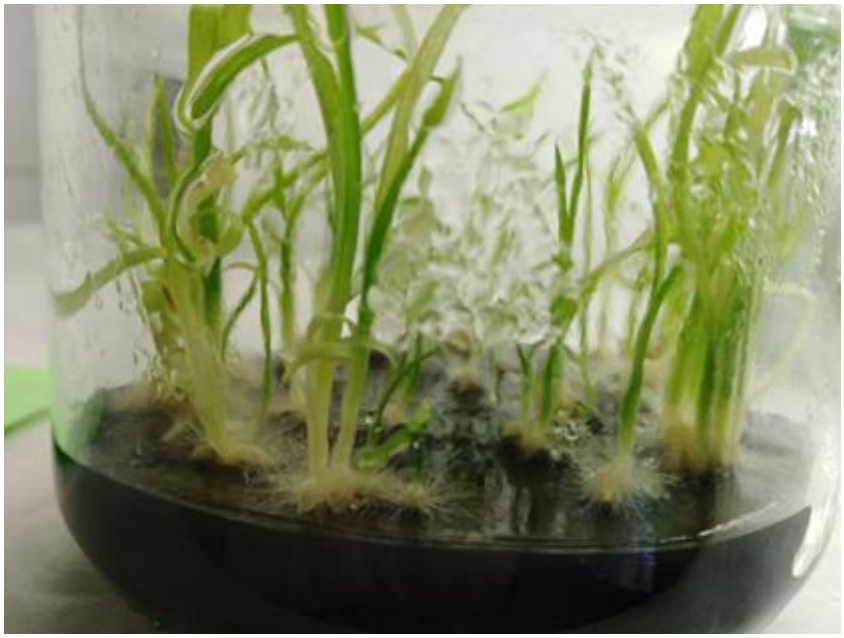

Figure 2. *In vitro* growing of orchid seedlings

4.3. A special focus on terrestrial Mediterranean orchids

4.3.1 Ethnobotany

Orchids are widely represented in the floricultural trade, where they are appreciated for their ornamental value, but they are also known in the folk traditions of many countries all over the globe (Hossain, 2011). Since ancient times, orchids have been used as source of nourishment and were also widely employed in many medicinal preparations. The first descriptions of orchids and their therapeutic utilizations were provided by Chinese even since the 28th century B.C (Teoh, 2016); other ancient reports are for example uses suggested by Theophrastus (371 B.C. –287 B.C) and Dioscorides (40 A.C.- 90 A.C.). In the ancient Ayurvedic preparation Ashtavarga, four terrestrial orchids were included (*Habenaria edgeworthii, H. intermedia, Malaxis acuminata* and

M. muscifera) (Pant & Raskoti, 2013; Teoh, 2016; Arora et al., 2017). Many orchids were implied for the treatment of various diseases and ailments, for example tuberculosis, paralysis, gastrointestinal problems, chest pains, syphilis, arthritis, cholera, cancers, piles, boils, muscular pains, menstrual disorders, diarrhoea, leucorrhoea, hepatitis, spermatorrhea, rheumatism, wounds, sores, and others (Hossain, 2011; Pant & Raskoti, 2013; Teoh, 2016; Arora et al., 2017).

If traditional uses, phytochemicals, and biological properties of some exotic orchids have been reported, information about terrestrial European and Mediterranean species is limited.

For thousands of years, in the popular tradition of Europe (as well as of Indian region and Middle East) terrestrial orchids were harvested to ground their tubers and produce the Salep (or Sahlep) (Tamer et al., 2006; Kreziou, et al., 2015; Matović, et al., 2010; Teoh, 2016 and references therein; Ghorbani et al., 2014; Molnár et al., 2017; Mincheva et al. 2019; Charitonidou et al., 2019). This preparation (originated in the Middle East) known since antiquity has returned to be quite famous in the Europe of Renaissance after the publication of *Gerard's Herbal* in 1633 (Teoh, 2016). Tamer et al. (2006) stated that 90 different orchid species are harvested to make Salep: tubers belonged to various genera, mainly *Orchis, Anacamptis* and *Ophrys* but also *Dactylorhiza, Neotinea, Himantoglossum, Serapias, Platanthera*. In the Indian tradition, *Dactylorhiza hatagirea* and species of *Eulophia* were employed (Jalal et al., 2008). This substance is used for the hot drink "Salep" or to make a derivate ice-cream, called in Turkey *Salepi dondurma* and in Greece *Kainaki* (Sezik, 2002a,b; Tamer et al., 2006; Kreziou, et al., 2015; Matović, et al., 2010).

This traditional preparation is still popular in Turkey, Greece, Balkans, and Iran, where, as a result, many species have become threatened with extinction (Molnár A. et al., 2017; Mincheva et al. 2019; Charitonidou et al., 2019). It has been estimated that about 30 tons of tubers are harvested every year, although the practice is now banned in many Mediterranean countries. However, illegal harvesting still occurs in Turkey and Iran (Teoh, 2016; Molnár A. et al., 2017). Since ancient times, eating orchid tubers (Salep preparation as practice included) was also considered useful for the treatment of different diseases (Teoh, 2016 and references therein).

In Europe and in the Mediterranean Area, tubers consumption other than in Salep preparation was carried out to recover some nutrients, especially in moments of war and famine: Mattirolo (1918), for example, listed various orchid species in his book *Phytoalimurgia Pedemontana,* useful to integrate the nutritional deficiencies of the Italian population, resulting from the economic crisis following the First World War. This practice continued in some areas until recently: e.g. tubers of *Himantoglossum robertianum* (syn. *Barlia robertiana*) were roasted and eaten by Sicilians (Lentini & Venza, 2007; Geraci et al., 2018); tubers of *Ophrys apifera, Neotinea ustulata* and other unspecified *Ophrys* and *Dactylorhiza* species were consumed in the traditional preparation *Kasùgottu* (literally, "cooked cheese") in Sardinia (Guarrera, 2007; Atzei, 2009)

In the Mediterranean traditional medicine, since the tubers are the most used parts, only a few reports concern the utilization of other portions of these plants. In Northern Italy, in some cases flowers were used in infusion to product decoctions or distillate. For example, fragrant orchids from the genus *Gymnadenia* (*G. nigra* and related, *G. odoratissima, G. conopsea*) were annotated for this purpose by Mattioli in the 1577. The inflorescence of *Dactylorhiza maculata* (syn. *Orchis maculata*) was thought to be psychoactive and was employed in rituals in Northern Italy (Credaro, 1990). Flowers and leaves of *D. sambucina* were harvested in Liguria (Praglia; Genoa, Italy) to prepare a decoction against coughs (Gastaldo et al., 1978). In Sardinia (Italy), leaves of *Epipactis helleborine* were used for the treatment of wounds (Atzei, 2009).

In addition, medicinal uses of orchid species present in the European and Mediterranean flora have been reported in other extra-continental countries: e.g., leaves and rhizome of *Goodyera repens* and related *Goodyera spp* were used by native Americans for skin and menstrual disorders (Yonzone et al., 2009) and by Nepal people against syphilis (Pant, 2013), while leaves of *A. palustris* were utilized for the treatment of respiratory diseases in Iran (Naghibi et al., 2014).

4.3.2. Phytochemistry and pharmacology

Several authors assess that orchids possess high medicinal potential as a source of drug (Hossain, 2011; Teoh, 2016, and references therein; Arora et al., 2017, and references therein). Orchids

produce a large spectrum of secondary metabolites: alkaloids, carotenoids, anthocyanins and sterols, terpenoids, flavonoids and other phenolics have been widely recorded (Lüning, 1967; Williams, 1976; Hossain, 2011). However, despite the large number of species in the family, few studies dedicated to their phytochemistry and/or biological activities have been carried out (Pant & Raskoti, 2013; Arora et al., 2017), especially concerning terrestrial European species.

In tissues of several orchid species, phytoalexins have been frequently found (Teoh, 2016 and references therein). These secondary metabolites belong to several classes of compounds (such as terpenoids, glycosteroids and alkaloids) and play a key role in the resistance against groups of microorganisms: hircinol, loroglossol and orchinol are among the widespread molecules in a spectrum that encloses at least 40 different compounds (Stoessl and Arditti, 1984). These compounds are known for exerting bacteriostatic and fungistatic activity, possibly supporting the traditional use of tubers and rhizomes from *Gymnadenia*, *Dactylorhiza* (Teoh, 2016), *Anacamptis* (Mustafa et al., 2015) for the treatment of skin lesions.

In general, terpenes (molecules composed by one to eight units of isoprene, a ring composed by five carbons) and phenanthrenes (polycyclic aromatic hydrocarbons) are important metabolites which possess various physiological, ecological, and even therapeutic functions. For example, orchid-derived compounds belonging to diterpenoids, sesquiterpenoids and triterpenoids have shown interesting antiviral activities, including anti-SARS CoV 3CL properties due to the inhibitive competition of a viral protease (Teoh, 2016). On the other hand, phenanthrenes have revealed antiallergic, anticancer, antimicrobial, antioxidant, anti-inflammatory or antithrombotic properties (Kovacs et al. 2007): , diphenanthrenes (less common compounds among the class of phenanthrenes), with anti-allergic properties, were isolated from *Gymnadenia conopsea*, an orchid species also spread in the Euromediterranean area (Matsuda et al. 2004). Alkaloids, which are a group of heterocyclic compounds with an aromatic ring enclosing one or more nitrogen atoms, are known for their activity on the nervous system. They have been found in different portions of several orchids, leaves and flowers included (Table 1). Comparative studies on the presence of important medicinal flavonoids such as quercetin and kaempferol in terrestrial orchids

were previously provided by Williams (1978) and Pagani (1976). These compounds, found also in the leaves of the species analysed by the above mentioned authors, possess different activities: antioxidant, sedative, antidepressant, anticonvulsant, anti-proliferative, anti-inflammatory, anti-microbial, anticancer, cardioprotective, antihypertensive, antiulcerogenic, antidiabetic and hepatoprotective. Another study provided by Strack et al. (1989), which aimed to compare the anthocyanin content of inflorescences in a phylogenetical key, reported phytochemical information about this water-soluble class of flavonoids for dozens of species.

Coupled with the general lack of phytochemical data, pharmacological activities of Orchidaceae have been rarely investigated (Hossain, 2011). This is also due to the difficulties in finding vegetal material originating from eco-sustainable practices like *ex-situ* production to be used for therapeutic purposes (Pant & Raskoti, 2013; Pant, 2013). This problem is particularly evident in the case of terrestrial orchids, which are more difficult to obtain by *in vitro* propagation, and whose phytochemical and biological activities have only been determined for a few species (see Tab. 1). Some information is available concerning epiphyte entities, such as *Vanda spp.* Hadi et al. (2015) reported the cosmeceutic potential of compounds obtained from stem and flowers of *Vanda coerulea* and *V. teres.* These extracts showed high antioxidant and anti-inflammatory activity and improved skin hydration: polyphenols including anthocyanins have been linked to these beneficial effects.

Table 1. Phytochemistry and evaluation of biological activities of terrestrial orchids present in Europe

Species	Compounds	Experimental	References
Anacamptis coriophora s.l. (L.) R.M.Bateman, Pridgeon & M.W.Chase	*p*-hydroxybenzylalcohol (tuber); methyl-(E)-p-methoxycinnamate, 13-heptadecyn-1-ol, 2,5-dimethoxybenzyl alcohol, 4-(1,1,3,3-tetramethylbutyl)-phenol (flower EOs); saturated and unsaturated hydrocarbons; nonanal, phenylacetaldehyde, anisaldehyde; thymol, α-copaene (flower EOs);	antioxidant activity; anti-proliferative effect (flower EOs)	Strack et al., 1989; El Mokni et al., 2016; Teoh, 2016 and references therein; Robustelli della Cuna et al., 2019

	chrysanthemin, ophrysanin (flower)		
Anacamptis morio (L.) R.M.Bateman, Pridgeon & M.W.Chase	quercetin-3-glucoside, quercetin; caffeic acid, *p*-coumaric acid, chlorogenic acid (leaves); *p*-coumaric acid, chlorogenic acid, coumarin derivatives (flowers) orchinol, p-hydroxybenzylalcohol (tuber); chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin II, serapianin, orchicyanin I (flowers)		Pagani, 1976; Strack et al., 1989
Anacamptis papilionacea (L.) R.M.Bateman, Pridgeon & M.W.Chase	quercetin (leaves); loroglossin (unspecified); chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin II, serapianin, orchicyanin I (flowers-variable content)		Williams, 1978; Strack et al., 1989
Anacamptis pyramidalis (L.) Rich.	*p*-hydroxybenzyl alcohol, orchinol, gastrodin, oxylipins oxo-dihydroxy-octadecenoic acid, trihidroxy-octadecenoic acid, dihydroxybenzoic acid derivatives, caffeic acid derivatives, gastrodin, gastrodin derivatives (tuber); saturated hydrocarbons, nonanal, heptanal, phenylacetaldehyde, octadecanal, 2-phenylethanol, benzyl alcohol; unsaturated hydrocarbons; heptanoic acid, nonanoic acid; α-copaene, thymol, α-cadinene (flower EOs); chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin II, serapianin, orchicyanin I (flowers)	Antioxidant activity (flower EOs); Antioxidant and scavenging activity (ethanol extract of flowers and above-ground portions); anti-diabetic, neuroprotective, skin whitening properties (methanol and water extract of tuber)	Strack et al., 1989; Štajner et al., 2010; Robustelli della Cuna et al., 2019; Mahomoodally et al., 2020
Cypripedium calceolus L.	alkaloids (unspecified)		Lüning, 1967
Cephalanthera damasonium (Mill.) Druce.	quercetin (leaves), loroglossin (tuber (?) - unspecified)		Williams, 1978; Veitch & Grayer, 2007b

Cephalanthera longifolia (L.) Fritsch	quercetin (leaves), kaempferol-O-glycosides, alkaloids (unspecified)		Williams, C. 1978. Lüning, 1967
Cephalanthera rubra (L.) Rich.	quercetin (leaves); loroglossin (unspecified); chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin II, serapianin, orchicyanin I (flowers)		Williams, C. 1978.Veitch & Grayer, 2007b; Strack et al., 1989
Dactylorhiza sambucina (L.) Soó	quercetin-3-glucoside; 7-glucoside; 3,7-diglucoside, caffeoil-1-glucose, chlorogenic acid, *p*-cumaroil-1-glucose; coumarin derivatives in traces (leaves); alkaloids (unspecified); isoquercitrin, quercetin 3,7-diglucoside, chlorogenic acid, *p*-coumaric acid and caffeic acid; 6-metossi-7-idrossi-coumarin (flowers); orchinol, *p*-hydroxybenzylalcohol (tuber); cyanin, seranin, ophrysanin, orchicyanin II, serapianin, orchicyanin I (flowers)		Pagani, 1976 Lüning,1967; Strack et al., 1989
Dactylorhiza romana (Sebast.) Soó	mucilage, sugar, water, starch and ash (tuber); cyanin, seranin, orchicyanin II, serapianin, orchicyanin I		Sezik, 1967; Strack et al., 1989
Dactylorhiza viridis (L.) R.M.Bateman, Pridgeon & M.W.Chase	quercetin, kaempferol (leaves); 4-(4-hydroxyphenyl) methoxybezenemethanol, 4,40-dihydroxydiphenyl methane, 4,40-dihydroxybenzyl ether, gastrodin, 4-hydroxy benzenemethanol, 4-hydroxybenzaldehyde, beta-sitosterol and beta-daucosterol; (ethanol extract) -Dactylorhin B, loroglossin, dactylorhin A, militarine, coelovirin A, gastrodin, thymidine, quercetin-3,7-di-O-beta-glucopyranoside; 1-4-beta-D-glucopyranosyloxybenzyl)-(2R,3S)-2-isobutyltatrate (1), 4-(4-beta-glucopyranosyloxybenzyl)-(2R,3S)-2-isobutyltatrate (2),1-(4-beta-D-glucopyranosyloxybenzyl)-	neuroprotective effects, prevention of memory loss; motor functional disability and brain cell loss	Williams, C. 1978; Huang et al. 2002a, 2002b; Zhang et al. 2006c and references therein); Li et al. 2009

	(2R,3S)-2-beta-D-glucopyranosyl-2-isobutyltartrrate(3), 4-(4-beta-D-glucopyranosyloxybenzyl)-(2R,3S)-2-beta-D-glucopyranosyl-2-isobutyltartrrate(4), (2R,3S)-2-beta-Dglucopyranosyl-2-isobutyltartaric acid (5) bis(4-beta-D-glucopyranosyloxybenzyl)-(2R,3S)-2-(beta-D-glucopyranosyl-(1!4)- beta- D glucopyranosyl)-2-isobutyltartrate (6) bis(4-beta-D-glucopyranosyloxybenzyl)-(2R)-2- (beta-D-glucopyranosyl-(1!4)-beta-D glucopyranosyl)-2-isobutylmalate (7): 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, 4,40-dihydroxydibenzyl ether, 4,40-dihydroxydiphenylmethane, 4-(4-hydroxybenzyloxy)benzyl alcohol, Gastrodin, Quercetin-3-7-diglucoside, Thymidine, loroglossin, Militarine, Dactylorhin A, Dactylorhin B, beta-sitosterol, Daucosterol.		
Epipactis atrorubens (Hoffm.) Besser	quercetin, kaempferol (leaves); chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin II, serapianin, orchicyanin I (flowers)		Williams, C. 1978; Strack et al., 1989
Epipactis helleborine (L.) Crantz	quercetin(leaves) Mannose-specific lectins; alkaloids (unspecified; flowers); 3-{2-{3-{3-(benzyloxy) propyl}-3-indol, 7,8-didehydro-4,5-epoxy-3,6-d-morphinan and oxycodone (flower nectar)	antiviral and antifungal activity; psychoactive and narcotic activity	Williams, C. 1978. Balzarini et al. 1992; Van Damme et al. 1994; Lüning, 1964; Jakubska et al, 2005
Epipactis leptochila (Godfery) Godfery	quercetin (leaves)		Williams, C. 1978.
Epipactis palustris (L.) Crantz	quercetin (leaves)		Williams, C. 1978.

Epipactis veratrifolia Boiss. & Hohen	quercetin (leaves)		Williams, C. 1978.
Gymnadenia conopsea (L.) R. Br	quercetin, kaempferol (leaves); dihydrophenanthrenes, gymconopins A–D; gymnosides I-X; phenanthrenes, dihydrostilbenes, alpha-tocopherol and catechin; 6 different cyclopeptides and 2 cyclopeptide derivatives (tuber); lignans, arctigenin, lappaol A and lappaol; dactylorhin A-B and E, loroglossin, militarine (unspecified); chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin II, serapianin, orchicyanin I (flowers)	anti-allergic effect on cutaneous anaphylaxis; scavenging activity on radicals (methanol extract of tuber); antioxidant properties and anti-fibrosis effect in lungs (ethanol extract of tuber)	Williams, C. 1978; Strack et al., 1989; Matsuda et al., 2004; Yang et al., 2009; Zi et al., 2010; Yue et al., 2010; Cai et al. 2006; Li, et al. 2009
Gymnadenia nigra (L.) Rchb.	chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin II, serapianin, orchicyanin I (flowers); benzyl alcohol, 2-phenylethanol, vanillin (inflorescence EOs), phenylacetaldehyde (leaf volatiles)		Strack et al., 1989; Tava et al, 2012
Goodyera repens (L.) R. Br	alkaloids (unspecified); loroglossin (rhizome)		Lüning, 1964; Aasen et al., 1973; Veitch and Grayer 2003
Himantoglossum robertianum (Loisel) P. Delforge	chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin II, serapianin, orchicyanin I (flowers); flavones, flavan-3-ol, scopoletin, phenolic acids	antioxidant, cicatrizing, anti-inflammatory, skin protective (hydroalcoholic flower extract)	Strack et al., 1989; Bazzicalupo et al., 2019
Himantoglossum hircinum (L.) Spreng	2,5-dihydroxy-4- methoxy-9, 1 0-dihydrophenanthrene [hircinol]; 5-hydroxy-2, 4-dimethoxy-9,10-dihydrophenanthrene [loroglossol] (tuber)		Arditti, et al.,1975 and references therein
Neottia ovata (L.) Bluff & Fingerh	luteolin 3,4-diglucoside (leaves)		Williams, C. 1978.
Neotinea ustulata	quercetin (leaves); chrisanthemin, seranin,		Williams, C. 1978; Strack et al., 1989

(L.) R.M.Bateman, Pridgeon & M.W.Chase	ophrysanin, serapianin (flowers)		
Orchis anatolica Boiss.	palmitic acid, di-butylphtalate, ethyl ester of palmitic acid, 9,12-Octadecadienoic acid, 9,12,15-Octadecatrienoic acid, ethyl ester of 9,12-Octadecadienoic acid (leaves); orchicyanin I (flower)	effect on the reproductive system (leaves ethanol extract)	Strack et al., 1989; Nawasreh & Tahtamouni, 2017
Orchis anthropophora (L.) All.	quercetin, kaempferol (leaves)		Williams, C. 1978.
Orchis italica Poir.	quercetin (leaves)		Williams, C. 1978.
Orchis mascula (L.) L	quercetin, kempferol (leaves); abundant mucilages (tuber); alkaloids, saponins, tannins, phenolics, terpenes, sterols and flavonoids (dried tubers-whole plant); loroglossin, *p*-hydroxybenzylalcohol, orchinol (tuber); chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin II, serapianin, orchicyanin I (flowers)		Williams, 1978; Strack et al., 1989Veitch and Grayer 2001; Aasen et al. 1973; Teoh, 2016; Al-Snafi, 2020*
Orchis militaris L.	2,4-dimethoxy-7- hydroxy-9,10-dihydrophenanthrene (orchinol); *p*-hydroxybenzylalcohol, orchinol, loroglossin, militarine (tuber); chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin II, serapianin, orchicyanin I (flowers)		Arditti et al., 1975; Strack et al., 1989; Teoh, 2016
Orchis simia Lam.	loroglossin, mucilage, starch, sugar (tuber), coumarin precursors, anthocyanins, cyanin, orchicyanins I and II (flower)		Ernst and Rodriguez 1984; Sezik, 1967; Strack et al., 1989; Teoh, 2016 and references therein
Ophrys apifera Huds.	quercetin, kaempferol (leaves); kaempferol 3-O-b-D-glucoside, kaempferol 3-O-b-D-rutinoside, kaempferol 3-O-b-D-rhamnoside (flowers and pollinia)		Williams, C. 1978; Karioti et al., 2008.

Ophrys argolica H.Fleischm	kaempferol 3-O-b-D-glucoside, kaempferol 3-O-b-D-rutinoside, kaempferol 3-O-b-D-rhamnoside (flowers and pollinia)		Karioti et al., 2008
Ophrys bombyliflora Link.	quercetin, kaempferol (leaves)		Williams, C. 1978.
Ophrys holosericea (Burm. f.) Greuter	Saturated and unsaturated hydrocarbons; nonanal, phenylacetaldehyde, heptanal, octadecanal; benzyl-alcohol, thymol, α-copaene, γ -muurolene (flower EOs)	Antioxidant activity	Robustelli della Cuna et al., 2019
Ophrys scolopax subsp. *cornuta* (Steven) E.G.Camus	kaempferol 3-O-b-D-glucoside, kaempferol 3-O-b-D-rutinoside, kaempferol 3-O-b-D-rhamnoside (flowers and pollinia); chrysanthemin, seranin, ophrysanin, serapianin (flowers)		Karioti et al., 2008
Ophrys sphegodes Mill.	loroglossin (tuber); chrysanthemin, seranin, ophrysanin, serapianin (flowers)		Strack et al., 1989; Veitch & Grayer, 2001
Ophrys tenthredinifera Willd.	quercetin, kaempferol (leaves); chrysanthemin, cyanin, ophrysanin, orchicyanin II, serapianin (flowers)		Williams, C. 1978; Strack et al., 1989
Platanthera bifolia (L.) Rich.	quercetin, kaempferol, 6-hydroxy-C-glycosides (leaves); benzyl benzoate, benzyl salicylate, cinnamyl alcohol, lilac aldehydes, methyl benzoate, methyl salicylate (flowers)		Williams, C. 1978; Veitch & Grayer, 2001; Plepys et al., 2002
Pseudorchis albida (L.) Á. Löve & D. Löve	quercetin, kaempferol (leaves)		Williams, C. 1978.
Serapias cordigera L.	quercetin, kaempferol (leaves); chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin II, serapianin, orchicyanin I (flowers-variable content)		Williams, C. 1978; Strack et al. 1989
Serapias lingua L.	quercetin (leaves); chrysanthemin, cyanin, seranin, ophrysanin, orchicyanin II, serapianin,		Williams, C. 1978; Strack et al., 1989

	orchicyanin I (flowers-variable content)		
Serapias vomeracea (Burm. f.) Briq	quercetin, kaempferol (leaves); saturated and unsaturated hydrocarbons, nonanal, phenylacetaldehyde, heptanal, undecanal, octadecanal; palmitic acid, nonanoic acid, heptanoic acid; *trans*- β -farnesene, γ -muurolene, thymol (flower EOs); chrysanthemin, seranin, ophrysanin, orchicyanin II, serapianin, orchicyanin I (flowers)	Antioxidant activity (flower EOs)	Williams, C. 1978; Strack et al., 1989; Robustelli della Cuna et al., 2019
Spiranthes aestivalis (Poir.) Rich	quercetin, kaempferol (leaves)		Williams, C. 1978

***reported pharmacological activities refer actually to *Dactylorhiza hatagirea* (syn. *Orchis latifolia*) or were included in not peer-reviewed studies.**

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4.4 Results: Published article (Plants, 2019)

The full text is available at the following link: https://www.mdpi.com/2223-7747/8/11/502/htm

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Article

Polyphenol Characterization and Skin-Preserving Properties of Hydroalcoholic Flower Extract from *Himantoglossum robertianum* (Orchidaceae)

Miriam Bazzicalupo [1], Bruno Burlando [2,3], Marcella Denaro [4], Davide Barreca [4], Domenico Trombetta [4], Antonella Smeriglio [4] and Laura Cornara [1,*]

1 Department of Earth, Environment and Life Sciences, University of Genova, 16132 Genova, Italy; miriamb91@hotmail.it
2 Department of Pharmacy, University of Genova, 16132 Genova, Italy; burlando@difar.unige.it
3 Institute of Biophysics, National Research Council (CNR), 16149 Rome, Italy
4 Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, 98168 Messina, Italy; mdenaro@unime.it (M.D.); dbarreca@unime.it (D.B.); dtrombetta@unime.it (D.T.); asmeriglio@unime.it (A.S.)
* Correspondence: cornaral@gmail.com; Tel.: +39-010-209-9364

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Abstract: *Himantoglossum robertianum* (Loisel.) P. Delforge is a Mediterranean orchid whose propagation in vitro has been achieved, making it eligible as a source of bioactive substances. Flowers were analyzed by light and SEM microscopy and used to obtain a polyphenol-rich, hydroalcoholic flower extract (HFE). HFE was characterized for total phenols, flavonoids and proanthocyanidins, and for polyphenol profile by RP-LC-DAD. Antioxidant assays, in vitro collagenase and elastase inhibition, and MTT and cell motility assays on HaCaT keratinocytes were done. Microscopy showed epidermal cells containing anthocyanins in the flower labellum. Flavonoids (flavones and flavan-3-ols) represented the most abundant compounds (42.91%), followed by scopoletin (33.79%), and phenolic acids (23.3%). Antioxidant assays showed strong activities, rating ORAC > FRAP > TEAC > β-carotene bleaching > DPPH > iron-chelation. Biological assays showed elastase and collagenase inhibition (up to 42% and 78%, respectively), improvement of HaCaT cell viability after treatment with 500 µM H_2O_2 (from 30% to 84% of control), and stimulation of cell migration rate up to 210% of control. In summary, HFE counteracted different free radicals, while protective properties were shown by cell-free and cell-based bioassays, suggesting the possible use of *H. robertianum* flowers for skin-preserving, repair, and anti-aging applications.

Keywords: antioxidants; collagenase; elastase; flavonoids; keratinocytes; skin aging; *Himantoglossum robertianum*

Some representative figures from the published article

Figure 1. (**A**) Flower spike of *H. robertianum*. (**B**) Plant habitus in olive grove environment.

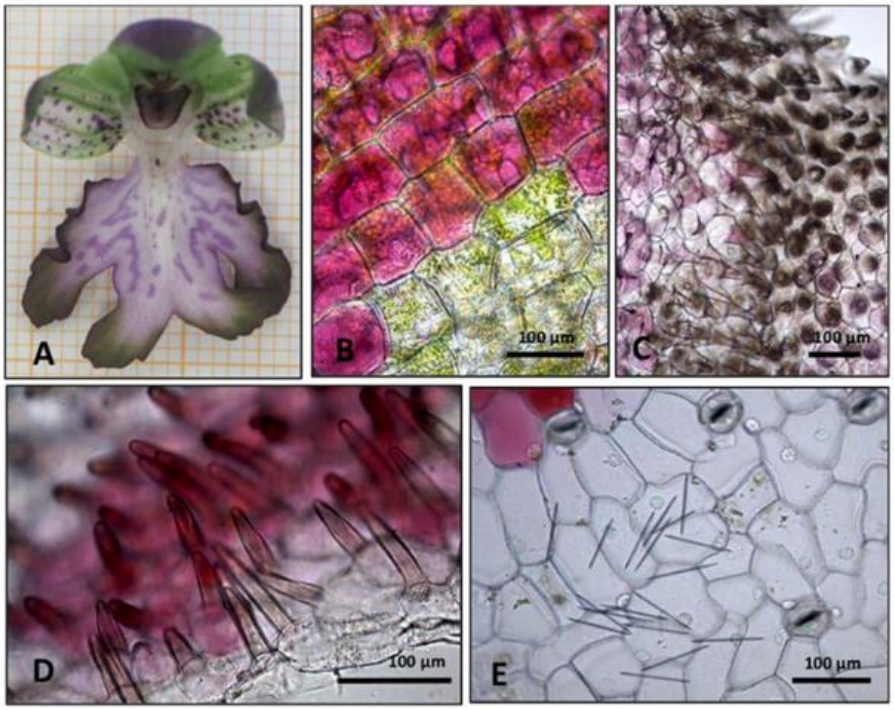

Figure 2. Stereomicroscope (**A**) and light microscope (**B**–**E**) pictures of the flower. (**A**) Total view showing three sepals, two petals, and a labellum. (**B**) Central portion of the labellum, showing purple, anthocyanin-rich cells interspersed among unpigmented cells. (**C**) Short papillose cells in an invagination of the medium-high portion of the labellum lateral arm. (**D**) Elongated pigmented papillae in the sub-stigmatic zone of the central labellum. (**E**) Stomata and raphides in the sepal.

4.5 Results: Published article (Plant Biosystems, 2020)

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The use of a new culture medium and organic supplement to improve *in vitro* early stage development of five orchid species

Jacopo Calevo[a,b], Andrea Copetta[b], Ilaria Marchioni[b,c], Miriam Bazzicalupo[b,d], Marta Pianta[d], Niktà Shirmohammadi[d], Laura Cornara[d] and Annalisa Giovannini[b]

[a]DBIOS - Department of Life Sciences and Systems Biology, University of Turin, Turin, Italy; [b]CREA - Research Centre for Vegetable and Ornamental Crops, Council for Agricultural Research and Economics, Sanremo (IM), Italy; [c]DAFE - Department of Agriculture, Food and Environment, University of Pisa, Pisa, Italy; [d]DISTAV - Department of Earth, Environment and Life Sciences, University of Genoa, Genoa, Italy

ABSTRACT

Due to the high demand, orchid propagation by *in vitro* techniques has lately increased, with an emphasis on the ways to improve germination and seedling development. In this work we compare the efficiency of three classic media for orchid propagation with a newly formulated medium supplemented with almond milk. Our target species responded differently to both the media and the supplement. However, with the exception of *Bulbophyllum plumatum* that did not show statistical differences with the most common media, a beneficial effect on orchid development growing on the new supplemented medium was observed for *Cymbidium tracyanum, Dendrobium wardianum* and for the two European orchid species *Dactylorhiza praetermissa* and *Himantoglossum robertianum*, speeding up the development of the latter two species into photosynthetic stages. The new medium could be, therefore, suitable for improving the development of some ornamental orchids and terrestrial orchid seedlings included in conservation projects that need an *ex situ* approach.

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KEYWORDS
Ex situ; germination; seedling development; organic supplement; chlorophyll content

Figure 1. Flowers of the Asian orchids *Cymbidium tracyanum* L.Castle (a) and *Dendrobium wardianum* var. *giganteum* R. Warner (b). Pictures by J. Calevo and F.S. Robustelli della Cuna, respectively.

Table 1. Chemical composition and pH of the media. Quantities are expressed as mg (L^{-1}) if not indicated otherwise.

Compound	¼MS	CG0	CG50	CG100	KC	MM
$Ca_3(PO_4)_2$	–	75	75	75	–	75
KH_2PO_4	42.5	150	150	150	250	75
$MgSO_4 \cdot 7H_2O$	45.135	250	250	250	–	–
$MgSO_4$	–	–	–	–	122.15	97.69
NH_4NO_3	412.5	410	410	410	500	–
$NH_4H_2PO_4$	–	150	150	150	–	–
$(NH_4)_2SO_4$	–	–	–	–	500	–
KNO_3	475	80	80	80	–	–
$CaCl_2$	83.005	–	–	–	–	–
$Ca(NO_3)_2$	–	150	150	150	241.3	–
$ZnSO_4 \cdot 7H_2O$	2.15	–	–	–	–	–
$Na_2MoO_4 \cdot 2H_2O$	0.062	–	–	–	–	–
$MnSO_4 \cdot H_2O$	4.225	8.45	8.45	8.45	5.68	1.54
KCl	–	–	–	–	250	–
KI	0.207	–	–	–	–	–
H_3BO_3	1.55	–	–	–	–	–
FeNa EDTA	9.175	–	–	–	–	–
$FeSO_4 \cdot 7H_2O$	–	25	25	25	25	27.8
Na_2EDTA	–	22.3	22.3	22.3	–	37.26
$CuSO_4 \cdot 5H_2O$	0.006	–	–	–	–	–
$CoCl_2 \cdot 6H_2O$	0.006	–	–	–	–	–
Glycine	0.5	–	–	–	–	2
myo-Inositol	25	–	–	–	–	100
Nicotinic acid	0.012	–	–	–	–	5
Pyridoxine HCl	0.012	–	–	–	–	5
Thiamine HCl	0.025	–	–	–	–	10
GA3	–	1	1	1	–	–
BAP	–	–	–	–	–	1
D-Biotin	–	–	–	–	–	0.05
Folic acid	–	–	–	–	–	0.5
Casein	–	500	500	500	–	400
Coconut water	20 mL	–	–	–	–	–
Pineapple powder	–	–	–	–	–	$20 \cdot 10^3$
Almond milk	–	–	50 mL	100 mL	–	–
Activated charcoal	$2 \cdot 10^3$	$2 \cdot 10^3$	$2 \cdot 10^3$	$2 \cdot 10^3$	$2 \cdot 10^3$	$2 \cdot 10^3$
Sucrose	$10 \cdot 10^3$	$10 \cdot 10^3$	$5 \cdot 10^3$	–	$10 \cdot 10^3$	–
Agar	$7 \cdot 10^3$	$7 \cdot 10^3$	$7 \cdot 10^3$	$7 \cdot 10^3$	$7 \cdot 10^3$	$7 \cdot 10^3$
pH	5.8	5.8	5.8	5.8	5.8	5.8

MS- Murashige & Skoog (1962)
CG- Calevo & Giovannini (new)
KC- Knudson C orchid medium - Morel (1965)
MM- Malmgren Modified medium – Malmgren (1996)

Table 2. Percentage of seed germination for *Cymbidium tracyanum*. Data were analyzed by ANOVA followed by Fisher's probable least-squares difference test. $P \leq 0.0085$.

Medium	*C. tracyanum*
¼ MS	75.6 ± 5.2 a
CG0	66.1 ± 4.6 a
CG50	67.5 ± 5.8 a
CG100	51.9 ± 2.8 b
MM	67.5 ± 3.3 a
KC	73.5 ± 4.0 a

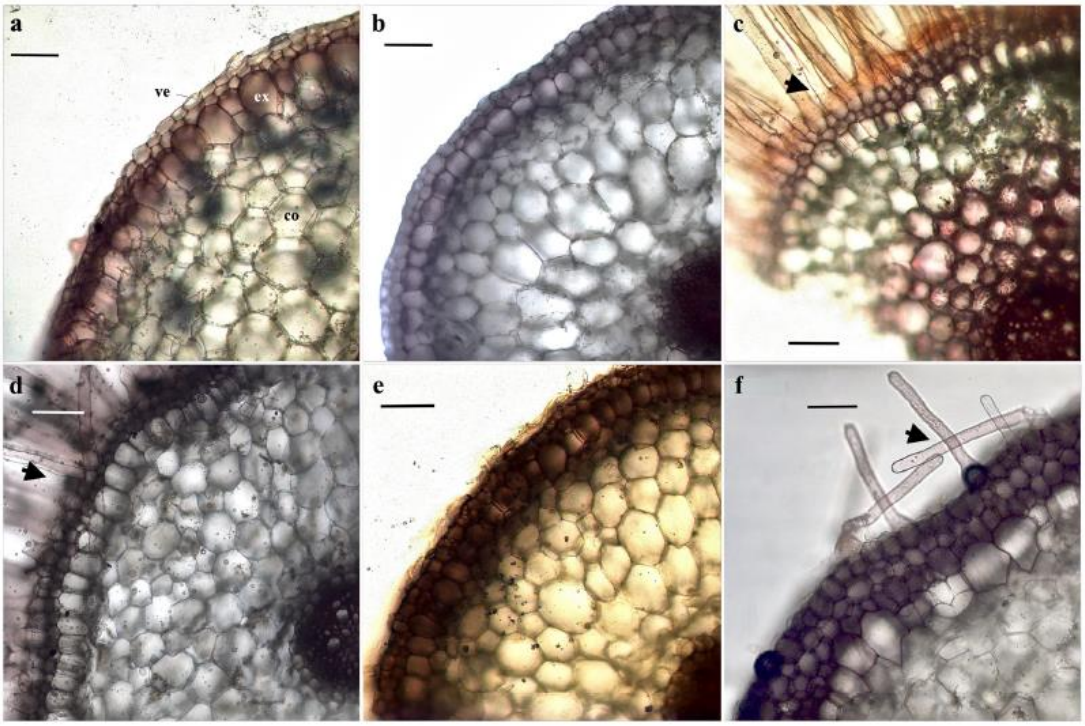

Figure 2. Hand-cut sections from roots of *Cymbidium tracyanum*. Sections stained with phloroglucinol/HCl, at T1, grown on 1/4MS (a), CG0 (b), CG50 (c), CG100 (d), KC (e), MM (f); scale bar = 100 µm. ve = velamen, ex = exodermis, co = cortex, arrows indicate presence of root hairs.

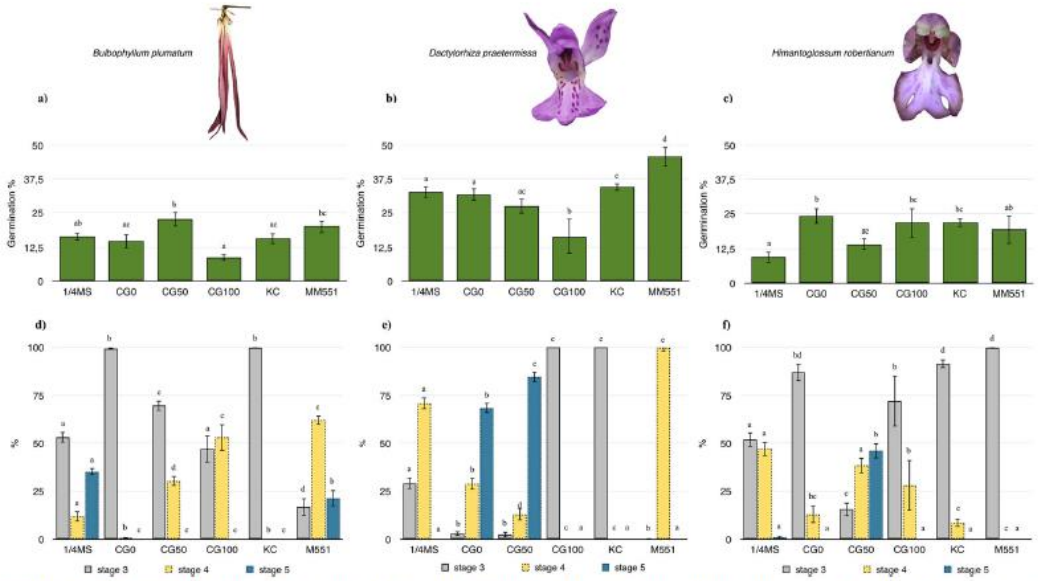

Figure 4. Germination percentage and percentage of developmental stages of *Bulbophyllum plumatum* (a;d), *Dactylorhiza praetermissa* (b;e) and *Himantoglossum robertianum* (c;f), respectively. Data were analyzed by ANOVA followed by Fisher's probable least-squares difference test with cut-off significance at $p \leq 0.05$ (four Petri dishes and 1600 seeds per species).

5. Citrus by-products as a source of bioherbicides

For the fight against pests and weeds, several research efforts are dedicated to the finding of eco-sustainable alternatives to common chemical pesticides, which cause damage to environment, the biota and human health. This Chapter discusses the possibility of utilizing by-products deriving from industrial processes for both reducing pollution and waste and for exploiting abundant sources of compounds with selective phytotoxicity and null or low toxicity. We therefore present the work, published in *Molecules* (2020), dedicated to the investigation of biological activities of essential oils from peels of three citrus species, which are widely cultivated and sold in the Mediterranean Area.

5.1 Bioherbicides: bioactive compounds and mechanisms of action

As briefly described in Chapter 1.3, the use of pesticides seriously threatens the health of the biotic component at all levels, and there is a constant risk of weeds developing resistance to chemicals (Holt & Lebaron, 1990). Therefore, research aimed at finding environmentally friendly alternatives to use chemical pesticides is constantly increasing. The problem of sustainability is very topical indeed: it is both a challenge and an opportunity for several sectors. The possibility of using eco-friendly by-products deriving from the agri-food chain is therefore of primary interest for the perspective of reducing waste and also to find natural substances to be used as bioherbicides (Iriondo-De Hond et al., 2018).

Bio-herbicides are in general considered a safe tool for biological control. They can be used in cultivated fields, but also in pastures, roadsides, forests, and can be applied in the form of sprays or granules (Bailey, 2014). There is a growing need for new bio-herbicides with safer toxicological and environmental profiles. However, before the commercialization of bioactive compounds of natural origin potentially useful for this purpose, their phytochemical and phytotoxic profile must be assessed. Evaluation of phytotoxicity is commonly investigated by genotoxicity tests and by seed germination bioassay (Verdeguer et al., 2020). Furthermore, the possible toxic effect on the

biotic components of aquatic systems is also verifiable with *in vitro* tests, for example, using a model species such as *Artemia salina* (Rajabi et al., 2015).

Plant products used as bio-herbicide often target specific weeds in specific situations (Bailey, 2014). Plant extracts or bioactive secondary metabolites exhibit a range of biological activities on seed germination and seedling growth. There are different types of damages, like loss of DNA/cell membrane integrity, or interference in mitosis, amylase activity and related biochemical processes, resulting in delays or inhibition of seed germination. Seedling growth is retarded by low rates of root-cell division, nutrient uptake, photosynthetic pigments and plant growth hormone synthesis; in parallel, the production of reactive oxygen species (ROS) and stress-mediated hormones increase.

Other plant products highly considered for the fight against weed diffusion are essential oils (Kaur & Singh, 2018). These volatile compounds contain natural flavours and fragrances contributing to their characteristic odour, but also allelochemicals able to inhibit weed germination and seedling growth (Mutlu et al., 2010; Batish et al., 2012; Hazrathi et al., 2017). It has been observed that terpenoids, particularly monoterpenes and sesquiterpenes which are the main components of essential oils, are at the basis of this inhibitory activity (Weston & Duke, 2003; Kaur & Singh, 2018).

5.2 Uses of by-products from the agri-food chain in the weed management

Every year, it has been estimated that hundreds of tons of by-products and waste are produced, deriving from the agricultural sector and the agro-industrial processing (Torres-León et al., 2018). Concerning fruit production, during industrial processing and packaging commonly discarded parts are pruning residues, stems, peels, bran, fruit shells and seeds. These latter portions often constitute more than the 50% of the fruit itself (Ayala et al., 2011). In addition, damage during products transportation, storage, and processing contribute to the generation of additional food and fruit waste. Plant by-products cause several environmental, economic and social problems, like costs of man-power to transport solid wastes in landfills, problems of fermentations during

degradability processes of the raw materials, ethical debates about global hunger suffering, etc (Novoa-Muñoz et al., 2008; Torres-León et al., 2018). However, many agri-food wastes and by-products could represent a valuable and abundant source of bioactive compounds, proteins, lipids, starch, micronutrients and fibers. Some by-products obtained from fruit industries are indeed highly appreciated as novel food in the diet of infants, adults, and animals, mainly in developing countries (Schieber et al., 2002).

Furthermore, numerous researches are being carried out on the possibility of using agri-food by-products to obtain bio-herbicides for agricultural practices, as substitutes for chemical ones. Natural compounds or allelochemicals deriving from plants, including weeds or food wastes, can exert their activities through mechanisms of phytotoxicity, by targeting new sites of action and by displaying new molecular structures (Duke et al., 2020; Verdeguer et al., 2020). On the other hand, they generally show low toxicity on other organisms, and can be rapidly degraded in the soil where they remain and tend not to be leached (Araniti et al., 2012; Kaur & Singh, 2018).

Plant-derived products that show to be active in prevention and control of weeds, pests and diseases would enhance sustainable organic agriculture, reducing crop loss in terms of both quality and production. Therefore, these bioactive compounds are suitable to be evaluated as possible bio-herbicides, or to be used as a template for synthetic eco-friendly new products.

Citrus spp are among the most diffused crops worldwide, every year generating hundreds of tons of fruit that are widely utilized either as fresh fruit or processed into juice, and also used in food flavourings, fragrances and cosmetics (Duarte et al., 2016; Denaro et al., 2020). Native to South-East Asia, members of genus *Citrus* (Rutaceae) have colonized Mediterranean basin for centuries. Citrus nowadays are very important species for the economy of some Mediterranean countries, where climatic and soil conditions allow for the production of fruits having superior quality (Duarte et al., 2016). Health properties and nutritional benefits of citrus bioactive compounds are well documented, with the high content in Vitamin C as the most well-known example (Miguel et al., 2009; Duarte et al., 2010; Barreca et al., 2017; Smeriglio et al., 2020; Denaro et al., 2020; Denaro et al., 2021).

Genus *Citrus* accounts for several species varying from sixteen to more than one hundred depending on the phylogenetic analysis carried out (Mabberley, 2008; Duarte et al., 2016): the taxonomic partitioning is complicated by low reproductive barriers that contribute to the formation of several interspecific and even intergeneric crosses. Furthermore, mutations occur frequently and may lead to minor or major changes in respect to the mother plant, complicating taxonomical identification. As stated by Denaro et al. (2020), notwithstanding the large literature regarding *Citrus* genus and its bioactive compounds, there is a general paucity of information on botanical features, phytochemistry, and biological activities for many citrus species and hybrids. In addition, some researchers (Smeriglio et al., 2018; Smeriglio et al., 2019) have highlighted that even portions commonly discarded during fruit consumption or industrial processing, like albedo and flavedo (which constitute the citrus peel), possess interesting biological activities like antiperoxidase, neuroprotective and cytoprotective activities (Denaro et al., 2020).

Large amounts of by-products are generated as result of citrus fruit processing and, in particular fruit juice industries produce every year several tons of citrus peel. The deriving Citrus processing waste, including peel, seed, cell and membrane residues, is generally known in Italy as “pastazzo” (Fig. 4).

Figure 3. A) discarded Citrus peels; B) detail of the peel. The orange arrow indicates the portion of the peel known as “flavedo”, rich in secretory pockets.

According to Calabretta et al. (2004), in Southern Italy, solid biowaste generated by orange, lemon and bergamot fruit processing average up to 0.72 x 10^6 mg per year, starting from 1.2 x 10^6 mg of citrus fruit. Pastazzo is mainly composed by water, mono- and di-saccharides, and oils (Sharma et al., 2017; Raimondo et al., 2018).

Several technologies have already been developed, aiming at the valorisation of pastazzo (Mamma et al., 2014; Wikandari et al., 2015; Raimondo et al., 2018), since citrus peels are in general well known for containing bioactive essential oils in their schizo-lysigenous pockets. Plant essential oils (EOs) have shown to be effective against a wide range of weeds and are a potential alternative to non-selective herbicides (Benvenuti et al., 2017). Therefore, the possibility of using essential oils obtained from these waste products for an integrated weed management is of great interest for the improvement of bioeconomy and for generating new market and non-market values (Marotta & Nazzaro, 2012; Chinnici et al., 2018; Raimondo et al, 2018). With this in mind, in the context of the sustainable use of local plant resources, we focused our study on the pharmacognostic and phytochemical characterization of three *Citrus* fruit peels widely exploited in Italian cosmetic and food industries, i.d. *C. x bergamia, C. x myrtifolia* and *C. limon*. The phytotoxic activities of the essential oil obtained from the fruit peels of these taxa has been tested for the possible use as safer and more environmentally friendly herbicides.

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Chemical Composition and Biological Activities of Essential Oils from Peels of Three *Citrus* Species

Lucia Caputo [1], Laura Cornara [2], Miriam Bazzicalupo [2], Clara De Francesco [3], Vincenzo De Feo [1,*], Domenico Trombetta [3] and Antonella Smeriglio [3]

1 Department of Pharmacy, University of Salerno, via Giovanni Paolo II 132, 84084 Fisciano, Salerno, Italy; lcaputo@unisa.it
2 Department for the Earth, Environment and Life Sciences, University of Genova, Corso Europa 26, 16132 Genova, Italy; cornara@dipteris.unige.it (L.C.); miriam.bazzicalupo@gmail.com (M.B.)
3 Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale Palatucci, 98168 Messina, Italy; clara904@hotmail.it (C.D.F.); dtrombetta@unime.it (D.T.); asmeriglio@unime.it (A.S.)
* Correspondence: defeo@unisa.it; Tel.: +39-089-969751

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Abstract: Background: Fruit peels are generally underutilized byproducts of the food industry, although they are valuable sources of bioactive compounds. The aim of this study is to evaluate a new application for three *Citrus* peel EOs as bio-herbicides. Methods: After a micro-morphological evaluation of *Citrus* peels by SEM analysis, the phytochemical composition of the EOs of *Citrus* × *bergamia* Risso & Poit., *Citrus* × *myrtifolia* Raf., and *Citrus limon* (L.) Osbeck was characterized by GC/FID and GC/MS analyses. The in vitro phytotoxicity against germination and initial radical elongation of several crop and weed species was evaluated. Furthermore, the eco-compatibility of these EOs has been assessed by the brine shrimp (*Artemia salina*) lethality assay. Results: SEM analysis highlighted the morphometric differences of the schizolysigenous pockets among the peels of the three *Citrus* species. Oxygenated monoterpenes are the main constituents in *C.* × *bergamia* (51.09%), whereas monoterpene hydrocarbons represent the most abundant compounds in *C.* × *myrtifolia* (82.15%) and *C. limon* (80.33%) EOs. They showed marked and selective phytotoxic activity in vitro, often at very low concentration (0.1 µg/mL) against all plant species investigated, without showing any toxicity on *Artemia salina*, opening the perspective of their use as safe bio-herbicides.

Keywords: *Citrus limon*; *Citrus* × *bergamia*; *Citrus* × *myrtifolia*; essential oil; phytotoxicity; eco-compatibility

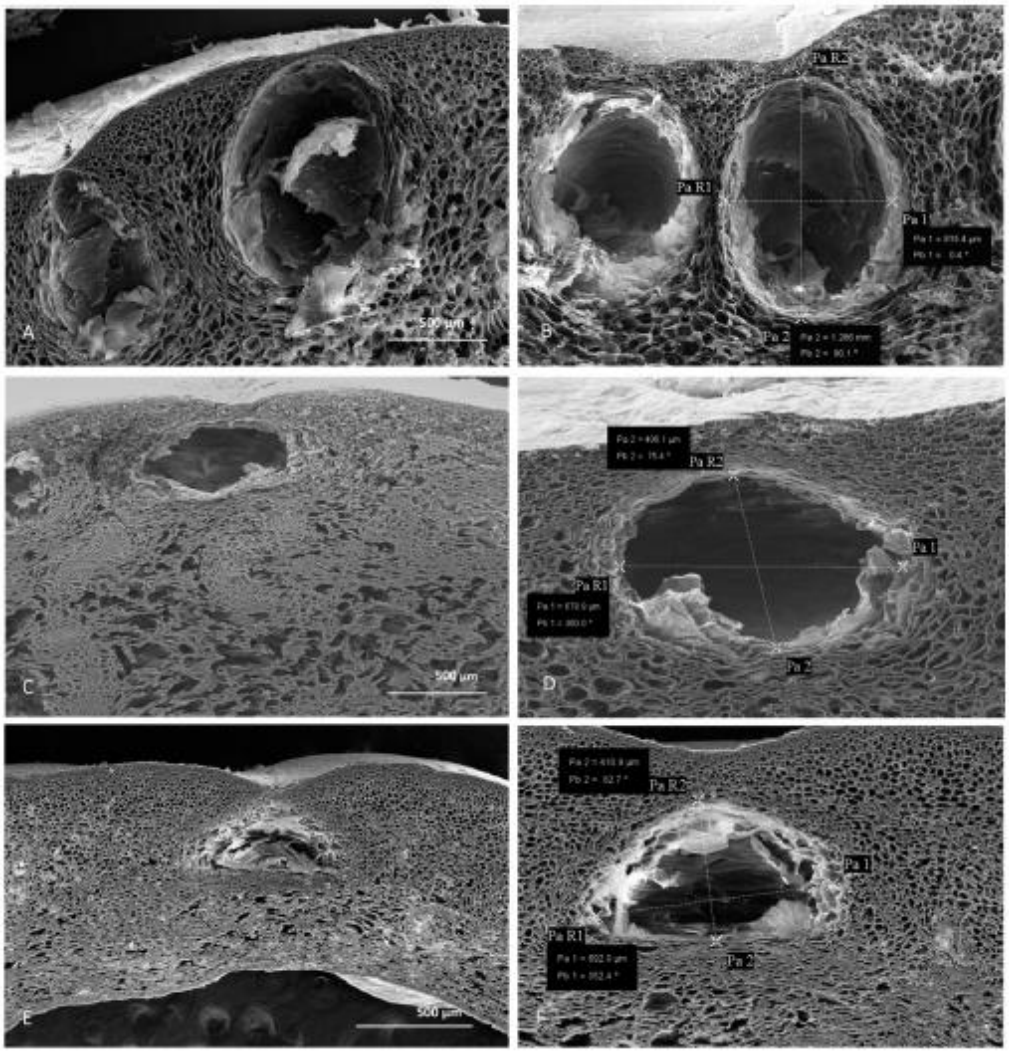

Figure 3. SEM micrographs of peel cross sections from *C. lemon* (**A**,**B**), *C.* × *bergamia* (**C**,**D**), and *C.* × *mytifolia* (**E**,**F**). Measures of the oil glands from the three different species are shown: lemon (**B**), bergamot (**D**) and chinotto (**F**).

6. Sustainable use of invasive species as sources of bioactive compounds for skin-care

Invasive alien species are a global emergency due to the damage they can cause to biodiversity, environment, human activities and health. However, the possibility of using raw material obtained from their eradication is seen with great interest, for the chance of obtaining bioactive compounds with high value for various health and environmental applications. In this Chapter, we briefly introduce invasive species, their mechanisms of invasion, policies dedicated to fight their spreading, and their involvement in folk tradition together with possible therapeutic utilizations. We then present our study, accepted for the publication by *Journal of Integrative Medicine* (2021, in press), dedicated to the validation of the anti-inflammatory, cicatrizing and skin-preserving properties of *Carpobrotus edulis*, invasive alien entity native to South Africa and now widely spread along the Mediterranean coasts.

6.1 Invasive alien species (IAS)

One of the major threats to biodiversity, both at local and global scale, is the spreading of Invasive Alien species (IAS) (IUCN, 2000). Invasive is defined as an organism of any taxonomic group which has been moved by human, intentionally or unintentionally, in a different area in respect to that of its origin. These species can have severe effects to the environment, human health and the economy (Howe and Smallwood, 1982; Villamagna and Murphy, 2010; Shackleton et al., 2018; Lagmaier & Lapin, 2020).

According to the concept of naturalization-invasion continuum (Richardson et al., 2010), an IAS needs to overcome a sequence of barriers to get naturalized or invasive: firstly, it has to be introduced in a new region, exploiting various pathways, by means of human activities which comprises both deliberate and non-intentional introduction/spreading into the wild. Not all introduced species successfully invade the new zones (Caley et al., 2008): however, if their biological traits are suitable for adaptation to the new environment, then they will begin to reproduce and spread, competing with the native entities. Common characteristics underlying the

invasiveness of a certain species are high reproduction and growth rates, efficient dispersal ability, high plasticity in physiological (and even genetical) adaptations to new conditions, high ability to survive in a variable range of ecological conditions (Pyšek & Richardson, 2010). Also hybridization mechanisms, which often lead to the origin of allopolyploid entities, can be at the basis of the invasiveness of a given species (Ellstrand & Schierenbeck, 2000; Verlaque et al., 2011).

Moreover, following the colonization of an environment by an invasive species there is a significant increase in biomass, which can also lead to an increase in the expansion of fires (Dogra et al., 2010).

So, coupled to the general increase of the awareness of this global problem, the study of invasive species has gradually embraced different disciplines like biogeography, conservation biology, epidemiology, human history, population ecology (Pyšek & Richardson, 2010), and even phytochemistry, pharmacology and medicine. The analysis of the phytochemical composition of an IAS could indeed help in understanding the invasion phenomenon, but could also bring to the discovering of new/abundant sources of bioactive compounds, which could be used for therapeutic or agronomic applications.

6.1.1. Strategies for the management of IAS

The seriousness of the problem represented by invasive plants (IAS) can also be perceived considering the great economic resources that are invested every year to fight them. Several countries all over the globe are indeed working on improving policies to address the problems linked to IAS diffusion. In Europe, there are groups specifically created for these purposes: the European Commission Committee on IAS, Invasive Alien Species Expert Group (IASEG), the Scientific Forum on IAS, the Working Group on IAS.

Among the actions undertaken by these groups is the compilation and continuous updating of the list of invasive species to be controlled. In general, there is a lack of knowledge of IAS and the socio-economic and environmental risks related to their spreading, and conflicts of interests often

put obstacles in the way of decisional processes (https://ec.europa.eu/environment/nature/invasivealien/index_en.htm). The EU also provides for the settlement of strategical measures, which can be resumed in a) prevention b) early detection and rapid eradication c) management. Actions are supported by EU through a series of financing programs, such as LIFE or Horizon 2020.

6.1.2 A case from the Mediterranean Basin: *Carpobrotus* genus.

The spread of invasive species is particularly frequent on the coasts and islands of the Mediterranean Basin. In recent decades these areas have undergone deep socio-economic and environmental transformations in response to the abandonment of traditional management practices and due to tourism activities (Simberloff et al. 2013; Lapiedra et al. 2015; Médail 2017). The most problematic plant entities in these habitats belong to the genus *Carpobrotus* N.E.Br, commonly named "iceplants" or "Hottentot figs", from the family Aizoaceae (Chenot et al., 2014; Celesti-Grapow et al., 2016; Celesti-Grapow et al., 2017; Campoy et al., 2018). These species, native from South Africa, are appreciated as ornamentals in Europe, where they have been artificially introduced at the beginning of the seventeenth century for the stabilization of sand coastal dunes (Campoy et al., 2018). *Carpobrotus* presents high dispersal rate, due to the ability of both clonal propagation and reproduction by seeds. The seed bank is large and persistent; the fruits are eaten and dispersed by animals. These species form dense monospecific mats that disturb pre-existing vegetation modifying soil characteristics like pH and nutrient content (Celesti-Grapow et al., 2016). *C. acinaciformis* and *C. edulis* have been recognized as the most invasive entities of the genus outside South Africa: in addition, they often hybridize also in the native area, leading to various taxonomic uncertainties that show greater adaptability in the newly colonized zones (Campoy et al., 2018).

Focused projects of eradication of such coastal IAS have shown positive results (Ruffino et al., 2015; Celesti-Grapow et al., 2017): for example, the removal of *Carpobrotus* spp. was the main objective of the project RESTO CON LIFE; other interventions on these species were carried out

in the contest of LIFE-ASAP, PonDerat and CSMon. Common good practices for successfully removing the plants foreseen herbicide applications and physical removal (Campoy et al. 2018).

6.1.3 Traditional uses of IAS and their phytochemical/pharmacological potential

Natural products obtained from plants used in folk traditions have gradually increased their socio-economic importance, especially concerning developing countries which often still rely only on these resources for their sustainment (Chen et al., 2016; Teoh, 2016).

Although IAS have negative effects on the ecosystems surrounding a given community, the people who live there often use these plants as food, as medicinal or for other purposes (Rahman & Bishwajt, 2014). IAS can be used in substitution of medicinal indigenous plants, a promising fact considering that many native species exploited in folk therapies are endangered or in decline (Semenya et al., 2012; Maema et al., 2016). The exploitation of IAS has been proposed as an adaptive strategy adopted by men to amplify knowledge for the treatment of diseases (Madeiros et al., 2017). However, the field is quite uninvestigated, with few targeted ethnobotanical studies. Maema et al. (2016) reported examples of invasive plants from Limpopo province (South Africa) proved to be potential sources of bioactive compounds for the treatment of asthma, toothache, hypertension, diabetes and viral infections (*Argentone ochroleuca*, *Catharanthus roseus* (L.), *Eriobotrya japonica*, *Datura stramonium* L., *Melia azedarach* L., *Opuntia ficus-indica*, *Schinus molle* L., *Ricinus communis* L., *Sambucus canadensis* (L.) and *Opuntia stricta* (Haw.) Haw.).

Fabaceae, Asteraceae, Rosaceae, Solanaceae, Lamiaceae, Anacardiaceae, Poaceae, Amaranthaceae, Apocynaceae, Brassicaceae, Cactaceae, Euphorbiaceae, Moraceae, and Myrtaceae are among the most utilized families in the folk tradition, in a ranking that sees IAS belonging to Asteraceae family as the most cited for having medicinal properties. The major medicinal properties reported are antipyretic, antirheumatic, anthelmintic, antimicrobial, antiulcerative, anticancerous, anti-inflammatory (Saxena, 2018 and references therein).

These latter authors and Maxima et al. (2020) highlighted that, due to the presence of specific compounds recognized for their biological activities, some IAS could be exploited at larger scale for therapeutic purposes. For example, metabolites like parthenin from *Parthenium*

hysterophorous revealed interesting activities against melanoma, and pancreatic or hepatic tumours (Saxena et al., 2018).

As recently reported, the potential use of waste resulting from the eradication of the halophyte *Carpobrotus edulis* is promising for medicinal applications (Maximo et al., 2020). This species is quite famous in the traditional medicine of South Africa, where traditional healers employ roots and leaves for the treatment of various infections, circulatory diseases, and gastrointestinal/skin problems. In addition, it has been used for the treatment of skin conditions also in some of the countries where this species has been naturalized, for example in Central Italy and Tunisia. (Motti et al., 2009; Meddeb et al., 2017).

Figure 4. *Carpobrotus sp* in an Italian coastal environment

Due to extensive studies dedicated to its ethnobotanical uses (summarized in Tab.2), in the final part of my PhD an extensive review has been carried out on the medicinal properties of this species (see Table 3).

The scientific literature reports the use of five types of *C. edulis* extracts: ethanolic, methanolic, in acetone, in hexane and aqueous. For some of these extracts both quantitative and qualitative phytochemical characterization was already performed. Also aspects linked to cytotoxicity, antibacterial activity, antifungal, antiviral, antioxidant, anti-proliferative, anti-cholinesterase properties were previously investigated (Mudimba et al, 2019 and references therein).

Table 2. Traditional uses of *C. edulis*

Ailment/use	Portion used	Region	World reference
Gastrointestinal disorders	Leaves juice/sap	South Africa	Watt & Breyer-Brandwijk, 1962; Matsiliza & Barker, 2001; Thring & Weitz, 2006: Van Wyk et al., 2008; Omoruyi et al., 2012b[a]; Festi & Samorini, 1995; Davids et al., 2016: Bisi-Johnson et al., 2010
Inflammations	Leaves juice	South Africa, Tunisia	Watt & Breyer-Brandwijk, 1962; Matsiliza & Barker, 2001; Thring & Weitz, 2006; Van Wyk et al., 2008; Deutschländer et al., 2009; Omoruyi et al., 2012b; Bisi-Johnson et al., 2010[e]; Meddeb et al., 2017
Respiratory diseases	Leaves juice/sap	South Africa	Van Wyk et al., 2008; Davids et al., 2016
Fungal or bacterial infections	Leaves juice, leaves pulp/sap	South Africa	Watt & Breyer-Brandwijk, 1962; Thring & Weitz, 2006; Omoruyi et al., 2012b[a/c], Davids et al., 2016
Throat, mouth infections	Leaves juice, leaves pulp	South Africa	Rood, 1994; Van Wyk et al., 2008; Deutschländer et al., 2009
Diuretic	Leaves juice	South Africa	Watt & Breyer-Brandwijk, 1962; Deutschländer et al., 2009; Festi & Samorini, 1995
Pregnancy, easy birth	Fruit decoction	South Africa	Festi & Samorini, 1995
Sedative, narcotic, psychoactive	Leaves, roots (chewed and smoked)	South Africa	Festi & Samorini, 1995
Ritual	Roots, leaves juice	South Africa	Festi & Samorini, 1995
Styptic	Leaves juice	South Africa	Festi & Samorini, 1995
Astringent	Leaves juice	South Africa	Watt & Breyer-Brandwijk, 1962; Rood, 1994; Van Wyk et al., 2008; Deutschländer et al., 2009; Festi & Samorini, 1995;
Skin disorders	Leaves juice, leaves pulp	South Africa, Tunisia, Italy	Watt & Breyer-Brandwijk, 1962; Thring & Weitz, 2006; Van Wyk et al., 2008; Deutschländer et al., 2009; Scherrer et al., 2005[b]; Meddeb et al., 2017
Oral and vaginal thrush	Leaves juice	South Africa, Tunisia	Watt & Breyer-Brandwijk, 1962: Van Wyk et al., 2008; Deutschländer et al., 2009; Meddeb et al., 2017

Diabetes mellitus	Leaves juice	South Africa	Matsiliza & Baker, 2001: Van Huyssteen, 2003; Deutschländer et al., 2009; Bisi-Johnson et al., 2010[e]
Mucosal ulcers and disorders	Leaves juice	South Africa, Italy	Watt & Breyer-Brandwijk, 1962; Matsiliza' & Barker, 2001; Thring & Weitz, 2006; Motti et al., 2009[d]
Allergies	Leaves juice	South Africa	Matsiliza' & Barker, 2001; Bisi-Johnson et al., 2010[e]
Viral infections	Roots and leaves, bolus leaves	South Africa	Omoruyi et al., 2012[b]; Omoruyi et al., 2020
Constipation	Roots and leaves	South Africa	Thring & Weits, 2006; Omoruyi et al., 2012b[a]
High blood pressure	roots	South Africa	Omoruyi et al., 2012b[a]
Animal stings and bites	Leaves juice and pulp	South Africa	Roberts, 1995
Food	Leaves, fruit	South Africa	Omoruyi et al., 2012b; Festi & Samorini, 1995; Campoy et al., 2018

Table 3. Biological properties and phytochemical characterization of *C. edulis*

Investigation	**Portion and preparation**	**Reference**	**Plant-derived product**
Antioxidant capacities	Leaves extract, stem extract**	Chokoe et al., 2008; Bouftira et al., 2009; Falleh et al., 2011a*; Falleh et al., 2011b; Hafsa et al., 2016; Meddeb et al., 2017; Rocha et al., 2017; Omoruyi et al., 2012a; Hafsa et al., 2018; Ondua et al., 2019;	Aqueous, water/ethanol, water/methanol,aqueous/acetone, methanolic, ethyl acetate extracts
Antibacterial properties against gram-	Leaves extract	Van der Watt & Pretorius, 2001; Chokoe et al., 2008; Buwa & Alfolayan, 2009; Martins et al., 2011*; Cock & Van Vuuren, 2014a; Cock & Van Vuuren, 2014b	Ethanolic, ethyl acetate, acetone, methanolic, aqueous extracts
Antibacterial properties against gram+	Leaves extract	Van der Watt & Pretorius, 2001*; Ordway et al., 2003; Martins et al., 2005; Chokoe et al., 2008; Buwa & Alfolayan, 2009; Martins et al., 2011; Meddeb et al., 2017	Aqueous/acetone, methanolic, dichloromethane extracts
Antifungal	Leaves essential oil	Omoruyi et al., 2014	Essential oil

Wound healing	Leaves extract	Meddeb et al., 2017	Aqueous/acetone extract
Antiproliferative	Leaves extract	Hafsaa et al., 2016	Aqueous, ethanol/water extracts
Anti-inflammatory	Leaves extract	Martins et al., 2011; Cock & Van Vuuren, 2014a; Cock & Van Vuuren, 2014b; Ondua et al., 2019	Aqueous, methanol, hexane, acetone, ethanol
Immune-modulating activity	Leaves, extract	Ordway et al., 2003	Methanolic extract
Anti-glycation properties	*C. edulis* polysaccharides	Hafsa et al., 2016; Hafsa et al., 2018	Aqueous, methanol/water extracts
Anti-neuronflammatory	Leaves extract	Rocha et al., 2017	Methanolic extract
Anti-cancer activity	Leaves extract	Ordway et al., 2003; Martins et al., 2010*; Hafsa et al., 2016	Aqueous, ethanol/water, methanolic
Inhibition of cholinesterases	Leaves extract	Custódio et al., 2012; Rocha et al., 2017	Methanolic extract
Antiviral	Leaves extract	Omoruyi et al., 2020	Aqueous extract

Phytochemical characterization of plant-derived products tested	
hexane, acetone, aqueous acetone, water/ethanol, ethyl acetate, methanolic, ethanolic, aqueous, petroleum/ether, chloroform; essential oils	Van der Watt & Pretorius, 2001; Chokoe et al., 2008; Bouftira et al., 2009; Falleh et al., 2011a; Falleh et al., 2011b; Omoruyi et al., 2012a; Omoruyi et al., 2014; Cock & Van Vuuren, 2014b; Hafsa et al., 2016; Meddeb et al., 2017; Rocha et al., 2017; Hafsa et al., 2018; Ondua et al., 2019

It therefore seemed necessary to analyze aspects documented in ethnobotany but not yet scientifically validated. My study, therefore, focused on the evaluation of skin-preserving, wound healing, and anti-age properties of an aqueous extract of *C. edulis*. For this purpose, phytochemical and antioxidant screening, and *in vitro* cell (wound healing activity) and *cell-free* tests (collagenase, elastase, and hyaluronidase) were employed, to assess the healing effect of this extract on the human skin. Moreover, *C. edulis* use as a natural resource for skin care could be an eco-friendly solution to reduce its invasiveness in the environment.

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6.2 Results: article accepted by Journal of Integrative Medicine

Title: *"Carpobrotus edulis* (L.) N.E.Br. extract as skin preserving agent: from traditional medicine to scientific validation"

Authors: Miriam Bazzicalupo[a], Laura Cornara[a], Bruno Burlando[b], Alberta Cascini[a], Marcella Denaro[c], Antonella Smeriglio[c], Domenico Trombetta[c]

[a]Università degli Studi Genova, Dipartimento di Scienze della Terra dell'Ambiente e della Vita, laura.cornara@unige.it, miriam.bazzicalupo@gmail.com, albertacascini@gmail.com; [b]Università degli Studi Genova, Dipartimento di Farmacia, burlando@difar.unige.it; [c]Università degli Studi di Messina, Dipartimento di Scienze Chimiche, Biologiche, Farmaceutiche e Ambientali, dtrombetta@unime.it, mdenaro@unime.it, asmeriglio@unime.it

Abstract

Objective: *Carpobrotus edulis* (L.) N.E.Br. is a succulent perennial plant native to South Africa but growing invasively in the Mediterranean basin. It is popularly used for the treatment of different diseases, including skin wound healing and regeneration, for which experimental validation is lacking. We therefore evaluated *C. edulis* skin preserving activity by testing an aqueous leaf extract (CAE) on cell cultures and in enzymatic assays.

Methods: Micro-morphological analysis of leaves was carried out by SEM and epifluorescence microscopy. Phytochemical features and antioxidant activity of CAE were evaluated by reversed-phase liquid chromatography coupled with diode array detection and electrospray ion trap mass spectrometry (RP-LC-DAD-ESI-MS), and in vitro cell-free assays, respectively. Biological properties were tested on keratinocytes and fibroblasts, and on elastase, collagenase, and hyaluronidase.

Results: CAE showed high carbohydrates (28.59 ± 0.68 %), total phenols (10.19 ± 0.60 g gallic acid equivalents/100 g dry extract), and flavonoids (54.59± 2.60 g rutin equivalents/100 g DE). RP-LC-DAD-ESI-MS revealed the predominant presence of hydroxycinnamic acids (51.96%), followed by tannins (14.82%) and flavonols (11.32%). The extract was not cytotoxic, had a strong and dose-dependent antioxidant activity, and inhibited collagenase (>90% at 500 µg/mL) and

hyaluronidase (100% at 1000 µg/mL). Cell culture experiments showed that CAE increases wound closure and collagen production, which correlate with high polyphenols.

Conclusion: Our data support the use of the plant for skin care and the treatment of skin problems. Moreover, use of *C. edulis* for skin care purposes could be an eco-friendly solution to reduce its invasiveness in the environment.

Keywords: Traditional medicine; Carbohydrates, Polyphenols, Antioxidant; Wound healing.

Abbreviations: SEM: Scanning Electron Microscopy; TBO: Toluidine Blue O; CAE: *Carpobrotus* Aqueous Extract; DPPH: 2,2-diphenyl-1-picrylhydrazyl; TEAC: Trolox Equivalent Antioxidant Capacity; FRAP: Ferric reducing Antioxidant Power; ORAC: Oxygen Radical Absorbance Capacity; MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RP-LC-DAD-ESI-MS: Reversed-phase liquid chromatography coupled with diode array detection and electrospray ion trap mass spectrometry;

- **A brief introduction, together with Materials & Methods relative to this study, have been reported in previous sections of this thesis.**

Results

3.1. Leaf micromorphology

The leaf of *C. edulis* is triangular in cross-section and possesses a palisade chlorenchyma surrounding a water storage parenchyma. Micromorphological analyses allowed us to localize condensed phenolic compounds within several specialized tannin cells, scattered in the photosynthetic parenchyma and also at the leaf tip (Fig. 1A arrows). These cells appeared blue/green with TBO staining, indicating the presence of phenols, flavonoids, and tannins. When observed in autofluorescence, these cells appeared light blue, confirming the presence of phenolic acids (Fig. 1B arrows), while the surrounding chlorenchyma cells showed red autofluorescence.

SEM micrographs showed the presence of large water-storage cells surrounding the central rib (Fig. 1C). Calcium oxalate druses (not shown) and raphide bundles (Fig. 1D, arrow) were also observed widespread in the parenchyma. In the chlorenchymatic zone, cells with condensed phenolic compounds were frequently found (Fig. 1E, arrows), corresponding to those stained in blue green by TBO under light transmission.

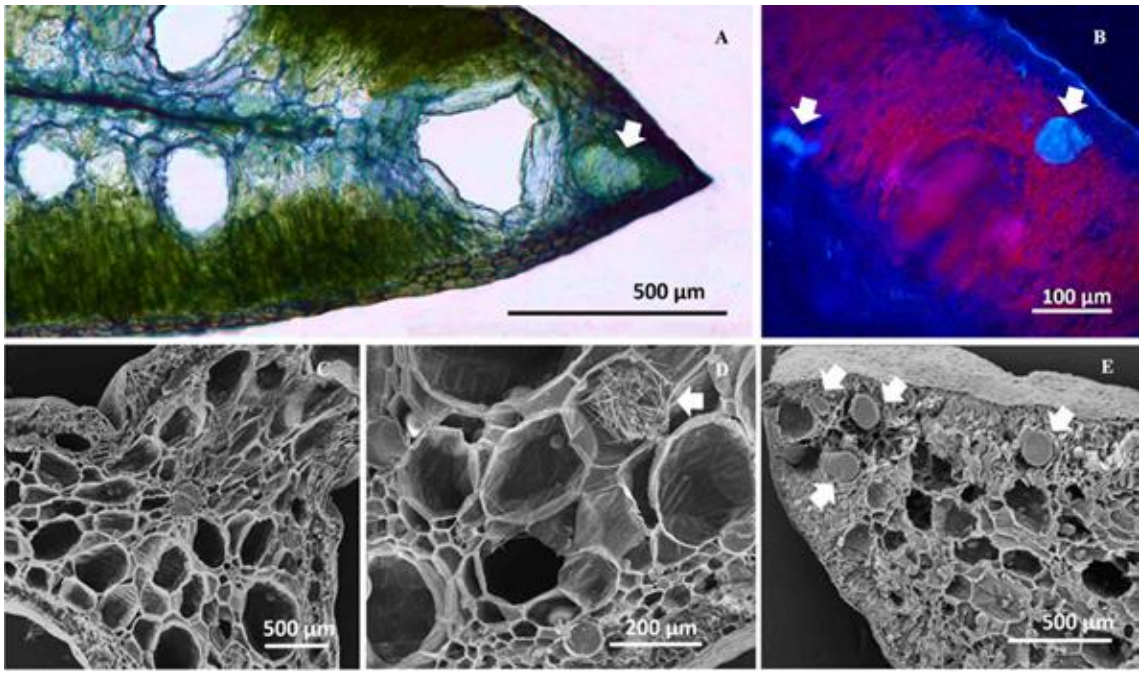

Figure 1. A-B. Light microscopy. A) Portion of a transverse section of *C. edulis* leaf, showing a palisade chlorenchyma surrounding a reserve water storage parenchyma. A polyphenol/tannin cell stained in blue green by TBO staining is visible at the leaf tip (arrow). B) The red autofluorescence indicates chlorophyll in chlorenchyma cells, while light-blue autofluorescence indicates the presence of phenolic compounds within the polyphenol/tannin cells (arrows). C-E. SEM micrographs. C) Large water-storage cells surrounding the central rib. D) Detail of leaf section showing a raphide bundle within a parenchyma cell (arrow). E) Several cells with condensed phenolic compounds are scattered in the mesophyll (arrows).

3.2. Phytochemical characterization and antioxidant properties

The preliminary phytochemical screening highlighted a good carbohydrate (28.59 ± 0.68 %) as well as a high total phenols and flavonoids content (10.19 ± 0.60 g GAE/100 g DE and 54.59 ± 2.60 g RE/100 g DE).

These preliminary observations were corroborated by RP-LC-DAD-ESI-MS analysis, which showed a very rich (Fig. 2) and heterogeneous polyphenol profile (Tab. 1) as previously observed by micromorphological analyses (see Section 3.1). Twenty-nine compounds were identified as showed in Fig. 2. Peaks' numbers refer to elution order and correspond to the compounds listed in Tab. 2.

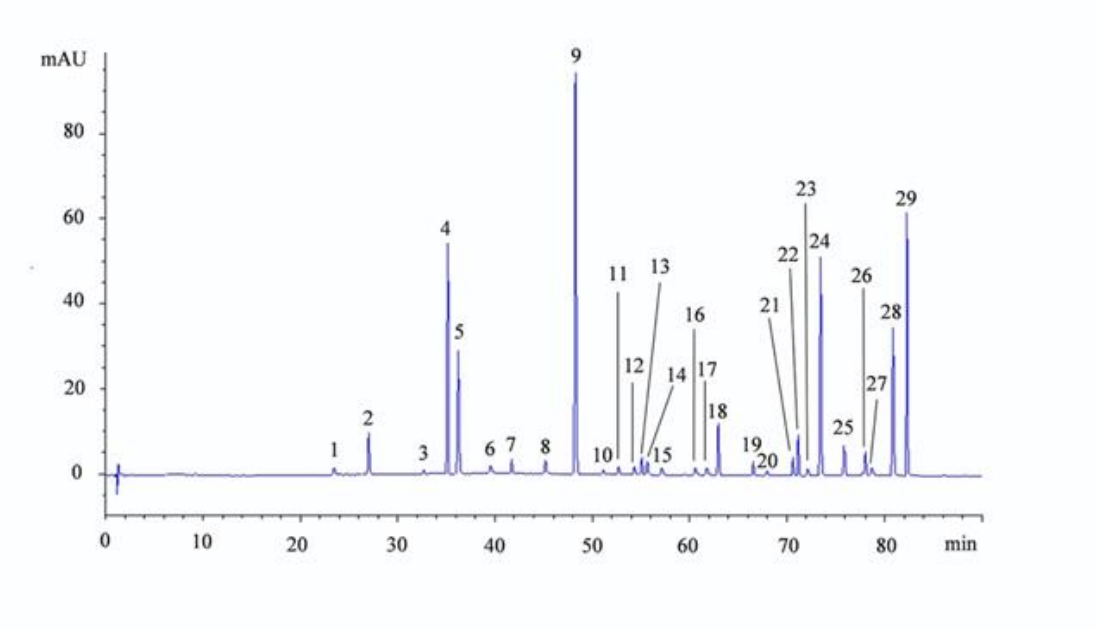

Figure 2. Representative LC-DAD chromatogram of CAE acquired at 260 nm, wavelength at which it is possible to observe all the classes of compounds identified. Peak numbers, which follow the elution order, correspond to compounds listed in Tab. 2.

Table 1. Phytochemical profile of *C. edulis* leaf water extract (CAE) by RP-LC-DAD-ESI-MS analysis. Results are expressed as mean relative area percentage of each compound with respect to total polyphenols.

Peak n.	RT	Putative identification	MF	Area%	$[M-H]^-$	m/z
		Flavones				
1	23.645	Hydroxyflavone	$C_{15}H_{10}O_3$	0.56 ± 0.02	237	238
8	45.095	Geranylchrysin	$C_{25}H_{26}O_4$	0.84 ± 0.03	389	390
		Pirymidine derivatives				
5	36.217	Amino-(4-benzamidoanilino) methylidene-(4,6 dimethylpyrimidin-2-yl) azanium	$C_{20}H_{21}N_6O$	8.18 ± 0.12	360	361
		Flavonols				
10	51.062	Kaempferol-3-*O*-arabinoside	$C_{20}H_{18}O_{10}$	0.10 ± 0.00	417	418
11	52.697	Kaempferol 3-*O*-α-L-rhamnoside	$C_{21}H_{20}O_{10}$	0.67 ± 0.02	430	431
12	54.196	Kaempferol 7-*O*-rhamnoside	$C_{21}H_{20}O_{10}$	0.49 ± 0.01	431	432
14	55.642	Isorhamnetin-*O*-glucuronide	$C_{22}H_{20}O_{13}$	0.89 ± 0.04	491	492
28	80.815	Quercetin 3'-*O*-sulfate	$C_{15}H_{10}O_{10}S$	9.18 ± 0.24	381	382
		Anthocyanins				
15	57.277	Delphinidin 3-*O*-(6"-*O*-malonyl)-β-D-glucoside-3'-*O*-β-D-glucoside	$C_{24}H_{23}O_{15}$	0.46 ± 0.02	712	713
		Isoflavones				
3	32.682	Genistein-4',7-disulfate	$C_{15}H_{10}O_{11}S_2$	0.22 ± 0.01	427.3	428
7	41.805	Glycitein-*O*-glucuronide	$C_{22}H_{20}O_{11}$	0.77 ± 0.03	459	460
16	60.781	Daidzin	$C_{21}H_{20}O_9$	0.33 ± 0.01	415	416
		Hydroxybenzoic acids				
18	63.094	Methylgallic acid-O-sulphate	$C_8H_8O_8S$	3.05 ± 0.12	264	265
		Flavanols				
19	66.642	(-)-Epigallocatechin 3-*O*-glucuronide	$C_{21}H_{22}O_{13}$	1.25 ± 0.03	480	482
		Hydroxycinnamic acids				
4	34.944	Dihydrocaffeic acid 3-sulfate	$C_9H_{10}O_7S$	12.89 ± 0.56	261	262
6	39.732	Sinapoyl-2-feruloyl gentiobiose	$C_{33}H_{40}O_{18}$	0.49 ± 0.02	723	724
9	48.246	Dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	19.45 ± 0.68	515	516
20	68.298	Sinapine	$C_{16}H_{24}NO_5$	0.29 ± 0.01	309	310
21	70.551	Chlorogenic acid hemihydrate	$C_{32}H_{38}O_{19}$	1.63 ± 0.03	725	726
23	72.265	1-Caffeoyl-5-feruloylquinic acid	$C_{26}H_{26}O_{12}$	0.53 ± 0.02	529	530
24	73.503	p-Coumaric acid 4-*O*-glucoside	$C_{15}H_{18}O_8$	12.30 ± 0.28	325	326
25	76.005	Sinapic acid (S)-malate ester	$C_{15}H_{16}O_9$	1.97 ± 0.04	339	340
26	77.934	Caffeoyl-2-hydroxyethane-1,1,2-tricarboxylic acid	$C_{14}H_{16}O_8$	1.87 ± 0.06	339	340
27	78.571	Sinapoyl malate	$C_{15}H_{16}O_9$	0.55 ± 0.02	339	340
		Triterpenes				
22	71.353	Uvaol	$C_{30}H_{50}O_2$	2.29 ± 0.11	441	442
		Tannins				
29	82.473	Valoneic acid dilactone	$C_{21}H_{10}O_{13}$	14.82 ± 0.62	469	470
		Others				
2	27.219	5-(3',5'-Dihydroxyphenyl)-γ-valerolactone 3-*O*-glucuronide	$C_{17}H_{20}O_{10}$	2.23 ± 0.05	383	384
13	54.905	3,5-Dihydroxyphenyl 1-*O*-(6-*O*-galloyl-β-D-glucopyranoside)	$C_{19}H_{20}O_{12}$	1.35 ± 0.06	439	440
17	61.817	Inositol 1,3,4,5-tetrakisphosphate	$C_6H_{15}O_{15}P_3$	0.40 ±0.01	499	500

RT, retention time; MF, molecular formula; Peak n., peak numbers refer to the elution order showed in Figure 2.

Expressing the results as relative mean area percentages (Tab. 1), phenolic acids were the most abundant compounds (55%) followed by flavonoids (15.74%), tannins (14.82%), pirymidine derivatives (8.18%), triterpenes (2.29%) and other compounds (3.97%). Among phenolic acids, the most abundant class is that of hydroxycinnamic acids (51.96%) with dicaffeoylquinic acid (19.45%) as the most abundant compound followed by dihydrocaffeic acid 3-sulfate (12.89%) and p-coumaric acid 4-*O*-glucoside (12.30%).

Flavonols were the most abundant flavonoids identified in CAE (15.74%) with the quercetin 3'-*O*-sulfate as the most abundant compound (9.18%), followed, for abundance, by the flavanol (-)-epigallocatechin 3-*O*-glucuronide (1.25%) (Tab. 1).

According to these results, a strong antioxidant and free-radical scavenging activity was detected. In particular, CAE showed the highest antioxidant activity in the ORAC test (IC_{50} 4.41 µg/mL), a hydrogen atoms transfer-based assay, followed by FRAP (IC_{50} 30.37 µg/mL), TEAC (IC_{50} 36.52 µg/mL) and DPPH (IC_{50} 112.89 µg/mL) (Tab. 3). Moreover, a concentration-dependent behavior was highlighted in all assays carried out (Fig. 3), showing a strong ability of CAE to scavenge several charged radicals.

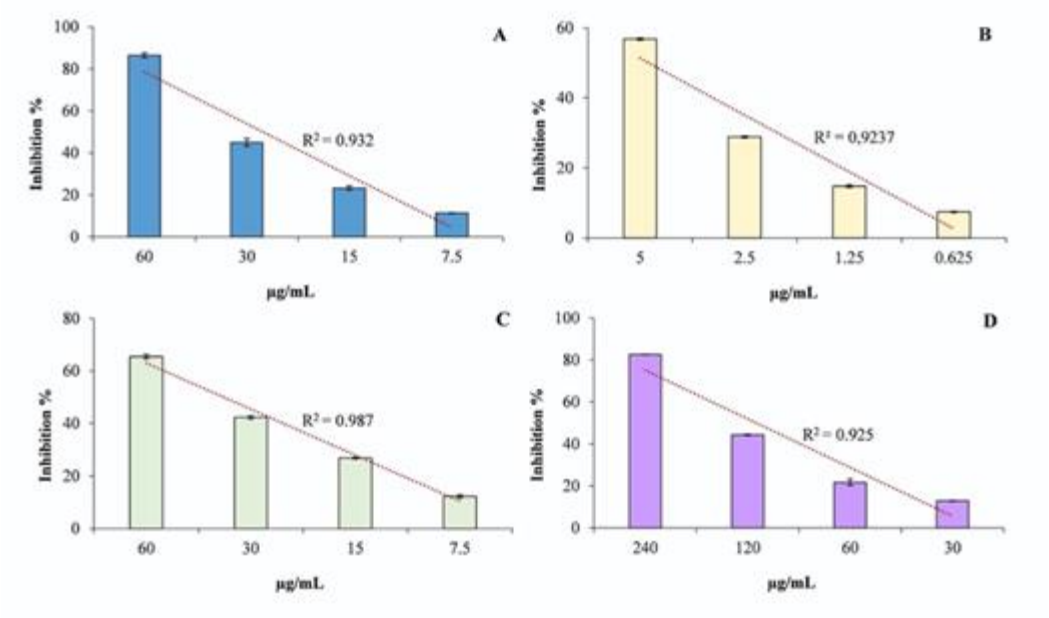

Figure 3. Antioxidant activities of CAE as evaluated by FRAP (A), ORAC (B), TEAC (C), and DPPH (D) assays. Results were expressed as mean inhibition percentages ± standard deviation of three independent experiments in triplicate (*n*=3).

Table 2. Antioxidant activity of *C. edulis* aqueous extract (CAE) in comparison with the reference standard (trolox). Results are expressed as half-maximal inhibitory concentration (IC_{50}, μg/mL) with confidence limits (C.L.) at 95%. $^{*}p<0.001$

Antioxidant assay	CAE	Trolox
	IC_{50} (μg/mL)	
FRAP	30.37 (10.94 - 84.35)*	3.80 (1.72 - 8.39)
ORAC	4.41 (3.56 - 5.46)*	0.71 (0.29 - 1.69)
TEAC	36.52 (30.85 - 43.24)*	2.97 (2.53 - 3.47)
DPPH	112.89 (59.23 - 215.18)*	3.84 (1.52 - 9.71)

3.3. Wound repair, collagen production and enzyme inhibition

The MTT assay showed that CAE induced only a weak cytotoxicity on HaCat keratinocytes at the 1000 μg/mL dose, with an estimated IC_{50} > 1000 μg/mL. The *in vitro* wound healing test performed for 24 h showed that the extract significantly increased the wound closure rate with respect to controls in a dose-dependent manner up to 83% wound closure at 500 μg/mL, with respect to 100% induced by allantoin (Fig. 4A, Fig. 5).

Cell-free enzymatic tests were carried out to evaluate the potential targets of CAE in the inhibition of skin extracellular matrix degradation. No inhibitory effect was observed on elastase (not shown). A significant inhibition was induced by 50, 500, and 1000 μg/mL CAE on collagenase, up to a value of 91%, all statistically equivalent to the inhibition of 1,10-phenanthroline (Fig. 4B). Inhibitions of 20% at 500 μg/mL, and of 100% at 1000 μg/mL CAE were found for hyaluronidase, with the last one being statistically equivalent to that of the positive control EGCG (Fig. 4C).

The ELISA assay allowed to verify that fibroblast collagen production was dose-dependently stimulated up to 50 μg/mL CAE, and then a plateau was maintained at 500 μg/mL (Fig. 4D).

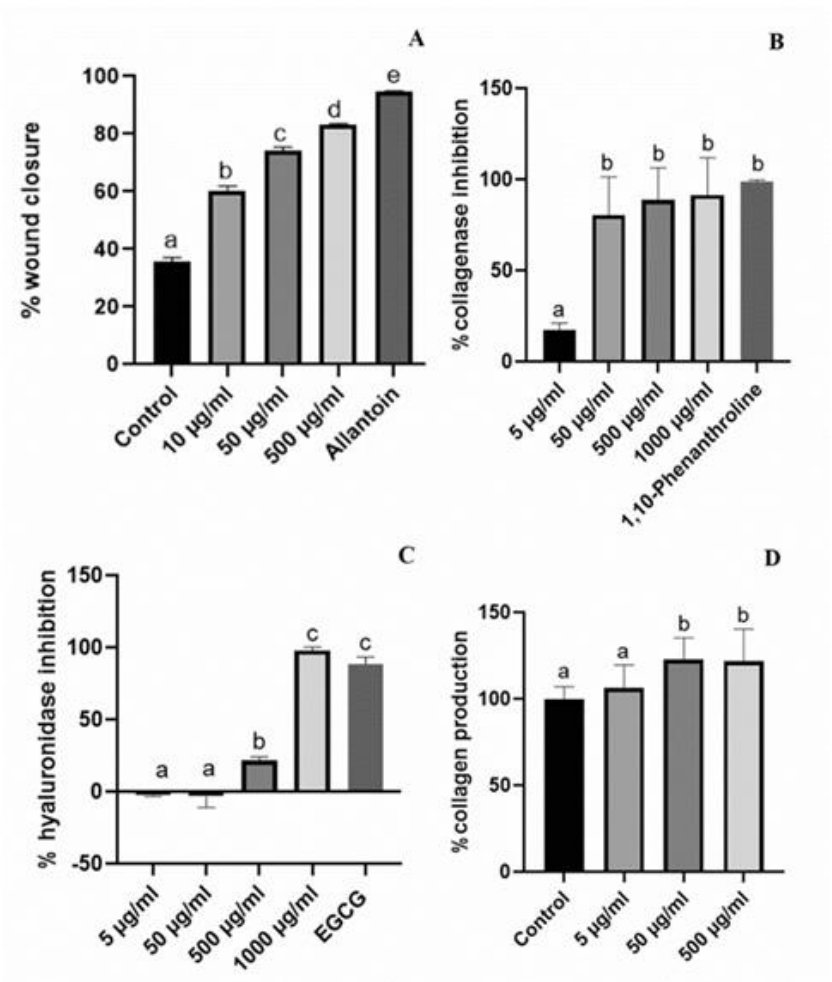

Figure 4. Biological activities of CAE assessed by means of *in vitro* cell cultures and enzymatic assay methods. A) Percentage of wound closure after exposure of keratinocytes to different doses of CAE, or to the positive control, 50 µg/mL allantoin. Data are means ± SEM (*n*=200-400 wound width measurements in 3 independent experiments). B) Percentage of collagenase inhibition exerted by CAE or by the positive control, 1 mM 1,10-phenanthroline. Data are means ± SD (*n*=4 independent samples). C) Percentage of hyaluronidase inhibition by CAE, or by 0.2 mg/mL epigallocatechin gallate (EGCG) used as positive control. Data are means ± SD (*n*=4 independent samples). D) Percent induction of collagen production by L929 fibroblasts after exposure to CAE with respect to the control (*n*=8 independent samples). Data are means ± SEM. Letters on bars indicate the result of Bonferroni's test ($p<0.01$) (A-C), and Dunnett's test (D) ($p<0.05$). Groups labelled with different letters show significant differences with respect to each other.

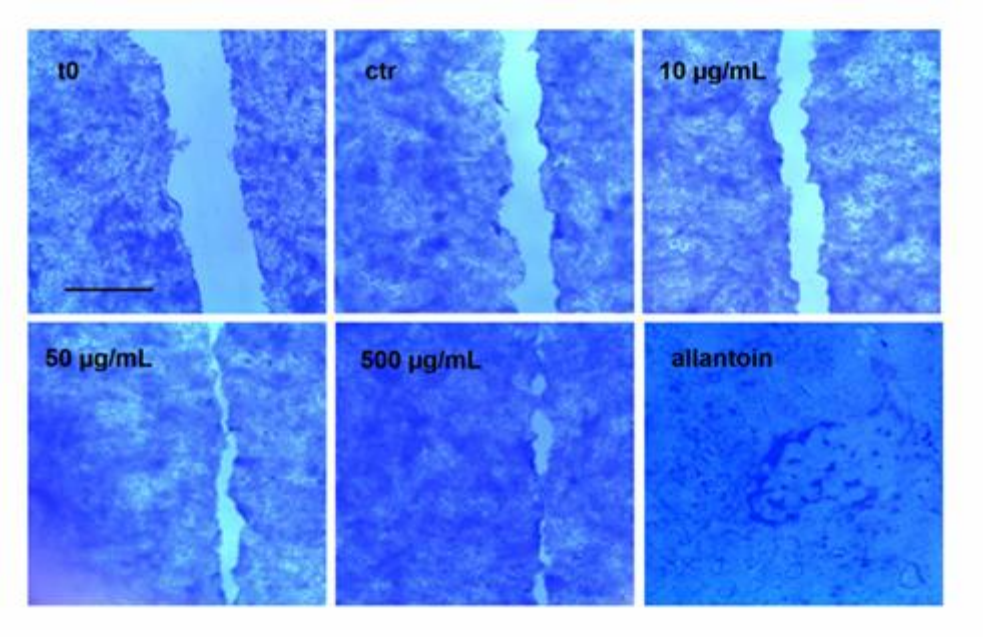

Figure 5. Representative pictures of wound closure after keratinocyte exposure to different doses of CAE, or to the positive control allantoin (50 µg/mL). Scale bar = 200 µm.

4. Discussion

In this work we focused on the evaluation of antioxidant and skin-preserving properties of an aqueous extract obtained from the leaves of *C. edulis* harvested in Liguria, Italy. The extraction method adopted led to a high extraction yield (5.19%). The use of liquid nitrogen allowed to block enzymatic activities, preserving the plant matrix from oxidation after cutting, and to obtain an impalpable powder from which bioactive compounds were easily extracted with high yield.

Our micromorphological analyses revealed the presence of cells responsible for the production and storage of secondary metabolites, among which tannins and polyphenols could be recognized. According to Talamond et al. (2015) and Dmitruk et al. (2019), phenols, flavonoids and coumarins emit in the blue region of the spectrum, and therefore, by observing these signals we could find a correspondence with the polyphenol/tannin cells described in *C. edulis* by Earnshaw et al. (1987). Moreover, cells with condensed phenolic compounds could also be identified in our SEM analyses, as reported by Sajo and Machado (2001) in other xeromorphic plants, e.g. *Xyris* species. The complex of morphological features observed in the present study fits with the typical adaptation of a succulent halophyte plant. The occurrence within the leaf mesophyll of cells rich

in phenolic compounds can be considered an adaptation to xeromorphitic conditions to reduce the damage caused by high light intensity (Mole et al., 1988; Fahn & Cutler, 1992).

Microscopic data were substantiated by the preliminary phytochemical screening as well as by the RP-LC-DAD-ESI-MS analysis, which revealed a complex and rather heterogeneous polyphenol profile. This is at present the most accurate phytochemical profiling of *C. edulis* made available (see Table 2), and therefore, it could provide a basis for bioassay-guided, drug discovery studies. The CAE showed a good carbohydrate content (28.59 ± 0.68 %), although leaves, stem and flowers are generally among the low-carbohydrate organs (Mudimba & Nguta, 2019). Moreover, the extract showed a high total phenols (10.19 ± 0.60 g GAE/100 g DE) and flavonoids (54.59 ± 2.60 g RE/100 g DE) content, comparing with previous results (6.87 ± 0.11 g GAE/100 g DE and 2.22 ± 0.05 g CE/100 g DE, respectively) (Falleh et al., 2011). Accordingly, the free-radical scavenging activity of CAE was higher than previously reported for similar extracts of the plant (Hafsa et al., 2016), possibly due to our specific extraction protocol. Notably, the ORAC test showed an IC_{50} about 6 times that of Trolox ($p<0.001$), which is the strongest synthetic compound used as reference standard. Such a result confirms the general notion that plant ethanol and aqueous extracts show best antioxidant activity due to the high presence of phenols, flavonoids, and carbohydrates (Omoruyi et al., 2012; Mudimba & Nguta, 2019). Also it is in agreement with previous observations showing that aerial parts of this and other halophytic species exhibit strongest antioxidant activities (Falleh et al., 2011).

Given its high content in phenolics and high antioxidant power, CAE is a promising plant complex for the treatment of inflammatory conditions, especially in the skin tissue. In the traditional medicine, *C. edulis* has been widely used for skin ailments, but only few works have been dedicated to validating this activity. We showed here for the first time that CAE possesses wound healing properties that approach those of the positive control allantoin. Similar properties were observed by using an acetone extract of *C. edulis* in a wound healing experiment conducted in vivo on the planaria worm *Dugesia sicula* (Meddeb et al., 2017). However, our data have been collected on a human skin in vitro model, and moreover, they confirm the presence of wound

healing properties in an aqueous extract, thus avoiding the problems that the use of an organic solvent raises e.g. in skin care applications.

A further strong indication about the use of CAE in skin care derives from enzymatic assays, revealing that the percent inhibition of collagenase and hyaluronidase activities observed in our experiments rate among the highest ones found with halophyte plant extracts (Jiratchayamaethasakul et al., 2020). This was especially evident with collagenase inhibition, which was detectable starting from a dose as low as 50 μg/mL. Moreover, the ability of CAE to enhance fibroblast collagen production, revealed by ELISA, could act synergistically with collagenase inhibition for skin applications aimed at dermal matrix reconstitution. The matrix production and skin protease activities of dermal tissue are involved in matrix turnover, preventing skin aging, while during inflammation an increase of protease activities and a decrease of collagen deposition generally occur. Therefore, proper modulatory effects on these processes can be an effective strategy in the prevention of inflammatory skin injury and aging (Lee & Kim, 2010).

Several studies are currently available on the wound-healing activity of flavonoids, allowing a correlation between observed biological activities of CAE and its constituents. Recently, numerous studies showed that topical administration of flavonoid-based formulations, with particular reference to the flavonols quercetin and kaempferol, accelerates wound-healing by regulating collagen formation, increasing the hydroxyproline levels of collagen fibrils, and decreasing significantly the wound closure time (Ozay et al., 2019; Jangde et al., 2018). Moreover, Özbilgin et al. (2018) showed that quercetin glycosides have strong collagenase and elastase inhibitory activities, which could explain their pivotal role in speeding up the healing process. Similarly, flavanols such as (−) epigallocatechin gallate have been shown to inhibit membrane type I matrix metalloproteinase, which hydrolyzes type I collagen (Stipcevic et al., 2016). These mechanisms of action, together with the well-known antimicrobial and astringent properties of flavonoids, seem to be responsible for wound contraction and rapid re-epithelization (Özbilgin et al., 2018). However, a positive effect on the migration of human keratinocyte cells was also observed after treatment with kaempferol-3-*O*-rutinoside, which was higher with respect

to that induced by the corresponding aglycone, underlining an important role of the sugar moiety in this biological activity (Petpiroon et al., 2015).

Other CAE compounds that could promote wound healing are phenolic acids, among which we showed the presence in CAE of two major hydroxycinnamic acids, namely caffeic and chlorogenic acids. Chen et al. observed that topic application of chlorogenic acid increased the synthesis of TNF-α, a proinflammatory cytokine (Chen et al., 2013), which is considered a critical step in the healing process for wound clearance and induction of collagen synthesis. Moreover, chlorogenic acid upregulated the expression of TGF-β1, increasing remarkably the expression of collagen VI, and providing a rationale for tissue repair and regeneration. The milieu of wounds is characterized by an overproduction of reactive oxygen species (ROS), Chlorogenic acid has also been shown to restore redox balance, normally altered in the wound milieu, by inducing superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) enzymes, thus reducing thiobarbituric acid reactive substance (TBARS) levels (Chen et al., 2013). Likewise, also the caffeic acid phenethyl ester showed a strong wound-healing activity through anti-inflammatory and antioxidant effects, enhancing the rate of contraction and granulation tissue formation of burn wounds (dos Santos et al., 2013). Such a complex of evidence highlights a possible major role of specific phenolics in conferring antioxidant and healing activities to the plant phytocomplex investigated in this study. However, synergistic interactions cannot be excluded, whereby minor constituents can also contribute to the biological activity of CAE.

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7. Discussion and Conclusions

This PhD research has been focused on the study of extracts and essential oils from plants adapted to the Mediterranean climate, with the purpose of identifying bioactive compounds which could be employed for health and environmental applications.

The research evaluated the possibility of obtaining bioactive compounds from different types of plants according to bio-sustainability criteria. In particular, very different species have been selected from the point of view of both the state of conservation and the possibility of using the raw material derived from them.

Portions/ species analyzed were:

- Inflorescences of *Himantoglossum robertianum*, a terrestrial orchid listed as Least Concern according to IUCN, that is progressively expanding in the Mediterranean environment
- Peels from different *Citrus* species, widely cultivated in Southern Italy
- Leaves of *Carpobrotus edulis*, an invasive alien species spread on the Mediterranean coasts.

As stated in the previous chapters, for several plants famous in folk tradition there is poor information on botanical aspects and biological activities. However, these under-investigated species could reveal valuable compounds with interesting biological properties. On the other hand, it is also intriguing the possibility of studying plants or derived compounds already known by the scientific community, evaluating their bioactive potential in unexplored contexts.

In these years we are witnessing an ongoing increase of awareness in environmental "hot" themes, such as Climate Change, biodiversity loss, ozonosphere reduction, and waste production. Consequently, on both national and international scales, many projects (for example those deriving from the Green Deal Plan actions) have been developed or are being defined, with the aim of valorizing biodiversity, contrasting invasive species diffusion, and reducing waste production and environmental impact. Hence, the research carried out in this thesis could give an important contribution in terms of valorization of biodiversity and natural resources, through the

sustainable employment and valorization of plant by-products deriving from industrial processes or invasive species eradication.

Orchid flowers as source of bioactive compounds.

As reported in the third chapter, family Orchidaceae accounts thousands of members: most of them have high conservation value due to orchid's peculiar biology, that drives species in a strict dependency on pollinators and mycorrhizae, and due to various threatening factors like habitat degradation, climate change, anthropogenic pressure, and herbivory.

Ethnobotanical surveys have elucidated that many orchids are used in the folk medicine of many regions in the world. However, little information about their phytochemistry and biological activities is available, especially concerning European Mediterranean species. In addition, orchids are threatened by illegal harvesting and commerce, and for this, are all protected and included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), reaching in these lists a coverage of about 70% of the total species.

Considering the little data available on the phytochemistry of European terrestrial orchids (Table 1 in Chapter 4), if it is possible to obtain material in a sustainable way, a more in-depth study of their bioactive compounds becomes interesting. As previously noted, the traditional use of orchids mainly concerns their tuber. The over-utilization of Salep (the most famous preparation made with orchid hypogean portions), that even nowadays lead to indiscriminate plant harvesting, is partially linked to the symbolic significance which centuries since have attributed to the testicle-like appearance of tubers. However, apart from Salep preparation (which use is now prohibited in many countries) and the use of tuber/rhizome as nutritional supplement, these portions have been employed also for the treatment of some skin affections, for wound healing, coughs, gastrointestinal problems, circulatory and neurological disorders. Phytochemical analysis found indeed that tubers of some of these species are rich in interesting compounds such as *p*-hydroxybenzylalcohol, gastrodin, and phytoalexins (like hircinol, loroglossol, and orchinol), already known for their biological activities (Kovács et al., 2008; Luo et al., 2017; Yan et al., 2019).

Respect to tubers, orchid leaves and flowers are less cited in the folk traditional medicine, but recent studies have evidenced the potential use of exotic orchid flowers for pharmaceutical applications (i.e. species of *Vanda*).

Concerning the leaves and flowers of terrestrial European orchids, some preliminary phytochemical screenings evidenced the presence of compounds having high therapeutic value, like quercetin, kaempferol, chlorogenic acid, coumarins, alkaloids, anthocyanins (Table 1 in Chapter 4). Considering this, the possible utilization of leaves and flowers in medicine or in cosmesis is therefore intriguing. In particular, the possibility of deepening the study of orchids without inducing excessive pressure on individuals by harvesting, but on the contrary by obtaining material from *in vitro* micropropagation, opens up new possibilities for research and applications. Our study analyzed the phytochemical composition and the biological properties of a hydroalcoholic flower extract obtained from the inflorescence of the Giant orchid, *Himantoglossum robertianum* (Loisel) P. Delforge. In addition, we also included this entity in a study concerning the optimization of a new culture medium to improve *in vitro* orchid propagation.

Figure 5. The showy inflorescences of the Giant orchid, *H. robertianum*

The extract of *H. robertianum* showed a high content in total phenols, mostly flavonoids, followed by scopoletin and phenolic acids, well-known antioxidant, anti-inflammatory, nootropic and neuroprotective compounds. The essays on antioxidant and radical scavenging properties of the extract have confirmed a high antioxidant activity. *Cell-free* enzymatic experiments have highlighted a high inhibitory activity of collagenase, while cell-based tests with keratinocytes also showed that it provides a significant protection against damage from oxidizing substances (H_2O_2). A significant increase in cell migration in the wound healing test was also recorded.
The pharmacognostic analysis and the study of biological properties allowed for the implementation of the knowledge related to this species, and also to validate the use of its flowers in pharmacological applications.

Concerning the micropropagation techniques, the new medium proposed results suitable for a sustainable production of plants for commercial and therapeutic aims, as well as for conservation purposes.

Phytotoxic activity of essential oils from citrus peels

Residues deriving from fruit industrial processing are often considered waste, causing several environmental and socio-economic problems. On the contrary, they represent a valuable source of bioactive compounds (Dilucia et al., 2020; Denaro et al., 2020), so the possibility of exploiting industrial by-products is receiving increasing attention by researchers.

Concerning Mediterranean, this aspect is particularly interesting in the case of lemon, oranges, and other citrus entities. Citrus fruits are indeed widely cultivated and sold in this area and they are highly consumed in the Mediterranean diet. As previously highlighted by Smeriglio et al. (2018; 2019), the portion generally discarded during the processing of citrus fruits, the peel, presents a wide spectrum of bioactive compounds, which biological activities could be potentially exploited for various health and environmental applications.

Essential oils (EOs) are well known as allelochemicals and as potential bioherbicides for weed control. Therefore, in our study we started from the schizolysigenous pockets containing oils

present in the peels of *Citrus x bergamia* Risso & Poit., *C.* x *myrtifolia* Raf. and *C. limon* (L.) Osbeck. Then we carried out the chemical characterization and studied the biological activities of EOs extracted from the peel of these three species.

Micro-morphological analyses allowed to add information on the sites of production and secretion mechanisms of essential oils in the flavedo of these species, which were understudied from this point of view.

EOs extracted were for the first time evaluated for their phytotoxicity and were found to be differently selective against crops and weeds. *C.* x *myrtifolia* EO inhibited germination of *Portulaca oleracea* and stopped radical elongation of *Lolium multiflorum,* while *C.* x *bergamia* EO inhibited only the germination of *P. oleracea*. EOs from *C. limon* were selectively active against radical elongation of *Lepidium sativum* and *Solanum lycopersicum*, while inhibited germination of *Raphanus sativus.* Toxicity assessment performed by tests with *Artemia salina* show that none of the three EOs show ecotoxicity.

Our study confirmed the potentiality of EOs obtained from citrus peels as useful tools for weed control. The use of natural substances as bioherbicides has many advantages such as no side effect on human health, the reduced risk of generation of weed resistance and the lack of pesticide residue in the environment.

Carpobrotus edulis extract as skin preserving agent

Members of genus *Carpobrotus* are known globally as widespread invasive plants that have a severe impact on biodiversity and the integrity of colonized native ecosystems (Campoy et al., 2018). However, numerous studies have shown that *Carpobrotus* extracts and bioactive compounds can be useful for human well-being and health, especially in the case of *C. edulis* (L.) N. E. Br.

Nevertheless, as evidenced by the review by Mudimba & Nguta (2019), the traditional use for the treatment of skin conditions and ailments remained although yet to be scientifically validated.

Within this framework, the results of our work have given confirmations on the excellent cicatrizing and skin-preserving properties of this plant.

Morphological and histochemical analyses conducted on the leaf allowed to localize the sites of production of secondary metabolites. Phytochemical screening of the aqueous leaf extract revealed a complex and heterogeneous polyphenolic profile, with 29 different compounds and a higher content in phenols, flavonoids, and carbohydrates in respect of previous data (Falleh et al., 2011). The extract showed a high antioxidant activity and no cytotoxicity was observed. Wound healing properties approached that of the allantoin, the reference molecule commonly used as cicatrizing drug. Percentage inhibitions of hyaluronidase and collagenase were among the highest recorded for plant extracts (Thring et al., 2009; Pientaweeratch et al. 2016), allowing us to hypothesize a possible synergistic effect with the increased collagen production observed by ELISA assay. Chlorogenic acid and caffeic acid, among the most abundant compounds recorded, are well-known for their wound healing potential that exert through antioxidant and anti-inflammatory activities, but we cannot exclude that the biological activities of the phyto-complex could be influenced also by less abundant compounds.

The use of an aqueous extract also prevents possible negative interactions between the solvent and biological substrates or cells. For this reason, *C. edulis* extract can be potentially used as a base for cosmetic products and as a template for the development of topical healing formulations.

In addition, the possibility of exploiting the raw material deriving from eradication projects (related to the conservation of biodiversity) to be used in cosmetic or medicinal applications, is important in a circular economy perspective. Furthermore, other entities from the genus *Carpobrotus* and other invasive species could be object of similar studies, with several advantages such as reduction of their pressure on native ecosystems and the generation of new incomes from their presence, an issue particularly interesting for “invaded” countries.

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8. Other collateral projects

During my PhD, I collaborated in other projects whose results have been included in the following articles published in international peer-reviewed journals:

Copetta A, **Bazzicalupo M**, Cassetti A, Marchioni I, Mascarello C, Cornara L, Pistelli L, Ruffoni B. (2021). Plant Production and Leaf Anatomy of *Mertensia maritima* (L.) Gray: Comparison of In Vitro Culture Methods to Improve Acclimatization. Horticulturae. 7(5):111. https://doi.org/10.3390/horticulturae7050111

Calevo J, **Bazzicalupo M**. (2020). Less is more: low-cost *in vitro* propagation of an Endangered Italian orchid. Nature Conservation Research 5 (Supplement 1).

Clericuzio M, Hussain FH, Amin HIM., Bona E, Gamalero E, Novello G, Lappano R, Talia M, Maggiolini M, **Bazzicalupo M**, Cornara L. (2020). Cytotoxic, Anti-bacterial, and Wound-healing Activity of Prenylated Phenols from the Kurdish Traditional Medicinal Plant *Onobrychis carduchorum* (Fabaceae). Planta Medica International Open, 07(03): e106-e113. DOI: 10.1055/a-1174-1197

Malaspina P, Catellani E, Burlando B, Brignole D, Cornara L, **Bazzicalupo M**, Candiani S, Obino V, De Feo V, Caputo L, Giordani P. (2020). Depigmenting potential of lichen extracts evaluated by *in vitro* and *in vivo* tests. PeerJ 8(7):e9150