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TITOLO TESI

Natural and Synthetic Sources as Antioxidant and Inhibitors of Tyrosinase

SSD BIO/10

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Declaration

I declare that this thesis represents my own work, except where due acknowledgement is made, and that it has not been previously included in a thesis, dissertation or report submitted to this University or to any other institution for a degree, diploma or other qualifications.

Signed ……

Amalia Di Petrillo

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Riassunto

La melanogenesi è il processo fisiologico che porta alla formazione di melanina, un pigmento che svolge un ruolo importante nella protezione contro i danni generati dai raggi ultravioletti e rappresenta un importante sistema di difesa della pelle.

La sovrapproduzione e l'accumulo di melanina si verificano in diversi disturbi della pelle, tra cui la melanosi solare, il melasma, le lentigo senili e l'iperpigmentazione post-infiammatoria. Poiché la tirosinasi è l'enzima chiave della melanogenesi, i suoi inibitori sono diventati sempre più importanti come agenti depigmentanti nei disturbi dell'iperpigmentazione.

Dal momento che molti agenti sbiancanti noti sono tossici, c'è stato un crescente interesse nell'identificare inibitori alternativi della tirosinasi, in particolare da fonti naturali. In questa tesi è stata studiata l'attività inibitoria della tirosinasi di diversi estratti naturali (*Asphodelus microcarpus, Sarcopoterium spinosum, Phytolacca dioica* e diversi mieli) e di eteroarilumarine neo-sintetizzate. Inoltre, degli stessi è stata valutata l'attività antiossidante e la citotossicità.

I risultati hanno mostrato che tutti gli estratti hanno un'attività inibitoria sulla tirosinasi dose dipendente. L'estratto etanolico delle foglie dell' *Asphodelus microcarpus* e l'estratto in etilacetato della *Phytolacca dioica* hanno mostrato la migliore attività inibitoria. Gli stessi estratti hanno mostrato la più alta attività antiossidante e livelli elevati di polifenoli e flavonoidi. Per quanto riguarda i composti neo-sintetizzati, due hanno mostrato attività inibitoria sulla tirosinasi anche su cellule B16F10. Questi risultati incoraggiano a continuare la ricerca su composti sintetici derivati dalle cumarine. Per quanto riguarda gli estratti, invece, questi potrebbero essere un ottima fonte di composti bioattivi da utilizzare come agenti depigmentanti nei disturbi della pelle.

Abstract

Melanogenesis is a physiological pathway for the formation of melanin, a pigment which plays an important role in the protection against UV damage and represents an important defense system of the skin against harmful factors.

Overproduction and accumulation of melanin occur in several skin disorders including solar melanosis, ephelides, melasma, senile lentigos and postinflammatory hyperpigmentation. Since tyrosinase is the limiting step enzyme in melanogenesis, its inhibitors have become increasingly important as depigmenting agents in hyperpigmentation disorders.

Since (or considering that) many known whitening agents have been proven to be toxic, there has been increasing impetus to identify alternative tyrosinase inhibitors, especially from natural sources. In this thesis has been investigated the inhibitory activity on tyrosinase of different natural extracts (A*sphodelus. microcarpus, Sarcopoterium spinosum, Phytolacca dioica* and several honeys) and of synthetic heteroarylcoumarins. It has been also evaluated the antioxidant activity and the citotoxicity of several compounds and extracts.

The results showed that all extracts have a direct inhibitory anti-tyrosinase activity with flowers ethanol extract of *Asphodelus microcarpus* and ethylacetate extract of *Phytolacca dioica* exhibiting the stronger effect. The same extracts showed the highest antioxidant activity and an elevated levels of total phenolics and flavonoid content.

As for the neo-synthesized compounds, two of them have shown inhibitory activity on tyrosinase even on B16F10 cells. These results encourage the the further deepening of research both on synthetic compounds derived from coumarins and on the extracts, that could be a good source of bioactive compounds useful as depigmenting agents in skin disorders.

Resumen

La melanogénesis es una vía fisiológica para la formación de melanina, un pigmento que juega un función importante en la protección contra los daños por UV y representa un importante sistema de defensa de la piel contra factores nocivos.

La sobreproducción y la acumulación de melanina ocurren en varios trastornos de la piel incluyendo melanosis solar, ephelides, melasma, lentigos seniles y hiperpigmentación postinflamatoria. Dado que la tirosinasa es la enzima de paso limitante en la melanogénesis, sus inhibidores se han vuelto cada vez más importantes como agentes despigmentantes en trastornos de hiperpigmentación.

Se ha demostrado que muchos agentes blanqueadores conocidos son tóxicos, esto ha aumentado el impulso para identificar inhibidores de tirosinasa alternativos, especialmente de fuentes naturales. En esta tesis se ha investigado la actividad inhibidora de la tirosinasa de diferentes extractos naturales (*Asphodelus microcarpus, Sarcopoterium spinosum, Phytolacca dioica* y varias mieles) y de heteroarilcumarinas sintetizadas. También se ha evaluado la actividad antioxidante y la citotoxicidad.

Los resultados mostraron que todos los extractos tenían una actividad anti-tirosinasa inhibitoria dependiente de la dosis. Con FEE de *Asphodelus microcarpus* y AE de *Phytolacca dioica* que exhiben el efecto más fuerte. Los mismos extractos mostraron la mayor actividad antioxidante y niveles elevados de fenoles y flavonoides. En cuanto a los compuestos neo-sintetizados, dos han mostrado actividad inhibidora sobre tirosinasa incluso en células B16F10. Estos resultados fomentan la continuación de la investigación sobre compuestos sintéticos basados en cumarina. Respecto a los extractos en su lugar, estos podrían ser una buena fuente

de compuestos bioactivos que podrían ser utilizados como agentes despigmentantes en trastornos cutáneos.

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List of abbreviations

***α*-MSH**: α-Melanocyte Stimulating Hormone

β-FGF: basic Fibroblast Growth Factor

AAPH: 2,2'-Azobis-2-Methyl-Propanimidamide, Dihydrochloride

ABTS: 2,2′-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid)

AE: Ethyl acetate extract

ASP: Agouti Signal Protein

AW: Aromatic Water

BE: *n*-Butanol Extract

BSA: Bovine Serum Albumin

CE: Crude Extract

DHI: 5,6-Dihydroxyindole

DHICA: 5,6-Dihydroxyindole-2-Carboxylic Acid

DOPA: 3,4-Dihydroxyphenylalanine

DPPH: 2,2-Diphenyl-1-Picrylhydrazyl Radical

DAD: Diode Array Detector

DQ: Dopaquinone

ET-1: Endothelin-1

FBS: Fetal Bovine Serum

FEE: Flowers Ethanol Extract

FL: Fluorescein Sodium Salt

GAE: Gallic Acid Equivalent

HD: Hydrodistillation

HE: Hexane Extract

HQ: Hydroquinone

HSCCC: High Speed Counter Current Chromatography

LEE: Leaves Ethanol Extract

MBC: Minimum Bactericidal Concentration

MBIC: Minimal Biofilm Inhibitory Concentration

MC1-R: Melanocortin 1 Receptor

MIC: Minimal Inhibitory Concentration

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

ORAC: Oxygen Radical Absorbance Capacity

QE: Quercetin Equivalent

ROS: Radical oxygen species

RW: Residual Water

SE: Soxhelet extract

SFE: Supercritical CO_2 Extractions

TEE: Tubers Ethanol Extract

TLC: Thin-layer chromatography

Trolox: 6-Hydroxy-2,5,7,8-Tetramethylchromane-2-Carboxylic Acid

TRP-1: Tyrosinase Related Protein-1

TRP-2: Tyrosinase-Related Protein-2

TYR: Tyrosinase

UVR: Solar Ultraviolet Radiation

WE: Water Extract

XO: Xanthine Oxidase

CHAPTER 1 – INTRODUCTION

1.1. Melanin pigment

Melanins are natural pigments, derivatives of the amino acid tyrosine, commonly distributed found in bacteria, fungi, plants and animals.

Structurally, melanins are a group of complex pigments with a structure relative diverse and undefined. They have been classified in several ways during the last 50 years (d`Ischia M. et al., 2013), based on their precursor molecules, melanins are classified into four groups:

- Eumelanin;
- Pheomelanin;
- Neuromelanin;
- Allomelanin.

All these pigments have in common their arrangement of different units linked by carbon-carbon bonds (C-C), but differ from each other in chemical composition, as well as structural and physical properties.

Eumelanin and pheomelanin are the main pigment responsible for the various pigmentations found in animal and human skin, hair, and eyes. Eumelanin provides primarily dark colors, from brown to black. Small amounts of eumelanin can give place to grey colors, as in human hair at mature age (Robbins R., 2012). Pheomelanin produces yellowish or reddish colors, and it is found in relatively large quantities in red hair, freckles, and feathers of fowls and other birds (Rorsman H. et al., 1979.). Both types of melanin are generated from a common precursor, dopaquinone (DQ), produced from tyrosine by the action of tyrosinase (TYR) (EC 1.14.18.1) (d`Ischia M. et al., 2009).

Eumelanin (Fig. 1A) is a chromophore, which is a cross-linked polymer molecule, insoluble in most solvents and tightly associated with proteins through covalent bonds. Moreover, eumelanins behave like polyanions with the capability to reversibly bind cations, anions, and polyamines in reactions facilitated by their high carboxyl group content. The semiquinone units that form during melanine

biosynthesis are also responsible for eumelanin actions as redox pigment with both reducing and oxidizing capabilities towards oxygen radicals and other chemical redox systems (Ito S., 2003). Eumelanin is the most important photoprotective factor, since melanins show a broadband UV-visible absorption spectrum (Kollias N., 1995), and they are able to dissipate up to 90% of the absorbed energy from the sunlight radiation. Thus, melanin prevents the skin from the potentially damaging effects of UV light. Besides, many epidemiological studies have shown a lower incidence for skin cancer in individuals with darker skin compared to those with fair skin (Diffey B.L., et al., 1995). It has been suggested that UV-induced photodamage and its repair are signals that induce melanogenesis.

Pheomelanin (Fig. 1B) synthesis pathway proceeds spontaneously, it is controlled by the concentrations of metabolites and inherent pH. This melanin is a benzothiazin monomer derived dopaquinone following nucleophilic addition of glutathione or cysteine; it consists of a lightly colored pigment (Costin & Hearing, 2007) and it is alkali soluble. Pheomelanin is tightly bound to proteins, indicating that in vivo it occurs as a chromoprotein, with high variability in nitrogen and sulfur content (C/N and C/S ratios). Furthermore, it can also act as a binding agent for drugs and chemicals and, like eumelanin, contains semiquinones with their associated paramagnetic properties. Despite of eumelanin, photolysis of pheomelanins determine products like superoxide, hydroxyl radicals and hydrogen peroxide so it may easily become a photosensitized agent rather than a photoprotector.

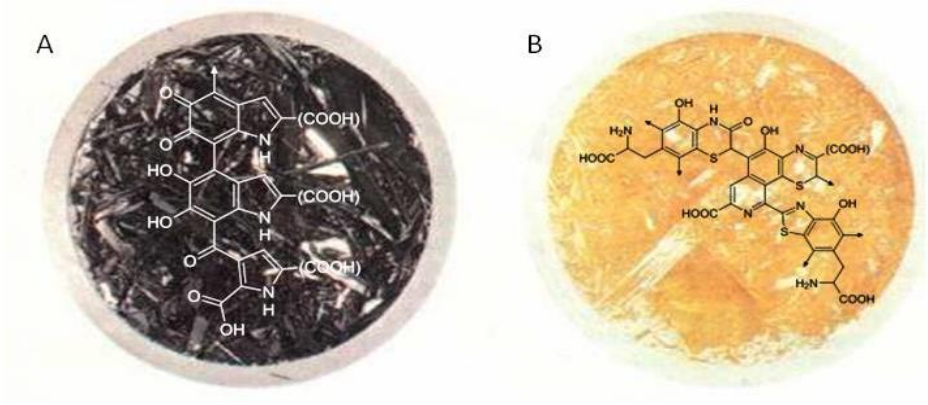


Figure 1: Part of the structural formula of eumelanin (A) and pheomelanin (B), "(COOH)" can be COOH or H, or (more rarely) other substituents.

Neuromelanin is a dark pigment occurs in some groups of neurons of vertebrate nervous systems with mixed properties of both eu- and pheomelanins. It differs from melanin pigment occurring elsewhere in its mode of formation and for its chemical composition.

They are macropolymers composed of aminochromes and noradrenalinochromes and originate from the oxidation of catecholamines and the subsequent interactions with other components of the cell such as proteins, lipids, and metals (Zecca L. et al., 2001).

It is hypothesized that neuromelanin, in physiological conditions, can protect the neurons by removing excess of reactive catecholamines from the cytosol, chelating metals, and binding toxic compounds. On the other hand, in oxidative stress conditions, such as iron overload or increased reactive oxygen species, neuromelanin can contribute to the formation of reactive compounds. The decrease in neuromelanin, as observed in Parkinson's disease may be the cause of the associated degenerative symptoms. (Zucca F.A. et al., 2003.).

The allomelanins, produced by higher plants and fungi, is formed by the oxidation of polyphenols, such as catechols and 1,8-dihydroxynaphtalene. Its color is always from dark brown to totally black and its structure depends on the nature of the main unit oxidized. Some vegetables use just normal

catechol, but others use different catecholic acids (such as caffeic, chlorogenic, protocatechuic, or gallic acids) (Solano F., 2014).

Allomelanins in plants are formed after minor cuts or wounds as a result of bird or insect bites. The reaction that conduces to allomelanins is a consequence of phenolic compounds oxidation by polyphenol oxidase (PPO). These mechanism of action is considered a primitive form of immunity but these reactions is cause of deterioration and loss of food quality (Yoruk R. and Marshall M.R., 2003). In this regard, polyphenol oxidase inhibitors to prevent browning are much more important than the melanin responsible of the browning.

1.2. Melanogenesis

Melanins pigments molecules are endogenously synthesized in melanosomes by melanocytes in a complex process called melanogenesis.

Melanocytes, specialized skin cells located in the basal layer of the epidermis, produce dermal melanin. Melanocytes insert granules of melanin into specialized cellular vesicles called melanosomes, and, under physiological conditions, melanin synthesis in melanocytes is restricted to these. In general, melanosome structure correlates with the type of melanin produced and its development involves four steps. Melanin is formed at III stage and in stage IV melanosomes are fully melanized. Under pathological conditions (e.g., melanoma), this orderly process is deregulated (Jimbow K et al., 1984).

The melanosomes mature within the melanocytes and pass to the outer tips of the dendrites, where they are transferred into the other skin cells of the epidermis. Melanosome transfer is a cytophagic process during which a portion of a melanocyte dendrite is pinched off by the epidermal cell so that melanosomes and melanocyte cytoplasm are incorporated into the keratinocyte.

Once the keratinocytes receive these melanosomes, they incorporate the granules and aggregate them over the nucleus, and degrade them as the keratinocytes undergo terminal differentiation.

Melanogenesis is a process formed by a combination of chemical and enzymatically catalyzed reactions that convert the amino acid tyrosine to melanin pigments through a series of intermediates

The melanogenesis process is initiated with the oxidation of L-tyrosine or L-DOPA to DQ by the key enzyme, TYR. Once produced, DOPA can auto-oxidize and cyclize spontaneously to produce DHI (Fig. 2); however, TYR is not the only melanogenic enzyme involved in the pathway and there are at least three other melanosomal proteins that in part determine melanin production.

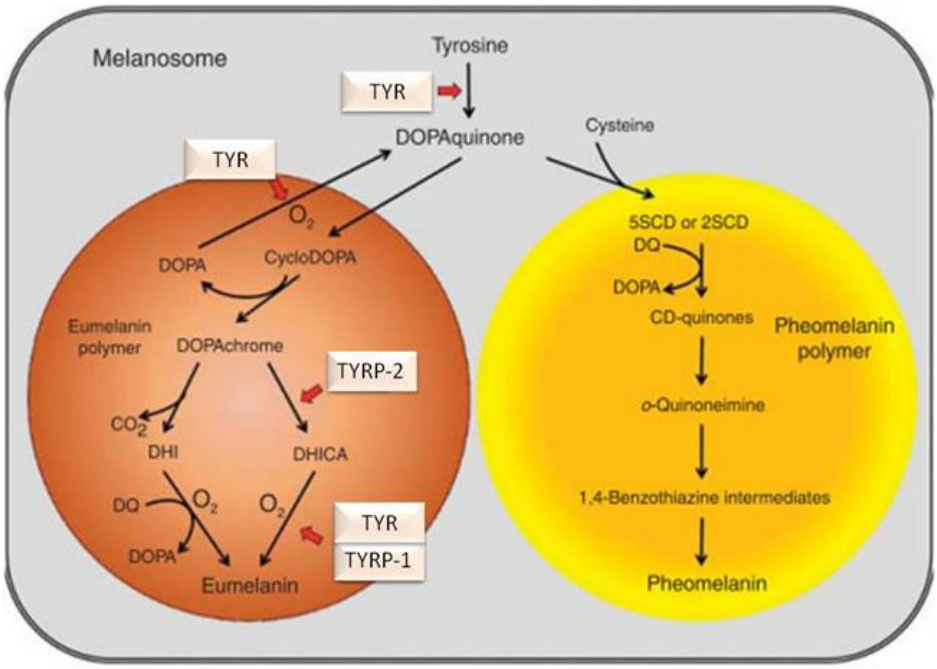


Figure 2: Melanin biosynthetic pathway.

The first of these is DOPAchrome tautomerase, also known as tyrosinase related protein 2 (TRP2); TRP2 functions specifically to tautomerize DOPAchrome to DHICA. DHICA is a melanogenic intermediate that still contains the carboxylic acid group, which is spontaneously lost in the absence of TRP2 catalytic activity upon which DHI is produced. The tyrosinase-related protein 1 (TRP1), functions as a DHICA oxidase, and promotes further oxidation and polymerization of eumelanin.

(Hearing V. J., 1999). The other major diversion in the melanogenic pathway occurs upstream in the pathway immediately following the production of DOPAquinone from DOPA. It is not yet known how this switch to produce pheomelanin is regulated, but if a sulfhydryl donor, probably cysteine (Potterf S.B., 1998), is available when DOPAquinone is generated, the latter will be stoichiometrically converted to cysteinylDOPA. Once this sulfur group has been incorporated into the melanin polymer, further oxidation, cyclization, and polymerization leads to the production of pheomelanin.

Skin pigmentation is influenced by many factors amongst these: ultraviolet light (UV), basic fibroblast growth factor (β-FGF), genetic components α-melanocyte stimulating hormone (α-MSH), agouti signal protein (ASP), and endothelin-1 (ET-1) and drugs. (Lam Do PhuongUyen, et al., 2008)

UV is a major environmental factor that dramatically alters the homeostasis of the skin by affecting the survival, proliferation and differentiation of various cutaneous cell types. The effects of UV on the skin include direct damage to DNA, apoptosis, growth arrest and stimulation of melanogenesis. Long-term effects of UV include photoaging and photocarcinogenesis. As reported above, eumelanin is much more efficient at blocking UV photons than pheomelanin, thus the more eumelanin in the skin, the less UV-permeable is the epidermis. UV rays penetrate into the deep layers of the subcutaneous tissue and its absorption is increased by the content of aromatic acids, such as tyrosine, tryptophan, and phenylalanine and urocanic acid. Urocanic acid, which forms in the keratinocyte by the process of keratinization, is present mainly in the stratum corneum and is important for skin moisture maintenance, and stimulation of stratum corneum thickening and melanin synthesis by melanocytes (Stanojevic, M., 2004).

UV can increase the synthesis of β-FGF in keratinocytes, which in turn stimulates proliferation and melanogenesis of epidermal melanocytes.The exposure of keratinocytes to UV results in the upregulation of β-FGF and other keratinocyte-derived cytokines such as ET-1, which is a small peptide originally isolated from endothelial cells. It plays an important role in stimulating

melanocyte proliferation and melanization through the G protein coupled endothelin B receptor-mediated signal transduction pathway and can also lead to an increase in TYR activity and increase in melanin production (Halaban R., et al., 1988).

Interactions between α-MSH and ASP are critical for the switch to produce eumelanin or pheomelanin. α-MSH produced from UV stimulated keratinocytes promotes eumelanin synthesis, whereas ASP promotes pheomelanin synthesis.

The effects of α-MSH are mediated by its binding to the MSH receptor (MSH-R), which is known as the melanocortin 1 receptor (MC1-R) (Fig.3). This binding, through various step, determines an increase in the intracellular concentration of cAMP, which causes an increase in TYR activity and eumelanin production. If the MC1-R is dysfunctional and fails to initiate a significant rise in the intracellular level of cAMP, pheomelanins are produced (Valverde P., et al., 1995). ASP acts as a competitive antagonist of α-MSH for MC1-R binding. MC1-R in melanocytes is considered to be a control point for pigmentation. MC1-R is also present on other cells such as monocytes, endothelial cells, and keratinocytes. When ASP is present melanocytes switch into their pheomelanogenic mode (Furumura M., et al., 1996).

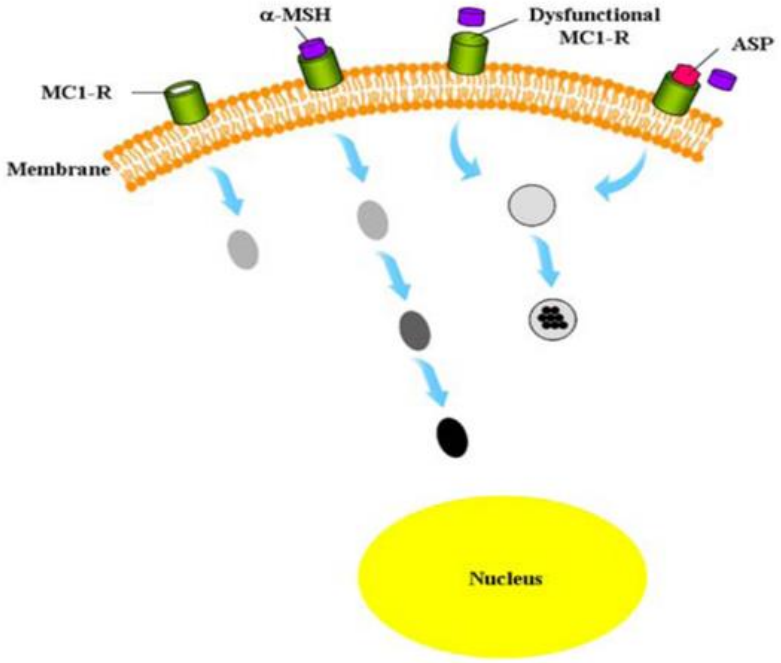


Figure 3: Interactions that control melanin production

Pigmentation may be induced by a wide variety of drugs; the most common ones include non-steroidal anti-inflammatory drugs (NSAIDs), phenytoin, antimalarials, amiodarone, antip- sychotic drugs, cytotoxic drugs, tetracyclines, and heavy metals. Drug-induced skin pigmentation may result from increased melanin synthesis, increased lipofuscin synthesis or cutaneous deposition of drug-related material. Certain heavy metals, such as iron, may be deposited in the dermis following damage to dermal vessels. If deposited in sufficient quantities a distinctive change in skin color may be observed without any significant increase in melanin. Some drugs react with melanin in different ways, including to form a drug-pigment complex, induce accumulation of melanin as a non-specific post-inflammatory change in predisposed individuals or induce pigmentation directly by accumulating and reacting with other substances in the skin.

Disorders of pigmentation

Skin color is highly individual and the variations are controlled by numerous factors like previously mentioned. Disorders of pigmentation can result from migration abnormalities of melanocytes from the neural crest to the skin during embryogenesis. In addition, impairment of melanosome transfer to the surrounding keratinocytes, an alteration in melanin synthesis and a defective degradation or removal of melanin may lead to abnormal skin pigmentation. Immunologic or toxic mediated destructions of melanocytes can end in pigmentation disorders. Disorders of pigmentation are classified in hypo- or hyperpigmentation which can occur as a genetic or acquired disease.

Among the most common skin disorder we have:

- *Melasma* (*chloasma*)

  Melasma is characterized by symmetrically distributed macules with irregular borders ranging from light brown to dark brown to gray-brown, which is the most common pigmentation disorders involving the face. Genetic backgrounds, chronic exposure to UV radiation, and female sex hormones have been implicated as the main causes of melasma, but exposure to the sun is necessary for its development. (Vaneeta et al., 2011)

- *Freckles*

  Freckles are a small brown patch on the skin, coloured by abundant epidermal melanin without any increase in the number of melanocytes. Freckles occur only on light-exposed skin and tend to darken in summer and fade in winter. The brown to black colour of lentigines is also produced by epidermal melanin, but in the lentigo there is an increased number of melanocytes scattered along the basal layer. (Launey & Land, 1984)

- *Lentigines*

  Lentigines are small, rounded and brown to black areas, which may coalesce to form patches or slightly elevated lesions, a centimeter or more across. Unlike freckles, lentigines do not darken on exposure to sunlight, but elderly people often develop large lentigines on light-exposed areas (Burton, 1979).

- *Vitiligo*

Vitiligo is a common, acquired diseases affecting 1% to 2% of the population with women being more affected than men. Fifty percent develop their lesions before age of 20 and 25% before the age of 10. It is likely that predisposition to Vitiligo is inherited as an autosomal dominant characteristic. Localized or generalized areas of the skin completely lack melanin pigmentation. Pigment cells (melanocytes) cannot be detected in depigmented areas, even on inspection by electron microscopy. This finding is in contrast to albinism, in which melanocytes are present but there is little or no pigmentation because of faulty or absent melanin synthesis. The cause of Vitiligo is unknown, an abnormal neurogenic stimulus, intrinsic genetic defect of melanocytes as an enzymatic selfdestruction mechanism involving a deficiency of a melanocytic growth (Arndt & Bowers, 2002).

- *Albinism*

Albinism is a heterogeneous cluster of disorders linked by an impaired capacity for melanin synthesis. The skin and hair are pigmented normally but affected males have translucency of the iris, defective pigmentation of the retina, nystagmus and photophobia. Female heterozygotes are less severely affected (Launey & Land, 1984).

1.3. Tyrosinase

Tyrosinase (EC 1.14.18.1) (Fig.4) is a dinuclear copper-containing multifunctional enzyme widely distributed in nature.

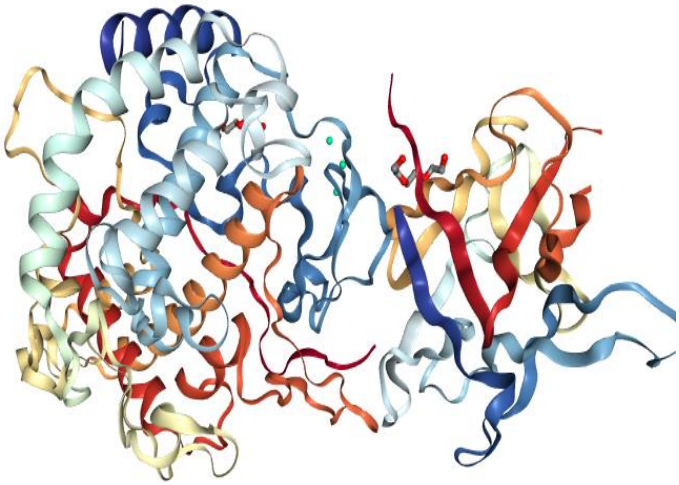


Figure 4: crystal structure of ppo3, a tyrosinase from agaricus bisporus, (pdb code: 2y9w)

The best-characterized tyrosinases are derived from *Streptomyces glaucescens*, *Neurospora crassa* and *Agaricus bisporus*. The enzyme extracted from the champignon mushroom *A. bisporus* is highly homologous with the mammalian ones and this renders it well suited as a model for studies on melanogenesis (Te-Sheng Chang, 2009). TYR from *A. bisporus* was reported to be a heterotetramer comprising two heavy (H) and light (L) chains with a molecular mass of 120 kDa. The core has an α-helical structure made up of a four-helix bundle (α2, α3, α6, α7). Two copper ions (called CuA and CuB) are located in the active site, CuA is coordinated by three histidine residues His 38, His 54 and His 63 and the CuB is coordinated by His 194, His 216 and His 190, respectively. The human TYR gene family has three members: TYR, TRP-1, and TRP-2 o DCT1. They bind different divalent metal cations and have different catalytic properties. They act in a complex; DCT1 binds zinc, tyrosinase binds copper, and it is unclear which cation is bound by TRP-1. TRP-1 and TYR share 40% AA identity. DCT1 and TYR share 32% identity; both were

identified because tyrosinase antibodies recognize them. During the process of melanin-pigments formation are involved three forms of the enzyme (Espín J.C., 2000)

The principal endogenous substrates of mushroom tyrosinase are L-tyrosine, p-aminophenol, and its condensation product with glutamate-glutaminyl-4-hydroxybenzene (GHB), all three being derived from the shikimate pathway (Stüssi and Rast 1981). Tyrosinase substrates can be divided into three groups, depending upon the nature of the quinonoid intermediate:

- o-quinone products contain a sidechain capable of intramolecular 1,4-addition to the quinone double bond;
- o-quinone products do not contain a sidechain and are uncyclizable but can undergo addition of water to the quinone double bond (Dawson and Tarpley 1951);
- o-quinone products are highly stable through the reaction and do not undergo nucleophilic attack (Ros-Martinez et al 1993).

Mono, di and trihydroxyphenols are tyrosinase substrates, however Tyr has greater affinity for dihydroxyphenols. Also, among the monohydroxyphenols (p-cresol and tyrosine), dihydroxyphenols (catechol, L-DOPA, D-DOPA, catechin and chlorogenic acid) and trihydroxyphenols (pyrogallol), catechol showed maximum activity, indicating that the enzyme is most active with catechol as a substrate (Zhang et al 1999).

Tyrosinase exhibits a characteristic feature of a lag period related to its monophenolase activity. The lag period is an autocatalytic process, which depends on the generation of the dihydric phenol substrate. Moreover, the lag period also depends on other factors such as enzyme concentration, enzyme source, pH of the medium, the presence of a hydrogen donor such as catechols and transition metal ions. Adding reducing agents, such as ascorbate, hydroxylamine and hydroxyquinone, can shorten the lag period, but they are less effective than *o*-dihydroxyphenols.

1.4. Tyrosinase Inhibitors

Although three enzymes (Tyr and TRP-1/2) are involved in the melanogenesis signaling pathway, Tyr is most critical and rate-limiting enzyme due to its key role in the process. Its inhibitors have become increasingly important as depigmenting agents that may be used as skin-whitening agents for treating skin disorders (Kim and Uyama, 2005; Wang et al., 2011) that represent one of the major dermatological concerns.

As mentionated above, photoaging, melasma and post-inflammatory conditions may lead to accumulation of abnormal amounts of melanin in the skin, causing dyspigmentation or hyperpigmentation and esthetic problems. Many approaches to inhibit melanin synthesis may act by way of the regulation of tyrosinase, i.e. inhibition of tyrosinase mRNA transcription, aberration of tyrosinase glycosylation, inhibition of tyrosinase catalytic activity and acceleration of tyrosinase degradation. Among them, inhibition of tyrosinase activity is the most common approach to control abnormal melanin synthesis. Cosmetic and medicinal fields use of tyrosinase inhibitors is becoming increasingly important in the cosmetic and medicinal industries due to prevent pigmentation disorders in dermatology and cosmetics for a long time. In the last few decades, a huge number of compounds from natural and synthetic sources have been tested as inhibitors of melanin synthesis, but a small number are used in skin-whitening products, primarily due to safety and efficacy considerations. Moreover, considerable interest in tyrosinase inhibitors exists also in the food industry because the activity of this enzyme is responsible for the browning of fruit and vegetables.

Agricultural and food fields browning of plant-derived foods and beverages occurs due to enzymatic oxidation of phenols by tyrosinases, which can cause the destruction of essential amino acids, the impairment of digestibility and nutritional quality, the formation of toxic compounds. (Kim & Uyama, 2005). Enzymatic browning can lead to deleterious changes in the appearance and organoleptic properties of food products, resulting in nutritional loss and shorter shelf-life. The rates of enzymatic browning in food are influenced by many factors, including the concentration of tyrosinase, phenolic substrates, oxygen availability, pH, temperature, etc. Physical and chemical

approaches have been extensively tested and applied for a long time to inhibit enzymatic browning. For this purpose, some physical methods, such as low temperature (refrigeration and freezing), autoclave and blanching, microwave energy, high pressure treatment, atmosphere packaging, vacuum packaging, edible films and coatings, have been proposed.. Chemical approaches include reducing agents (sulphiting agents, ascorbic acid and derivatives, thiol compounds), acidulants (citric acid and fumaric acid), chelating agents (phosphates, EDTA and organic acids), complexing agents (cyclodextrins and its derivatives), andenzyme inhibitors (aromatic carboxylic acids, aliphatic alcohol, anions, peptides and substituted resorcinols.

Inhibitors in the usual sense, working by a definite chemical action, may be reversible or irreverible.

In reversible inhibition, which is further subdivided into**: competitive**, **uncompetitive, mixed types** (competitive/uncompetitive)**, non-competitive inhibitors**, the activity of the enzyme is fully restored when the inhibitor is removed from the system in which the enzyme functions (Fig. 5)

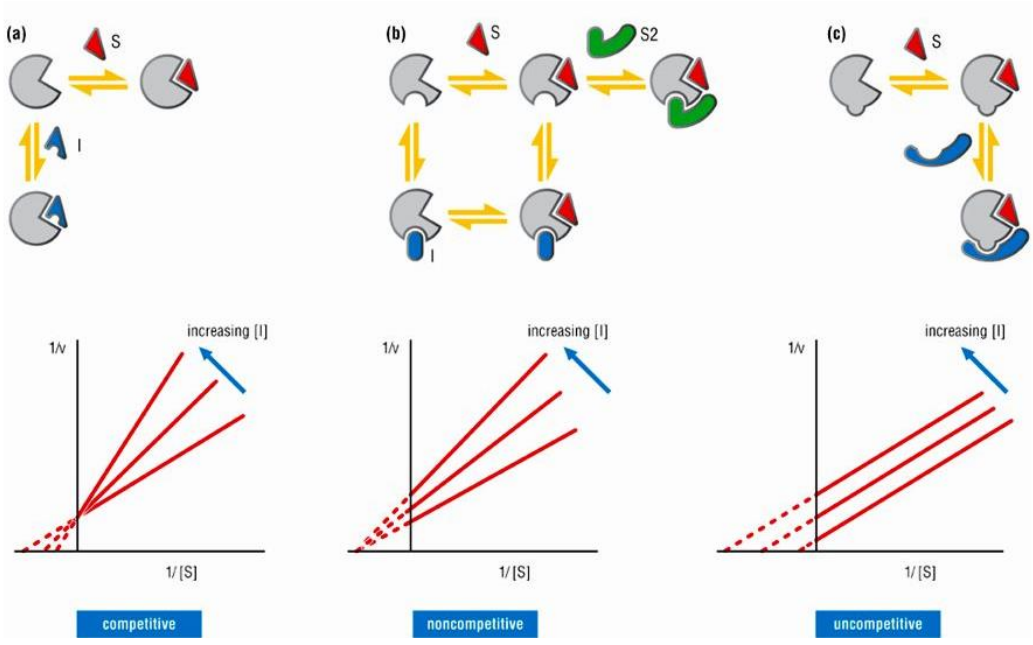


Figure 5: Action mechanism of inhibitors

Competitive inhibitors work by binding at the active site on the enzyme. They compete with substrate for the active site and prevent the substrate from binding. Their structure is similar to that of the substrate since they are binding at the same site. The presence of these inhibitors causes an increase in Km of the enzyme, but leaves Vmax unaffected. In contrast, an uncompetitive inhibitor can bind only to the enzyme-substrate complex. A mixed (competitive and uncompetitive mixed) type inhibitor can bind not only with a free enzyme but also with the enzyme-substrate complex. For most mixed-type inhibitors, their equilibrium binding constants for the free enzyme and the enzyme-substrate complex, respectively, are different. However, a special case among the mixed inhibitors is the non-competitive inhibitors, which bind to a free enzyme and an enzyme-substrate complex with the same equilibrium constant. In addition to the inhibitory mechanism, inhibitory strength is the primary criterion of an inhibitor. Inhibitor strength is usually expressed as the inhibitory IC_{50} value, which is the concentration of an inhibitor needed to inhibit half of the enzyme activity in the tested condition.

In most studies conducted to discover new tyrosinase inhibitors, a well-known tyrosinase inhibitor such as kojic acid is often used as a positive standard at the same time in order to compare the inhibitors described in different literature (Te-Sheng Chang, 2009).

Kojic acid (5-hydroxy-2 hydroxymethyl-4-pyrone) (Fig. 6) is a naturally occurring hydrophilic fungal product derived from certain species of Acetobacter, Aspergillus, and Penicillium. It shows a competitive inhibitory effect on monophenolase activity and a mixed inhibitory effect on the diphenolase activity of mushroom tyrosinase. Kojic acid has also been used as a skin lightening or bleaching agents in cosmetic industry and as food additive for preventing enzymatic discoloration of vegetables, crabs and shrimps.

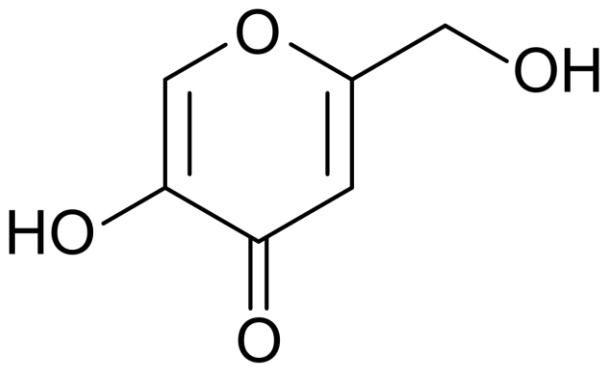


Figure 6: Kojic acid structure

However some side effects, such as allergy and thyroid adenomas have been reported with the use of kojic acid in mice and it is associated with sensitization, contact dermatitis and erythema.

Another common compound used for treatment of hyperpigmentation is hydroquinone and its derivates.

Hydroquinone **(**1,4-dihydroxybenzene) (Fig. 7) is a phenolic compound which is considered to be one of the most studied tyrosinase inhibitors up to date because is a potent inhibitor of melanogenesis both in vitro and in vivo. It act has a competitive inhibitors. However, it can cause DNA damage and this carcinogenic effect has raised concerns regarding its use. Due to this, the International Agency for Research on Cancer has placed hydroquinone as not classifiable as to its carcinogenicity in human. The Food and Drug Administration (FDA) has even proposed banning over-the counter skin bleaching agents containing hydroquinone. Due to the side-effect and safety profile, hydroquinone is not used as a component of cosmeceuticals available in the market for the treatment of hyperpigmentation. (Palumbo A. et al., 1990).

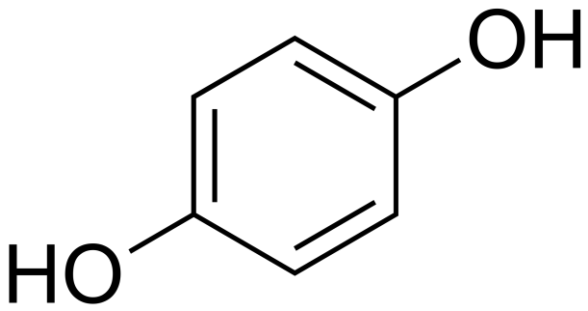


Figure 7: Hydroquinone structure

One of hydroquinone derivates the most widely prescribed as skin-lightening and de-pigmenting agent is the Arbutin (hydroquinone-O-β-d-glucopyranoside). Arbutin is a naturally occurring plant derived compound found in the dried leaves of a number of different plant species including, bearberry (Arctostaphylos uva-ursi), blueberry, cranberry and pear trees. Arbutin has two forms (α-, β-form): β-arbutin inhibits both tyrosinase activity from mushroom and mouse melanoma noncompetitively, but α-arbutin inhibits only tyrosinase from melanoma by mixed-type inhibition. Despite its potent tyrosinase inhibitory effect, there is still concern about the cancer risk associated with its aglycone hydroquinone, which might be formed by biotransformation in human body (Maeda K. and Fukuda M., 1996).

Due to instability, poor penetration of the skin, irritation and mutagenic effects of the above tyrosinase inhibitors, many studies are aimed at finding new tyrosinase inhibitors, both from natural and synthetic sources, with fewer unwanted effects.

1.5. Objective of this thesis

The objective of this thesis is to find innovative tyrosinase inhibitors as anti-melanogenic agents both synthetic compounds and plants extracts. Melanogenesis, as already mentioned above, is a physiological process, involving tyrosinase, resulting in the synthesis of melanin pigments which are responsible for skin pigmentation and provide a beneficial effect in preventing skin damage

under normal condition. Therefore, research of tyrosinase inhibitors have become increasingly important in medicinal and cosmetic products in relation to hyperpigmentation. Moreover tyrosinase is responsible for enzymatic browning reactions in fruits and vegetables. However, in this case, food browning is undesirable and reduces nutritional and commercial value of the products. In order to prevent browning, enzyme inhibitors have a key role as food additives. In this study we tested different type of plants extracts (*Asphodelus microcarpus, Sarcopoterium spinosum and Phytolacca dioica*), honey extracts from different botanical origin (*Arbutus unedo, Asphodelus microcarpus, Eucaliptus globulus, Carduus nutans and Hedysarum coronarium*) and synthetic compounds with heteroarylcoumarin scaffold.

1.6. Selected plants, honeys and synthetic compounds

1.6.1. *Asphodelus microcarpus*

Asphodelus microcarpus Salzm.et Vivi (Asphodeliaceae) (Fig. 8) is a perennial and a stout robust herb, commonly distributed over the coastal Mediterranean region.

The bulbs and roots of *A. microcarpus* are used to treat ectodermal parasites, jaundice, psoriasis and by Bedouins as an antimicrobial agent. It has been also reported in ethobotanical literature its use for otitis, toothache in Algeria (Sarri, M. et al., 2014) and for lung diseases in Sardinia (Loi, M.C. et al., 2005). Asphodel leaves are also used for the processing of wicker baskets and over the centuries has contributed to the poor balance of families of pastors, masses and farmers in Sardinia. Several studies were performed in order to verity its antimicrobical activity. Recently, antimicrobial activity of areal part of *A. microcarpus* was evaluated on Propionibacterium acnes and on methicillin resistant Staphylococcus aureus MRSA (Di Petrillo at al., 2017)

Phytochemical studies on *A. microcarpus* revealed the presence of lipids, carbohydrates, sterols, anthraquinones and arylcoumarins (Ghoneim MM et al., 2014). It is well known that the last two compounds have tyrosinase inhibitory activity (Fais A. et al., 2009; Leu I.Y. et al, 2009) and plant extracts with antimelanogenic activity typically possess polyphenols such as flavonoids, which are

usually the factors responsible for the activities in plant extracts. For these reason and for its wide diffusion in Sardinia, this plant was chosen for my study.

Figure 8: *Asphodelus microcarpus*

1.6.2. Sarcopoterium spinosum

Sarcopoterium is a genus of flowering plants in the rose family. The genus is synonymous to *Poterium*. The sole species within this genus, *Sarcopoterium spinosum* (Fig. 9), is common to the southeast Mediterranean region and Middle East. It is a perennial bush with small flowers in inflorescence from February to April and its fruits mature in autumn, then fall to earth to germinate with the rain water. The ethnobotanical survey reported that *S. spinosum* is used in traditional medicine primarily for the management of diabetes (P. Smirin,et al., 2010). *Sarcopoterium spinosum* extract as an antidiabetic agent: *in vitro* and *in vivo* study and secondary therapeutic

application are for pain relief or digestive problems. Recent publications concern its antioxidant and hypoglycaemic activity (Rosenzweig T., et al., 2007).

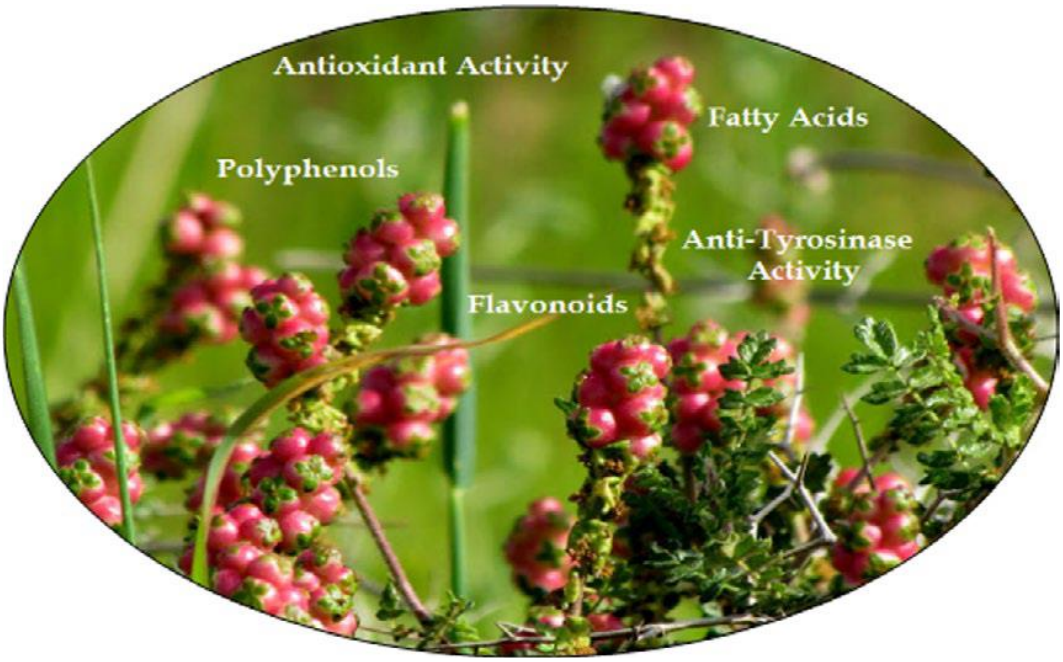


Figure 9: *Sarcopoterium spinosum*

1.6.3. *Phytolacca dioica*

The plant genus Phytolacca encloses 35 species with a close relativity and similar characteristics. Poke plants are shrubs or trees, annual, likewise perennial and the stems are erect up to 1-3 m height. It is an easily grown plant, succeeding in most soils and full sun or partial shade.

P. dioica (Fig. 10) is otherwise known as Belhambra (English) or Belhambraboom (Afrikaans) tree and can easily be recognized by its massive trunk, simple and somewhat fleshy leaves borne on pinkish stalks with a pendulous cluster of berries. The leaves and berries of *P. dioica* have been reported to be rich sources of triterpenoid saponins, which have been described as displaying important biological activities such as molluscicidal, anti-inflammatory, antifungal and antibacterial effects (Escalante et al., 2002; Di Maro et al., 2007). Ethnopharmacological information also revealed that *P. dioica* is used for healing skin wounds (Quiroga et al., 2001). Also, a number of ribosomeinactivating proteins (RIPs) that are potentially useful for the development of

immunotoxins for tumor therapy and the production of transgenic plants endowed with specific parasite resistance have been isolated from the plant (Blanco et al., 1997, 1998).

Figure 10: *Phytolacca dioica*

1.6.4. *Sardinian Honeys*

Honey is a supersaturated solution of fructose and glucose and contains a wide range of minor constituents such as minerals, proteins, vitamins, organic acids, enzymes, and phenolic compounds (Ferreira, et al., 2009). This natural product has several properties such as antibacterial, antioxidants, anti-inflammatory, antithrombotic and antiallergic (Molan PC, 2013). Moreover, the medicinal use of honey for skin disease is cited in a number of ethnopharmacological and

ethnomedical surveys. According to traditional Chinese medicine, honey prevents scars, removes discoloration and freckles, and improves the general appearance of skin. In Arab medicine, honey is used for fungal infections of the skin.

The composition of honey is tightly associated to its botanical source and also to the geographical area from where it originated (Soares S. et al., 2017).

Five variety of honey were used in this study: arbutus, asphodelus, eucalyptus, thistle and sulla.

- Strawberry tree (*Arbutus unedo* L., Ericaceae) unifloral honey is a typical product of some Mediterranean regions, Sardinia in particular (Spano N., 2006). It is dark amber when young and light brown when crystallized. The scent is pungent while its flavor has aspects of green ivy, coffee dregs, leather, burnt, and bitter herbs. Due to the characteristic taste, sweet initially but rapidly reveals its strong complex bitter flavor, this honey is known as ‘‘bitter honey’’ and it has been traditionally employed mainly for curative aims (Tuberoso et al., 2010).
- *Asphodelus microcarpus* Salzm. et Viv. monofloral honey is produced during springtime; it is light-colored, with medium-fine size crystals. The smell is delicate and the taste weak and immediately sweet. Of the Sardinian melliferous species, Asphodelus is the first to come into flower, starting to bloom in February-March and, in relation to the altitude, continuing to bloom until May. Asphodel honey is very similar to sulla (Hedysarum coronarium L.) honey, but the latter has a slightly sour taste.
- *Eucaliptus* honey is usually a honey harvested from *Eucalyptus globulus*, probably the most well-known evergreen tree from all *Eucalyptus* species. Honeys of this botanical origin have amber to clear amber color, a slightly waxy smell and a persistent, slightly acidic taste. Eucalyptus honey is the largest studied honey, is well known its antioxidant, antimicrobial and balsamic activity.
- Thistle honey is a common unifloral honey and, depending on the area of production, several species belonging to the Asteraceae family can be linked to the botanical origin of this honey. Star thistle (*Centaurea solstitialis* L.), nodding thistle (*Carduus nutans* L.), Mediteranean thistle

(*Galactites tomentosa* Moench), and other plants belonging to the genus Carduus or Cirsium, are the main sources of thistle honey.

- Sulla (*Hedysarum coronarium* L.) is a legume well adapted to semi-arid Mediterranean environments and represents an effective example of multiple-uses species exploited for environmental protection, landscape enhancement and honey production. Considerable amount of sulla unifloral honey is produced in Southern and Central Italy.

1.6.5. Synthetic compounds

Coumarins represent a class of compounds of interest for a long time due to their biological activities: they have been shown to be useful as antitumoural and anti-HIV agents. In addition, they have show to possess cardioprotective properties: many of them are selective coronary vasodilators, an effect that may be related to a Ca^{2+}-antagonistic activity. In addition, Masamoto et al. (Masamoto Y. Et al., 2004). investigated the structure-activity relationship of 18 coumarins for their inhibitory activity on mushroom tyrosinase, and they find that esculetin exhibited the strongest inhibitory activity. Recently, in contrast with the findings of Masamoto, Sollai et al. (Sollai, F. et al., 2008) have shown that esculetin is nevertheless to be considered a tyrosinase substrate rather than an inhibitor, whereas umbelliferone appears to be an inhibitor of the mentioned oxidase.

In this thesis was added strategically hydroxyl groups in different positions of the coumarin core and in addition, 4-(6-hydroxy-2-naphthyl)-1,3-bezendiol, in order to find news potent inhibitors.

CHAPTER 2: MATERIALS AND METHODS

2.1. Chemical and instruments

All chemicals for antioxidant and enzyme activity were obtained as pure commercial products from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification. HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany) and formic acid was purchased from Prolabo (VWR International, France). Water was treated in a Milli-Q water purification

system (TGI Pure Water Systems, USA). The phenolic compounds standards (5-O-caffeoylquinic acid, luteolin-6-C-glucoside, luteolin-7-O-glucoside and apigenin) were from Extra synthese (Genay, France). CO_2 purity 99 % was from Air Liquide Italia, Cagliari, Italy.

B16F10 mouse melanoma cells (CRL-6475) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, NY, USA).

As regard the microbical activity were used: *Staphylococcus aureus* ATCC 6538 (American Type Culture Collection), *Staphylococcus haemoliticus* clinical isolate NC1, *Streptococcus uberis* human clinical isolate NC20, *Streptococcus faecalis* ATCC 29212, *Streptococcus mutans* CIP103220 (Collection Institut Pasteur), *Streptococcus salivarius*, strain k12 (from a commercial product, Bactoblis®), *Streptococcus pyogenes* human clinical isolate NC4, *Streptococcus intermedius* DSMZ 20573 (German Collection of Microorganism and cell culture), *Bacillus clausii*, (isolated from a commercial product, Enterogermina®); *Escherichia coli* ATCC 7075 *Candida albicans*, *Candida kruseii*, *Candida glabrata*, human oral clinical isolates, designed from BF1 to BF3 respectively. Bacterial growth agar mediums manufactured by Microbiol (Uta, Italy) and Phosphate-buffered saline GIBCO®PBS (ThermoFisher).

- HPLC-DAD Varian system ProStar and ThermoSeparation diode array detector SpectroSystem UV 6000lp (ThermoSeparation, San Jose, CA), Phenomenex Kinetex XB-C18 100 Å column (150 mm x 4.60 mm, 5 µm, Chemtek Analitica, Anzola Emilia, Bologna, Italy);
- HPLC–DAD–ESI/MS Hewlett–Packard 1200 chromatograph (Agilent Technologies, Waldbronn, Germany), an Aqua C18 125 Å column (5 µm, 250 × 4.6 mm I.D.; Phenomenex), plate reader spectrophotometer (SLT-Spectra II, SLT Instruments, Germany;
- ^{1}H NMR (300 MHz) and ^{13}C NMR (75.4 MHz) spectra were recorded using a Bruker AMX spectrometer;
- preparative TLC uniplate 20x20 cm analtech,
- microplate reader Fluostar Optima BMGLabtech

- preparative High Speed Counter Current Chromatography (HSCCC) INTROPREP HT QUATTRO CCC. high-speed counter-current chromatograph equipped with a 136 mL coil column made of PTFE tubing (i.d. of the tubing = 2.0 mm, total volume = 136 mL) and a 6 mL sample loop. The HSCCC system was connected to a pump

- NMR Agilent Technologies 400 MR using standard software programs and deuterated mehanol

2.2. Extraction procedure

2.2.1. *Asphodelus microcarpus*

Asphodelus microcarpus subsp. *microcarpus* Salzm. et Viv. leaves, flowers and tubers (L, F and T respectively) were collected in southern Sardinia (Quartu Sant'Elena, Cagliari, Italy). The GPS coordinates were 39° 22′41.5″ N and 09° 19′62.3″ E. The plant was identified by Dr. Cecilia Loi, Department of Life and Environmental Sciences, Section of Botany, University of Cagliari, Italy. A voucher specimen (1405/16 Herbarium CAG) has been deposited in the Museum Herbarium CAG (Life and Environmental Sciences Department).

Plant materials were washed with deionized water, frozen at -80 °C and then lyophilized. The dried plant was stored at -80 °C until required.

The lyophilized plant materials (1 g): flowers, leaves and tubers, were extracted in 10 mL of water, or ethanol, or methanol for 24 h at room temperature under continuous stirring. After filtration, ethanol or methanol extracts were diluted 10-fold with water and then all extracts were lyophilized.

For biological activity dried powders (1 mg) were dissolved in 1 mL of the apposite solvent (water or 1% ethanol:water or 1% methanol:water before use.

For HPLC–DAD–ESI/MS analyses dried extract was dissolved in 1 mL of 0.1% formic acid:acetonitrile (70:30, v/v) and filtered through a 0.22 μm disposable LC filter disk for HPLC analysis.

2.2.2. *Sarcopoterium spinosum*

Sarcopoterium spinosum fruitswere collected in Sant'Elia, Cagliari (Sardinia, Italy) in June 2014. A voucher specimen (Herbarium CAG 479) has been retained in the General Herbarium of the Botanical Garden of Cagliari. Fruits were dried in anovenat 40 °C with forced ventilation for two days. Then, fruits were ground in a blender. Vegetable material was subjected to three different extraction methodologies: supercritical fluid extraction, solvent extraction and hydrodistillation.

Supercritical fluid extraction

Supercritical CO_2 extractions (SFE) was performed in a laboratory apparatus equipped with a 320 mL extraction vessel. Extractions were carried out in a semi batch mode: batch charging of vegetable matter and continuous flow solvent, adopting an experimental arrangement that leave out the first separator. About 150 g of *S. spinosum* were charged in each run. Operative conditions were: 250 bar and 40 °C in the extraction section and 20 bar and 15 °C in the second separator. The extract obtained was assayed diluted in DMSO 10 mg/mL for the assays: total polyphenols, total flavonoids, antioxidant and anti-tyrosinase activities.

Solvent extraction

Solvent extraction was performed with *n*-hexane in a conventional Soxhlet extraction apparatus (SE). The sample was concentrated on a rotary evaporator, under vacuum. The concentrated extract was completely dried in an oven, then diluited in DMSO 10 mg/mL for the assays: total polyphenols, total flavonoids, antioxidant and anti-tyrosinase activities.

Hydrodistillation

Hydrodistillation (HD) was performed in a circulatory Clevenger-type apparatus according to the procedure described in the European Pharmacopoeia (Council of Europe 1997).

The fruits of *S. spinosum* did not contain measurable amount of essential oil and HD is not suitable to extract fixed oil. After the hydrodistillation, the aromatic water (AW) and the residual water (RW) were collected separately and tested. The aromatic water was used as such for the assays;

while the residual water was lyophilized and diluted in water to 10 mg/mL, for total polyphenol and flavonoids content and for antioxidant and anti-tyrosinase activities.

2.2.3. Phytolacca dioica

The fruits of *Phytolacca dioica Linn* were collected from Cagliari, Italy (coordinate 39.224195, 9.105899). The plant was identified by Dr. Cecilia Loi, Department of Life and Environmental Sciences, Section of Botany, University of Cagliari, Italy. A voucher specimen (1233/A Herbarium CAG) has been deposited in the Museum Herbarium CAG (Life and Environmental Sciences Department). Freshly collected were washed with running water to remove glochids and impurities, air-dried and hand-peeled. The seeds were separated from the juicy pulp, washed abundantly with distilled water then dried at room temperature for 24 h and weighed to be at end reduced into a fine powder using a blender type A11 basic (IKA, Germany). The powdered seeds samples were stored at -20°C prior to analysis.

The powdered seeds (20 g) were extracted with 70% ethanol (crude extract, CE). The CE extracts were filtered and centrifuged at 12,000g × for 20 min at 4ºC and then evaporated under reduced pressure to dryness. This extract was suspended in distilled water (water extract, WE) and sequentially fractionated by hexane (hexane extract, HE), ethyl acetate (ethyl acetate extract, EAE) and *n*-butanol (butanol extract, BE). After separation of the phases, the solvents were removed in a rotary evaporator at 45°C under vacuum. All extracts and fractions were submitted to biological assays. All analyses were performed using triplicate samples.

2.2.4. Sardinian Honeys

Five Sardinian honeys types were obtained from a local farm (Zafferano e Spezie di Sardegna srl Agricola). Honey samples were as follow: Arbutus (*Arbutus unedo*), Asphodelus (*Asphodelus spp*), Thistle (*Cardus f.*), Eucalyptus (*Eucalyptus spp.*) and Sulla (*Hedysarum coronarium*).

After sampling, honey was stored in the dark at 4 °C until analysis, in order to preserve its chemical composition.

For antioxidant activity, total phenolic and flavonoid content and inhibitory activity, each honey (5 g) was dissolved in 25 mL of distilled water and immediately tested.

2.3. Biological activity

2.3.1 Antioxidant activity

2.3.1.1. $ABTS^{\bullet+}$ radical scavenging activity

The $ABTS^{\bullet+}$ method is based on the capacity of an antioxidant to scavenge the free radical $ABTS^{\bullet+}$ (Delogu et al., 2016). $ABTS^{\bullet+}$ reagent (2,2′-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid)) is produced by reacting 7 mM ABTS with 2.45 mM potassium persulfate (final concentration) in aqueous solution, kept in the dark at room temperature for 24 h before use. The concentration of the blue-green ABTS radical solution was adjusted to obtain an absorbance of 0.700 ± 0.02 (mean ± SD) at 734 nm.

Sample (10 µL) was added to $ABTS^{\bullet+}$ solution and incubated in the dark at room temperature for 1 min. Afterwards the decrease in A734 was calculated and compared to the Trolox standard curve. The activity was expressed as concentration of sample necessary to give a 50% reduction in the original absorbance (IC_{50}).

2.3.1.2. DPPH radical scavenging activity

The DPPH radical scavenging activity of the samples was analyzed according to the procedure previously described (Pintus et al., 2015). Sample solution (20 µL) was added to a mixture of 100 mM acetate buffer (pH 6.5, 630 µL) and 0.3 mM DPPH (2,2-Diphenyl-1-Picrylhydrazyl Radical) in ethanol (350 µL) and left to stand at room temperature in the dark for 15 min. The absorbance of the resulting solutions was measured at 515 nm and compared to the Trolox standard curve. The

activity was expressed as concentration of sample necessary to give a 50% reduction in the original absorbance (IC_{50}).

2.3.2. Total polyphenol content

Total polyphenol content in the extracts was determined by the Folin-Ciocalteu reagent in accordance with the method described by Alhakmani (Alhakmani et al., 2013) with slight modifications.

Ten µL of the extracts or compounds was dissolved in 50 µL of the Folin-Ciocalteu reagent and 790 µL of distilled water. The solutions were mixed and incubated at room temperature for 1 min. After 1 min, 150 µL of 20% sodium carbonate solution was added. The final was shaken and the then incubated for 45 min in the dark at room temperature. The absorbance of all samples was measured at 750 nm. A calibrating curve was plotted using gallic acid as standard.. Results were expressed as mg of gallic acid equivalents (GAE) per g of dry weight (dw).

2.3.3. Total flavonoids content

The flavonoids content in extracts was determined by aluminum nitrate colorimetric method described by Bekir (Bekir et al., 2013), with some modifications. Briefly, an aliquot of 0.50 mL of sample solution was mixed with 0.10 mL of 10% (w/v) aluminum nitrate, 0.10 mL of 1 M potassium acetate and 4.30 mL of 80% ethanol. After incubation at room temperature for 40 min, the absorbance of the reaction mixture was measured at 415 nm. Different concentrations of quercetin solution were used for calibrations and results were expressed as mg of quercetin equivalents (QE) per g of dw.

2.3.4. In vitro enzymatic inhibition

2.3.4.1. Mushroom Tyrosinase

Tyrosinase inhibition assays were performed with L-DOPA as substrate. w. The reaction mixture (200 μL) contained 80 μL of phosphate buffer (0.5 M, pH 6.5), 60 μL of mushroom tyrosinase (240 U mL^{-1}), 20 μL of inhibitor dissolved in DMSO at the different concentrations or DMSO (control), were mixed. The assay mixture was then incubated at 37°C for 10 min. Finally, 40 μL of 2.5 mM L -DOPA in phosphate buffer were added and immediately monitored (t=0) at at 492 nm for dopachrome formation in reaction mixuture.

Kojic acid was used as a positive control. Each measurement was made in triplicate.

The percentage of inhibition of tyrosinase activity was calculated as inhibition (%) = (A - B)/A x 100, where A represents the difference in the absorbance of control sample between an incubation time of 0.5 and 1.0 min, and B represents the difference in absorbance of the test sample between an incubation time of 0.5 and 1.0 min.

The IC_{50} value, a concentration giving 50% inhibition of tyrosinase activity, was determinate by interpolation of dose-response curves.

The mode of inhibition of the enzyme was performed using the Lineweaver-Burk plot. The assay was performed varying the concentration of inhibitor and L-DOPA. Kinetics constants were determined by the second plots of the apparent K_m/V_{max} and/or $1/V_{max}$ *versus* the inhibitor concentration.

2.3.4.2. Xanthine Oxidase

Xanthine oxidase (XO) activity was determined by measuring the formation of uric acid from xanthine. The xanthine solution was prepared dissolving xanthine in a minimal volume of NaOH, and adjusting pH to 7.5. The XO solution was prepared by diluting XO from cow's milk to a final concentration of 0.5 U/ml in cold 0.1 M phosphate buffer (pH 7.5).

The assay mixture consisted of 200µl of sample, 689µl 0.1 M phosphate buffer (pH 7.5) and 61µl of XO solution. The reaction was initiated by adding 61µl of 0,82 mM xanthine solution.The change in absorbance was recorded at 295 nm for 3 minutes at room temperature.

Allopurinol was used as a standard inhibitor. XO activity was expressed as percent inhibition of xanthine oxidase, calculated as [1-(B/A)]x100, where A is the change in absorbance of the assay without the plant extract, and B is the change in absorbance of the assay with the plant extract. The IC_{50} value, a concentration giving 50% inhibition of XO activity, was determined by interpolation of dose-response curves. The mode of inhibition on the enzyme was performed using the Lineweaver–Burk plot. Different concentrations of substrate (20-70 µM) were used for the assa

2.3.5. Cell culture

B16F10 mouse melanoma cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Cells were seeded in 6-well plates (10^5 cells/mL). After 24 h, the medium was substituted by fresh one supplemented with 100 nM α-MSH and different concentration of plant extract (0.05-0.15 mg/mL) and incubated for 48 h. Cells treated with 100 nM α-MSH and kojic acid were used as positive control and for comparing the inhibitory strength of the extracts.

2.3.5.1. Cell viability

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine cell viability (Mosmann, 1983). Briefly, cells were plated in a 96-well plates (10^4 cells/well) and these were treated for 48 h with various concentration of samples (0.05-0.4 mg/mL). After incubation at 37°C MTT solution were added to cells and for 3 h at 37 °C. The MTT solution was then removed, the resulting violet formazan precipitates were dissolved in isopropanol and the absorbance was determined at 590 nm using a microplate reader with a 630 nm reference.

2.3.5.2. Intracellular tyrosinase activity

The tyrosinase activity in B16F10 cells was performed following a previously described method (Pintus et al., 2015). α-MSH-stimulated cells were plated in 60π-dishes (10^5 cells/mL) and incubated for 48 h in absence or presence of sample (0.05-0.15 mg/mL). The cells were lysed in 50 mM phosphate buffer (pH 6.8) containing 1% Triton X-100 and 0.1 mM phenylmethyl-sulfonyl fluoride. Cellular lysates were clarified by centrifugation at 12,000 rpm for 20 min at 4 °C. The protein content were calculated by the Bradford method using Bovine serum albumin (BSA) as a standard (Bradford, 1976). The cellular extract (3 µg of protein) was mixed with L-DOPA substrate solution (1.25 mM) in 25 mM phosphate buffer (pH 6.8) and the absorbance at 475 nm was read until the reaction has finished.

2.3.5.3. Melanin content assay

α-MSH-stimulated cells were plated on 60π-dishes (10^5 cells/mL) and incubated for 48 h in absence or presence of sample (0.05-0.15 mg/mL). After washing with PBS, cells were harvested and an aliquot was used for protein quantification. The remaining cells were centrifuged and lysed with NaOH 1 M at 100° C for 1 h. Melanin concentrations ware determined at 405 nm and compared with a standard curve of synthetic melanin.

2.3.5.4. L-DOPA staining assay

The DOPA-staining assay was performed as reported by Sato (Sato et al., 2008) with some modifications. B16F10 cells were treated for 48 h with either α-MSH alone or α-MSH plus *Asphodelus microcarpus* flowers extracts at different concentration or kojic acid (100 or 150 µg/mL) as positive control. After treatment, cells were lysed in 50 mM phosphate buffer (pH 6.8) containing 1% Triton X-100 and 0.1 mM phenylmethyl-sulfonyl fluoride. Protein extracts (5 µg) were then mixed with 10 mM Tris–HCl buffer, pH 7.0, containing 1% SDS, without mercaptoethanol or heating, and resolved by 8% SDS-polyacrylamide gel electrophoresis. After

running,gel was rinsed in 0.1 M phosphate buffer (pH 6.8) and equilibrated for 30 min twice with gentle shaking at room temperature. The gel was then transferred in a staining solution containing 0.1 M phosphate buffer (pH 6.8) with 5 mM L-DOPA, and incubated in the dark for 1 h at 37 °C. Tyrosinase activity was visualized in the gel as dark melanin-containing bands.

2.3.6. Identification, Extraction and Isolation of phytochemicals

2.3.6.1. HPLC-DAD fingerprinting

The analyses were performed using HPLC-DAD fitted with a pump module 230, an autosampler module 410 and ThermoSeparation diode array detector SpectroSystem UV 6000lp set at 210 and 280 nm. The gradient elution was performed on Phenomenex Kinetex XB-C18 100 Å column using 0.2 M phosphoric acid (solvent A), and acetonitrile (solvent B) as mobile phase at a constant flow rate of 1.0 ml/ min. The gradient (v/v) was formed as follows: 100% of solvent A for 5 min, then decreasing of solvent A to 80% in 20 min, to 50% in 40 min, to 10% in 50 min and finally increasing to 100 % of solvent A until 60 min. The injection volume was 10 µl. The obtained chromatograms and spectra were elaborated with a ChromQuest V. 4.0 data system (ThermoQuest, Rodano, Milan, Italy). The standard solutions were prepared in methanol and working standard solutions were prepared in ultrapure water. The calibration curves were plotted according to the external standard method, correlating the analyte peak areas with the corresponding concentrations. The honey samples were diluted in ultrapure water 1:5 (w/v), vortexed and filtered through RC membrane syringe filter (0.45 µm, Ø 15 mm, Phenomenex) prior to the analysis.

2.3.6.2. HPLC–DAD–ESI/MS analyses

HPLC–DAD–ESI/MS analyses was performed using a Hewlett–Packard 1200 chromatograph equipped with a binary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. The HPLC system was connected via the DAD cell outlet to an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) mass spectrometer (MS) consisting

of an ESI source and a triple quadrupole-ion trap mass analyzer, which was controlled by the Analyst 5.1 software. An Aqua C18 125 Å column thermostated at 35 °C was used. The solvents were: (A) 0.1% formic acid, and (B) acetonitrile.

The elution gradient established was isocratic 15% B for 5 min, 15–20% B over 5 min, 20–35% B over 10 min, 35–50% B over 10 min, 50–60% B over 2 min, isocratic 60% B for 5 min and re-equilibration the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in the MS operated in the negative ion mode. Spectra were recorded between *m/z* 100 and 1000. Zero grade air served as the nebulizer gas (30 psi) and as turbo gas (400 °C) for solvent drying (40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). Both quadrupols were set at unit resolution and EMS and EPI analyses were also performed. The EMS parameters were: ion spray voltage 4500V, DP -50 V, EP -6 V, CE -10 V and cell exit potential (CXP) -3 V, whereas EPI settings were: DP -50 V, EP -6 V, CE -25 V and CES 0 V.

The phenolic compounds present in the samples were identified according to their UV and mass spectra and by comparison with commercial standards when available.

2.3.6.3. High Speed Counter Current Chromatography

The preparative High Speed Counter Current Chromatography (HSCCC) was carried out in order to isolate compounds from several plant extracts. The first step of HSCC is the choice of the solvent system. The solvent system was decided after comparing two-phase solvent systems containing n-hexane, ethyl acetate, methanol and water (H:E:M:W) in different proportions (3:5:3:5 and 1:4:2:3 (v/v/v/v)). The partition coefficients (K_D) of the compounds in the specific solvent system were evaluated by a UV detector at 254 nm in a Spectroline CX-20 UV Fluorescence Analysis Cabinet, followed by spraying TLC plates with vanillin (2% in methanol) and sulfuric acid (1% in methanol). The system HEMW 1:4:2:3 (v/v/v/v) was chosen because it showed a better distribution of the compounds in the two phases solvent.

The lower aqueous phase was used as the stationary phase, and the upper organic phase as the mobile phase. The sample solution was prepared by dissolving 500 mg of the dried *P. dioica* seeds extract into 6 mL of two-phase solvent, filtered through 0.45 µm membrane filter prior to injection into the HSCCC system.

The column was first filled with the lower stationary phase, subsequently, the apparatus was rotated at 850 rpm while the upper phase was pumped into the inlet of the column as the mobile phase at a flow rate of 2 mg/mL. After that equilibrium was established in the column, 6 mL of the extract were injected. After 100 min the rotation was stopped and the lower phase was pumped into the column and separation was carried out for 100 minutes more. The fractions were manually collected. The machine was used in the tail to head mode all the time.

2.3.6.4. Preparative TLC

Isolated fractions from HSCCC not completely pure, were purified by preparative TLC. A streak of fraction was applied manually on a preparative TLC glass plate. After air drying, the plate was developed in a solvent system of chloroform, methanol and water mixture (6:4:1). The chromatogram was visualized by observing under ultraviolet lamp at 254 nm and 365 nm or spraying with 10% sulphuric acid (H_2SO_4) followed by heating on a hot plate. The scratched sample was dissolved in chloroform and filtrated in order to remove silica (Rajauria and Abu-Ghannam, 2013). Isolated compound were identified with 1H NMR.

2.3.7. Antimicrobial activity

2.3.7.1. Microbial strains

To evaluate the antimicrobial profile of *Asphodelus microcarpus* leaves ethanol extract, 13 different microbial strains have been used. We selected three different sets of microorganisms: (i) Gram positive_bacteria, *Staphylococcus aureus*, *Staphylococcus haemoliticus*, *Streptococcus uberis*, *Streptococcus faecalis*, *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus pyogenes*,

Streptococcus intermedius, *Bacillus clausii*; (ii) Gram Negative bacteria, *Escherichia coli*; (iii) Yeasts, *Candida albicans*, *Candida kruseii*, *Candida glabrata*. *E. coli*, *B. clausii*, *S. haemolyticus* and *S. aureus* were plated in Muller Hinton agar. Shaedler agar for Streptococci and Sabouraud dextrose agar for fungi. These microbial strains were used for an in vitro susceptibility test: (a) the agar diffusion method , (b) Minimal Inhibitory Concentration (MIC), (c) Minimum Bactericidal Concentration (MBC), which were determined in accordance with the Committee for Clinical Laboratory Standards, NCCLS. In addition, the Minimal Biofilm Inhibitory Concentration (MBIC) was used to evaluate the *Asphodelus microcarpus* leaves ethanol extract (LEE) antibiofilm activity (Sepandj et al., 2004)

2.3.7.2. Antimicrobial Susceptibility Testing

The Agar diffusion method was performed by using the Kirby-Bauer (KB) procedure and used as preliminary antimicrobial test to reveal the entire antimicrobial susceptibility profile for the examined *Asphodelus microcarpus* leaves ethanol extract. $1 \cdot 10^7$ cells/mL were inoculated onto the surface of an agar plate containing one of the subsequent bacterial growth agar mediums: (i) Muller-Hinton agar for aerobic bacteria, (ii) Shaedler agar for Streptococcus spp., (iii) Fungi on Sabouraud agar. This antimicrobial activity test was performed following the NCCLS protocol by using a paper filter disc (Ø = 6mm) impregnated with AE work solution (1000 µg/mL). MIC and MBC were performed only in susceptible microbial strains with KB test and they were performed according to the Micro-broth dilution method (Langfield et al., 2004; Wiegand et al., 2008) by using a ½ serial dilution, from 500 to 3.9 µg/mL of the LEE; the positive controls were performed with a Chlorhexidine digluconate solution (CHX), Sigma-Aldrich, ranged a concentration from 500 to 0.48 µg/mL (Table 1).. The cultures were incubated in air at 37 °C for 24 h for the aerobic strains and in 5% CO2 at 37 °C for the Streptococcal species.

For the biofilm evaluation, we used the protocol described by Montana University's Center for Biofilm Engineering (Merritt et al., 2005). A microplate containing serial concentrations of the

compound, inoculated with the bacterial strains as previously described for MIC and MCB evaluation, was incubated at 37 °C for 6 days, to permit the biofilm formation. The plate samples were subsequently washed three times with Phosphate-buffered saline to eliminate planktonic cells; thus, the biofilm was stained with 100 µL of 0.1 % w/v of crystal violet solution for 10 minutes at 25 °C. After three washes with PBS solution, 200 µL of 30% v/v acetic acid was added in every well to solubilize the dye from the bacterial biomass. The biofilm amount was measured with a plate reader spectrophotometer at 450 nm. The experiment was performed in triplicate and the MBIC represented the lowest concentration showing a 450 nm absorbance comparable with negative control, > 95% (sample without bacteria).

2.4. Synthetic compounds

2.4.1. General procedure for the synthesis of compounds based on 3-heteroarylcoumarine scaffold

Selected compounds were synthesized in the Department of Organic Chemistry, University of Santiago de Compostela. Compounds **1**–**12** were efficiently synthesized according to the synthetic strategy outlined in Fig. 11. The synthesis of these compounds was carried out by two different methodologies. The first one occurred in two different steps: the first step was a Perkin–Oglialoro condensation of different commercially available hydroxybenzaldehydes and thiophenylacetic acids, using potassium acetate (CH_3CO_2K) in acetic anhydride (Ac_2O), under reflux, for 16 h, to obtain the acetoxy-3-thiophenylcoumarins. Acetylation of the hydroxy groups and pyrone ring closure occurs simultaneously. The second step was the hydrolysis of the obtained acetoxy derivatives in the presence of aqueous HCl solution and MeOH, under reflux, for 3 h, to achieve the hydroxy substituted 3-thiophenylcoumarins (**1**–**6** and **8**–**11**). Structures of the synthesized compounds were established on the basis of their spectral data (Hashimoto K, et al., 1994). The second methodology is a one-step traditional Perkin reaction starting from the commercially available *ortho*-hydroxybenzaldehyde and the corresponding thiophenylacetic acid, using *N*,*N*′-

dicyclohexylcarbodiimide (DCC) as dehydrating agent, DMSO as solvent, at 110°C, for 24 h, that allows obtaining compounds **7** and **12**.

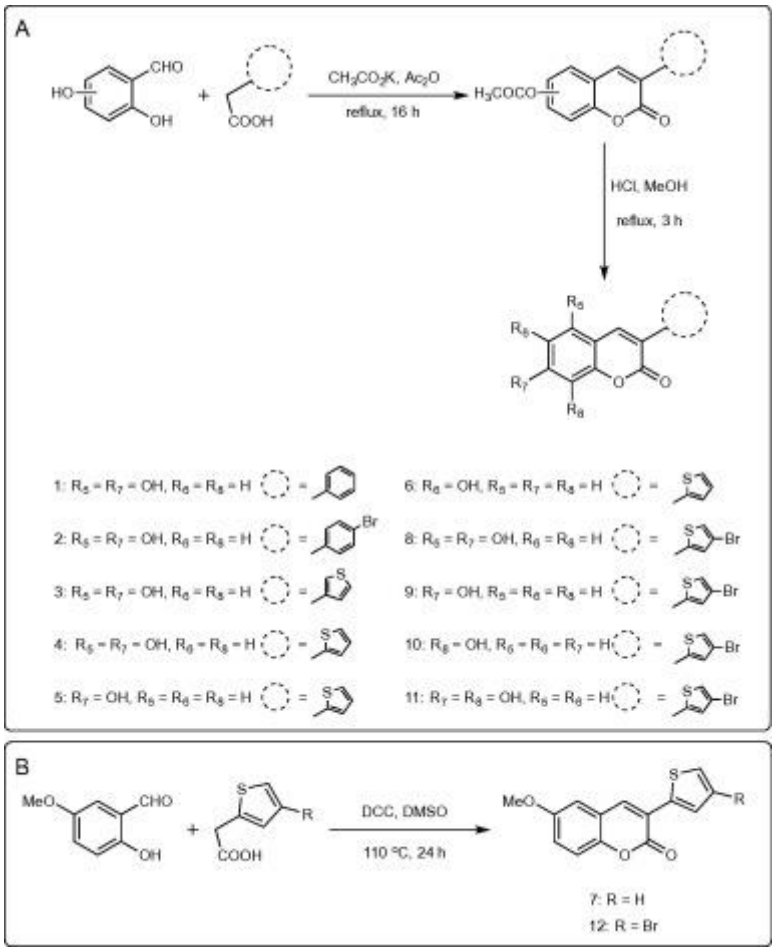


Figure 11: Synthetic route for the obtention of the derivatives represented in this study. (A) Perkin–Oglialoro reaction and chemical structure of the compounds **1–6** and **8–11**; (B) Perkin reaction and chemical structure of compounds **7** and **12**.

2.4.2. Molecular docking

Molecular docking was figured out in the Department of Biomedical Informatics, Columbia University Medical Center, NewYork. Some compounds were studied with molecular docking

techniques to understand the possible key interactions responsible for the protein–ligandbinding. For this purpose, it has been used the crystal structure 2Y9W (PDB code)45 in which the crystallized tyrosinase belongs to the mushroom *A. bisporus,* the same organism used in our experimental in vitro tyrosinase inhibition. The initial docking was performed with the 2Y9W structure in which there is no ligand bound to the protein. The protein structure contains a water molecule or hydroxyl ion coordinated with the copper ions. It has been considered the oxy form of the enzyme as a suitable template for docking calculations. Moreover, an additional docking using the structure 2Y9X (PDB code), epresenting the deoxy state of the enzyme, was performed to evaluate the stability of the results. The ligands were docked to the protein through glide in the Schrodinger package .

2.5. Statistical analysis

Data are expressed as mean ± SD from three independent experiments. The analysis average of the treatment using multiple comparisons was determined by using Duncan's multiple range tests, and the data were compared using the p values: $p < 0.05$ was considered statistically significant. The least significant difference (LSD) was used to determine the difference between the methods used to the investigation of the various antioxidant capacities, polyphenols content and enzymatic activity. The statistical analysis of differences between various treatments on cells was determined by the Student's *t*-test. Values of $p < 0.05$ were considered statistically significant. Statistical analysis was performed with SPSS v.18.0 (IBM corp., Armonk, NY, USA)

Chapter 3: Results and discussion

3.1. Asphodelus microcarpus

3.1.1. Results

3.1.1.1. Phenolics and flavonoids content

The phenolic and flavonoid amount of leaves, flowers and tubers extracts of *A. Microcarpus* are showed in Table 1. For each part of the plant, the best results were obtained with ethanolic extracts with a total phenolic contents of 39.35 ± 4.2, 54.44 ± 13.6 and 68.62 ± 9.8 mg GAE/g dw, for TEE (tubers ethanol extract), LEE (leaves ethanol extract) and FEE (flowers ethanol extract) respectively. Tubers extracts displayed the lowest level of total phenolics. There were significant differences in the plant extracts in terms of contents of total flavonoid, which varied from 1.4 (TEE) to 31.13 (LEE) mg QE/g dw. The highest flavonoid content was found in LEE (31.13 ± 1.96 mg QE/g dw) followed by FEE (27.28 ± 2.33 mg QE/g dw). Tubers are the part of the plant with lowest polyphenol and flavonoids contents.

Table 1: Polyphenol and flavonoid content in leaves, flowers and roots extracts from *Asphodelus microcarpus*.

Extracts		**Total polyphenols(*)**	**Flavonoids(**)**
Leaves	Aqueous	36.83 ± 0.6^{d}	5.90 ± 2.52^{c}
	Ethanolic	54.44 ± 13.6^{e}	31.13 ± 1.96^{g}
	Methanolic	35.48 ± 0.4^{d}	17.27 ± 1.28^{e}
Flowers	Aqueous	36.58 ± 1.2^{d}	3.33 ± 0.41^{b}
	Ethanolic	68.62 ± 9.8^{f}	27.28 ± 2.33^{f}
	Methanolic	26.37 ± 1.2^{c}	11.43 ± 1.07^{d}
Tubers	Aqueous	5.10 ± 0.5^{a}	1.99 ± 0.42^{ab}
	Ethanolic	39.35 ± 4.2^{d}	1.4 ± 0.33^{a}
	Methanolic	15.31 ± 7.8^{b}	3.94 ± 1.05^{b}

(*) mg GAE/g of dry weight
(**) mg QE/g of dry weight

3.1.1.2. Antioxidant activity of the extracts

The IC_{50} values of each extracts and positive control (trolox) were calculated in the present study and are depicted in Table 2. The extracts scavenged the ABTS and DPPH radicals in a concentration dependent manner. For DPPH assay, ethanolic extracts from flowers and leaves (FEE and LEE respectively) showed the best activity ($IC_{50} = 28.4 \pm 0.85$ µg/mL for FEE and $IC_{50} = 55.9 \pm 1.55$ µg/mL for LEE) compared to the other extracts ($p < 0.05$). Likewise, for ABTS radical scavenging assay, FEE and LEE showed an IC_{50} of 33.1 ± 1.55 µg/mL and 74.5 ± 7.77 µg/mL, respectively. The water extracts of tubers showed the smallest scavenging capacity. The IC_{50} values are high if compared with the standard ($IC_{50} \sim 3.3$ µg/mL) but the crude extracts examined probably contain pro-oxidants agents, which may compete with the antioxidants in the reaction with ABTS and DPPH radicals.

Table 2: Free radical-scavenging content of *A. microcarpus* extracts.

Extracts		**IC_{50} values (µg/mL)**	
		ABTS scavenging	**DPPH scavenging**
Leaves	Aqueous	174.35 ± 7.99^{e}	134.75 ± 7.85^{b}
	Ethanolic	74.5 ± 7.77^{b}	55.9 ± 1.55^{a}
	Methanolic	131.1 ± 1.27^{d}	140.85 ± 0.49^{b}
Flowers	Aqueous	126.4 ± 5.09^{c}	108 ± 2.83^{b}
	Ethanolic	33.1 ± 1.55^{a}	28.4 ± 0.85^{a}
	Methanolic	107.6 ± 1.27^{de}	113.25 ± 1.77^{b}
Tubers	Aqueous	720.45 ± 13.5^{h}	670.4 ± 27.72^{e}
	Ethanolic	257.75 ± 10.96^{f}	360 ± 56.57^{c}
	Methanolic	680.25 ± 14.35^{g}	579 ± 29.7^{d}
Trolox		3.4 ± 0.3	3.2 ± 0.4

3.1.1.3. Tyrosinase inhibitory activity of the extracts

Table 3 shows the inhibition of tyrosinase activity by extracts and kojic acid (positive control) at 0.2 mg/mL. The results indicated that the ethanolic extracts showed good activity, while the water and methanolic extracts showed only moderate activity at the concentration tested. The attention was, therefore, focused on ethanolic extract from flowers that show the best enzyme inhibition when compared with other extracts ($p < 0.05$). An IC_{50} value of 0.27 mg/mL and the kinetic behaviour of tyrosinase at different concentration of L -DOPA was also investigated (Fig. 12). The Lineweaver-Burk plot gave a family of parallel straight lines with the same slope (Fig 12a). With the increasing of the inhibitor concentration, the values of both K_m and V_{max} are reduced, while the ratio K_m/V_{max} remains quite the same. The slopes are independent from the concentration of FEE, which indicates that the compound is an uncompetitive inhibitor of the enzyme. The inhibition constant (Ki) of 0.19 mg/mL was obtained from a plot of the vertical intercept ($1/V_{max}$) versus the inhibitor concentration (Fig. 12b).

Table 3: Inhibition of tyrosinase by *A. microcarpus* extracts.

Extracts		**% Inhibition at 0.2 mg/mL**
Leaves	Aqueous	9.85 ± 0.21^{b}
	Ethanolic	29.9 ± 0.14^{d}
	Methanolic	20.4 ± 1.4^{c}
Flowers	Aqueous	6.55 ± 0.21^{b}
	Ethanolic	40.25 ± 4.4^{e}
	Methanolic	13.9 ± 2.4^{b}
Tubers	Aqueous	10.65 ± 1.34^{b}
	Ethanolic	8.4 ± 1.3^{b}
	Methanolic	2.25 ± 1^{a}
Kojic Acid		97.4±2.8

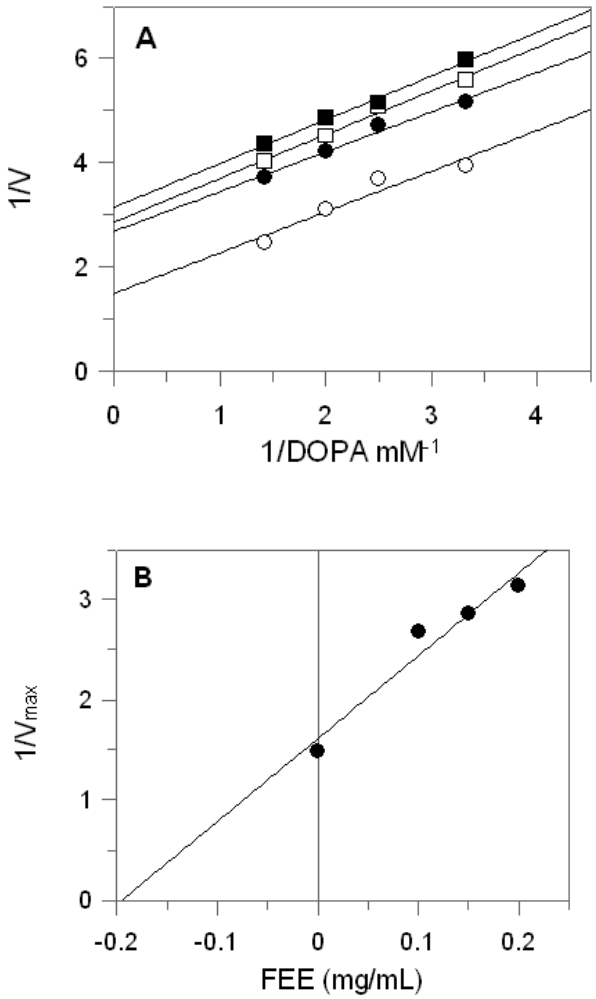


Figure 12: Kinetic behaviour of tyrosinase of *Asphodelus microcarpus* flowers ethanol extract from 0 to 0,2 mg/mL. (A)Lineweaver-Burk plot, (B) Inhibition constant

3.1.1.4. Cell viability

The results showed that FEE did not have a significant cytotoxic effect until 150 µg/mL (viability of 80 %), while 200 and 400 µg/mL resulted in a loss of viability of 35 and 50 % respectively (Fig. 13). Thus, further experiments using up to 150 µg/mL extract concentration were performed.

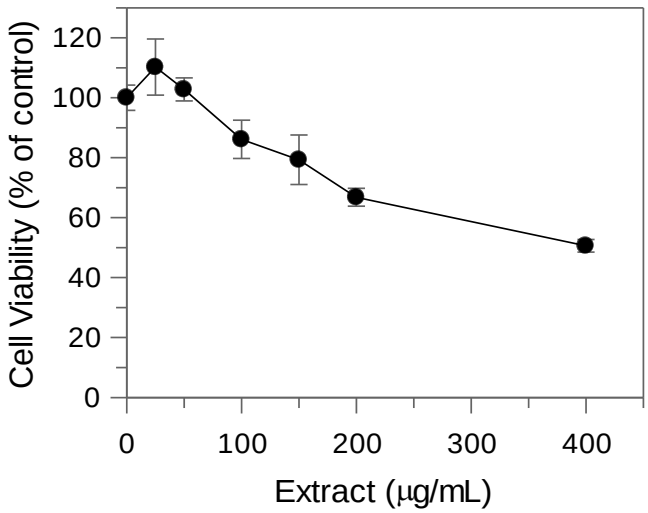


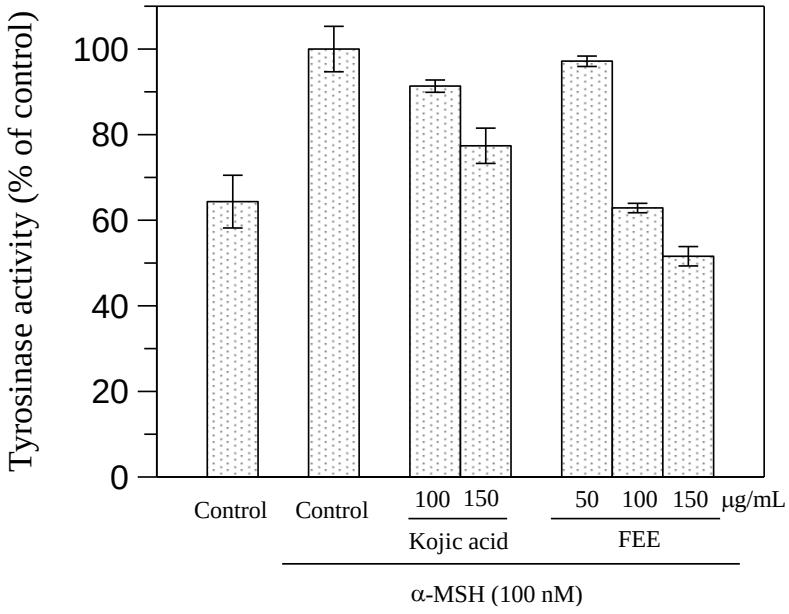
Figure 13: Effect of *A. microcarpus* extracts on cell viability in B16F10 melanoma cells. After 48 h incubation with ethanolic (●) extracts, cell viability was determined by MTT assay. Data are expressed as mean ± SD from three independent experiments.

3.1.1.5. Intracellular tyrosinase activity and melanin content

To obtain information about the inhibiting potency of FEE in the cellular model, the inhibitory effect of the extract on the tyrosinase activity of B16F10 cells treated with 100 nM α-MSH was examined. Upon exposure to α-MSH alone, the tyrosinase activity was significantly increased, compared to untreated cells (Fig. 14 A). After 48 h of incubation with FEE, tyrosinase inhibition at 50, 100 and 150 µg/mL was 2.85 ± 1.2, 37.14 ± 1.1 and 48.4 ± 2.26 % respectively. Thus, FEE significantly reduced the tyrosinase activity in murine cells in a concentration-dependent manner. The inhibitory effect of the ethanolic extract was even much stronger than that of kojic acid, the positive control, that showed a tyrosinase inhibition of 8.65 ± 1.44 and 22.61 ± 4.13 % at 100 and 150 µg/mL respectively. The melanin content of α-MSH-stimulated B16F10 cells in presence of the extract was also evaluated. FEE also reduced cellular melanin in a concentration-dependent manner (Fig. 14 B). Comparing the results obtained with the extracts and with kojic acid at the same concentrations of 100 µg/mL and 150 µg/mL, FEE exerted the highest cellular antimelanogenesis effect with an inhibition of 35.92 ± 2.77 and 48.77 ± 2.11 % versus 15.45 ± 0.64 and 41.2 ± 2.12 % of the standard inhibitor. Effect of FFE on the intracellular tyrosinase activity was also confirmed

by tyrosinase zymography. B16F10 cells were treated with α-MSH alone or α-MSH plus substances (extract or kojic acid). Tyrosinase activity in non-treated cells was very low while α-MSH-stimulated cells showed dark band with higher activity. FEE seemed to be almost ineffective at the concentration of 50 μg/mL while at 100 and 150 μg/mL of extracts, activity of tyrosinase decreased and lighter bands were observed (Fig. 15). Results are in agreement with the data of intracellular tyrosinase inhibition, confirming the anti-melanogenic effect of the extract, ever better than kojic acid.

A



B

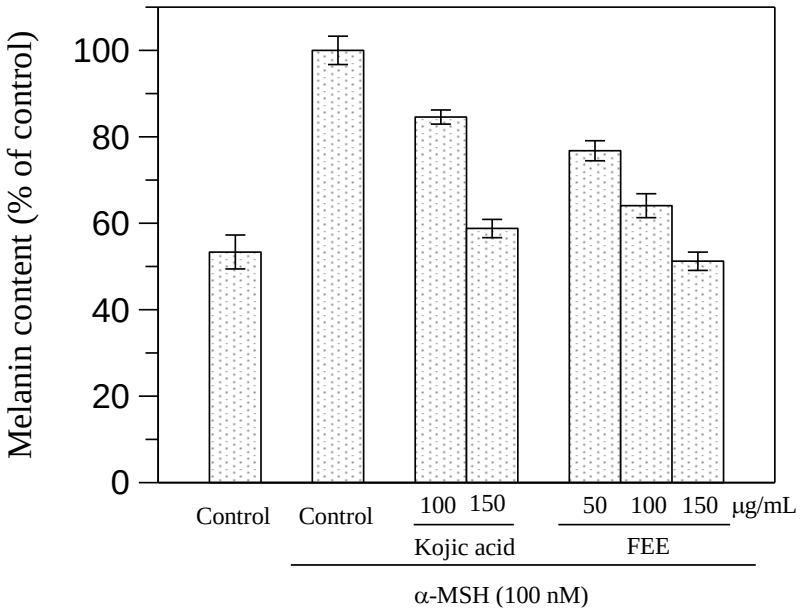


Figure 14: Effect of flowers extract on B16F10 melanoma cells.
Tyrosinase activity (A) and melanin production (B) are expressed as percentage of the control and the effects of FEE were compared with Kojic acid as standard inhibitor.

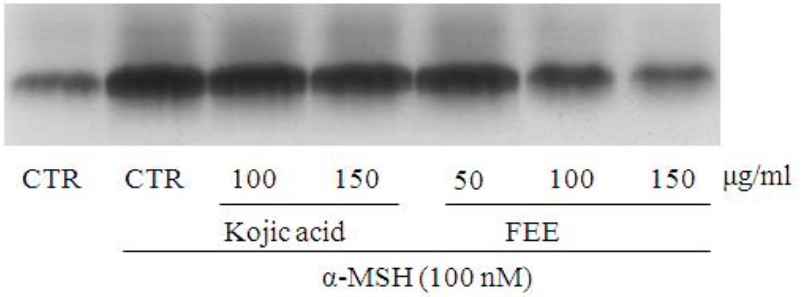


Figure 15: Tyrosinase activity was estimated by zymography

3.1.1.6. Characterization of phenolic compounds

The HPLC phenolic profile of *A. microcarpus* FEE recorded at 330 nm is shown in Fig. 16. The data of retention times (Rt), wavelengths of maximum absorbance (λmax), pseudomolecular ions ($[M-H]^-$), diagnostic fragments, and tentative identification for each peak of the phenolic compounds detected are listed in Table 4. Peaks 1 and 2 were tentatively assigned as 3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid based on their chromatographic characteristics and fragmentation patterns according to the identification keys described by Clifford (Clifford et al., 2003). The identity of 5-*O*-caffeoylquinic acid was further confirmed by comparison with an authentic standard. Peak 3 showed a molecular ion $[M-H]^-$ at *m/z* 447 giving place to four MS^2 fragment ions, a major one at *m/z* 357 $[M-H-90]^-$, and other three at *m/z* 327 $[M-H-120]^-$, m/z 285 $[M-H-162]^-$ and at *m/z* 429 $[M-H-18]^-$. This fragmentation pattern is characteristic of *C*-glycosylated flavones at C-6/C-8, according to the data reported by Ferreres (Ferreres et al., 2003). Thus, the peak was identified as luteolin-6-*C*-glucoside, which was also confirmed by comparison with a standard. The same pseudomolecular ion $[M-H]^-$ was found for peaks 4 and 5 ($[M-H]^-$ at *m/z* 447), but in both cases only a characteristic fragment at *m/z* 285 $[M-162]^-$ was produced, indicating the correspondence to *O*-hexosides. They were tentatively assigned as luteolin *O*-hexoside and luteolin 7-*O*-glucoside, respectively; the identity of this latter was confirmed by comparison with a standard. Peaks 6 and 8 presented pseudomolecular $[M-H]^-$ ions at *m/z* 489 and 593, respectively, and were identified as luteolin derivatives owing to the production of a common luteolin MS^2 fragment at *m/z* 285. They were assigned as luteolin *O*-acetylglucoside and luteolin *O*-deoxyhesylhexoside, respectively, based on the losses of 204 mu (162+42 mu) and 380 mu (146+162 mu) to produce the MS^2 fragment The remaining four peaks were assigned as different flavonoid aglycone based on their UV spectra and mass characteristics. Thus, the majority peak 7 was identified as luteolin, peak 9 as the flavanone narigenin, and peak 10 as apigenin, as also confirmed by comparison with standards. Finally, peak 11, with a pseudomolecular ion $[M-H]^-$ at

m/z 299 releasing a fragment at *m/z* 285 from the loss of a methyl residue (14 mu), was assigned as methyl-luteolin.

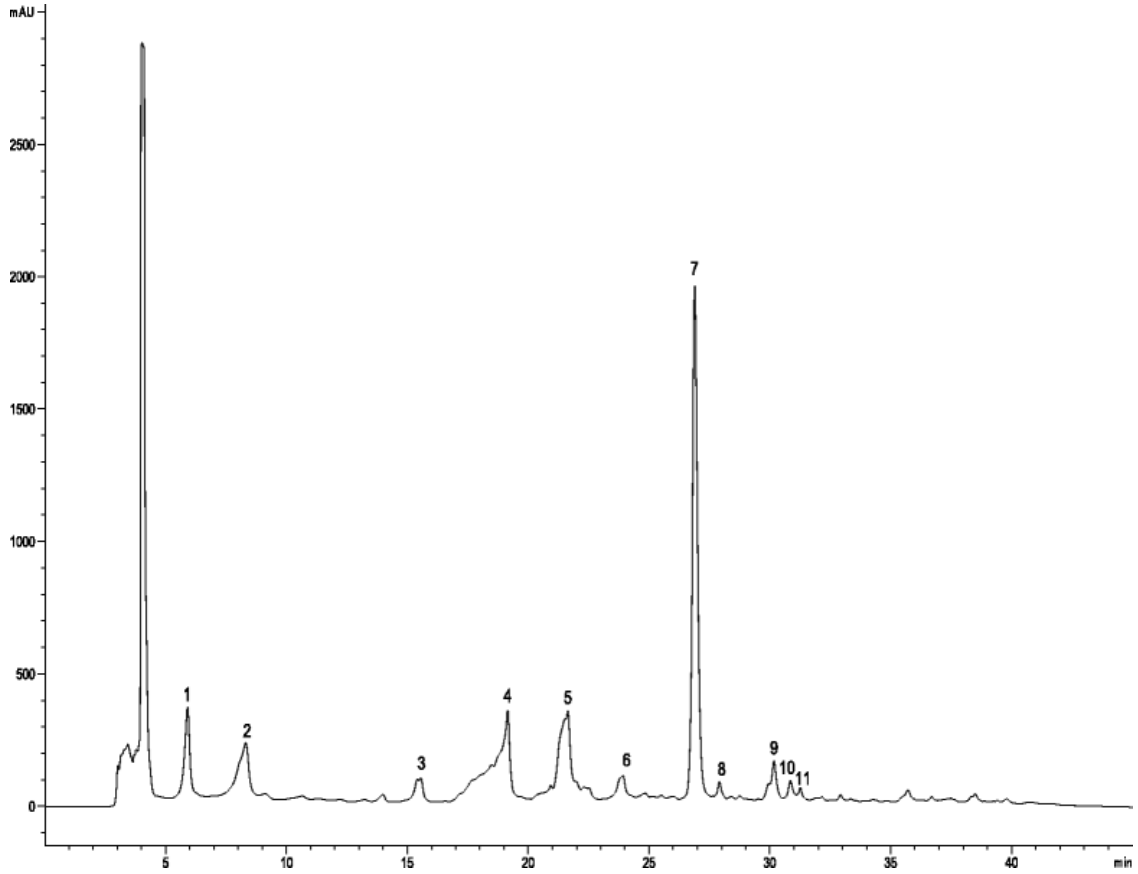


Figure 16: HPLC phenolic profile of *A. microcarpus* FEE recorded at 330 nm

Table 4: Identification of polyphenolic compounds in *A. microcarpus* flowers ethanolic extract by HPLC-DAD-ESI/MS analysis

Peak	**Rt (min)**	**λmax (nm)**	**Pseudomolecular ion $[M-H]^-$ (*m/z*)**	**MS^2 (*m/z*), (%)**	**Tentative identification**
1	5.9	326	353	191(100) 353 (94) 179(71) 135 (48) 173(9)	3-*O*-caffeoylquinic acid
2	8.3	326	353	191 (100)	5-*O*-caffeoylquinic acid
3	15.4	346	447	447(100) 357(98) 327(97) 285(25) 429(20)	Luteolin-6-*C*-glucoside
4	19	340	447	447(100) 285(40)	Luteolin-*O*-hexoside
5	21.5	338	447	285(100)	Luteolin-7-*O*-glucoside
6	23.9	338	489	285(100)	Luteolin *O*-acetylglucoside
7	26.8	345	285	285(100)	Luteolin
8	27.9	324	593	285(100)	Luteolin *O*-deoxyhexosylhexoside
9	29.9	288	271	151 (100) 119(56)	Naringenin
10	30.2	338	269	269(100) 151(36)	Apigenin
11	30.8	348	299	300(100) 285(88)	Methyl-luteolin

3.1.1.7. Antimicrobial activity of leaves extract

When tested on 13 different microorganisms, AE showed an inhibition effect on Gram-positive bacteria while lower inhibition was observed on the Gram negative bacteria *E. coli*, even at high extract concentration (500 µg/mL), Table 5. When tested for antibiofilm activity, AE showed an interesting effect on various bacterial strains (*E. coli, S. aureus, S. haemolyticus and B. clausii*). The MBIC was shown to be, except for *B. clausii*, 2 or 4 fold lower than the respective MIC value, Figure 17. This result suggests that AE is able to counteract the biofilm formation by the probable presence of anti-attachment or quorum quenching substances (Zhu, 2013). No activity was detected against Yeasts.

Table 5: Antibacterial profile of *A. microcarpus* leaves ethanol extract on a set of different microorganisms.

Strains	**Kirby Bauer**	**MIC**	**MBC**
Gram positive bacteria	Ø mm	µg/mL	µg/mL
Bacillus clausii	**4**±1	**250**	**>500**
Staphylococcus aureus	**10**±2	**250**	**>500**
Streptococcus salivarius	-	-	-
Streptococcus mutans	-	-	-
Staphylococcus haemolyticus	**6**±1	**250**	**>500**
Streptococcus faecalis	-	-	-
Streptococcus intermedius	-	-	-
Streptococcus pyogenes	-	-	-
Streptococcus uberis	-	-	-
Gram negative bacteria			
Escherichia coli	**4**±1	**500**	**>500**

Legend: (-) strain that resulted no sensitive with preliminary Kirby Bauer antimicrobial test Ø = 0 mm, conc. 1000 µg/mL.

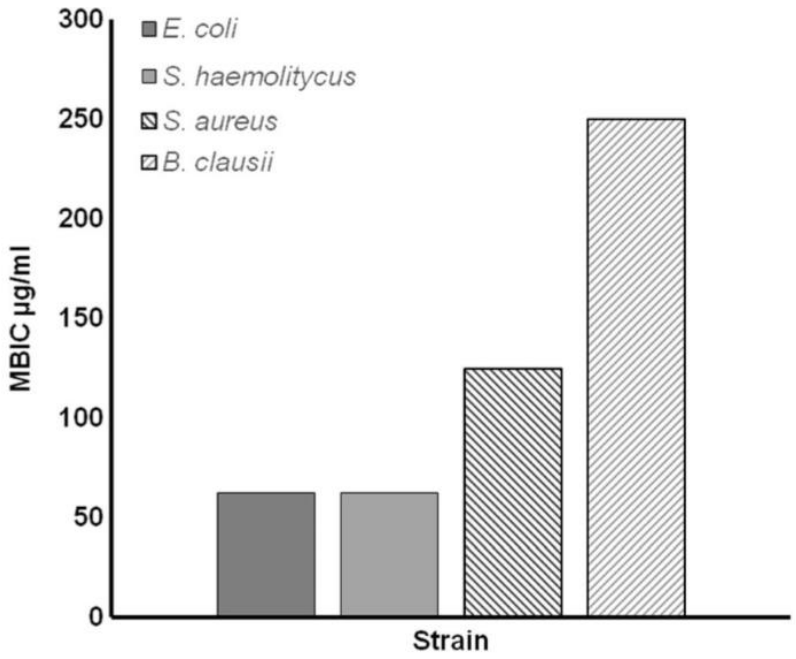


Figure 17: Minimal biofilm inhibitory concentration (MBIC) on four bacterial strains resulted biofilm sensitive at *Asphodelus microcarpus* extract. MBIC represent the lowest concentration showing a 450 nm absorbance > 95% comparable with negative control, sample without bacteria (ABS, SD ±15%).

3.1.2. Discussion

The interest in finding novel antimelanogenic agents from natural sources with antioxidant activity is of great interest. Since the key role of tyrosinase in melanin pathway, research of molecules that inhibit tyrosinase have become increasingly important for medicinal and cosmetic products that may be used as powerful skin-whitening agents for treating skin disorders.

In this study the antioxidant capacity of different extracts of *A. microcarpus* was analyzed and their effects on the tyrosinase activity and melanin synthesis was evaluated.

A. microcarpus extracts showed significant antioxidant activity. In particular, the ethanolic extract of the flowers showed the highest scavenging activity, which could be attributed mainly to its high levels of total polyphenols and flavonoids. Inhibitory effects of *A. microcarpus* extracts on mushroom tyrosinase were evaluated, and also in this case FEE revealed the highest inhibitory activity with an uncompetitive mode of inhibition. Antimelanogenic effect of this extract was confirmed in assays in B16F10 cells, being even more active than the positive control (kojic acid).

It is well known that polyphenols, and namely flavonoids, behave as inhibitors of ROS generation and could be responsible for the antimelanogenic activity of plant extracts (Choi et al., 2008; Shan et al., 2009; Ye et al., 2010). Up to eleven phenolic components are detected and tentatively identified in the ethanolic flowers extract, i.e., two chlorogenic acids, six luteolin derivates, naringenin, apigenin and methyl-luteolin. The major compound in the extract, was the aglycone luteolin, a compound that in a previous study was reported to show whitening activity. This compound did not inhibit directly tyrosinase and its activity was attributed to the inhibition of adenyl cyclase involved in the signalling pathway of α-MSH in B16F10 melanoma cells (Choi et al., 2008). This might explain the greater inhibition of FEE on B16F10 cells compared to the effect towards mushroom tyrosinase.

The inhibition of tyrosinase has an important role in order to prevent melanin accumulation in skin. Therefore, tyrosinase inhibitors are an attractive target in cosmetics and treatments for pigmentation disorders. The *A. microcarpus* extract may be used for the production of herbal preparations containing phytochemicals with significant bioactivity or as a source of inspiration for the development of new drug with less toxic side effects. In conclusion, our results demonstrated that ethanolic flower extract of *A. microcarpus* has strong tyrosinase inhibitory activity. This effect is even better than the standard inhibitor in cellular system. Moreover, it also shows the highest scavenging activity, which could be attributed mainly to its high levels of total polyphenols and flavonoids. These results suggest that FEE may be helpful such as source of bioactive compounds for controlling hyperpigmentation and as skin whitening agents.

Plant are rich of polyphenols, flavonoids and a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, glycosides, it has been shown that these compounds also have an antimicrobial activity (Abdollahzadeh S., et al. 2011; Pisano M.B. et al., 2016.). In fact, they could be a good alternative to synthetic chemical antimicrobial agents and antibiotics, because of the serious side effects, antimicrobial resistance and the emergence of previously uncommon

infections, which have been increasing due to inappropriate or widespread overuse of antimicrobials.

Regarding antimicrobial activity of *Asphodelus microcarpus,* its antimicrobial activity is previously published (Ghoneim MM, 2014) but only about the tuber extract. Moreover, have been reported the antimicrobial activities of methanolic extract of *A. microcarpus* leaves (El-Ghaly, 2017).

In this work ethanol leaves extract showed the ability to inhibit some Gram-positive and, with lower potency, Gram-negative bacteria but displayed no activities against *Streptococci spp*. and Yeasts. If we compare two Gram positive bacteria groups, our data demonstrate that extract is more active with aerobic strains (Staphylococcus) than with facultative anaerobic strains (Streptococcus).

MBIC test was carried out to evaluate the possible effects of the same extract on biofilm-mediated diseases: results showed an antibiofilm effect against some pathogens (*E. coli, S. aureus, S. haemolyticus and B. clausii*). Further studies are required to explain if the extract acts against biofilm formation through the quorum-sensing pathway or it acts as an anti-attachment agent.

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BMC Complementary and Alternative Medicine

RESEARCH ARTICLE Open Access

Tyrosinase inhibition and antioxidant properties of *Asphodelus microcarpus* extracts

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Abstract

Background: *Asphodelus microcarpus* belongs to the family Liliaceae that include several medicinal plants. In the traditional medicine plants of the genus *Asphodelus* are used to treat skin disorders such as ectodermal parasites, psoriasis, microbial infection and for lightening freckles. In order to find novel skin depigmenting agents, the present work was carry out to evaluate antioxidant activity and tyrosinase inhibitory potential of leaves, flowers and tubers extracts of *A. microcarpus*. The phytochemical composition of the active extract was also evaluated.

Methods: Three different extracts (water, methanol and ethanol) from leaves, flowers and tubers of *A. microcarpus* were evaluated for their inhibitory effect on tyrosinase activity using L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate. Inhibition of cellular tyrosinase activity and melanin production was also investigated in melanoma B16F10 cells. Antioxidant activity, total phenolic and flavonoids contents were determined using standard in vitro methods. HPLC-DAD-MS was used to identify phenolic profile of the active extract.

Results: The results showed that all extracts have a direct inhibitory anti-tyrosinase activity, with ethanolic extract from flowers (FEE) exhibiting the stronger effect. Kinetic analysis revealed that FEE acts as an uncompetitive inhibitor with a Ki value of 0.19 mg/mL. The same effect was observed in murine melanoma B16F10 cells. Cellular tyrosinase activity as well as melanin content were reduced in FEE-treated cells. The results were comparable to that of the standard tyrosinase inhibitor (kojic acid). Furthermore, the same extract showed the highest antioxidant activity and an elevated levels of total phenolics and flavonoid content. Eleven phenolic components were identified as chlorogenic acid, luteolin derivates, naringenin and apigenin.

Conclusions: Our findings showed that FEE from *A. microcarpus* inhibits tyrosinase and exerted antimelanogenesis effect in B16F10 cells. This extract also showed the highest scavenging activity, which could be mainly attributed to its high levels of total polyphenols and flavonoids. These results suggest that *A. microcarpus* has a great potential as sources of bioactive compounds which could be used as depigmenting agents in skin disorders.

Keywords: Antioxidants, *Asphodelus microcarpus*, B16F10 melanoma cells, Flavonoids, Polyphenols, Tyrosinase inhibitors

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Broad-range potential of *Asphodelus microcarpus* leaves extract for drug development

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Abstract

Background: Many plants have been used in traditional medicine for their antibacterial, antifungal, antiprotozoal, antiviral, antidiarrhoeal, analgesic, antimalarial, antioxidant, anti-inflammatory and anticancer activities.
In order to find novel antimicrobial and antiviral agents, the aim of the present study was the evaluation of the antibacterial and antibiofilm susceptibility of *Asphodelus microcarpus* leaves extract. Moreover, the antiviral activity and the phytochemical composition of the active extract were also determined.

Methods: Antimicrobial and antibiofilm activities of leaves ethanol extract of *A. microcarpus* were evaluated on 13 different microbial strains. We selected three different sets of microorganisms: (i) Gram-positive bacteria, (ii) Gram-negative bacteria and (iii) yeasts. The potential antiviral activity of *A. microcarpus* leaves ethanol extract was evaluated with a luciferase reporter gene assay in which the dsRNA-dependent RIG-I-mediated IFN-β activation was inducted or inhibited by the Ebola virus VP35 protein. HPLC-DAD-MS was used to identify phenolic profile of the active extract.

Results: *A. microcarpus* leaves extract showed a potent inhibitory activity on Gram-positive bacteria while only a reduced inhibition was observed on Gram-negative bacteria. No activity was detected against Yeasts. The extract also showed an interesting antibiofilm motif on various bacterial strains (*E. coli, S. aureus, S. haemolyticus* and *B. clausii*). Moreover, this extract significantly affected the Ebola virus VP35 inhibition of the viral RNA (vRNA) induced IFN response.

Conclusions: The overall results provide supportive data on the use of *A. microcarpus* as antimicrobial agent and a potential source of anti-viral natural products.
Data collected set the bases for further studies for the identification of single active components and the development of new pharmaceuticals.

Keywords: *Asphodelus microcarpus*, Plant extract, Antibiotics, Biofilm, Antimicrobial activity, Antiviral response, Ebola virus

3.2. Sarcopoterium spinosum

3.2.1 Results

3.2.1.1. Phenolics and flavonoids content

Table 6 shows the phenolic and flavonoid content of each extract, expressed as mg of gallic acid for phenols (mg GAE) or quercetin for flavonoid (mg QE) per g of extract, on dry basis. There are significant differences in the plant extracts in terms of contents of total phenols and flavonoids. Residual water (RW) extract showed the highest values with a total phenol content of 378.43±4.34 mg GAE/g and a total flavonoid content of 25.72±2.63 mg QE/g, followed by SFE extract and for last SE extract that showed, statistically, the lowest, total phenol content ($p < 0.05$). The solvents of the extraction influence the content of bioactive compounds, and the hexane does not promote the solubilisation of phenolic compounds (Bae et al. 2012).

Table 6: Total phenolic and flavonoid content in extracts of *S. spinosum* fruits.

Extract	**Total polyphenols** (mg GAE/g of dry weigh)	**Flavonoids** (mg QE/g of dry weight)
Supercritical CO_2 Extractions	10.3 ± 1.6^{b}	6.04 ± 0.96^{a}
Residual water extract	378.43 ± 4.34^{c}	25.72 ± 2.63^{b}
Soxhelet extract	3.9 ± 0.9^{a}	ND

3.2.1.2. Antioxidant activity of the extracts

The radical scavenging abilities of the extracts were evaluated using ABTS and DPPH assays. The antioxidant activity is given as EC_{50} value. The results (Table 7) show that AW and SE exhibited the lowest EC_{50} value of radical scavenging activity, while RW resulted in the highest EC_{50} value: 0.0056±0.0001 mg/mL for ABTS radical cation and 0.0048±0.0004 mg/mL for DPPH radical

concentration. The antioxidant activity value obtained showed that the RW extract has a similar antioxidant potential to Trolox ($p < 0.05$).

3.2.1.3. Tyrosinase inhibitory activity of the extracts

All the plant extracts were subjected to tyrosinase assay with L-DOPA as substrate. As shown in Table 7 tyrosinase activity was inhibited by extracts: at 0.4 mg/mL of SFE, SE and RW and 0.4 mL/mL for AW, the tyrosinase inhibition ranged from 2.7±0.7% to 59±1%. The results indicated that RW had statistically the highest activity ($p < 0.05$), while SFE, AW and SE extracts showed only moderate activity at the concentration tested. The highest scavenging activity and the inhibitory activity of RW, could be attributed mainly to its high levels of total polyphenols and flavonoids. Literature data reported that polyphenols possess strong antioxidant activity and behave as inhibitors of ROS (reactive oxygen species) generation and tyrosinase (Ranilla et al. 2010; Bouzaiene et al. 2016).

Table 7: Anti-tyrosinase and antioxidant activities of *S. Spinosum.*

Extract	**% Inhibition Tyrosinase (0.4 mg/mL)**	**Antioxidant activity (EC_{50} values mg/mL)**	
		ABTS	**DPPH**
Supercritical CO_2 Extractions	13.81±1.30[b]	1.30 ±0.03[b]	1.14±0.01[b]
Soxhelet extract	2.7±0.7[a]	>2	>2
Residual water extract	59.14±1.14[c]	0.0056±0.0001[a]	0.0048±0.0004[a]
Aromatic water	12.71±0.24[b*]	>0.5[**]	>0.5[**]

[*] at 0.4 mL/mL; [**] EC_{50} value expressed as mL/mL.

3.2.1.4. Characterization of phenolic compounds

The HPLC phenolic profile of *S. spinosum* RW recorded at 330 nm is shown in Fig. 18. and identification for each peak of the phenolic compounds detected are listed in Table 8.The majority peak 1 was identified as quercetin glucuronide with m/z 477 and MS^2 fragment 301 after elimination of a glucurone unit (176 amu). (Kajdžanoska M., 2010)

Peak 2 showed a molecular ion $[M-H]^-$ at m/z 447 and only a characteristic fragment at m/z 285 $[M-162]^-$ was produced, indicating the correspondence to O-hexosides. This was tentatively assigned as luteolin 7-O-glucoside; the identity of this latter was confirmed by comparison with a standard (Di Petrillo et al., 2016). Peak 3 was tentatively assigned as isorhamnetin 3-O-glucuronide according to their UV-vis and mass spectra (all of isorhamnetin derivatives released an MS^2 product ion at m/z 315) (Barros L., et al., 2012). Peak 4 was tentatively assigned as quercetin sulphate based on the losses of 80 mu to produce the MS^2 fragment (m/z 301) indicating the correspondence to sulphate. The last peak was identified as quercetin according to their UV-vis and mass spectra and was also confirmed by comparison with standards.

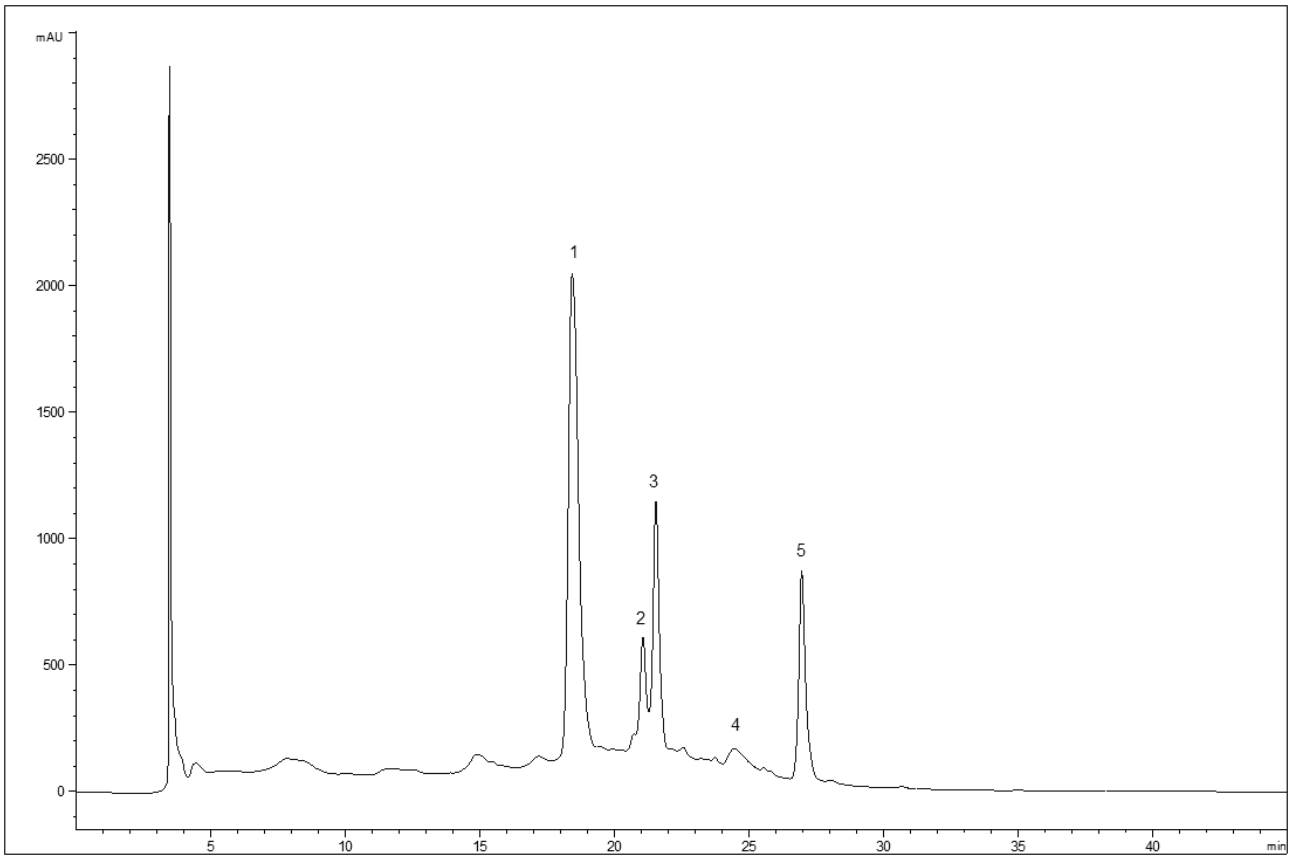

Figure 18: The HPLC phenolic profile of *S. spinosum* RW recorded at 330 nm

Table 8: Identification of polyphenolic compounds in *S. spinosum* RW extract by HPLC-DAD-ESI/MS analysis

Peak	**Rt (min)**	**λmax (nm)**	**Pseudomolecular ion $[M-H]^-$ (*m/z*)**	**MS^2 (*m/z*), (%)**	**Tentative identification**
1	18.5	357	477	301(100) 477(93)	Quercetin glucuronide
2	21.1	348	461	285(100) 461(85) 153(35)	Luteolin 7-O-glucuronide
3	21.6	355	491	300(100) 491(99) 301(66) 315(30)	Isorhamnetin 3-O-glucuronide
4	24.6	370	381	301(100) 381(76)	Quercetin suphate
5	27	370	301	301	Quercetin

3.2.2. Discussion

Sarcopoterium spinosum is a poorly studied Mediterranean plant so we thought it would be interesting to find out its new uses, especially medicinal and cosmetic industry. The main objective of this study is to investigate the antioxidant and tyrosinase inhibitory properties of extracts of *Sarcopoterium spinosum* fruit. The extracts were obtained by supercritical CO_2 (SFE), at 250 bar and 40 °C, hexane in a Soxhlet apparatus (HE) and hydrodistillation (HD). The plant did not contain measurable amount of essential oil, but the aromatic and the residue water were tested. The supercritical and the hexane extracts were characterized, respectively, by palmitic acid (7.7 %, SFE vs 12.7 %, HE), oleic acid (9.4 % vs 9.3 %), linolenic acid (44.6 % vs 39.5 %) and α-linolenic acid (25.9 % vs 20.6 %). In the residue water of HD have been identified: quercetin glucuronide, luteolin 7-O-glucuronide, isorhamnetin 3-O-glucuronide, quercetin sulphate and quercetin.

The *S. spinosum* extracts were evaluated for total phenolic, flavonoid, and *in vitro* antioxidant activity DPPH and ABTS scavenging assays. The results indicated that HD residue possessed potent antioxidant activity, comparable to the Trolox. The HD residue has the highest polyphenol content (378±4 mg GAE/g of weight) and flavonoids (26±3 mg QE/g of weight) among all plant extracts tested. Tyrosinase activity assays were performed with L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate. The HD residue is the most active extract of this series (IC_{50} = 0.292 mg/ml). This result may be due to the presence of polyphenols in it as quercetin and luteolin derivates. This first work on its tyrosinase inhibitory activity could be a starting point for future experiments focused on its use like skin-whitening agents.

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Evaluation of antioxidant and tyrosinase inhib activities of the extracts of Sarcopoterium spinosum (L.) Spach fruits

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3.3. Phytolacca dioica

3.3.1. Results

3.3.1.1. Characterization of phenolic compounds

In order to identify the nature of phenolic compounds contained in AE (ethilacetate extract) of *P.dioica* seeds HPLC-DAD-ESI/MS analyses was carried out. The HPLC separation profile revealed a high complexity in the seed phenolic composition as demonstrated by the presence of many peaks recorded at 330 nm (Fig.19).

The phenolic composition of AE is summarized in Table 9 with the relative reference for each compound previously identified.

Relevant peaks in the extract were assigned as neo-lignans. Peaks 5 and 6 were tentatively identified AS different isomers of isoamericanol (B1, B2, C1 and C2) with the molecular mass of 434 (Waibel et al, 2003). This identification was further confirmed from the MS^2 fragments at *m/z* 329 that might correspond to (iso)americanol A (also associated to peak 2) and *m/z* 327 to (iso)americanin A (Waibel et al., 2003; Tanaka, 1987).

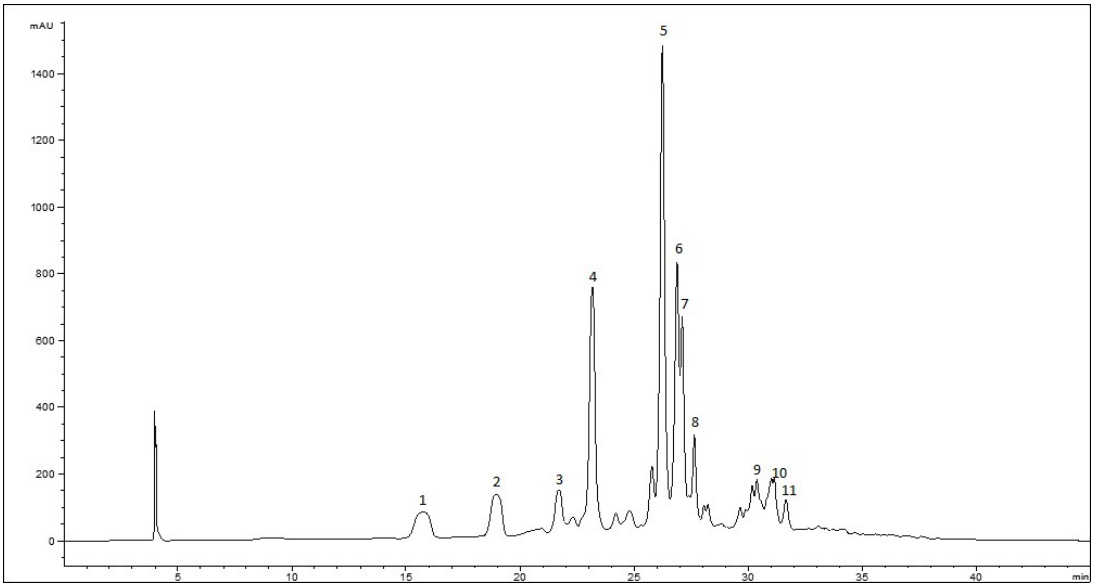

Figure 19: HPLC chromatogram of *P. dioica* seeds ethyl acetate extract recorded at 330 nm for phenolic compounds. Peak identification is given in Table 9.

Table 9: Identification of polyphenolic compounds in *Phytolacca dioica* seeds ethyl acetate extract by HPLC-DAD-ESI/MS analysis

Peak	**Rt (min)**	**λmax (nm)**	**Pseudomolecular ion $[M-H]^-$ (*m/z*)**	**MS^2 (*m/z*), (%)**	**Tentative identification**
1	15.7	342	163	135(100) 121(42) 145(41) 119(20)	p-coumaric acid
2	19	330	329	137(97)	(iso)americanol A
3	21.7	324	297	135(41) 179(23)	caffeoyl-threonic acid (isomer I)
4	23.1	320	687	179(100) 133(100) 269(28)	Unknown
5	26.2	334	493	329(100) 327(100) 165(64) 175(35) 137(32)	isoamericanol B1 or related isomers (B2 / C1 / C2 / (iso)princepin)
6	26.8	338	493	165(100) 298(75) 329(40) 147(23)	isoamericanol B1 or isomers (B2 / C1 / C2)
7	27.1	320	297	135(36) 179(16)	caffeoyl-threonic acid (isomer II)
8	27.6	320	462	289(93) 165(100) 147(32)	Unknown lignan
9	30.3	334	657	493(100) 327(41) 165(25)	Unknown lignan
10	31.1	324	329	211(32) 229(31) 171(18)	trihydroxy-octadecenoic acid

3.3.1.2. HSCCC and structure identification of isolated compounds from Phytolacca dioica seeds extracts

Two compounds were isolated from AE extract using HSCCC. Structure of purified compounds was elucidated by interpretation of spectroscopic experiments (UV, MS, and ^{1}H NMR) and by comparison with literature.

Compound **1**: Mixture of Isoamericanol B1, B2, C1 and C2 UV (MeOH) λ_{max} nm: 334; HRESI–MSm/z$[M-H]^-$: 493; ^{1}H NMR (Methanol-d4, 400 MHz) – Table 10 (Waibel, 2003)

Compound **2**: UV (MeOH)λ_{max}(MeOH)nm: 210; HRESI–MSm/z$[M-H]^-$: 6 6 5 [M + H] +, 5 1 5 [M+H-150]+;^{1}H NMR (Methanol-d4, 400 MHz): 0.78 (3H, s, H26), 0.91 (3H, s, H29), 1.12 (3H, s, H27), 1.15 (3H, s, H24), 1.26 (3H, s, H25), 2.68 (2H, brd, H-18), 3.66 (3H, s, COOMe), 4.35 (1H, d, J=8Hz, H3), 5.31 (1H, brs, H12), 5.32 (1H, d, 8Hz, H1 Xyl). Compound **2** was identified as phytolaccoside B.

Table 10: NMR compound 1 – Mixture of Isoamericanol B1, B2, C1 and C2

Isoamericanol (B1, B2, C1, C2)	**^{1}H-NMR**	**^{13}C-NMR**
1	-	129.2
2	6.90 (d, 2.5 Hz)	115.9
3	-	146.2
4	-	146.3
5	6.83 (d, 8.5 Hz)	115.7
6	6.76 (dd, 2.5, 8.5 Hz)	119.4
7	4.80 (d, 8 Hz)	76.2
8	3.97 (m)	78.7
9	3.46 (dd, 12.5, 5.5) 3.65 (dd, 12.5, 3)	60.6
1’	-	130.7
2’	7.00 (m)	117.2
3’	-	145.7
4’	-	145.7
5’	6.94 (m)	119.2
6’	6.94 (m)	119.8
7’	-	76.1
8’	3.97 (m)	78.6
9’	3.46 (dd, 12.5, 5.5)	60.6
1”	3.65 (dd, 12.5, 3)	131.0
2”	6.94 (m)	115.9
3”	-	144.0
4”	-	145.6
5”	6.90 (d, 2.5)	117.5
6”	4.17 (dd, 6, 1.5 Hz)	119.2
7”	6.47 (d, 16 Hz)	129.8
8”	6.17 (dt, 16.6, 7.8 Hz)	128.5
9”	-	62.0

3.3.1.3. Phenolics and flavonoids content

To elucidate the composition of the extracts from *P. dioica* seeds the polyphenolic component of the samples was quantified (Table 11).

The AE extract exhibited the highest phenolic content. In fact, it was respectively 2.8 and 3.9 folds higher than that determined in BE and CE extracts. The AE also yielded the highest amount of flavonoids, followed by WE, CE and BE. As it was expected, HE was the poorest in both phenolic and flavonoid compounds.

3.3.1.4. Antioxidant activity of the extracts

P. dioica extracts were evaluated for their antioxidant activity using two different methods, leading to quite similar values for each extract analysed. In agreement with the polyphenolics amount, the HE showed the poorest antioxidant activity, whereas the AE and BE showed the highest antioxidant activity (Table 11). EC_{50} values of AE and BE are comparable to that of Trolox, used as reference compound.

Compound 1 (mixture of Isoamericanol B1, B2, C1 and C2) has showed for ABTS and DPPH 7 and 6.5 µg/mL EC_{50} (Table 11) respectively, explaining partly the antioxidant activity shown.

Table 11: Free radical scavenging activity of *P. dioica* extracts

Extract	**Total polyphenols(*)**	**Flavonoids(**)**	**EC_{50} values (µg/mL)**	
			ABTS scavenging	**DPPH scavenging**
Hydroalcolic extract	102.07±2.30^{c}	18.43±1.22^{bc}	16.97±0.32^{a}	20.4±1.23^{a}
Hexane extract	2.61 ± 0.21^{a}	1.82±0.42^{a}	>500	>500
Ethylacetate extract	396.41±4.21^{e}	43.57±1.26^{d}	3.1±0.04^{a}	4.85±0.11^{a}
Butanol extract	143.64±1.35^{d}	15.19±0.54^{b}	5.64±0.26^{a}	6.28±0.91^{a}
Water extract	46.34±0.42^{b}	19.66±0.39^{c}	127.48±8.37^{b}	137.79±5.53^{b}
Mixture of isoamericanol B1, B2, C1 and C2	-	-	7.1±0.4	6.51±0.74
Phytolaccoside B	-	-	>500	>500
Trolox	-	-	3.4±0.3	3.2±0.4

3.3.1.5. Enzyme inhibitory activity of the extracts and isolated compounds

All extracts were also evaluated for their inhibitory proprieties on TYR and XO enzymes (Table 12).

Results showed that AE exhibited the most potent inhibitory activity against mushroom tyrosinase, with 50.83% inhibition at 200 µg/mL. BE and CE were less potent and none activity was showed by HE and WE.

AE was also the most potent inhibitor of xanthine oxidase among all extracts; it exhibited 99.93±1.94% inhibition at a concentration at 200 µg/mL. CE and BE showed 50.5±0.99% and 23.47±1.66 % inhibition, respectively, at same conditions, whereas the remaining extracts showed no XO inhibitory activity.

Taking into account the complete inhibition of XO by AE, further attention was focused on this mode of inhibition of the enzyme, which was determined by Lineweaver-Burk plot analysis, as shown in Figure 20. The kinetic analysis revealed that the extract acts as a mixed-type inhibitor. In fact, increasing the concentration of extract resulted in a family of lines, which intersected in the second quadrant. To calculate the equilibrium inhibition constants for the inhibitor binding with the free enzyme (K_I) and the enzyme-substrate complex (K_{IS}), two secondary graphs were obtained plotting the K_m/V_{max} (slope) and $1/V_{max}$ (intercept) versus the concentration of AE. The values of K_I and K_{IS} were determined to be 0.68 and 0.007 mg/mL respectively.

The mode of inhibition of this extract on tyrosinase was also determined using Lineweaver-Burk plot which displayed a uncompetitive inhibition of the enzyme, see Figure 20. The equilibrium constant for binding with enzyme–substrate complex (KIS) was calculated to be 0.1135 mg/mL.

The isolated mixture of isoamericanol B1, B2, C1 and C2 showed a good inhibitory activity on tyrosinase and xanthine oxidase enzymes with IC_{50} of 0.110 ± 0.02 mg/mL and 0.145 ± 0.05 mg/mL respectively (Table 12). No activity was detected for the phytolaccoside B.

Table 12: Inhibitory activity of the extracts of *P. dioica* seeds against Tyrosinase and Xanthine Oxidase at 200 µg/mL.

Extract	**Inhibitory activity (Inhibition % ± SD)**	
	Tyrosinase	**Xanthine oxidase**
Hydroalcoli extract	22.35±0.49[a]	50.5±0.99[b]
Exane extract	ND	ND
Ethylacetate extract	50.83±0.84[c]	99.93±1.94[c]
Butanol extract	29.23±0.90[b]	23.475±1.66[a]
Water extract	ND	ND

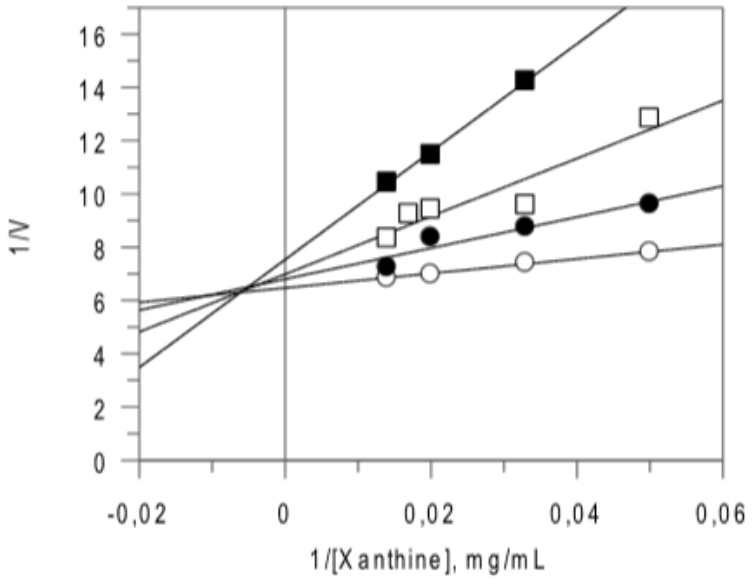

Figure 20: Lineawear-Burk plot for inhibition of AE extract on xanthine oxidase

3.3.2. Discussion

In view of the limited published information regarding chemical composition of *Phytolacca dioica* seeds we have obtained new information about their phenolic compounds. Moreover has been evaluated the bioactive potential of seed extracts of *Phytolacca dioica* in inhibiting the enzyme tyrosinase and xanthine oxidase as well as evaluating their antioxidant activities.

The ethyl acetate extract exhibited highest antioxidant capacity if compared with other extracts.The EC50 values were strongly correlated with total phenolic contents, suggesting the contribution of these compounds to the antioxidant properties of the extracts. Moreover, most of the polyphenols identified were lignans with well-known antioxidant activity (Xiao-Xiao Huang et al, 2014).

The results of this study showed that the ethyl acetate extract of P. dioica seeds possessed a good XO and tyrosinase inhibitory activity. The isolated mixture of isoamericanol (B1, B2, C1 and C2) showed a good inhibitory activity on tyrosinase and xanthine oxidase enzymes.This is the first time that these isoamericanols have been described as an inhibitor of the above-mentioned enzymes and the presence of these compounds in the thyl acetate extract may contribute to its the activity. These findings suggest that *P. dioica* extract could be a source of bioactive molecules.

3.4. Sardinian honey

3.4.1. Results

3.4.1. Physicochemical parameters

Honeys from different botanical origin showed different physicochemical characteristics (colour, moisture, pH, electrical conductivity and total acidity) due to the presence of diverse types of compounds (Table 13). The CIE *L*a*b** parameters showed colours variability: *L** (lightness) ranged from 22.11 (Eucaliptus) to 33.94 (Arbutus); the parameters a^* and b^*, colorimetric coordinates, showed that green components (negative *a** values) were present in Asphodelus and Sulla honeys. Chrome value C* was highest in Arbutus honey while Sulla and Asphodelus honeys had lowest values. This parameter expresses the saturation of colors, with high-saturated colors

being vivid and low-saturated colors dull (Voss 1992; Hoppula and Karhu, 2006). The moisture of honey samples ranged from 16.24 ± 0.21 to 17.19 ± 0.26% and the pH varied from 3.65 ± 0.04 to 4.55 ± 0.02 (Table 13). The pH value is not directly related to free acidity because of the buffering action of various acids and minerals present. As shown in Table 13, total acidity ranged from 5.61 ± 0.99 to 32.97 ± 1.41 meq/kg. Finally, the electrical conductivity of honeys was from 0.06 ± 0.01 to 0.943 ± 0.03 mS/cm.

Table 13: Physicochemical parameters of Sardinian honeys

Honeys	**Colour**					**Moisture (% w/w)**	**Electrical conductivity (mS/cm)**	**pH**	**Total acidity (meq/kg)**
	L*	**a***	**b***	**C***	**h°**				
Arbutus	33.94 ± 0.01	+1.51 ± 0.01	+8.26 ± 0.01	15.09 ± 0.02	81.66° ±0.11	17.19 ± 0.26	0.37 ± 0.03	4.31 ± 0.02	20 ± 0.2
Asphodelus	25.4 ± 0.05	-0.14 ± 0.03	+1.8 ± 0.02	3.11 ± 0.03	93.9° ± 0.1	17.03 ± 0.05	0.06 ± 0.01	3.88 ± 0.11	5.61 ± 0.99
Eucaliptus	22.13 ± 0.01	+0.89 ± 0.01	+8.13 ± 0.01	8.18 ± 0.02	69.3° ± 0.36	15.03 ± 0.05	0.93 ± 0.03	4.55 ± 0.02	32.97 ± 1.41
Thistle	25.23 ± 0.05	+0.72 ± 0.03	+8.68 ± 0.12	8.72 ± 0.1	85.22° ± 0.2	16.83 ± 0.15	0.43 ± 0.01	4.12 ± 0.09	25.8 ± 0.28
Sulla	32.56 ± 0.06	-0.53 ± 0.02	4.18 ± 0.02	3.02 ± 0.05	96.34° ± 0.24	16.24 ± 0.21	0.12 ± 0.01	3.65 ± 0.04	15.1 ± 1.7

3.4.2. Phenols and flavonoids content

Concerning the quantification of the total phenolic compounds in Sardinian honeys, the results revealed that the total polyphenol content ranged from 200 ± 2 to 969.7 ± 8.5 mg GAE/kg (Table 14) and the honey from Arbutus had the highest total phenolic content when compared to other, followed by Eucalyptus. Meanwhile, the honey from Sulla showed the lowest value. The total flavonoid content of the honey samples were in the range of 10.4 ± 1.4 to 96.5 ± 3.3 mg QE/kg of honey (Table 14). The honey from Arbutus, also in this case, gave the best results, showing a significantly high flavonoid content ($p < 0.05$).

Table 14: Total phenol and flavonoid contents of honey samples.

	Total Phenol Content (mg GAE*/kg)	**Total Flavonoid Content (mg QE**/kg)**
Arbutus	969.7 ± 8.5^{e}	96.5 ± 3.3^{d}
Asphodelus	401.5 ± 4.6^{b}	70.2 ± 4.8^{c}
Eucaliptus	828.8 ± 6.4^{d}	56.3 ± 2.3^{b}
Thistle	448.2 ±2 .6^{c}	15.1 ± 1.8^{a}
Sulla	200 ± 2^{a}	10.4 ± 1.4^{a}

*GAE: gallic acid equivalent. **QE: quercetin equivalent.

The data are given as mean ± standard deviation (SD) of triplicate experiments. The statistical comparison between values from the different types of honey applied using the post hoc Duncan test. Means followed by distinct letters in the same column are significantly different ($p < 0.05$).

3.4.3. Antioxidant activity of the extracts

The antioxidant activities of honey from different types of pollen were evaluated using ABTS and DPPH assays (Table 15). The results of ABTS ranged from 4.2 ± 0.2 to 57.8 ± 4.4 and of DPPH from 6.12 ± 1.2 to 54.25 ± 3.4 mg/mL. Both assays showed that honey from Arbutus flower had the highest antioxidant capacity compared to other ($p < 0.05$), while the honeys from Sulla and Asphodelus had the lowest activity.

3.4.4. Tyrosinase and Xanthine Oxidase inhibitory activity of the extracts

All five honeys were also investigated for their inhibitory activity toward xanthine oxidase and tyrosinase enzymes (Table 15). Eucalyptus honey exhibited the highest degree of inhibition of xanthine oxidase with IC_{50} of 38.2 ± 3.3 mg/mL followed by Thistle honey and Arbutus honey (IC_{50} values of 40.2 and 57.9 mg/mL respectively). Sulla honey had the lowest degree of inhibition (IC_{50} values of 83.9 mg/mL), while Asphodelus honey showed no inhibitory effect.

Table 15 also show the tyrosinase inhibitory activity of honeys. Honey from Thistle flowers had the highest anti-tyrosinase activity (IC_{50} 64.3 ± 1.6 mg/mL) when compared to the other types of tested honey ($p < 0.05$) followed by Arbutus and Eucalyptus honeys (IC_{50} 119.7 ± 5.2 and 157.7 ± 2.5 respectively), while Sulla and Asphodelus honeys showed the lowest activity.

Table 15: Antioxidant properties and inhibitory activity of honey samples.

Honey	**EC_{50}**			
	ABTS (mg/mL)	**DPPH (mg/mL)**	**Xanthine Oxidase (mg/mL)**	**Tyrosinase (mg/mL)**
Arbutus	4.2 ± 0.2[a]	6.1 ± 1.2[a]	57.9±2.3[c]	119.7±5.2[b]
Asphodelus	57.8 ± 4.4[d]	54.3 ± 3.4[d]	-	>200
Eucaliptus	9.1 ± 2.5[b]	21.4 ± 1.7[b]	38.2±3.3[a]	157.7±2.5[c]
Thistle	20 ± 1.7[c]	23.3 ± 0.4[b]	40.2±1.8[b]	64.3±1.6[a]
Sulla	56.8 ± 8.3[d]	45.3 ± 1.9[c]	83.9±3.7[d]	>200
3-Phenyl lactic acid			>200	>200
Lumichrome			>200	>200
Allopurinol			12 ± 1.7	
Kojic Acid				$(0.8 \pm 0.03) \times 10^{-3}$

The data are given as mean ± standard deviation (SD) of triplicate experiments. The statistical comparison between values from the different types of honey applied using the post hoc Duncan test. Means followed by distinct letters in the same column are significantly different ($p < 0.05$).

3.4.5. HPLC-DAD fingerprinting

The HPLC-DAD fingerprinting confirmed the typical marker for asphodel, Arbutus, and Thistle unifloral honeys (Tuberoso et al, 2009; Tuberoso et al, 2010; Tuberoso et al, 2011) (Table 16). For Eucalyptus and Sulla flower unifloral honeys there are no proposed polar marker compounds, but HPLC-DAD fingerprinting well-match with the average profiles of these unifloral honeys. Table 16

shows quantitative data for the selected marker compounds. Some amino acids were also detected. Asphodel honey is characterized by high amount of methyl syringate (174.5 ± 9.2 mg/kg), higher than the minimum level of 122.6 mg/kg proposed for this unifloral honey (Tuberoso et al., 2009). Arbutus honey presents all the four typical markers proposed for this unifloral honey (Tuberoso et al., 2010): homogentisic acid (346.8 ± 17.2 mg/kg) unedone (166.2 ± 9.6 mg/kg) t,t-abscisic acid (ABA) (109.6 ± 4.2 mg/kg), and c,t-ABA (116.9 ± 4.7 mg/kg). Lumichrome and 3-phenyl lactic acid represent the chemical markers of Thistle honey and their amount in the analysed honey (68.3 ± 2.9 and 265.6 ± 15.0 mg/kg, respectively) were on the average for this unifloral honey (Tuberoso et al., 2011). Small amount of kojic acid (1.7 ± 0.0 mg/kg) were identified only in Thistle honey. This compound is a degradation product of carbohydrates and previously it was found in Manuka honey (Alvarez-Suarez et al., 2014).

Table 16: Compounds identified by targeted HPLC-DAD analysis (mg/kg)

	Compound	***Asphodelus***	***Arbutus***	***Eucalyptus***	***Thistle***	***Sulla***
1	Kojic acid	nd	nd	nd	1.7 ± 0.0	nd
2	Tyrosine	130.0 ± 4.1	nd	2.5 ± 0.1	14.8 ± 0.3	7.0 ± 0.1
3	HMF	nd	nd	4.8 ± 0.1	6.7 ± 0.2	nd
4	Homogentisic acid	nd	346.8 ± 17.2	nd	nd	nd
5	Phenylalanine	348.1 ± 22.8	nd	tr	31.5 ± 1.9	8.8 ± 0.2
6	Unedone	nd	166.2 ± 9.6	nd	nd	nd
7	3-Phenyl lactic acid	nd	nd	nd	265.6 ± 15.0	nd
8	Lumichrome	nd	nd	0.6 ± 0.0	68.3 ± 2.9	nd
9	Methyl syringate	174.5 ± 9.2	nd	nd	nd	nd
10	*t,t*-Abscisic acid	nd	109.6 ± 4.2	nd	nd	nd
11	*c,t*-Abscisic acid	nd	116.9 ± 4.7	nd	nd	nd

All measurements were performed in triplicate, the results are presented as average ± standard deviation out of n = 3 samples; nd: not detected. *: dosed as *c,t*-abscisic acid

3.4.2. Discussion

In this study Sardinian honeys were analysed for their physicochemical characteristics and biological activities. The pH values and electrical conductivity of the honey samples were not different from each other and can be compared with honey samples from other geographical locations reported for Argentine, Spanish, and Italian honeys (Cantarelli et al 2008). All the samples had their acidity values within the limits of <50 meq/kg, as specified by the international standards. Several researchers have reported different values of acidities for some honeys from various locations around the world (Omafuvbe et al 2009; Jilani et al 2008). All the results were within the limits allowed by the European Council Directive and the Codex Standard for Honey. The colour of honey, in addition to the flavour and aroma, is one of the features that could allow a first classification of the honey by botanical origin. In fact, Chrome value C*, which expresses the saturation of colors, was very different depending of the type of honeys; Arbutus honey showed the highest value while Sulla and Asphodelus honeys had lowest values. Several studies reported a correlation between color of unifloral and multifloral honeys, phenolic concentration and antioxidant capacity (Ferreira et al., 2009). Analysis of Brazilian honeys revealed that the darkest honey samples had the highest antioxidant capacity and highest phenolic, flavone, and flavonol contents (Pontis et al., 2014). These data are in accordance with our results, in fact Sardinian honeys have shown a high correlation between the Chrome value (C*), antioxidant activity and total phenol content. The Arbutus honey had the highest phenolic content, antioxidant capacity and color intensity and, on the other hand, Sulla honey showed the lowest phenolics content and lowest color intensity.

The phenolic and flavonoid composition of honey depends on floral sources, environmental and seasonal factors. These compounds are considered beneficial for human health due to their wide range of biological activities, including antimicrobial, anticarcinogenic, antioxidant activity, and skin whitening (Kubo et al., 2000; Ulusoy and Kolayli, 2014).

Anti-tyrosinase activity of honey was tested and Thistle honey showed the highest percentage of inhibition ($\rho < 0.05$) (Table 15). The HPLC analysis of the honeys revealed that the majority compounds of Thistle honey were 3-phenyl lactic acid and lumichrome. In literature there are no evidence about the anti-tyrosinase activity of lumichrome, while anti-melanogenic activity of 3-phenyl lactic acid in cell system have been reported in a previous study (Kim et al., 2011). Therefore, we performed anti-tyrosinase assay using commercial lumichrome and 3-phenyl lactic acid. These compounds showed no tyrosinase inhibition. Thus anti-tyrosinase activity observed in Thistle honey can not be attributed to lumichorme or 3-phenyl lactic acid. Even if they are present in a lower concentration, the presence of kojic acid and 5-(hydroxymethyl)-2-furfural could contribute to anti-tyrosinse effect, as reported in literature (Sharma, 2004). Furthermore, it is known that a lot of phenol compounds have ability to inhibit tyrosinase activity. The honeys from Thistle, Arbutus and Eucalyptus had the highest concentration of phenols and highest anti-XO and anti-tyrosinse activity. Thus, it could explain the better results for what concern the enzyme inhibition capacity by these three honeys.

This study revealed that these honeys could be a nutrition source of antioxidant molecules and may be used as potential components in cosmetic products. Moreover, they could provide novel compounds useful for the treatment of various diseases such as gout or hyperpigmentation.

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Sardinian honeys as sources of xanthine oxidase and tyrosinase inhibitors

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Abstract Sardinian honeys obtained from different floral sources (Arbutus, Asphodelus, Eucalyptus, Thistle, and Sulla) were evaluated for their ability to inhibit tyrosinase and xanthine oxidase enzymes and for their antioxidant activity. Physicochemical parameters, total phenolic, and flavonoids content were also determined. Honey from Arbutus flowers had the highest antioxidant activity followed by Eucalyptus and Thistle ones. These three honeys showed good tyrosinase and xanthine oxidase inhibition properties. Thus, these Sardinian honeys could have a great potential as antioxidant sources for pharmaceutical and cosmetic applications.

Keywords Enzyme inhibitors · Honey · Tyrosinase · Xanthine oxidase · Antioxidant

3.5. Synthetic tyrosinase inhibitors based on a coumarin scaffold

3.5.1. Results

7.3.1. Tyrosinase inhibitory activity of synthetic compounds

The inhibition of tyrosinase displayed by the studied compounds is shown in Table 17, and the inhibitory activity was compared to that of the kojic acid (positive control). The experimental results showed that the studied compounds present different profiles of inhibition of mushroom tyrosinase activity. Among the new tested compounds (Table 17), compounds **4** and **8** appeared to be strong inhibitors, with IC_{50} values of 0.15 and 0.38 μM respectively. In fact, these compounds proved to be approximately 120 and 50 times more active than the standard inhibitor (IC_{50} of kojic acid = 17.90 μM). The inhibitory mechanisms of compounds **4** and **8** on mushroom tyrosinase were determined from Lineaweaver-Burk plots. The plots of the initial rates of tyrosinase activity in the presence of increasing concentrations of compound **4** yielded a family of straight lines with different slopes that crossed the Y-axis at similar points, indicating that compound **4** is a competitive inhibitor (Fig. 21). Figure 22 shows the double-reciprocal plots of the enzyme inhibition by compound **8**. Results show that the plots 1/V *versus* 1/[S] gave a family of parallel straight line with the same slopes. As the inhibitor concentration increased, the values of both K_m and V_{max} are reduced, but the ratios of K_m/V_{max} are unchanged. The slopes are independent of the concentration of compound **8**, which indicates that it is an uncompetitive inhibitor of the enzyme. Results revealed that inhibitor binds at the site distinct from the substrate and combines with enzyme-substrate complex (ES) but not with enzyme free (E). The equilibrium constant for binding with ES complex, K_{IS}, is obtained from a plot of the vertical intercept ($1/V_{max}$) *versus* the inhibitor concentration. The value of K_{IS} of compound **8** was determined to be 0.128 μM.

Table 17: Half maximal inhibitory concentration (IC_{50}) values of 3-aryl and 3-heteroarylcoumarins against mushroom tyrosinase activity.

Compound	IC_{50}µM
1	408.37 ± 19.66
2	1.05 ± 0.056
3	0.19 ± 0.016
4	0.15 ± 0.028
5	>1000
6	>1000
7	>1000
8	0.38 ±0.084
9	31.8 ± 10.89
10	>1000
11	259.03 ± 1.62
12	>1000
Kojic acid	17.9±0.98

Data represent the mean (± standard deviation), SD of three independent experiments.

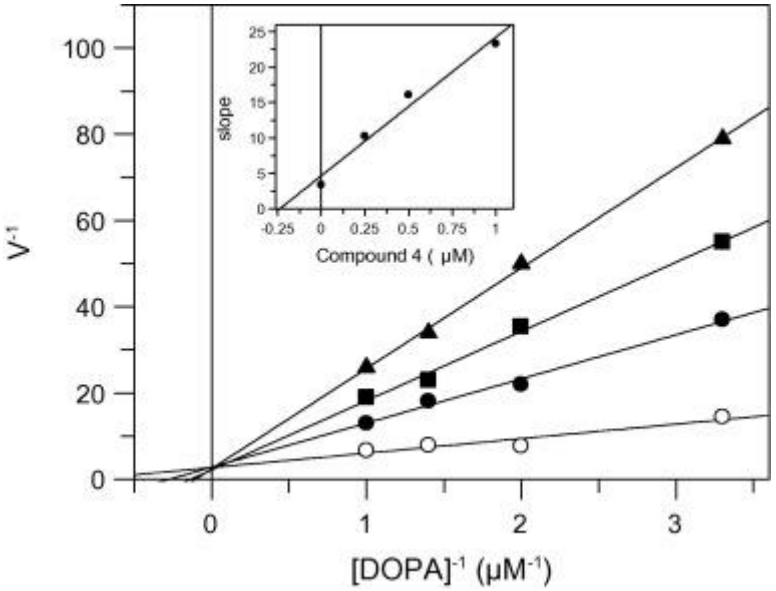


Figure 21: Lineweaver–Burk plots for the inhibition of compound 4 on mushroom tyrosinase. The concentrations of inhibitor were 0 (○), 0.25 (●), 0.5 (■), and 1 (▲) µM. The secondary plot of slope (Km/Vmax) versus concentration of compound 4, to determine the inhibition constant (KI) is showed as inset.

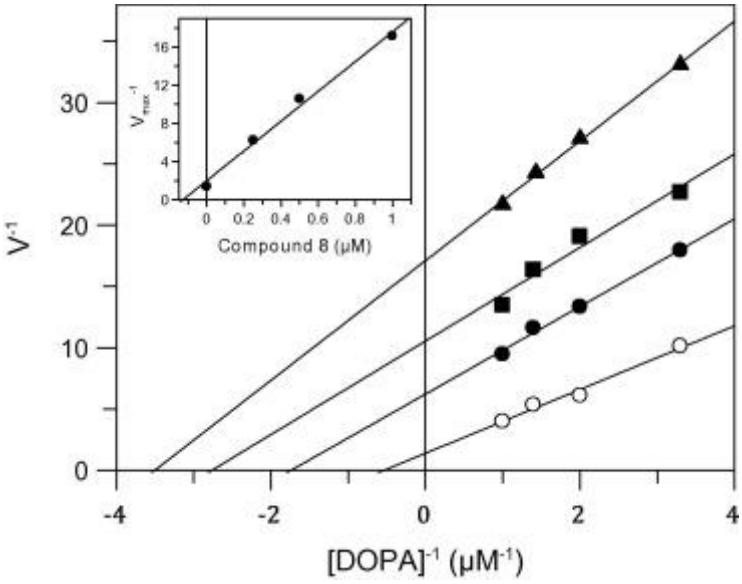


Figure 22: Lineweaver-Burk plots for inhibition of compound 8 on mushroom tyrosinase. The inhibitor concentrations were 0 (○), 0.25 (●), 0.5 (■) and 1 (▲) μM, respectively. Inset: plot of 1/Vmax versus concentrations of compound 8 to determine the inhibition constant (KIS).

7.3.2. Antioxidant activity

The free radical scavenging activity of hydroxylated compounds was carried out using ABTS and DPPH methods and Trolox was used as the positive control to compare the antioxidant capacity of the studied molecules.

Half maximal effective concentration (EC_{50}) values for both assays are reported in Table 18.

Antioxidant capacity of compounds without hydroxy groups has been also determined but they did not exert any antioxidant activity Based on the experimental results, compounds **5**, **6**, **9**-**11** did not exert antioxidant activities in both assays. Regarding ABTS·assay, compounds **1**-**4** and **8** showed the best EC_{50} values, in the same range (EC_{50} = 7.08, 20.77, 8.4, 8.51 and 11.69 μM, respectively) as Trolox (EC_{50} = 5.28 μM). The same results were obtained in the DPPH·assay, except for compound **1**, that showed a higher value (47.11 μM) than the other studied compounds. The radical-scavenging activity of the compounds **2**-**4** and **8** proved to be even better than the positive control (EC_{50} Trolox = 28.27 μM), with EC_{50} values rangingbetween 9.42 and 18.65 μM.

Table 18: Antioxidant activity of a selected series of compounds (**1**–**6** and **8**–**11**).

Compound	**EC_{50}(μM)**	
	ABTS	**DPPH**
1	7.08±0.035	47.11±10.09
2	20.77±0.42	18.65±0.85
3	8.40± 0.20	9.42±0.31
4	8.51 ± 1.70	15.83±0.55
5	>200	>200
6	>200	>200
8	11.69 ± 3.83	14.13±0.44
9	>200	>200
10	>200	>200
11	>200	>200
Trolox[a]	5.28 ± 0.15	28.27±0.45

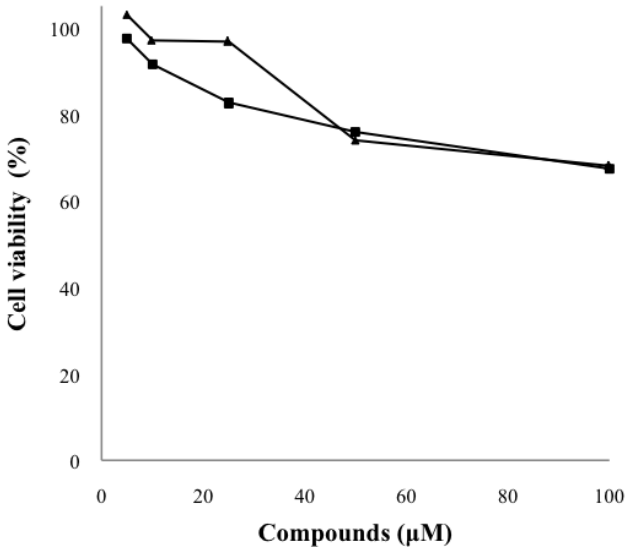
Data represent the mean (± standard deviation, SD) of three independent experiments.

[a] Positive control.

7.3.3. Cytotoxicity

Among all the tested compounds, we selected the best tyrosinase inhibitors to evaluate their depigmenting activity in B16F10 cells. The compounds that showed the highest inhibition effect on mushroom tyrosinase were compounds **2**, **3**, **4** and **8** (Fig. 23 A and B). First, we evaluated the potential cytotoxicity of these compounds on melanoma cells by measuring cell viability. Cells were treated with different concentration of each compound (0–100 μM) for 48 h and were examined using MTT test (Mosmann T., 1983). Results also indicate that all the compounds exhibited no considerable cytotoxic effect in B16F10 melanoma cells at the concentration in which tyrosinase activity is inhibited. Cell viability was slightly decreased at compounds concentration above 50 μM. Thus, the following experiments were performed using up to this concentration.

A



B

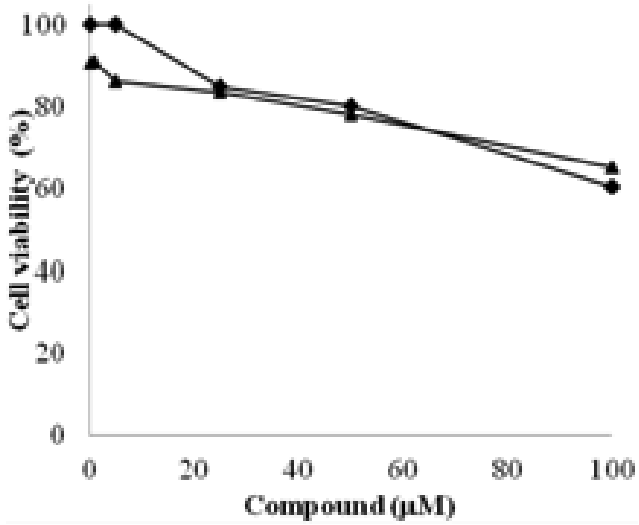


Figure 23: Cell viability of compounds **2**(■)and **3**(▲) in section A; cell viability of compounds 4(♦) and 8(▲) in section B

7.3.4. Intracellular tyrosinase activity and melanin content

We examined the inhibitory effect of the selected compounds on cellular tyrosinase activity and melanin content of B16F10 melanoma cells treated with 100 nM α-MSH. Compounds **3** and **4** did not exert any effect on both tyrosinase activity and melanin production in cellular system. On the other hand, cellular tyrosinase activity, as well as levels of melanin content, was reduced in a dose-dependent manner in presence of compounds **2** or **8**. Fig. 24A shows tyrosinase activity of cells

treated with 100 nM α-MSH and compounds **2** or **8** at different concentration (10, 25 and 50 μM). After treatment with compound **8**, tyrosinase activity decreased to 94.3, 78.5 and 27.7 % of the control. Compound **2** showed a higher inhibitory effect with 18.1 % inhibition at 10 μM, 51.8 % at 25 μM and 86.8 % at 50 μM. Similar effects were observed on melanin production (Fig. 24B). Also in this case, both compounds reduced melanin content with compound **2** being more active regarding to compound **8**. Moreover, all these data showed that compound **2** and **8** resulted to be more effective as antimelanogenic agents than kojic acid, the standard tyrosinase inhibitor, which did not showed any inhibition at the highest sample concentration (50 μM) as compared with the control (Fig. 24 A and B). The inhibitory effect on melanogenesis of compound **2** and **8** was confirmed through the results of tyrosinase zymography. DOPA staining assay was carried out with lysates of α-MSH-stimulated B16F10 cells treated with or without compounds (Fig. 25 and 26). Tyrosinase activity is very low in non-stimulated cells while α-MSH treatment created a dark band compared to that of untreated control. Upon incubation with compounds, activity of tyrosinase decreased and lighter bands were observed.

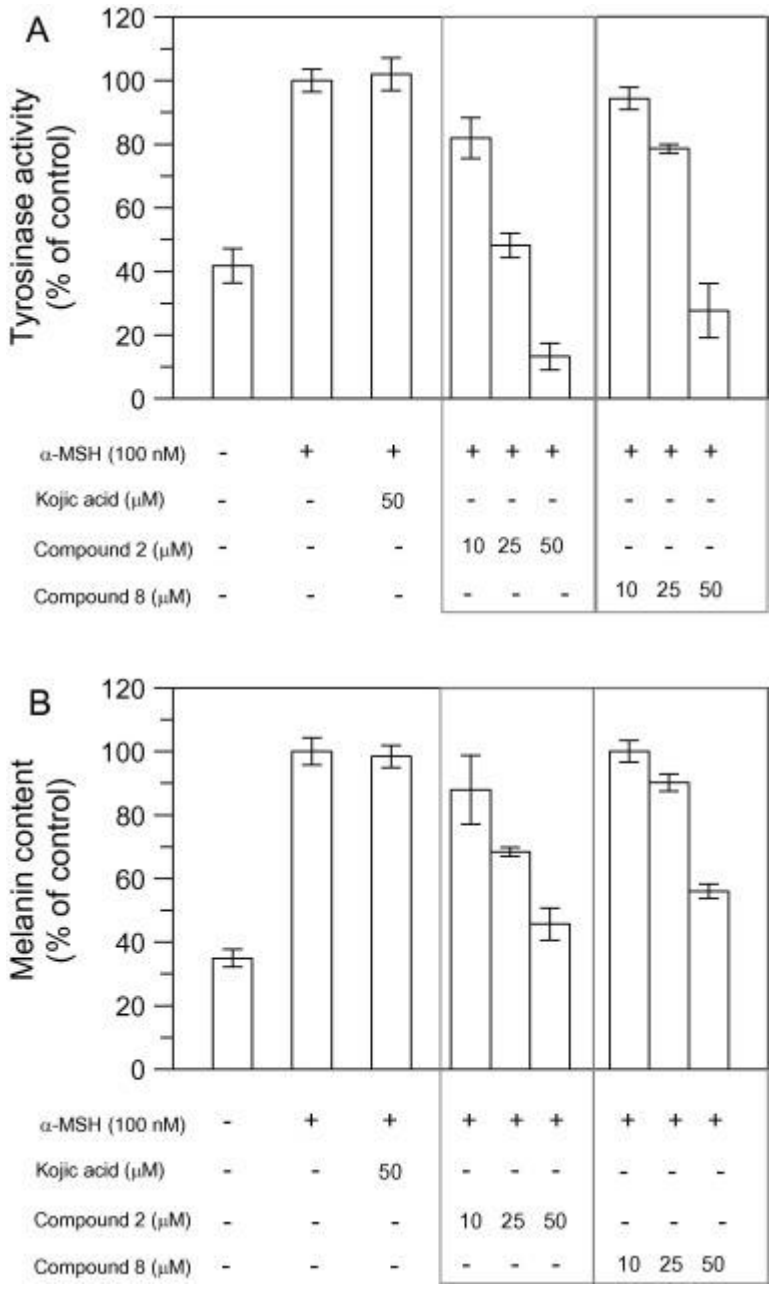


Figure 24: Effects of compound **2** and **8** on cellular tyrosinase activity (A) and melanin content (B) in α-MSH stimulated B16F10 cells.

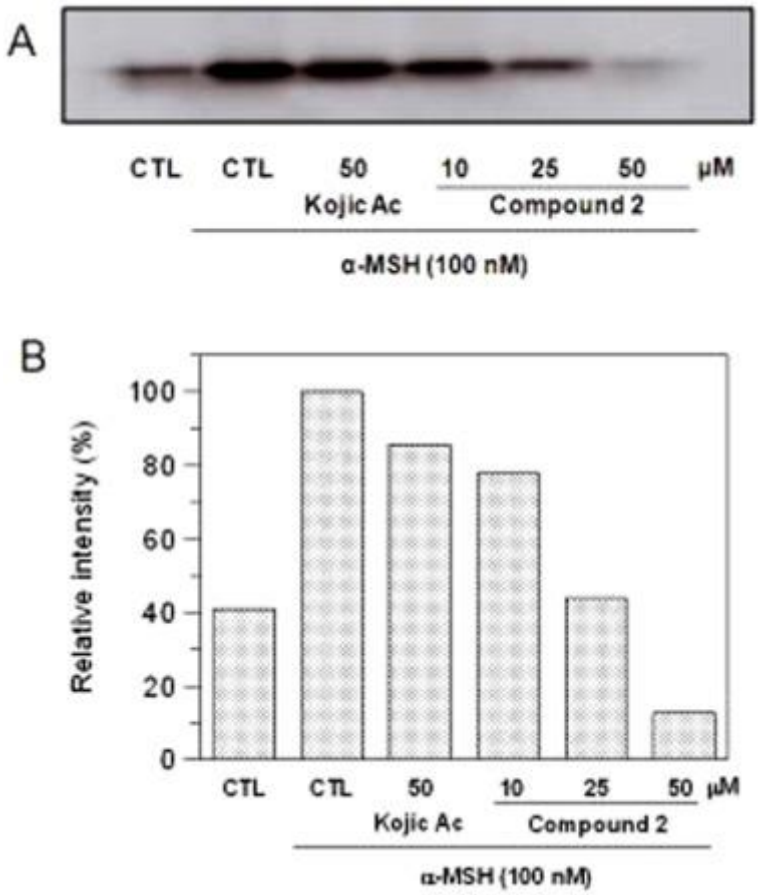


Figure 25: Effects of compound **2** on B16F10 cells, by L-DOPA staining. Tyrosinase activity was estimated by zymography (A) and the relative intensity of bands was determined with ImageJ software (B).

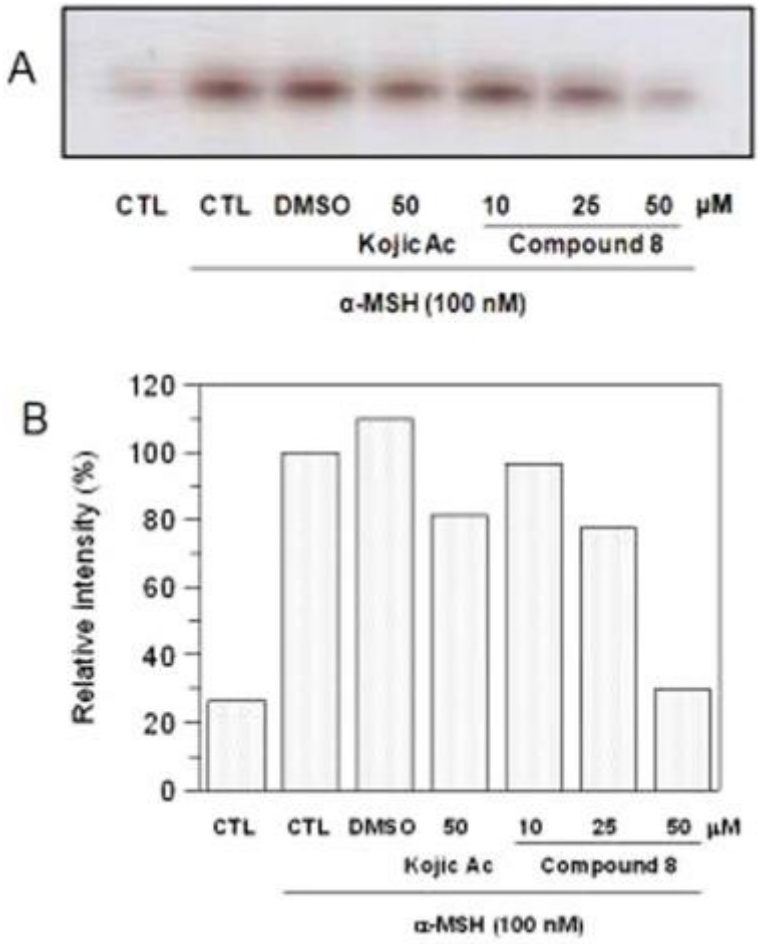


Figure 26: Effects of compound **8** on B16F10 cells, by L-DOPA staining. Tyrosinase activity was estimated by zymography (A) and the relative intensity of bands was determined with ImageJ software (B).

7.3.5. Molecular docking

It has been studied the most active compounds using molecular docking simulations to determine the key residues important for the ligand-protein interaction. Compound **4** showed a binding mode in the 2Y9W where the coumarin ring is directed towards the bottom of the cavity whereas the 3-heteroaryl substituent is placed close to the Arg268 towards the protein surface. The hydroxy groups placed at positions 5 and 7 of the coumarin skeleton play an important role for the anchoring with the enzyme. Hydroxyl at position 5 established a hydrogen bond with the residue Met280. The compound also established a hydrogen bond with the amide moiety of the residue Asn260 using the 7-hydroxy group. Moreover, residues His263 and Phe264 established π-π stacking interactions with

the coumarin scaffold. Additionally, it has been calculated the residue energy contribution for ligand binding. The residues that play an important role in stabilizing the complex are: Asn260, Met280, Phe264 and Val283. Compounds that do not present hydroxy substituents at the mentioned positions showed lower tyrosinase activity with the limitation to establish the described H-bonds with both residues. Similar results for compound **4** were obtained using the alternative 2Y9X crystallized enzyme in the docking. The proposed binding mode for compound **4** is in accordance with our experimental assays in which the compound did not interact with the copper but showed competitive inhibition.

3.5.2. Discussion

In the current study, a series of heteroarylcoumarins have been synthesized and studied for their inhibitory activities on mushroom tyrosinase and murine melanoma B16F10 cells, as well as for their antioxidant capacity.

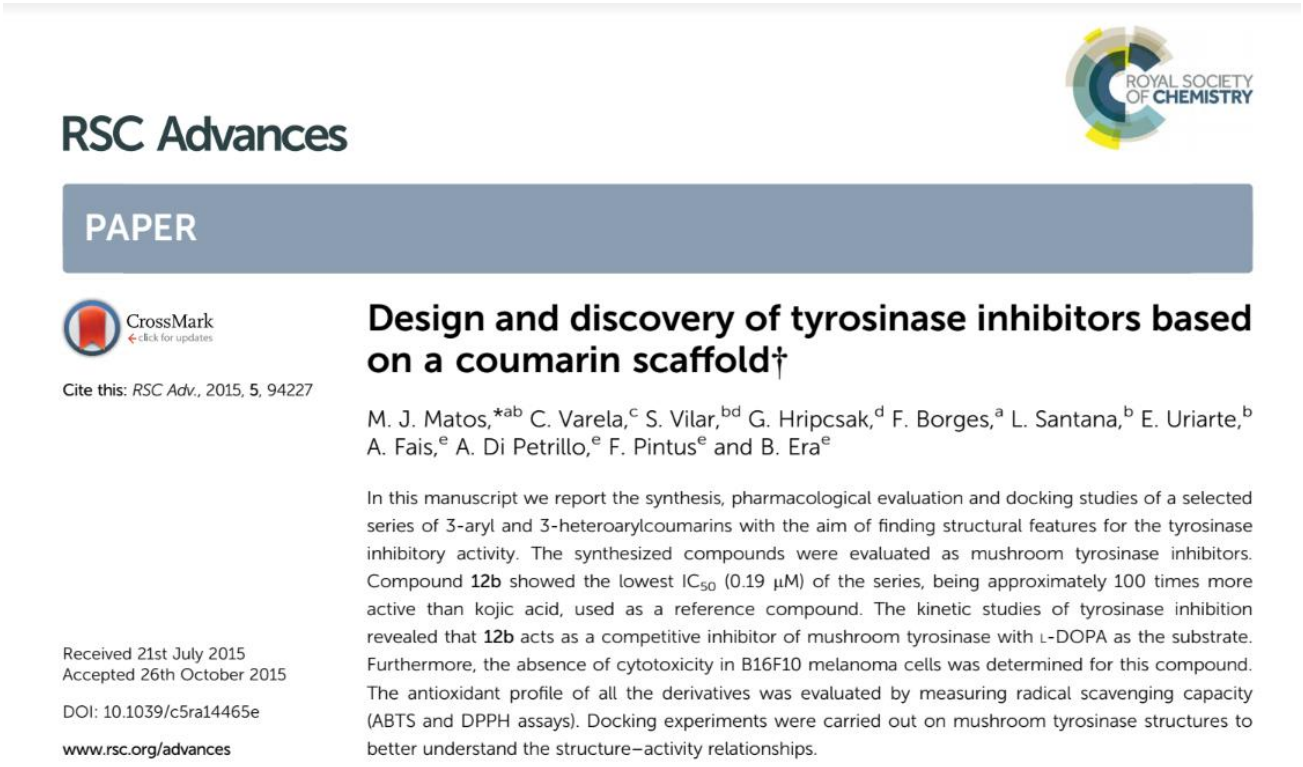
The radical-scavenging activity of the compounds **2-4** and **8** proved to be even better than the positive control. This effect can be explained due to the presence of two hydroxy groups on their structures. Comparing the EC_{50} values of compounds **8** and **11**, which differ only in the position of these groups in the coumarin scaffold (5,7 *vs* 7,8, respectively), the position 5,7 seems to be important for the antioxidant activity of this scaffold.

Among these coumarin derivatives, compounds **4** and **8** exhibited higher tyrosinase inhibitory activities than kojic acid. The kinetic studies of tyrosinase inhibition revealed that compound **4** acts as a competitive inhibitor, while compound **8** proved to be an uncompetitive inhibitor of mushroom tyrosinase. Furthermore, compounds **2** and **8** inhibited cellular tyrosinase activity and melanin production in B16F10 cells.

Docking experiments were carried out in order to compare the theoretical and experimental affinity of these coumarin derivatives to mushroom tyrosinase.

The remarkable results found for these compounds encourage us to continue our research based on the coumarin scaffold. The analyzed data so far is very important to define which compound will be

the best candidate for further *in vivo* studies. In summary, this study is a noteworthy contribution in the area and allows the scientific community a new approach in the optimization of new molecules as depigmenting agents.



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Design and discovery of tyrosinase inhibitors based on a coumarin scaffold†

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In this manuscript we report the synthesis, pharmacological evaluation and docking studies of a selected series of 3-aryl and 3-heteroarylcoumarins with the aim of finding structural features for the tyrosinase inhibitory activity. The synthesized compounds were evaluated as mushroom tyrosinase inhibitors. Compound **12b** showed the lowest IC_{50} (0.19 μM) of the series, being approximately 100 times more active than kojic acid, used as a reference compound. The kinetic studies of tyrosinase inhibition revealed that **12b** acts as a competitive inhibitor of mushroom tyrosinase with L-DOPA as the substrate. Furthermore, the absence of cytotoxicity in B16F10 melanoma cells was determined for this compound. The antioxidant profile of all the derivatives was evaluated by measuring radical scavenging capacity (ABTS and DPPH assays). Docking experiments were carried out on mushroom tyrosinase structures to better understand the structure–activity relationships.

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New insights into highly potent tyrosinase inhibitors based on 3-heteroarylcoumarins: Anti-melanogenesis and antioxidant activities, and computational molecular modeling studies

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ABSTRACT

Melanogenesis is a physiological pathway for the formation of melanin. Tyrosinase catalyzes the first step of this process and down-regulation of its activity is responsible for the inhibition of melanogenesis. The search for molecules capable of controlling hyperpigmentation is a trend topic in health and cosmetics. A series of heteroarylcoumarins have been synthesized and evaluated. Compounds **4** and **8** exhibited higher tyrosinase inhibitory activities (IC_{50} = 0.15 and 0.38 μM, respectively), than the reference compound, kojic acid (IC_{50} = 17.9 μM). Compound **4** acts as competitive, while compound **8** as uncompetitive inhibitor of mushroom tyrosinase. Furthermore, compounds **2** and **8** inhibited tyrosinase activity and melanin production in B16F10 cells. In addition, compounds **2**–**4** and **8** proved to have an interesting antioxidant profile in both ABTS and DPPH radicals scavenging assays. Docking experiments were carried out in order to study the interactions between these heteroarylcoumarins and mushroom tyrosinase.

CHAPTER 4: Conclusions

The interest in finding novel antimelanogenic agents from natural or synthetic sources with antioxidant activity associated with inhibitory properties is of great interest. Considering the key role of tyrosinase in melanin pathway, research of molecules that inhibit tyrosinase have become increasingly important for medicinal and cosmetic products that may be used as powerful skin-whitening agents for treating skin disorders. In addition, enzymatic browning in fruit and fungi is undesirable in, for example, fresh fruits, beverages, vegetables, and mushrooms. Browning after harvest is a common phenomenon in crops such as mushrooms, which decreases the commercial value of the products. These phenomena have encouraged researchers to seek new potent tyrosinase inhibitors for use in antibrowning of foods and skin whitening.

The work was focused in analysis of extracts of *Asphodelus microcarpus, Phytolacca dioica, Sarcopoterium spinosum* and Sardinian honeys as antioxidant, tyrosinase inhibitors and antimicrobial activity. Moreover synthetic compounds derived from the coumarin, compound naturally present in the plants, have been also tested for their inhibitory activity on tyrosinase and their antioxidant activity. The results showed that all extracts have a direct inhibitory anti-tyrosinase activity with flowers ethanol extract of *Asphodelus microcarpus* and ethylacetate extract of *Phytolacca dioica* exhibiting the stronger effect. The same extracts showed the highest antioxidant activity and an elevated levels of total phenolics and flavonoid content.

As for the neo-synthesized compounds, two of them have shown inhibitory activity on tyrosinase even on B16F10 cells.

The results achieved open promising perspectives for further studies of the biological effect and medicinal use of these natural and synthetic source, and the possibility to isolate bioactive compounds from the natural extracts. However, further research will be required before the inhibitors (coumarins analogues or bioactive compounds isolated from natural extracts) may be shown to have clinical application. This goal might be reached with the cooperation and help of cosmetic or biotechnology companies.

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