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Valorizzazione di leguminose foraggere per i settori nutraceutico, farmaceutico e cosmetico.

Evaluation of fodder legumes for nutraceutical, pharmaceutical and cosmetic applications.

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To my family...

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The bioactivity of *Hedysarum coronarium* extracts on skin enzymes and cells correlates with phenolic content Bruno Burlando, Giulia Pastorino, Annalisa Salis, Gianluca Damonte, Marco Clericuzio, Laura Cornara*

Posidonia oceanica (L.) Delile ethanolic extract modulates cell activities with skin health applications Laura Cornara, Giulia Pastorino, Barbara Borghesi ,Annalisa Salis, Marco Clericuzio, Carla Marchetti, Gianluca Damonte, Bruno Burlando*

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Abstract

Since the beginning of human cultivation, the role of plants in medicine has been of huge importance. The aim of this PhD Project is to investigate some forage plants belonging to the family of Fabaceae to gain knowledge concerning their possible use in nutraceutical, pharmaceutical and cosmetics field. These plants have been chosen because they are characterized by great productive potential, but they are still under-exploited from the officinal and industrial point of view. They are also a potential source for the extraction of bioactive compounds with possible pharmaceutical and cosmetic applications (Rodrigues et al., 2013; Cornara et al., 2016), being generally rich in secondary metabolites such as alkaloids, amines, flavonoids, coumarins, condensed tannins and saponins (Burlando et al., 2010; Pastorino et al., 2017). To increase their use also in the industrial sector and, consequently, their commercial value, the study has focused on the identification of bioactive compounds with possible interest in human health. In this study a multidisciplinary approach was carried out, investigating several aspects such as antioxidant, antilipidemic and cytotoxicity strength, content of total phenols and flavonoids. In this PhD Project we studied four species of legumes: *Melilotus officinalis* (L.) Pall., *Lespedeza capitata* Michx., *Sulla coronaria* (L.) Medik and *Glycyrrhiza glabra* L. Thanks to a collaboration with Egadi S.r.l., Favignana, Italy, we also concentrated our study to another kind of plant, belonging to the family of Potamogetonaceae: *Posidonia oceanica* (L.) Delile. The species has shown bioactivities suitable for the development of cosmetic and dermatologic applications. In summary, the results of these investigations show new opportunities to exploit the plants studied plants in bioindustry processes, possibly increasing their commercial value.

1. Introduction

In the last years consumers are paying much more attention in the use of natural principles and in the source of new therapeutic compounds. Fabaceae are used by humans since ancient times for forage, soil improvement, and food. These plants are known for their ability to fix nitrogen by symbiosis with *Rhizobium*-type bacteria hosted at their radical nodules so they use, also today, in the agricultural field. This symbiosis is very important because the bacteria can fix atmospheric nitrogen converting it into ammonia that can be incorporated by the plant and then used for protein synthesis. A greater interest in the study of plants used in traditional medicine, is growing and the use of natural principles is attracting interest in the care of the person and in the prevention of disease (Capecka et al., 2005). Unfortunately, despite a long tradition of using legumes as fodder, their biological effects, particularly on skin cells, were almost unknown. During the first year of my PhD Project (2014/2015), commercial extracts of *Melilotus officinalis* and *Lespedeza capitata* were tested on different cell types to evaluate effects on cell proliferation. During the second year of the research, we carried out other types of tests on these two plants and we added to our research a third forage legume collected in two distinct locations in Italy: *Sulla coronaria* L. Medik. (basionym of *Hedysarum coronarium*). To have a complete vision we studied extracts from the plant, evaluating their cytotoxicity and cell-proliferation induction-inhibition test, and in addition enzymatic assays, induction of type I collagen production by ELISA method and antilipidemic activity. Also in the second year, thanks to a collaboration with the cosmetic manufacturer Egadi S.r.l., Favignana, Italy, an Italian industry, Maressentia (www.maressentia.it), we studied an extract from the seagrass *Posidonia oceanica*. During the last year we started a study of another plant belonging to the Fabaceae's family: *Glycyrrhiza glabra* L. In addition, we evaluated antioxidant properties and HPLC-MS of *Lespedeza capitata* and *Sulla coronaria*, especially thanks to the collaboration with the University of Pharmacy of Porto, Portugal (https://sigarra.up.pt/up/pt/web_base.gera_pagina?p_pagina=ffup), and the CEBR Center at the University of Genova (<http://www.cebr.unige.it/>).

2. Background

Fabaceae is a family of great interest, which is second only to that of Gramineae for economic importance. The use of legumes in the pastures and the improvement of the land is traced back to the time of Romans with Varrone, who in 37 a.C said: "Legumes should be planted in light soils, not so much for their own production as for the good they make for subsequent crops". In addition to traditional food and feed, legumes can be ground in flour for culinary use. Cohen (1977) reported the lentil domestication (*Lens esculenta*) in a site dating back to 9,500-8,000 years ago in Iran; Roosevelt et al. (1996) reported the use of *Hymenaea* as a source of food in Amazonian prehistory. Bean (*Phaseolus vulgaris*) and soybean (*Glycine max*), respectively basic cultures in the Americas and Asia, were domesticated more than 3,000 years ago (Kaplan and Lynch, 1999). Some legumes are industrially used to prepare biodegradable plastics, oils, rubbers, dyes and inks. Some legume fodder species contain a high percentage of proteins, while others are also known for medicinal properties (Duke, 1992; Kennedy, 1995; Molteni et al., 1995; Mucciarelli, 2011; Stoddard, 2013).

3. Aims

This PhD Project, aims to identify the biological properties of legume extracts and to increase the use of these plants for nutraceutical, pharmaceutical and cosmetic purposes. We know that plants combining ethnobotanical background, phytochemical pedigree, and large biomass availability are an obvious first choice in this kind of studies. Over the last years, the herbal market has rapidly increased with the search for bioactive secondary metabolites from botanical sources for health care purposes. Such a tendency is further supported by the idea that many natural compounds, due to the huge chemodiversity of plants, may be more biocompatible and involve less adverse effects than synthetic or toxic drugs. For these reasons many surveys have been conducted that aim at finding natural ingredients with possible applications as food additives or medicine. The search for natural principles, is attracting also an ever-growing interest in the field of skin care. In a screening of new possible active principles for the development of cosmetics, this study has been focused on legume fodder crops and other high-productivity plants. For our studies we used the extracts of four plants belonging to the Fabaceae's family: *M. officinalis*, *L. capitata*, *S. coronaria* and *G. glabra*. We focused the research also on *Posidonia oceanica* (L.) Delile, family Potamogetonaceae. In order to study the properties of extracts, we selected a battery of analyses oriented to characterize the chemical profile of plant extracts and to reveal biological activities inherent to skin care applications. Chemical characterizations were conducted by evaluating antioxidant properties, radical scavenging and phenolic contents. More detailed analyses were carried out by HPLC-MS technique. Biological properties were explored by first assessing cytotoxicity thresholds of extract doses and then evaluating extract effects on main enzymes and components involved in skin matrix homeostasis and skin pigmentation. In addition cell motility and lipolysis activities were also tested.

4. Materials and methods

Cell culture reagents and other chemicals were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated. For our tests *in vivo*, we used different kinds of cells cultivated at the laboratories of CNR, Genova (IBF-CNR), Italy, or at the Department of Pharmacy, University of Porto, Porto, Portugal. For this research we used *M. officinalis*, *L. capitata* and *G. glabra*'s industrial extracts.

- 70% ethanolic industrial powder extract from aerial parts of *M. officinalis* (CAS 84082-81-5) were purchased from Farmalabor Srl (Canosa di Puglia, Italy). According to the manufacturer, *M. officinalis* extract contains 1% of coumarin and undetermined flavonoids and saponins.
- 70% ethanolic industrial powder extract from aerial parts of *L. capitata* (CAS 84837-05-8) were purchased from Farmalabor Srl (Canosa di Puglia, Italy). According to the manufacturer, *L. capitata* extract contains 4% of flavonoids and undetermined catechols and condensed tannins.
- 70% ethanolic industrial powder extract from the root of *G. glabra* (CAS 84775-66-6), were purchased from EPO Istituto Farmochimico Fitoterapico S.r.l (Milano, Italy). According to the manufacturer, *G. glabra* extract contains 10% of triterpenes saponins (especially glycyrrhizin) and undetermined flavonoids and coumarins.
- Samples of *S. coronaria* were collected from two different localities in Italy: Pisa and Ventimiglia.
- Samples of *P. oceanica* were collected from Egadi islands, Sicily.

4.1 Plants of investigation

For our studies we used four plants belonging to the Fabaceae's family: *M. officinalis*, *L. capitata*, *S. coronaria* and *G. glabra*. The Fabaceae include many species (over 10000), widespread in temperate-cold and tropical regions. These are trees, shrubs, grasses, with leaves in general alternate, provided with stipules, which can be modified. The flowers, gathered in indefinite inflorescences, have a chapeau-shaped or zygomorphous aspect and a pentameral corolla, formed by a larger banner, two wings, and two petals partially welded in a hull. The fruit is a legume or modification of this: for example, a loment when the seeds are separated by transverse septa, an achene in the case of monospermous fruits, a indehiscent legume.

For this project of PhD, we used industrial extracts of *M. officinalis*, *L. capitata* and *G. glabra*. Thanks to a collaboration with the University of Pisa (Pisa, Italy) and the Hanbury Botanical Gardens (Ventimiglia, Italy), we collected plants of *S. coronaria* from two different localities in Italy: Pisa and Ventimiglia. This plant was included in the study because many species of its genus have long-term use in Traditional Chinese Medicine, and 155 different compounds of biological interest have been identified in the genus (Dong et al., 2013). During my PhD Project I also studied *Posidonia oceanica*, belonging to the family of Potamogetonaceae, collected from Egadi islands, Sicily thanks to a collaboration with the cosmetic manufacturer Egadi s.r.l.

4.1.1 *Melilotus officinalis* (L.) Pall

M. officinalis (yellow sweet clover) is a biennial herbaceous plant, 50-150 cm tall, endowed with a taproot and an erect stem, alternate trifoliate leaves and yellow, fragrant flowers (Fig.1). Its typical aroma is due to the presence of coumarins. In addition to coumarin, the plant produces other known bioactive compounds, including: scopoletin, umbelliferone, melilotin, kaempferol, quercetin and various phytosterols and triterpenic sapogenins (Yang et al., 2014). The species is spontaneous in Europe and is one of the main plants cultivated in Italy, with a production of about 205,000 kg per year. It has been included in the Belfrit list, an agreement between the governments of Belgium, France and Italy, concerning the use of substances in food supplements and herbal preparations. The plant is extensively studied and traditionally used as an anti-inflammatory, phlebotonic, spasmolytic, diuretic and sedative (Burlando et al., 2010). It is used for the functionality of venous circulation and microcirculation, for the drainage of body fluids, as urinary disinfectant, to facilitate the digestive process, in anxiety states and to promote sleep. It contains melilotoxin and coumarin acting as anticoagulants (Chae et al., 2003), while the coumarin derivative dicumarol has been used as a model molecule for the development of Warfarin, an anticoagulant rodenticide and widely used drug (Kresge et al., 2005). *M. officinalis* has also skin soothing and anti-inflammatory effects (Dweck, 2011), making it a commonly used remedy for skin health in Russia and Central Asia (Mamedov et al., 2005). Finally, data have been provided claiming for benefits in the treatment of diabetes (Chorepsima et al., 2013).

4.1.2 *Lespedeza capitata* Michx.

Lespedeza capitata (roundhead bushclover), native to eastern North America, is a perennial herb plant up to 1.5m tall, widely used as livestock forage (Fig.2). This plant was a common medicine in the tradition of North American natives. According to ISMEA (2013), it is included in the list of species grown in Italy by companies of the Federation of Italian Manufacturers of Medicinal Plants FIPPO (2012), and is also included in the Belfrit list. Some industries also use this plant as food supplement for its diuretic action, drainage of body fluids, purifying functions, regulation of urinary tract and cardiovascular function, and lipid metabolism. Different authors have reported the richness of *L. capitata* in flavonoids and tannins (Calo et al. 1969; Glyzin et al. 1970; Wagner et al., 1972). These compounds are presumably responsible of the plant's positive effects on tissue draining, kidney and cardiovascular diseases (Wagner et al., 1972; Yarnell, 2002), and diabetes (Linard et al. 1982). *L. capitata* has been traditionally used by American natives since ancient times (Linard et al. 1982; Moerman 1998; Haddock 2005). The Omaha and Ponca tribes burned pieces of moistened stem on the skin as a counter-irritant to treat rheumatism and neuralgia (Linard et al., 1982). The Comanches boiled the leaves for a beverage tea beneficial to sick people (Carlson and Jones 1940), while the Meskwaki used the root as an antidote against poison (Smith 1928; Linard et al., 1982; Foster et al., 1990). The Iroquois used the whole plant in combination with *Euonymus obovata* for stricture (Herrick 1995). In the early 18th century the plant was officially prescribed for nephritis in the United States, while it had been included for centuries in the traditional pharmacopoeia as a remedy for kidney diseases (Burlando et al., 2010). In fact, different authors had reported that *L. capitata* has the ability to lower blood cholesterol levels, as well as to remove nitrogenous compounds from the blood (Gilmore 1977; Kindscher 1992). *L. capitata* also exerts positive effects on diabetes (Jorge, Horst et al. 2004) and tissue draining, kidney and cardiovascular diseases (Wagner et al., 1972; Yarnell 2002). In the "Preliminary listing of Medicinal and Economic Kansas Plants" Smythe studied this plant as a diuretic and emetic (Smythe 1901). Different authors have reported that *L. capitata* promotes the renal excretion of urea and chloride (Yarnell 2002; Gwaltney-Brant 2016). Pastorino et al. evaluated the skin effects of *L. capitata* (Pastorino et al., 2017) while Villa reported that its flavonoids exert a free radical scavenging activity that protects collagen from injurious processes induced by chronoaging and UV exposure

(Villa 2002). Antitumoral activity against Walker-256 carcinosarcoma has been reported by Fong et al. (Fong et al., 1972). Actually, in Europe, different food supplements are available with this plant, mainly due to its diuretic action, however, the individual bioactive compounds responsible for this action, as well as their mechanisms of action are almost unknown.



Fig.1 Plant of *Melilotus officinalis* (L.) Pall with particular of stomata in the leaves (Leica M205C stereo microscope 20x)



Fig.2 Plant of *Lespedeza capitata* Michx.

4.1.3 *Sulla coronaria* (L.) Medik

Sulla coronaria (L.) Medik (synonym *Hedysarum coronarium* L.) is native to the western Mediterranean area. Italy is the only Mediterranean country where this plant is widely cultivated as fodder, especially in the central-southern area, while it is also frequent as adventitia (Fig.3). The plant can be found in different Italian regions, including Liguria, Emilia-Romagna, Tuscany, Umbria, Marche, Lazio, Abruzzo, Molise, Campania, Puglia, Basilicata, Calabria, Sicily, and Sardinia. It is a legume very appreciated from the agricultural point of view for soil improvement and fertilization. Species allied to *S. coronaria*, of Asian origin, show a vast variety of secondary metabolites (Dong et al., 2013), while *S. coronaria* has not been studied yet in this sense. Therefore I started my research on this species characterizing it from the chemical point of view. Due to its high protein value and tannin content, the plant is used to reduce gastrointestinal infections of pasture animals such as cattle or poultry (Bonanno et al., 2010). The *Hedysarum* genus has a long history of use in TCM, indicating these plants as adaptants and for treating female disorders (Dong et al., 2013; Li Zhang et al., 2013). Today *S. coronaria* is used in herbal medicine for its astringent, vitaminizing, and anti-hypercholesterolemic properties. Leaves and flowers, especially in Sicily, are used for mixed raw salads, which have nourishing properties, to prepare flans, omelettes and various soups. It is also considered to be an excellent melliferous plant. The honey deriving from it has a very light color and delicate smell and taste, because it contains high quality fructose and numerous trace elements, such as zinc, copper, magnesium, iron, and manganese. Sulla honey has also other positive effects, such as laxative properties, facilitates diuresis and is particularly suitable for babies and nursing mothers. The presence of pollen of *S. coronaria* in honey is considered a marker of Italian origin of the product.

4.1.4 *Glycyrrhiza glabra* L.

Glycyrrhiza glabra is known from ancient times due to its ethnopharmacological value and its therapeutic properties, which have been documented since the ancient Egyptian age (Fig.4). This species is native from the Mediterranean areas, but it is also present in India, Russia and China. *G. glabra* is a typical herbaceous perennial, growing to 1.0 m in height, with pinnate leaves about 7–15 cm long. The flowers are purple to pale whitish blue, forming a hermaphroditic inflorescence, and are pollinated by insects. The fruit is an oblong legume, 2–3 cm long, containing several seeds. The roots of *G. glabra* are the most used parts, while leaves are considered an agrochemical waste. *G. glabra* present different phytochemicals, such as glycyrrhizin (GA, glycyrrhizic acid or glycyrrhizinic acid), 18 β -glycyrrhetinic acid (18 β -GA, enoxolone), glabrin A and B (GL), and isoflavones, which are responsible of several pharmacological activities (Wang et al., 2013). In folk medicine, *G. glabra* is used as anti-inflammatory (Harwansh et al., 2011; Yang et al., 2017), anti-bacterial (Wang et al., 2015), anti-fungal (Motsei et al., 2003), anti-diabetic, antiviral (Wang et al., 2013; Wang et al., 2015), anti-ulcer (Shalaby et al., 2004; Jalilzadeh et al., 2015), hepatoprotective (Huo et al., 2011; Li et al., 2011), anticancer (Ohtsuki et al., 1992; Sheela et al., 2006; Lee et al., 2008), antitussive (Jahan and Siddiqui 2012; Damle 2014), anti-oxidant (Chin et al., 2007; Rackova et al., 2007; Varsha et al., 2013), skin whitening, and antidiuretic agent (Saeedi et al., 2003). In addition, liquorice extracts may have potential therapeutic value for the management of depressive disorders. Recent studies have shown that the extract produces significant antidepressant-like effect in mice in forced swim test (FST), and tail suspension test (TST) (Dhingra and Sharma 2006). Thanks to her taste, liquorice has also been traditionally used as an artificial sweetener, thus helping such conditions as diabetes mellitus (non-insulin dependent) (Liu et al., 2013; Xie et al., 2015). Actually, the most important industrial use of *G. glabra* is in the production of additives such as flavours and sweetening agents. Its roots are commonly used as food flavoring for American-type tobaccos, chewing gums, candies, baked goods, ice cream, and soft drinks (Wang et al., 2013; Saucedo et al., 2016; Rizzato et al., 2017). In beers and fire extinguishers root extracts are used as foaming agents. The fibers obtained from the roots are used for insulation, wallboard and boxboard, after removing medicinal and flavoring constituents.

Fig.3 Plant of *Sulla coronaria* (L.) Medik, details of leaves and pollen (Leica M205C stereo microscope 20x)

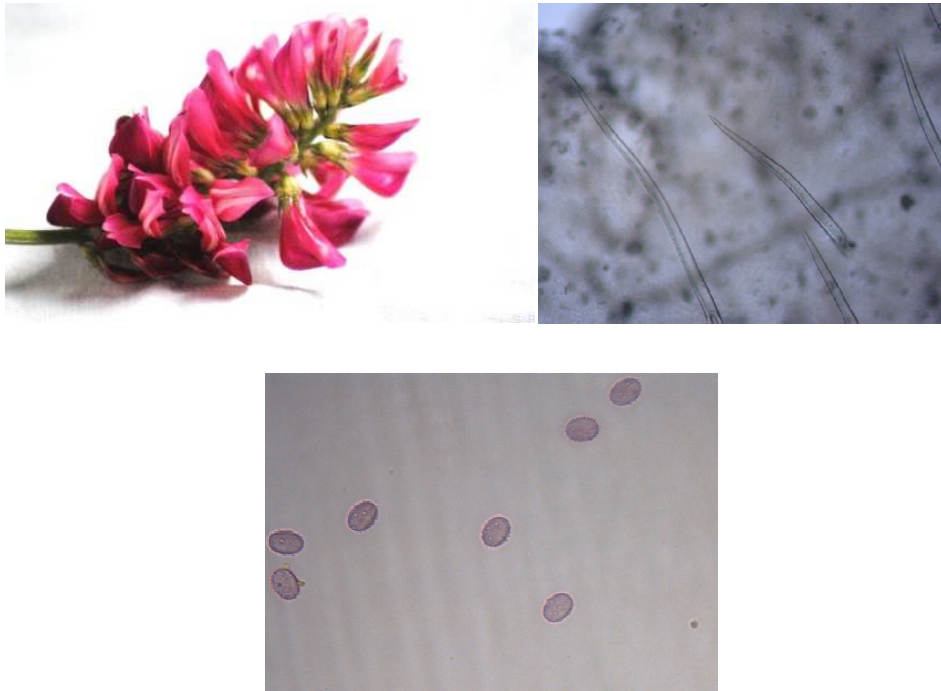


Fig.4 Plant of *Glycyrrhiza glabra* L.

4.1.5 *Posidonia oceanica* (L.) Delile

Posidonia oceanica (L.) Delile, family Potamogetonaceae, is a long-living, slow-growing, endemic Mediterranean seagrass forming extensive meadows in coastal shallow waters and tolerating temperatures ranging from 10 to 28 °C (Fig.5). *Posidonia* meadows cover an area between 25,000 and 50,000 km² of coastal sea bottom, which corresponds to about 25% of the seabed from 0 to 45 meters depth. The species has characteristics similar to terrestrial plants, with roots, rhizomatous stem, and ribbed leaves. The roots are 3-4 mm thick, are long up to 40 cm, and very branched. The leaves, bright green, become brown with growth, and when they are old and partially degraded, remain wrapped to rhizomes forming a hairy sheath. Leaf remains can be frequently found beached in the form of roundish fibrous formations, technically called “egagropili”. The plant blooms in October, when inflorescences can be observed underwater, even if little showy; fruits (sea olives) detach at ripening and fall to the bottom where they are degraded and seeds can sprout. The plant undergoes massive leaf loss in autumn, giving rise in some areas to conspicuous beach deposits. The generic name *Posidonia* derives from the Greek Poseidon (Ποσειδών), the god of sea, while the specific *oceanica* epithet indicates the wider distribution that the species had in the past compared to the current one. It is surprising to think that in the past this natural resource was exploited in multiple ways: Pope Julius III spread the use of *posidonia* leaves as a padding of cushions and mattresses, which were thought to be repellent for pests, and seemed to have beneficial effects on irritated skin and respiratory infections. In Venice, dry *posidonia* was used as a packaging to protect the famous Murano glassware during transport. The plant has a considerable ecological importance, since it provides a suitable habitat for a community of organisms that could not survive on sandy bottoms, also protecting them from predation. The covering of the leaves and the intertwining rhizomes and roots stabilize the seabed and create an ideal microhabitat for flora and fauna, increasing the biodiversity of coastal areas (Borum et al., 2004). Studies conducted with HPLC have shown that in the young leaves there is a high concentration of chicoric, p-coumaric, vanillic and ferulic acids, while mature leaves contain higher amounts of gentisic, caffeic and cinnamic acids (Haznedaroglu and Zeybek, 2007).



Fig.5 Underwater meadows of *Posidonia oceanica* (L.) Delile

4.2 Extraction Protocols

A good part of my PhD project was based on the setup of extraction protocols to be applied to *S. coronaria* and *P. oceanica* samples collected in the field, in order to obtain fractions to be tested for their bioactivity on skin cells and enzymes. Of three plant species (*M. officinalis*, *L. capitata*, *G. glabra*) I used commercially-available ethanol extracts suitable for the experimental activities.

4.2.1 Extraction of *Sulla coronaria*

S. coronaria has been collected at two different locations in Italy: Ventimiglia and Pisa. This was made possible thanks to the collaboration with the Hanbury Botanical Gardens and the University of Pisa. The protocol of extraction of this plant is based on literature reports, based on a mixture of solvents (Terrill et al., 1992). Samples of *S. coronaria* were first cleaned and dried, at room temperature in a protected place. For the extraction only aerial parts were used (flowers, leaves): 25 g of plant material, 2ml of water, 100ml of MTBE, 70ml of ethyl acetate, 60ml of acetone. The total extraction yield was about 4% (dw/dw) (Fig.6).

4.2.2 Extraction of *Posidonia oceanica*

Fresh, beached residues of *P. oceanica* leaves were collected in autumn 2015 at Favignana island, Sicily, under the supervision of the “Area Marina Protetta Isole Egadi” natural reserve (Favignana, Italy). Soon after collection, leaves were separated from shoots, cleaned manually of the basal sheath and epiphytes and rinsed in seawater, dehydrated for 36 h in a forced-ventilation oven at 42 °C, and grounded to a particle size of about 1–2 mm. The pulverized material (100 g) was put in a beaker and extracted under shaking at RT for 4 h in 60% aq. ethanol (1 L) acidified with formic acid (pH 4.0). The residual of the first extraction was separated from the supernatant and subjected to a second extraction as above. The supernatants of the two extraction steps were mixed together, cloth filtered, filtered again through a Duran® sintered glass filter disc, and vacuum-dried in a Buchi Rotavapor R-114 (Buchi Italia s.r.l.) under controlled temperature (<45 °C). The dried *P. oceanica* ethanolic extract (PEE) was finely pulverized with a mortar and stored at –20 °C until use. The total extraction yield was about 10% (dw/dw).



Fig.6 Different steps of the extraction of *S.coronaria*. (dried samples, extraction with solvents and Rotavapor)

4.3 In vitro antioxidant activity

My analyses of extracts were first focused on biochemical bioactive constituents with antioxidant and free-radical neutralizing activity, which are commonly found in plants (Balasundram et al., 2006). In particular, phenolic compounds are natural sources of antioxidants (Balasundram et al., 2006). As antioxidants, phenolics have been reported to be able to interfere with the activities of enzymes involved in reactive oxygen species generation.

4.3.1 DPPH radical scavenging assay

The DPPH radical scavenging assay is mostly applied to antioxidant capacity of plant extracts (Guimaraes et al., 2010). DPPH (DPPH •, 2,2-diphenyl-1-picrylhydrazyl) is known as a stable free radical possessing a characteristic maximum absorption between 515 and 517 nm. Its stability is due to the delocalization of the unpaired electron present on the nitrogen atom of the molecule. When DPPH• reacts with an antioxidant compound, which can donate hydrogen, it is reduced and changes its color (from violet to yellow). A calibration curve was prepared with Trolox (linearity range: 2.5–100 µg/mL, $R^2 > 0.996$). The antioxidant capacity based on the DPPH free radical scavenging ability of the extract was expressed as µmol Trolox equivalents per gram of plant material on db. For this experiment 18,25 mg of DPPH dissolved in a solution of EtOH/acetate of sodium 2:1. The reaction mixture on 96 wells plate consisted of a solution of the serial dilutions of the different samples (30 µL) and a methanol solution (270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 525 nm (Guimarães et al., 2010) in a microplate reader. (BioTeck Synergy HT).

4.3.2 Ferric reducing antioxidant power assay (FRAP)

The FRAP assay is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+} -TPTZ) to the ferrous form (Fe^{2+} -TPTZ) (Benzie and Strain 1996; Pellegrini et al., 2003). The analysis was conducted in 96 well plates and the reaction mixture consisted of extracts with appropriate dilution (35 μL) and 265 μL of FRAP reagent (10 mL of 0.3 M sodium acetate buffer at pH 3.6, 1 mL of 10 mM TPTZ solution, and 1 mL of 20 mM of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution). A calibration curve was prepared with 1 mM ferrous sulphate in distilled water. The mixture was incubated for 30 minutes at 37 °C, and the increase in absorbance was measured at 595 nm in the microplate reader (BioTeck Synergy HT).

4.3.3 Determination of total phenolic content (TPC)

Total phenolic content (TPC) was determined spectrophotometrically according to the Folin–Ciocalteu procedure (Singleton and Rossi 1965) with minor modifications (Alves et al., 2010). This assay is rapid and easy to perform, but it presents some drawbacks due to interference with some substances (Prior et al., 2005; Wong, et al., 2006). Stock solutions were prepared by dissolving gallic acid (1mg/mL) or extracts in distilled water. A solution of 7.5% of sodium carbonate was also prepared. In a microplate of 96well, 30ul of Acid gallic/extracts at different dilutions was mixed with 150ul of Folin-Ciocalteu (1:10) and 120ul of Na_2CO_3 7.5%. Water without Folin-Ciocalteu was used as a blank. A standard curve was obtained from gallic acid 1000ppm. The increase in absorbance was measured 765 nm in the microplate reader (BioTeck Synergy HT), the blue color of the reaction products has a maximum absorption around the wavelength of 760 nm. The total phenolic content (TPC) of the extracts was expressed as mg of gallic acid equivalents (GAE) per gram of plant material on dry basis.

4.3.4 Determination of total flavonoids content (TFC)

Total flavonoid content (TFC) was determined by a colorimetric assay according to Zhishen et al. (Zhishen et al., 1999). Stock solutions were prepared by dissolving 5 mg of catechin (500 ppm) or extracts in ultrapure water. Solutions 1% NaNO₂, 5% AlCl₃, and 1M NaOH were also prepared. The reaction mixture on 96 well plate consisted of 30 µl of catechin or extracts at different dilutions, 75 µl of ultrapure water and 45 µl of 5% NaNO₂. After 5 minutes 45 µl of 5% AlCl₃ were added and after 1 min, 60 µl of 1M NaOH and 45 µl of water were also added. Catechin was used as a reference standard. The absorbance was read at 510 nm using the microplate reader (BioTeck Synergy HT). Total flavonoid concentration (TFC) was expressed as mg of catechin equivalents (CAE) per gram of plant material.

4.3.5 Captation of superoxide anion radical

This assay was performed using a 96 well plate with different reagents: 19 mM phosphate buffer, pH 7.4, NADH (β-nicotinamide adenine dinucleotide, reduced dipotassium salt, Sigma N45005), NBT (nitrotetrazolium blue chloride, Sigma N6876), PMS (phenazine methosulfate, Sigma P9625). Quercetin, ascorbic acid and Tiron (4,5-Dihydroxy-1,3-benzenedisulfonic acid, Sigma 17, 255-3) were used as reference standards. The reaction mixture consisted in 50 µl of extracts at different dilution, 50 µl of NADH, 150 µl of NBT and 50 µl of PMS. A mixture of phosphate buffer, NADH, NBT, and PMS was used as control, while a mixture of phosphate buffer, NADH and NBT as blank. The absorbance was read at 560 nm for six minutes using the microplate reader (BioTeck Synergy HT).

4.3.6 Captation of hydrogen peroxide

This assay was performed on 96 well plates, using phosphate buffer 19 mM, pH 7.4, 50mM Tris-HCl, 800 µM lucigenin (N, N'-Dimethyl-9-9'-biacridium dinitrate-Sigma, M8010), and 30% hydrogen peroxide. Quercetin and ascorbic acid were used as reference standards. The reaction mixture consisted of 91.5 µl of Tris-HCl,

50 µl of extracts at different dilutions, 100 µl of lucigenin, and 8.5 µl of 30% hydrogen peroxide. A mixture of Tris-HCl, phosphate buffer, lucigenin and 30% hydrogen peroxide was used as control, while a mixture of Tris-HCl, phosphate buffer, and lucigenin was used as blank. The luminescence was read for 5 min using the microplate reader (BioTeck Synergy HT).

4.4 HPLC-MS

HPLC coupled with mass spectrometry analysis (HPLC-MS/MS) was performed using an Agilent 1100 HPLC-MSD Ion Trap XCT system, equipped with an electrospray ion source (HPLC-ESI-MS) (Agilent Technologies). Separation of PEE extract was performed on a Symmetry C18 column 1 × 150 mm with 3 µm particle size (Waters Corporation, Milford, MA, USA). Eluents used were water (eluent A) and MeOH (eluent B), both added with 0.1% formic acid. The gradient employed was: 5% eluent B for 5 min, linear to 40% eluent B in 35 min, then linear gradient to 95% in 15 min and finally hold at 95% eluent B for other 5 min. The flow rate was set to 30 µL/min and the column temperature was set at 30 °C (Fig.7).

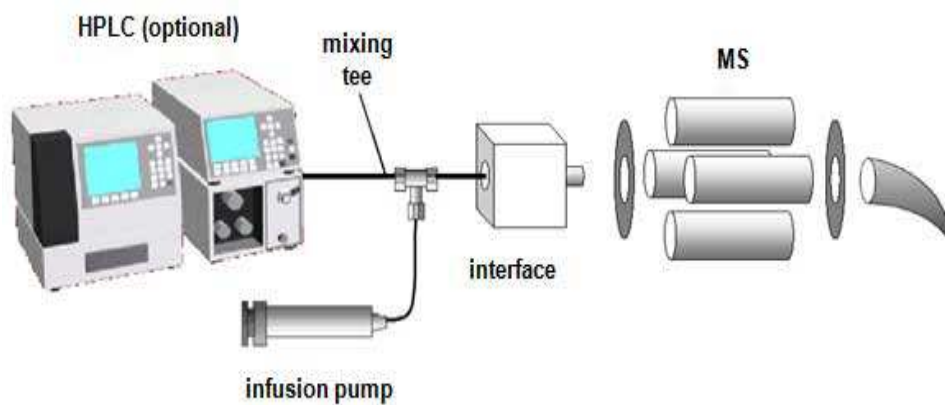


Fig.7 HPLC/MS pattern of the instrument

The injection volume was 8 µL. Ions were detected in the positive and negative ion mode, in the 200-1000 m/z range, and ion charged control with a target ion value of 200,000 and an accumulation time of 300 msec. A capillary voltage of 3300 V, nebulizer pressure of 15 psi, drying gas of 8 L/min, dry temperature of 325 °C and rolling averages 2 (averages: 5) were the parameters set for the MS detection. MS/MS analysis was conducted using amplitude optimized time by time for each compound. HPLC/MS analysis was conducted

only for *S. coronaria*, *L. capitata*, and *P. oceanica*, due to the abundance of data concerning *M. officinalis* and *G. glabra* present in the literature. The spectra resulting from all the acquired analyses were analysed qualitatively using the tools of the LC/MSD Trap software 5.3 (Fig.8).

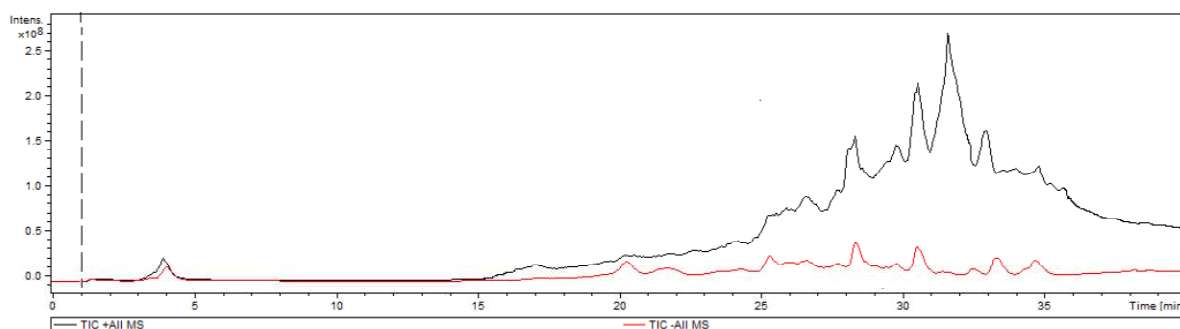


Fig.8 HPLC/MS Spectra of *S.coronaria* obtained with the LC/MSD Trap software

The identification was performed by taking bibliographic data as reference and comparing the molecular masses obtained from the extracts. Data present in online databases such as MassBank (High Quality Mass Spectral Database) were also used. Once the mass of the compounds was obtained, I tried to identify them based also on data reported in the literature (Dong et al., 2013; Brito et al., 2014). This has allowed to identify several characteristic compounds of *S. coronaria*.

4.5 Cell viability

The study of cell viability was performed on different kind of cells (e.g. Fig.9) using the MTT assay at the University of Genova, Italy, and the MTS assay at the University of Porto, Portugal. These methods are commonly used to quantify cell viability in multi-well plates, based on the measurement of a marker activity associated with viable cells (Sittampalam et al, 2004). A variety of tetrazolium compounds have been used to detect viable cells. The most commonly used include: MTT, MTS, XTT, and WST-1. MTT is positively charged and readily penetrates viable eukaryotic cells, while MTS, XTT, and WST-1 are negatively charged and do not readily penetrate cells. These latter are typically used with an intermediate electron acceptor that can transfer electrons from the cytoplasm or plasma membrane to facilitate the reduction of the tetrazolium into the colored formazan product.

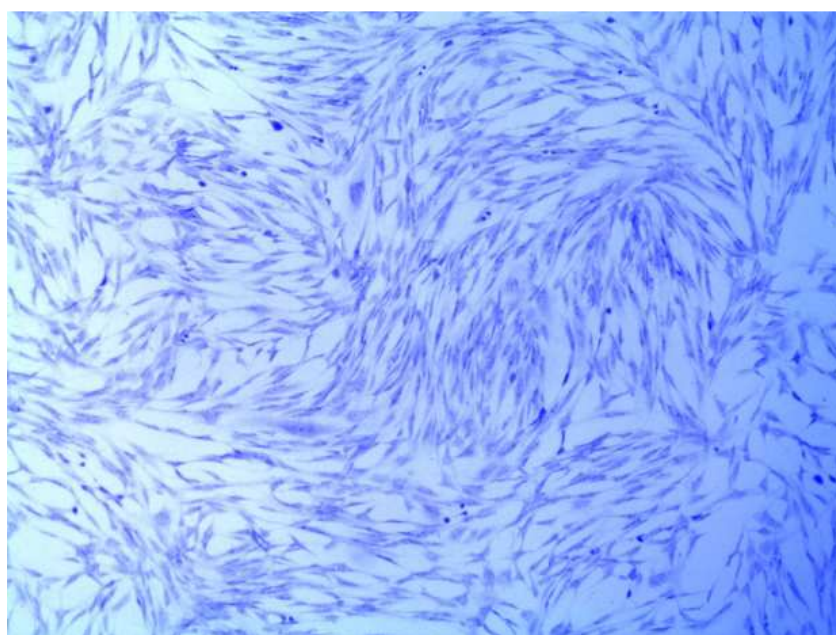


Fig.9 Stabilized human dermal fibroblasts SC587 colored with TBO (Leica M205C stereo microscope)

4.5.1 MTT assay

The study of cells viability was performed with (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolyl) assay on the cell lines HACAT (stabilized human dermal keratinocytes), 46BR1N (stabilized human dermal fibroblasts) , SC587 (stabilized human dermal fibroblasts), MEWO (stabilized melanoma cell line), MeCOP (stabilized melanoma cell line), REN (mesothelioma cell line), MCF7 (breast cancer), PC3 (prostate cancer), and adipocytes. The MTT test is a colorimetric assay used to measure the activity of enzymes that reduce MTT to formazan (Fig.10, 11).

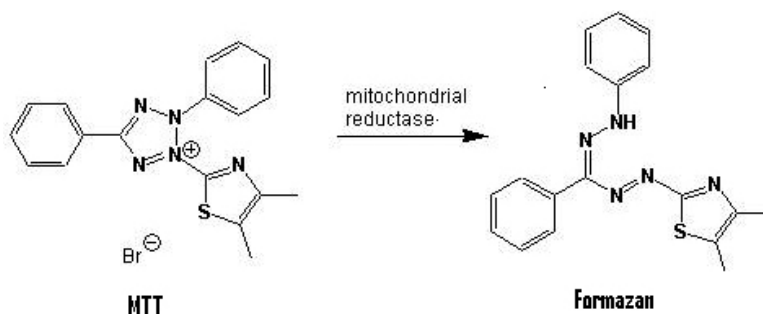


Fig.10 Reduction of MTT to formazan during the MTT assay

Cells were settled in 96-well plate (TPP, tissue culture testplate) at a density of 25×10^3 cells per mL culture medium for 24 h. Cells were incubated at 37 °C and exposed for 24 h or 48h to increasing concentrations of extracts dissolved in culture medium, ranging between 50µg/mL–1500 µg/mL. Thereafter, cells were washed with PBS and then the number of viable cells evaluated by adding the MTT reagent and incubating for 3 h at 37 °C. Control was determined by cells incubated with culture medium only. The absorbance was measured at 570 nm using a multi-plate reader (Molecular Devices V Max). The percentage of cell viability was computed by comparing the absorbance of the control (untreated cells) with that obtained from cells treated with

different concentrations of extract. The effect of extracts on cell viability was expressed as EC50.

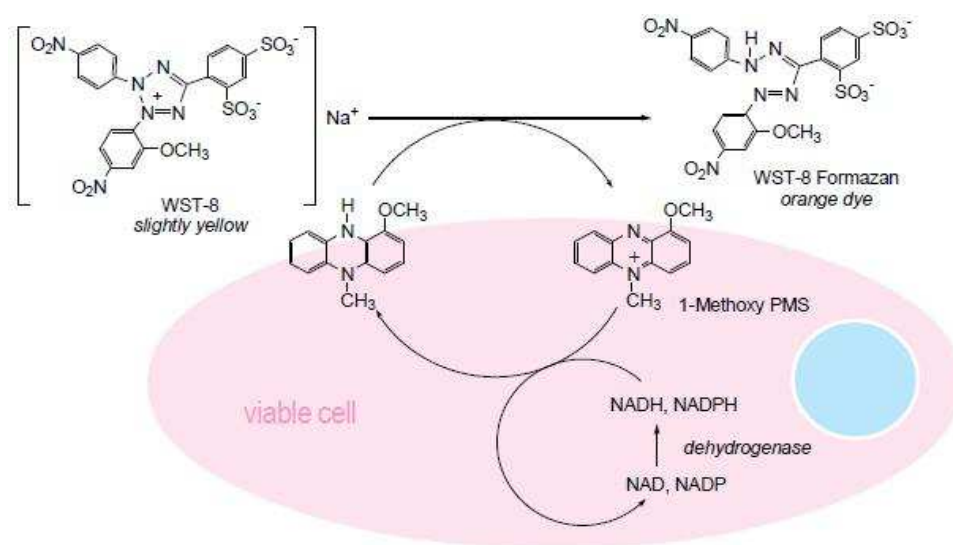


Fig.11 Mechanism of action of MTT assay

4.5.2 MTS assay

The study of cells viability was performed with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay on the Caco-2 and HT-29 cell lines. Cells were settled in 96-well plates and treated and incubated as above, except that MTS reagent was used and absorbance was measured at 490 nm with background subtraction at 630 nm in the microplate reader (BioTeck Synergy HT).

4.5.3 Proliferation assay

Cultured human fibroblasts and keratinocytes were treated with subtoxic doses of the extracts (0, 5, 10, 20 $\mu\text{g/ml}$) to evaluate effects on cell proliferation, by MTT assay. Cells were plated in 96-well plates at a density of 5,000 cells per well, and then exposed to different extract dilutions at 37 °C for a period of 3 and 10 days.

At the end of exposures tested with MTT, by using a multi-plate reader (Molecular Devices V Max), obtaining cell growth curves.

4.6 Enzymatic assay

Our enzymatic assays were conducted to test extract ability of modulating the activity of certain enzymes that play key roles in the skin. These tests included: collagenase, elastase, hyaluronidase and tyrosinase assays, and were conducted on 96-well plates.

4.6.1 Collagenase assay

The collagenase assay was conducted by following the protocol provided by Sigma-Aldrich with some modifications. Enzyme inhibition was evaluated at room temperature using FALGA (N-(3-[2-furyl] acryloyl)-Leu-Gly-Pro-Ala) as a substrate. The reaction mixture (final volume 225 μ l) was prepared by mixing 50 mM Tris pH 7.5, 10 mM CaCl_2 , 400 mM NaCl, 0.8 mM FALGPA (Sigma-Aldrich F5135), extracts at various final concentrations, and 0.16 U/mL collagenase from *Clostridium histolyticum* (Sigma-Aldrich C0130). After 10 min, plates were read at 345 nm in a Pro Tecan Genios plate reader (Tecan, Wien, Austria). Finally, the percentage of inhibitory activity was calculated.

4.6.2 Elastase assay

This enzyme assay was carried out at room temperature using N-succinyl-Ala-Ala-Ala-p-nitroanilide (Suc-Ala3-PNA) as a substrate. The reaction mixture (final volume 225 μ l) was prepared by mixing 200 mM Tris pH 8.0, 10 mM Suc-Ala3-pNA (Sigma-Aldrich S4760), extracts at various final concentrations as specified, and 2 U/mL elastase from pig pancreas (Sigma-Aldrich E1250). After 15 min, plates were read in the Tecan plate reader at 410 nm. Finally, the percentage of inhibition was calculated.

4.6.3 Hyaluronidase assay

The inhibition of type I hyaluronidase was evaluated using as hyaluronic acid substrate. A volume of 0.75 ml of enzyme solution containing about 5 units of hyaluronidase (Sigma-Aldrich H3506) in enzyme diluent (20 mM NaH₂PO₄, 77 mM NaCl, 0.01% BSA (w/v), pH 7.0, 37 ° C, was mixed with 0.25 mL of enzyme diluent containing extracts at various final concentrations. The reaction mixture and a blank were kept at 37 °C for 10 min, and then 1 ml of hyaluronic acid solution was additioned. The samples were then mixed and incubated at 37 °C for 45 min. Subsequently, 2.5 ml of acidic albumin solution (24 mM sodium acetate, 79 mM acetic acid, 0.1% BSA (w/v)), was added to 0.5 ml of each sample and blank at 25 °C. Thereafter, samples were kept at room temperature for 10 min, and then read at 600 nm in the Vmax Microplate Reader.

4.6.4 Tyrosinase assay

Aliquots of 10 µL of a solution composed of 125 U/mL mushroom tyrosinase (Sigma-Aldrich) in phosphate buffer (pH 6.8) were added to 96-well plates, followed by 70 µL of phosphate buffer and 60 µL of ultrapure water, or extract dissolved in ultrapure water, in order to obtain a series of final concentrations ranging between 5÷1000 µg/mL of extract. Kojic acid was used as positive control. Thereafter, 70 µL of 0.3 mg/mL L-tyrosine (Sigma-Aldrich) in ultrapure water were added. Blanks without enzyme were also included for all conditions. Plates were then incubated at 30 °C for 30 min and absorbance was read at 505 nm in the microplate reader. Percent inhibitory activity (IA%) was calculated according to the formula:

$$I\% = \left(1 - \frac{(A_{en/ex} - A_{ex})}{(A_{en} - A_{bk})} \right) \cdot 100$$

where $A_{(ex+en)}$ = absorbance of assay mixture with extract and enzyme; $A_{(ex)}$ = absorbance of assay mixture with extract and without enzyme; $A_{(en)}$ = absorbance of assay mixture with enzyme and without extract; $A_{(bk)}$ = absorbance of assay mixture without enzyme and without extract (blank).

4.7 Collagen production

The effect of extracts on the production of type I collagen on fibroblasts was evaluated by the ELISA technique. Cells were cultured in 96-well plates (15,000 cells/well), and incubated with 5, 10, or 20 µg/mL of extract for 48 h. After treatment, the medium was removed and cells were washed with PBS (100 µl/well), fixed with 3,7% paraformaldehyde for 10 min, washed 3 times with wash buffer (0.5 mM CaCl₂, 1 mM MgCl₂, 0.1% Triton in PBS, 100 µl/well), incubated with 3% BSA in wash buffer for 30 min, washed with wash buffer, incubated with the primary antibody (ab6308, Abcam, Cambridge, UK diluted 1: 300 in wash buffer containing 1% BSA, 50 µl/well) for 2 h under agitation, washed three times with wash buffer, incubated with secondary antibody (Ab97046, Abcam diluted 1: 1000 in wash buffer containing 1% BSA 50 µl/well) for 60 min under agitation and washed 3 times with wash buffer. The solution was carefully removed from the wells, the plates were incubated for 15 min with 50 µl/well TMB ELISA solution, and then blocked with 2 M sulfuric acid. All experimental phases occurred at room temperature and the reading was carried out at 620 nm in the VMax microplate reader.

4.8 Melanin assay

This assay was carried out on MeWo melanoma cells. Confluent cells were suspended and plated in 24-well plates (100,000 cells/well), allowed to settle for 24 h, and then exposed to PEE extract at a dose of 50 µg/mL for 72 h. Treatment with arbutin (1 mg/mL) was used as positive control. Thereafter, the culture medium was removed, cells were washed with PBS, trypsinized, centrifuged, and the pellet was subjected to freeze-thawing. The pellet was then dissolved in 100 µL of 1 N NaOH and read at 505 nm to determine the melanin content.

4.9 Cellular mobility

The analysis was conducted using the scratch wound assay on keratinocytes and fibroblasts. Cells were cultured on 24-well plates to confluence. Thereafter, a cut was made in the cell monolayer with a 0.1-10 μ L sterile pipette tip. After being cut, cells were washed in PBS and incubated with different concentrations of extracts at 37 °C for 24 to 48 h. Thereafter, cells were fixed with 3.7% paraformaldehyde for 10 min, washed in PBS, and stained with blue toluidine dye for 30 min. Cells were photographed using a stereomicroscope (Leica Microsystems, Wetzlar, Germany), and images were analyzed using the NIH Image J software (Fig.12).

4.10 Lipolysis assay

Lipolysis induction was evaluated in vitro on differentiated human adipocytes the ZenBio Human Adipocyte Lipolysis Assay Kit (ZenBio, cat # LIP-1-L1; LIP-1-NCL1) for the detection of free glycerol. Pre-adipocytes were grown in pre-adipocyte medium provided by the manufacturer, settled in 96-well plates, differentiated into adipocytes for one week in Adipocyte Differentiation Medium (cat# DM-2), and maintained for a further week in Adipocyte Medium (cat# AM-1). Fully differentiated adipocytes were incubated for 3 h with 10, 100, or 200 μ g/mL PEE extract, and samples of conditioned medium were then assayed for glycerol according to the manufacturer protocol. Samples were read in the microplate reader at 550 nm. Absorbance increase is proportional to glycerol concentration in the sample. Otherwise, lipid droplets in adipocytes were stained with Oil Red O following cell exposure to the extract. Cells were photographed under a microscope and reduction in lipid stain was quantified by image analysis, as a measure of lipid degradation (Fig. 13). In both analyses, 1 μ M isoproterenol was used as positive control. These tests were carried out at the DISTAV Laboratory, University of Genoa.

Fig.12 Scratch wound assay in fibroblasts colored with TBO: Control and 10 μ g/mL. Pictures take by Leica M205C stereo microscope

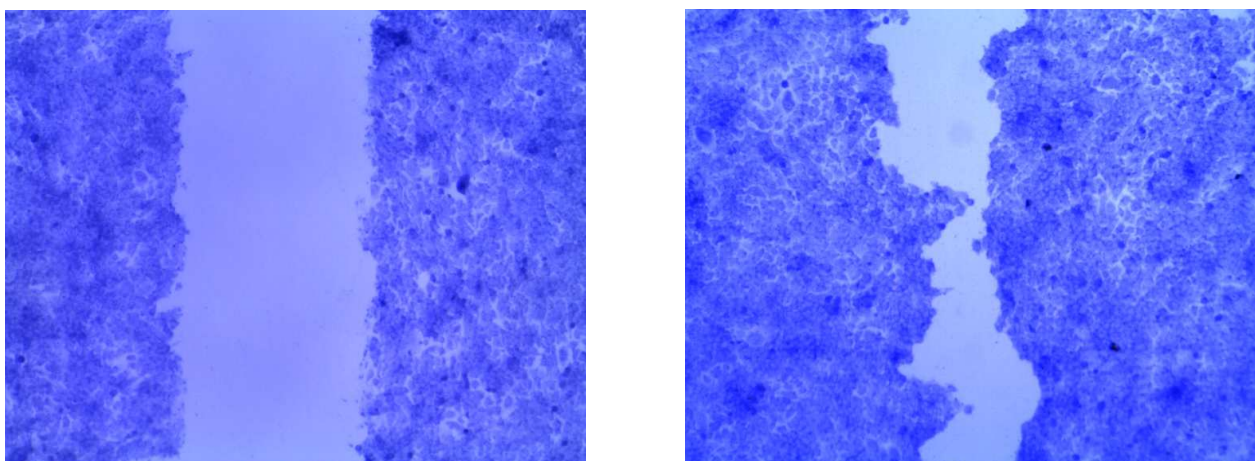
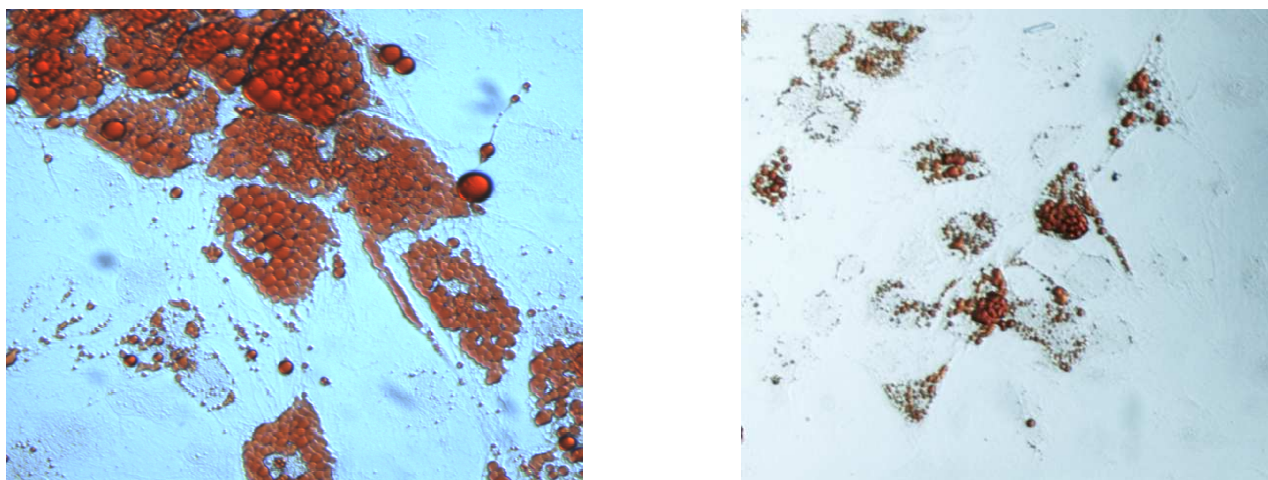


Fig.13 Lipolysis assay in adipocytes. Control and 1 μ M isoproterenol used as positive control. Pictures take by Leica M205C stereo microscope

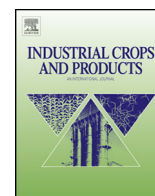


5. Results

Profiles of antioxidant activities of plant extracts have been obtained for *M. officinalis*, *L. capitata*, and *G. glabra* (Table 1), and also for *H. coronarium* and *P. oceanica*. HPLC/MS characterization has been conducted on *H. coronarium* and *P. oceanica*. Biological activities of extracts have been determined on *M. officinalis*, *L. capitata*, *H. coronarium*, and *P. oceanica*, using both cell-free and cell-based systems. The results of these analyses are reported in three studies, as follows.

Table 1 - DPPH• scavenging activity (DPPH• SA) , Ferric Reducing Antioxidant Potential assay (FRAP), Total Polyphenol Content (TPC) and Flavonoid assay of *Melilotus officinalis*, *Lespedeza capitata* and *Glycyrrhiza glabra* extracts. * Denote a significant difference between mean values, Repeated measures ANOVA followed by Turkey's post-test, n=3 independent experiments.

ASSAY	<i>M. officinalis</i>	<i>L.capitata</i>	<i>G.glabra</i>
DPPH• SA (%)	13.41 ± 2.50	18.14±2.93	16.05 ± 1.01
FRAP (μmol Fe ²⁺)	35.24 ± 2.06	97.01 ± 5.64*	59.29 ± 2.94*
Flavonoid (μg cathequin equivalent)	3.36 ± 0.36	11.61 ± 0.58*	5.81 ± 0.66*
TPC (mg gallic acid equivalent)	10.41 ± 0.67	19.62 ± 1.15*	20.33 1.03*



Biological activities of the legume crops *Melilotus officinalis* and *Lespedeza capitata* for skin care and pharmaceutical applications

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ABSTRACT

The search for natural principles is attracting much interest in the field of skin care. Fabaceae are major agricultural crops and potential sources of bioactive compounds with possible applications in human health and skin care. This study concerns the biological activities of the legume crops *Melilotus officinalis* (L.) Pall. and *Lespedeza capitata* Michx. for their potential use in skin care applications. The effects of plant ethanolic extracts at doses ranging from 0.25 to 50 µg/mL (from 1 to 5000 µg/mL in cell viability assays) were evaluated using in vitro tests on HaCaT human keratinocytes, 46BR.1N fibroblasts, and adipocyte cell cultures, and on matrix-degrading enzymes. MTT assay revealed weak effects on cell viability (IC₅₀ > 1000 µg/mL) and significant increase of fibroblast growth rate with both extracts. Similar induction of cell motility by the two extracts was observed on keratinocytes, while on fibroblasts *M. officinalis* induced a stronger effect with respect to *L. capitata*. Cell-free enzymatic assays showed stronger collagenase inhibition by *L. capitata*, while an ELISA assay revealed more efficient stimulation of fibroblast collagen production by *M. officinalis*. Oil-Red-O adipocyte staining showed more pronounced lipolytic effect of *M. officinalis* with respect to *L. capitata*. Both extracts showed the ability of stimulating skin cells in order to promote tissue regeneration, prevent skin aging, and reduce fat deposition. In most cases, different patterns of activation/inhibition were observed. Data indicate that these legume crops could be profitably exploited in skin care applications, possibly in combined formulations for the development of antiaging and anticellulite products.

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1. Introduction

The search for natural principles is attracting an ever-growing interest in the field of skin care. Plants combining ethnobotanical background, phytochemical pedigree, and large biomass availability are an obvious first choice in this kind of studies. In a screening of new possible active principles for the development of cosmetics, this study has been focused on legume fodder crops. Forage legumes, belonging to the family Fabaceae, are interesting agricultural crops due to their ability of fixing nitrogen by bacterial symbioses hosted in rhizobium root nodules (Frame et al., 1998). These plants are generally rich in secondary metabolites, such as

alkaloids and amines, cyanogenic glycosides, flavonoids, coumarins and other phenolics, condensed tannins, triterpenoid saponins, and lectin peptides. Hence, they are a potential source for the extraction of bioactive compounds with possible applications in human health and skin care (Cornara et al., 2016; Rodrigues et al., 2013). In this study, two major legume crops, viz. the yellow sweet clover, *Melilotus officinalis* (L.) Pall., and the roundhead lespedeza, *Lespedeza capitata* Michx have been considered.

The medicinal virtues of *M. officinalis* are well known, as testified by its inclusion in the EMA catalogue (European Medicines Agency) (www.ema.europa.eu/ema/pages/includes/document/open_document.jsp?webContentId=WC500059264). *L. capitata* has been less studied on the medicinal ground, but this species, together with *M. officinalis*, has been included in the BELFRIT list for plants used in food additives, originating from an agreement among the governments of Belgium, France and Italy. BELFRIT claims for *M. officinalis* address to blood circulation and tissue drainage, while *L. capitata*,

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is indicated for drainage, cardiovascular and kidney functions and lipid metabolism (<http://www.trovanorme.salute.gov.it/norme/renderNormsanPdf?anno=0&codLeg=48636&parte=2&serie=>).

M. officinalis is an herbaceous plant with a typical odorous smell for the presence of coumarins. It has been used traditionally as anti-inflammatory, antioedematous, phlebotonic, spasmolytic, diuretic and sedative (Burlando et al., 2010). The plant rose to prominence in 1930s for anticoagulant effects on cattle causing internal hemorrhage, which was shown later to depend on coumarin conversion into the anti-vitamin K dicoumarol by *Aspergillus* molds, due to bad hay storage. (Chae et al., 2003). Thereafter, dicoumarol led to the development of Warfarin, a widely used rodenticide and anticoagulant drug (Kresge et al., 2005).

Due to the high presence of coumarins and flavonoids, *M. officinalis* extracts have been tested experimentally and clinically for tissue drainage, specifically in the treatment of postoperative circulatory problems (Consoli, 2003; Xu et al., 2008), and problematic wounds (Bakhshayeshi et al., 2011). Besides coumarin, the plant produces other well-known bioactive compounds, including the coumarin derivatives scopoletin, umbelliferone and melilotin, the flavonoids kaempferol and quercetin, various phytosterols and triterpene sapogenins (Yang et al., 2014).

Properties useful for skin care have been ascribed to *M. officinalis* on empirical basis, including soothing, lenitive and possibly anti-inflammatory and antioedema effects (Dweck, 2011). The plant is listed among species popularly used in Russia and central Asia for skin conditions (Mamedov et al., 2005), while its clinical use in the treatment of diabetic foot ulcer has been reviewed (Chorepsima et al., 2013).

L. capitata is a perennial shrub native to eastern North America and used as forage for livestock. Records of folk medicine from native North Americans report the use of the root as an antidote to poisoning, and of the stems as moxibustion in the treatment of neuralgia and rheumatism (Glyzin et al., 1973; Linard et al., 1978). Flavonoids and tannins are abundant in the plant, and are presumed to account for its therapeutic virtues (Linard et al., 1978). In experimental and clinical studies, *L. capitata* was reported to exercise positive effects on tissue draining, kidney and cardiovascular diseases (Wagner and Elbl, 1992; Yarnell, 2002), and on diabetes (Jorge et al., 2004). The Asian congener *L. cuneata* (Dum. Cours.) G. Don has been studied for skin moisturizing properties and protection against photoaging (Kim et al., 2012; Lee et al., 2012). These data suggest that also *L. capitata* could exert positive effects on skin.

Despite a long tradition of use of these plants, their active principles, mechanisms of action, and specific effects on skin cells, are almost unknown. This study was therefore aimed to disclose biological activities of *M. officinalis* and *L. capitata* on skin cells and on extracellular matrix-degrading enzymatic activities, potentially useful for skin care application. To this purpose, commercially available, dry ethanolic extracts of the two species have been used. These extracts have been certified for use on humans as food and cosmetic grade (Farmalabor Srl, www.farmalabor.it). Ethanolic extracts represent a possible choice of manufacturers, especially for the production of eco-compatible and biocompatible cosmetics. Inhibitory activity on collagenase, elastase, and hyaluronidase were screened by cell-free enzymatic assays. Cell viability and induction of cell proliferation were assessed by MTT assay on human keratinocytes and fibroblasts, cell motility induction by scratch wound assay on keratinocytes, induction of collagen synthesis by ELISA technique on fibroblasts, and lipolytic activity by Oil Red O staining of adipocytes. Both species showed bioactivities potentially exploitable for the development of skin care and pharmaceutical applications, but with different patterns of skin cell activation.

2. Materials and methods

2.1. Materials

Culture media and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated. Food/cosmetic grade, ethanolic extracts of *M. officinalis* (CAS 84082-81-5) and *L. capitata* (CAS 84837-05-8), in the form of dry powder, were purchased from Farmalabor Srl (Canosa di Puglia, Italy). *M. officinalis* extract contains 1% coumarin glycosides, and undetermined kaempferol derivatives, quercetin derivatives, and sapogenins. *L. capitata* extract contains 4% flavonoids, and undetermined catechols and condensed tannins. For use in experiments, extracts were dissolved in cell culture medium or in proper buffer, as indicated.

2.2. Cell culture

Stabilized human epidermal keratinocyte (HaCaT) and dermal fibroblast (46BR.1N) cell lines were grown in DMEM, supplemented with 10% (v/v) FBS, 1% glutamine and 1% antibiotic, at 37 °C, in a 5% CO₂, fully humidified atmosphere.

2.3. Cell viability and proliferation assays

The effect of extracts on keratinocyte and fibroblast cell viability was determined by the MTT assay, based on the reduction of the tetrazolium dye MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, to its insoluble formazan by mitochondrial enzymes. Cells were settled in 96-well plates for 24 h, 10,000 cells per well, and then exposed for 48 h to increasing log concentrations of extracts dissolved in culture medium, ranging between 1 and 5000 µg/mL. Thereafter, cells were incubated for 3 h at 37 °C with a solution consisting of 100 µL MTT (5 mg/mL in PBS) per mL of cell culture medium without serum, and then treated with a mix of 1 N HCl–isopropanol (1:24, v/v), followed by stirring to dissolve the dark-blue formazan crystals formed. After a few minutes at RT, the plates were read at 570 nm in a VMax microplate reader (Molecular Devices, Sunnyvale, CA). Dose-response curves obtained by MTT data were analyzed by a logistic regression model, yielding IC₅₀ and IC₀₅ values that were assumed as median and threshold levels of cell viability inhibition, respectively.

Effects on fibroblast growth rates were assessed by settling cells in 96-well plates at 5000 cells per well, and then exposing them for up to 10 days to the indicated extract doses. At the end of exposures, the MTT assay was applied to evaluate cell densities, obtaining cell growth curves.

2.4. Cell motility

Stimulation of cell motility was evaluated by a scratch wound assay. Keratinocytes or fibroblasts were settled in 24-well plates and grown to confluence. Scratch wounds were created in confluent monolayers by using a sterile 0.1–10 µL pipette tip. After washing away suspended cells, cultures were incubated for 24 h with medium containing extracts at the specified concentrations. Thereafter, cells were fixed in 3.7% formaldehyde in PBS for 30 min, and then stained with 0.1% toluidine blue for 30 min. A series of samples were fixed and stained just after wounding for t = 0 measurements. Digitized pictures of wounds were taken using a stereomicroscope equipped with digital camera (Leica Microsystems, Wetzlar, Germany). Wound width was measured at wounding (t = 0) and at the end of treatments with the NIH Image J software. Wound closure rates were determined as the difference between wound width at 0 and 24 h.

2.5. Collagen production

The effect of extracts on collagen type I production by fibroblasts was evaluated by ELISA. Fibroblasts were settled in 96-well plates (15,000 cells/well) for 24 h, and then exposed to extracts as indicated. After treatments, the medium was removed and cells were washed with PBS (100 μ L/well), fixed with 3.7% paraformaldehyde for 10 min, and washed 3 times with washing buffer (0.5 mM CaCl_2 , 1 mM MgCl_2 , 0.1% Triton in PBS, 100 μ L/well). Cells were then incubated for 30 min with blocking buffer (3% bovine serum albumin (BSA) in washing buffer), washed, and incubated for 2 h under agitation with mouse anti-human collagen type I primary antibody (ab6308, Abcam, Cambridge, UK) diluted 1:300 in washing buffer containing 1% BSA (50 μ L/well). Thereafter, cells were 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 μ L/well), and washed 3 times with washing buffer. The washing solution was carefully removed from wells, plates were incubated for 15 min with 50 μ L/well of the Pierce 1Step™ Ultra TMB ELISA Substrate Solution (Thermo Fisher Scientific, Waltham, MA), blocked with 2 M sulfuric acid, and read at 620 nm in the VMax microplate reader. All steps were carried out at RT.

2.6. Enzymatic assays

Enzymatic assays were conducted in 96-well plates. Collagenase (EC 3.4.24.3) inhibition was evaluated at RT using *N*-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA) as the substrate (<http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-collagenase-using-n-3-2furylacryloyl-leu-gly-pro-ala.html>). The reaction mixture (final volume 225 μ L) was prepared by mixing 50 mM tricine, pH 7.5, 10 mM CaCl_2 , 400 mM NaCl, 0.8 mM FALGPA (Sigma-Aldrich F5135), extracts at various final concentrations, as specified, and 0.16 u/mL collagenase from *Clostridium histolyticum* (Sigma-Aldrich C0130). After 10 min, plates were read at 345 nm in a Tecan Genios Pro plate reader (Tecan, Wien, Austria). Percent inhibitory activity (I%) was calculated according to the formula:

$$I\% = \left(1 - \frac{(A_{\text{en/ex}} - A_{\text{ex}})}{(A_{\text{en}} - A_{\text{bk}})} \right) \cdot 100$$

where: $A_{\text{en/ex}}$ = absorbance of assay mixture with enzyme and extract; A_{ex} = absorbance of assay mixture with extract and without enzyme; A_{en} = absorbance of assay mixture with enzyme and without extract; A_{bk} = absorbance of assay mixture without enzyme and extract (blank).

Enzymatic assay of elastase (EC 3.4.21.36) was done at RT using *N*-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (Suc-Ala₃-pNA) as the substrate (<http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-elastase.html>). The reaction mixture (final volume 225 μ L) was prepared by mixing 200 mM TRIS, pH 8.0, 10 mM Suc-Ala₃-pNA (Sigma-Aldrich S4760), extracts at various final concentrations, as specified, and 2 u/mL of elastase from porcine pancreas (Sigma-Aldrich E1250). After 15 min, plates were read in the Tecan plate reader at 410 nm. Percent inhibitory activity was calculated as above.

Hyaluronidase (EC 3.2.1.35) inhibition was tested in vitro on hyaluronidase type I from bovine testes using bovine hyaluronic acid as the substrate (<http://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-hyaluronidase.html>). A volume of 0.75 mL of enzyme solution, containing about 5 units of hyaluronidase (Sigma-Aldrich H3506) in enzyme diluent (20 mM NaH_2PO_4 , 77 mM NaCl, 0.01% BSA (w/v), pH 7.0 at 37 °C), was mixed with 0.25 mL of enzyme diluent containing extracts at

various final concentrations, as specified. A blank containing 1 mL of enzyme diluent only was also prepared. The reaction mixture and blank were equilibrated at 37 °C for 10 min, supplemented with 1 mL hyaluronic acid solution, consisting of 0.015% (w/v) bovine hyaluronic acid sodium salt (Sigma-Aldrich H7630) in phosphate buffer (300 mM NaH_2PO_4 , pH 5.35 at 37 °C), mixed by swirling and incubated at 37 °C for 45 min. Thereafter, 0.5 mL of each test sample and blank were vigorously mixed with 2.5 mL of acidic albumin solution (24 mM sodium acetate, 79 mM acetic acid, 0.1% BSA (w/v), pH 3.75 at 25 °C), allowed to stand for 10 min at RT, and read at 600 nm in the VMax microplate reader. By using values of transmittance as a measure of hyaluronidase activity, percent hyaluronidase inhibition by extracts was calculated according to the formula:

$$I\% = \left(1 - \frac{(T_{\text{en/ex}} - T_{\text{ex}})}{(T_{\text{en}} - T_{\text{bk}})} \right) \cdot 100$$

where: $T_{\text{en/ex}}$ = transmittance of assay mixture with enzyme and extract; T_{ex} = transmittance of assay mixture with extract and without enzyme; T_{en} = transmittance of assay mixture with enzyme and without extract; T_{bk} = transmittance of assay mixture without enzyme and extract (blank).

2.7. Lipolysis assay

Lipolytic activity of extracts was assessed by Oil red O staining of human adipocytes cultured in vitro (Zen-Bio Inc., Research Triangle Park, NC). Following the manufacturer's protocol, pre-adipocytes were grown in Preadipocyte Medium (cat# PM-1, Zen-Bio Inc.), settled in 96-well plates, differentiated into adipocytes for one week in Adipocyte Differentiation Medium (cat# DM-2), and maintained for a further week in Adipocyte Medium (cat# AM-1). Thereafter, fully differentiated adipocytes were incubated for 3 h with extracts in Adipocyte Medium. After incubation, cells were washed thrice in PBS, fixed with FineFix® working solution (Milestone Srl, Sorisole, Italy) for 20 min, washed once in deionized water and then twice in PBS, and stained with Oil red O working solution for 20 min. Oil Red O working solution was prepared by adding 3 parts of Oil Red O stock solution (3 mg/mL in isopropanol) to 2 parts of deionized water, and then filtering with a 0.2 μ m syringe filter. After staining, cells were washed thrice with PBS, and then photographed under an Olympus IX71 inverted microscope. By using the NIH ImageJ program (imagej.nih.gov/ij/), picture areas covered by stained lipid droplets were selected in fields of fixed size with threshold function and measured.

2.8. Statistics

Data were analyzed with the R package, version 3.0.1 (<http://www.r-project.org/foundation/>), by using Dunnett's post-hoc test for multiple comparisons. The difference between two conditions was considered significant if $p < 0.05$. Cell viability data were analyzed using a logistic dose–response curve as reported in Ranzato et al. (2014).

3. Results

3.1. Effects of extracts on cell viability and proliferation

The effects on cell viability of *M. officinalis* and *L. capitata* ethanolic extracts were determined by the MTT assay on keratinocytes and fibroblasts, after 48 h incubations with increasing extract concentrations up to 5000 μ g/mL. Dose-response data yielded in all cases IC_{50} values greater than 1000 μ g/mL, showing very low cell viability inhibition for these extracts.

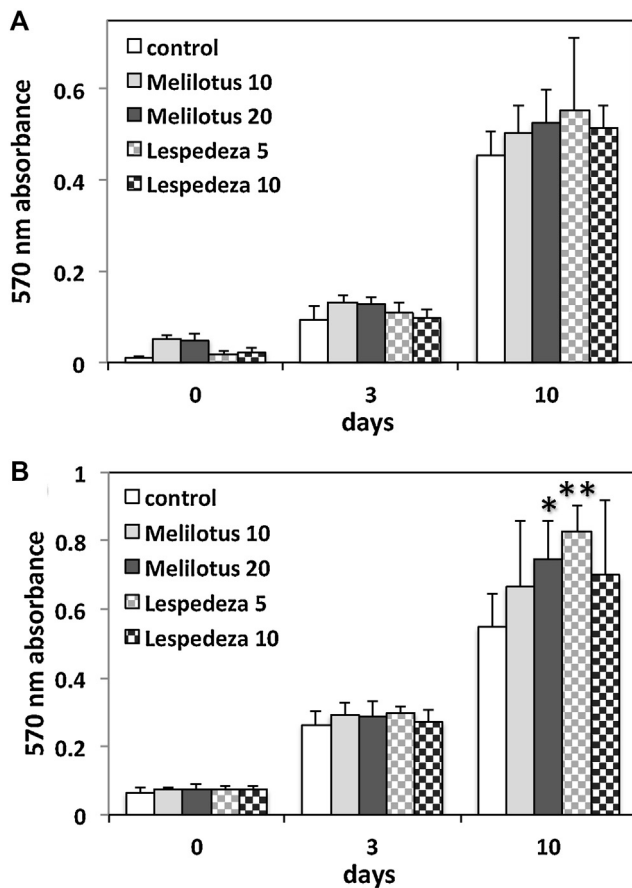


Fig. 1. Cell growth data derived from MTT cell viability assay conducted at different endpoints on HaCaT keratinocytes (A) and 46BR.1N fibroblasts (B), maintained under control conditions or under continuous incubation with 10 or 20 $\mu\text{g/mL}$ *Melilotus officinalis*, or with 5 or 10 $\mu\text{g/mL}$ *Lespedeza capitata*. Data are mean absorbances $\pm$ s.d. of 570 nm readings from 12 replicate wells in two independent experiments. Asterisks indicate the results of statistical comparisons against control: * = $p < 0.05$; ** = $p < 0.01$.

Keratinocytes and fibroblasts were also treated with subtoxic doses of extracts for 10-days to evaluate effects on cell proliferation. In order to individuate minimum effective doses, preliminary tests were carried out, revealing that in long-term treatments the *L. capitata* extract affects cell viability starting from a concentration of 20 $\mu\text{g/mL}$. Hence, in these experiments doses of 5 and 10 $\mu\text{g/mL}$ for *L. capitata*, and of 10 and 20 $\mu\text{g/mL}$ for *M. officinalis* have been used. Use of MTT assay at different endpoints showed a slight stimulation of keratinocyte proliferation with both extracts after 10 days of treatment (Fig. 1A). Conversely, on fibroblasts there was a highly significant increase ($p < 0.01$) of growth rate with *L. capitata* used at 5 $\mu\text{g/mL}$, and a significant increase ($p < 0.05$) with *M. officinalis* at 20 $\mu\text{g/mL}$ (Fig. 1B).

3.2. Cell motility

Cell motility was evaluated by a scratch wound assay, a basic in vitro test for wound healing potential. The assay conducted on keratinocytes, specifically a test of re-epithelialization, showed a significant induction of cell motility. Up to nearly a 600% increase of wound closure rate was observed for both extracts, with a similar trend in the range 10–50 $\mu\text{g/mL}$ (Fig. 2A). The effect of extracts on fibroblasts was significant but weaker than on keratinocytes, while the effect of *M. officinalis* was stronger than that of *L. capitata*. *M. officinalis* induced about a 200% increase of wound closure rate in the range 10–50 $\mu\text{g/mL}$, while *L. capitata* induced a signifi-

cant increase of cell motility only at the highest dose of 50 $\mu\text{g/mL}$ (Fig. 2B).

3.3. Enzyme inhibition

Cell-free assays of matrix-degrading enzyme activities showed no recordable effect of both extracts for hyaluronidase and elastase. By contrast, significant inhibition was observed on collagenase activity, with a significantly stronger effect of *L. capitata* with respect to *M. officinalis*. The inhibitory effect of *M. officinalis* showed a dose-dependent trend in the range 0.25–25 $\mu\text{g/mL}$, up to about 10% inhibition, whereas *L. capitata* showed a maximum average 30% inhibition at 2.5 $\mu\text{g/mL}$ (Fig. 3). The flavan-3-ol derivative epigallocatechin-3-gallate, used as positive control, induced a 35% average inhibition at a dose of 10 $\mu\text{g/mL}$.

3.4. Collagen production

The effect of extracts on the production of collagen type I was evaluated on fibroblasts by an ELISA assay. Both extracts induced a dose-dependent stimulation of collagen production by cells, but only exposure to 50 $\mu\text{g/mL}$ *M. officinalis* resulted in a significant increase of collagen production with respect to control (Fig. 4).

3.5. Lipolysis

The induction of lipolysis was evaluated on in vitro differentiated human adipocytes by staining cell lipid droplets with Oil Red O after cell exposure to extracts (Fig. 5A). The reduction of cell staining, quantified by image analysis, was taken as a measure of lipid degradation. Data showed dose-dependent lipolysis induced by both extracts, with a stronger effect of *M. officinalis* with respect to *L. capitata*. Significant reduction of cell lipid droplets was observed with doses of 20 and 50 $\mu\text{g/mL}$ *M. officinalis*, whereas the effect of *L. capitata* was significant only at the dose of 50 $\mu\text{g/mL}$ (Fig. 5B). The maximum lipolytic effects induced by extracts were comparable to those obtained with 1 μM isoproterenol, used as positive control.

4. Discussion

The experiments carried out in this study show that both *M. officinalis* and *L. capitata* extracts possess bioactivities potentially exploitable for skin care applications. The richness in flavonoids of both extracts could account for the observation of parallel trends in biological responses. However, in most cases different patterns of activation/inhibition have been verified, most likely depending on differences in the phytocomplexes of the two plants. *M. officinalis* extract contains glycosides of coumarin and of the common flavonoids kaempferol and quercetin, and in addition sapogenins. *L. capitata* extract is mainly composed of unspecified flavonoids, possibly including kaempferol and quercetin, and in addition catechols and condensed tannins.

In our experiments both extracts were found to stimulate fibroblast growth, as revealed by MTT measurements. This effect may be linked to similar observations with *Ginkgo biloba*, which revealed that various flavonoids, including kaempferol and quercetin, induce proliferative effects on human skin fibroblasts (Kim et al., 1997).

Stimulation of cell motility is important for regenerative processes such as epidermis re-epithelialization and dermal tissue reconstitution and turnover. Our experiments demonstrated that these processes can be strongly stimulated by *M. officinalis*, and *L. capitata* extracts, as shown by comparison with similar experiments in a study on Brazilian plants used as traditional wound healing agents (Schmidt et al., 2009). Also in this case, flavonoids could play a significant role, given that this class of compounds is well known for its wound healing properties (Ambiga et al., 2007;

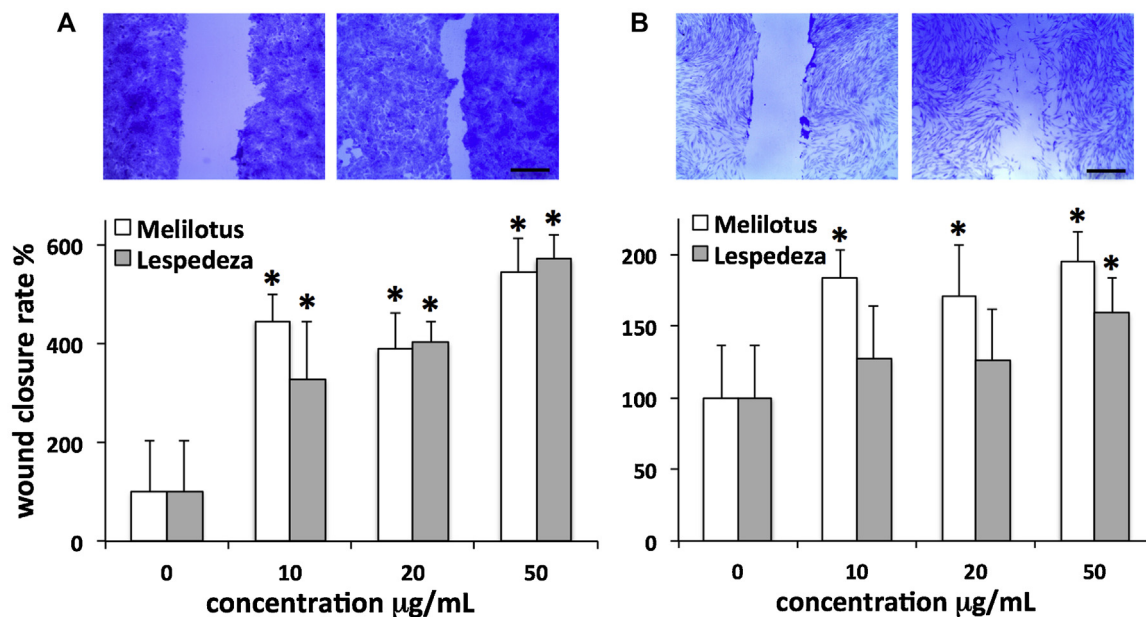


Fig. 2. Scratch wound assays conducted on keratinocytes (A) and fibroblasts (B) along 24 h incubation with different concentrations of *Lespedeza* or *Melilotus* extracts. Upper panels: representative light micrographs of cell layers stained with blue toluidine 24 h after wounding. Each couple of pictures shows the results of control (left) and 10 µg/mL *Melilotus* (right) incubations. Bar 200 µm. Lower panels: mean $\pm$ s.d. wound closure rates from 6 replicates in two different experiments, expressed as differences between wound width at 0 and 24 h after wounding. * = $p < 0.01$ with respect to control.

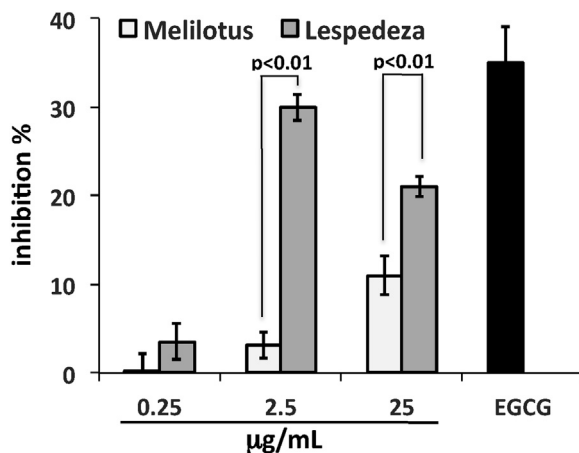


Fig. 3. Inhibition of *Clostridium histolyticum* collagenase activity by *Lespedeza* and *Melilotus* extracts, determined in a cell-free, in vitro assay using FALGPA as a substrate. Data are expressed as mean $\pm$ s.e.m. percent inhibition ($n=4$) exerted by extracts and 10 µg/mL epigallocatechin-3-gallate (EGCG). See Section 2.6 for further details.

Clericuzio et al., 2012). However, the stronger activity of the *M. officinalis* extract, detected on fibroblasts, could be related to the presence of saponins. This is suggested by previous findings showing that the saponin astragaloside IV significantly promotes cell migration in a scratch wound assay (Chen et al., 2012).

Preservation of matrix homeostasis depends largely on degradation/biosynthesis balance, which tends to be biased by environmental stress factors or aging. Positive effects of our extracts could derive from two kinds of action. On one side, enzyme assays showed inhibitory effects of extracts on collagenase. These data are quantitatively comparable with similar results obtained with grape extracts (Bharti et al., 2013), and moreover are in line with reported collagenase inhibition by flavonols such as quercetin and kaempferol (Lim and Kim, 2007). On the other side, a dose-dependent induction of type I collagen production by fibroblasts was also observed. In particular, the effect of

50 µg/mL *M. officinalis* exceeds what reported in similar experiments for a traditional Korean herbal formula (Kim et al., 2016). Screenings of flavonoids able to induce collagen biosynthesis in human fibroblasts have revealed stimulatory properties, neutral effects, or even detrimental activity. However, the list of flavonoids that activate collagen production by human fibroblasts is conspicuous, including the commercially available flavonols morin and rutin, and the flavone chrysin (Stipevic et al., 2006), the flavones 6-hydroxyluteolin-7-O-glucoside, pedalitin, apigenin-7-O-glucuronide, apigenin-7-O-methylglucuronide, and pectolinarin isolated from Asteraceae plants (Galicka and Nazaruk, 2007; Nazaruk and Galicka, 2014), and the flavonol kaempferol-3-O-sophoroside isolated from *Sambucus sieboldiana* (Yim et al., 2015).

Lipolytic properties are attracting much interest in the field of skin care. In our study, the induction of lipolysis by both extracts could reveal again a role of flavonoids. In support to this view, lipolytic activities have been reported for the common flavonol

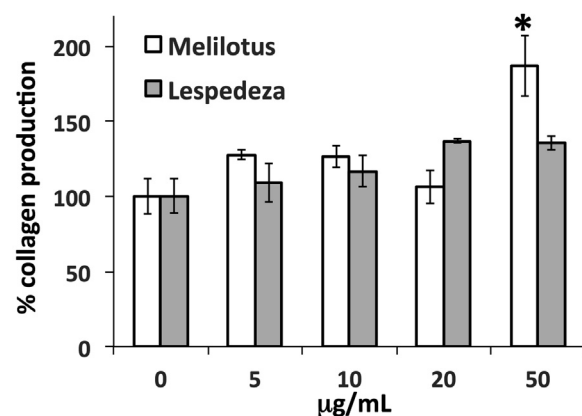


Fig. 4. Induction of fibroblast collagen production determined by an ELISA assay after 48 h incubations with increasing concentrations of *Lespedeza* and *Melilotus* extracts. Data are mean absorbances $\pm$ s.e.m. of 620 nm readings from 6 replicate wells in two independent experiments. * = $p < 0.01$ with respect to control.

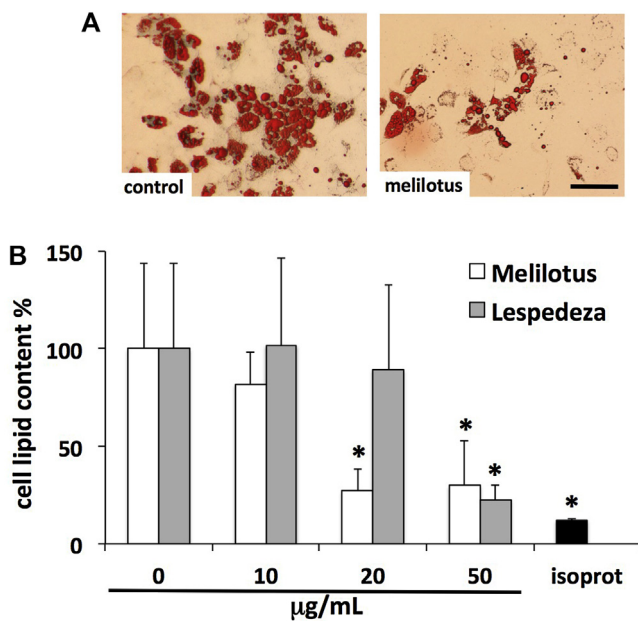


Fig. 5. Induction of lipolysis in human adipocytes determined through Oil Red O staining after 3 h incubations with increasing concentrations of *Lespedeza* and *Melilotus* extracts, or with 1 µM isoproterenol (isoprot) as positive control. Upper panel: representative light micrographs of adipocytes showing lipid droplets stained in red, after incubation under control conditions (left), or with 20 µg/mL *Melilotus* extract (right). Bar 100 µm. Lower panel: data of cell lipid content expressed as mean ± s.d. measurements of areas covered by stained lipid droplets in $n = 4$ fields of fixed size. * = $p < 0.01$ with respect to control. See Section 2.7 for further details.

myricetin (Wang et al., 2015), and for flavonoids from *Nelumbo nucifera*, including the flavonol derivatives quercetin-3- O - α -arabinopyranosyl-(1 $\rightarrow$ 2)- β -galactopyranoside, isoquercitrin, and astragalin, and the flavan-3-ol catechin and its derivative hyperoside (Ohkoshi et al., 2007). However, the stronger induction of lipolysis by *M. officinalis* at intermediate doses suggests a supplemental role for coumarins. Consistent with this hypothesis, the coumarin aculeatin, derived from *Toddalia asiatica*, has been shown to induce adipocyte differentiation and lipolysis in 3T3-L1 preadipocytes (Watanabe et al., 2014).

5. Conclusion

The complex of data suggests that both extracts possess the ability to stimulate skin cells and tissue regeneration, to prevent skin aging, and to reduce fat deposition beneath the skin, frequently leading to cellulite. It is possible to speculate that the mechanisms of action for these effects could be related to the presence in both extracts of flavonoids, which have been previously reported to mediate similar effects. On the other hand, differences in the strength of effects were also observed, possibly depending on the presence of specific constituents, such as coumarin and saponins in *M. officinalis*.

The doses at which the effects were observed ranged from 5 to 50 µg/mL. Assuming that the active principles should have an approximate molecular weight around 300–600, the above doses correspond roughly to 1–100 µM for single major compounds, values that are within the range of active concentrations reported in literature. Moreover, these doses are compatible with pharmacological uses, and therefore, both extracts could be profitably exploited in various kinds of skin care and pharmaceutical applications, possibly in combined formulations, e.g. for the development of antiage and anticellulite products.

Conflict of interest

The authors report no conflict of interest.

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References

- Ambiga, S., Narayanan, R., Gowri, D., Sukumar, D., Madhavan, S., 2007. Evaluation of wound healing activity of flavonoids from *Ipomoea carnea* Jacq. *Anc. Sci. Life* 26, 45–51.
- Bakhshayeshi, S., Madani, S., Hemmatbadi, M., Heshmat, R., Larijani, B., 2011. Effects of Semelil (ANGIPARS) on diabetic peripheral neuropathy: a randomized, double-blind Placebo-controlled clinical trial. *Daru* 19, 65–70.
- Bharti, P., Seasotiya, L., Bai, S., Malik, A., Dalal, S., 2013. Antibacterial, antioxidant, collagenase inhibition, urease inhibition activities and chemical composition of the essential oil and extracts of *Cinnamomum zeylanicum* bark. *Int. J. Phytomed.* 5, 36–45.
- Burlando, B., Verotta, L., Cornara, L., Bottini-Massa, E., 2010. *Herbal Principles in Cosmetics. Properties and Mechanisms of Action*. CRC Press, Taylor & Francis Group, Boca Raton.
- Chae, H.N., Cho, M.G., Kim, D.J., Koo, B.Y., Lee, J.B., 2003. A cosmetic composition containing a mixture extract of *Melilotus officinalis* and *Trichosanthes kirilowii* Maxim is provided with anti-inflammatory, moisturizing and abirritant effects. *Pat n* KR1020030032377.
- Chen, X., Peng, L.H., Li, N., Li, Q.M., Li, P., Fung, K.P., Leung, P.C., Gao, J.Q., 2012. The healing and anti-scar effects of astragaloside IV on the wound repair in vitro and in vivo. *J. Ethnopharmacol.* 139, 721–727.
- Chorepsima, S., Tentolouris, K., Dimitroulis, D., Tentolouris, N., 2013. *Melilotus*: contribution to wound healing in the diabetic foot. *J. Herb. Med.* 3, 81–86.
- Clericuzio, M., Tinello, S., Burlando, B., Ranzato, E., Martinotti, S., Cornara, L., La Rocca, A., 2012. Flavonoid oligoglycosides from *Ophioglossum vulgatum* L. having wound healing properties. *Planta Med.* 78, 1639–1644.
- Consoli, A., 2003. Chronic venous insufficiency: an open trial of FLEBS Crema. *Minerva Cardioangiol.* 51, 411–416.
- Cornara, L., Xiao, J., Burlando, B., 2016. Therapeutic potential of temperate forage legumes: a review. *Crit. Rev. Food Sci. Nutr.* 56 (Suppl. 1), S149–S161.
- Dweck, A.C., 2011. *Formulating Natural Cosmetics*. Allured Books, Carol Stream, IL.
- Frame, J., Charlton, J.F.L., Laidlaw, A.S., 1998. *Temperate Forage Legumes*. CAB International, Wallingford, UK.
- Galicka, A., Nazaruk, J., 2007. Stimulation of collagen biosynthesis by flavonoid glycosides in skin fibroblasts of osteogenesis imperfecta type I and the potential mechanism of their action. *Int. J. Mol. Med.* 20, 889–895.
- Glyzin, V.I., Bankovskii, A.I., Zhurba, O.V., Sheichenko, V.I., 1973. Flavonoids of *Lespedeza bicolor*. *Chem. Nat. Compd.* 6, 487–488.
- Jorge, A.P., Horst, H., de Sousa, E., Pizzolatti, M.G., Silva, F.R., 2004. Insulinomimetic effects of kaempferitrin on glycaemia and on 14C-glucose uptake in rat soleus muscle. *Chem. Biol. Interact.* 149, 89–96.
- Kim, S.J., Lim, M.H., Chun, I.K., Won, Y.H., 1997. Effects of flavonoids of *Ginkgo biloba* on proliferation of human skin fibroblast. *Skin Pharmacol.* 10, 200–205.
- Kim, H.J., Kim, K.S., Kim, D.I., 2012. Inhibitory effects of *Lespedeza cuneata* ethanol extract on ultraviolet-induced photo aging. *J. Korean Soc. Food Sci. Nutr.* 41, 1540–1545.
- Kim, M.K., Bang, C.Y., Yun, G.J., Kim, H.Y., Jang, Y.P., Choung, S.Y., 2016. Anti-wrinkle effects of Seungma-Galgeun-Tang as evidenced by the inhibition of matrix metalloproteinase-1 production and the promotion of type-I procollagen synthesis. *BMC Complement. Altern. Med.* 16, 116.
- Kresge, N., Simoni, R.D., Hill, R.L., 2005. Hemorrhagic sweet clover disease, dicumarol, and Warfarin: the work of Karl Paul Link. *J. Biol. Chem.* 280, e5.
- Lee, H.J., Gu, H.A., Park, S.N., 2012. A determination of the moisturizing effect and stability of a cream containing *Lespedeza cuneata* G. Don extracts. *Korean J. Microbiol. Biotechnol.* 40, 237–242.
- Lim, H., Kim, H.P., 2007. Inhibition of mammalian collagenase, matrix metalloproteinase-1, by naturally-occurring flavonoids. *Planta Med.* 73, 1267–1274.
- Linard, A., Delaveau, P., Paris, R.R., 1978. Sur les flavonoides du *Lespedeza capitata* Michaux. *Plantes medicinales et phytothérapie* 12, 144–147.
- Mamedov, N., Gardner, Z., Craker, L.E., 2005. Medicinal plants used in Russia and central Asia for the treatment of selected skin conditions. *J. Herbs Spices Med. Plants* 11, 191–222.
- Nazaruk, J., Galicka, A., 2014. The influence of selected flavonoids from the leaves of *Cirsium palustre* (L.) Scop. on collagen expression in human skin fibroblasts. *Phytother. Res.* 28, 1399–1405.
- Ohkoshi, E., Miyazaki, H., Shindo, K., Watanabe, H., Yoshida, A., Yajima, H., 2007. Constituents from the leaves of *Nelumbo nucifera* stimulate lipolysis in the white adipose tissue of mice. *Planta Med.* 73, 1255–1259.
- Ranzato, E., Magnelli, V., Martinotti, S., Waheed, Z., Cain, S.M., Snutch, T.P., Marchetti, C., Burlando, B., 2014. Epigallocatechin-3-gallate elicits Ca²⁺ spike

- in MCF-7 breast cancer cells: essential role of Cav3.2 channels. *Cell Calcium* 56, 285–295.
- Rodrigues, F., Palmeira-de-Oliveira, A., das Neves, J., Sarmiento, B., Amaral, M.H., Oliveira, M.B., 2013. *Medicago* spp. extracts as promising ingredients for skin care products. *Ind. Crops Prod.* 49, 634–644.
- Schmidt, C., Fronza, M., Goettert, M., Geller, F., Luik, S., Flores, E.M., Bittencourt, C.F., Zanetti, G.D., Heinzmann, B.M., Laufer, S., Merfort, I., 2009. Biological studies on Brazilian plants used in wound healing. *J. Ethnopharmacol.* 122, 523–532.
- Stipcevic, T., Piljac, J., Vanden Berghe, D., 2006. Effect of different flavonoids on collagen synthesis in human fibroblasts. *Plant Foods Hum. Nutr.* 61, 29–34.
- Wagner, H., Eibl, G., 1992. ACE-inhibitory procyanidins from *Lespedeza capitata*. *Planta Med.* 58, 297.
- Wang, Q., Wang, S.T., Yang, X., You, P.P., Zhang, W., 2015. Myricetin suppresses differentiation of 3 T3-L1 preadipocytes and enhances lipolysis in adipocytes. *Nutr. Res.* 35, 317–327.
- Watanabe, A., Kato, T., Ito, Y., Yoshida, I., Harada, T., Mishima, T., Fujita, K., Watai, M., Nakagawa, K., Miyazawa, T., 2014. Aculeatin, a coumarin derived from *Toddalia asiatica* (L.) Lam., enhances differentiation and lipolysis of 3T3-L1 adipocytes. *Biochem. Biophys. Res. Commun.* 453, 787–792.
- Xu, F., Zeng, W., Mao, X., Fan, G.K., 2008. The efficacy of melilotus extract in the management of postoperative ecchymosis and edema after simultaneous rhinoplasty and blepharoplasty. *Aesthetic Plast. Surg.* 32, 599–603.
- Yang, J., Wang, L.-L., Zhou, X.-T., Yuan, J.-Z., Zhang, T.-J., Liu, F., 2014. Chemical constituents from *Melilotus officinalis*. *Chin. Tradit. Herb. Drugs* 45, 622–625.
- Yarnell, E., 2002. Botanical medicines for the urinary tract. *World J. Urol.* 20, 285–293.
- Yim, J.H., Jang, M.S., Moon, M.Y., Lee, H.Y., Kim, S.C., Lee, N.H., 2015. Constituents from the branches of *Sambucus sieboldiana* var. *pendula* with the properties of collagen synthesis activation. *J. Appl. Pharm. Sci.* 5, 119–122.

The bioactivity of Hedysarum coronarium extracts on skin enzymes and cells correlates with phenolic content

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ORIGINAL ARTICLE



The bioactivity of *Hedysarum coronarium* extracts on skin enzymes and cells correlates with phenolic content

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ABSTRACT

Context: The search for bioactive compounds from botanical sources is attracting much interest. However, differences in chemical composition may occur within the same species depending on different geographical origins.

Objectives: We evaluated the properties on skin enzymes and cells of extracts from sulla legume crop *Hedysarum coronarium* L. (Fabaceae), collected at two Italian sites near Pisa and Ventimiglia, for possible dermatological and cosmetic applications.

Material and methods: Plant aerial portions were extracted in MTBE/ethyl acetate/acetone, obtaining two extracts named Pisa sulla extract (PSE) and Ventimiglia sulla extract (VSE). Extracts were subjected to chemical characterization, LC-MS/MS analysis and biological assays.

Results: PSE showed stronger antiradical scavenging and higher phenolic and flavonoid contents with respect to VSE. LC-MS/MS analysis revealed similar composition for the two extracts, but PSE was richer in condensed tannins and flavonoids, principally rhoifolin, quercetin, naringenin and derivatives. PSE induced stronger inhibition on collagenase and elastase by *in vitro* enzyme assays, possibly due to higher levels of condensed tannins and quercetin. ELISA bioassay on human dermal fibroblasts revealed stronger PSE induction of collagen production. Determination of glycerol release from adipocytes disclosed stronger stimulation of lipolysis by PSE, allegedly ascribed to higher charge of quercetin and derivatives. In summary, the higher richness in phenolics of PSE is strictly related to stronger bioactivity.

Discussion and conclusions: Data indicate that aerial *H. coronarium* material is suitable for the development of dermatological and cosmeceutical products, but the geographical origin is an important factor for maximally exploiting the biological properties of this species.

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Introduction

The search for bioactive secondary metabolites from botanical sources for health care purposes is attracting an ever-increasing interest due to the huge chemodiversity of plants. Such a tendency is further supported by the idea that many natural compounds may be more biocompatible and involve less adverse effects than synthetic or toxic drugs. However, significant differences in chemical composition may occur among closely related taxonomical entities, such as species, subspecies or varieties, but also within these taxonomical entities, depending on different geographical origins (Carmona et al. 2007). The variability in the phytocomplex of a plant species can be characterized in terms of chemotypes, suggesting that the geographical origin of plant materials is to be carefully considered in the search for new bioactive compounds (Kaiser et al. 2016).

In a survey of legume crops scarcely investigated for health and skin care potential (Pastorino et al. 2017), we focused here on *Hedysarum coronarium* L. [syn. *Sulla coronaria* (L.) Medik] (Fabaceae), commonly known as sulla or French honeysuckle. This is a short-lived, perennial legume present as both wild

herb and cultivated forage throughout the Mediterranean (Annicchiarico et al. 2014). Aerial portions of the plant are known to contain good levels of protein, as well as condensed tannins ranging at about 20–50 mg/kg (Terrill et al. 1992). Phenolics reported to be present in this species include glycosylated derivatives of the flavonoids kaempferol, quercetin, genistein, formononetin and afmosin, and the anthocyanins peonidin 3-monoglucoside, peonidin 3,5-diglucoside and malvidin 3,5-diglucoside, as flower pigments (Chiki and Harborne 1983; Tibe et al. 2011).

The high content in tannins of sulla is thought to increase the performance of meat and milk in dairy sheep and cattle feeding on the plant, and in addition to induce resistance to gastrointestinal parasites (Molle et al. 2003; Hoste et al. 2006). Anthelmintic activity has been confirmed by *in vitro* experiments (Aïssa et al. 2015). Besides veterinary uses, sulla has also been consumed by humans as a vitaminic vegetable and as an astringent, anti-hypercholesterolaemic, laxative, refreshing and soothing herb (Lentini and Venza 2007). Scientific studies on medicinal properties are lacking, but its chemical composition rich in phenolic

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compounds suggests that the plant deserves attention for possible curative effects.

Our study was conducted on aerial portions of *sulla* collected at two different sites, located nearby the cities of Pisa and Ventimiglia. Pisa is in central Italy, well inside the geographic range of the species, while Ventimiglia is in western Italy, close to the boundary of the species range. Plant extracts obtained in methyl *tert*-butyl ether/ethyl acetate/acetone were chemically characterized by LC-MS and assayed for antioxidant power, total phenolics and flavonoid contents. Extracts were used for *in vitro* bioassays aimed at assessing modulatory effects on extracellular matrix turnover and on lipolysis. Positive correlation between phenolic content and bioactivity strength was observed.

Materials and methods

Materials

Cell culture reagents and other chemicals were from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. Plant specimens of *Hedysarum coronarium* were collected in May 2015, during the flowering period, from two wild Italian populations, growing at the Hanbury Botanical Gardens, La Mortola, Ventimiglia (43°46'57"N 07°33'20"E), and in the southern surroundings of Pisa (43°41'32"N, 10°23'06"E). Aerial portions were cleaned, dried at air, and used for extraction procedure. Taxonomical identification was carried out by one of us (LC) and voucher specimens of plants from both geographical sites were deposited at the Herbarium of DISTAV, University of Genova (GE 20052015).

Extraction

Specimens of *H. coronarium* were air-dried in greenhouse at ~25–30 °C. Aerial portions of dried material were cleaned, chopped and extracted according to Tibe et al. (2011). The dried material (25 g) was put in a beaker containing 100 mL methyl *tert*-butyl ether (MTBE), 70 mL ethyl acetate, 60 mL acetone and 2 mL distilled water at RT, sonicated for 15 min, cloth filtered, transferred to a Buchi Rotavapor R-114 (Buchi Italia s.r.l.) to remove organic solvents and then dried under nitrogen stream. Dried extracts were stored at –20 °C until use. For biological assays, dimethyl sulphoxide (DMSO) stock solutions of extracts were prepared in order to obtain a maximum DMSO concentration of 0.1% (v/v) at the highest extract incubation doses.

Radical scavenging assay

Radical scavenging activity of extracts was quantified by the DPPH (2,2-diphenyl-1-picrylhydrazide) assay, using the method described by Chung et al. (2002). Briefly, a volume of 0.5 mL DPPH dissolved in EtOH (40 µg/mL) was mixed with 0.5 mL of serial dilutions of extracts in EtOH, incubated for 30 min in the dark at RT and then read in an Agilent Cary 60 spectrophotometer (Agilent Technologies, Palo Alto, CA) at 517 nm. EtOH without DPPH was used as blank. Results were expressed as percent DPPH inhibition.

Quantification of total phenolics

The total phenolic content of extracts was determined by the common Folin–Ciocalteu method. Stock solutions were prepared by dissolving gallic acid or extracts in EtOH at 5 mg/mL.

Aliquots of 2.4 mL distilled water, 0.15 mL Folin–Ciocalteu reagent and 0.45 mL Na₂CO₃ (20% in distilled water) were combined in 3 mL cuvettes with 30 µL of serial water dilutions of gallic acid or extract stock solutions. The contents of the cuvettes were mixed, incubated at 40 °C for 30 min and read at 765 nm in the spectrophotometer. A standard curve was obtained from gallic acid samples and total phenolic contents were expressed as mg gallic acid equivalents (GAE) per g of extract.

Determination of total flavonoids

The total flavonoid content of extracts was determined according to Zhishen et al. (1999). Stock solutions were prepared by dissolving quercetin or extracts in EtOH as above. Aliquots of 100 µL of serial water dilutions of extract stock solutions were placed in cuvettes with 6 µL of aqueous NaNO₂ (1:20, w/v). After 5 min incubation, 6 µL of aqueous AlCl₃ (1:10, w/v) was added and the mixture was vortexed, and then, after 1 min, 40 µL of 1 M NaOH was added. The absorbance was measured against a blank at 510 nm. A calibration curve was prepared using proper dilutions of a quercetin stock solution, and the total flavonoid content of samples was given as g quercetin equivalent per g of plant extract.

HPLC-MS

HPLC coupled with mass spectrometry analysis (HPLC-MS/MS) was performed using an Agilent 1100 HPLC-MSD Ion Trap XCT system, equipped with an electrospray ion source (HPLC-ESI-MS) (Agilent Technologies). Separations of extracts were performed on a Symmetry C18 column 1 × 150 mm with 3 µm particle size (Waters Corporation, Milford, MA). Eluents used were water (eluent A) and MeOH (eluent B), both added with 0.1% formic acid. The gradient employed was as follows: 50% eluent B for 3 min, then linear to 95% eluent B in 25 min and finally hold at 95% eluent B for other 15 min. The flow rate was set to 30 µL/min and the column temperature was set at 25 °C. The injection volume was 8 µL. Ions were detected in the positive and negative ion mode, in the 200–1000 *m/z* range and ion charged control with a target ion value of 200,000 and an accumulation time of 300 msec. A capillary voltage of 3300 V, nebulizer pressure of 15 psi, drying gas of 8 L/min, dry temperature of 325 °C and rolling averages 2 (averages: 5) were the parameters set for the MS detection. MS/MS analysis was conducted using amplitude optimized time by time for each compound.

Enzyme assays

Enzymatic assays were conducted in 96-well plates according to a previously described method (Pastorino et al. 2017). Collagenase (EC 3.4.24.3) inhibition was evaluated in a reaction mixture containing 50 mM tricine, pH 7.5, 10 mM CaCl₂, 400 mM NaCl, 0.8 mM FALGPA (Sigma-Aldrich, F5135) as the enzyme substrate, 0.16 units/mL collagenase from *Clostridium histolyticum* (Sigma-Aldrich, C0130) and serial water dilutions of extract stock solutions in DMSO, as indicated. The mixture was incubated for 10 min at RT, and then plates were read at 345 nm in a Tecan Genios Pro plate reader (Tecan, Wien, Austria).

Elastase (EC 3.4.21.36) inhibition was measured in a mix containing 200 mM TRIS, pH 8.0, 10 mM Suc-Ala₃-pNA (Sigma-Aldrich, S4760) as the enzyme substrate, 2 units/mL of elastase from porcine pancreas (Sigma-Aldrich, E1250) and extracts as

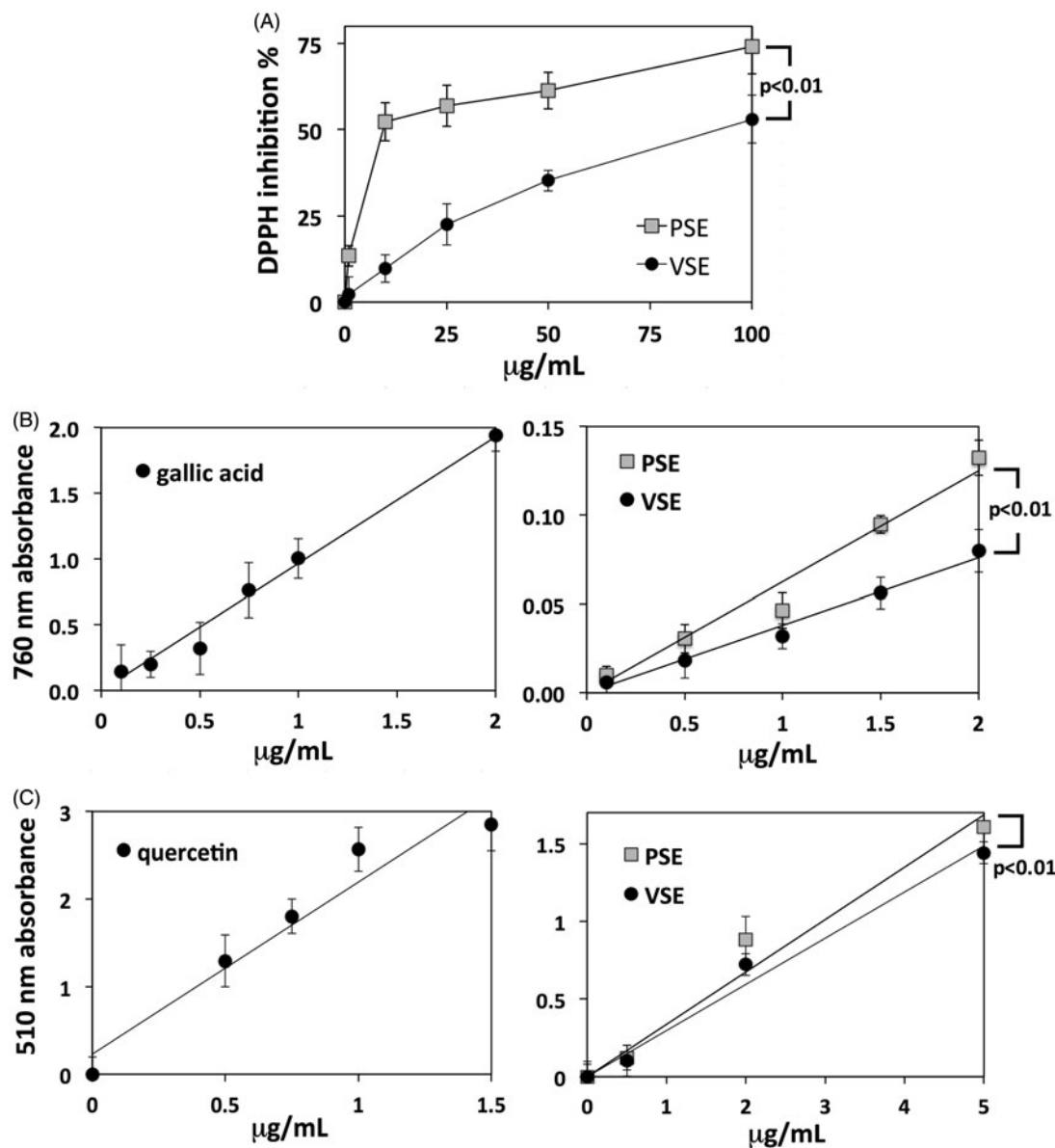


Figure 1. (A) Percentage inhibition of DPPH scavenging activity exerted by increasing concentrations of PSE and VSE. Data are means $\pm$ S.D. of $n = 3$ distinct determinations. (B) Folin-Ciocalteu assays of total phenolic content. Standard calibration curve obtained with gallic acid (left) and curves obtained with PSE and VSE (right). Data are means $\pm$ S.D. of $n = 4$ distinct determinations. In the right panel, statistical comparison between the two slope coefficients is shown. The phenolic contents of PSE and VSE expressed as GAE are reported in Table 1. (C) Assays of total flavonoids. Standard calibration curve obtained with quercetin (left) and curves obtained with PSE and VSE (right). Data and statistics as above. The flavonoid contents of PSE and VSE expressed as quercetin equivalents are reported in Table 1.

Table 1. Quantification of total phenolics and flavonoids in sulla extracts.

Extract	Total phenolics	Total flavonoids
PSE	62 $\pm$ 1	13 $\pm$ 8
VSE	38 $\pm$ 1	11 $\pm$ 4

Data are means $\pm$ S.E.M of triplicate determination. Total phenolics are expressed as gallic acid equivalents (mg/g). Total flavonoids are expressed as quercetin equivalents (mg/g).

above. After 15 min incubation, plates were read at 410 nm in the plate reader.

Cell culture

Stabilized human dermal fibroblasts (46BR.1N, Sigma-Aldrich) were grown in DMEM supplemented with 10% (v/v) FBS, 1%

glutamine and 1% antibiotic, at 37 °C, in a 5% CO₂, humidified atmosphere. Subcutaneous human preadipocytes (Zen-Bio Inc., Research Triangle Park, NC) were grown in preadipocyte medium (cat# PM-1, Zen-Bio Inc.) according to the manufacturer's protocol.

Cell viability and proliferation assays

Effects of extracts on cell viability were determined on fibroblasts by the MTT assay, as previously reported by Pastorino et al. (2017). Cells were settled in 96-well plates, exposed for 48 h to increasing concentrations of extracts (1–1000 µg/mL), processed for MTT assay and read at 570 nm in a VMax microplate reader (Molecular Devices, Sunnyvale, CA). Absorbance values were used to obtain dose–response curves and IC₅₀ values.

Table 2. LC-MS identification of major compounds in PSE and VSE extracts.

Compound	^a RT	^b [M + H] ⁺	^c [M – H] [–]
Paratocarpin E/hedysarumine B	22.4	409.3	
Naringenin-5,7-di-O-β-D-glucopyranoside	22.0	599.3	
Quercetin-3-O-β-D-glucopyranoside-7-O-α-rhamnofuranoside/rutin	28.9	611.2	
Isoquercitrin/hyperoside	29		463.2
3-Sitosterol	29.9	415.4	
Betulinic acid/ursolic acid	29.9	457.3	
Narcissin/isorhamnetin-3-O-rutinoside	30.9	625.2	
Galactose/glucose	31.8	181.2	
Hedysarimcoumestan G/hedysarimcoumestan H	31.8	383.3	
Quercetin	32.7	303.4	
Squasapogenol	33.3	441.5	
1,3,9-Trimethoxycoumestan	33.4		325.5
Sucrose	33.4		343.9
Methyl-hedysarimcoumestan H	33.4		394.7
(-)Vestitol/naringenin	34.3	273.4	
Epicatechin/catechin	34.4	291.5	
Prenylfuran- [3,2-g]-isoflavone	34.7		376.6
Isoformononetin/formononetin	34.8	269.4	
Hedysarimcoumestan B/4',6-dimethoxy-7-hydroxy isoflavone/afroformosin	34.8	299.4	
Rhoifolin	35.0	579.5	
Hexadecanoic acid 2,3-dihydroxypropyl ester	35.3	331.6	
Oleic acid	35.5	283.4	
n-Tetracosanoic acid	35.5	369.5	
Kaempferide	36.8	301.5	
Hedysarimpterocarpene C	36.8	627.5	
Isorhamnetin	37.1	317.6	
Hexacosyl acetate	37.1	425.5	
Diosmin	38.7	609.9	607.7

^aRT: retention time.^b[M + H]⁺: *m/z* in positive ion mode.^c[M – H][–]: *m/z* in negative ion mode.**Table 3.** LC-MS relative quantification of major flavonoids from PSE and VSE.

Compound	RA
Epicatechin/catechin	+++
Rhoifolin	+++
Naringenin-5,7-di-O-β-D-glucopyranoside	++
Quercetin	++
Quercetin-3-O-β-D-glucopyranoside-7-O-α-rhamnofuranoside	+

RA: relative abundance of the compounds in PSE compared to VSE (+ ≈ 20%, ++ ≈ 40%, +++ ≈ 60%).

Collagen production

The effect of extracts on collagen type I production was evaluated by ELISA technique as previously reported (Pastorino et al. 2017). Briefly, fibroblasts were settled in 96-well plates, incubated with extracts for 48 h, fixed with 3.7% paraformaldehyde, blocked with BSA, probed with mouse anti-human collagen type I primary antibody (ab6308, Abcam, Cambridge, UK), then probed with HRP-conjugated rabbit anti-mouse IgG secondary antibody (ab97046, Abcam), incubated with Pierce 1-StepTM Ultra TMB ELISA Substrate Solution (Thermo Fisher Scientific, Waltham, MA), blocked with 2 M sulphuric acid and read at 620 nm in the microplate reader.

Lipolysis assay

The lipolytic activity of extracts was assessed on adipocytes by measuring glycerol released into the medium from triglyceride breakdown. Human subcutaneous pre-adipocytes were grown in preadipocyte medium (cat# PM-1, Zen-Bio Inc.) following the manufacturer's protocol, settled in 96-well plates, differentiated into adipocytes for one week in adipocyte differentiation medium (cat# DM-2) and maintained for a further week in adipocyte medium (cat# AM-1). Thereafter, fully differentiated adipocytes

were incubated for 3 h with extracts in adipocyte medium, and the conditioned medium was then assayed for glycerol using the Adipocyte Lipolysis Assay Kit (cat# LIP-1-L1; LIP-1-NCL1). The procedure involves glycerol phosphorylation by ATP, glycerol-1-phosphate oxidation by glycerol phosphate oxidase to dihydroxyacetone phosphate and H₂O₂, and peroxidase-catalyzed quinoneimine dye production, showing maximum absorbance at 540 nm. Samples were read in the microplate reader at 550 nm. The increase in absorbance is directly proportional to glycerol concentration in the sample.

Statistics

Data were analyzed with the R package, version 3.0.1 (<http://www.r-project.org/foundation/>), by using Student's *t*-test with Bonferroni's correction for multiple comparisons. The difference between two conditions was considered significant if *p* < 0.05. Inhibition of cell viability was determined using a logistic dose-response curve as reported by Ranzato et al. (2014).

Results

Chemical characterizations

The extraction procedure, aimed at optimizing phenolic yield, allowed to obtain two dried extracts named Pisa sulla extract (PSE) and Ventimiglia sulla extract (VSE). Extracts were used for general chemical characterizations and chemical fingerprinting. The assay of DPPH scavenging activity was used as a measure of extract antioxidant power. Concentration-dependent curves of DPPH inhibition showed a significantly stronger activity of PSE with respect to VSE (Figure 1(A)).

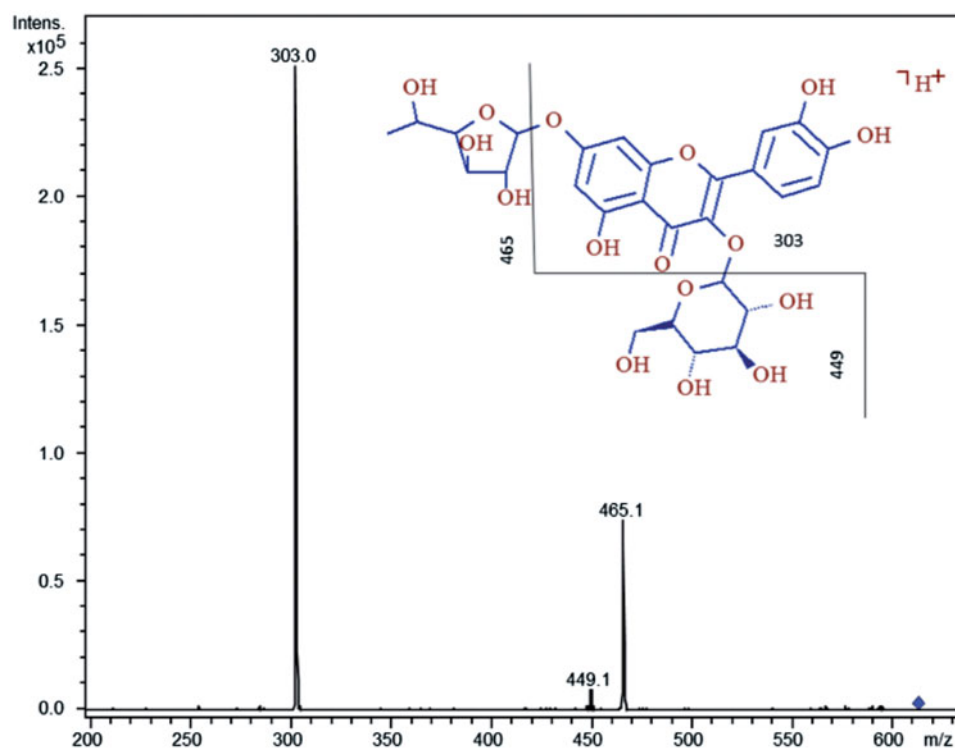


Figure 2. MS/MS spectrum of quercetin-3-O-β-glucopyranoside-7-O-α-rhamnofuranoside. The hypothesized fragments and their m/z ratios are indicated with the broken line in the structure.

Total phenolic contents were determined by the Folin–Ciocalteu method, obtaining a gallic acid standard curve at 765 nm that allowed converting absorbance data into gallic acid equivalents (GAE). Thereafter, concentration-dependent curves at 765 nm were obtained for extracts, in order to compare their regression line slopes (Figure 1(B)). PSE showed a higher phenolic content than VSE (Table 1), while regression line comparison showed that the difference is statistically significant (Figure 1(B)).

A similar approach was used for the colorimetric quantification of total flavonoid content, by using quercetin as the reference standard (Figure 1(C)). Consistent with the analysis of total phenolics, PSE showed a higher flavonoid content than VSE (Table 1). Such a difference was smaller than that found for phenolics, but it resulted statistically significant according to regression line comparison (Figure 1(C)).

Compound identification

HPLC-MS/MS analysis of PSE and VSE extracts allowed the identification and relative quantification of different compounds. The MS/MS molecular masses obtained in our analyses were compared with online libraries (MassBank, <http://www.massbank.jp/>) and previously published MS data concerning different species of the genus *Hedysarum*, synonymous of *Sulla* (Dong et al. 2013). PSE and VSE showed similar composition in terms of major constituents, belonging to simple sugars, isoprenyl chalcones, coumestans, pterocarpenes, flavanones, flavones, flavonols, isoflavanes, isoflavones, flavan-3-ols, triterpenoids, phytosterols, fatty acids and esters (Table 2). However, a comparison of the relative abundances of these compounds showed that some molecules were significantly more abundant in PSE than in VSE, confirming the data obtained by general chemical characterizations. In Table 3, five flavonoid molecules are reported whose

chromatographic peak areas were ~20% (+), ~40% (++) and ~60% (+++), higher in PSE with respect to VSE. Each molecule was confirmed for its structure by mean of MS/MS analysis, and a typical spectrum thereof is reported in Figure 2.

Effects of extracts on extracellular matrix

Extracellular matrix turnover is essential for the maintenance of a healthy skin dermal layer, involving both degradation and neo-synthesis processes. We evaluated the *in vitro* inhibition of extracts on collagenase and elastase, two main dermal enzyme activities involved in the degradation of collagen and elastin, respectively. The effects on collagenase of the two extracts showed different patterns. VSE induced a biphasic effect, with inhibition rise at lower concentrations followed by a decrease in the effect at higher concentrations (Figure 3(A)). PSE showed strong inhibition at the lowest concentration and a progressive decline for higher doses (Figure 3(A)). The effect of the two extracts on elastase was inhibitory and significantly stronger for PSE than for VSE (Figure 3(B)).

Before using extracts on *in vitro* grown fibroblasts, we made a check for possible deleterious effects on cell viability. The MTT assay showed low inhibition of cell viability for both extracts, with $IC_{50} > 150 \mu\text{g/mL}$ at 48 h, warranting that any effect on cell viability in the tests conducted on cells was negligible. By using an ELISA assay, we measured the modulatory effect of extracts on collagen type I production in fibroblasts. The two extracts showed a dose-dependent stimulatory effect, and also in this case, the activity of PSE was stronger than that of VSE (Figure 3(C)).

Effects of extracts on lipolysis

Fat tissue turnover is essential for the maintenance of the subdermal skin layer, while its impairment may concur to the

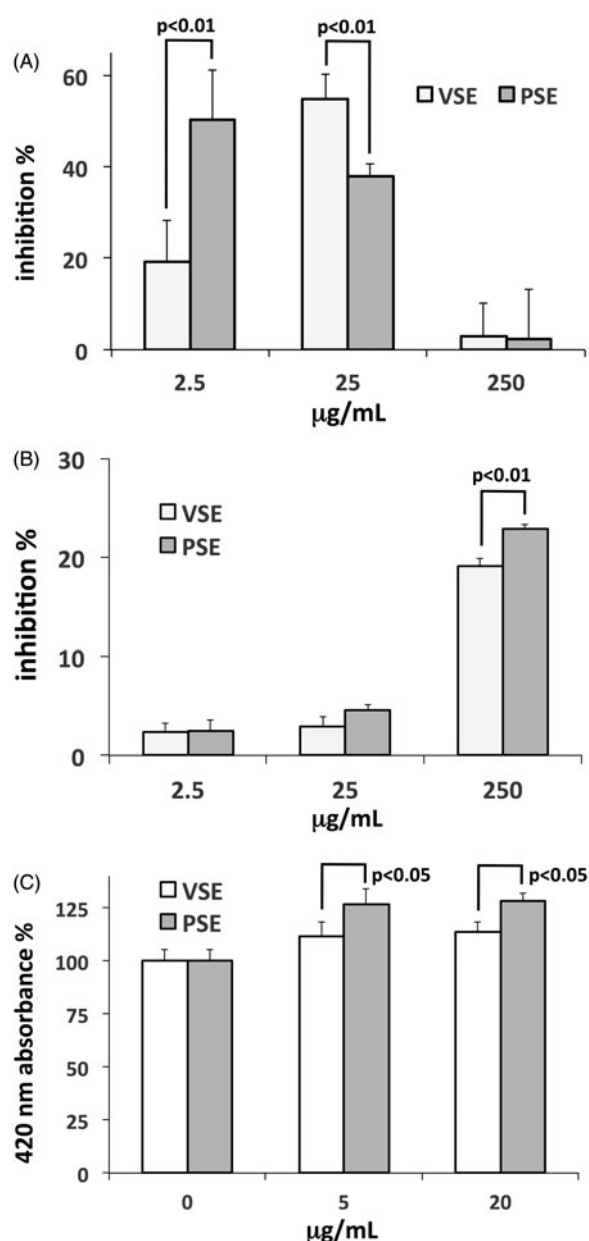


Figure 3. (A) Percent inhibition of *Clostridium histolyticum* collagenase activity exerted by PSE and VSE, determined by *in vitro* assay and expressed as means $\pm$ S.E.M. ($n = 3$). (B) Percent inhibition of elastase from porcine pancreas by PSE and VSE. Data as above. (C) Induction of fibroblast collagen production by PSE and VSE, determined by ELISA after 48 h incubations. Data are expressed as means $\pm$ S.D. ($n = 6$).

development of cellulitis. We verified the effects of extracts on the lipolytic activity of *in vitro* cultured adipocytes (Figure 4, upper panel). Measurements of glycerol release from cells, used as an index of lipolytic activity, revealed a dose-dependent stimulatory effect induced by both extracts. However, a steeper rise in lipolysis was observed with PSE, showing maximum difference with VSE at 100 μ g/mL, followed by an upward convergence of effects at 200 μ g/mL, reaching the lipolytic effect of isoproterenol used as positive control (Figure 4, lower panel).

Discussion

Data of chemical characterizations consistently indicated a higher content of phenolic compounds in PSE with respect to VSE,

allegedly linked to the stronger radical scavenging activity of the former extract. Moreover, the semiquantitative analysis conducted on LC-MS data indicated that the difference in phenolic contents is maximally accounted for by catechins. The identification of these compounds as major extract constituents could actually reflect the presence of condensed tannins, of which catechins are monomer units. Condensed tannins are known to be abundant in *sulla* and would undergo in-source collision-induced dissociation (CID) during MS analysis. Hence, the comparison of MS data from the two extracts suggests that the higher abundance of phenolics in PSE is primarily accounted for by tannins. However, total flavonoid content and MS data consistently showed that also the flavonoid component contributes to the difference in phenolic composition between the two extracts.

It is well known that phenolics, especially flavonoids, play an important role in determining the biological properties of herbal products. Therefore, a higher content of these compounds in a phytocomplex is predictive of stronger bioactivity. Our data are consistent with this view, as we found similar composition in the two *sulla* extracts and similar effects in different bioassay, but the higher content in phenolics of PSE went together with the stronger activity of this extract in all bioassays.

Inhibition of collagenase and elastase is generally considered to prevent unbalanced extracellular matrix turnover due to rapid breakdown of collagen type I in inflamed skin. Inhibitors are therefore sought for in the development of skin preserving and antiageing remedies. By using *in vitro* enzyme assays, we found biphasic effects of extracts on collagenase, characterized by inhibitory activities at very low concentration of PSE and at relatively low concentration of VSE. These results suggest the presence of strong inhibitors, more concentrated in PSE than in VSE, and of other compounds interfering with inhibition at higher extract doses. Based on the composition of our extracts, possible inhibitors could be condensed tannins and quercetin, previously found to exert inhibitory effects on collagenase (Lim and Kim 2007; Diaz-Gonzalez et al. 2012). Data of elastase inhibition revealed a weaker activity with respect to collagenase inhibition, but the effect of PSE was stronger. Also in this case, a role could be played by condensed tannins that have been previously shown to bind to pancreatic elastase, the enzyme used in our tests (Bras et al. 2010). However, the effect could be ascribed at least in part also to quercetin (Park et al. 2016).

The stimulatory effect of PSE on collagen synthesis is particularly interesting from pharmaceutical and pharmacological points of view, because it was observed at very low concentrations, suggesting the presence of very strong inducers in the phytocomplex. Condensed tannins, quercetin and other major polyphenols of *sulla* extracts are not known to induce this kind of effect. However, collagen induction has been reported for flavonoids and triterpenoids from other plants (Maquart et al. 1990; Pastorino et al. 2017). Therefore, it can be speculated that the effect observed with PSE could be due to compounds belonging to either of these classes.

The lipolytic effect of *sulla* extracts testifies their possible use in anticellulite products. This activity can be allegedly ascribed to quercetin and its derivatives, since these compounds are well represented in *sulla* extracts and their lipolytic effect has been pharmacologically characterized in rat adipocytes (Kuppusamy and Das 1994). It has also been shown in OP9 mouse stromal cells, which can differentiate into adipocytes, that quercetin prevents adipogenesis and stimulates lipolysis through the regulation of transcriptional factors and lipases (Seo et al. 2015). The stronger effect of PSE observed in our study is in line with these

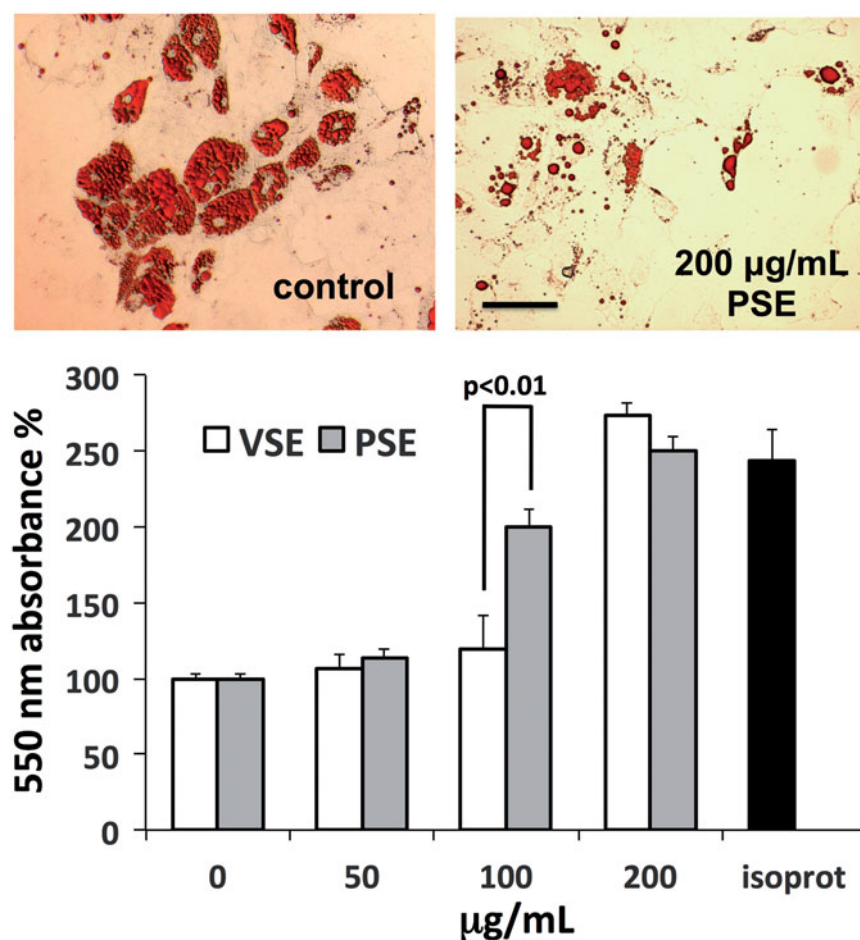


Figure 4. Induction of lipolysis in human adipocytes by PSE and VSE. Upper panel: microscope views of adipocytes exposed or not to PSE, fixed with FineFix[®], stained with Oil Red O and then photographed under an Olympus IX71 inverted microscope. Bar = 100 µm. Lower panel: assay of glycerol released from adipocytes after exposure for 3 h to extracts or to 1 µM isoproterenol (isoprot) as positive control. Data are standardized as percent of control and expressed as means ± S.D. ($n = 6$).

reports, because quercetin is a major compound accounting for the difference in polyphenols between PSE and VSE.

Conclusions

This study was aimed at verifying the possible use in dermatologic and cosmeceutical products of herbal extracts obtained from *sulla*. This species is a legume crop widely cultivated in the Mediterranean area and potentially available in amounts compatible with industrial use. Moreover, due to the known variability of plant phytocomplexes, even within a species or variety, depending on the geographical origin of plants, we have compared extracts from individuals sampled at two distant sites in the geographic range of the species.

Our data showed marked differences in total phenolic content between the two extracts, most likely depending on different abundances of condensed tannins and flavonoids, mainly rhoifolin, quercetin, naringenin and derivatives. In addition, the higher richness in phenolics of the PSE extract, deriving from Pisa, was in conjunction with a stronger bioactivity of this extract in all the conducted bioassays. These tests collectively showed that *sulla* plant material is suitable for the development of pharmaceutical and cosmeceutical products targeting major skin problems, such as inflammatory, degenerative and ageing processes, or the insurgence of cellulite. Hence, our

data provide a strong indication that the legume crop *H. coronarium* can be exploited for these purposes, but they also show that the geographical origin of plants is an important factor to be considered for maximally exploiting the biological properties of the species.

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Disclosure statement

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References

- Aïssa A, Manolaraki F, Ben Salem H, Kraiem K, Hoste H. 2015. *In vitro* anthelmintic activity of Tunisian Fabaceae (*Hedysarum coronarium* L, eco-type Bikra 21) against haemonchus contortus. *Int J Agron Agr Res*. 7:103–110.
- Annicchiarico P, Ruissi P, Di Miceli G, Pecetti L. 2014. Morpho-physiological and adaptive variation of Italian germplasm of sulla (*Hedysarum coronarium* L.). *Crop Pasture Sci*. 65:206–213.
- Bras NF, Gonçalves R, Fernandes PA, Mateus N, Ramos MJ, de Freitas V. 2010. Understanding the binding of procyanidins to pancreatic elastase by experimental and computational methods. *Biochemistry*. 49:5097–5108.
- Carmona M, Sanchez AM, Ferreres F, Zalacain A, Tomas-Barberan F, Alonso GL. 2007. Identification of the flavonoid fraction in saffron spice by LC/DAD/MS/MS: comparative study of samples from different geographical origins. *Food Chem*. 100:445–450.
- Chiki A, Harborne JB. 1983. Anthocyanins of *Hedysarum coronarium* and their contribution to flower colour variation. *Phytochemistry*. 22:2322–2323.
- Chung YC, Chang CT, Chao WW, Lin CF, Chou ST. 2002. Antioxidative activity and safety of the 50 ethanolic extract from red bean fermented by *Bacillus subtilis* IMR-NK1. *J Agric Food Chem*. 50:2454–2458.
- Diaz-Gonzalez M, Rocasalbas G, Francesko A, Tourino S, Torres JL, Tzanov T. 2012. Inhibition of deleterious chronic wound enzymes with plant polyphenols. *Biocatal Biotransfor*. 30:102–110.
- Dong Y, Tang D, Zhang N, Li Y, Zhang C, Li L, Li M. 2013. Phytochemicals and biological studies of plants in genus *Hedysarum*. *Chem Cent J*. 7:124.
- Hoste H, Jackson F, Athanasiadou S, Thamsborg SM, Hoskin SO. 2006. The effects of tannin-rich plants on parasitic nematodes in ruminants. *Trends Parasitol*. 22:253–261.
- Kaiser S, Carvalho AR, Pittol V, Dietrich F, Manica F, Machado MM, de Oliveira LF, Oliveira Battastini AM, Ortega GG. 2016. Genotoxicity and cytotoxicity of oxindole alkaloids from *Uncaria tomentosa* (cat's claw): Chemotype relevance. *J Ethnopharmacol*. 189:90–98.
- Kuppusamy UR, Das NP. 1994. Potentiation of beta-adrenoceptor agonist-mediated lipolysis by quercetin and fisetin in isolated rat adipocytes. *Biochem Pharmacol*. 47:521–529.
- Lentini F, Venza F. 2007. Wild food plants of popular use in Sicily. *J Ethnobiol Ethnomed*. 3:15.
- Lim H, Kim HP. 2007. Inhibition of mammalian collagenase, matrix metalloproteinase-1, by naturally-occurring flavonoids. *Planta Med*. 73:1267–1274.
- Maquart FX, Bellon G, Gillerly P, Wegrowski Y, Borel JP. 1990. Stimulation of collagen synthesis in fibroblast cultures by a triterpene extracted from *Centella asiatica*. *Connect Tissue Res*. 24:107–120.
- Molle G, Decandia M, Fois N, Ligios S, Cabiddu A, Sitzia M. 2003. The performance of Mediterranean dairy sheep given access to sulla (*Hedysarum coronarium* L.) and annual ryegrass (*Lolium rigidum* Gaudin) pastures in different time proportions. *Small Ruminant Res*. 49:319–328.
- Park CH, Ahn MJ, Hwang GS, An SE, Whang WK. 2016. Cosmeceutical bioactivities of isolated compounds from *Ligularia fischeri* Turcz leaves. *Appl Biol Chem*. 59:485–494.
- Pastorino G, Marchetti C, Borghesi B, Cornara L, Ribulla C, Burlando B. 2017. Biological activities of the legume crops *Melilotus officinalis* and *Lespedeza capitata* for skin care and pharmaceutical applications. *Ind Crops Prod*. 96:158–164.
- Ranzato E, Magnelli V, Martinotti S, Waheed Z, Cain SM, Snutch TP, Marchetti C, Burlando B. 2014. Epigallocatechin-3-gallate elicits Ca²⁺ spike in MCF-7 breast cancer cells: essential role of Cav3.2 channels. *Cell Calcium*. 56:285–295.
- Seo YS, Kang OH, Kim SB, Mun SH, Kang DH, Yang DW, Choi JG, Lee YM, Kang DK, Lee HS, et al. 2015. Quercetin prevents adipogenesis by regulation of transcriptional factors and lipases in OP9 cells. *Int J Mol Med*. 35:1779–1785.
- Terrill TH, Douglas GB, Foote AG, Purchas RW, Wilson GF, Barry TN. 1992. Effect of condensed tannins upon body growth, wool growth and rumen metabolism in sheep grazing sulla (*Hedysarum coronarium*) and perennial pasture. *J Agric Sci*. 119:265–273.
- Tibe O, Meagher LP, Fraser K, Harding DR. 2011. Condensed tannins and flavonoids from the forage legume sulla (*Hedysarum coronarium*). *J Agric Food Chem*. 59:9402–9409.
- Zhishen J, Mengcheng T, Jianming W. 1999. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem*. 64:555–559.

Article

Posidonia oceanica (L.) Delile Ethanolic Extract Modulates Cell Activities with Skin Health Applications

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Abstract: Seagrasses are high plants sharing adaptive metabolic features with both terrestrial plants and marine algae, resulting in a phytocomplex possibly endowed with interesting biological properties. The aim of this study is to evaluate the in vitro activities on skin cells of an ethanolic extract obtained from the leaves of *Posidonia oceanica* (L.) Delile, family Potamogetonaceae, herein named *Posidonia* ethanolic extract (PEE). PEE showed high radical scavenging activity, high phenolic content, and resulted rich in chicoric acid, as determined through HPLC-MS analysis. The use of MTT assay on fibroblasts showed a PEE cytotoxicity threshold (IC₀₅) of 50 µg/mL at 48 h, while a sub-toxic dose of 20 µg/mL induced a significant increase of fibroblast growth rate after 10 days. In addition, an ELISA assay revealed that PEE doses of 5 and 10 µg/mL induced collagen production in fibroblasts. PEE induced dose-dependent mushroom tyrosinase inhibition, up to about 45% inhibition at 1000 µg/mL, while 50% reduction of melanin was observed in melanoma cells exposed to 50 µg/mL PEE. Finally, PEE lipolytic activity was assessed by measuring glycerol release from adipocytes following triglyceride degradation. In conclusion, we have collected new data about the biological activities of the phytocomplex of *P. oceanica* seagrass on skin cells. Our findings indicate that PEE could be profitably used in the development of products for skin aging, undesired hyperpigmentation, and cellulite.

Keywords: adipocytes; chicoric acid; collagen type I; fibroblasts; lipolysis; melanin; tyrosinase

1. Introduction

Over the last years, the herbal market has rapidly increased and many surveys have been conducted that aim at finding natural ingredients with possible applications as food additives or medicine. In this area, special attention has been given to the sea environment as a rich source of new active compounds [1]. Among marine botanical organisms, algae have been deeply investigated and exploited [2]; in contrast, the phytopharmaceutical profile of seagrasses is still almost unknown.

However, these latter are high plants sharing adaptive metabolic features with both terrestrial plants and seaweed, resulting in a peculiar phytocomplex possibly endowed with interesting biological properties [3].

Posidonia oceanica (L.) Delile, family Potamogetonaceae, is a long-living, slow-growing, endemic Mediterranean seagrass forming extensive meadows in coastal shallow waters [4]. The plant undergoes massive leaf loss in autumn, giving rise in some areas to conspicuous beach deposits. The profile of secondary metabolites is mostly characterized by phenolic compounds, as also testified by the presence of so-called tannin cells [5]. Chicoric acid is generally reported as the major constituent of leaves [6,7], caftaric and gentisic acids are also abundant in leaves, and other phenolic constituents include the aldehyde vanillin, and *p*-coumaric, ferulic, caffeic, and cinnamic acids [8]. Other chemical constituents isolated from the plant include phenol derivatives (e.g., phloroglucinol), benzoic acid derivatives (e.g., *p*-hydroxybenzoic and vanillic acids), calchones, and proanthocyanidins [9]. Lignin is present in all tissues [10], while major flavonoids include the flavonols kaempferol, quercetin, isorhamnetin (about 1–3 $\mu\text{g g}^{-1}$ dw), and myricetin (about 20 $\mu\text{g g}^{-1}$ dw) [11]. The lipid fraction has been found to contain prevalently palmitic, palmitoleic, oleic, and linoleic acids, in addition to the phytosteroids campesterol, stigmasterol, and β -sitosterol [12]. A novel sesquiterpenic alcohol named posidozinol has also been isolated from the leaves [13].

The plant exploitation has mostly concerned beached leaf litter, used in the past as packing material for glassware and pottery, roof insulation, shipping of fishery products, cattle bedding, mattress and pillow filling, and still used in some cases as cattle forage and compost [14]. News on medicinal uses date back to ancient Egypt, where it was supposedly used for skin disease [15], while more recently in the southwestern Mediterranean region the plant has been used as a remedy for acne, leg pain, diabetes, respiratory infections, hypertension, and colitis [16].

Very little scientific data on *P. oceanica* bioactivities and therapeutic properties are available. Antibacterial and antimycotic activities have been found in a rhizome extract [17], while antidiabetic and vasoprotective effects on alloxan diabetic rats have been observed upon treatment with an ethyl acetate fraction from the aqueous residue of a hydroethanolic leaf extract [18].

We obtained a *P. oceanica* ethanolic leaf extract (PEE) characterized by a high concentration of chicoric acid, as determined through HPLC-MS analysis. PEE was subjected to *in vitro* cell-free and cell-based tests, aimed at showing possible applications for skin care and disease. These experiments revealed stimulation of fibroblast proliferation and collagen production, anti-melanogenic activities, and the stimulation of lipolysis in adipocytes, suggesting that PEE could find applications in skin anti-aging, anti-cellulite and depigmenting products.

2. Results

2.1. Chemical Characterizations

The PEE extract showed an IC_{50} value of $32 \pm 2 \mu\text{g/mL}$ in the DPPH radical scavenging assay, corresponding to 6.5 mM (1.14 mg/mL) ascorbic acid equivalents (AAE). The extract was found to contain $126 \pm 3 \mu\text{g g}^{-1}$ dw total polyphenols, expressed as gallic acid equivalents (GAE), and total iodine at $60 \pm 10 \mu\text{g g}^{-1}$ dw, according to ICP-MS quantification. The HPLC-MS analysis of PEE showed, at a retention time (RT) of 32.5 min (66% solvent B), a major chromatographic peak whose full scan and fragmentation mass spectra were consistent with those expected for chicoric acid (Figure 1A,B). Quantification of the compound was achieved using a calibration curve obtained by injecting chicoric acid standard in concentrations ranging from 5 to 25 μM (linearity $r^2 = 0.999$), yielding a value of $55.8 \pm 7 \text{ mg g}^{-1}$ dw of chicoric acid in PEE. Other major PEE compounds identified by HPLC-MS and tandem MS/MS were flavonoid molecules including procyanidin C2, procyanidin B2, isorhamnetin-3-*O*-glucoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-malonylglucoside, and isorhamnetin-3-*O*-malonylglucoside (Figure 1C).

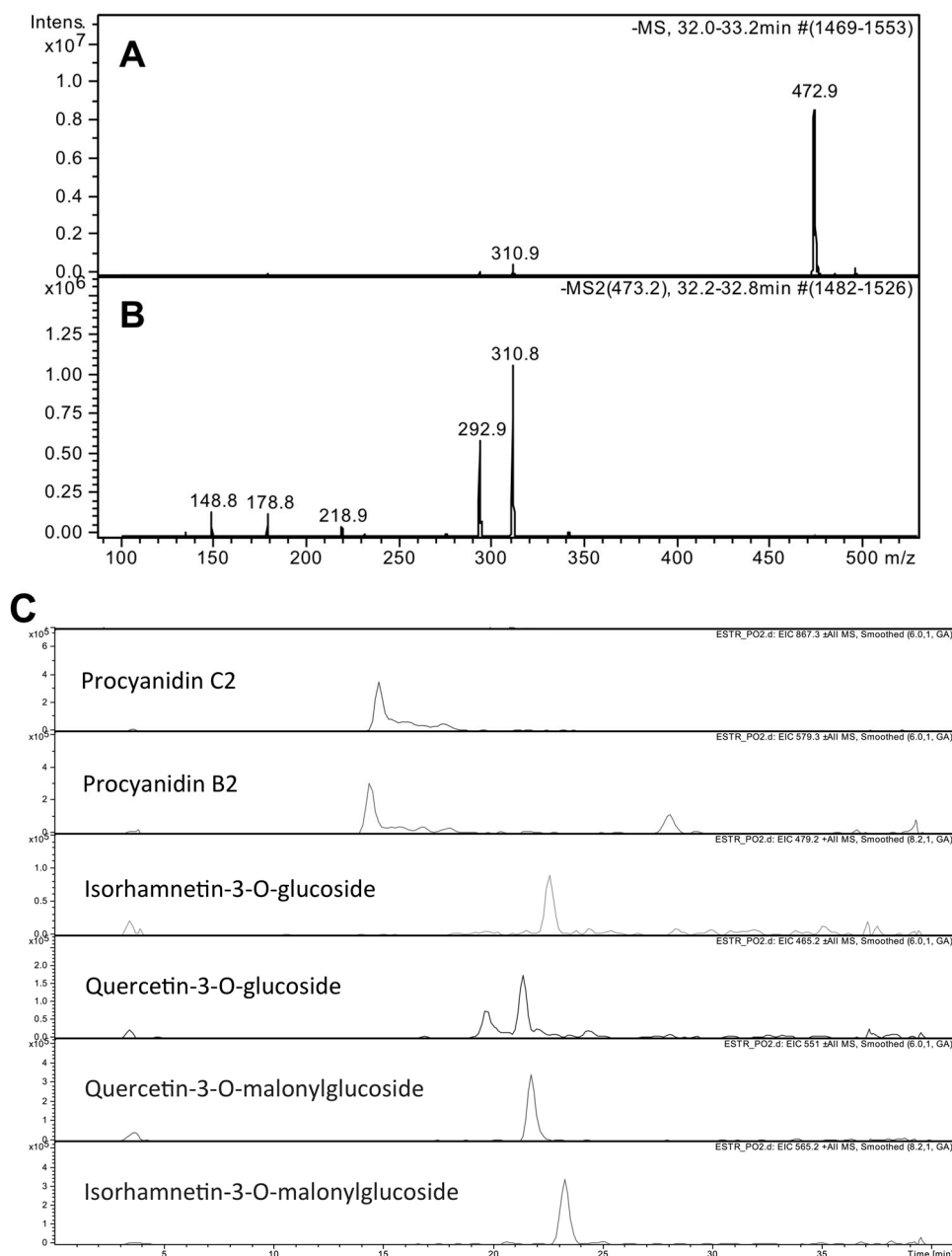


Figure 1. (A) Full scan acquired in negative ion mode and (B) tandem mass spectra relative to the peak at retention time (RT) = 32.5 min, in the HPLC-MS total ion chromatogram of *Posidonia* Ethanolic Extract (PEE). The molecular mass and fragment ions present are consistent with those expected for chicoric acid. (C) Extracted ion chromatograms acquired in positive ion mode, relative to other major PEE constituents identified by MS/MS spectra.

2.2. Fibroblast Activation

Dose-response curves obtained by MTT data were analyzed by a logistic regression model as previously reported [19], allowing the estimation of the PEE effect on fibroblast viability at 48 h, with a median inhibitory concentration of $IC_{50} = 170 \mu\text{g/mL}$ (95% CI = 146–197), and a toxicity threshold of $IC_{05} = 50 \mu\text{g/mL}$ (95% CI = 33–73) (Figure 2A).

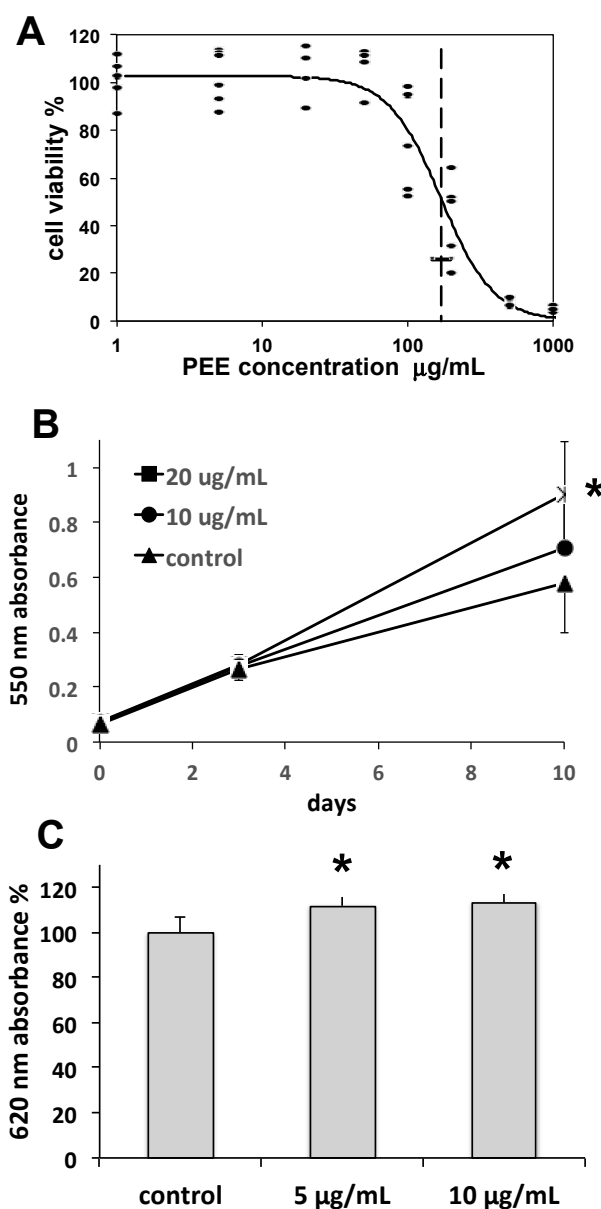


Figure 2. (A) Dose-response curve of the effect of PEE on fibroblast cell viability. Data are percent cell viabilities recorded in $n = 6$ replicates of MTT assay after 48-h incubations with different PEE concentrations. Downhill logistic best fit (continuous line), IC_{50} value (dashed vertical line), and its 95% CI (horizontal bar) are shown. (B) Fibroblast growth rate curves derived from the MTT assay and expressed in terms of cell densities. Data are mean 550 nm absorbances $\pm$ SD from six replicates in two independent experiments. * = $p < 0.01$ with respect to other groups at the same endpoint. (C) Induction of fibroblast collagen production determined by an ELISA assay after 48-h incubations with 5 and 10 $\mu\text{g/mL}$ PEE. Data are mean 620 nm absorbances $\pm$ SEM from six replicates in two independent experiments. * = $p < 0.01$ with respect to the control.

MTT data concerning fibroblast cell growth showed a dose-dependent increase of growth rate in fibroblasts exposed for up to 10 days to sub-toxic 20 $\mu\text{g/mL}$ PEE (Figure 2B). Moreover, a significant increase of collagen production was observed in fibroblasts exposed to 5 and 10 $\mu\text{g/mL}$ PEE, with respect to cells maintained under control conditions (Figure 2C).

2.3. Demelanizing Activity

PEE modulatory effects on skin melanization were evaluated in free solution on mushroom tyrosinase using L-tyrosine as a substrate, as well as on MeWo melanoma cells growing in vitro. The positive control kojic acid induced dose-dependent inhibition of tyrosinase activity with an estimated IC_{50} of 14.7 $\mu\text{g/mL}$, or 2.06 μM (Figure 3A). Incubation with PEE also induced dose-dependent tyrosinase inhibition, starting from about 20% inhibition at 5 $\mu\text{g/mL}$ up to about 45% inhibition at 1000 $\mu\text{g/mL}$ (Figure 3A). For all PEE concentrations, the use of the *t*-test with Bonferroni correction yielded $p < 0.01$ ($n = 6$) with respect to the control (no inhibition).

Data obtained with mushroom tyrosinase were confirmed by experiments on melanoma cells, showing an about 50% reduction of melanin content induced by 50 $\mu\text{g/mL}$ PEE at 72 h. Such a depigmenting effect was not statistically different ($p > 0.05$) from that obtained with the positive control arbutin (1 mg/mL) (Figure 3B). MTT analysis of PEE cytotoxicity on these cells revealed a toxicity threshold of $IC_{05} > 100$ $\mu\text{g/mL}$ at 72 h, showing that the above melanin reduction was not due to aspecific injurious effects.

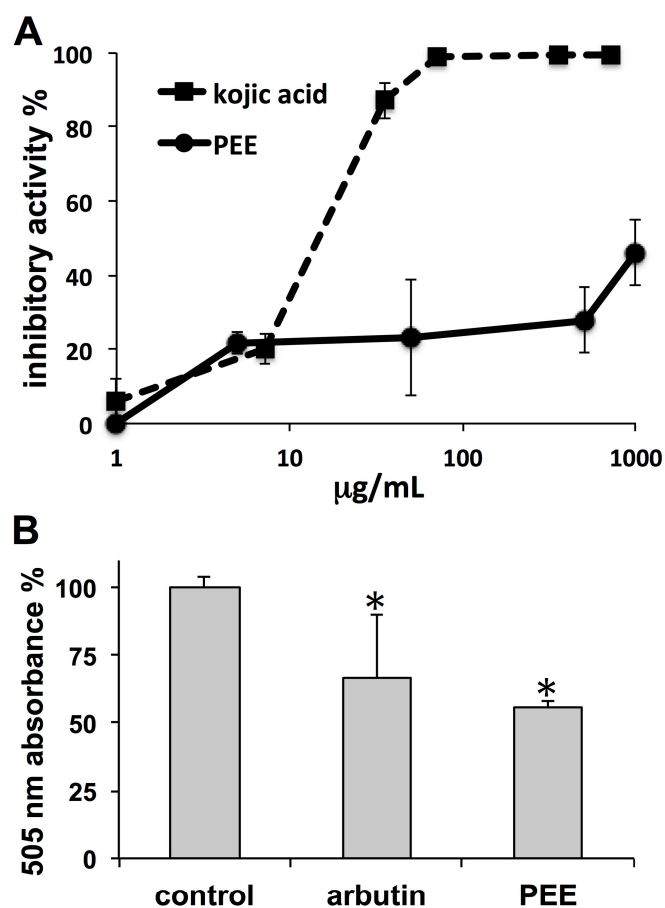


Figure 3. (A) Dose-dependent curves of mushroom tyrosinase inhibition exerted by kojic acid as a positive control (dashed line) and by PEE (continuous line). Tyrosinase activity was determined in a cell-free, in vitro assay using L-tyrosine as a substrate. Data are mean percent inhibitions $\pm$ SD from six replicates in two independent experiments (see Materials and Methods). (B) Inhibition of melanin production in MeWo melanoma cells after 72 h incubation with 1 mg/mL arbutin, or with 50 $\mu\text{g/mL}$ PEE. Cell melanin production was quantified as mean 505 nm absorbances $\pm$ SD from three replicates in two independent experiments. * = $p < 0.01$ with respect to the control.

2.4. Lipolysis Activation

PEE lipolytic activity was assessed in vitro by measuring glycerol released by adipocytes following triglyceride degradation. Determination of a standard curve for glycerol (Figure 4A) allowed for the quantification of the induction of lipolysis by PEE in terms of glycerol released by cells (Figure 4B). Data showed a dose-dependent increase of lipolysis in the range of 10–200 $\mu\text{g/mL}$ PEE, reaching a significant induction at the highest concentration, though lower than that induced by the positive control isoproterenol. The MTT analysis of PEE cytotoxicity on adipocytes revealed a toxicity threshold of $\text{IC}_{05} > 200 \mu\text{g/mL}$ at 48 h, showing that the observed lipolytic effect was not due to cell damage.

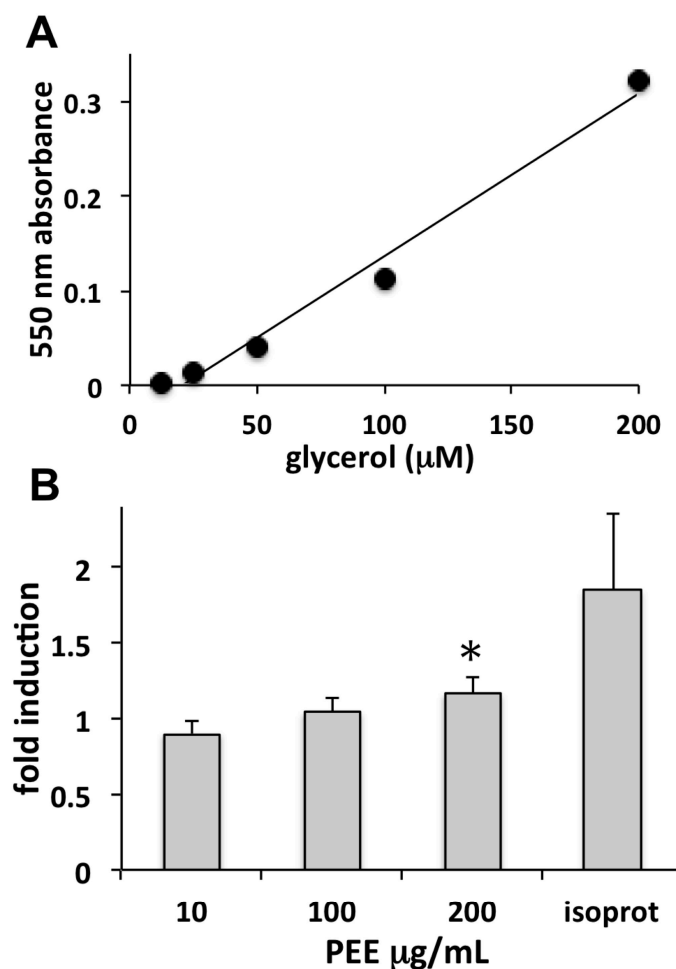


Figure 4. Induction of lipolysis in human adipocytes evaluated by glycerol release. (A) Standard curve of glycerol determined by the ZenBio Kit Human Adipocyte Lipolysis Assay Kit (see Materials and Methods). (B) Assay of glycerol released from adipocytes determined as above after exposure for 3 h to different concentrations of PEE, or to 1 μM isoproterenol (isoprot). Data are mean fold inductions of lipolysis $\pm$ SD, calculated as the ratio between $\mu\text{moles/L}$ of glycerol released by treated cells and by controls, obtained from three replicates in two independent experiments (* = $p < 0.05$).

3. Discussion

This is the first study concerning the effects of a *P. oceanica* extract on skin cells, and one of the very few scientific reports about the biological properties of the *P. oceanica* phytocomplex. In agreement with literature reports about this plant, our HPLC-MS characterization showed that the major compound of PEE was chicoric acid. The resultant extract was enriched in chicoric acid, with amounts 4–5-fold higher than those reported for *P. oceanica* leaves [7]. The quantification of PEE radical scavenging

activity was remarkably high among plant extracts [20], consistent with the richness in chicoric acid and other phenolic compounds typical of *P. oceanica*. Our analyses also confirmed the chemical affinity of seagrass with seaweed, since the iodine content of PEE rated within the range reported for seaweed [21]. Considering the usual doses of extracts from natural sources used in products for humans (e.g., 0.1–1%), the PEE iodine content is quite compatible with a maximum tolerable daily intake of 1.0 mg iodine set by the World Health Organization [22].

Collagen is the main constituent of the dermal matrix, while collagen type I is the most abundant isoform, forming collagen bundles. Collagen is produced by fibroblasts, is essential for skin tone and turgor, and undergoes physiological turnover through continuous degradation by matrix metalloproteinases and replacement by fibroblast neosynthesis. During skin aging, collagen degradation tends to overwhelm renewal, resulting in the formation of fine lines, wrinkles, and other alterations. Hence, the maintenance of fibroblast function is a prerequisite for contrasting skin aging. In our study, the complex of effects induced by PEE on fibroblast growth rate and collagen synthesis indicate a positive stimulation of fibroblast activity, suggesting the possible use of PEE in anti-wrinkle and anti-aging skin care formulations.

The observed PEE inhibitory activities on both tyrosinase and melanoma pigmentation indicate skin whitening properties. In tyrosinase activity assays, the strength of PEE inhibition was much lower than that of the reference compound kojic acid. Even though a different inhibition profile could be possible using L-dopa instead of L-tyrosine as a substrate, the dose-dependent curve of kojic acid fits a logistic trend, consistent with the effect of a single agent, whereas that of PEE diverges from this model, suggesting that the dose-dependent tyrosinase inhibition of PEE could be the result of multiple agents with different inhibitory patterns. In tests on melanoma cells, the PEE demelanizing effect was stronger than that of the reference compound arbutin, confirming that the PEE whitening effect could occur through a complex mechanism, with a component involving tyrosinase inhibition, and another one targeting pathways that regulate cell melanization such as MC1R-MITF [23]. However, regardless of the mechanism, the data indicate the possible use of PEE in formulations aimed at contrasting hyperpigmentation conditions, such as melasma associated with age, freckling, age spots, post-inflammatory melanization, and sites of actinic damage.

Cellulite is a skin condition associated with hypodermal fat accumulation, commonly occurring as lumps and dimples on women's thighs. Adipocytes are fat storage cells of adipose tissue that may undergo excessive fat load, leading to the appearance of cellulite. The lipolytic induction of PEE observed in our study is indicative of a possible use in topically applied products aimed at reducing fat accumulation in adipocytes and unaesthetic cellulite.

Among the compounds of PEE that could be responsible for the observed effects, chicoric acid, the most abundant acid found in PEE, has been shown to possess antioxidant, antiangiogenic, anti-inflammatory, antiallergic, antidiabetic, and anti-HIV activities [24–27]. Among these, only the strong antioxidant power of chicoric acid can be matched to our data, whereas the other findings from our experiments are completely new for *P. oceanica*, and have not been reported for chicoric acid so far. Due to its abundance in the plant and in PEE, the complex consisting of chicoric acid and flavonoids is a major candidate for at least part of the observed PEE effects on skin cells and activities.

In conclusion, we have collected data concerning the biological effects on skin cells of a chicoric acid-rich, ethanolic extract of the seagrass *P. oceanica*. The obtained results represent a novel discovery among the studies on the biological effects of plant phytocomplexes, since they concern a seagrass that is poorly investigated from this point of view. Our findings indicate that the *P. oceanica* phytocomplex can be profitably used in the development of products for contrasting wrinkle formation and skin aging, undesired hyperpigmentation, and cellulite.

4. Materials and Methods

4.1. Reagents and Plant Material

Cell culture reagents and other chemicals were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated. Fresh, beached residues of *Posidonia oceanica* (L.) Delile seagrass were collected in autumn 2015 at Favignana Island, Sicily, under the supervision of the “Area Marina Protetta Isole Egadi” natural reserve (Favignana, Italy). Plants were determined by one of the authors (LC) and voucher specimens were deposited at the Herbarium of DISTAV, University of Genova, Genova, Italy (GE s.n.).

4.2. Extraction

Soon after collection, leaves were separated from shoots, cleaned manually of basal sheath and epiphytes and rinsed in seawater, dehydrated for 36 h in a forced-ventilation oven at 42 °C, and grounded to a particle size of about 1–2 mm. The pulverized material (100 g) was put in a beaker, extracted under shaking in 60% aq. ethanol (1 L), and acidified with formic acid (pH 4.0) at room temperature for 4 h. The residual of the first extraction was separated from the supernatant and subjected to a second extraction as above. The supernatants of the two extraction steps were mixed together, cloth filtered, and then filtered through a Duran[®] sintered glass filter disc (DURAN Group GmbH, Wertheim, Germany) and vacuum-dried in a Buchi Rotavapor R-114 (Buchi Italia s.r.l., Cornaredo, Italy) under controlled temperature (<45 °C). The dried *P. oceanica* ethanolic extract (PEE) was finely pulverized with a mortar and stored at −20 °C until use. The total extraction yield was about 10% (dw/dw).

4.3. Radical Scavenging and Total Polyphenol Assays

Radical scavenging activity of PEE was quantified by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, following a well-established protocol [20]. We used an EtOH solution of DPPH 10^{-4} M; a 4:1 DPPH: sample ratio, a reaction time of 30 min, and the absorbance was recorded at 516 nm. After linear regression, the antioxidant activity was calculated as IC₅₀, i.e., the concentration needed to reduce the initial DPPH absorbance value to half. The above data were also compared to those recorded for a standard solution of ascorbic acid, in order to express the antioxidant power of extracts as ascorbic acid equivalents (AAE) [28].

The total polyphenol content of PEE was determined by the Folin-Ciocalteu method as previously reported [29]. A standard curve was obtained from gallic acid samples and total polyphenols in PEE were expressed as mg GAE per g of dry extract.

4.4. ICP-MS

Total iodine determination in PEE was performed by inductively coupled plasma-mass spectrometry, using a Thermo Scientific X Series 2 ICP-MS equipped with a perfluoroalkoxy (PFA) micro-flow concentric nebulizer (Thermo Fisher Scientific, Waltham, MA, USA). The inlet system included a PC3 Peltier chiller (Elemental Scientific, Omaha, NE, USA) and a cyclonic spray chamber. Plasma worked at the power of 1400 W; coolant, auxiliary, and nebulizer flows were set at 14.0, 0.82, and 0.96 L/min, respectively. The peristaltic pump ran at 30%, except for uptake and washout of the sample, set as fast pump (100%). The Collision Cell Technology-Kinetic Energy Discrimination (CCT-KED) mode used an H₂/He 8/92 mixture, set at a flow of 5.0 mL/min.

An aliquot of 10 mg of PEE was weighed and put in a glass test tube with 0.15 mL of 69% HNO₃, 1.0 mL of 30% H₂O₂, and 3.0 mL of ultrapure H₂O. The test tube was heated at 90 °C in a quartz sand bath for 2 h, and then the volume of the digested solution was increased to 5.0 mL with ultrapure water. Quantitative analysis was performed by means of calibration standard solutions in the range of 1–200 µg/L.

4.5. HPLC-MS

High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS/MS) was performed using an Agilent 1100 HPLC-MSD Ion Trap XCT system, equipped with an electrospray ion source (HPLC-ESI-MS) (Agilent Technologies, Santa Clara, CA, USA). Separation of PEE was performed on a Symmetry C18 column 1 mm × 150 mm with 3 µm particle size (Waters Corporation, Milford, MA, USA). Eluents used were water (eluent A) and MeOH (eluent B), both added with 0.1% formic acid. The gradient employed was: 5% eluent B for 5 min, linear to 40% eluent B in 35 min, then linear gradient to 95% in 15 min, and finally hold at 95% eluent B for another 5 min. The flow rate was set to 30 µL/min and the column temperature was set to 30 °C. The injection volume was 8 µL. Ions were detected in the positive and negative ion mode, in the 200–1000 *m/z* range, and ion charged control with a target ion value of 200,000 and an accumulation time of 300 ms. A capillary voltage of 3300 V, nebulizer pressure of 15 psi, drying gas of 8 L/min, dry temperature of 325 °C, and 2 rolling averages (averages: 5) were the parameters set for the MS detection. MS/MS analysis was conducted using an amplitude optimized time by time for each compound.

4.6. Cell Culture

Stabilized human dermal fibroblasts (46BR.1N, Sigma-Aldrich, St. Louis, MO, USA) were used for cell proliferation and collagen production assays. Fibroblasts were grown in DMEM, supplemented with 10% fetal bovine serum (FBS) at 37 °C, in a 5% CO₂, fully humidified atmosphere.

Cells of the MeWo human melanoma cell line (ATCC, HTB-65) were used for melanin assays. These cells were cultured in RPMI supplemented with 10% FBS, 1% glutamine, and 1% antibiotic mix, at 37 °C in humidified 5% CO₂.

Subcutaneous human preadipocytes (Zen-Bio Inc., Research Triangle Park, NC, USA) were used for lipolysis assays. Preadipocytes were grown in Preadipocyte Medium (cat# PM-1, Zen-Bio Inc.) according to the manufacturer's protocol, at 37 °C in humidified 5% CO₂.

4.7. Cell Viability and Proliferation Assays

PEE effects on cell viability were determined on 46BR.1N fibroblasts, MeWo cells, and adipocytes by the MTT assay, as previously reported [30]. Cells were settled in 96-well plates for 24 h, 10,000 cells per well, and then exposed for 48 h to a logarithmic series of PEE concentrations in DMEM, ranging between 1 and 1000 µg/mL. After the MTT reaction, plates were read at 550 nm in a VMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Cell growth rates were determined on 46BR.1N fibroblasts by settling cells in 96-well plates at a density of 5000 cells per well, and then exposing them for periods of 3 and 10 days to 10 or 20 µg/mL PEE. At the end of exposures, an MTT assay was carried out as above, obtaining cell growth curves.

4.8. Collagen Production

The production of collagen type I by 46BR.1N fibroblasts was evaluated by ELISA as previously reported [30]. Briefly, cells were settled in 96-well plates and incubated with 5, 10, or 20 µg/mL PEE for 48 h. Thereafter, cells were fixed with 3.7% paraformaldehyde, blocked with BSA, probed with mouse anti-human collagen type I (ab6308, Abcam, Cambridge, UK), and then with HRP-conjugated rabbit anti-mouse IgG (ab97046, Abcam), incubated with Pierce 1Step™ Ultra TMB ELISA Substrate Solution (Thermo Fisher Scientific), blocked with 2 M sulfuric acid, and read at 620 nm in the microplate reader.

4.9. Cell-Free Tyrosinase Inhibition Assay

Depigmenting properties of PEE were assessed by the use of an in vitro mushroom tyrosinase inhibition assay [31]. Aliquots of 10 µL of a solution composed of 125 U/mL mushroom tyrosinase in phosphate buffer (pH 6.8) were added to 96-well plates, followed by 70 µL of phosphate buffer and 60 µL of ultrapure water, or PEE dissolved in ultrapure water, in order to obtain a series of final

concentrations ranging between 5 and 1000 µg/mL of PEE. Kojic acid was used instead of PEE as a positive control. Thereafter, 70 µL of 0.3 mg/mL L-tyrosine in ultrapure water was added. Blanks without enzyme were also included for all conditions. Plates were then incubated at 30 °C for 30 min and absorbance was read at 505 nm in the microplate reader. Percent inhibitory activity ($I\%$) was calculated according to the formula:

$$I\% = \left(1 - \frac{(A_{\text{en/ex}} - A_{\text{ex}})}{(A_{\text{en}} - A_{\text{bk}})} \right) \times 100, \quad (1)$$

where $A_{\text{ex/en}}$ = absorbance of assay mixture with extract and enzyme; A_{ex} = absorbance of assay mixture with extract and without enzyme; A_{en} = absorbance of assay mixture with enzyme and without extract; A_{bk} = absorbance of assay mixture without enzyme and extract (blank).

4.10. Melanin Assay

Confluent MeWo cells were suspended and plated in 24-well plates (100,000 cells/well), allowed to settle for 24 h, and then exposed to PEE at a dose of 50 µg/mL for 72 h. Treatment with arbutin (1 mg/mL) was used as a positive control. Thereafter, the culture medium was removed, cells were washed with PBS, trypsinized, centrifuged, and the pellet was subjected to freeze-thawing. The pellet was then dissolved in 100 µL of 1 N NaOH and the crude extract was assayed with the microplate reader at 505 nm to determine the melanin content. All tests were performed in triplicate.

4.11. Lipolysis Assay

The lipolytic effect of PEE was evaluated on primary human adipocytes using the ZenBio Cellulite Treatment Screening Kit Human Adipocyte Lipolysis Assay Kit (cat# LIP-1-L1; LIP-1-NCL1, Zen-Bio Inc., Research Triangle Park, NC, USA) for the detection of free glycerol. Pre-adipocytes were grown as above, settled in 96-well plates, differentiated into adipocytes for one week in Adipocyte Differentiation Medium (cat# DM-2), and maintained for a further week in Adipocyte Medium (cat# AM-1). Fully differentiated adipocytes were incubated for 3 h with 10, 100, or 200 µg/mL PEE, and samples of conditioned medium were then assayed for glycerol as previously reported [29]. Samples were read in the microplate reader at 550 nm. Absorbance increase is proportional to glycerol concentration in the sample.

4.12. Statistics

Data were analyzed with the R package, version 3.0.1 (<http://www.r-project.org/foundation/>), using Student's *t*-test with Bonferroni's correction for multiple comparisons. The difference between two conditions was considered significant if $p < 0.05$.

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References

1. Martins, A.; Vieira, H.; Gaspar, H.; Santos, S. Marketed marine natural products in the pharmaceutical and cosmeceutical industries: Tips for success. *Mar. Drugs* **2014**, *12*, 1066–1101. [[CrossRef](#)] [[PubMed](#)]
2. Thomas, N.V.; Kim, S.K. Beneficial effects of marine algal compounds in cosmeceuticals. *Mar. Drugs* **2013**, *11*, 146–164. [[CrossRef](#)] [[PubMed](#)]

3. Papenbrock, J. Highlights in seagrasses' phylogeny, physiology, and metabolism: What makes them special? *ISRN Bot.* **2012**, *2012*, 103892. [[CrossRef](#)]
4. Vacchi, M.; De Falco, G.; Simeone, S.; Montefalcone, M.; Morri, C.; Ferrari, M.; Bianchi, C.N. Biogeomorphology of the Mediterranean *Posidonia oceanica* seagrass meadows. *Earth Surf. Process. Landf.* **2017**, *42*, 42–54. [[CrossRef](#)]
5. Dumay, O.; Costa, J.; Desjobert, J.M.; Pergent, G. Variations in the concentration of phenolic compounds in the seagrass *Posidonia oceanica* under conditions of competition. *Phytochemistry* **2004**, *65*, 3211–3220. [[PubMed](#)]
6. Cariello, L.; Zanetti, L. Distribution of chicoric acid during leaf development of *Posidonia oceanica*. *Bot. Mar.* **1979**, *22*, 359–360. [[CrossRef](#)]
7. Grignon-Dubois, M.; Rezzonico, B. Phenolic fingerprint of the seagrass *Posidonia oceanica* from four locations in the Mediterranean Sea: First evidence for the large predominance of chicoric acid. *Bot. Mar.* **2015**, *58*, 379–391. [[CrossRef](#)]
8. Haznedaroglu, M.Z.; Zeybek, U. HPLC determination of chicoric acid in leaves of *Posidonia oceanica*. *Pharm. Biol.* **2007**, *45*, 745–748. [[CrossRef](#)]
9. Heglmeier, A.; Zidorn, C. Secondary metabolites of *Posidonia oceanica* (Posidoniaceae). *Biochem. Syst. Ecol.* **2010**, *38*, 964–970. [[CrossRef](#)]
10. Klap, V.A.; Hemminga, M.A.; Boon, J.J. Retention of lignin in seagrasses: Angiosperms that returned to the sea. *Mar. Ecol. Prog. Ser.* **2000**, *194*, 1–11. [[CrossRef](#)]
11. Cannac, M.; Ferrat, L.; Pergent-Martini, C.; Pergent, G.; Pasqualini, V. Effects of fish farming on flavonoids in *Posidonia oceanica*. *Sci. Total Environ.* **2006**, *370*, 91–98. [[CrossRef](#)] [[PubMed](#)]
12. Viso, A.C.; Pesando, D.; Bernard, P.; Marty, J.C. Lipid Components of the Mediterranean Seagrass *Posidonia-Oceanica*. *Phytochemistry* **1993**, *34*, 381–387. [[CrossRef](#)]
13. Hammami, S.; Salem, A.B.; Ashour, M.L.; Cheriaa, J.; Graziano, G.; Mighri, Z. A novel methylated sesquiterpene from seagrass *Posidonia oceanica* (L.) Delile. *Nat. Prod. Res.* **2013**, *27*, 1265–1270. [[CrossRef](#)] [[PubMed](#)]
14. Burlando, B.; Cornara, L. *Posidonia*, la custode della biodiversità del Mediterraneo. *Erbor. Domani* **2016**, *394*, 36–45.
15. Batanouny, K.H. *Wild Medicinal Plants in Egypt*; Academy of Scientific Research and Technology: Cairo, Egypt, 1999.
16. El-Mokasabi, F.M. Floristic composition and traditional uses of plant species at Wadi Alkuf, Al-Jabal Al-Akhder, Libya. *Am. Eur. J. Agric. Environ. Sci.* **2014**, *14*, 685–697.
17. Bernard, P.; Pesando, D. Antibacterial and Antifungal Activity of Extracts from the Rhizomes of the Mediterranean Seagrass *Posidonia oceanica* (L.) Delile. *Bot. Mar.* **1989**, *32*, 85–88. [[CrossRef](#)]
18. Gokce, G.; Haznedaroglu, M.Z. Evaluation of antidiabetic, antioxidant and vasoprotective effects of *Posidonia oceanica* extract. *J. Ethnopharmacol.* **2008**, *115*, 122–130. [[CrossRef](#)] [[PubMed](#)]
19. Martinotti, S.; Ranzato, E.; Burlando, B. In vitro screening of synergistic ascorbate-drug combinations for the treatment of malignant mesothelioma. *Toxicol. In Vitro* **2011**, *25*, 1568–1574. [[CrossRef](#)] [[PubMed](#)]
20. Nikolova, M. Screening of radical scavenging activity and polyphenol content of Bulgarian plant species. *Pharmacogn. Res.* **2011**, *3*, 256–259. [[CrossRef](#)] [[PubMed](#)]
21. Yeh, T.S.; Hung, N.H.; Lin, T.C. Analysis of iodine content in seaweed by GC-ECD and estimation of iodine intake. *J. Food Drug Anal.* **2014**, *22*, 189–196. [[CrossRef](#)]
22. Misurcova, L.; Machu, L.; Orsavova, J. Seaweed minerals as nutraceuticals. *Adv. Food Nutr. Res.* **2011**, *64*, 371–390. [[PubMed](#)]
23. Garcia-Borrón, J.C.; Abdel-Malek, Z.; Jimenez-Cervantes, C. MC1R, the cAMP pathway, and the response to solar UV: Extending the horizon beyond pigmentation. *Pigment Cell Melanoma Res.* **2014**, *27*, 699–720. [[CrossRef](#)] [[PubMed](#)]
24. Tusch, D.; Lajoix, A.D.; Hossy, E.; Azay-Milhau, J.; Ferrare, K.; Jahannault, C.; Cros, G.; Petit, P. Chicoric acid, a new compound able to enhance insulin release and glucose uptake. *Biochem. Biophys. Res. Commun.* **2008**, *377*, 131–135. [[CrossRef](#)] [[PubMed](#)]
25. Lee, N.Y.; Chung, K.S.; Jin, J.S.; Bang, K.S.; Eom, Y.J.; Hong, C.H.; Nugroho, A.; Park, H.J.; An, H.J. Effect of Chicoric Acid on Mast Cell-Mediated Allergic Inflammation in Vitro and in Vivo. *J. Nat. Prod.* **2015**, *78*, 2956–2962. [[CrossRef](#)] [[PubMed](#)]

26. Reinke, R.A.; Lee, D.J.; McDougall, B.R.; King, P.J.; Victoria, J.; Mao, Y.; Lei, X.; Reinecke, M.G.; Robinson, W.E., Jr. L-Chicoric acid inhibits human immunodeficiency virus type 1 integration in vivo and is a noncompetitive but reversible inhibitor of HIV-1 integrase in vitro. *Virology* **2004**, *326*, 203–219. [[CrossRef](#)] [[PubMed](#)]
27. Kimura, T.; Yamamoto, S.; Ogawa, I.; Miura, H.; Hasegawa, M. Antioxidant ability of chicoric acid and its analogous compounds. *Nippon Kagaku Kaishi* **1999**, *1999*, 739–750. [[CrossRef](#)]
28. Hartwig, V.G.; Brumovsky, L.A.; Fretes, R.M.; Sánchez Boado, L. A novel procedure to measure the antioxidant capacity of yerba maté extracts. *Ciênc. Tecnol. Aliment.* **2012**, *32*, 126–133. [[CrossRef](#)]
29. Burlando, B.; Pastorino, G.; Salis, A.; Damonte, G.; Clericuzio, M.; Cornara, L. The bioactivity of Hedysarum coronarium extracts on skin enzymes and cells correlates with phenolic content. *Pharm. Biol.* **2017**, *55*, 1984–1991. [[CrossRef](#)] [[PubMed](#)]
30. Pastorino, G.; Marchetti, C.; Borghesi, B.; Cornara, L.; Ribulla, C.; Burlando, B. Biological activities of the legume crops Melilotus officinalis and Lespedeza capitata for skin care and pharmaceutical applications. *Ind. Crops Prod.* **2017**, *96*, 158–164. [[CrossRef](#)]
31. Kobayashi, Y.; Kayahara, H.; Tadasa, K.; Nakamura, T.; Tanaka, H. Synthesis of amino acid derivatives of kojic acid and their tyrosinase inhibitory activity. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 1745–1746. [[CrossRef](#)]



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6. Other Projects

Lespedeza capitata, a possible use in human's health.

Giulia Pastorino, Diana Pinto, Annalisa Salis, Lizzianne de Francisco, Laura Cornara, Francisca Roudrigues*

Interest is growing in the study of bioactive molecules from plants used in traditional medicine, flanked by new demand by industry (Capecka et al., 2005). The market request is to search natural principles as nutraceuticals, pharmaceuticals and cosmetics. The aim of this study is to explore *Lespedeza capitata* Michx, a plant from Fabaceae's family, for its possible use as medicine, pharmaceutical, and food supplement for human's health. *L. capitata* was commonly used in the tradition of native Americans and today some industries use this plant as food supplement for its diuretic action. The objectives of this study are the research of total phenols, flavonoids, the antioxidant power, the characterization of constituents with HPLC-MS, and also the effect on viability of gut cells. Results from this study will provide a better understanding of the antioxidant properties of this plant for further investigation and development of value-added food and nutraceuticals. In this article we focused on biochemical bioactive constituents with antioxidant activity, that were found in high concentrations in plants (Balasundram et al., 2006). In particular phenolic compounds are natural sources of antioxidants (Balasundram et al., 2006) and present an antioxidant activity, based on their action against free radicals (Baydar 2013). As antioxidants, phenolics have been reported to interfere with the activities of enzymes involved in reactive oxygen species generation (Heim et al., 2002). Some of these compounds have an antioxidant capacity stronger than those of vitamins C and E (Prior 2000).

***Glycyrrhiza glabra*: A review of current uses and future perspectives**

Giulia Pastorino, Francisca Rodrigues, Laura Cornara, M. Beatriz P.P. Oliveira*

In the last years consumers are paying much more attention in the use of natural principles and in the source of new therapeutic compounds. There is a growing demand by industry for plants used in traditional medicine as basic medicines, pharmaceuticals, and food supplements for human's health. *Glycyrrhiza glabra* Linn belongs to the Fabaceae family and has been known since ancient times due to its ethnopharmacological value (Fiore et al., 2005). *G. glabra* presents different phytochemicals, such as glycyrrhizin (GA, glycyrrhizic acid or glycyrrhizinic acid), 18 β -glycyrrhetinic acid (18 β -GA, enoxolone), glabrin A and B (GL), and isoflavones, which are related with several pharmacological activities (Wang et al., 2013). In folk medicine, *G. glabra* has been as anti-inflammatory, antibacterial, antifungal, antidiabetic, antiviral, antiulcer, antitussive, antioxidant, skin whitening, and antidiuretic. The aim of this review is to provide an overview of *G. glabra* in terms of traditional uses, bioactive constituents, and pharmacologic activities of. Moreover, challenges of using this legume in the formulation of new compounds for human's health are also considered.

6.1 Science Events

In parallel to the PhD project, I also worked to the realization of popular science events at the Festival della Scienza (Fig.14). The Festival della Scienza is held yearly in Genova, between the end of October and the beginning of November, including conferences, workshops and exhibitions aimed at bringing the public closer to science.



Fig.14 Festival della Scienza's logo

In collaboration with my tutor Laura Cornara, I realized two events related to botanical topics. The first one was related to my PhD research, and was called “Is the bean magic?” (Fig.15). This laboratory was suitable for all age groups and was centered on the variety and biodiversity of Leguminosae. It was divided into three parts, dedicated to the description of the plant family, of the flower, and of the fruit, respectively. There was also an exhibition where people could see few known Italian products and participate to a short laboratory experience on bean's proteins. The second event was inspired by my feeling for the Japanese culture, and was called “Japanese tradition and....vegetables!”. This exhibition was focused on important plants of the Japanese culture, especially the properties of tea and a simulation of the tea ceremony (Fig.16).

Thousands of people visited these workshops and this made me very proud.

Fig.15 Is the bean magic?



Fig.16 Japanese tradition and...vegetables!

7. Discussion and conclusions

I have focused my research first, on the study of plants belonging to the Fabaceae family and later, during the second year of the Phd project on the seagrass *P.oceanica*. Fabaceae have been used by humans since ancient times for forage, soil improvement, and food. Some species of legumes are also known for their therapeutic virtues but others are used only as a crop, while being underexploited as regards their medicinal potential. Recently, some plants belonging to Fabaceae have been included in the Belfrit list. The "Belfrit Project" (from the initials of the three countries) is an agreement between the governments of Belgium, France and Italy, concerning the use of substances in food supplements and herbal preparations. The competent authorities of Belfrit have defined, based on current scientific evidence, a common list of plant substances and preparations ("Botanicals") that can be used in food supplements. This list is open to updating, with the inclusion of plants not included yet, but admitted in at least one of the three countries. Three of the species studied in my PhD project, namely *M. officinalis*, *L.capitata*, and *G.glabra*, are included in the Belfrit list. *M. officinalis* has been extensively studied and is used for the functionality of venous circulation, as an anti-inflammatory, phlebotonic, spasmolytic, diuretic, anticoagulant, sedative, and as an adjuvant in the treatment of diabetes (Cornara et al., 2016). *L. capitata*, is a plant widely used in the tradition of native americans (Glyzin et al, 1973). It is known for its action on the drainage of body fluids, and for purifying functions, functionality of the urinary tract, cardiovascular regulation, and lipid metabolism. *G.glabra* has been known since ancient time for different therapeutic properties (Wang et al., 2013): anti-inflammatory, antibacterial, antifungal, antidiabetic, antiviral, antiulcer, hepatoprotective, anticancer, antitussive, antioxidant, skin whitening, and antidiuretic agent. Although *H. coronarium* is not present in the Belfrit list, this species is used in herbal medicine too, for astringent, vitaminizing and cholesterol-regulating properties. The genus *Hedysarum* (=Sulla) has a long history of use in TCM, which indicates these plants as adaptogenic and for the treatment of

female disorders (Dong et al., 2013; Li Zhang et al., 2013). Asiatic species akin to *S. coronaria* show an enormous variety in secondary metabolites, while *S. coronaria* had not been studied in this sense. Therefore, this species has been included and chemically characterized in this PhD project. Plant extracts are a rich source of compounds with properties useful for human health (Duque et al., 2017; Marques et al, 2017) and therefore, in the last years they have been used in a growing number of cosmetic preparations, also due to restrictions of use for ingredients of animal origin (Fonseca et al., 2015). Plant secondary metabolites like phenols, polyphenols, and flavonoids serve as sources of antioxidants and perform free radical scavenging (Adedapo et al, 2008; Shridhar et al, 2017). In recent years, the search for phytochemicals possessing antioxidant properties has been on the rise due to the potential use of these agents in the therapy of various chronic and infectious diseases. In the present Phd project, different methods were used to evaluate the total antioxidant capacity of extracts. Total antioxidant activity was analyzed by DPPH (Djacbou et al., 2014), while more specific activities were measured by TPC, TFC, FRAP, captation of superoxide anion, and captation of hydrogen peroxide. Moreover, plants extracts were also evaluated in different kinds of cell-based analyses, such as: cell viability, cell proliferation assay, enzymatic assays, collagen production, melanin assay, cellular mobility, and lypolysis assay. All these assays were performed on extracts from *M. officinalis*, *L. capitata*, *H. coronarium*, and *P. oceanica*. Moreover, in *L. capitata*, *H. coronarium*, and *P. oceanica* we also characterized the chemical composition of the extract with HPLC-MS. For the study of *G. glabra*, I focused my analysis on the antioxidant properties, because this plant has been broadly used as traditional medicine and an ingredient in food industry, particularly as flavour and sweetener agent. For each extract, investigations started with cell viability assays in order to individuate toxicity thresholds for extract doses, or to screen potentially toxic compounds that could affect cell functions and morphology. By using subtoxic doses on cells, I was able to detect the induction of collagen production and proliferation in fibroblasts, depigmentation in

melanoma cells, and lipolysis in adipocytes. In conclusion, the experiments carried out in this PhD project have shown that all the examined extracts possess bioactivities potentially exploitable for skin care applications, suggesting that these plants are suitable for the development of pharmaceutical and cosmetic products. In particular, the complex of data suggests that *L. capitata* and *M. officinalis* extracts possess the ability to stimulate skin cells and tissue regeneration, to prevent skin aging, and to reduce fat deposition beneath the skin, frequently leading to cellulite. It is possible that these effects could be related to the presence in both extracts of flavonoids, which have been previously reported to mediate similar actions. On the other hand, differences in the strength of effects were also observed, possibly depending on the presence of specific constituents in *M. officinalis* and *L. capitata*. The doses at which the effects were observed ranged from 5 to 50 g/mL., which is compatible with pharmacological uses. Therefore, both extracts could be profitably exploited in skin care and pharmaceutical applications, possibly in combined formulations, e.g. for the development of antiage and anticellulite products. Our data also provide that *H. coronarium* is suitable for the development of pharmaceutical and cosmeceutical products targeting major skin problems, such as inflammatory, degenerative and ageing processes, or the insurgence of cellulite. However, the results concerning *H. coronarium* show that the geographical origin of plants is an important factor to be considered for maximally exploiting the biological properties of the species. Finally, data obtained with ethanolic extract of the seagrass *P. oceanica* indicate that this plant can be profitably used in the development of products for contrasting wrinkle formation and skin aging, undesired hyperpigmentation, and cellulite. These results are completely new in the framework of studies concerning the biological properties of phytocomplexes and their applications, given that seagrasses are poorly investigated from this point of view.

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9. References

- Adedapo, A. (2008) Antibacterial and antioxidant properties of the methanol extracts of the leaves and stems of *Calpurnia aurea*. BMC Complement Altern Med, 8 (2008), p. 53
- Alves, R. (2010). Antiradical activity, phenolics profile, and hydroxymethylfurfural in espresso coffee: influence of technological factors. J Agric Food Chem 58(23): 12221-12229.
- Benzie, I. F. and J. J. Strain (1996). The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power": the FRAP assay. Anal Biochem 239(1): 70-76.
- Balasundram, Sundram, Samir (2006) Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. Food Chemistry 99:191-203
- Bonanno, A.; Di Miceli, G.; Di Grigoli, A.; and Amato, G. (2010). Effects of feeding green forage of sulla (*Hedysarum coronarium* L.) on lamb growth and carcass and meat quality. Animal 2011, 5:1, pp 148–154
- Brito; Ramirez; Areche; Sepúlveda; Simirgiotis, (2014). HPLC-UV-MS profiles of phenolic compounds and antioxidant activity of fruits from three citrus species consumed in Northern Chile. Molecules. 2014 Oct 29;19(11):17400-21.
- Burlando, B., Verotta, L., Cornara, L., Bottini-Massa, E., (2010). Herbal Principles in Cosmetics. Properties and Mechanisms of Action. CRC Press, Taylor & Francis Group, Boca Raton.
- Calo, A., et al. (1969). Separation, detection, and determination of *Lespedeza capitata* flavones. Boll Chim Farm 108(10): 587-593.
- Capecka, E., et al. (2005). Antioxidant activity of fresh and dry herbs of some Lamiaceae species. Food Chemistry 93(2): 223-226.
- Carlson and Jones (1940). Some notes on uses of plants by the Comanche Indians. Papers of the Michigan Academy of Science, Arts and Letters 25:517-542.
- Chae, H.N., Cho, M.G., Kim, D.J., Koo, B.Y., Lee, J.B., (2003). A cosmetic composition containing a mixture extract of *Melilotus officinalis* and *Trichosanthes kirilowii* Maxim is provided with anti-inflammatory, moisturizing and abirritant effects. Pat n KR1020030032377
- Chin, Y. W., Jung, H. A., Liu, Y., Su, B. N., Castoro, J. A., Keller, W. J., Kinghorn, A. D. (2007). Anti-oxidant constituents of the roots and stolons of licorice (*Glycyrrhiza glabra*). J Agric Food Chem, 55(12), 4691-4697. doi:10.1021/jf0703553
- Chorepsima, S., Tentolouris, K., Dimitroulis, D., Tentolouris, N., (2013). Melilotus: contribution to wound healing in the diabetic foot. J. Herb. Med. 3, 81–86.
- Cornara, L., Xiao, J., Burlando, B., (2016). Therapeutic potential of temperate forage legumes: a review. Crit. Rev. Food Sci. Nutr. 56 (Suppl. 1), S149–S161.
- Cohen M.N. (1977). The Food Crisis in Prehistory: Overpopulation and the Origins of Agriculture. Yale University Press, New Haven, CT.

- Damle, M. (2014). *Glycyrrhiza glabra* (Liquorice) - a potent medicinal herb. International Journal of Herbal Medicine.
- Dhingra, D., & Sharma, A. (2006). Antidepressant-like activity of *Glycyrrhiza glabra* L. in mouse models of immobility tests. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 30(3), 449-454. doi:http://doi.org/10.1016/j.pnpbp.2005.11.019
- Djabou, D; et al. (2014) Comparison of in vitro antioxidant properties of extracts from three plants used for medical purpose in Cameroon: *Acalypha racemosa*, *Garcinia lucida* and *Hymenocardia lyrata*. Asian Pacific Journal of Tropical Biomedicine. Volume 4, Supplement 2, July 2014, Pages S625-S632
- Dong Y, Tang D, Zhang N, Li Y, Zhang C, Li L, Li M. (2013). Phytochemicals and biological studies of plants in genus *Hedysarum*. Chem Cent J. 7:124.
- Duke JA (1992) Handbook of Legumes of Economic Importance. Plenum Press, New York.
- Duque, L., Bravo, K., Osorio, E., (2017). A holistic anti-aging approach applied in selected cultivated medicinal plants: a view of photoprotection of the skin by different mechanisms. Ind. Crops Prod. 97, 431–439.
- Dweck, A.C., (2011). Formulating Natural Cosmetics. Allured Books, Carol Stream, IL.
- Fiore, C., Eisenhut, M., Ragazzi, E., Zanchin, G., & Armanini, D. (2005). A history of the therapeutic use of liquorice in Europe. Journal of Ethnopharmacology, 99(3), 317-324.
- Fong, H. H. S., et al. (1972). Antitumor Activity of Certain Plants Due to Tannins. Journal of Pharmaceutical Sciences 61(11): 1818.
- Fonseca, S; et al. (2015). Sustainability, natural and organic cosmetics: consumer, products, efficacy, toxicological and regulatory considerations. Braz. J. Pharm. Sci. 51, 17–26.
- Foster, et al. (1990). A Field Guide to Medicinal Plants. Eastern and Central N. America. Houghton Mifflin Co.
- Gilmore (1977). Uses of plants by the Indians of the Missouri River region. University of Nebraska Press, Lincoln, Nebraska.
- Glyzin, et al. (1970). Flavonoids of *Lespedeza bicolor*. Chem Nat Compd 6: 487.
- Guimaraes, R., et al. (2010). Targeting excessive free radicals with peels and juices of citrus fruits: grapefruit, lemon, lime and orange." Food Chem Toxicol 48(1): 99-106.
- Gwaltney-Brant, S. M. (2016). Chapter 8 - Nutraceuticals in Renal Diseases A2 - Gupta, Ramesh C. Nutraceuticals. Boston, Academic Press: 101-108.
- Haddock (2005). Wildflowers and Grasses of Kansas: A Field Guide.
- Harwansh, R. K., Patra, K. C., Pareta, S. K., Singh, J., & Biswas, R. (2011). Pharmacological studies of *Glycyrrhiza glabra*: a review. Pharmacologyonline, 2: 1032-1038.
- Herrick (1995). Iroquois medical botany. Syracuse University Press, Syracuse, New York.

- Huo, H. Z., Wang, B., Liang, Y. K., Bao, Y. Y., & Gu, Y. (2011). Hepatoprotective and Antioxidant Effects of Licorice Extract against CCl₄-Induced Oxidative Damage in Rats. *Int J Mol Sci*, 12(10), 6529-6543. doi:10.3390/ijms12106529
- Kaplan L, Lynch TF. (1999). Phaseolus (Fabaceae) in archeology: AMS radiocarbon dates and their significance in pre-Colombian agriculture. *Econ Bot* 53: 261–272
- Kennedy AR (1995) The evidence for soybean products as cancer preventative agents. *J Nutr* 125: S 733–S743
- Kresge, N., Simoni, R.D., Hill, R.L., (2005). Hemorrhagic sweet clover disease, dicumarol, and Warfarin: the work of Karl Paul Link. *J. Biol. Chem.* 280, e5.
- Kindscher (1992). Medical wild plants of the prairie. ISBN 9780700605279."
- Jahan, Y., & Siddiqui, H. H. (2012). Study of antitussive potential of *glycyrrhiza glabra* and *adhatoda vasica* using a cough model induced by sulphur. *International Journal Of Pharmaceutical Sciences And Research*.
- Jens Borum, Carlos M. Duarte, Dorte Krause-Jensen and Tina M. Greve, (2004). European seagrasses: an introduction to monitoring and management. http://www.seagrasses.org/handbook/european_seagrasses_high.pdf.
- Jorge, et al. (2004). Insulinomimetic effects of kaempferitrin on glycaemia and on ¹⁴C-glucose uptake in rat soleus muscle. *Chem. Biol. Interact* 149, pp. 89-96.
- Linard, A., et al. (1982). Isocarlinoside, a di-C-glycosylflavone from *Lespedeza capitata*. *Phytochemistry* 21(3): 797-799.
- Li Zhang W. et al. (2013). Can Hedysari Radix replace Astragali Radix in Danggui Buxue Tang, a Chinese herbal decoction for woman aliment? *Phytomedicine* 20:1076– 1081
- Lee, C. S., Kim, Y. J., Lee, M. S., Han, E. S., & Lee, S. J. (2008). 18β-Glycyrrhetic acid induces apoptotic cell death in SiHa cells and exhibits a synergistic effect against antibiotic anti-cancer drug toxicity. *Life Sciences*, 83(13–14), 481-489. doi:http://dx.doi.org/10.1016/j.lfs.2008.07.014
- Mamedov, N., Gardner, Z., Craker, L.E., (2005). Medicinal plants used in Russia and central Asia for the treatment of selected skin conditions. *J. Herbs Spices Med.Plants* 11, 191–222.
- Marques P; et al. (2017) *Cynara scolymus* L.: A promising Mediterranean extract for topical anti-aging prevention *Industrial Crops and Products*. Volume 109, 15 December 2017, Pages 699-706
- Mehmet Zeki Haznedaroglu and Ulvi Zeybek (2007) HPLC Determination of Chicoric Acid in Leaves of *Posidonia oceanica* .*Pharmaceutical Biology* 2007, Vol. 45, No. 10, pp. 745–748
- Moerman (1998). Native American ethnobotany. Timber Press, Portland, Oregon. ISBN 0881924539.
- Molteni A. et al. (1995). In vitro hormonal effects of soybean isoflavones. *J Nutr* 125: S751–S756
- Motsei, M. L., Lindsey, K. L., van Staden, J., & Jäger, A. K. (2003). Screening of traditionally used South African plants for antifungal activity against *Candida albicans*. *Journal of Ethnopharmacology*, 86(2–3), 235-241. doi:http://dx.doi.org/10.1016/S0378-8741(03)00082-5

- Mucciarelli M. & Maffei M. (2011). Piante foraggiere alimentari e medicinali - Atlante iconografico Ed. Levotto & Bella ISBN/EAN: 9788882181536. 2011 pp 241.
- Ohtsuki, K., Oh-Ishi, M., & Nagata, N. (1992). The stimulatory and inhibitory effects of glycyrrhizin and a glycyrrhetic acid derivative on phosphorylation of lipocortin I by A-kinase in vitro. *Biochem Int*, 28(6), 1045-1053.
- Pastorino, G., Marchetti, C., Borghesi, B., Cornara, L., Ribulla, C. and Burlando, B. (2017). Biological activities of the legume crops *Melilotus officinalis* and *Lespedeza capitata* for skin care and pharmaceutical applications. *Industrial Crops and Products*. 96:158–64
- Pellegrini, N., et al. (2003). Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. *J Nutr* 133(9): 2812-2819.
- Prior, R. L., et al. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agric Food Chem* 53(10): 4290-4302.
- Quirós-Sauceda, A. E., Ovando-Martínez, M., Velderrain-Rodríguez, G. R., González-Aguilar, G. A., & Ayala-Zavala, J. F. (2016). Chapter 60 - Licorice (*Glycyrrhiza glabra* Linn.) Oils A2 - Preedy, Victor R Essential Oils in Food Preservation, Flavor and Safety (pp. 523-530). San Diego: Academic Press.
- Rackova, L., Jancinova, V., Petrikova, M., Drabikova, K., Nosal, R., Stefek, M., Kovacova, M. (2007). Mechanism of anti-inflammatory action of liquorice extract and glycyrrhizin. *Nat Prod Res*, 21(14), 1234-1241. doi:10.1080/14786410701371280
- Rizzato, G., Scalabrin, E., Radaelli, M., Capodaglio, G., & Piccolo, O. (2017). A new exploration of licorice metabolome. *Food Chem*, 221, 959-968. doi:10.1016/j.foodchem.2016.11.068
- Rodrigues, F., Palmeira-de-Oliveira, A., das Neves, J., Sarmento, B., Amaral, M.H., Oliveira, M.B., (2013). Medicago spp. extracts as promising ingredients for skin care products. *Ind. Crops Prod*. 49, 634–644.
- Roosevelt AC. et al. (1996). Paleo-indian cave dwellers in the Amazon: the peopling of the Americas. *Science* 272:373–384.
- Saeedi, M., Morteza-Semnani, K., & Ghoreishi, M. R. (2003). The treatment of atopic dermatitis with licorice gel. *J Dermatolog Treat*, 14(3), 153-157.
- Shalaby, M., Ibrahim, H. S., Mahmoud, E. M., & Mahmoud, A. F. (2004). Some effects of *Glycyrrhiza glabra* roots extract on male rats. *Egyptian Journal of Natural Toxins*, Vol. 1: 83-94.
- Sheela, M. L., Ramakrishna, M. K., & Salimath, B. P. (2006). Angiogenic and proliferative effects of the cytokine VEGF in Ehrlich ascites tumor cells is inhibited by *Glycyrrhiza glabra*. *Int Immunopharmacol*, 6(3), 494-498. doi:10.1016/j.intimp.2005.07.002
- Shridhar, C; et al. (2017) . In vitro antioxidant and anticancer activity of *Leea indica* leaf extracts on human prostate cancer cell lines. *Integrative Medicine Research* Volume 6, Issue 1, March 2017, Pages 79-87
- Singleton, V. L. and J. A. Rossi (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture* 16(3): 144.

- Sittampalam GS, Coussens NP, Brimacombe K, et al., editors (2004) Assay Guidance Manual Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences
- Smith (1928). Ethnobotany of the Meskwaki. Bulletin of the Public Museum of the City of Milwaukee 4:2(175-326).
- Smythe (1901). Preliminary listing of medicinal and economic plants.
- Stoddard & De Laujudie (2013). Legume futures Report 1.3. Novel feed and non-food uses of legumes. European's Union's Seventh Programme for research.
http://www.legumefutures.de/images/Legume_Futures_Report_1.3.pdf
- Tian, J., Liu, W., Zhen, Z., & Tong, X. (2013). Successful treatment of latent autoimmune diabetes in adults with Traditional Chinese Medicine: a case report. Journal of Traditional Chinese Medicine, 33(6), 766-769. doi:[http://dx.doi.org/10.1016/S0254-6272\(14\)60010-8](http://dx.doi.org/10.1016/S0254-6272(14)60010-8)
- Terrill TH, Douglas GB, Foote AG, Purchas RW, Wilson GF, Barry TN. (1992). Effect of condensed tannins upon body growth, wool growth and rumen metabolism in sheep grazing sulla (*Hedysarum coronarium*) and perennial pasture. J Agric Sci. 119:265–273.
- Tong, X., Xie, Q., Rong, G., Zhou, S., & Meng, Q. (2015). Detection of consensus and treatment principles of diabetic nephropathy in traditional Chinese medicine: A new approach. Journal of Traditional Chinese Medical Sciences, 2(4), 270-283. doi:<http://dx.doi.org/10.1016/j.jtcms.2016.02.001>
- Varsha, S., & Sonam, P. (2013). Phytochemical screening and determination of anti-bacterial and anti-oxidant potential of *Glycyrrhiza glabra* root extracts. Journal of Environmental Research And Development.
- Villa (2002). An anti-edema composition containing rutin. EP 1226827 A1 20020731.
- Yang, J., Wang, L.-L., Zhou, X.-T., Yuan, J.-Z., Zhang, T.-J., Liu, F., (2014). Chemical constituents from *Melilotus officinalis*. Chin. Tradit. Herb. Drugs 45, 622–625.
- Yang, R., Yuan, B. C., Ma, Y. S., Zhou, S., & Liu, Y. (2017). The anti-inflammatory activity of licorice, a widely used Chinese herb. Pharm Biol, 55(1), 5-18. doi:10.1080/13880209.2016.1225775
- Yarnell, E. (2002). Botanical medicines for the urinary tract. World J. Urol 20, pp. 285–293.
- Wagner, H., et al. (1972). Flavonoids in *Lespedeza capitata*. Phytochemistry 11(4): 1518.
- Wang, J., Chen, X., Wang, W., Zhang, Y., Yang, Z., Jin, Y., Yang, G. (2013). Glycyrrhizic acid as the antiviral component of *Glycyrrhiza uralensis* Fisch. against coxsackievirus A16 and enterovirus 71 of hand foot and mouth disease. J Ethnopharmacol, 147(1), 114-121. doi:10.1016/j.jep.2013.02.017
- Wang, L., Yang, R., Yuan, B., Liu, Y., & Liu, C. (2015). The antiviral and antimicrobial activities of licorice, a widely-used Chinese herb. Acta Pharmaceutica Sinica. B, 5(4), 310-315. doi:10.1016/j.apsb.2015.05.005
- Wong, S. P., et al. (2006). Antioxidant activities of aqueous extracts of selected plants. Food Chemistry 99(4): 775-783.
- Zhishen, J., et al. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chemistry 64: 555-559

