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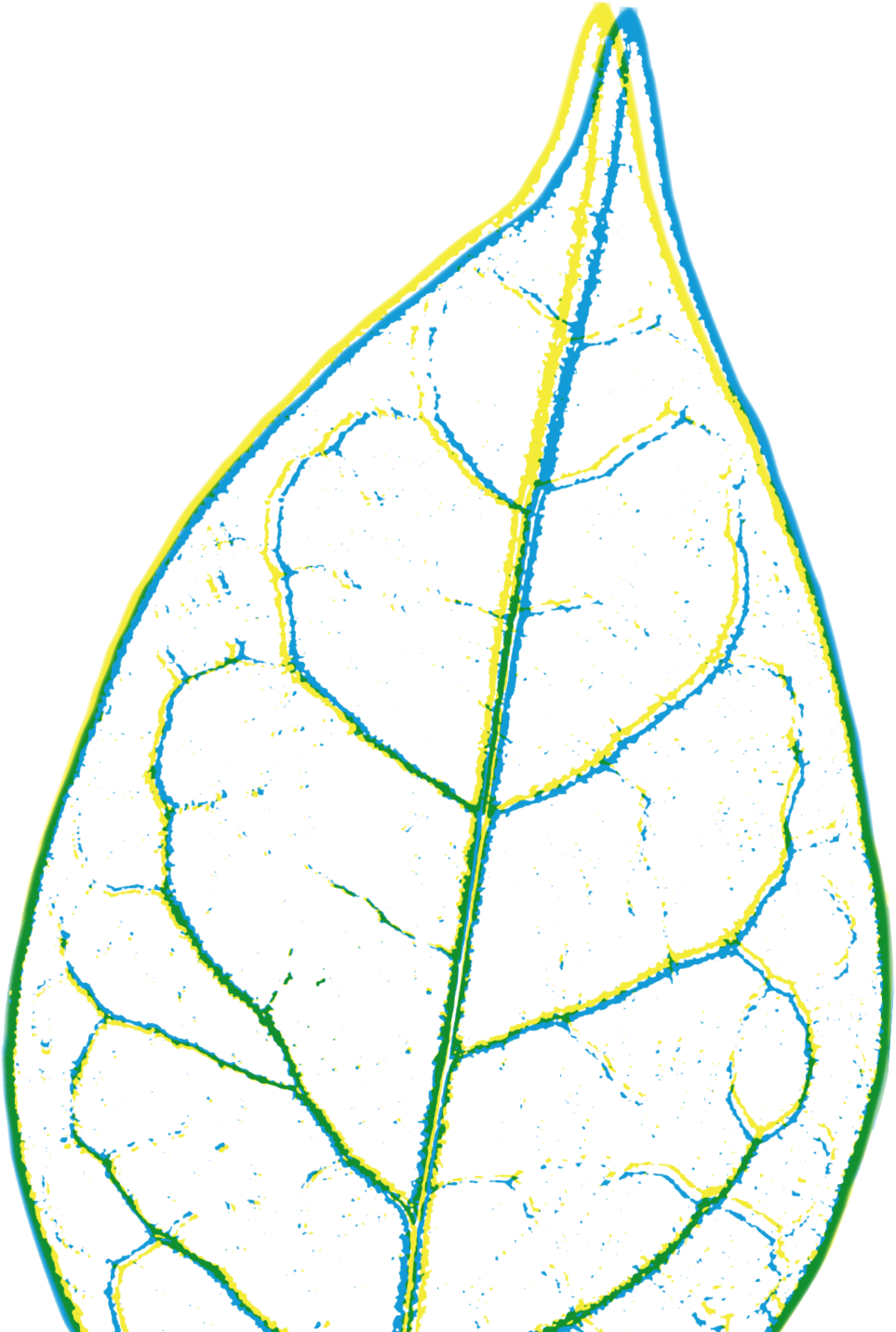
Secondary metabolism in evergreen species with high tolerance to osmotic stress: what's the role for these species as biofactories?



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Riassunto

Le specie mediterranee sono sottoposte a una moltitudine di stress abiotici, soprattutto durante la stagione estiva, in cui si ha una combinazione di elevate temperature, elevata radiazione solare e bassa disponibilità di acqua nel terreno. Le piante che si sono evolute in questo ambiente, tuttavia, hanno a disposizione una serie di caratteristiche morfo-anatomiche, biochimiche e fisiologiche che contribuiscono ad aumentare la loro tolleranza al "drought". In particolare, queste piante sono in grado di sintetizzare un'ampia gamma di metaboliti secondari. Tra questi ci sono i polifenoli che svolgono un ruolo chiave nel mantenimento dell'omeostasi redox all'interno delle cellule, dalla cui stabilità dipende la sopravvivenza delle piante in questi ambienti. Numerosi studi hanno inoltre dimostrato come i polifenoli abbiano una funzione antiossidante anche nell'uomo e negli ultimi anni il loro utilizzo in campo medico e nutraceutico è notevolmente aumentato.

In questo contesto diventa fondamentale conoscere l'ecologia, la fisiologia e la biochimica delle piante mediterranee che rivestono un interesse farmacologico, per poterle valorizzare e utilizzare in questo ambito.

Gli obiettivi di questa tesi, quindi, sono essenzialmente due: da un lato valutare le strategie di acclimatazione messe in atto da tre specie della macchia mediterranea (*Phillyrea latifolia* L., *Pistacia lentiscus* L. and *Cistus incanus* L.) nel loro habitat naturale; dall'altro lato, si vuole valutare la possibilità di utilizzare queste specie come "biofactories", ovvero come fonti di molecole (polifenoli) di potenziale interesse farmacologico.

A questo scopo, nel 2014 e nel 2015 è stato condotto un esperimento *in situ* sulle dune costiere di Castiglione della Pescaia (Gr). Sono state effettuate sia misure fisiologiche (scambi gassosi, relazioni idriche e fotochimica del fotosistema) che biochimiche (polifenoli, carotenoidi, ABA e ABA-GE) su base annuale, stagionale e giornaliera. Inoltre, per studiare l'effetto degli stress abiotici sulla funzionalità dei processi fisiologici e biochimici delle tre specie, tali misurazioni sono state correlate con i parametri climatici (temperatura, radiazione globale e precipitazioni).

Successivamente, le specie sono state valutate come fonte di composti bioattivi. In particolare, dopo una caratterizzazione strutturale dei loro componenti polifenolici (ottenuta tramite l'utilizzo della spettrometria di massa), gli estratti fogliari sono stati investigati per la loro attività antiossidante e per la loro citotossicità. Queste analisi sono state condotte in collaborazione con il dipartimento di medicina molecolare e dello sviluppo dell'Università di Siena. Specificatamente, l'attività antiossidante degli estratti è stata testata tramite il saggio *in vitro* del radicale DPPH (1,1-diphenyl-2-picrylhydrazyl), mentre la loro citotossicità è stata valutata tramite un test *ex vivo* utilizzando una linea cellulare di fibroblasti murini immortalizzati (NIH 3T3).

Complessivamente, i risultati ottenuti da questo progetto di dottorato sono stati i seguenti:

1. descrizione dei principali meccanismi di risposta ecofisiologici e biochimici di *P. latifolia*, *P. lentiscus* e *C. incanus* a stress multipli tipici dell'ambiente mediterraneo su scala giornaliera, stagionale e annuale;
2. ottenimento degli estratti arricchiti in polifenoli da *C. incanus* e *P. lentiscus* con successiva caratterizzazione fitochimica;
3. quantificazione dell'attività antiossidante *in vitro* degli estratti e della loro citotossicità con test *ex vivo*, in modo da valutarne un possibile utilizzo per un futuro impiego in campo farmacologico.

Abstract

Vegetation in Mediterranean areas is exposed to different sources of environmental stresses, especially during the summer when high temperatures, high solar irradiance and low water availability in the soil prevail for long periods. To cope with such combination of stresses, which are commonly defined as drought, Mediterranean plants have evolved a suite of morpho-anatomical, biochemical, and physiological mechanisms. In particular, these plants are able to synthesize a broad spectrum of secondary metabolites. Among these, polyphenols play a key-role in the defence against reactive oxygen species (ROS), which are inevitably produced when aerobic or photosynthetic metabolism is impaired by environmental stresses. The antioxidant activity of polyphenols is also maintained in human cells and in the last years their use in the medicinal and nutraceutical fields has considerably grown.

In this context, the knowledge of Mediterranean plants of pharmaceutical interest becomes crucial and represents a prerequisite for their rational valorization and protection.

The present PhD research was developed under this scenario. In particular, the general objectives of this thesis are:

1) to explore the physiological and biochemical responses of three selected Mediterranean species (*Phillyrea latifolia* L., *Pistacia lentiscus* L. and *Cistus incanus* L.) in their natural habitat;

2) to assess the relative significance of these species as “biofactories”, i.e. potential sources of health-promoting polyphenols.

In order to achieve these goals, a comparative field study was carried out in 2014 and 2015 on the coastal dunes of Castiglione della Pescaia (Gr), where all the above species co-occurred. We measured both physiological (gas exchange, water relations and PSII photochemistry) and biochemical parameters (carotenoids, polyphenols and abscisic acid in its free and conjugated form) on a daily and seasonal basis. Furthermore, the effects of climatic factors (temperature, global irradiance and precipitation) on the physiological and biochemical traits were also assessed.

Then, to investigate the examined species as “biofactories”, we identified the most abundant polyphenols present in their leaves throughout the HPLC–DAD–MS/MS (Liquid Chromatography-tandem Mass Spectrometry) technique. Moreover, we optimized an extraction protocol to obtain different polyphenolic-rich extracts (flavonoid-rich fractions and tannin-rich fractions) from each plant.

Finally, in order to compare their antioxidant and cytotoxic effects, the total extracts and the obtained fractions were tested throughout *in vitro* and *ex vivo* assays. These analyses were conducted in collaboration with the Department of Molecular Medicine and Development at the University of Siena. Specifically, the antioxidant activity was examined using the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical

scavenging test, while the study of cytotoxicity was carried out on mouse fibroblast (NIH 3T3) cell line.

Overall, the results obtained from this PhD project were the following:

1. definition of the main physiological and biochemical response mechanisms of *P. latifolia*, P*. lentiscus* and *C. incanus* to multiple stress typical of the Mediterranean environment on a daily, seasonal and inter-annual basis;
2. phytochemical characterization of the polyphenolic-enriched extracts obtained from *C. incanus* and *P. lentiscus* leaves;
3. characterization of the *in vitro* antioxidant activity and the *ex vivo* cytotoxicity of the extracts, in order to evaluate their pharmacological potential.

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1. **General Introduction**

1.1 **How plants cope with drought stress in the Mediterranean climate**

Vegetation in Mediterranean areas is exposed to different sources of environmental stresses. In particular, during the summer season, high temperatures, high solar irradiance and low water availability in the soil prevail for long periods (Munné-Bosh *et al.* 1999). To cope with such combination of stresses, which are known as drought, Mediterranean plants have evolved a suite of morpho-anatomical, biochemical, and physiological features that enable their survival in such harsh conditions (Galmés *et al.* 2007).

Mediterranean vegetation mostly consists of deep rooted evergreen sclerophyll trees, semi-deciduous shrubs and winter annual herbs (Medrano *et al.* 2009). Evergreen sclerophylls go through the stress conditions with intact green leaves, by exhibiting a variety of morphological adaptations such as small leaves, thick cuticles, and a deep root systems (Karavatas and Manetas 1999). On the other hand, semi-deciduous species partially avoid water stress through a reduction of their foliage area, thus restricting their growth to the more favourable seasons (Werner *et al.* 1998). Finally, winter annual herbs escape seasonal water limitation by finishing their annual cycle before summer (Galmés *et al.* 2007, Ain-Lhout *et al.* 2001, Medrano *et al.* 2009).

Mediterranean species also display different water-use behaviours to withstand drought periods. In particular, these plants could be classified in drought avoiding and drought tolerant species (Lo Gullo and Salleo 1988).

Drought avoiding plants undergo limited changes in leaf water potential and/or relative water content during water stress. This is achieved by either restricting water losses from the plant body (water saving) or by increasing water absorption to replace losses by transpiration (water spending) (Kozlowski and Pallardy 2002). By contrast, drought tolerant plants can survive at low internal water potential and either maintain high relative water content (drought-tolerance dehydration-avoidance) or tolerate low RWC (drought-tolerance dehydration-tolerance) (Levitt 1980, Kozlowski and Pallardy 2002). This classification roughly corresponds to the actually widespread categorisation of plants in “isohydric” vs “anisohydric” species (Tardieu and Simonneau 1998). The former group maintains plant water potential relatively constant under drought stress conditions, while the latter displays large water potential fluctuations while maintaining relatively high gas exchange rates even during arid season (Nardini *et al.* 2014).

As stated above, in the Mediterranean summer, plants experience the overlapping of multiple stressors, such as high light, water and heat stress. Under such conditions, leaves cannot utilize all the photosynthetically active radiation (PAR) absorbed for photosynthesis and this results in what is often described as excess excitation energy (Müller and Niyogi 2001). Increases in electron transport rates in the absence of any increase in CO_2 assimilation may lead to the reduction of oxygen and therefore to the formation of reactive oxygen species (ROS) (Smirnoff 1993). ROS play a role in intra- and intercellular signalling at low concentrations, but damage

several cellular components (e.g. lipids, proteins, nucleic acids) when present at high concentrations (Hernández *et al.* 2004).

Biochemical adjustments aimed at inhibiting the generation of and at detoxifying ROS once formed operate effectively to preserve sensitive organs from photo-oxidative damage (Foyer and Noctor 2000, Mittler *et al.* 2004).

These include, e.g, alterations of pigment composition (Logan *et al.* 1998, Lu *et al.* 2003), by decreasing the total chlorophyll concentration or increasing the ratio of violaxanthin-cycle pigments to chlorophyll total (Havaux and Tardy 1999, Melgar *et al.* 2009). These biochemical mechanisms effectively reduce the risk of photodamage and enhance the dissipation of excess energy via the so-called non-photochemical quenching (NPQ) (Demmig-Adams and Adams 1996, Kyparissis *et al.* 1995, Havaux and Niyogi 1999, Muller *et al.* 2001). Moreover, some carotenoids may effectively function as ROS scavengers. For example, zeaxanthin behaves as a chloroplast antioxidant, by conferring rigidity to thylacoid membranes and hence limiting lipid peroxidation (Havaux 1998), while β-carotene is both a physical and a chemical quencher of singlet oxygen (Ramel *et al.* 2012).

Similarly to carotenoids, polyphenols display a great potential to reduce various forms of reactive oxygen species. For example, UV-absorbing flavonoids located in the epidermal cells strongly reduce highly energetic solar wavelengths from reaching ROS-generating cells, and the consequential photo-oxidative stress and damage (Brunetti *et al.* 2015). Furthermore, vacuolar flavonoids (in conjunction with guaiacol peroxidases) may help maintaining whole-cell H_2O_2 within a sub-lethal concentration range (Ferreres *et al.* 2011). Finally, flavonoids located in the outer chloroplast envelope and in the cell wall may improve membrane stability and tightly control retrograde signals to the nucleus (Agati *et al.* 2013).

Other biochemical mechanisms which are known to confer to plants the capacity of living under drought are the synthesis and the accumulation of osmoprotectans, also known as compatible solutes. Compatible solutes include carbohydrates as glucose, fructose, sorbitol, mannitol, sucrose; proline, malic acid and inorganic ions, depending on plant species (Wang and Stutte 1992, Guicherd *et al.* 1997, Guidi *et al.* 2008). The active accumulation of these solutes in the symplast, as opposed to passive dehydration, has been proposed to play a major role in turgor maintenance and therefore maintenance of stomatal opening and gas exchange (Hsiao *et al*. 1976). Furthermore, some osmolytes, such as sugars, have been proposed to act as signalling molecules under stress and interact with hormones as part of the sugar-signalling network in plants (Chaves *et al.* 2003).

Finally, the plant hormone ABA (abscisic acid) is produced *de novo* under drought conditions and plays a major role in response and tolerance to dehydration (Shinozaki and Yamaguchi-Shinozaki 1999).

The cellular and molecular mechanisms underlying ABA-induced stomatal closure have been extensively investigated (Tardieu and Davies 1992, Wilkinson and Davies 2002). Recent evidence suggests that ABA promotes stomatal closure in a dual way, through the well-known effect on guard cell metabolism as well as through an 'indirect' hydraulic effect, inactivating bundle sheath

aquaporins and decreasing water permeability within the leaf vascular tissues (Parent *et al.* 2009, Tardieu *et al.* 2010, Brunetti *et al.* 2014).
Furthermore, recent studies suggest that ABA-induced H_2O_2 production mediates the generation of NO, an extensive signal molecule capable to up-regulate plant antioxidant defences (Tossi *et al.* 2009).

1.2 **Polyphenols of Mediterranean woody species as antioxidants**

As mentioned earlier, Mediterranean-type ecosystems are characterized by extended periods of water deficit and high temperature during the summer, and high sun irradiance becomes excessive light under such circumstances (Agati *et al.* 2012). Under such conditions the biosynthesis of polyphenols is strongly up-regulated (Close and McArthur 2002).
These secondary metabolites are utilized for many functions within the plant cell but the broad consensus is that polyphenols are aimed at maintaining cellular redox homeostasis in response to changes in environmental conditions by inhibiting the generation of reactive oxygen species (ROS) and quenching ROS once they are formed (Brunetti *et al.* 2013).
The antiradical capacity of polyphenols is strongly associated with their chemical structure (Rice-Evans *et al.* 1996). Specifically, the hydrogen-donating ability of polyphenols usually encountered in plant cells resides almost exclusively on the presence of catechol group in the B-ring of the flavonoid skeleton (Tattini *et al.* 2004). This evidence is consistent with the very high concentration of dihydroxy B-ring-substituted flavonoids usually detected in plants adapted to high sunlight irradiance (Brunetti *et al.* 2013).
Leaving aside the antioxidant role of polyphenols in plants, these compounds have long been suggested to perform the same reducing functions in humans (Brunetti *et al.* 2013b). However, the antioxidant potentials of polyphenols have been tested through *in vitro* and *ex vivo* experiments and much uncertainty persist regarding their real mechanisms of action in human cells (Scalbert *et al.* 2005).
Recent studies have speculated that their classical hydrogen-donating antioxidant activity is unlikely to be the sole explanation for cellular effects (Williams *et al.* 2004, Spencer 2007, Upadhyay and Dixit 2015). This premise is based on a number of observations. Firstly, polyphenols are extensively metabolised in the human body (Williams *et al.* 2004). This leads to structural modifications that effectively decrease the ability of polyphenols to donate hydrogen atoms. For example, phenolic compounds may undergo methylation, sulfation or glucuronidation, or a combination of these processes that alter their “classical” antioxidant nature (Balasundram *et al.* 2006, Yang *et al.* 2001). Secondly, polyphenols are poorly soluble in the aqueous cellular milieu and their concentrations accumulated *in vivo*, for example, in the plasma or most tissues, are very low (from high nanomolar to low micromolar) (Brunetti *et al.* 2013b). This low bioavailability leads to a kinetically unfavourable situation compared to other compounds with similar free radical scavenger capabilities that are present in our blood in concentrations in the micromolar range, such as tocopherols and ascorbic acid (Fraga 2007).

Finally, both antioxidant and prooxidant effects of polyphenols have been described *in vivo*, with contrasting effects on cell physiologic processes (Halliwell 2008). Consequently, it has been suggested that other mechanisms may explain the observed changes in cell or tissue oxidation beyond the ability of polyphenols to directly prevent free radical-mediated tissue damage (Fraga 2007).

In recent years, polyphenols have been reported to play key roles as signalling molecules in mammals through their ability to interact with a wide range of receptors and enzymes, which, in turn, are responsible for mediating ROS-induced signalling cascades vital to cell growth and differentiation (Scalbert *et al.* 2005).

This inductive or signalling effects may occur at low concentrations (nanomolar) and are effectively serve by ROS-quenching polyphenols (Stevenson and Hurst 2007).

For example, the flavonoid quercetin can form hydrogen bonds with Ser212 through the 3'-OH group, thus showing the capacity to inhibit mitogen-activated protein kinase (MEK1) activity (Lee *et al.* 2008). Mitogen-activated protein kinases (MAPK) can control the expression of antioxidant enzymes, inhibit cell cycle progression and cell proliferation, and the expression and functional activation of oncogenes (Hu and Kong 2004). Therefore, protein kinases have been now suggested as molecular targets for chemoprevention by flavonols (Surh 2003, Brunetti *et al.* 2013b).

Although further research is needed to conclusively address the actual significance of polyphenols as antioxidant in humans, the current evidence for protective effects of polyphenols against various diseases linked to oxidative stress has generated new expectations for improvements in health, with great interest from pharmaceutical industry regarding promotion and development of plant polyphenol-rich products (Scalbert *et al.* 2005).

In this context, Mediterranean plants are an attractive source of polyphenolic compounds. Since most of these secondary metabolites are synthesized in response to multiple stress factors, the "wild-neglected" species of Mediterranean maquis, which grow spontaneously in harsh environments, may represent unrivalled and innovative "biofactories", with great potentials in pharmacological and nutraceutical fields.

1.3 **Botanical description and pharmacological properties of the study species**

1.3.1 *Phyllirea latifolia* L.

Phillyrea latifolia L., commonly known as green olive tree or mock privet, is an evergreen shrub of the Oleaceae family (Ayranci and Erkan 2013). The Oleaceae consist of 25 genera and some 600 species that are distributed world-wide in temperate to tropical areas. The genus *Phillyrea* includes two species, *Phillyrea latifolia* L. and *Phillyrea angustifolia* L., both present in the Mediterranean region (which comprises the Northwest Africa, the Eastern Mediterranean, the Iberian Peninsula, the Apennine Peninsula and the Balkan Peninsula). The former has a circum-Mediterranean distribution, the latter is widespread in the western Mediterranean (Tutin *et al.* 1993). *Phillyrea latifolia* L. is a small deep-rooted tree that can be up to 8 m tall, but which often

occurs as a dense, branching shrub, with a smooth, greyish-brown trunk and erect branches. Its leaves are simple, coriaceous, opposite and varied in shape - lanceolate, elliptical, ovate or sharply-serrated, 2-5 cm long, 1-3 cm wide (Fig. 1).

The stomata are usually restricted to the abaxial surface (Sachse 2001). Leaves also have glandular trichomes, that generally occur alone or in pairs on both cuticles (less numerous on the adaxial surface) (Gravano *et al.* 1998). These trichomes consist of a multicellular secretory head, a unicellular stalk and a collecting cell surrounded by epidermal cells and spongy mesophyll cells (Gravano *et al.* 1998).

Phillyrea latifolia L. produces hermaphrodite flowers, which grow together in short (up to 10 mm) axillary panicles or spikes on the previous year's shoots (Pollack *et al.* 2001). The calyx has 4 triangular-acute sepals. The corolla has 4 greenish-white petals and 2 stamens with short filaments and exserted anthers. The stigma is 2-lobed (Aronne and Wilcock 1994). The fruits are bluish-black drupes up to 4 mm in diameter, usually containing a single seed enclosed by a lignified endocarp (Mira *et al.* 2015). *P. latifolia* blooms in April-May and it is fructiferous in the period from August to November (Longo *et al.* 2007) (Fig. 1).

Figure 1. Leaves, flowers and fruits of *Phillyrea latifolia* L.

P. latifolia leaves extracts are well known in the Mediterranean folk medicine (Carreteo *et al.* 2001). For example, in Italy, Spain and in the north of Africa, people have used infusions and decoctions prepared from its leaves and fruits as diuretic, antipyretic (Pieroni and Pachaly 2000),

antispasmodic (Merzouki *et al.* 1997) and for the treatment of ulcers and mouth inflammations (Lanza *et al.* 2000).

In recent years, many investigations have been conducted in order to confirm the pharmacological properties of this species and several studies concerning the polyphenolic composition of leaves of *P. latifolia* were published (Romani *et al.* 1996, Agati et al 2002, Lanza *et al.* 2001, Azaizeh *et al.* 2013). As reported by Romani *et al.* (1996) phenolic acids, the secoiridoids oleuropein and oleuropein aglycone, verbascoside and the flavonol glycosides (i.e. glycosides of luteolin, apigenin, and quercetin) are the major phenylpropanoids in *P. latifolia* leaves.

Some of the phenylpropanoids already mentioned have interesting antioxidant properties (Takahama, 1989; Yamasaki *et al.* 1997).

Some of these compounds have demonstrated to have intensive antiradical and antioxidant properties (Baghel *et al.* 2012, Tattini *et al.* 2000, Galardi *et al.* 2003, Rice-Evans *et al.* 1995). In particular, oleuropein have been shown to possess several pharmacological properties, including anti-cancer (Owen *et al.* 2000), cardioprotective (Andreadou *et al.* 2007), anti-ischemic and hypolipidemic activities (Andreadou *et al.* 2006).

Lanza *et al.* (2001) isolated four polyphenols (salidroside, syringing, coniferin and phillyrin) and tested their *in vitro* anti-inflammatory activity, demonstrating that these compounds can exert inhibitory actions on the enzymes of the arachidonate cascade in calcium-stimulated mouse peritoneal macrophages and human platelets. Particularly, phillyrin and salidroside exert a preferential effect on the cyclo-oxygenase pathway, inhibiting the release of prostaglandin E2 and reducing thromboxane B2 levels (Pan *et al.* 2003).

In addition, Hussain and Tobji (1997) showed that the aerial parts of *P. latifolia* have intense antibacterial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis*.

Finally, polar fractions and flavonoids isolated from methanolic extracts of the leaves of *P. latifolia* showed significant *in vitro* complement inhibiting effect on the classical pathway of the complement system (Pieroni *et al.* 2000).

Thus, in conclusion, *P. latifolia* leaf polyphenols possess an array of potentially beneficial properties which contribute to confirm the old ethno-medical uses of this species in the Mediterranean region.

1.3.2 *Pistacia lentiscus* L.

The genus *Pistacia* (also known as “mastic tree”) belongs to the Anacardiaceae family, which comprises of about 70 genera and over 600 species. These species are distributed from the Mediterranean basin to central Asia (Bozorgi *et al.* 2013). In Italy, *Pistacia lentiscus* L. is the most widespread species, growing along the coast from sea level to 700 meters above sea level (Barra *et al.* 2007).

Near the seashore *Pistacia lentiscus* L. is present as a multi-branched evergreen shrub, from 1 to 3 m high, with separate male and female plants and a strong smell of resin (Iauk *et al.* 1996). Its leaves are alternate, compound paripinnate, 1.5-3.5 cm long, 0.5-2 cm wide, with 6 leaflets. The

leaflets are leathery, rounded at their tip with an entire margin (Imtiyaz *et al.* 2013, Saghir and Porter 2012).

Pistacia lentiscus blooms in the months of April-May. The flowers are reddish and apetalous, arranged as a panicle-like inflorescence (consisting of 8-10 flowers, 1-4 cm long) produced in the axils of persistent foliage leaves that developed in the previous year, mostly in the distal region of the shoot (Bachelier and Endress, 2007). The male flower has 5 sepals and 5 stamens with basifixed anthers and short and red filaments (Jimenez *et al.* 2015). Female flowers have a tricarpellar and unilocular ovary filled by one anatropous ovule (Scaramuzzi 1957, Verdù and García-Fayos 1997). The fruit is a spherical or ovoid drupe, 4-5 mm in diameter, which is initially reddish and then black in autumn (Verdú and García-Fayos, 2002) (Fig. 2).

Figure 2. Leaves, flowers, fruits and bark of *Pistacia lentiscus* L.

Different parts of *Pistacia lentiscus* have been traditionally used for a wide range of purposes. For example, in Greece, lentisk is cultivated for its aromatic resin. The product, which is obtained by making small vertical incisions in the bark, is commonly known as mastic and it is used in the production of chewing gum, in perfumery, in dentistry and in the distillery industry (Zakynthinos and Rouskas 2001, Nahida *et al.* 2012) (Fig. 2).

Analysis of chemical composition of mastic revealed that is a complex mixture of volatile compounds, mainly terpenes (Magiatis *et al.* 1999, Papageorgiou *et al.* 1991). Numerous studies have demonstrated that it possesses antimicrobial properties against a broad range of bacterial and fungal pathogens and antioxidant activity as well (Loutrari *et al.* 2006). Furthermore, mastic gum extracts were demonstrated to induce apoptosis in HCT116 human colon cancer cells (Balan

et al. 2005) and inhibit growth and survival of human K562 leukemia cells, attenuate angiogenesis and reduce vascular endothelial growth factor and chemokine release by Lewis lung carcinoma (LLC) cells in mice (Loutrari *et al.* 2006, Magkouta *et al.* 2009).
In the same way, berries of *P. lentiscus* are rich in essential oils and fatty acids and are traditionally used in the treatment of ulcers and heal psoriasis (Mahenni *et al.* 2008, Trablesi *et al.* 2012, Trabelsi *et al.* 2015). They also contain high amounts of anthocyanins, which confer antioxidant capacity and induce autophagy, a mechanism to enhance chemoprevention (Longo *et al.* 2007, Bhouri *et al.* 2010).
The most important components of *P. lentiscus* leaves are polyphenols representing the 7.5 % of leaf dry weigh. They include phenolics acids, flavonols glycosides (mostly myricetin derivatives), hydrolizable tannins and anthocyanins (Romani *et al.* 2002, Rodríguez-Pérez *et al.* 2013). Furthermore, they are rich in essential oils and fatty acids (Lo Presti *et al.* 2008). Aqueous extracts of *P. lentiscus* leaves have traditionally been used in folk medicine for their diuretic properties, as anti-inflammatory, antiseptic as well as for the treatment of various diseases, such as hypertension, stomach aches and kidney stones (Gardeli *et al.* 1980, Bampouli *et al.* 2014; Ljubunic *et al.* 2005, Benhammou *et al.* 2008).
Recent phytochemical investigations provided evidence for these traditional applications. Many studies were carried out on the activity of *P. lentiscus* leaves considering their polyphenolic fractions. These polyphenolic extracts have displayed powerful antioxidant capacity (Baratto *et al.* 2003, Piluzza and Bullitta 2011) as well as hepatoprotective, anti-inflammatory and anticancer effects (Ljubuncic *et al.* 2005, Abdelwahed *et al.* 2007, Esmat *et al.* 2011, Remila *et al.* 2015, Ghenima *et al.* 2015, Janakat and Al-Merie 2002).

1.3.3 *Cistus incanus* L.

The Cistaceae family is formed by different genus, including *Helianthemum*, *Halimium* and *Cistus*.The genus *Cistus* comprises about 20 shrub species, of which 16 occur in Europe and 12 in Morocco (Guzmàn and Vargas 2005).
The taxonomy of this genus has traditionally been mostly based on reproductive characters. In particular, previous phylogenetic studies revealed the separation of *Cistus* species in two major groups: one of purple-flowered species and another containing the white-flowered species (Guzmàn and Vargas 2005, Guzmàn *et al.* 2009). Moreover, the white-flowered lineage is divided in two groups: the *C. clusii* and the *C. salviifolius* groups.
According to Demoly (1996) *Cistus incanus* L. (syn. *C. creticus* subsp. *eriocephalus*) is a hybrid between *Cistus albidus* and *Cistus crispus* (Greuter *et al.* 1984, Demoly 1996, Guzmàn and Vargas 2005). In Italy, *Cistus incanus* L. (also known as “pink rockrose” or “hairy rockrose”) is widespread in dry rocky places, from sea level to 800 m (Thanos *et al.* 1992).
This shrubby plant, about 1 m tall, possess crowd branches that are thickly clothed with short grey hairs (Sweet 1830). It is a seasonally dimorphic species, in which brachyblasts with small xeromorphic leaves develop in summer, while dolichoblasts with large mesomorphic leaves in

winter (Aronne and De Micco 2009). Summer leaves are sessile, ovatel spathulate, attenuated towards the base, 3-nerved, rugose and undulate (Sweet 1830). Both leaf surfaces are covered by a thick layer of white stellate trichomes hairs consisting of a stalk and eight-18 long branches (Aronne and De Micco 2001). Some of these thrichomes are glandular and secret a brownish resin consists mainly of terpenoids (Demetzos *et al.* 1990).

C. incanus blooms in May and has five sepals and five pink petals on its bisexual flowers, generally solitary, sometimes in pairs (Herrera 1991). The stamens are yellow and very numerous with anthers fixed by their bases to the filaments and opening by longitudinal slits to liberate the orange color pollen. The ovary is superior, usually with three carpels (Herrera 1991). The fruit is a woody capsule and the seeds are small (1 – 2 mm) (Thanos *et al.* 1992) (Fig. 3).

Figure 3. Leaves, flowers and fruits of *Cistus incanus* L.

C. incanus has a long history in ethnic and popular medicine. The medicinal benefit of this plant has been known since the 4th century BC, when its herbal extracts were used for treating skin diseases and gastric problems (Petereit *et al.* 1990, Danne *et al.* 1993, Riehle *et al.* 2014). Moreover, its aqueous extracts have been used for the prevention and therapy of coughs and common colds (Kalus *et al.* 2009, 2010).

Recently, aqueous extracts of its leaves have been shown to have anti-bacterial, anti-oxidant and anti-mycotic effects (Lendeckel *et al.* 2002, Bouamama *et al.* 1998, Wittpahl *et al.* 2015, Droebner et al 2011). Furthermore, the CYSTUS052 aqueous extract derived from *C. incanus* aerial parts was shown to exhibit potent anti-influenza virus activity in mice (Droebner *et al.* 2007) and to inhibit human immunodeficiency virus (HIV) *in vitro* (Rebensburb *et al.* 2016).

All these extracts are rich in various polyphenols, including flavonoids and tannins; thus, it was tempting to assign the bioactivities of *C. incanus* to these compounds.
A study performed by Danne *et al.* in 1993 showed that *C. incanus* leaves contain four different proanthocyanidins. Subsequently, some flavonols glycosydes have also been identified in this species (Santagati *et al.* 2008 and Barrajón-Catalán *et al.* 2011).
However, the polyphenolic composition of *C. incanus* is complex and contains other molecules that could contribute to the its health benefits.
Therefore, further detailed studies are needed to identify these compounds and other *in vitro* and *in vivo* investigations should be conducted to evaluate the efficacy of *C. incanus* as biofactory.

1.4 **Objectives of the thesis**

As stated in the first paragraph, plants inhabiting the Mediterranean region are subjected to a wide range of environmental stresses, especially during the summer when low water availability is superimposed on high light and high temperatures.
Such stressful conditions may have acted as driving forces for their evolution, leading these plants to develop a suite of morpho-anatomical, physiological and biochemical features which allow their persistence in a such harsh environment.
In particular, these plants are able to synthesize a broad spectrum of secondary metabolites. Among these, polyphenols play a key-role in counteracting the deleterious effects of reactive oxygen species (ROS) in plant cell metabolism. The antioxidant activity of polyphenols is also maintained in human cells and in the last years the use of these compounds in the medicinal field has considerably grown.
The present PhD project was developed under this scenario. In particular, the objectives of the present work are: 1) to explore the physiological and biochemical responses of three selected Mediterranean species in their natural habitat; 2) to assess the relative significance of these species as “biofactories”, i.e. potential sources of health-promoting polyphenols.

1.5 **References**

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2. Dynamics of physiological and biochemical traits in three co-occurring Mediterranean shrubs: a daily, seasonal and interannual study

2.1 Abstract

Under natural conditions the overlapping of multiple stressors may induce a wide range of ecophysiological and biochemical responses in Mediterranean plants. In particular, during summer drought, the ability of different species to avoid damaging effects determines their survival. There is a vast body of evidence on water relations and photosynthetic performances of co-occurring woody species under the Mediterranean climate. Nonetheless, only few studies combined their physiological and biochemical responses, whereas no research examined inter-specific variations at different timescales. In this context, we carried out a comparative field study to investigate the physiological and biochemical strategies used by three dominant woody species (*Phillyrea latifolia*, *Pistacia lentiscus* and *Cistus incanus*), which co-occur on coastal dunes in Southern Tuscany. We measured both physiological (gas exchanges, water relations and PSII photochemistry) and biochemical parameters (carotenoids, polyphenols and abscisic acid in its free and conjugated form) on a daily and seasonal basis during two consecutive years (2014 and 2015).

Our study on inter-specific responses along different timescales permitted a better understanding of the mechanisms adopted by these Mediterranean plants in field conditions. In particular, on a diurnal scale, water relation patterns allowed us to recognise the different water-use behaviours of the species, while daily variation in F_v/F_m clarified the distinction among photoinhibition-avoider and photoinhibition-tolerant plants. On a seasonal scale, as shown by the principal component analysis, each species could be characterised by different physiological and biochemical traits. Furthermore, on interannual scale, species-specific variations in leaf carotenoid concentrations were observed. Finally, multiple regression analysis showed that temperature was the most important climatic factor in affecting both physiological and biochemical parameters in all three species. Taken together, our results confirm a remarkable ability of the investigated plants to optimize multiple mechanisms and withstand environmental conditions in their natural habitat.

2.2 **Introduction**

Plants growing in the Mediterranean basin overcome relatively long drought periods with low precipitation coinciding with high irradiance and high temperatures (Peñuelas *et al.* 1998; Lo Gullo and Salleo, 1988). When extended and severe droughts occur, the capacity of species to avoid damaging effects determines their survival (Filella *et al.* 1998).

Physiological and morphological traits play a key-role in determining the acclimation of Mediterranean plants to limited water supply (Bombelli and Gratani 2003, Munné-Bosch 1999). In this context, Mediterranean plants have been included in different functional classifications, based on their leaf habit and their water-use strategy (Hernández *et al.* 2010). Particularly, on the basis of the leaf habit, two main functional groups of woody plants have been identified in Mediterranean ecosystems: evergreen-sclerophyllous and drought-semideciduous plants. The first group is characterised by xeromorphic leaf traits (e.g. dense pubescence, thick cuticle and epicuticular wax layer) (Karavatas and Manetas 1999), whereas plants belonging to the second group reduce transpiring surface through partial fall of their leaves during dry periods (Werner *et al.* 1998). In addition, on the basis of their water-use strategies, two contrasting mechanisms have been proposed: drought-tolerance and drought-avoidance. Drought tolerant species are able to maintain physiological processes at declining leaf water potentials, whereas drought-avoiders endure drought conditions by avoiding tissue dehydration. Physiological features conferring drought tolerance include osmotic adjustments, low turgor loss point, and low vulnerability to cavitation (Fini *et al.* 2013). On the other hand, plants that avoid drought can minimise water loss by reducing stomatal opening (water savers) or by extracting water from the soil to a similar extent of water evaporation (water spenders) (Lo Gullo and Salleo 1988, Chaves *et al.* 2002). This classification may be approximately associated to the distinction between “anisohydric“ and “isohydric” species introduced by Tardieu and Simmoneau (1998) (Nardini *et al.* 2014). However, these behaviors are not mutually exclusive and, in practice, plants may switch between these two strategies during drought progression (Domec and Johnson 2012).

Acclimation to summer drought also involves changes in plant biochemistry. Particularly, secondary metabolites, especially carotenoids and polyphenols, have major roles against photo-oxidative stress in field conditions (Hernández *et al.* 2012, Tattini *et al.* 2015, Smirnoff 1993).

In all photosynthetic organisms, the xanthophylls-carotenoid complement of light-harvesting complexes protect photosystem II dissipating as heat the excess of light energy (NPQ, non-photochemical quenching) (Demmig-Adams and Adams 1996). In addition, β-carotene and zeaxanthin contribute to limit lipid peroxidation by reducing ROS (reactive-oxygen species) production and regulating the fluidity of photosynthetic membranes (Havaux 1998, Ramel *et al.* 2012)

Apart from carotenoids, other important molecules are present in chloroplasts to control oxygen toxicity (Foyer *et al.* 1994; Asada 1999, Munné-Bosh and Peñuelas 2003). Among the latter, polyphenols may play a key-role in counteracting the deleterious effects of excess light stress associated with drought and high temperature, following the depletion in the activity of antioxidant enzymes (primary antioxidant defenses of the plants) (Tattini *et al.* 2015).

The antioxidant activity of polyphenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers. Moreover, depending on their chemical structure and their peculiar location in the leaf, polyphenols also exhibit others wide range of ecological properties. For example, they are implicated in responses to UV radiation (Agati *et al.* 2013), resistance to pathogens and nutrients deficiency (Hichem and Mounir 2009).

Lastly, drought may also affect other biochemical mechanisms, such as those linked to the increase in ABA (abscisic acid) biosynthesis, a phytohormone that induces stomatal closure (Wilkinson and Davies 2010). ABA can be considered as the end-product of MEP carotenoid pathway (plastidial methylerythritol phosphate pathway), because it is synthesized from violaxanthin in the plastid and then converted in ABA in the cytosol (Milborrow 2001). Lee *et al.* (2006) showed that ABA can be produced also from its conjugated form with glucose (ABA-GE). Particularly, the biosynthesis of free-ABA from ABA-GE could increase in response to drought (Lee *et al.* 2006, Xu *et al.* 2012).

The strong seasonality of Mediterranean climate, have often raised the question of how distinct are species' responses to drought.

Previous comparative field studies on Mediterranean plants have been aimed at underlying their morphological and physiological characteristics (David *et al.* 2007, Ciccarelli *et al.* 2016, Filella *et al.* 1998, Peñuelas *et al.* 1998, Gratani *et al.* 2016, 2013, Llorens *et al.* 2003). To date, most of the studies that have considered physiological and biochemical responses were performed on one species (Nogués *et al.* 2012, 2014), and none of these studies considered inter-specific variations at daily, seasonal and annual timescales.

In order to fill this gap, our study explores the physiological and biochemical strategies used by three co-occurring maquis plants in a Mediterranean costal dune ecosystem. The study species are two evergreens sclerophyllous, *Pistacia lentiscus*, *Phillyrea latifolia*, and a semi-deciduous, *Cistus incanus*. In previous ecophysiological surveys, *P. latifolia* was described as a drought tolerant or an anisohydric species (Barbeta *et al.* 2015), *P. lentiscus* as a drought avoider-water spender or isohydric species (Ozturk *et al.* 2010, Trifilò *et al.* 2015) and *C. incanus* as a drought avoider – water saver plant (Gratani and Bombelli 2000, 2003 and Sánchez-Blanco *et al.* 2002).

The aims of this work are 1) to characterize the physiological and biochemical responses of the three species 2) to elucidate the mechanisms underlying them, and 3) to evaluate their dynamics at diurnal, seasonal and interannual timescales.

Knowledge of species-specific differences in physiological and biochemical responses, as well as their variation at different timescales, is a crucial issue considering the forecasted increases of daily, seasonal and annual mean temperatures due to global warming (Puglielli *et al.* 2016).

This is of particular importance for those species distributed in the Mediterranean basin, which is one of the most prominent "hot spot" in future climate change projections (Giorgi 2006, IPCC 2013).

2.3 **Materials and methods**

2.3.1 Experimental site and study species

For this study, we selected three species with contrasting morphological and physiological traits, inside the most widespread and dominant species in the Mediterranean basin: *P. latifolia*, *P. lentiscus* and *C. incanus*. Measurement campaigns were carried out in spring (May), summer (July) and autumn (October) 2014 and 2015.
Particularly, measurements were conducted on 27 –29 May 2014, 23-25 July 2014, 04-06 October 2014, 29-30 May 2015, 07-09 July 2015 and 04-06 October 2015.
A homogeneous area was delimited in the coastal dunes of Castiglione della Pescaia (Gr) (42° 46'N, 10°53'E), where all the above species co-occurred.
Measurements of leaf water relations, gas exchange and chlorophyll fluorescence parameters were carried out on daily basis (8:00, 12:00,15:00, 18:00) and taken on four individuals per species, on fully-expanded and sunny-exposed leaves of the upper part of the crown. At all four sampling times, several leaves from each selected plant were collected, rapidly frozen in liquid nitrogen and stored at -80°C. These samples were used for the secondary metabolite analyses.
Meteorological parameters (precipitation, air temperatures and global irradiance) were obtained from the weather station "Ponti di Badia", located 8 km from the study site.

2.3.2 Water relations, gas exchanges and chlorophyll fluorescence

8 leaves of each species were collected in the field and transported to the laboratory in sealed zip-lock, tared plastic bags. The whole leaves were weighted (fresh weight, FW). Then, they were hydrated until saturation (constant weight) for 48 h in darkness (turgid weight, TW). The value of Ψ_w was remeasured to check that it was higher than -0.05 MPa with no leaf oversaturation. Finally, leaves were dried in an oven at 105°C for 24 h (dry weight, DW).
Relative Water Content (RWC) was calculated according to this expression (Guidi *et al.* 2008):

$$RWC = (FW - DW)/(TW - DW)$$

Leaf water potentials (Ψ_w) were measured on eight leaves per species (two leaves per each plant) from 8:00 to 18:00 h using a Scholander-type pressure chamber (PMS Instruments, Corvallis, OR) (Scholander *et al.* 1965), following the methodology previously described in Tattini *et al.* (2002). After measuring Ψ_w, the leaves were frozen in liquid nitrogen until measurement of osmotic potential (Ψ_π).
$\Psi\pi$ was measured on sap extracted from leaf material (~300 mg FW) by centrifugation (5 min at 12,000 rpm). A 10 µl aliquot of the sap was used to measure total concentration of solutes (in mOsm) with a vapour pressure osmometer (Wescor 5520, Wescor Inc., Utah, USA) and converted to Ψ_π according to Van't Hoff equation (Gucci *et al.* 1997, Nobel 1999).
Gas exchanges were measured by a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE, USA) operating at ambient CO_2. The central unit recorded incident PPFD and cuvette temperature. Chlorophyll fluorescence was measured using a portable PAM-2000 Chl

fluorometer (Heinz Walz, Effeltrich, Germany). Minimum fluorescence (F_0) was measured with a 0.8 µmol m^{-2} s^{-1} measuring light beam on dark-adapted leaves. Maximum fluorescence in the dark-adapted state (F_m) was determined using saturating pulses (0.5 s) of red light (8000 µmol m^{-2} s^{-1}), thus allowing calculation of maximum quantum yield of photosystem II (F_v/F_m = (F_m − F_0)/F_m). Leaves were then exposed to actinic light and a second saturating pulse was applied. This allowed us to determine the maximum fluorescence in light-adapted state (F_m') and the steady-state fluorescence (F_s). These data were used to calculate non-photochemical quenching (NPQ = (F_m − F_m')/F_m'; Schreiber *et al.* 1986) and actual quantum yield of photosystem II (PSII; ϕ_{PSII} = (F_m' − F_s)/F_m'; Genty *et al.* 1989).

2.3.3 Analysis of photosynthetic pigments and polyphenols

Individual carotenoids were identified and quantified using the protocol reported in Beckett *et al.* (2012). Fresh leaf material (300 mg) was extracted with 2 x 2.5 ml acetone (added with 0.5 g l^{-1} $CaCO_3$) and injected (15 µl) into a Perkin Elmer Flexar liquid chromatograph equipped with a quaternary 200Q/410 pump and an LC 200 diode array detector (DAD) (all from Perkin Elmer, Bradford, CT, USA). Photosynthetic pigments were separated in an Agilent Zorbax SB-18 (250 × 4.6 mm, 5 µm) thermostated at 30 °C using a 18-min run and a linear gradient solvent system from 100% of solvent A (methanol/water 95/5) to 100% solvent B (methanol/ethylacetate 6.8/3.2) with a flow rate of 0.8 mL min^{-1}. Individual carotenoids and chlorophylls were identified and quantified using retention times and UV spectral characteristics of authentic standards from Extrasynthese (Lyon-Nord, Genay, France). De-epoxidation state of the xanthophyll cycle was calculated as: DES = (*A* + *Z*)/ (*A* + *Z* + *V*) where *A, Z* and *V* represent violaxanthin, antheraxanthin and zeaxanthin concentrations, respectively (Tattini *et al.* 2014, 2015).
Individual polyphenols were analyzed following the protocol of Tattini *et al.* (2004). Fresh leaf material (300 mg) was extracted twice with 5 mL of ethanol/water (75/25) adjusted at pH 2.5 with formic acid and the supernatant partitioned with 3 × 5 mL of *n*-hexane. The ethanol fraction was reduced to dryness, and the residue was rinsed with 1 mL of methanol/water (90/10). Aliquots of 10 µL were injected into the Perkin Elmer liquid chromatography unit reported earlier. Phenylpropanoids were separated using a Agilent Zorbax SB-18 (250 × 4.6 mm, 5 µm), operating at 30 °C with a flow rate of 1 mL min^{-1} and eluted with a linear gradient solvent system from 100% solvent A (water adjusted to pH 2.5 with HCOOH/acetonitrile (90/10)) to 100% solvent B (acetonitrile/water adjusted to pH 2.5 with HCOOH (90/10)) over a 45-min run. Identification and quantification of these metabolites was carried out using retention times and UV spectral characteristics of authentic standards, as well as based on literature data (Tattini *et al.* 2000, Romani *et al.* 2002, Gori *et al.* 2016).

2.3.4 Analysis of ABA (abscisic acid) and ABA-GE (abscisic acid glucose ester)

Fresh leaf tissue (300 mg) was grinded in liquid nitrogen and added with 40 ng of deuterium-labeled internal standards (d_6-ABA and d_5-ABA-GE from the National Research Council of Canada). The extraction solvent was CH_3OH/H_2O (80/20) adjusted to pH 2.5 with formic acid. Samples were extracted with 3 mL of extraction solvent and the supernatant was defatted by

normal hexane extraction (2 x 3 mL). The aqueous-methanolic phase was then collected and purified through Sep-Pak C18 cartridges (Waters, Massachusetts, USA). The sample solutions were loaded on the cartridges and washed with 2 mL of water (pH 2.5). ABA and ABA-GE were eluted by 1.2 mL of ethylacetate. The eluate was reduced to dryness under nitrogen and rinsed with 250 µL of CH_3OH/H_2O (50/50). Finally, 3 µL of sample solution were injected into the LC–ESI–MS/MS system consisting of an UPLC (Nexera UPLC Shimadzu Corporation) coupled with a MS/MS detector (TQ 8030) equipped with an ESI source (all from Shimadzu Corporation, Kyoto, Japan) operating in negative ion mode. Compounds were separated using a Poroshell C18 column (3.0 × 100 mm, 2.7 µm i.d., Agilent, USA). Gradient elution was performed with water acidified with 0.1% formic acid (solvent A) and acetonitrile/methanol (1/1) added with 0.1% of formic acid (solvent B) at a constant flow-rate of 300 µL min^{-1} ranging from 95% solvent A to 100% solvent B during a 30-min run (Velikova *et al.* 2016). Quantification was conducted in multiple reaction mode (MRM) as reported in López-Carbonell *et al.* (2009).

2.3.5 Statistical analysis

Data were analysed with repeated-measures ANOVA (SPSS v.20, IBM, Chicago, IL, USA) where species, year and month were the between-subject factor and the daily variation of each parameter was the within-subject factor (each hour as a single level for a total of 4 levels) (Potvin *et al.* 1990).

ANOVA outliers were identified and removed from the dataset using scatter plots and residuals plot to respect assumption of normality distribution.

In order to verify whether variability between species were mainly induced on annual, monthly or daily basis, the variance explained (r^2) of "species x year", "species x month" and "species x daily" interactions was calculated as the ratio between the total sum of squares of the considered interaction and the sum of the total sum of squares of the model.

For parameters showing significant "species x time" (daily, seasonal and inter-annual) interactions and high r^2, differences between species were analysed using one-way ANOVA. Post-hoc comparisons were carried out using Tukey's test.

Based on the results of r^2 calculation, which indicated a higher source of variability between species deriving from the "species x month" interaction, three different principal component analysis (PCA) were performed maintaining separated data of different months (May, July and September).

This analysis was performed on the basis of a correlation matrix with the aim of summarising the several studied parameters determining most of the variation between species (Zunzunegui *et al.* 2011) and, at the same time, of obtaining a graphical differentiation between species which could simplify the identification of their different behaviours (STATGRAPHICS Centurion XV.II, StatPoint Inc., Warrenton, Virginia, USA).

A preliminary selection of physiological and biochemical parameters to include in PCA was carried out, excluding parameters on average or highly correlated each other (r≥0,6) and deriving by the calculation of other parameters. From 17 initial parameters, 12 were maintained (P_n, g_s, Ψ_w, Ψ_π, NPQ, ϕ_{PSII}, F_v/F_m, PP_{Tot}, DES, Car_{Tot}, Chl*a*/Chl*b*, ABA). Correlation between physiological and biochemical traits was tested using Pearson product moment correlation coefficients.

Finally, in order to investigate the influence of air temperature, radiation and precipitation on the measured parameters, multiple regression analysis (MRA) was carried out for each species and for each parameter, setting meteorological parameters as independent variables and physiological and biochemical parameters as dependent variables. Graphics were designed using SigmaPlot 11.0, Systat Software Inc. (San Jose, California, USA).

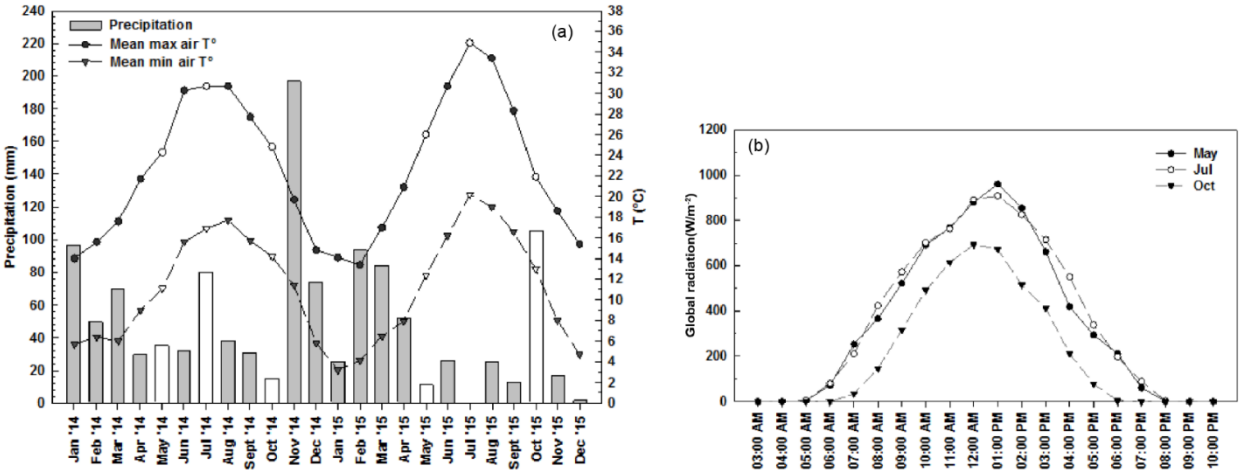
2.4 **Results**

2.4.1 Climatic conditions

The average precipitation and temperatures during the experimental period are shown in Fig. 1a. Concerning the weather conditions, the two study years differed in the seasonal distribution and the total amount of rainfall and temperature fluctuations. In 2014, cumulative rainfall during the 3 months preceding measurements were 245 mm (February-April) and 200 mm (July-September), while in 2015 were 241 mm from February to April and 64 mm from July to September.

In 2014 the average minimum and maximum temperatures were 11.1 °C and 24.3 °C in May, 16.9 °C and 30.7 °C in July and 15.7 °C and 27.7 °C in October, while in 2015 were 12.4°C and 26 °C in May, 20.2 °C and 34.9 °C in July, 16.6 °C and 28.3 °C in October.

Year averages of mean daily global irradiance (W/m^2) during the days of measurements were 298.7 ± 32.6 W/m^2 in May, 303.9 ± 33.04 W/m^2 in July and 176.4 ± 28. W/m^2 in October. Global radiation reached maximum values of 990 W/m^2 in May at 12:45 pm (Fig. 1b).



igure 1a. Monthly total precipitation (mm) and daily average of maximum and minimum air temperature throughout the study period. **Figure 1b**. Year averages of daily global irradiance (W/m^2) during the days of measurements (data of the Meteorological Station of Ponti di Badia, Grosseto).

2.4.2 Physiological traits

Gas exchange parameters (P_n and $g_{s,}$) and water relations (Ψ_w and Ψ_π) significantly differed between species at daily, seasonal and annual timescale ("species x year", "species x month" and "species x daily" interactions) (Table SM1). For all these measurements, "species x month" and "species x daily" interactions showed the higher r^2 (Table SM2), indicating a greater contribution of months and hours in determining differences between species. All chlorophyll fluorescence parameters (F_v/F_m, ϕ_{PSII} and NPQ) showed significant inter-specific differences at seasonal and daily timescale (Table SM1). The r^2 values suggested that, for NPQ and ϕ_{PSII} measurements, species mainly differed on monthly basis, while for F_v/F_m the differentiation between species was mostly observed at daily level.

2.4.2.1 Gas exchanges

-Seasonal variations

Figure 2 shows the seasonal patterns of photosynthesis (P_n, µmol m^{-2} s^{-1}) and stomatal conductance (g_s, mmol m^{-2} s^{-1}) of the study species. In July P_n declined in all three species (Fig. 2a). *P. latifolia* had the lowest decrease (-35%) and *C. incanus* the highest one (-80%). In October, after the first rainfalls, all species recovered and P_n values were similar to May. Leaf stomatal conductance (g_s) showed the same trend as P_n for *P. latifolia*. *C. incanus* showed the highest value of g_s in October, whereas *P. lentiscus* maintained fairly constant g_s during the whole experimental campaign (Fig. 2b).

-Daily variation

In order to highlight the daily differences between species, Fig. 2 also shows the July diurnal patterns of P_n (Fig. 2c) and g_s (Fig. 2d) as the average of the two years of measurements (2014 and 2015). *P. lentiscus* did not show marked changes in gas exchanges during the day (P_n and g_s dropped after 17:00, when g_s was light limited). In contrast, *P. latifolia* and *C. incanus* showed a significant daily pattern, with the lowest P_n and g_s values between 12 and 15 pm and at 18.00.

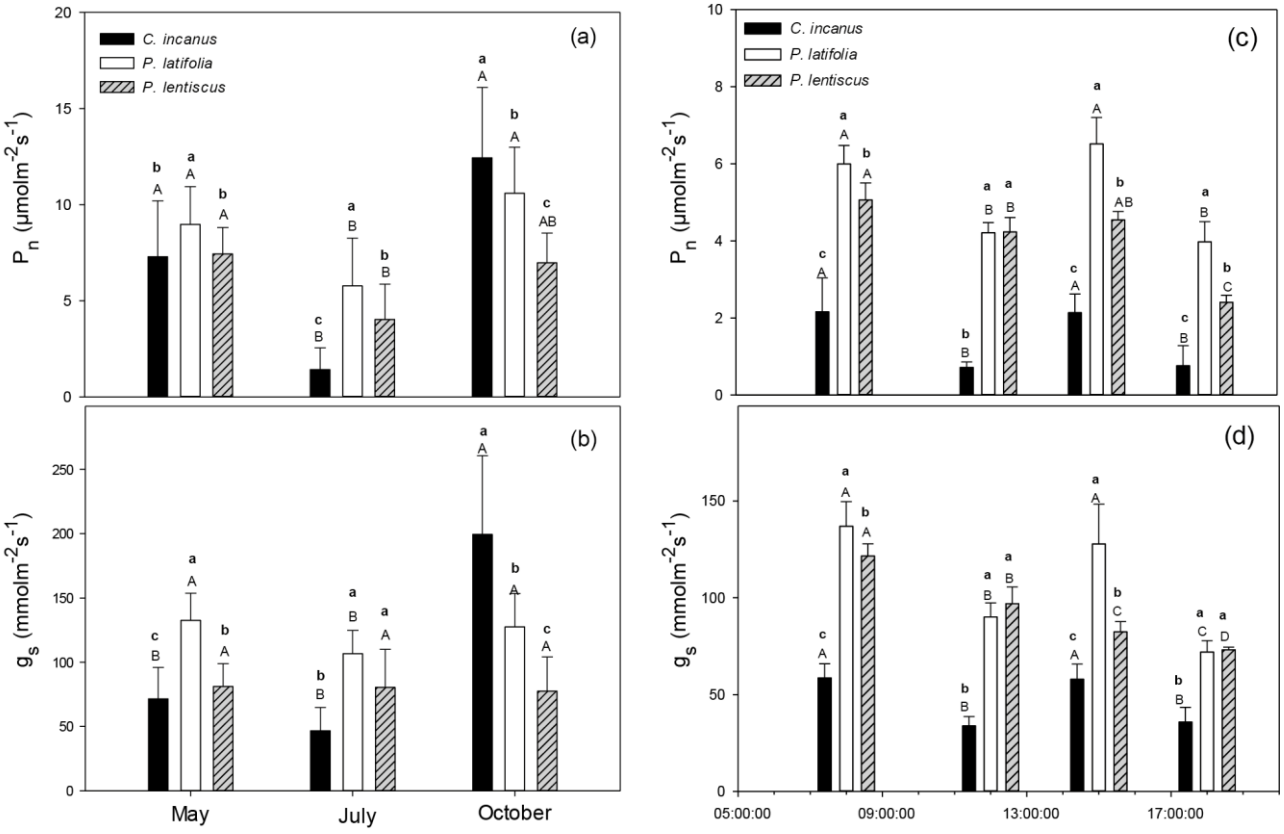


Figure 2. *Seasonal and July daily trends of (a, c) net photosynthetic rate (P_n) and (b, d) stomatal conductance (g_s) in* C. incanus*,* P. latifolia *and* P. lentiscus*. The mean values for each hour or month (±SD) are shown (n=8). Mean values with the same letters are not significantly different (p≥0.05). Lowercase letters indicate the differences among the species for each sampling point, capital letters indicate the intra-specific differences during the study period.*

2.4.2.2 Water relations

-Seasonal variations

Fig. 3 shows the seasonal trends of (a) water potential (Ψ_w) and (b) osmotic potential (Ψ_π) in the study species.

The course of water potential (Ψ_w) during seasons was similar for *C. incanus* and *P. latifolia*. In both species, seasonal variations in Ψ_w showed a typical pattern with higher values in May followed by a decline during the summer drought (July) and a recovery in response to autumn rains (October). Conversely*, P. lentiscus* did not show significant change in Ψ_w among seasons. Furthermore, Ψ_w was always lower in *C. incanus* than in *P. latifolia*, reaching values of – 3.3 MPa in July, while in autumn the two species showed similar values of Ψ_w (Fig. 3a).

Concerning the osmotic potential (Ψ_π), *P. latifolia* and *P. lentiscus* did not show consistent variations in Ψ_π at seasonal timescale. Conversely, Ψ_π of *C. incanus* decreased from May (-1.99 MPa) to July (-2.82 MPa) (Fig. 3b).

-Daily variations

In July, the diurnal time course of Ψ_w confirmed clear differences between the species (Fig. 3c). *P. lentiscus* showed higher values and lower daily variation of Ψ_w than *C. incanus* and *P. latifoli*a. Furthermore, this species did not show any daily changes in Ψ_π (Fig. 3d). In *P. latifolia* and *C. incanus*, Ψ_w decreased during the warmest hours of the day (12:00) and remained low (-3.43 MPa in *P. latifolia* and -3.2 MPa in *C. incanus*) during the early afternoon (15:00). At sunset, Ψ_w recovered only partially (-2.74 MPa for *C. incanus* and -2.55 MPa in *P. latifolia*). Similarly to Ψ_w, Ψ_π of both species declined substantially during the day (Fig. 3d).

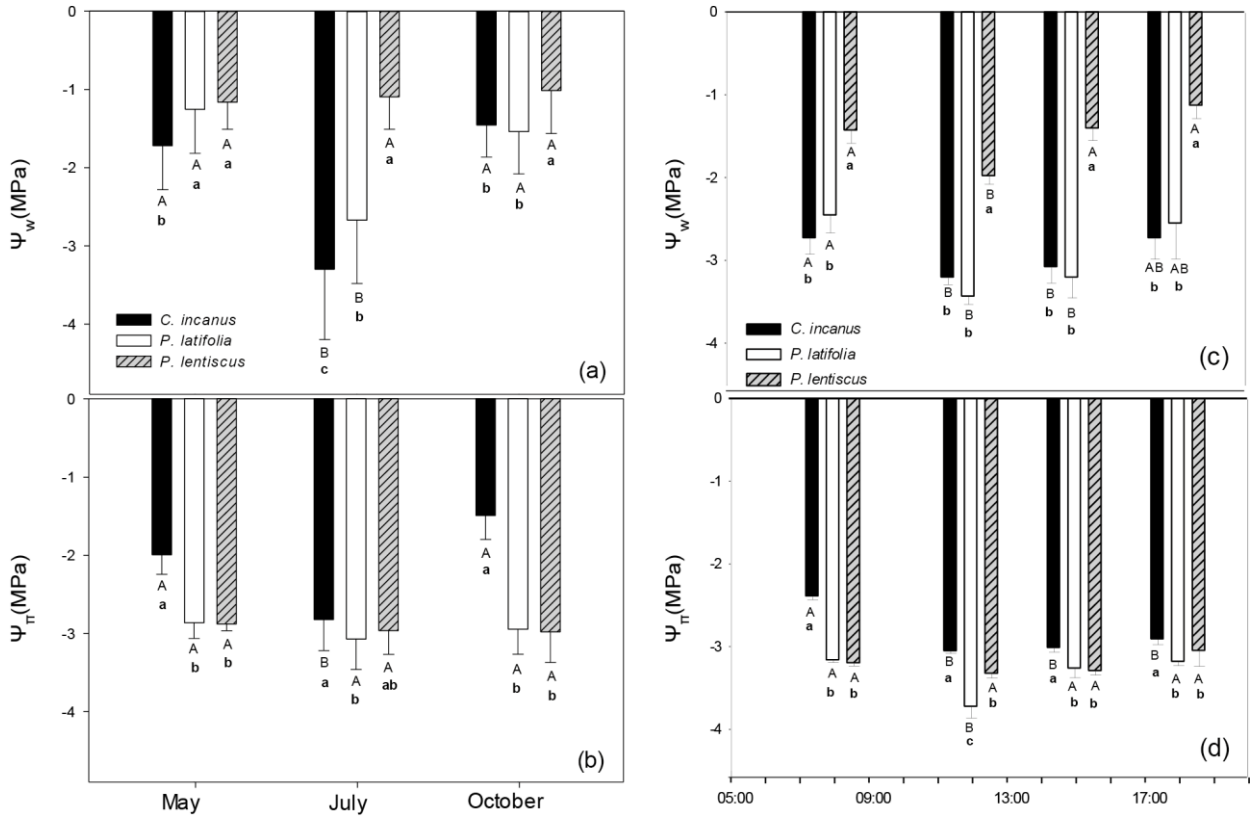


Figure 3. Seasonal and July daily trends of (a, c) water potential (Ψ_w) and (b, d) osmotic potential (Ψ_π) in *C. incanus*, *P. latifolia* and *P. lentiscus*. The mean values (±SD) are shown (n=16). Mean values with the same letters are not significantly different ($p \geq 0.05$). Lowercase letters indicate the differences among the species for each sampling point, capital letters indicate the intra-specific differences during the study period.

2.4.2.3 Chlorophyll fluorescence parameters

-Seasonal variations

F_v/F_m ratio of *C. incanus* and *P. lentiscus* leaves of were around 0.84 during the whole seasonal cycle (indicating optimal maximum efficiency of PSII), whereas *P. latifolia* displayed a statistically significant decrease in F_v/F_m in July (Fig. 4a).

Similarly to F_v/F_m, ϕ_{PSII} did not change significantly between months in *P. lentiscus* and *C. incanus* (Fig. 4b), whereas *P. latifolia* exhibited a decrease in ϕ_{PSII} in July.
Non-photochemical quenching (NPQ) did not display significant seasonal variations in all three species and was significantly higher in *P. latifolia* in July and October *(Fig. 4c).*

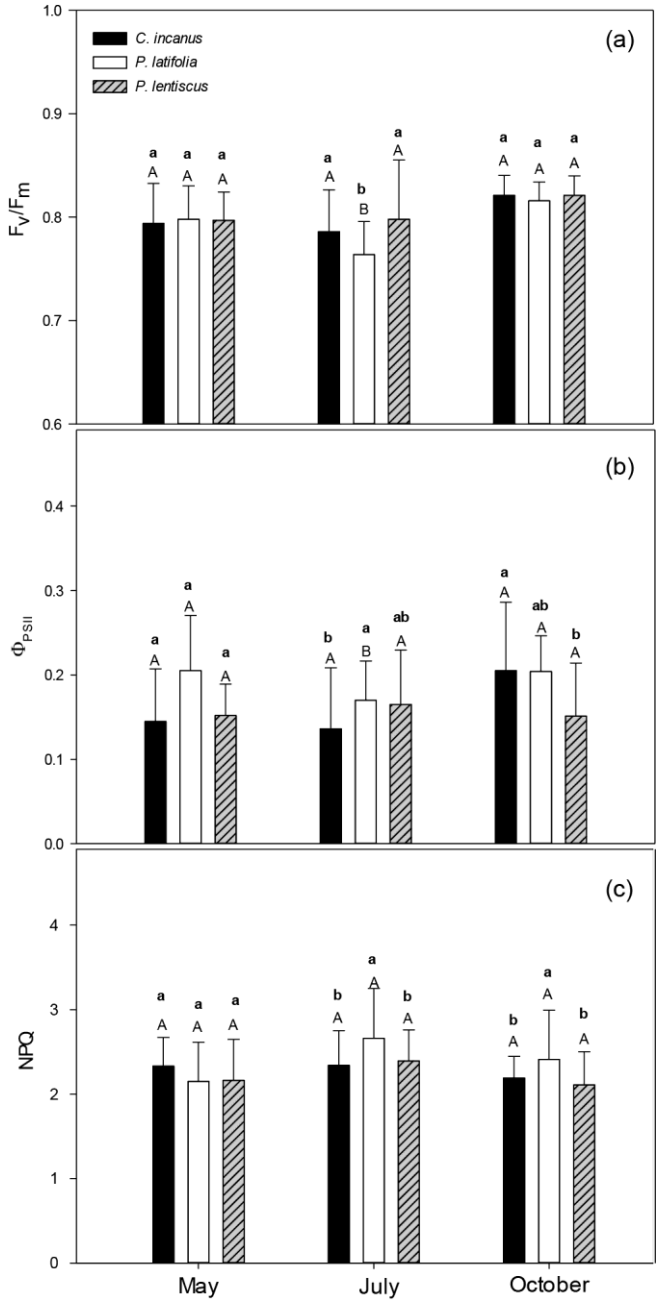


Figure 4. Seasonal trends of maximal efficiency of PSII (F_v/F_m) (a), actual efficiency of PSII (Φ_{PSII}) (b), and non-photochemical quenching (NPQ) (c) in *C. incanus*, *P. latifolia* and *P. lentiscus*. The mean values for each month (±SD) are shown (n=8). Mean values with the same letters are not significantly different ($p \geq 0.05$). Lowercase letters indicate the differences among the species for each month, capital letters indicate the intra-specific differences during the study period.

-Daily variations

In July all the species exhibited a significant diurnal decrease of the F_v/F_m ratio, reaching minimum values at 15:00 (Fig. 5). However, this decrease was higher in *P. latifolia* than *C. incanus* and *P. lentiscus*.

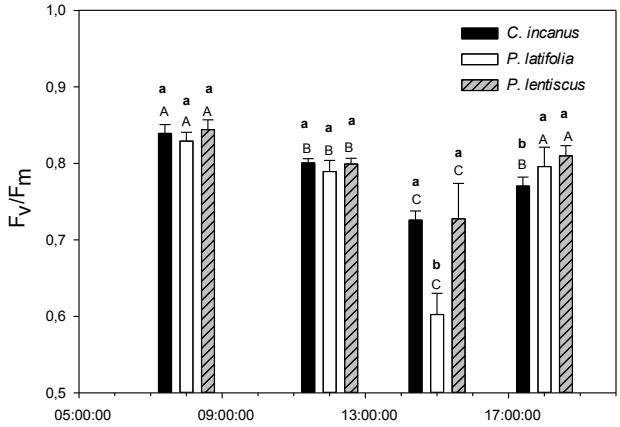


Figure 5. July daily trend of maximal efficiency of PSII (F_v/F_m) in *C. incanus*, *P. latifolia* and *P. lentiscus*. The mean values for each hour (±SD) are shown (n=8). Mean values with the same letters are not significantly different (p≥0.05). Lowercase letters indicate the differences among the species for each hour, capital letters indicate the intra-specific differences during the day.

2.4.3 Biochemical traits

When comparing biochemical traits (carotenoids, polyphenols, ABA and ABA-GE) by RP-ANOVA, we found that all the interactions were significant ("species x year", "species x month" and "species x daily" interactions) (Table SM1). Nevertheless, VAZ/Chl_{Tot} and ABA did not significantly differ between species at daily and annual timescales, respectively (Table SM1). The r^2 values suggested that, for polyphenols and ABA measurements, species mainly differed on a monthly basis, while for carotenoids the differentiation between species was mostly observed at annual level. High values of r^2 were also found in "daily x species" interaction for free ABA (Table SM2).

2.4.3.1 Carotenoids

-Seasonal variations

There were not significant changes in the concentration of total carotenoids (Car_{Tot}) and violaxanthin-cycle pigments (VAZ=Violaxanthin + Antheraxanthin + Zeaxanthin) between months in all three species (Fig. 6a, 6b). *C. incanus* was the only species which displayed a significant change in chlorophyll total content (Chl_{Tot}), with lower values in May and July than in October (Fig. 6c). Furthermore, for all species, the VAZ de-epoxidation state (DES) was higher in May and July and lower in October (Fig. 6d). VAZ/Chl_{Tot} was lower in spring relative to summer

and autumn in *P. lentiscus*, higher in spring and summer than in autumn in *C. incanus*, whereas did not change significantly between seasons in *P. latifolia* (Fig. 6e).
P. lentiscus displayed a statistical significant decrease in Chl*a*/Chl*b* ratio in July (Fig. 6f)
The concentration of Car_{Tot}, VAZ, and Chl_{Tot} in *C. incanus* was constantly around the double of that of the evergreen species (Fig. 6a, 6b, 6c). By contrast, in C. *incanus,* DES was lower than in *P. lentiscus* and *P. latifolia* in May, higher in July, and did not differ from them in October (Fig. 6d).
Finally, in *C. incanus*, VAZ/Chl_{Tot} ratio was higher than *P. lentiscus* in May and than *P. lentiscus* and *P. latifolia* in July (Fig. 6e).

-*Interannual variations*

The concentration of Car_{Tot} of *P. latifolia* and *P. lentiscus* were similar in different years, whereas *C. incanus* contained lower concentration of Car_{Tot} in 2015 than in 2014 (Fig. 7a). In *C. incanus* this decrease was marked and occurred in parallel with the drop in Chl_{Tot} content (from 7.69 ± 1.63 µmol/gDW to 5.28 ± 1.56 µmol/gDW) and in VAZ content (from 0.5 µmol/gDW to 0.2 µmol/gDW) (Fig. 7b, 7c), while there were not changes in the VAZ/Chl_{Tot} ratio in this species (Fig. 7e). In *P. lentiscus* VAZ and VAZ/Chl_{Tot} were slightly higher in 2015 than in 2014 (+42% and +20%, respectively) (Fig. 7b, 7e), whereas Chl_{Tot} and Chl*a*/Chl*b* ratio were significantly lower in 2015 than in 2014 (Fig. 7c, 7f).

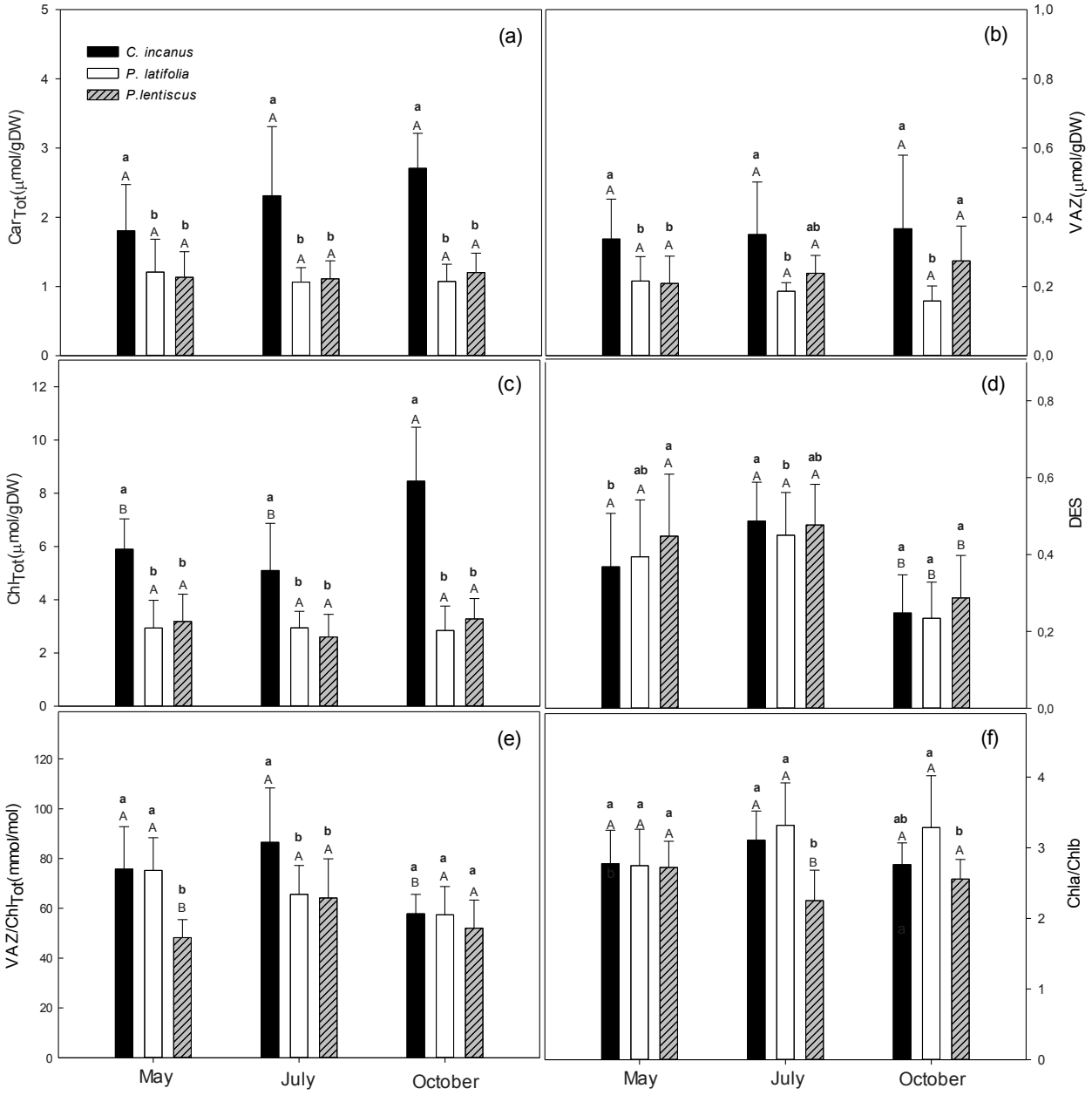


Figure 6. Seasonal variation in carotenoid content (Car_{Tot}) (a), xanthophyll cycle pigment (VAZ) content (b), chlorophyll content (Chl_{Tot}) (c), conversion state of xanthophyll cycle pigments (DES) (d), xanthophyll cycle pigments to chlorophyll total ratio (VAZ/Chl_{Tot}) (e) and Chl*a*/Chl*b* ratio (f) in *C. incanus, P. latifolia* and *P. lentiscus*. The mean values for each month (±SD) are shown (n=8). Mean values with the same letters are not significantly different (p≥0.05). Lowercase letters indicate the differences among the species for each month, capital letters indicate the intra-specific differences during the study period.

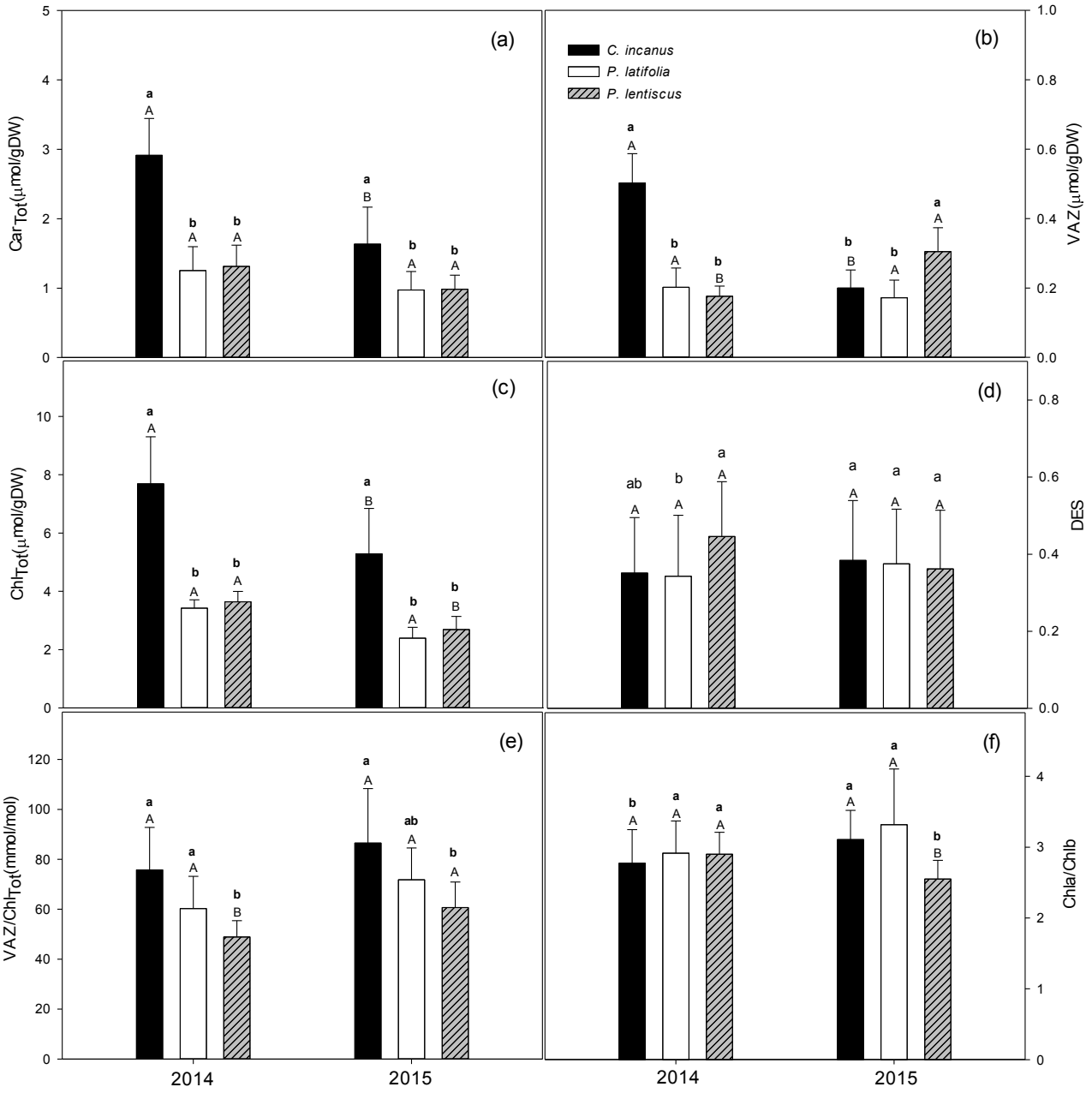


Figure 7. Interannual variation in carotenoid content (Car_{Tot}) (a), xanthophyll cycle pigment (VAZ) content (b), chlorophyll content (Chl_{Tot}) (c), xanthophyll cycle pigments to chlorophyll total ratio (VAZ/Chl_{Tot}) (d) and Chla/Chlb ratio (e) in *C. incanus, P. latifolia* and *P. lentiscus*. Mean values with the same letters are not significantly different ($p \geq 0.05$). Lowercase letters indicate the differences among the species, capital letters indicate the intra-specific differences.

2.4.3.2 Polyphenols

-Seasonal variations

Proanthocyanidins, i.e. condensed tannins, represent the major group of polyphenols in *C. incanus* leaves, followed by flavonol glycosydes (i.e. myricetin and quercetin glycosides) (data not shown). In this species concentration of total polyphenols (POL_{Tot}, represented by the sum of proanthocyanidins and flavonol glycosides, µmol/gDW) increased from May to July and decreased in October (Fig. 8a) whereas total phenylpropanoids (PP_{Tot}) were higher in spring and summer than autumn (8b).

In *P. lentiscus* the most abundant compounds were hydrolisable tannins (galloyl derivatives of quinic acid), whereas the phenylpropanois are mostly composed of flavonol glycosides (myricetin derivatives) and gallic acid derivatives (data not shown). In this species, the sum of all polyphenols did not display a significant seasonal change (Fig. 8a). By contrast, total phenylpropanoids increased in July and remained high in October. No detectable levels of tannins were found in *leaves* of *P. latifolia*. Thus, in this species, the sum of total polyphenols corresponds to the total concentration of phenylpropanoids, which are composed of flavonol glycosides (i.e. quercetin and luteolin glycosides) and hydroxycinnamic acid derivatives (mostly caffeic acid derivatives) (data not shown). In this species, PP_{Tot} raised in July and persisted in October (Fig. 8b).

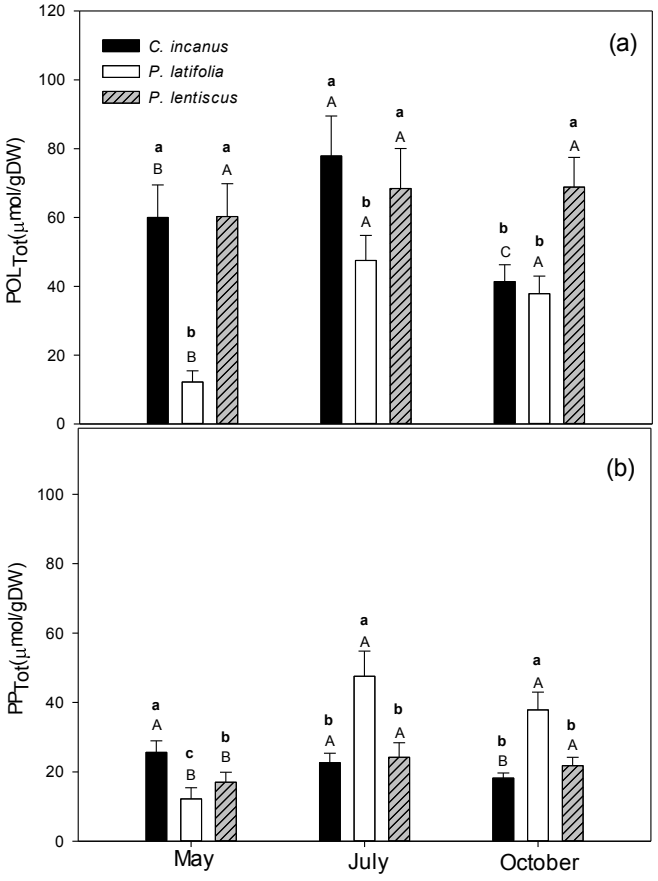


Figure 8. Seasonal variation in polyphenolic (POL_{Tot}) (a) and phenylpropanoid (PP_{Tot}) content (b) in *C. incanus, P. latifolia* and *P. lentiscus*. The mean values for each month (±SD) are shown (n=8). Mean values with the same letters are not significantly different (p≥0.05). Lowercase letters indicate the differences among the species for each month, capital letters indicate the intra-specific differences during the study period.

2.4.3.3 ABA

-Seasonal variations

Seasonal patterns and the absolute leaf contents in ABA and ABA-GE significantly differed between species (Fig. 9a and 9b). Leaves of *P. lentiscus* were characterised by concentrations of ABA and ABA-GE around 10-fold higher than the other species. In this species, May values of both ABA and ABA-GE were around 12 nmol/gDW and 64 nmol/gDW, respectively. These levels were maintained constant in July, whereas in October ABA concentration significantly decreased (~3 nmol/gDW) concomitantly with a significant increase in ABA-GE (~107 nmol/gDW).
In *P. latifolia*, ABA and ABA–GE concentrations had a similar seasonal course, increasing from May to July, and decreasing in October.
In *C. incanus*, ABA did not change from May to July and decreased in October, while ABA-GE reached the highest value in July.

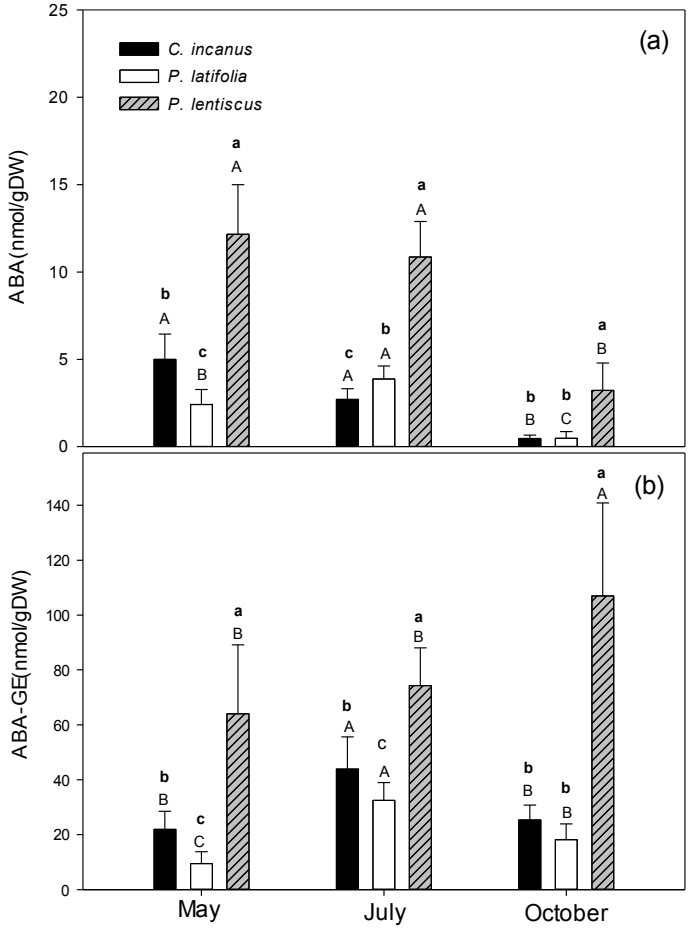


Figure 9. Seasonal variation in ABA (a) and ABA-GE content (b) in *C. incanus, P. latifolia* and *P. lentiscus*. The mean values for each month (±SD) are shown (n=8). Mean values with the same letters are not significantly different (p≥0.05). Lowercase letters indicate the differences among the species for each month, capital letters indicate the intra-specific differences during the study period.

-Daily variations

Considering the daily patterns, in *P. lentiscus* ABA remained almost constant during the daily cycle, whereas in *P. latifolia* and C. *incanus* ABA concentrations increased by 12:00 in both species. However, from 12:00 to 18:00, ABA remained unchanged in *P. latifolia*, whereas in *C. incanus* decreased from 12:00 to 15:00 and, then, from 15:00 to 18:00 to similar morning values (Fig. 10).

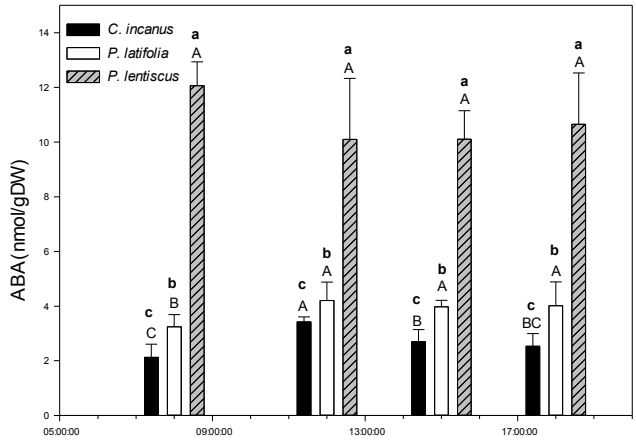


Figure 10. July daily variation in ABA content in *C. incanus*, *P. latifolia* and *P. lentiscus*. The mean values for each hour (±SD) are shown (n=8). Mean values with the same letters are not significantly different (p≥0.05). Lowercase letters indicate the differences among the species for each hour, capital letters indicate the intra-specific differences during the day.

2.4.4 Principal Component Analysis

Individuals of the same species remained very close in all the three monthly PCA (Fig. 11).
In May PCA (Fig. 11a), the first axis, which accounted for 46% of the total variance, was defined by the opposition between *C. incanus* (associated with Car_{Tot}, Ψ_π, NPQ and DES) and *P. latifolia* (associated with P_n, g_s, ϕ_{PSII} and F_v/F_m) on the positive side, and *P. lentiscus* on the negative side (characterised by ABA, Chl*a*/Chl*b*, and PP_{Tot}). The second component accounted for the 29.2 % of the variance and opposed *P. lentiscus* and *C. incanus* on the positive side and *P. latifolia* on the negative side (associated with P_n, g_s, ϕ_{PSII} and F_v/F_m)
In July PCA (Fig. 11b), the first and the second principal components explained the 50.7 and 27.3% of the total variance, respectively. *C. incanus* data constituted the most segregated group, characterised by the highest content in Car_{Tot} and DES value, and the lowest values of osmotic potential (Ψ_π). Axis II divided the evergreens into two groups: *P. lentiscus*, with positive scores, characterised by high values of Ψ_w and ABA and low values of Chl*a*/Chl*b* and *P. latifolia*, with negative scores, with high P_n, g_s, and PP_{Tot} and low F_v/F_m and ϕ_{PSII}.
Finally, In October PCA (Fig. 11c), the first axis (56.1% of the total variance) divided *C. incanus* from the evergreen species, whereas the second axis (26.1% of the total variance) separated *P. latifolia* (positive scores) from *P. lentiscus. C. incanus* was characterized by g_s, P_n, Car_{Tot}, *P. latifolia* by NPQ and PP_{Tot} and P. *lentiscus* by Ψ_w, ABA and DES.

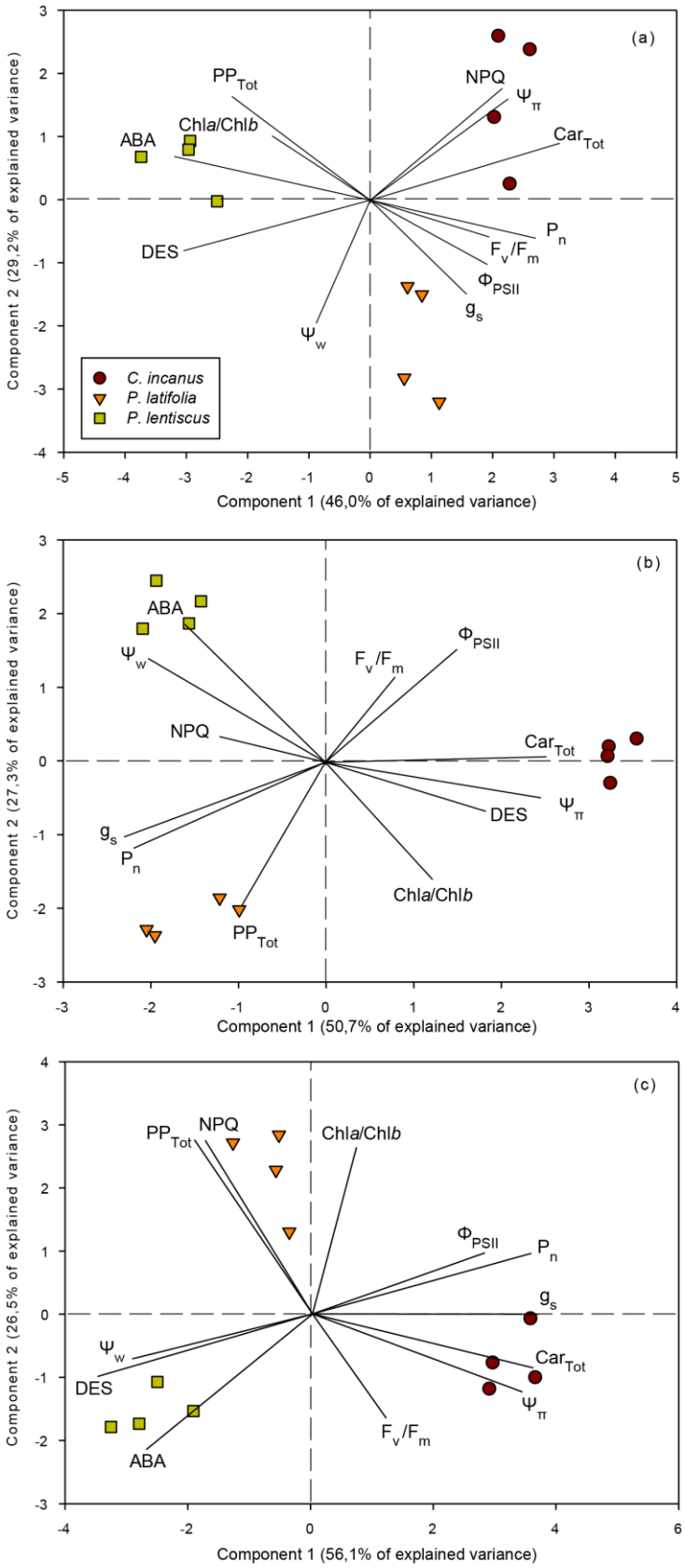


Figure 11. PCA analysis.

2.4.5 Multiple regression analysis

The effects of the climatic factors (temperature, global irradiance and precipitation) on the physiological and biochemical traits of the study species were assessed through multiple regression analyses (MRA) and the data are given in Table 1.

MRA highlighted the main role of temperature (T) in affecting both physiological and biochemical parameters in all three species. Particularly, temperature tended to increase NPQ and DES in all species (r^2≥0.67 for NPQ, and r^2≥0.59 for DES) and decrease Ψ_w in *C. incanus* and *P. latifolia* (r^2=0.70 and r^2= 0.75, respectively).

Furthermore, temperature negatively affected F_v/F_m (r^2=0.65), Chl_{Tot} (r^2=0.86) and Car_{Tot} (r^2=0.50) in *C. incanus* and positively affected PP_{Tot} (r^2=0.68) and ABA (r^2=0.54) in *P. latifolia*. Finally, both P_n (r^2=0.54) and Chl*a*/Chl*b* (r^2=0.51) decreased with increasing T in *P. lentiscus*.

Species	Parameter	F	P-Value	r^2	T(°C)	GI(W/m^2)	P (mm)
C. incanus	P_n	27.74	0.00	0.48	-0.8511	0.0024	0.0308
	g_s	9.57	0.00	0.24	-7.0521	-0.0100	0.3846
	Ψ_w	71.23	0.00	0.70	-0.1943	-0.0006	0.0018
	Ψ_π	62.39	0.00	0.67	-0.1266	-0.0001	0.0021
	F_v/F_m	56.52	0.00	0.65	-0.0024	-0.0001	0.0002
	Φ_{PSII}	13.66	0.00	0.31	-0.0055	-0.0001	-0.0002
	npq	60.89	0.00	0.67	0.0530	0.0011	0.0003
	DES	150.45	0.00	0.83	0.0197	0.0003	-0.0002
	Chl_{Tot}	195.85	0.00	0.86	-0.1618	-0.0018	0.0325
	Chl*a*/Chl*b*	28.70	0.00	0.48	0.0540	-0.0009	-0.0036
	Car_{Tot}	31.27	0.00	0.50	-0.0134	-0.0002	0.0021
	Pol_{Tot}	10.94	0.00	0.26	1.1034	0.0301	-0.0387
	PP_{Tot}	10.20	0.00	0.25	-0.4291	0.0067	-0.0316
	ABA	17.53	0.00	0.36	-0.1357	0.0004	-0.0280
P. latifolia	P_n	14.60	0.00	0.32	-0.4795	0.0031	0.0075
	g_s	0.82	0.48	0.03	-0.0598	0.0146	-0.0792
	Ψ_w	90.25	0.00	0.75	-0.1859	-0.0008	-0.0039
	Ψ_π	18.53	0.00	0.38	-0.0223	-0.0005	-0.0013
	F_v/F_m	17.88	0.00	0.37	-0.0025	-0.0001	0.0001
	Φ_{PSII}	16.17	0.00	0.35	0.0005	-0.0002	-0.0007
	npq	108.88	0.00	0.78	0.1946	0.0014	0.0169
	DES	82.27	0.00	0.73	0.0117	0.0003	-0.0005
	Chl_{Tot}	12.83	0.00	0.30	0.0053	-0.0010	0.0069
	Chl*a*/Chl*b*	3.66	0.02	0.11	0.0486	0.0002	-0.0006
	Car_{Tot}	13.26	0.00	0.30	-0.0147	-0.0005	0.0006
	Pol_{Tot}	66.64	0.00	0.68	3.6434	-0.0050	0.2030
	PP_{Tot}	66.64	0.00	0.68	3.6434	-0.0050	0.2030
	ABA	36.00	0.00	0.54	0.2255	-0.0010	-0.0141
P. lentisco	P_n	36.57	0.00	0.54	-0.4812	0.0034	0.0009
	g_s	9.26	0.00	0.23	-0.8131	0.0140	-0.2490
	Ψ_w	27.45	0.00	0.47	0.0195	-0.0008	-0.0052
	Ψ_π	21.30	0.00	0.41	-0.0182	-0.0003	-0.0036
	F_v/F_m	17.81	0.00	0.37	0.0005	-0.0001	0.0001
	Φ_{PSII}	19.27	0.00	0.39	-0.0010	-0.0001	-0.0005
	npq	115.74	0.00	0.79	0.1202	0.0019	0.0088
	DES	43.40	0.00	0.59	0.0073	0.0004	0.0003
	Chl_{Tot}	33.77	0.00	0.16	-0.0034	-0.0010	0.0074
	Chl*a*/Chl*b*	1.93	0.01	0.51	-0.0395	-0.0003	0.0014
	Car_{Tot}	9.35	0.00	0.23	0.0037	-0.0001	0.0031
	Pol_{Tot}	4.51	0.01	0.13	0.6764	-0.0004	0.0828
	PP_{Tot}	11.55	0.00	0.27	0.4711	-0.0006	0.0476
	ABA	9.27	0.00	0.23	1.0000	0.0004	-0.0705

Table 1. Multiple regression analysis of physiological and biochemical traits in the study species as a simultaneous function of temperature (T), global irradiance (GI) and precipitation (P).

2.5 **Discussion**

2.5.1 Inter-specific differences in physiological traits

Responses of photosynthesis to drought in *P. latifolia* and *P. lentiscus* growing under natural conditions were reported in many studies (e.g., Kyparissis *et al.* 2000, Filella *et al.* 1998, Gratani and Varone 2004, Karavatas and Manetas 1999, Ogaya and Peñuelas 2003, Gratani *et al.* 2013.). The seasonal pattern of P_n and g_s was consistent with data reported in these previous surveys. In particular, *P. latifolia* had a lower decrease in P_n and g_s than *P. lentiscus* during drought periods (July) (Fig. 2a, 2b). These physiological features, combined with the complete recovery of photosynthetic capacity in October, attest a greater adaptation of *P. latifolia* than *P. lentiscus* to drought conditions (Bombelli and Gratani 2003).

The summer decrease in P_n and g_s in the semi-deciduous species were more dramatic as compared with the corresponding values for the evergreen species. However, in October, photosynthesis and stomatal conductance were higher in leaves of *C. incanus* than of *P. latifolia* and *P. lentiscus* (Fig. 2a, 2b). This higher seasonal variability in gas exchanges observed in *C. incanus* could be caused by its leaf longevity of less than one year and its shallow root system, which renders it more sensitive to drought periods, though responding faster after a rainfall event (Galle *et al.* 2011, Gulías *et al.* 2009).

Photosynthesis and stomatal conductance of the three species were in accordance with their different species-specific water-use behaviours. In particular, all three species confirmed their different water-use strategies previously reported by other authors under Mediterranean field conditions. *P. latifolia* followed a typical anisohydric behaviour (or drought tolerant strategy) manifested by a steep decrease of water potential during the hottest hours of the day (Fig. 3c). Furthermore, as previously observed by Borghetti *et al.* 2004, *P. latifolia* was able to adjust its osmotic potential on a daily basis under water stress (Fig. 3d). The capacity of leaves to adjust osmotically is considered an important physiological adaptation of drought tolerant plants, allowing them to maintain leaf turgor and gas exchanges and facilitating the extraction of water from soils in very dry environments (Liu *et al.* 2011). Conversely, the low decrease in Ψ_w of *P. lentiscus*, both on daily and seasonal timescales, could be explained through its capacity of extracting water from soil rapidly enough to compensate water loss by transpiration (Ozturk *et al.* 2010). This hypothesis is supported by previous studies on *P. Lentiscus*, in which isotopic discrimination analysis (using $\delta^{18}O$ and $\delta^{13}C$) confirmed that the deep root system of this species allowed the maintenance of a favourable plant water supply (Ehleriger and Dawson 1992, Valentini *et al.* 1992, Filella and Peñuelas 2003). Furthermore, no osmotic adjustments both on daily and seasonal timescales were observed in this species (Fig. 3d, 3b). Therefore, this plant could be defined as a drought avoider-water spender or as an isohydric species (Ozturk *et al.* 2010). Finally, according to Gratani and Bombelli (2000, 2003) and Sánchez-Blanco *et al.* (2002), C. *incanus* could be considered as a drought avoider – water saver species, trying to conserve water by progressive stomatal closure combined with a daily decrease in Ψ_w and a reduction of leaf evaporative surface through partial shedding of its small summer leaves. Moreover, in this last species, osmotic adjustments were observed at diurnal and seasonal timescales (Fig. 3d, 3b).

The divergent physiological behaviours of the three shrubs are also highlighted by their different susceptibly to photoinhibition. Martínez-Ferri (2000) compared the photoprotective mechanisms of four co-occurring Mediterranean species during summer and suggested that drought-avoiding species are able to avoid photoinhibition maintaining high F_v/F_m, whereas drought-tolerant species tolerate a more pronounced dynamic photoinhibition of PSII by increasing xanthophyll mediated NPQ mechanism. This hypothesis may be confirmed by our results showing photoinhibition-avoidance in the drought avoiding species (*C. incanus* and *P. lentiscus*) and photoinhibition-tolerance in the drought-tolerant species (*P. latifolia*).

Specifically, the drought tolerant *P. latifolia* was apparently the most affected by the severe environmental conditions of the Mediterranean summer. This species exhibited a daily significant reduction in F_v/F_m, concomitantly with a downregulation of ϕ_{PSII} in July (Fig. 5, 4b). Furthermore, among the study species, *P. latifolia* had the highest NPQ values in July. We hypothesize that, in this species, as in *Lavandula stoechas* (Munné-Bosch and Alegre 2000) and in *Rhamnus ludovici-salvatoris* (Gulías *et al.* 2002), NPQ represents a strategy for coping with rapidly induced drought events combined with high solar radiation, typical of the Mediterranean climates also on daily basis. Indeed, NPQ significantly increased in *P. latifolia* leaves throughout the day (data not shown). These data could indicate a process of dynamic photoinhibition in the photosynthetic apparatus of this species, in which the drop in F_v/F_m over the course of the day (Fig. 5) was concomitant with a thermal dissipation activity associated with the xanthophyll cycle rather than the result of photodamage to the leaves (Demmig-Adams and Adams 1992, Kyparissis *et al.* 2000). Indeed, the F_v/F_m ratio rapidly recovered late in the afternoon, indicating that there was not a permanent damage to the reaction centres. Consequently, as already shown for other Mediterranean evergreen sclerophyllous, this species could be classified as a photoinhibition tolerant (Martínez-Ferri *et al.* 2004, Gulías *et al.* 2002, Werner *et al.* 2002, Baquedano and Castillo 2007, Prieto *et al.* 2009).

P. lentiscus and C. incanus seem to be less susceptible to photoinhibition. In fact, although a significant slightly decrease in F_v/F_m were found at daily level (15:00 h) in both species, no statistically significant differences were found at seasonal timescale in this parameter (Fig, 4a, 5). Photoinhibition avoidance behaviour in *Cistus* spp. and P*. lentiscus* have previously been described by other authors (Oliveira and Peñuelas, 2000, Sánchez-Blanco *et al.* 2002, Hernández and Munné-Bosch 2004, Ciccarelli *et al.* 2016, Flexas *et al.* 2001, Gulías *et al.* 2002, Valladares and Sánchez-Gómez 2006, Vasques *et al.* 2016). Different mechanisms may contribute to photoprotection in these two species. In *Cistus*, this strategy can be probably attributable to some morphological (leaf dimorphism, acute leaf angles, leaf pubescence, partial leaf shedding during drought periods) and biochemical (reduction of chlorophyll content) features, that may increase leaf surface reflectance and reduce photon absorbance (Werner *et al.* 2002, Ain-Lhout *et al.* 2004, Zunzunegui *et al.* 2005). Similarly, the photoinhibition avoidance behavior of *P. lentiscus* could be linked to epidermal modifications (epicuticular waxes) and biochemical adjustments, such as the observed decrease in Chl*a*/Chl*b* ratio during summer drought (Fig. 6f). The chlorophyll loss might be one of the major photoprotective mechanism in this species, confirming the recent observation reported by Vasques *et al.* (2016).

2.5.2 Inter-specific difference in biochemical traits

Our analysis of seasonal dynamics in plant photoprotective pigments revealed similarities and differences between the considered species. In general, concentration of total carotenoids (Car_{Tot}) and chlorophylls (Chl_{Tot}) were around two-fold higher in *C. incanus* than in *P. latifolia* and *P. lentiscus* in all seasons (Fig. 6c). However, although the semi-deciduous species showed a higher VAZ pool size, its DES values did not differ from evergreens (Fig. 6b, 6d). Therefore, inter-specific differences in carotenoid concentrations do not inevitably imply a higher activity of the xanthophyll cycle. Furthermore, for all species, we found higher DES in May and July than in October, indicating that thermal dissipation of energy is a general pattern for all the investigated shrubs, as found in others Mediterranean species (Faria *et al.* 1996, 1998, Munné- Bosch and Alegre 2000, Ain-Lhout *et al.* 2004). In general, the flexibility of the conversion state of the xanthophyll cycle is controlled by changes in irradiance and temperature (Kyparissis *et al.* 2000). Our results confirmed a relevant contribution of temperature and irradiance (Table 1) to seasonal variations in the de-epoxidation of xanthophyll cycle pigments. In particular, plants of our study showed a higher DES when exposed to high light (in May) and to a combination of high light and high temperature (in July) (Fig. 6d). Thus, this seasonal variation in the de-epoxidate of xanthophyll cycle pigments could be attributed to seasonal variations in irradiance and temperature.

Differently from DES, modulations of chlorophyll contents are highly species-dependent. According to García-Plazaola *et al.* (2000), the high chlorophyll content of *Cistus* spp allowed to maintain high rates of photosynthesis under well-watered conditions. However, under high irradiance (May) or severe drought (July), chlorophylls decreased in this species (Fig. 6c). As previously observed in *Cistus clusii* and *C. salvifolius* (Hernández *et al.* 2004, Grant *et al.* 2014), this is not necessarily a symptom of unsuccessful adaptation to photoinhibitory and photo-oxidative conditions. The consequences of a chlorophyll loss under photoinhibitory conditions are a reduction of the photons absorbed by leaves and an increased ratio of xanthophyll to chlorophyll (VAZ/Chl_{Tot}), thus enhancing the capacity to dissipate excess excitation energy per amount of light intercepted (Demming -Adamas and Adams 1996, Munnè-Bosh and Alegre 2000, Galmés *et al.* 2007, Baquedano *et al.* 2007, Munné-Bosch *et al.* 2009). Consequently, *C. incanus* did not suffer from photo or oxidative damage as indicated by the absence of any seasonal change in ϕ_{PSII} or difference in the F_v/F_m ratio (Fig. 4a, 4b).

Modulation of Chl*a*/Chl*b* ratio could also be considered an indicator of plant adaptations to high light and high temperatures (Demmig-Adams and Adams 1996, Esteban *et al.* 2014). Consistent with the results of Munné-Bosh and Peñuelas (2003) and Vasques *et al.* (2016), a decrease of Chl*a*/Chl*b* was observed in *P. lentiscus* in summer (Fig. 6f). Furthermore, this ratio was also higher in 2015 than in 2014 (Fig. 7f). Chl*a* is present in the reaction centres and the antennae of both photosystems, whereas the presence of Chl*b* is restricted to light-harvesting systems (Esteban *et al.* 2014). Thus, the reduction in Chl*a* reflects changes in PSII/PSI balance and may indicate a protective mechanism against potentially damaging effects caused by high light and high temperature conditions (Zunzunegui *et al.* 2009).

Inter-specific differences in carotenoid concentrations were also observed at annual timescale.

Summer 2015 was exceptionally hot and dry. Particularly, July 2015 was characterized by exceptionally high temperatures in many parts of Europe, with monthly anomalies up to 4°C in Spain, France and Italy (Ionita *et al.* 2016).

The combination of drought, high temperatures and high light intensity may have a negative effect on *C. incanus* photosynthesis (Gratani and Varone 2004) and may have been one of the reasons for both lower carotenoid and chlorophyll contents observed in *this species* in summer 2015.

In *P. lentiscus*, VAZ pool adjustments also seemed to be important responses to changes in intensity of drought and heat stress. Specifically, the larger quantity of VAZ observed in 2015 than in 2014 in leaves of this species could have conferred greater stability to the membranes, reducing the deleterious effects of high temperatures through a structural photoprotective role (Ninemets *et al.* 2003, Tattini 2015) (Fig. 7b). This hypothesis was supported by VAZ/Chl_{Tot} value, that in summer 2015 was on average 60.7 mmol mol^{-1}(Fig. 6e), indicating the presence of a free VAZ pool in thylakoids (i.e. VAZ unbound to the light-harvesting protein complex, and hence not involved in NPQ) (Havaux *et al.* 2007, Esteban *et al.* 2015, Tattini *et al.* 2015) (Fig. 7e).

Differently from *C. incanus* and *P. lentiscus*, we only found slight differences in total carotenoid concentration and VAZ pool between both years in *P. latifolia* (Fig. 7a, 7b). These data might indicate that this Mediterranean evergreen underwent a fixed seasonal adjustment of carotenoid composition, but there were no further adjustments in response to the higher temperatures of summer 2015.

Mediterranean shrubs of our study may accumulate large amounts of polyphenol compounds, a large group of secondary metabolites which increase drought tolerance (Di Ferdinando *et al.* 2014). In general, polyphenols are well-known as high level antioxidant constituents because of their high ability to scavenge toxic free radicals and reactive oxygen species (Brunetti *et al.* 2013). However, each polyphenol class may serve different functions depending on species (Di Ferdinando *et al.* 2014). For example, in *Cistus* spp. tannins are located in the trichome channels from which they are released to the soil and may potentially control nitrogen- cycling processes, whereas in *P. lentiscus* are distributed through the whole-leaf depth, contributing to the reinforcement of the cell wall increasing sclerophylly (Castells *et al.* 2004, Di Ferdinando *et al.* 2014, Bussotti *et al.* 1998).

Recently, much attention has been given to the possible function of phenylpropanoids as selective filters against UV-B radiation damage. Their optical properties with high absorbance in the UV spectrum, combined with the mainly superficial location on the cuticles, trichomes or epidermis, make them useful in this respect (Agati and Tattini 2010).

In *P. latifolia* and *P. lentiscus*, total phenylpropanoids (PP_{Tot}) increased from May to July and remained high in October, whereas in *C. incanus* the higher PP_{Tot} concentrations were found in May and July than in October (Fig. 8b). Furthermore, leaves of *C. incanus* also showed higher levels of total polyphenols (POL_{Tot}) in July (Fig. 8a). Although direct experimental evidence is lacking, a high production of polyphenols (mainly phenylpropanoids), in summer, when plants are exposed to the concomitant action of different stress events (drought, high temperature and high solar radiation) may reflect an increased requirement for antioxidant function, suggesting

that polyphenols act as free radical scavengers in addition to the well-known as UV-screening function (Agati *and Tattini* 2010, Agati *et al.* 2012).
Furthermore, as previously observed in the Mediterranean evergreen *Arbutus unedo* (Nenadis 2015), the higher amount of phenylpropanoids compounds found in October in *P. lentiscus* and *P. latifolia* could be related to the lower temperature values registered during the night in this month, which may have induced an increase in phenylpropanoids to improve plant tolerance to cold stress. This is supported by data on other species studied at temperatures between 9 and 21 °C, in which low temperatures induced an increase in phenols in the absence of UV radiations (Liu *et al.* 2016). Although our results seem to indicate that concentrations of polyphenols fluctuate according to the seasonality of environmental stresses, we cannot exclude that the simultaneous influence of biotic factors (e.g. pathogens, insects, herbivores) likely contributed to these temporal patterns.
The involvement of ABA in mediating plant responses to drought stress has been extensively investigated. ABA plays a critical role in regulating plant water status through its well-known action on guard cells and by induction of genes that encode enzymes and other proteins involved in cellular dehydration tolerance (Zhu 2002, Zhang *et al.* 2006).
Furthermore, ABA is related to oxidative stress in plant cell (Jiang and Zhang 2002). Recent evidences suggest that ABA-induced H_2O_2 production mediates the generation of NO, an extensive signal molecule capable to up-regulate plant antioxidant defences (Tossi *et al.* 2009).
To our knowledge, little is known on variations of ABA contents in Mediterranean plants exposed to drought or to a combination of stressors in field conditions. In this work, the study species presented significant different levels of free ABA and of glucose-conjugated ABA (ABA-GE) in the leaves at seasonal and daily levels (Fig. 9a, 9b).
In this regard, the higher content of free ABA found in all species in May and July, seems to be related to the magnitude of the stress imposed to the plants in these months. On a diurnal basis, *P. lentiscus* showed no significant changes in free ABA content in July. Conversely, *C. incanus* and *P. latifolia* showed large diurnal fluctuations in free ABA (Fig. 10). In particular, increases in the midday concentrations of ABA were concomitant with the lowest daily value in g_s found in both species, confirming the well-known effect of ABA on stomatal functioning.
Lastly, all three species showed a significant seasonal variation in ABA-GE concentrations. ABA-GE is the preferred transport form of ABA and also a storage form, which can easily be converted to free ABA through β-glucosidase (Lee *et al.* 2006, Xu *et al.* 2012). Although, this pattern was not observed in all species, in *P. latifolia*, ABA-GE levels increased in parallel to ABA levels, leading to hypothesize that, under drought stress, the increase observed in free ABA in this plant could be partially related to its breakdown from the endogenous ABA-GE (López-Carbonell *et al.* 2006).

2.6 **Conclusions**

Principal component analysis highlighted the existence of significant differences between the study species at seasonal timescale, confirming the results of r^2 and providing a concise summary of the results obtained by ANOVAs (Fig. 9). In particular, PCA showed that the seasonality of the Mediterranean climate had an effect on the response in all studied parameters and that each species could be characterised by a different set of both physiological and biochemical traits in each month. This means that there is no single mechanism for acclimation in the Mediterranean field condition, and these mechanisms vary in response to natural seasonal changes in water, light and temperature regime. In this context, as inferred from multiple regression analysis, temperature was the environmental parameter which played a major role in the variation of responses among species (Table 1).

Furthermore, some of the variables directly related to temperature, as the de-epoxidation index, NPQ, chlorophyll content or Chl*a*/Chl*b* ratio, induced a greater interannual variation in the species. Thus, differences in both carotenoid compositions and concentrations found in 2014 and 2015, mostly depended on temperature variations between the two years.

These findings confirm the importance to address research on these metabolites, in order to understand the function that, plant pigments, may play in the acclimation of Mediterranean plants, and the influence of drought and extreme temperatures that prevail during summer seasons in this region.

Furthermore, our analysis of inter-specific responses along different timescales permitted a better understanding of the underlying mechanisms that govern these Mediterranean species in field conditions. Indeed, some species-specific behaviours could be better observed at daily timescale. For example, diurnal patterns of Ψ_w and Ψ_π allowed us to discriminate the different water-use behaviours of the species, while daily variation in F_v/F_m clarified the distinction among photoinhibition-avoider and photoinhibition-tolerant plants.

In conclusion, the approach used in this study, in which we examined important physiological and biochemical traits at different timescales and considering multiple shrub species in a costal dune ecosystem, may represent a first step in understanding Mediterranean plant responses to climate change. If climate warming will cause more frequent and severe drought events, the identification of some key-traits involved in Mediterranean plant responses to these environmental changes, could be important in order to understand which species will be adversely and selectively affected in this vulnerable area.

Table SM1. *Results of RM-ANOVA*

	P_n	g_s	Ψ_w	Ψ_π	F_v/F_m	Φ_{PSII}	NPQ	Car_{Tot}	Chl_{Tot}	VAZ	DES	VAZ/Chl_{Tot}	Chl *a*/Chl *b*	Pol_{Tot}	PP_{Tot}	ABA	ABA-GE
Species																	
C. incanus	7.05	105.91	-2.16	-2.10	0.80	0.16	2.28	2.28	6.49	0.35	0.37	73.35	2.89	60.76	21.51	2.72	30.39
P. latifolia	8.45	107.29	-1.82	-2.96	0.79	0.18	2.68	1.11	2.91	0.19	0.36	66.05	3.12	32.54	32.54	2.26	20.04
P. lentisco	6.15	83.08	-1.09	-2.94	0.81	0.16	2.60	1.15	3.17	0.24	0.40	54.79	2.68	65.87	21.01	8.75	81.74
Month																	
May	7.90	81.83	-1.38	-2.58	0.79	0.15	2.15	1.38	4.01	0.25	0.40	66.40	2.75	45.17	18.28	6.53	31.80
July	3.74	82.92	-2.36	-2.95	0.78	0.17	3.05	1.50	3.69	0.26	0.47	72.06	3.06	64.63	30.81	5.81	50.21
October	10.00	131.53	-1.34	-2.47	0.82	0.18	2.36	1.66	4.87	0.27	0.26	55.73	2.87	49.36	25.96	1.39	50.17
Year																	
2014	6.98	83.95	-1.74	-2.72	0.80	0.15	2.71	1.83	4.92	0.29	0.38	59.38	2.79	56.57	26.59	4.21	45.07
2015	7.45	113.57	-1.64	-2.62	0.79	0.19	2.33	1.20	3.46	0.23	0.37	70.08	3.00	49.54	23.45	4.94	43.05
Significance																	
Species	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Year	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.198	0.000	0.000	0.000	0.000	0.000	0.078
Species x Year	**0.000**	**0.000**	**0.000**	**0.000**	**0.025**	**0.865**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.073**	**0.000**
Month	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Species x Month	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Daily	0.000	0.000	0.000	0.000	0.003	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.253	0.000	0.000	0.000	0.000
Species x Daily	**0.000**	**0.000**	**0.000**	**0.000**	**0.005**	**0.007**	**0.005**	**0.000**	**0.000**	**0.000**	**0.009**	**0.450**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**

Table SM2. *Variance explained (r^2)*

r^2	P_n	g_s	Ψ_w	Ψ_π	F_v/F_m	Φ_{PSII}	NPQ	Car_{Tot}	Chl_{Tot}	VAZ	DES	VAZ/Chl_{Tot}	Chl *a*/Chl *b*	Pol_{Tot}	PP_{Tot}	ABA	ABA-GE
Species	0.065	0.047	0.224	0.455	0.019	0.028	0.039	0.501	0.564	0.278	0.017	0.198	0.121	0.422	0.227	0.354	0.650
Year	0.004	0.084	0.003	0.007	0.008	0.075	0.044	0.171	0.113	0.069	0.000	0.097	0.041	0.024	0.020	0.005	0.001
Species x Year	0.007	0.004	0.029	0.006	0.005	0.000	0.020	**0.091**	**0.024**	**0.469**	**0.011**	**0.035**	**0.099**	0.004	0.006	0.002	0.006
Month	0.488	0.206	0.249	0.121	0.108	0.019	0.188	0.023	0.053	0.002	0.362	0.156	0.062	0.137	0.214	0.210	0.067
Species x Month	**0.150**	**0.385**	**0.141**	**0.174**	**0.017**	**0.078**	**0.084**	**0.060**	**0.093**	**0.027**	**0.033**	**0.115**	**0.065**	**0.165**	**0.431**	**0.073**	**0.087**
Daily	0.166	0.150	0.216	0.129	0.439	0.589	0.397	0.019	0.040	0.013	0.409	0.115	0.004	0.037	0.027	0.037	0.010
Species x Daily	**0.021**	**0.017**	**0.048**	**0.095**	**0.038**	0.014	0.016	0.011	0.011	0.005	0.006	0.002	0.090	0.026	0.021	**0.082**	0.021

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Characterisation and Antioxidant Activity of Crude Extract and Polyphenolic Rich Fractions from *C. incanus* Leaves

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Abstract: *Cistus incanus* (Cistaceae) is a Mediterranean evergreen shrub. *Cistus incanus* herbal teas have been used as a general remedy in traditional medicine since ancient times. Recent studies on the antioxidant properties of its aqueous extracts have indicated polyphenols to be the most active compounds. However, a whole chemical characterisation of polyphenolic compounds in leaves of *Cistus incanus* (*C. incanus*) is still lacking. Moreover, limited data is available on the contribution of different polyphenolic compounds towards the total antioxidant capacity of its extracts. The purpose of this study was to characterise the major polyphenolic compounds present in a crude ethanolic leaf extract (CEE) of *C. incanus* and develop a method for their fractionation. Superoxide anion, hydroxyl and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assays were also performed to evaluate the antioxidant properties of the obtained fractions. Three different polyphenolic enriched extracts, namely EAC (Ethyl Acetate Fraction), AF1 and AF2 (Aqueos Fractions), were obtained from CEE. Our results indicated that the EAC, enriched in flavonols, exhibited a higher antiradical activity compared to the tannin enriched fractions (AF1 and AF2). These findings provide new perspectives for the use of the EAC as a source of antioxidant compounds with potential uses in pharmaceutical preparations.

Keywords: polyphenolic enriched fractions; flavonols; LC–MS/MS (liquid chromatography–tandem Mass Spectrometry); DPPH radical-scavenging activity

1. Introduction

Plants inhabiting Mediterranean-type ecosystems are usually challenged by multiple stressors, particularly during the summer, when water deficiency co-occurs with high solar irradiance and high temperatures. These environmental constraints induce severe photo-oxidative stress in Mediterranean plants [1,2], resulting in the formation of many reactive oxygen species (ROS). Reactive oxygen species include both radicals, such as superoxide anion and hydroxyl radicals, and non-free radicals, such as hydrogen peroxide and singlet oxygen. Within the plant cell, a first line of defense against reactive oxygen species is constituted by antioxidant enzymes [3]. In particular, superoxide dismutase (SOD) detoxifies superoxide anion ($O_2^{\cdot -}$) by converting two $O_2^{\cdot -}$ into H_2O_2 and O_2 [4]. Furthermore, in the presence of $O_2^{\cdot -}$ and transition metal ions, H_2O_2 can generate hydroxyl radical ($^{\bullet}OH$) via the superoxide-driven Fenton reaction [3]. The $^{\bullet}OH$ is highly reactive, causing damage to DNA and lipid peroxidation [5]. Alterations in cellular ROS/REDOX homeostasis induce the activation of additional antioxidant defense systems constituted by secondary metabolites [2]. In particular, polyphenols have been widely reported to protect plants against oxidative stress [6], neutralising ROS, chelating transition metals and reducing lipid peroxidation [7–9].

New evidence suggests that polyphenols also have "indirect" antioxidant effects both in plants and humans [10]. The mechanisms by which polyphenols express these beneficial effects in vivo is not yet clear but it appears to involve their interaction with cellular signaling pathways [11,12]. In particular, polyphenols are thought to have the ability to interact with a wide range of protein kinases that supersede key steps of cell growth and differentiation [13]. Interestingly, the same structural features conferring antioxidant activity to polyphenols are also responsible for their ability to regulate these developmental processes [10]. Though such functions have not been conclusively proven in plant cells, they form the basis of the beneficial effects exerted by polyphenols in a wide range of diseases in animals, including their anti-cancer properties.

Mediterranean shrub species, such as *Cistus incanus*, are naturally rich in polyphenols and might represent a source of bioactive compounds for the development of novel drugs [14]. In traditional medicine, *C. incanus* herbal infusions have been used as anti-inflammatory agents in the treatment of various skin diseases [15,16]. Furthermore, *C. incanus* polyphenolic-rich extracts have been reported to possess antimycotic, antibacterial and antiviral properties [17–21]. Recently, aqueous extracts of the aerial parts of this plant have been demonstrated to exert intense antioxidant capacities that could be attributed to their high polyphenol content [22,23].

To the best of our knowledge, a complete chemical characterisation of the polyphenolic composition of the leaves of *C. incanus* has not yet been reported. Moreover, detailed antioxidant activities of different enriched fractions have not been investigated. Consequently, limited data is available on the contribution of the different polyphenolic compounds to the total free radical scavenging activity of leaves of this species. This study aims to characterise the major polyphenolic compounds contained in a crude ethanolic leaf extract (CEE) of *C. incanus*, and to develop an extraction protocol to obtain tannin and flavonol enriched fractions. Finally, scavenging activity against superoxide anion, hydroxyl and DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals have been used to compare the antioxidative properties of CEE and its derived fractions.

2. Results and Discussion

2.1. Qualitative Characterisation of Phenolic Compounds Present in Crude Extract of Cistus incanus (C. incanus) Leaves

In our study, HPLC–DAD-MS/MS was performed to assess the polyphenolic composition of a crude ethanolic extract (CEE) of *C. incanus* leaves. Individual polyphenols were identified on the basis of their fragmentation patterns as well as by comparison of their retention time and UV–VIS spectra with those of authentic standards.

Our analytical conditions allowed the separation of a large percentage of compounds, as shown in Figure 1.

The MS data obtained by liquid chromatography–tandem mass spectrometry (LC–MS/MS) of the most representative phenolics present in the CEE of *C. incanus* are listed in Table 1, identified with the numbers 1–19 according to their elution order. The compounds identified were classified in to three main classes: gallic acid derivatives (peaks 1, 2), condensed tannins (peaks 3–8), also known as proanthocyanidins, and flavonol glycosides (peaks 9–19). Peak 1 was identified as monogalloyl glucose (m/z at 331), with the main fragments at m/z 169 (gallic acid) and 125 (loss of CO_2 from gallic acid).

Peak 2 was identified as gallic acid (m/z 169) as previously reported by [22,24]. Condensed tannins, both monomeric, dimeric and polymeric proanthocyanidins have been already reported in *C. incanus* extracts [16].

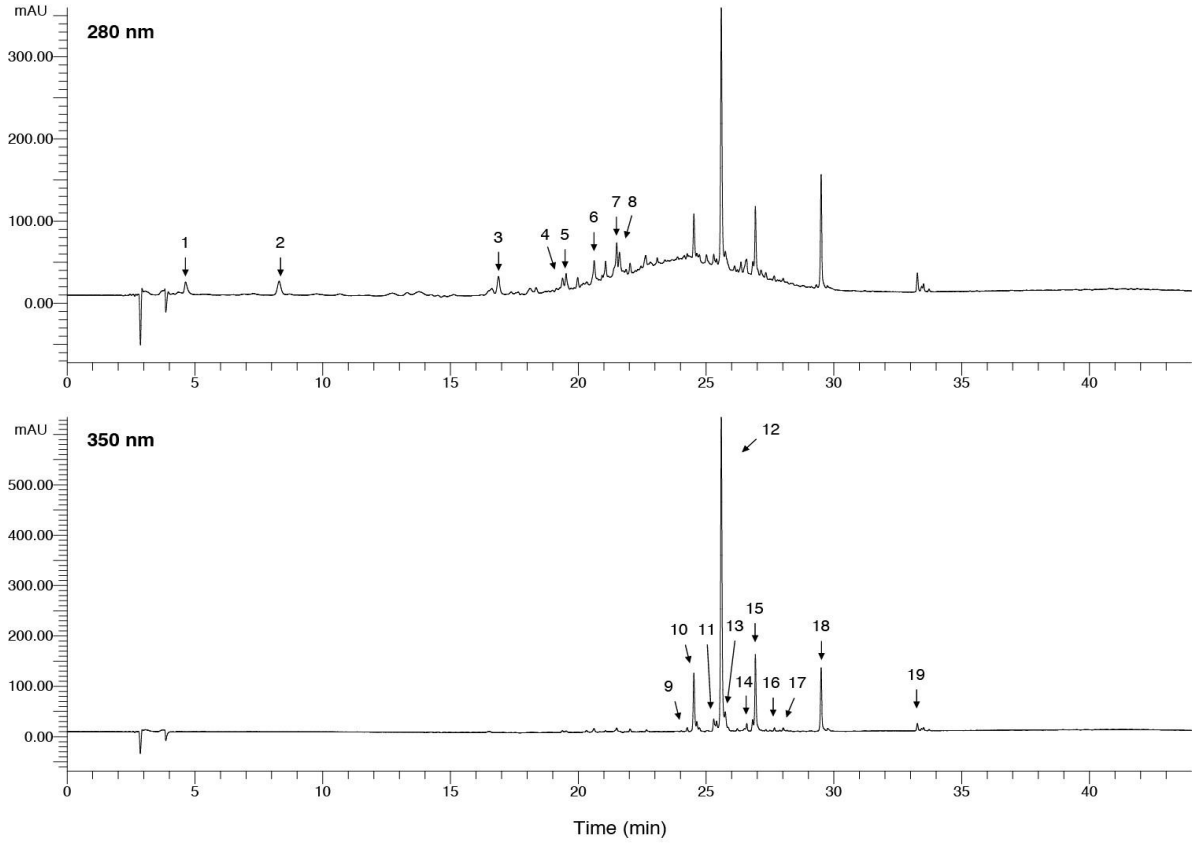

Figure 1. Chromatographic profile of crude ethanolic leaf extract (CEE) of leaves of *Cistus incanus* acquired by HPLC–DAD detected at the relative maxima of absorbance of proanthocyanins (280 nm) and flavonols (350 nm), respectively. Chromatographic conditions are given in the Materials and Methods section. For compound identification see Table 1.

Table 1. HPLC–DAD-MS/MS characterisation of main polyphenols present in crude ethanolic leaf extract (CEE) of *C. incanus*. Compounds numbers correspond to those indicated in Figure 1. (n.d *, not detected; sh, shoulder).

Peak *n*	t_R (min)	Λ (nm)	$[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative Assignement
1	4.6	234,270	331	125, 169	Monogalloyl glucose
2	8.3	234,272	169	125	Gallic acid
3	16.9	236,272	609	441, 423, 483, 305, 303	(Epi)Gallocatechin dimer
4, 5	19.5	234,272	305	611, 125, 137	(−)-Gallocatechin and (−)-epigallocatechin
6	20.6	236,276	593	407, 467, 425, 289, 285	(Epi)gallocatechin-(epi)catechin or (Epi)catechin-(epi)gallocatechin
7, 8	21.5	236,278	289	245, 205	(+) Catechin and (−) Epicatechin
9	24.2	260,360	n.d *	-	Myricetin derivative 1
10	24.5	254,362	479	316, 271	Myricetin-3-*O*-hexoside
11	25.4	260,360	n.d *	-	Myricetin derivative 2
12	25.6	260,358	463	316, 271, 179	Myricitrin
13	25.7	256,356	609	301	Rutin
14	26.6	265,355	433	301, 271	Quercetin-3-*O*-pentoside
15	26.9	256,350	447	301, 179	Quercitrin
16	27.8	264,352	n.d *	-	Quercetin derivative 1
17	28.2	264,352	n.d *	-	Quercetin derivative 2
18	29.5	264,314,346sh	593	285, 145	Kaempferol 3-*O*-rutinoside
19	33.3	268,314,348sh	739	285, 306, 145, 452	Kaempferol-3-(3”,6”-dicoumaroyl)-glucose

Our chromatographic method was suitable for the determination of two dimeric (3, 6) and two monomeric proanthocyanidins (4, 5). In particular, as expected by the general scheme proposed by [25,26], the loss of a phloroglucinol unit ($C_6H_6O_3$), as well as losses due to Retro-Diels-Alder (RDA) fission and interflavanoid cleavage, were the predominant fragmentation pathways of dimeric proanthocyanidins.

On this basis, the fragmentation pattern of the epigallocatechin dimer (peak 3, $[M-H]^-$ at 609) was consistent with an RDA fission of the heterocyclic ring resulting in the fragment ion at m/z 441 [23]. Furthermore, the fragments detected at m/z 303 (methylenic quinone) and m/z 305 (flavan-3-ol monomer) derived from an inter-flavanic bond cleavage, through the quinine methane (QM) cleavage mechanism, whereas the fragment ion at m/z 483 resulted from the loss of a phloroglucinol unit (Figure 2), [27]. According to [15], gallocatechin-(4α-8)-gallocatechin or the regio-isomer gallocatechin-(4α-6)-gallocatechin were strongly suggested as molecular structure for this dimeric proanthocyanidin.

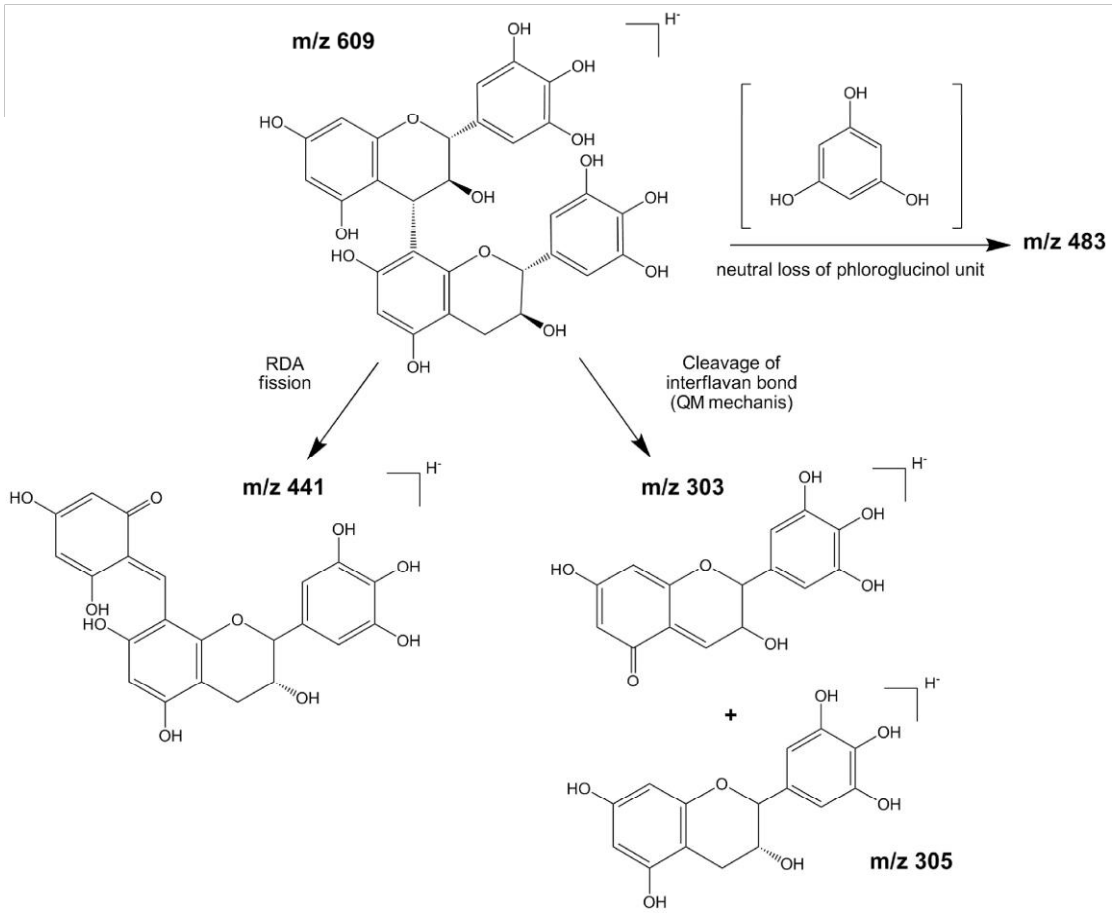

Figure 2. Hypothetical ESI(−)-MS/MS fragmentation pattern for Epigallocatechin dimer (peak 3, $[M-H]^-$ at m/z 609). RDA = Retro-Diels Alder fission, QM = Quinone Methide cleavage mechanism.

At 20.6 min (peak 6) another dimeric proanthocyanidin was recorded. Its pseudomolecular ion peak [M-H]$^-$ at m/z 593 suggested that this compound consisted of one (epi)gallocatechin and one (epi)catechin subunit [25]. MS/MS fragmentation of m/z 593 gave a fragment ion at m/z 425 from RDA rearrangement [28]. The sequential water elimination produced the ion at m/z 407 and the QM cleavage of the interflavonol bond produced a fragment ion at m/z 289. Finally, the ion at m/z 467 resulted from the loss of a $C_6H_6O_3^-$ fragment from the pseudomolecular ion. For this dimeric structure gallocatechin-(4α-8)-catechin or catechin-(4α-8)-gallocatechin is suggested according to [15].

Two monomeric gallocatechins were identified at 19.5 and 19.6 min (peaks 4 and 5). In particular, (−)-gallocatechin and its isomer (−)-epigallocatechin with [M-H]$^-$ at 305 m/z were detected. Their molecular weight was confirmed by the presence of the ion at m/z 611 corresponding to [M + M-H]$^-$. Further ions were detected at m/z 137, ([M-H-$C_8H_8O_4$]$^-$) resulting from retro RDA fission, and at m/z 125, corresponding to the loss of CO_2 from gallic acid. In addition, both (+)catechin and (−)epicatechin (289 m/z) were found in the CEE (peaks 7 and 8) by comparison with fragmentation patterns of commercial standards.

Polymeric proanthocyanidins could not be resolved by reversed-phase HPLC as revealed by the unresolved hump between 22 and 28 m (Figure 1), as also previously reported by other authors [27,29].

Ten compounds were identified as flavonols. As occurred in other members of *Cistus* subgenus [28], myricetin-3-*O*-hexoside (10) and myricitrin (12) were present in the CEE. Fragmentations of the precursor ions at m/z 479 (10) and at m/z 463 (12, Figure 3) had a common fragment at m/z 316 [M-H_2O-Hexose-2H]$^-$, which could be attributed to myricetin [30–33]. The neutral loss of sugar units (losses of 162 for the hexose and 146 for the rhamnose moieties from compounds 10 and 12, respectively) and the product ion at m/z 271, typical of 3-*O*-monoglycosides [34], confirmed the presence of these compounds.

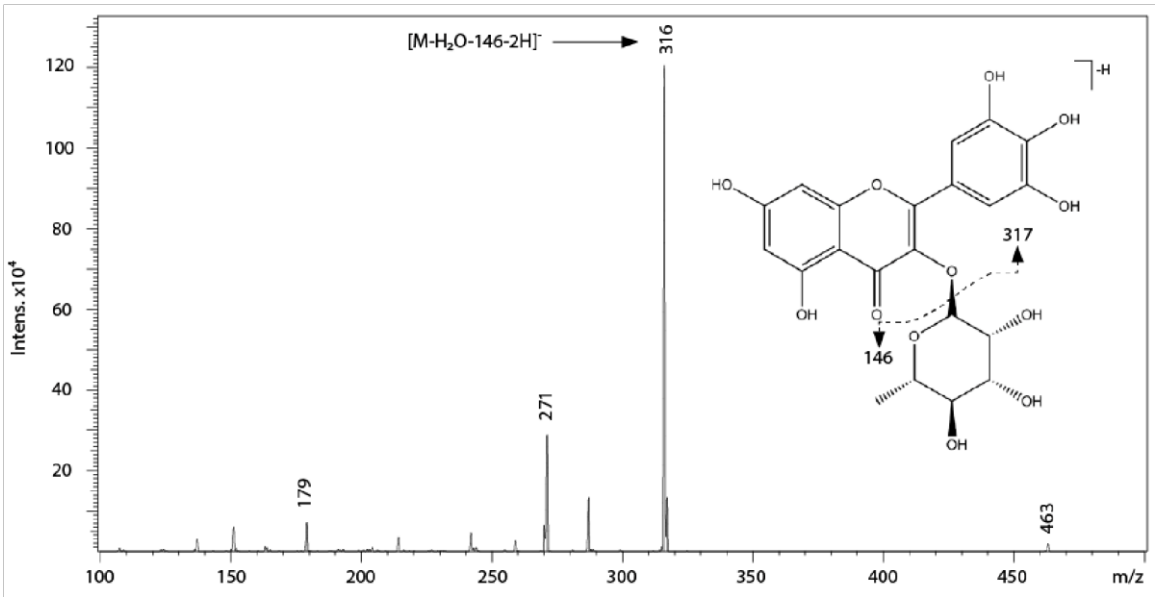

Figure 3. Structure, fragmentation and MS/MS spectrum of peak 12 (myricitrin). Solid arrow indicates the most abundant ion in myricitrin fragmentation; dashed arrow indicates the loss of rhamnose moiety.

Peaks 13, 14 and 15 with precursor ions at m/z 609, 433 and 447 respectively, were identified as quercetin derivatives on the basis of the presence of their aglycone fragment (m/z 301). Particularly, peak 13 was positively identified as rutin, peak 14 as quercetin-3-*O*-pentoside, and peak 15 as quercitrin [22,24]. Peak 18 was identified as a kaempferol 3-*O*-rutinoside, on the basis of the pseudomolecular ion $[M-H]^-$ at m/z 593 and the fragment at m/z 285 ($[M-146-162-H]^-$), due to the loss of a glucosyl and a rhamnosyl moiety in an unique fragment (Figure 4). This fragmentation pattern is characteristic of flavonol rutinosides, in which the linkage 1–6 between rhamnose and glucose, that forms rutinose, allows for free rotation and a more accessible fragmentation than other disaccharides [35,36]. In accordance with [19], peak 19 was assigned as kaempferol-3-(3″,6″-dicoumaroyl)-glucose with a molecular ion at m/z 739 and a fragment at m/z 285.

Other flavonols have been tentatively identified as myricetin derivatives (peaks 9 and 11) and as quercetin derivatives (peaks 16 and 17) based on their retention times and their UV–VIS spectra, in the absence of conclusive mass-spectrometric data.

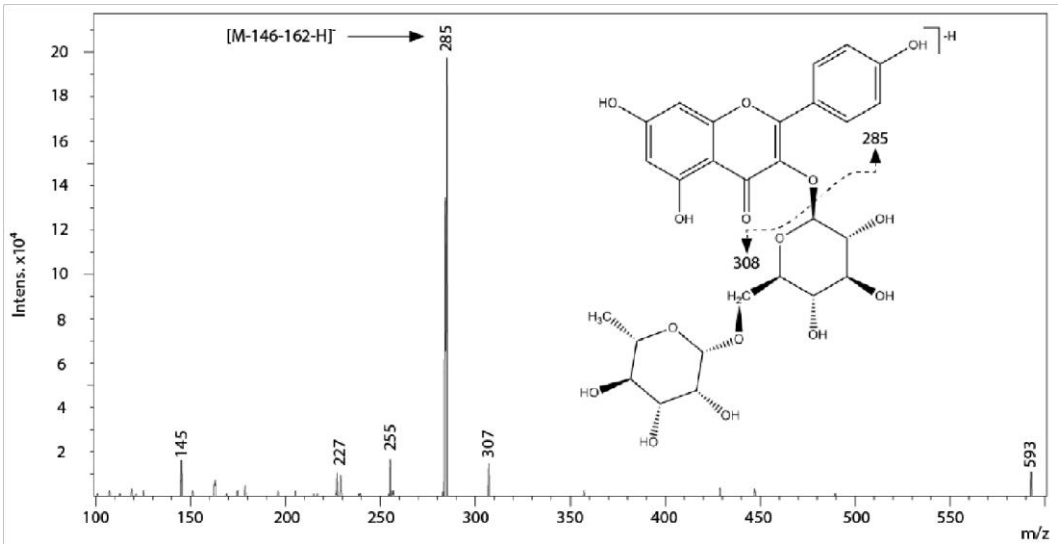

Figure 4. Structure, fragmentation and MS/MS spectrum of peak 18.

2.2. Antiradical Activity Evaluation of Different Extracts of Cistus incanus (C. incanus) Leaves

The CEE was partitioned following the protocol in Figure 5. The application of our partitioning process resulted in three different fractions enriched in distinct classes of polyphenols, one ethyl acetate flavonol enriched fraction (EAF) and two aqueous tannin enriched fractions (AF1 and AF2).

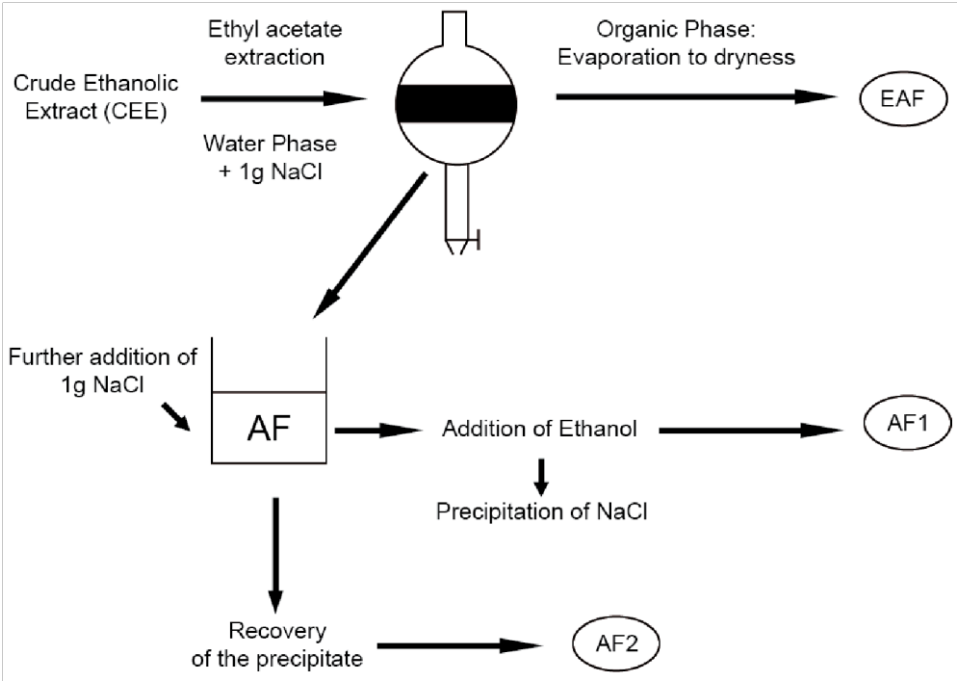

Figure 5. Scheme for fractionation of the CEE. EAF = Ethyl acetate Fraction, AF = Aqueous Fraction, AF1 = Aqueous Fraction 1, AF2 = Aqueous Fraction 2.

Compounds contained in the different extracts were identified and quantified by HPLC–DAD. The EAF was mainly composed of flavonol glycosides and oligomeric proanthocyanidins (monomers and dimers) whereas the two aqueous fractions contained only low and high polymeric proanthocyanidins (AF1 and AF2, respectively). These results are shown in Table 2. The potential antioxidant activities of the different fractions were compared using three in vitro assays based on the scavenging of reactive oxygen species or stable free radicals: superoxide anion radical-scavenging, hydroxyl radical-scavenging and DPPH-scavenging assay (Figure S1 in supplementary material). Table 3 illustrates the IC_{50} values. IC_{50} denotes the concentration of the sample required to scavenge 50% of free radicals. These values were obtained from the regression equations, plotting extract concentrations against inhibition percentages of free radical formation in the different assays.

Table 2. Mean concentration of phenylpropanoids (μmol/mL) in CEE and enriched fractions of *Cistus incanus* leaves (n = 3).

Sample	Monogalloyl Glucose and Gallic Acid	Catechins Derivatives [a]	Myricetin Derivatives [b]	Quercetin Derivatives [c]	Kaempferol Derivatives [d]	Proanthocyanidin Polymers
CEE	0.315 ± 0.024	2.256 ± 0.076	2.719 ± 0.148	3.578 ± 0.217	0.055 ± 0.009	55.376 ± 3.067
EAF	0.236 ± 0.019	1.647 ± 0.069	2.202 ± 0.127	3.140 ± 0.162	0.036 ± 0.004	nd
AF1	nd	nd	nd	nd	nd	25.193 ± 0.597
AF2	nd	nd	nd	nd	nd	31.037 ± 0.901

nd=notdetectable. [a](Epi)gallocatechin dimer, (−)-Gallocatechin,(−)-Epigallocatechin, (Epi)gallocatechin-(epi)catechin, (+)-Catechin, (−)-Epicatechin; [b] Myricetin derivative 1, Myricetin-3-*O*-hexoside, Myricetin derivative 2, Myricitrin; [c] Quercetin-3-*O*-pentoside, Quercitrin, Quercetin derivative 1, Quercetin derivative 2; [d] Kaempefol-3-*O*-rutinoside, Kaempferol-3-(3″,6″-dicoumaroyl)-glucose. EAF = Ethyl acetate Fraction, AF1 = Aqueous Fraction 1, AF2 = Aqueous Fraction 2.

Table 3. IC_{50} (half maximal inhibitory concentration, μM) of different extracts and standards in superoxide anion, hydroxyl and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assays. Each value in the table is represented as Mean ± SD (n = 3). Means not sharing the same letter are significantly different at $p < 0.05$ probability level in each column. CEE: Crude Ethanolic Extract; EAF = Ethyl acetate Fraction, AF1 = Aqueous Fraction 1, AF2 = Aqueous Fraction 2, MYR = Myricitrin Standard, EPI = Epicatechin Standard.

Sample	IC_{50} (μM)		
	Superoxide Anion Radical	Hydroxyl Radical	DPPH Radical
CEE	20.47 ± 1.05 [b]	0.68 ± 0.05 [c]	2.99 ± 1.18 [b]
EAF	5.47 ± 0.98 [d]	0.52 ± 0.05 [d]	0.92 ± 0.10 [c]
AF1	24.99 ± 2.10 [a]	0.99 ± 0.08 [a]	11.78 ± 0.85 [a]
AF2	22.80 ± 1.19 [a]	1.09 ± 0.12 [a]	10.92 ± 0.38 [a]
MYR	4.86 ± 0.86 [d]	0.44 ± 0.03 [d]	0.68 ± 0.07 [c]
EPI	12.20 ± 1.65 [c]	0.83 ± 0.07 [b]	1.49 ± 0.27 [b,c]

2.2.1. Superoxide Anion Radical ($O_2^{\cdot -}$) and Hydroxyl Radical Scavenging Activities

As shown in Table 3, the superoxide scavenging activity of different extracts of *C. incanus* leaves was found to occur in the following order: EAF >> CEE > AF1 and AF2. Our results indicate that lowest IC_{50} value is related to the highest concentration of flavonol compounds, as confirmed by the IC_{50} value of myricitrin standard. As already reported by Salaris *et al.* [37] polyphenols may act in two ways, by the direct scavenging of $O_2^{\cdot -}$ and by the inhibition of xanthine oxidase enzyme, thus preventing the generation of this radical. In particular, Cos *et al.* [38] showed that catechin derivatives are superoxide scavengers without inhibitory activity on xanthine oxidase, whereas myricitin and quercetin derivatives display both activities. Furthermore, these flavonols have lower IC_{50} values for the reduction of superoxide level than for the inhibition of xanthine oxidase [39].

The highest antiradical scavenging activity of EAF was confirmed also by the hydroxyl radical scavenging assay (Table 3). Among the various extracts tested, this fraction displayed the lowest IC_{50}, which is around half the values of the aqueous fractions (AF1 and AF2).

The ability of the EAF to quench hydroxyl radicals could be related to the capacity of some flavonols to form stable radicals. This mechanism has not been completely clarified; however, they could act as hydrogen donors, breaking radical chains through the formation of aroxyl radicals. The final products of these reactions are stable quinonic structures [40].

2.2.2. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

Results show that the highest DPPH radical scavenging activity was performed by EAF (IC_{50} = 0.92 ± 0.097), whereas the aqueous fractions had the highest IC_{50} values (11.78 ± 0.85 for AF1 and 10.92 ± 0.38 for AF2, respectively). The crude ethanolic extract exhibited an IC_{50} value of 2.99 ± 1.18, closer to EAF than to AFs (AF1 and AF2). Our results clearly indicate that the DPPH radical-scavenging activity was greatly influenced by the phenolic composition of the samples. In particular, flavonols (myricetin and quercetin derivatives) were dominant contributors to the DPPH radical scavenging activity of *C. incanus* extracts. Nevertheless, although high levels of proanthocyanidins were found in the aqueous extracts, these compounds did not seem to contribute significantly to the antiradical activity of the CEE measured by the DPPH method. Furthermore, no statistical difference was found between AF1 and AF2, suggesting that differences in the degree of polymerization of proanthocyanidins had relatively little effect on their overall quenching capacity.

2.2.3. Structural Aspects of in Vitro Antiradical Activity of *C. incanus* Leaf Extracts

Our data shows a stronger antiradical capacity of EAF than AFs in all the tested assays. Furthermore, the antiradical capacity of *C. incanus* extracts is largely influenced by their polyphenolic composition. These results are in agreement with previous studies on other members of *Cistus* subgenus. For example, n-butanolic and ethyl acetate fractions of *C. laurifolius* displayed the highest flavonol content and also exerted the highest antioxidant activity in DPPH and FRAP (Ferric Reducing Antioxidant Potential) assays [41]. Tomas *et al.* [42] observed that the antioxidant capacities of *C. salvifolius* extracts in FRAP and TBARS (Thiobarbituric Acid Reactive Substances) assays increased considerably when these were concentrated in some specific flavonols. Numerous authors have investigated the antioxidant activity of polyphenols and several studies have been undertaken to establish the relationship between their structure and their radical-scavenging activity. The radical-scavenging activity of polyhenols depends upon the substitution pattern of their hydroxyl groups, i.e., on the availability of phenolic hydrogens and on the possibility of stabilization of the resulting phenoxyl radicals via hydrogen bonding or by electron delocalization [43]. In particular, the structural requirements considered to be essential for effective radical scavenging are: (i) the presence of a ortho-OH structure (catechol group in the B ring); (ii) a 2,3- double bond conjugated with the 4-oxo group. Moreover, compounds that contain multiple hydroxyl substitutions showed stronger antiperoxyl radical activities [44–46]. Among the compounds identified in *C. incanus* leaf extracts, myricitrin satisfies all of these criteria. In contrast, a flavan-3-ol such as catechin, which lacks of the 2,3- double bond and the 4-oxo function, is unable to support electron delocalization between the A- and the B-rings limiting its radical scavenging potential. This is supported by the comparison of IC_{50} values

of myricitrin and epicatechin standards, since IC_{50} of myricitrin was approximately half the value of epicatechin in all the three assays (Table 3). Conversely, some galloylated catechins benefit from the contribution of esterification with gallic acid (3,4,5-trihydroxybenzoic acid), which compensates for the lack of electron delocalization with major electrondonating properties. This is the case of the (epi)gallocatechin dimer present in the EAF that could participate in the enhancement of its antioxidant activity. However, the presence of many hydroxyl groups in polymeric proanthocyanidins did not increase their scavenging capacity. As previously described by other authors [47–49], the chemical structure of polymeric proanthocyanidins may cause stereochemical hindrances, resulting in relatively high IC_{50} values of AF1 and AF2.

3. Materials and Methods

3.1. Plant Material and Extraction Procedure

Fully-expanded leaves from adult plants of *Cistus incanus* growing on seashore dunes in Southern Tuscany (42°46⁰ N, 10°53⁰ E) were harvested in July 2015. Plant material was rapidly frozen in liquid nitrogen and stored at −80 °C before proceeding with the analysis. 5 g of fresh plant tissue was ground in a mortar with liquid nitrogen. The obtained powder was extracted with 70% of aqueous ethanol acidified to pH 2.5 by HCOOH (50 mL × 5) and sonicated for 30 m. The solution was then partitioned with n-hexane (50 mL × 5) to completely remove lipophilic compounds, following the protocol previously reported by Romani *et al.* [50]. The ethanolic phase constituted the crude ethanolic extract (CEE). 125 mL of the CEE were then evaporated under vacuum (Rotavapor 144R, Buchi, Switzerland), re-dissolved in 250 mL of water and extracted five times with 50 mL ethyl acetate (*v*/*v*) (Figure 5). 1 g of NaCl was added to break down the emulsion and to accelerate the phase-separation process ("salting out" effect). The organic phase (ethyl acetate fraction, EAF) consisted mostly of flavonols, while the aqueous fraction (AF) contained tannins. Two more distinctive fractions (AF1 and AF2) were obtained by a successive precipitation through the addition of NaCl (1 g) to AF. This process was carried out to obtain the separation between low and high molecular weight polymeric proanthocyanidins following a modified protocol from Saucier *et al.* [51]. The precipitate formed was collected by filtration on glass filters (AF2), while the filtrate was added with ethanol to precipitate the salt and recover AF1. Finally, the CEE and AF1 were totally evaporated. All fractions were re-dissolved in 2.5 mL of water: ethanol, 80:20. An aliquot of each extract (300 μL) was diluted in 1.20 mL of methanol and acid water (pH 2 by HCOOH) 80:20 (*v*/*v*) and used for polyphenol analysis by HPLC–DAD and HPLC–MS.

3.2. Chemicals and Reagents

The phenolic standards gallic acid, epicatechin, myricetin 3-*O*-rhamnoside, quercetin 3-*O*-rhamnoside, rutine and kaempferol 7-*O*-glucoside were obtained from Extrasynthese (Genay Cedex, France). $FeSO_4$, hydrogen peroxide, sodium salicylate, xanthine, xanthine oxidase, nitro blue tetrazolium (NBT), ethylenediaminetetraacetic acid (EDTA), formic acid, ethanol, n-hexane, methanol and acetonitrile of HPLC purity were purchased from Sigma Aldrich (Milan, Italy). DPPH (2,2-diphenyl-1-picrylhydrazyl) was obtained from Merck (Darmstadt, Germany). Distilled water was purified in a milli-Q water purification system (Millipore Corporation, Bedford, MA, USA).

3.3. HPLC–DAD and LC–ESI-MS/MS Anlaysis of Phenolic Compounds

Identification of individual phenolics was carried out using their retention times and both UV–VIS, MS and MS/MS spectra. The LC–DAD-MS/MS system consisted of a Shimadzu LCMS-8030 quadrupole mass spectrometer (Kyoto, Japan) operated in the electrospray ionization (ESI) mode and a Shimadzu Nexera HPLC system (Kyoto, Japan) equipped with a diode array detector (DAD), a degasser, two eluent pumps, a column oven and an autosampler. The separation was performed on a reversed-phase Waters Nova-Pak C18 column (4.9 × 250 mm, 4 μm), (Water Milford, MA, USA). The mobile phase consisted of 1% aqueous formic acid (solvent A) and 1% formic acid in acetonitrile/methanol (25/75) (solvent B). Separation was obtained using the

following elution gradient: 2% B isocratic for 10 min, from 2% to 98% B linear for 30 min, 98% B isocratic for 7 min. The flow rate was 0.6 mL/min, and the injection volume was 10 μL. The column oven was set at 30 °C. The mass spectral data were acquired with the following ESI inlet conditions: nebulising gas and drying gas were nitrogen at a flow rate of 3.0 and 15.0 L/min, respectively; the interface voltage was set to −3.5 kV; desolvation line (DL) temperature was 250 °C and the heat block temperature was 400 °C. The mass spectrometer operated in Negative Ion Scan and in Product Ion Scan mode using analyte-specific precursor ions, with Argon as CID (Collision Induced Dissociation) gas at a pressure of 230 kPa. Quantification of the single phenolic compounds was directly performed by HPLC–DAD in triplicates. In particular, six individual compounds, i.e., gallic acid, epicatechin, myricetin 3-*O*-rhamnoside, quercetin 3-*O*-rhamnoside, rutine, were quantified with their own standard curves. Calibration of epicatechin, myricetin and kaempferol derivatives was performed at 280 and 350 nm using epicatechin, myricetin 3-*O*-rhamnoside and kaempferol 7-*O*-glucoside as reference compounds, respectively.

3.4. Superoxide Scavenging Activity

The scavenging activity of sample extracts on superoxide was measured according to a modified version of the method reported by Nishikimi, Rao and Yagi [52]. Superoxide anion was generated enzymatically by xanthine/xanthine oxidase system. Sample extracts were added in the concentration range of 1.95–40 μM to the reaction mixture consisting of xanthine 0.3 mM and 0.3 mM NBT dissolved in potassium phosphate buffer (pH 7.4) with 0.05 mM EDTA (PBE). Finally, 1 mL of xanthine oxidase (0.09 units/mL PBE) was added to the mixture and incubated at 37 °C for 20 min. The absorbance of NBT was measured at 560 nm. The superoxide scavenging activity was expressed as percent (%) superoxide quenching, which was calculated as (1 − B/A) × 100, where B and A are the activities of xanthine oxidase with and without the addition of sample extracts, respectively.

3.5. Hydroxyl Radical-Scavenging Activity

The scavenging activity of sample extracts on hydroxyl radicals was measured according to the method of Smirnoff and Cumbes [53]. The reaction mixture consisted of $FeSO_4$ (1.5 mM), hydrogen peroxide (6 mM), sodium salicylate (20 mM) and various concentrations of extracts (0.065–13 μM). The reaction mixture was incubated at 37 °C for 1 h in a water bath. After incubation the absence of the hydroxylated salicylate complex was measured spectrophotometrically at 562 nm. The percentage of hydroxyl radical scavenging activity was calculated by the following formula: % scavenging activity = $[1 - (A_1 - A_2)/A_0] \times 100$, where A_0 was absorbance of the control without extracts, A_1 was the absorbance in the presence of the extract, and A_2 was the absorbance without sodium salicylate.

3.6. DPPH Radical-Scavenging Activity

The extracts were tested for in vitro DPPH Radical-Scavenging activity following the protocol described by Baratto *et al.* [54] with some modifications. The EPR (Electron Paramagnetic Resonance) signal of the DPPH radical was monitored before and after the addition of extracts and standards. Measurements were performed on a X-band (ν = 9 GHz) Bruker Elexsys E500 Series spectrometer (BRUKER DALTONIK GmbH, Germany) with an ER4122SHQE cavity. Spectra were recorded using the following experimental conditions:

temperature 298 K, microwave frequency 9.865 GHz, central field 351.7 mT, scan width 10 mT, microwave power 4 mW, modulation frequency 100 kHz, modulation amplitude 0.1 mT. 0.1 mL of 0.2 mM ethanol solution of DPPH were mixed with 0.1 mL of ethanol (blank) or with an equal volume of each extract, in the concentration range of 0.065–13 μM. The obtained mixture was shaken and left at room temperature for 20 min. To determine the scavenging capacity, the area of the EPR radical signal was calculated through a double integral of the experimental spectrum. DPPH scavenging capacity was obtained by the following equation: % scavenger = (1 − A/A0) × 100 where A is the area of the DPPH signal in the presence of extract or standard and A0 is the area of the DPPH signal alone. IC_{50} values were calculated and compared with standards of myricitrin and epicatechin.

3.7. Statistical Analysis

All the experiments were conducted in triplicates, and the data were presented as mean ± SD (standard deviation). SPSS (version 23; SPSS Inc., Chicago, IL, USA) was used to process the results. For the DPPH assay a one-way ANOVA test followed by Tukey's test ($p < 0.05$) was used to analyze the differences among IC_{50} of the CEE and its various fractions.

4. Conclusions

The purpose of this study was to investigate the polyphenolic composition of a crude ethanolic leaf extract of *C. incanus*. We focused on obtaining three different polyphenolic enriched fractions in an attempt to make systematic comparisons among their antioxidant activities and to identify the major antioxidative components of *C. incanus* leaves. Among all the fractions analysed, the ethyl acetate fraction was found to be the most effective in terms of radical scavenging activity. These results offer clear evidence that the flavonol enriched fraction obtained from *C. incanus* leaves could be a suitable target for further in vivo antioxidant studies.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/1422-0067/17/8/1344/s1.

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4. Other data: Preliminary assessment of antioxidant activity and cytotoxicity of *P. lentiscus* and *C. incanus* polyphenolic-rich extracts: which species is the best biofactory?

4.1 Characterisation and antioxidant activity of polyphenolic extracts from *P. lentiscus* leaves

4.1.1 Qualitative characterisation of polyphenolic compounds present in crude extract of *P. lentiscus* leaves

Fully-expanded leaves from adult plants of *Pistacia lentiscus* L. were collected on seashore dunes in southern Tuscany in July 2015. Plant material was frozen in liquid nitrogen and stored at -80°C before proceeding with the analyses. A crude ethanolic leaf extract (CEE) was obtained following the same extraction procedure used for *C. incanus* leaves (Gori *et al.* 2016, chapter 3 of the present thesis). The CEE was then analysed by HPLC–DAD–MS/MS (Liquid Chromatography-tandem Mass Spectrometry). Chromatographic and mass spectrometry analyses were carried out as described by Gori *et al.* (2016).

The HPLC-DAD chromatogram of the crude ethanolic leaf extract of *P. lentiscus* is shown in Figure 1 (peaks cited correspond to those in Table 1). Polyphenolic compounds were identified by peak retention time, UV-VIS spectra, MS/MS fragmentation patterns and also taking into account the data from the literature (Table 1).

The compounds identified were classified into three main classes: 1) gallic acid derivatives (peaks 1 and 2), 2) gallotannins (galloyl derivatives of quinic acid) (peaks 3-11) and 3) flavonoids (peaks 12-24).

The presence of gallotannins (gallate esters of quinic acid) has been previously reported in *P. lentiscus* and in other plants of the Anacardiaceae family (Hou *et al.* 2000, Romani *et al.* 2002, Baratto *et al.* 2003, Rodríguez-Pérez *et al.* 2013, Erşan *et al.* 2016). Our chromatographic method allowed the identification of seven gallotannins, including two new compounds named tetragalloyl quinic acid (isomer 1) and tetragalloyl quinic acid (isomer 2) (peaks 10 and 11, respectively).

Among flavonoids, four peaks were identified as myricetin derivatives (peaks 13, 14, 16, 19), five as quercetin derivatives (12, 15, 17, 18, 20) and three as kaempferol derivatives (21, 22, 23) most of them previously reported in *P. lentiscus* leaves (Kawashty *et al.* 2000, Romani *et al.* 2002, Rodrìguez-Pérez *et al.* 2012).

Peaks 6 and 24 were identified as (+)-catechin and luteolin respectively, by comparison with authentic standards.

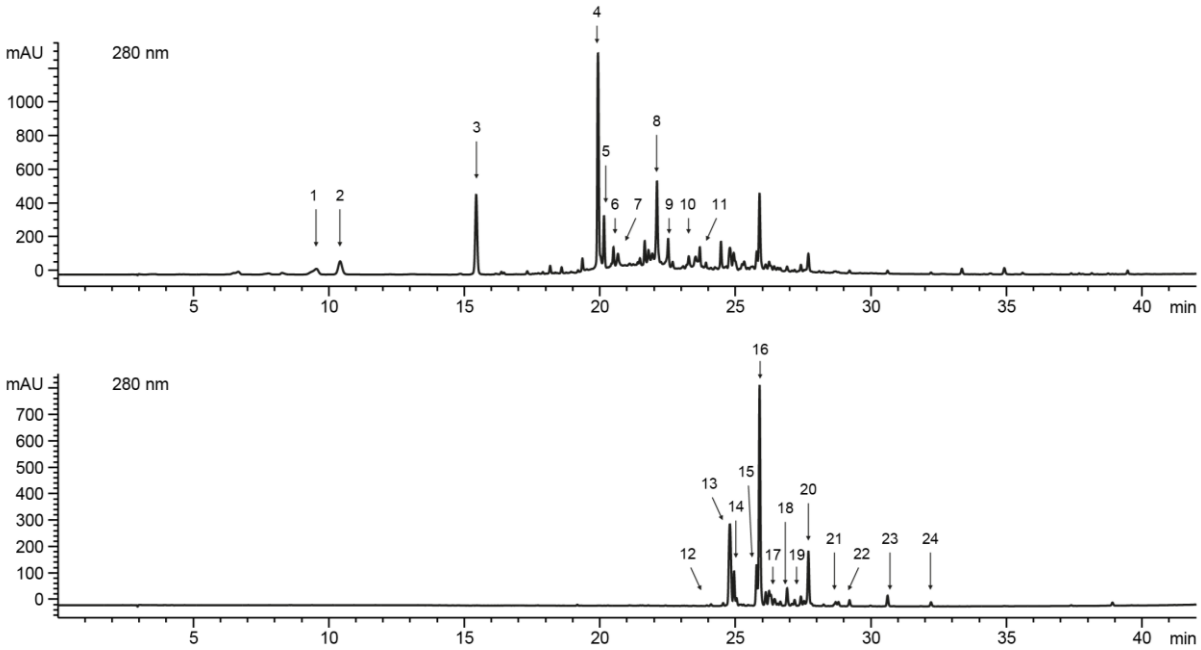

Fig. 1. Chromatographic profile of CEE of leaves of P. lentiscus acquired by HPLC-DAD detected at the relative maxima of absorbance of tannins (280 nm) and flavonoids (350 nm), respectively. Chromatographic conditions were given in the Experimental section. For compound identification see Table 1

Table 1. HPLC-DAD-MS/MS characterisation of main polyphenols present in a crude ethanolic leaf extract of *P. lentiscus* leaves. Compounds numbers correspond to those indicated in Fig. 1.

Peak n°	t_R (min)	λ(nm)	$[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative assignement
1	9.6	234,270	331	169,151,125	Monogalloyl glucose
2	10.4	234,272	169	125	Gallic acid
3	15.5	236,272	343	191	Monogalloyl quinic acid
4	20.0	236,276	495	169,191,125,343	Digalloyl quinic acid (isomer 1)
5	20.1	236,276	495	169,191	Digalloyl quinic acid (isomer 2)
6	20.6	236,278	289	245,203,109,125	(+) Catechin
7	20.7	254,362	367	183,124	Diquinic acid
8	22.2	256,356	647	343,191,169,495	Trigalloyl quinic acid (isomer 1)
9	22.5	256,356	647	343,169,191	Trigalloyl quinic acid (isomer 2)
10	23.4	265,355	799	495,343,191,169	Tetragalloyl quinic acid (isomer 1)
11	23.5	265,355	799	495,191,169	Tetragalloyl quinic acid (isomer 2)
12	24.7	256,350	493	301	Quercetin derivative 1
13	24.9	264,314,346sh	479	317,316	Myricetin-3-Ogalactoside
14	25	268,314,348sh	625	316,317	Myricetin-3-O-rutinoside
15	25.9	270,355,300sh	609	301	Quercetin-3-O-rutinoside
16	26.0	260,358,346sh	463	316	Myricetin-3-O-rhamnoside
17	26.2	270,350,300sh	463	301	Quercetin derivative 2
18	27	255,350,300sh	433	300,301	Quercetin-3-O-arabinoside
19	27.3	266,352	615	317	Myricetin-3-O-(O-galloyl)-rhamnoside
20	27.7	266,350,300sh	447	300,301	Quercetin-3-O-rhamnoside
21	28.8	265,348	417	285	Kaempferol-3-O-arabinoside
22	29.2	265,348	447	285	Kaempferol derivative
23	30.5	265,346	593	285	Kaempferol-3-O-rutinoside
24	33.4	280,340	285	203,123,109	Luteolin

4.1.2 Antiradical activity evaluation of different extracts of *P. lentiscus* leaves

The crude ethanolic leaf extract (CEE) was partitioned following a protocol similar to that used for the *C. incanus* crude extract (Gori *et al.* 2016) and two main fractions were obtained, namely EAF (Ethyl Acetate Fraction) and AF (Aqueous Fraction). These extracts were analysed by HPLC-DAD in order to quantify the single compounds. The EAF was mainly composed of flavonoids (mirycetin, quercetin and kaempferol derivatives), whereas the aqueous fraction (AF) mostly contained gallotannins. These results are shown in Table 2.

Then, the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of the CEE, fractions and standards, were investigated using EPR (Electron Paramagnetic Resonance) spectroscopy and these results expressed as IC_{50} values are shown in Fig. 2. IC_{50} values varied in the following order: ethyl acetate fraction (0.60 µM ± 0.048) < myricitrin standard (0.68 µM ± 0.072) < crude ethanolic extract (0.74 µM ± 0.075) < aqueous fraction (1.1 µM ± 0.110) ≤ gallic acid standard (1.20 µM ± 0.095).

This means that the DPPH radical-scavenging activity was greatly influenced by the polyphenolic composition of the different extracts. As a consequence, the high antioxidant potential of the ethyl acetate fraction may be attributed to its flavonoid compounds which possess ideal structural chemistry for free radical scavenging activity (Rice-Evans 1996). Our findings are in accordance with previous researchers who have reported that *P. lentiscus* extracts exhibited different antioxidant properties depending on their polyphenolic constituents (Vaya *et al.* 2006, Benhammou *et al.* 2008, Atmani *et al.* 2009, Saliha, *et al.* 2013). Specifically, Saliha *et al.* (2013) compared the antioxidant properties of an initial methanolic extract with its chloroform, ethyl acetate and aqueous fractions and found that the ethyl acetate extract displayed the highest antioxidant activity using DPPH scavenging, hydroxyl radical scavenging, reducing power and lipid peroxidation assays.Furthermore, the IC_{50} values of *P. lentiscus* samples compared favourably with that of *C. incanus* (Gori *et al.* 2016), indicating that ethyl acetate fractions of both species displayed the strongest free radical scavenging activity, and this was due to the presence of flavonoids.

Table 2. Mean concentration of polyphenols (µmol/mL) in CEE and enriched fractions of *P. lentiscus* leaves (n=3).

Sample	Gallic acid and Diquinic acid	Gallotannins[a]	Myricetin derivatives[b]	Quercetin derivatives[c]	Kaempferol derivatives[d]
CEE	0.374 ± 0.014	25.979 ± 2.244	5.098 ± 0.197	6.055 ± 0.879	1.376 ± 0.067
EAF	0.146 ± 0.039	1.202 ± 0.227	4.740 ± 0.102	4.256 ± 0.144	0.984 ± 0.047
AF	0.201 ± 0.058	19.012 ± 1.199	Nd	Nd	Nd

Nd = Not detectable

[a]Monogalloyl quinic acid, Digalloyl quinic acid (isomer 1 and 2), Trigalloyl quinic acid and (isomer 1 and 2), Tetragalloyl quinic acid (isomer 1 and 2);

[b]Myricetin galactoside, Myricetin-3-O-rutinoside, Myricetin-3-O-rhamnoside, Myricetin-3-O-(O-galloyl)-rhamnoside;

[c]Quercetin-derivative 1, Quercetin-3-O-rutinoside, Quercetin derivative 2, Quercetin-3-O-arabinoside, Quercetin-3-O-rhamnoside;

[d]kaempferol-3-O-arabinoside, kaempferol derivative, kaempferol-3-O-rutinoside.

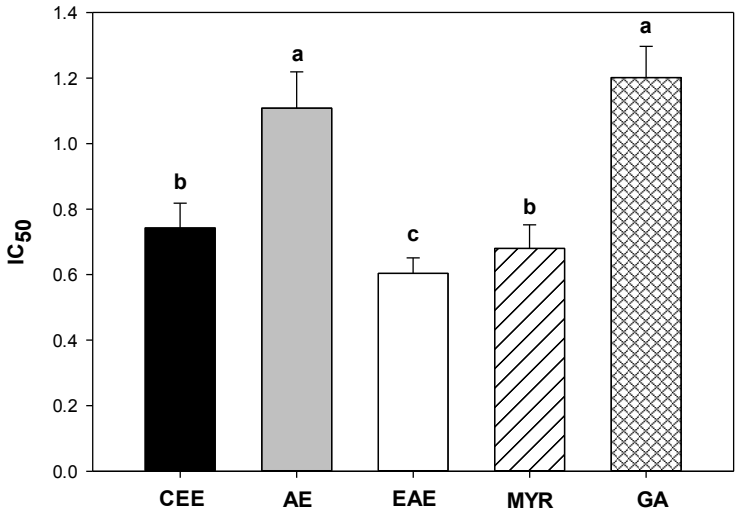

Fig. 2. IC_{50} values (µM) for DPPH scavenging of standards and sample extracts. Data are represented as the mean ± SD (n= 3). CEE: Crude Ethanolic Extract; EAF: Ethyl Acetate Fraction; AF: Aqueous Fraction; MYR: Myricitrin Standard; GA: Gallic Acid Standard

4.2 **Cytotoxic activity of polyphenolic extracts form *C. incanus* and *P. lentiscus* leaves on NIH 3T3 fibroblast cells**

4.2.1 Materials and Methods

-Culture of cell line and Neutral Red Uptake (NRU) assay

The cytotoxic effects of crude extracts and polyphenolic-rich fractions of the two species were tested with the Neutral Red Uptake (NRU) assay (Borenfreund and Puerner, 1985) using mouse immortalized NIH 3T3 fibroblast cells.

The NRU assay is based on the ability of viable cells to incorporate and bind the neutral red dye in the lysosomes. Specifically, the uptake of neutral red depends on the cell capacity to maintain pH gradients, through the production of ATP. At physiological pH, the dye presents a net charge close to zero, enabling it to penetrate the membranes of cells. Inside the lysosomes, there is a proton gradient to maintain a pH lower than that of the cytoplasm. Thus, the dye becomes charged and can be retained inside the lysosomes. When the cells die or the pH gradient is reduced, the dye cannot be retained. Therefore, the amount of retained dye is proportional to the number of viable cells (Repetto *et al.* 2008).

NIH 3T3 cells were grown in Dulbeccos's modified Eagle's medium (DMEM) (4.5 g/L glucose), supplemented with 10% fetal bovine serum, L-glutamine-streptomycin at 1%, and nonessential amino acids at 1% and incubated at 37°C in an atmosphere containing 5% CO_2.

At confluence, the cells were washed with a phosphate buffered saline solution (PBS) 0.1 M, detached from the support by means of a trypsin solution, centrifuged at 1500 rpm for 5 minutes at room temperature and resuspended in a complete medium (dilution 1:15). Cells (3×10^4) suspended in the medium were seeded in each well of a 24-well multiwell; the medium volume of each well was brought to 1 mL and the cells were left in an incubator at 37°C and 5% CO2 for 24 hours. At the end of incubation, the cells were tested with different concentrations of the extracts (0.065 µM, 0.65 µM, 6.5 µM, 65 µM and 0.65 mM) and the multiwells were left to incubate for further 24 hours at 37°C and 5% CO_2.

Then, the following solutions were prepared: (1) stock solution of neutral red: 0.33 g of neutral red in 100 mL of sterile distilled water; (2) medium with neutral red: 1.0 mL of stock solution + 99.0 mL of complete culture medium; (3) neutral red extraction solution: glacial acetic acid at 1% + ethanol at 50% + distilled water at 49% (Fiorenzani *et al.* 2014).

After the incubation, the culture medium was removed from each well and cells were washed with 1 ml of PBS solution (0.1 M). After, 1mL of medium containing the neutral red dye was added to each well. The plates were then incubated for another 3 h. Then, the medium was removed and the cells were again rapidly washed with 1 ml of PBS solution. At this point, the plates were placed on a shaker for 30 minutes at room temperature in order to extract the dye from the vital cells. During this phase, the plates were covered to protect them from light. Five minutes after the end of the shaking, the extraction solution was removed from each well and the absorbance was read at 540 nm in a UV-VIS spectrophotometer (Lambda 25, Perkin Elmer).

The percentage of cell viability compared to control wells was calculated, according to the following equation:

$$\text{dead cells (\%)} = (\text{absorbance of the control} - \text{absorbance of test}) \times 100 / \text{absorbance of the control}$$

These data were used to plot dose-response curves which determine the extract concentrations capable of killing 50% of the cell population tested, indicated by the LC_{50} value (Lethal Concentration).
Dulbecco's Modified Eagle's Medium (DMEM), trypsin solution, and all the solvents used for cell culture were purchased from Lonza (Belgium). Mouse immortalized NIH 3T3 fibroblasts were obtained from American Type Culture Collection (USA).

-Statistical Analysis

Each experiment was performed three times using three replicates for each concentration assayed. Results were expressed as the mean ± SD.
Statistical significance was determined by Student's t-test and one-way analysis of variance (ANOVA) using the SPSS software (version 23; SPSS Inc., Chicago, IL, USA). Statistical significance was considered $P<0.05$.

4.2.2 Results

The effects of the plant extracts on NIH 3T3 cells were shown in Table 3 in the form of LC_{50} values after 24 h of treatment.
The LC_{50} values of *P. lentiscus* extracts on mouse fibroblast cells ranged from 72.9 to 116.6 µM, whereas the concentration of *C. incanus* extracts required to produce a 50% cytotoxic effect on NIH 3T3 cells ranged from 3.2 µM to 12.4 µM. This means that *C. incanus* extracts are more cytotoxic towards the mouse fibroblast cells than *P. lentiscus* extracts. Furthermore, comparing the cytotoxicity of the different fractions obtained by *P. lentiscus* leaves, we found that ethyl acetate fraction exhibited a greater cytotoxicity compared with the aqueous fraction and the total extract (CEE) ($p < 0.05$). Among the *C. incanus* fractions, AF1 and AF2 exhibited the highest cytotoxicity activity and EAF had the lowest (Table 3).

Table 3. Cytotoxic activities (IC_{50}, µM) of the various fractions and extracts. Data are represented as the mean ± SD (n= 3). CEE: Crude ethanolic extract; EAF: Ethyl acetate Fraction; AF: Aqueous Fraction.

Plant	Extract/Fraction	LC_{50} ± SD (µM)
P. lentiscus	**CEE**	72.9 ± 3.7 c
	EAF	98.5 ± 5.9 b
	AF	116.6 ± 4.8 a
C. incanus	**CEE**	7.67 ± 1.2 b
	EAF	12.4 ± 1.7 a
	AF1	3.2 ± 0.8 c
	AF2	3.9 ± 0.7 c

4.2.3 Discussion and future directions

Results from *in vitro* scavenging capacity of polyphenolic extracts of *P. lentiscus* and *C. incanus* leaves suggested that both species contain constituents that are capable to exert high antiradical actions. Such antiradical activity may justify their future applications as therapeutic agents for the treatment of various human diseases associated with oxidative stress, including cancer, atherosclerosis, diabetes and chronic inflammations (Halliwell 1994).

However, an important preliminary step in pharmacological studies of these extracts is to evaluate their *ex vivo* cytotoxicity, in order to select the least toxic fractions from among the most antioxidant ones.

In this investigation, we clarified the *ex vivo* cell toxicity effects of our polyphenolic extracts throughout the neutral red uptake assay in murine fibroblast NIH 3T3 cell line. We selected this cell line because it is recommended by the U.S. National Institute of Environmental Health Science (NIEHS) Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM).

Our data showed that all the extracts obtained from *P. lentiscus* leaves are non-cytotoxic up to 72.9 µM, indicating that all extracts are safe at the concentration ranges in which they exert their antioxidant properties (0.60 – 1.1 µM). Conversely, the LC_{50} values of *C. incanus* extracts against the mouse fibroblasts (NIH 3T3) were found to be very low when compared to that of *P. lentiscus* (the LC_{50} value of the CEE of *C. incanus* was about 9 times lower than the cytotoxicity displayed by the CEE of *P. lentiscus*), suggesting that *C. incanus* leaves are potentially sources of toxic compounds. For example, the high cytotoxicity of *C. incanus* aqueous extracts could be due, at least in part, to the presence of proanthocyanidins. Similar results have been observed by other authors, as in the case of (–)-epigallocatechin-3-gallate (EGCG) presents in black tea which inhibited growth of NIH 3T3 fibroblasts (Liang *et al.* 1999).

However, further *ex vivo* experiments with other cell lines to ascertain their toxicological effects are in progress.

C. incanus ethyl acetate fraction is enriched in flavonoid compounds and displayed the highest antiradical capacity among all fractions analysed (Gori *et al.* 2016). Flavonoids have been previously reported as antiproliferative compounds (Huang *et al.* 1996, Kawaii *et al.* 1999, Kuntz *et al.* 1999, Pouget *et al.* 2001, Kaefer and Milner 2008). In particular, a large body of studies is evidencing the ability of these compounds to modulate uncontrolled proliferation pathways or protooncogene gene expression (Ferriola *et al.* 1989, De Azevedo *et al.* 1996, Kuo *et al.* 1995, Ahmad *et al.* 1998, Ramos *et al.* 2007, Xavier *et al.* 2009). In this sense, it is accepted that the chemopreventive and tumor-inhibitory effects associated to flavonoids could be due to their capability to inhibit oxygen reactive species (ROS) or free radicals (Halliwell 1996). Anticancer compounds are designed to kill cells, but this activity should be selective for tumor cells. Because of that, it is necessary to demonstrate that they are less toxic to normal cells than to tumoral cells. Therefore, the *C. incanus* ethyl acetate fraction, which displayed the lowest cytotoxicity against NIH 3T3 cells, could be capable to exert an antiproliferative activity by the presence of large amounts of flavonols and be used as a natural source of anticancer-preventive agents in future studies.

To conclude, our results suggest that both *C. incanus* and *P. lentiscus* leaves have therapeutical potentials but with different applications. Specifically, all polyphenolic extracts of *P. lentiscus* leaves displayed low cytotoxicity against NIH 3T3 cells and might be used in preventative treatments for oxidative stress-mediated diseases in both medicine and the food industry. Conversely, the ethyl acetate fraction of *C. incanus* leaves might be tested as potential anti-cancer agent.

Further research, however, will be conducted in order to confirm our findings and to elucidate the *in vivo* possible beneficial effects of *P. lentiscus* and *C. incanus* polyphenolic-rich extracts.

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5. **General conclusions and future directions**

In the Mediterranean basin, drought imposes a major environmental threat for plants and its adverse impact has become an urgent problem under the increasing aridity predicted for this region by most climate models.

Some woody species play a key-role in the maintenance of the Mediterranean ecosystem and act as "hotspots" of biodiversity in this area. These plants, over millennia, have developed a suite of morpho-anatomical, physiological, and biochemical mechanisms to cope with the severe stress conditions typical of the Mediterranean climate. These mechanisms include the ability to synthesise an extraordinary arsenal of secondary metabolites. In particular, some species are generally very rich in polyphenols which, in addition to protect plants from the detrimental effects of solar short-wave radiation, may also counter the negative actions of reactive oxygen species on plant cell metabolism. Apart from their roles in plants, polyphenols have an impressive array of health-promoting benefits and represent the basis for the use of these plants in the medical field. Consequently, Mediterranean species represent an extremely rich biological resource for the discovery of novel drugs and for developing innovative nutraceutical applications.

In this context, conservation of Mediterranean plants of pharmaceutical interest is therefore necessary and require a thorough knowledge of their ecology, physiology and biochemistry.

In the present thesis, the first objective was to characterize the mechanisms adopted by *P. latifolia, C. incanus* and *P. lentiscus* under the Mediterranean climate. To this end, we carried out a comparative field study in which a combination of physiological and biochemical parameters was observed at different timescales and related to variation in environmental conditions.

In our investigation, we found a wide diversity of physiological and biochemical responses of the investigated species. Particularly, inter-specific differences were observed in leaf water status, chlorophyll fluorescence and secondary metabolites, suggesting a remarkable ability of these plants to optimize survival strategies and withstand environmental stressful conditions on their natural habitat.In addition, our analysis of inter-specific responses along different timescales permitted a better understanding of the underlying mechanisms that govern the acclimation of these species to Mediterranean climate under field conditions. For example, diurnal patterns of water relations allowed us to discriminate the different water-use behaviours of the species, while daily variation in chlorophyll fluorescence parameters clarified the distinction among photoinhibition-avoider and photoinhibition-tolerant plants.

The second objective of this thesis was to assess the relative significance of the study species as "biofactories", i.e. potential sources of health-promoting polyphenols. Considering that *P. latifolia* was the minor species of interest because its polyphenolic composition as well as its therapeutic potentials have been already widely explored, only *P. lentiscus* and *C. incanus* were assessed as sources of bioactive polyphenols. To achieve this, we preliminarily identified the

most abundant polyphenols in their leaf tissues throughout the HPLC–DAD–MS/MS technique. Moreover, we optimized an extraction protocol and obtained different polyphenolic-rich extracts (flavonoid-rich fractions and tannin-rich fractions) from each plant. Then, the total extracts and the obtained fractions were tested throughout *in vitro* and *ex vivo* assays. These analyses were conducted in collaboration with the Department of Molecular Medicine and Development at the University of Siena. Specifically, the antioxidant activity was examined using the DPPH radical scavenging assay, while the study of cytotoxicity was tested with the Neutral Red Uptake (NRU) assay using NIH 3T3 fibroblast cells.

In our investigation, we found that the ethyl acetate fractions of both species displayed the strongest free radical scavenging activity. Furthermore, the NRU test showed that all *P. lentiscus* extracts were less cytotoxic than those of *C. incanus*.

Despite its preliminary characters, our research is new to literature and provides a scientific basis for further *ex vivo* and *in vivo* studies, which will contribute to evaluate the potential application of these plants in the pharmacological field. For instance, *P. lentiscus* extracts could be tested for their antioxidant activity on normal cell cultures, while *C. incanus* extracts could be cecked as anti-cancer agents on tumoral cells.

In conclusion, our results largely confirm the central role of these maquis plants in the conservation of biodiversity in the Mediterranean environment. Furthermore, *P. lentiscus* and *C. incanus* leaves can be considered good sources of phyto-pharmaceutical compounds. Consequently, valuable uses are possible for these plants and their cultivation could represent new economically sustainable and environmentally-sound opportunities for semi-arid areas.

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