UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO

DIPARTIMENTO DI
Innovazione dei sistemi biologici, agroalimentari e forestali

in convenzione con **UNIVERSITA` DEGLI STUDI MEDITERRANEA**
(REGGIO CALABRIA, ATENEO PARTNER)

Corso di Dottorato di Ricerca in

Scienze, Tecnologie e Biotecnologie per la sostenibilita' - XXIX Ciclo.

RECOVERY AND VALORIZATION OF LAVANDULA MULTIFIDA L. FOR THE BIODIVERSITY PRESERVATION IN CALABRIA REGION

(s.s.d. BIO/15)

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Data della discussione
24 luglio 2018

SHORT ABSTRACT

Lavandula genus, belonging to Lamiaceae family, possesses a remarkable importance since it includes numerous species and hybrids that have been widely cultivated since ancient times. Among Lavandula genus, *Lavandula multifida* L. is a plant of great interest which has been traditionally used in folk medicine due to its multiple beneficial properties.

Taking in consideration the condition of high extinction risk of this species in Southern Italy, and the need to preserve the biodiversity and revalorize this plant in this European coastal region, the environmental adaptability and also some beneficial and therapeutic properties of this plant were evaluated. In particular, the antioxidant pattern and flavonoid profile, the cytotoxic and cytoprotective effects of leaf-extracts, and the effects of leaf-extracts of *L. multifida* on innate immune system are analysed and discussed in the present thesis. The aim is to revalorize this important species which is critically endangered in the Southern regions of Italy, contributing to its preservation in the Mediterranean region.

KEYWORDS

Lavandula multifida L., leaf extracts, antioxidant pattern, flavonoids, cytotoxicity, cytoprotection, immunostimulation.

EXTENDED ABSTRACT

This PhD thesis dealt with the analysis of environmental adaptability and germination strategies, as well as with the study of antioxidant, cytotoxic, cytoprotective and immunostimulant properties of the plant *L. multifida* L. growing in Calabria region. The aim is to emphasize the economic and biological value of *L. multifida* which is at high extinction risk in Calabria and Sicily regions, in order to promote the preservation of biodiversity in Mediterranean regions, and also to evaluate the possibility of therapeutical and pharmaceuticals applications.

Nella presente tesi di Dottorato vengono affrontati alcuni aspetti sulle proprietà germinative e sull' adattamento ambientale di *L. multifida* L. che cresce in Calabria, valutandone anche le proprietà antiossidanti, citotossiche, citoprotettive e immunostimolanti dell'estratto fogliare. Trattandosi di una specie a rischio erosione genetica nelle regioni del sud Italia, l'obiettivo e' quello di contribuire alla salvaguardia della biodiversità nel Mediterraneo e di valutare le possibilità di applicazione in campo terapeutico e farmaceutico di *L. multifida* L., al fine di enfatizzarne l'importanza economica e biologica.

In accordance with the aim of this PhD thesis, the main results of the following analysis are reported:

- evaluation of the germination capacity of Calabrian *L. multifida* under different environmental conditions (temperature and photoperiod), in comparison with Spanish *L. multifida,* a very widespread plant in different Spanish regions
- activity of some hydrolyzing enzymes involved in germination and the antioxidative pathway in seeds of Calabrian *L. multifida*, compared to Spanish *L. multifida*

- analysis of some enzymatic and non enzymatic antioxidants and phenolic compositions in leaf extract of Calabrian *L. multifida*, in comparison with the commercial species *Lavandula angustifolia* (*L. officinalis*)
- determination of cytotoxic and cytoprotective effects of water and ethanolic leaf extracts of Calabrian *L. multifida* (between 10 µg/mL and 1000 µg/mL) on HeLa cell line, compared to extracts of Sicilian *L. multifida* and *L. angustifolia*
- assessment of the effects of water and ethanolic leaf extracts (between 10 µg/mL and 1000 µg/mL) of Calabrian *L. multifida* on innate immune system of Sparus aurata (phagocytosis, respiratory burst activity, peroxidase activity), in comparison with Sicilian *L. multifida* and *L. angustifolia*
- determination of cytotoxic effects of water and ethanolic leaf extracts (between 1 µg/mL and 1000 µg/mL) of Calabrian *L. multifida,* Sicilian *L. multifida* and *L. angustifolia* on SAF-1 cell line.
- assessment of bactericidal effects of water and ethanolic leaf extracts (between 10 µg/mL and 500 µg/mL) of Calabrian *L. multifida* on some fish pathogens (Vibrio harveyi, Vibrio anguillarum, Aeromonas salmonicida), in comparison with Sicilian *L. multifida* and *L. angustifolia.*

Results showed a lower germination percentage in seeds of Calabrian *L. multifida* if compared to Spanish ones, being Calabrian *L. multifida* more sensitive to temperature and photoperiod. Accordingly to this, a less efficient mobilization and use of reserves in germinating seeds of Calabrian *L. multifida* can also explain the diverse germination speed and germination percentages between the two populations. In addition, to prevent oxidative damage during the metabolic reactivation that characterizes the germination process, the two population of *L. multifida* have been demonstrated to possess a battery of antioxidant enzymes and antioxidant compounds which are differently involved in seed germination. The different activation of these antioxidant systems during germination can be considered a metabolic adaptation and may explain the diverse ability to respond to external environmental conditions. These results support the possibility of utilization of propagation from seeds under controlled environmental conditions as a viable method for the ex-situ conservation of Calabrian *L. multifida*.

Calabrian *L. multifida* leaves contain relevant quantity of enzymatic and non enzymatic antioxidants, suggesting a remarkable ability to optimize survival strategies in its natural habitat and to withstand environmental stress, in comparison with the commercial species *L. angustifolia.* Furthermore, Calabrian *L. multifida* leaves may be a good potential source of antioxidant substances for human health, as revealed by the flavonoid composition in its leaves.

Water and ethanolic extracts of Calabrian and Sicilian *L. multifida* exert a significant cytoprotective effect against a chemically-induced condition of oxidative stress on HeLa cells, so that such extracts may be considered a potential source of natural antioxidants to protect cells against oxidative damage.

Extracts from Calabrian and Sicilian *L. multifida* positively affect phagocytosis and respiratory burst activity of gilthead seabream head-kidney leucocytes, but have no bactericidal activity against the fish pathogens tested, thus supporting the possibility to use them as immunostimulant in aquaculture production of Sparus aurata, in order to achieve protection against pathogenic infections through enhancement of the innate immunity.

Results reported in this PhD thesis aim to support the biological value of the threatened species *L. multifida*, demonstrating the possibility of applying conservation programs for its population reinforcement and reintroduction in Southern Italy, and also highlighting its potential utilization as source of new active principles for new antioxidant formulations and as immunostimulant in aquaculture production.

INTRODUCTION

Gradual declining in plant biodiversity represents a worldwide problem. The rise in the global population, the rapid and sometimes unplanned industrialization, the indiscriminate deforestation, the overexploitation of natural resources, the climate change and the pollution have led to the degradation and fragmentation of natural habitats (Sen and Samanta, 2014). Since ancient times, people have gathered plant resources for their needs. Therefore, the necessity of preserving biodiversity is connected with the multiple uses of plants as source of food, fibers, building materials, as well as for medication and cultural needs (Shippmann et al, 2002). The natural diversity of plants is also considered to be a source of important traits, e.g., resistance to diseases and pests, resistance to drought, salting and to other abiotic stresses and also source of traits that allow to improve the quality, and nutritive value of cultivated plants.

Biodiversity contributes significantly towards human livelihood and development, and thus plays a predominant role in the well being of the global population. Around 80 % of the global population still relies on vegetal drugs, according to World Health Organization reports, and today several medicines owe their origin to medicinal plants (Sen and Samanta, 2014).

Many plants have been used for centuries as remedies for human diseases, because they contain natural bioactive compounds with therapeutic value. Lamiaceae (Labiatae) represents a very important medicinal plant family which stands for about more than 3000 species of plants. Plants belonging to this family are herbs or shrubs often with an aromatic smell and rich in medicinal properties of great worth in natural medicine and pharmacopoeia. Some examples of this family include mints, tulsi, thyme, spearmint, coleus and lavender. These plants are widely distributed in the Maltese Islands and other

Mediterranean countries because some of them produce a high amount of essential oils that enables them to survive to the hot summer season (Ramasubramania Raja, 2012). Plants belonging to this family are widely cultivated for medicinal, perfumery, culinary and ornamental purposes.

Among plants belonging to the Lamiaceae family, Lavandula (lavender, Lamiaceae) is a genus of 39 species of particular importance, since it includes numerous species and hybrids that have been widely cultivated since ancient times, and have been applied in food, pharmaceutical and agroindustries (Lis-Balchin, 2003). Lavender is native to the Mediterranean regions including France, Spain, Andorra and Italy, anyway it grows in many other countries of the world. The name lavender comes from the Latin verb lavo, lavare and means “to wash” or “to clean”. In fact, Lavender was used by the Romans as a bath additive, and it was the most used soap making during the Middle Ages. It was also used as food additive and as laxative (Prusinowska and Śmigielski, 2014). Its essential oil is of great economic importance in the perfumery and fragrance industry, and possess multidirectional biological activity. The material used for herbal purposes includes flowers and flowering aerial parts containing essential oil, anthocyanins, phytosterols, sugars, minerals and tannins. Its essential oil contains over 300 chemical compounds, and its qualitative and quantitative composition is variable depending on the genotype, climatic conditions, growing location, and morphological feature (Prusinowska and Śmigielski, 2014). This essential oil has significant antioxidant and antimicrobial activities, and it exerts a significant positive effect on the digestive and nervous system (Buchbauer et al, 1991; Dapkevicius et al, 1998; Guillemain et al, 1989; Mayaud et al, 2008; Wolfe et al, 1996). During the last few years, the exploitation of native lavender species has increased, due to the necessity of preserving their genetic heritage and also for a renewed interest in the use of naturally derived compounds.

Among Lavandula genus, *Lavandula multifida* L. is a rare and short living plant, 30 to 100 cm high, with a diploid genetic pool, equipped with triangular pinnatisect leaves , and with blue or white purple flowers which give off a strong smell (Photo 1). The species reproduces by seeds, with an outcrossing mating system (Larsen, 1960). It spontaneously grows along the Mediterranean coast in Egypt, Tunisia, Morocco, Algeria, Spain and Portugal. In Italy, it has been found in Calabria (Capo dell’Armi,

Photo 2), and in Sicily (Monte Pellegrino, Brucoli, Capo Sant'Alessio), where its populations are reduced and fragmented due to the human impact on its natural habitat (Galesi et al, 2005). The species has completely disappeared in Capo Scaletta and Taormina (Sicily) (Galesi et al, 2005). Its natural habitat is represented by coastal areas on poorly evolved limestone soils, and hot arid climatic conditions. Due to the rarity and the threats of this plant, *L. multifida* is included in the "Regional Red Lists of Italian Plants" under the status IUCN of "critically endangered" in Calabria and "endangered" in Sicily (Conti et al, 1997). For this reason, *L. multifida* has been involved in regional projects aimed to the recovery, the preservation of biodiversity and the revalorization of this plant species in Mediterranean region.

Photo 1. Leaves (on the left) and flower (on the right) of *Lavandula multifida.*

Photo 2. Calabrian *Lavandula multifida* (Capo dell'Armi, Reggio Calabria, Italy).

Calabrian *L. multifida* L. (Lamiaceae) spontaneously grows on the poorly evolved limestone soils in Capo dell'Armi (Reggio Calabria, Italy). The needs to preserve its genetic heritage and to revalorize *L. multifida* growing in Southern Italy led to the necessity of studying its environmental adaptability. Therefore, in the first part of the present thesis, the evaluation of the germination capacity, the activity of some hydrolyzing enzymes involved in germination and the antioxidative pathway in seeds of Calabrian *L. multifida* L. were investigated. The aim was the understanding of the environmental adaptability and of the germination strategies of this plant, with the objective of defining possible conservation programs for the population reinforcement and reintroduction in Southern Italy. The analysis were carried out in comparison with a Spanish core population of *L. multifida* growing in Almeria, as the Spanish species is very widespread on different types of substrates. The research of biological traits associated with reproduction is considered essential for the development of guidelines for conservation and management of endangered species (Evans et al, 2003).

The natural habitat of *L. multifida* is not favorable to the growth of many crop species because of stressful conditions that are potential sources of reactive oxygen species (ROS). ROS are able to cause oxidative damage by reacting with biomolecules. It is known that a complex network of enzymatic and small antioxidant molecules controls the concentration of ROS and repairs oxidative damage, contributing to the control of plant growth, development and response to the environment (Smirnoff, 2005). Therefore, it is thought that a fine regulation of antioxidant resources exists in *L. multifida* in order to enable the plant to face ROS overproduction. This mechanism is included in plant's physiological conditions and responses to different environmental stimuli. For this reason, in the present thesis some enzymatic and non enzymatic antioxidants and phenolic composition of Calabrian *L. multifida* L. leaves are evaluated and discussed. Among enzymatic antioxidants, catalase, peroxidase, dehydroascorbate reductase, and ascorbate reductase activities were assessed, while total phenols, reduced glutathione, ascorbic and dehydroascorbic acid, total antioxidant capacity, tocopherols, total carotenoids and anthocyanins were determined as non enzymatic antioxidants. Furthermore the presence of carvacrol, carvacrol methyl-ether, thymol and flavonoid components was determined in leaves of Calabrian *L. multifida* L. The analysis of

antioxidant components were performed in comparison with the widely distributed species *Lavandula angustifolia* Miller (*Lavandula officinalis*), in order to show possible differences in antioxidant contents and to improve the knowledge of bioactive compounds present in leaves which could be helpful for possible pharmaceutical and therapeutical applications.

Besides the need to preserve the biodiversity in the Mediterranean coastal regions, *L. multifida* L. needs to be revalorized, contributing to the knowledge of its possibilities of applications in pharmaceutical and cosmetical fields.

Recently there is a growing interest in searching for antioxidants of natural origin. Due to their high content in polyphenolic compounds, many herbs belonging to Lamiaceae family represent a rich source of health-beneficial antioxidant components (Dragland et al, 2003).

It is well known that living cells and organims are exposed to reactive oxygen species (ROS) as natural bioproducts of the normal metabolism of the oxygen, which play an important role in cell signaling and homeostasis (Hayyan et al, 2016). Under normal conditions, ROS levels are low enough to be neutralized and removed by the natural endogenous antioxidant cellular defence mechanisms (Halliwell and Gutteridge, 1985). However, several stress conditions, including UV radiation, exposure to different toxic compounds or xenobiotics, may enhance ROS production and free radicals to such an extent that cellular defence mechanisms are unable to cope. Oxidative stress arises as consequence of an imbalance between ROS formation and degradation. The clinical implications of these alterations can be severe; in fact, the accumulation of ROS in several cellular components is thought to be a major cause of molecular injury leading to cell aging and to age-related degenerative diseases such as cancer, brain dysfunction and coronary heart disease (Kehrer, 1993; Duthie et al, 2001; Valko et al, 2006). Recently there is a growing interest in finding antioxidants as well as in identifying natural products for use in the treatment of several oxidative stress-related diseases. Antioxidants, as molecules that inhibit the oxidation of other molecules, help to prevent or reverse the negative effects caused by oxidative stress due to their capability to quench free radicals and ROS. They can be natural or synthetic. However, the

widespread use of synthetic antioxidants used in food, drugs and cosmetics has led to concerns about their safety and toxicity (Kahl and Kappus, 1993). Therefore, a growing interest in finding antioxidants of natural origin exists. Besides this, many plants belonging to Lavandula genus have been chemically characterized and are known to present large amounts of secondary metabolites such as coumarins, terpenoids, triterpenes and sesquiterpenes (Areias et al, 2000). Especially abundant in these plants are flavonoids, a class of natural polyphenolic compounds known for their important antioxidant and free radical scavenging activities (Procházková et al, 2011), but also for their ability to inhibit the activity of several enzymes that regulate cell proliferation (Formica and Regelson, 1995), arrest the cell-cycle progression (Yoshida et al, 1990; Ranelletti et al, 1992; Lepley et al, 1996; Plaumann et al, 1996) and induce cell death by apoptosis (Wei et al, 1994; Plaumann et al, 1996; Ahmad et al, 1997). In our study,in order to demonstrate the possibilities of industrial applications of this plant species, the cytotoxic and antioxidant cytoprotective effects of extracts obtained from leaves of Calabrian *L. multifida* L. were assayed on the human cervical cancer cell line Hela. These assays were performed in comparison with extracts obtained from leaves of Sicilian *L. multifida* L. and from leaves of the commercial species *L. angustifolia.* These assays allowed to test the ability of *L. multifida* L. leaf extract to offer cytoprotection on Hela cells against oxidative stress, considering the possibility of these extracts to be included in antioxidant formulation for application in dermopharmaceutical and cosmetic fields. Beside this, HeLa cell line was used to carry on the cytotoxic assays as it represents the first continuous cell line used worldwide for scientific and medical research, because of its steady growth making it an excellent model for almost every process that occurs in human cells (Masters, 2002). Thanks to their rapid growing and stability in cell culture, Hela cells have been globally used by researcher not only to study cancer, viral infections, gene mapping, radiation effects, but also to investigate the effect of toxic substances and phytochemicals, as well as to test the human sensitivity to tape, glue and cosmetics (Masters, 2002).

Currently, there is a growing interest in screening medicinal plants extracts for their bactericidal, fungicidal and even immuno-stimulant properties, in order to exploit new biocompounds of natural origin which could be employed in the prevention and/or control of fish diseases in aquaculture (Reverter et al, 2014). The importance of using immunostimulants in aquaculture is known since many years (Sakai, 1999). The use of immunostimulants as an alternative to drugs, chemicals and antibiotics currently being used to control fish diseases in fish culture is attracting the attention of many researchers, and the use of medicinal plants-originated products as potential therapeutic measures for modulating the immune response and to prevent and control fish diseases represents a topic of great interest in research (Bairwa et al, 2012). In the intensive aquaculture system, application of antibiotics and chemotherapeutics as prophylactic measures has been widely criticized for their negative impacts on the environment and also on fish, such as immunodepression or residue accumulation in tissues, and besides this leads to the development of drug resistant pathogens (Harikrishnan et al, 2009; FAO, 2003; Rijkers et al, 1980; Smith et al, 1994). In general, immunostimulants represent a group of biological and synthetic compounds that enhance the non-specific defence mechanisms in animals, thus imparting generalized protection. This kind of protection is particularly important for fish living in environments where the nature of pathogens is unknown and the immunization by specific vaccine may be inappropriate. Immunostimulants promote a greater and more effective sustained immune response to those infectious agents such as viruses, bacteria, fungi, and parasites, producing subclinical disease without risks of toxicity, carcinogenicity or tissue residues (Bairwa et al, 2012).

Herbal extracts-originated products have a potential application as immunostimulants in fish culture, primarily because they can be easily obtained, are not expensive and act against a broad spectrum of pathogens. Most of the herbs and herbal extracts can be given orally, which is the most convenient method of immunostimulation (Bairwa et al, 2012). The use of plant extracts as immunostimulants has recently received increasing attention not only because they combine lower costs with their high level of biodegradability, but also because they may have additional physiological effects since they contain many nutrients, micronutrients as well as other immunostimulant substances (Cuesta et al, 2005). Looking for new applications of *L. multifida* extracts,

and taking in considerations the need of searching for new immunostimulants of natural origin, and also considering the importance of gilthead seabream (*Sparus aurata*) in Mediterranean diet and in marine fish aquaculture, the in vitro effects of Calabrian *L. multifida* leaf extracts on head kidney leucocytes activities (viability, phagocytosis, respiratory burst and peroxidase activity) were tested, making a comparison with similar extracts obtained from *L. multifida* collected from Sicily and from the widely-distributed species *L. angustifolia*. Moreover, the cytotoxic activity on SAF-1 cells (a cell line obtained from gilthead seabream) and the bactericidal activity of the extracts on *Vibrio harveyi*, *Vibrio anguillarum* and *Aeromonas salmonicida* were also checked.

All experiments performed and described in the present thesis aim to demonstrate the possibilities of applications of *L. multifida* extracts in pharmaceutical and cosmetic fields, and also in aquaculture system as immunostimulants, while promoting the preservation and conservation of this critically endangered plant species growing in Southern Italy.

STATE OF THE ART

The genus Lavandula L. comprises some of the most promising plants that have been used in screening assays to assess biological properties, such as antioxidant (Matos et al, 2009), antimicrobial (Moon et al, 2007; Roller et al, 2009), insecticidal (Pavela, 2005), antifeedant (González-Coloma et al, 2011), parasiticidal (Moon et al, 2006), and herbicidal (Haig et al, 2009) effects.

Among the plants belonging to Lamiaceae family, *Lavandula multifida* L. represents a plant species of remarkable importance because of its wide utilization in traditional and folk medicine to treat headaches, diabetes, depression and for its sedative properties (Gamez et al, 1987; Gilani et al, 2000), to prepare decoctions against rheumatism, chill and as digestive system benefic agent (El-Hilaly et al, 2003). Anyhow not all these therapeutic properties has been demonstrated and supported by experimental studies, and only two documents on pharmacological investigation were released to support the hypoglycemic action and the antinflammatory activity of *L. multifida*. The topical antinflammatory activity of both the ethanolic and acqueous extracts of *L. multifida* was evaluated by Sosa et al. (2005), as inhibition of the croton oil-induced ear edema in mice, supporting the traditional use of the plant as a remedy against inflammatory disorders such as rheumatism, while the hypoglycemic action was evaluated without evidencing any effect (Gamez et al, 1987).

Polyphenol-rich extracts of *L. multifida* L. collected from Morocco were tested for their antioxidant, hypocholesterolaemic and hypotriglyceridaemic activities, showing no significant effect on both plasma total cholesterol and triglycerides profiles in rat; on the contrary, the antioxidant activity was very high, being the half time hemolisis of erythrocytes increased by 479% in the test of inhibition of the AAPH (2, 2'-azobis (2-

amidinopropane) hydrochloride)-induced oxidative erythrocyte hemolysis (Ramchoun et al, 2009).

Many studies on *L. multifida* have been carried out on its essential oils. The antifungal activity of *L. multifida* L. essential oil collected from Portugal was determined against several pathogenic fungi responsible for candidosis, meningitis, dermatophytosis, and aspergillosis (Zuzarte et al, 2012), demonstrating the effectiveness of its essential oil in complementary therapy to treat disseminated candidosis, and suggesting a mechanism of action leading to cytoplasmic membrane disruption and cell death. Furthermore the chemical composition of its essential oils obtained from dried aerial parts was analyzed by gas chromatography/mass spectrometry (GC/MS), showing high contents of monoterpenes, with carvacrol and cis-β-ocimene being the main constituents, and predominance of linalool, camphene, linalyl acetate, α-thujene, bornyl acetate, β-caryophellene, nerol, and terpinolene (Msaada et al, 2012). Antibacterial activity of essential oil of *L. multifida* L. collected from Morocco was tested against human pathogenic gram-positive and gram-negative bacteria by the agar diffusion method, showing a significant inhibitory activity against Staphylococcus aureus, Bacillus subtilis, Proteus mirabilis, Proteus vulgaris, Listeria innocua, Listeria monocytogenes, Escherichia coli CECT and Escherichia coli K12 (Douhri et al, 2014).

The in vitro cytotoxic effect of various Moroccan *L. multifida* extracts (obtained through extraction with hexane, dichloromethane and methanol using Soxhlet) was evaluated on Embryonal Rhabdomyosarcoma cancerous cell lines (RD), Kidney adenocarcinoma of hamster (BSR), and on Monkey kidney cancerous cell lines (Vero). Results from MTT cytotoxicity test indicated different levels of cytotoxic activities towards the three cancer cell lines investigated, and in general a dose-dependent decrease in the survival of of the cancer cells was observed (M'hamed et al, 2016). Furthermore, the antibacterial activity of these extracts was evaluated against three bacterial strains of Genus Rhodococcus, showing a strong growth inhibition effect (M'hamed et al, 2016).

At present, *L. multifida* L. populations are being highly disturbed by increasing clearance, overgrazing and overexplotation, consequently leading to habitat fragmentation into small populations (less than 200 individuals for the most

encountered populations) and to a rapid reduction of genetic diversity (Franklin, 1980; Hamrich and Godt, 1996). The isolation of populations and the decrease of their size caused an increase in genetic drift and the gene fixation, leading to fitness loss (Pearson and Dawson, 2005; Byers et al, 2005; Korner and Jeltsch, 2008). Moreover, the lack of genetic variability caused the reduction of the ability of the plant to adapt to environmental changes (Chograni and Boussaid, 2010).

MATERIALS AND METHODS

In order to perform the analysis described in the present PhD thesis, the following materials and methods have been used, as described below.

Germination experiments

Seeds were collected in 2014, immediately after maturation from two different sites, where the plant spontaneously grows: Almeria (Southern Spain) and Capo dell'Armi (Reggio Calabria, Southern Italy). The sampling involved randomly throughout the subpopulation examined. Clean seeds were stored dry at 4°C. Germination experiments were carried out after 6 months of collection to ensure that the seeds were not dormant. First of all, steps were taken to test for the viability of the seeds; 50 seeds of each lot were selected and treated with a 1% solution of 2,3,5-triphenyltetrazolium chloride (ISTA, 2006). Results of this viability test showed that 80% of the selected seeds was viable. Seeds were selected and surface-sterilized with freshly-prepared 30% (v/v) commercial bleach solution for 15 minutes, followed by washing for several times with distilled and autoclaved water. Twenty seeds were placed on filter papers in Petri dishes (9 cm diameter) and then stratified at dark at 4°C for 72 hours, in order to synchronize germination process. Stratification procedure allows the break of dormancy, so improving the germination process (Budvytyte, 2001). After the stratification process, Petri dishes were placed in a growth chamber at different temperatures (15°C, 20°C, 25°C and 30°C), under a photoperiod of 12 hours light/12 hours-dark, to assess possible differences in germination percentage due to different temperature conditions. Some experiments were also performed at dark, at 20 °C and at 25°C, to evaluate the importance of the light on germination process. For all experimental conditions, five replicates were prepared and the number of germinated seeds was assessed every day over a period of 14 days. Seeds were considered germinated when the radicle was at

least 0.5 cm long. At a temperature of 15 ° C no seed germinated, or this reason, this temperature value was not included in the graphs.

- ✓ *Seed sample extraction*

Seeds at the end of stratification (Time 0) and 4 days after sown (4 days) were homogenized using chilled mortar and pestle, with 0.1 M K-phosphate buffer (pH 7.0) (1:5 w/v), 1 mM Na_2-EDTA, 10 mM $MgCl_2$, and 1% (w/v) PVPP. The extracts were centrifuged at 12.000 rpm for 20 minutes at 4°C. The resulting supernatant was used for all assays. All enzyme activities were measured at 25◦C on a UV, visible light spectrophotometer.

- ✓ *Determination of total proteins*

Total proteins were determined according to method of Bradford (1976) using bovine serum albumin as standard. The concentration of protein was obtained by reading the absorbance at 595 nm against blank and related to the calibration curve. Total proteins were expressed as mg proteins/g fresh weight.

- ✓ *Seed enzyme assay*

Alpha-Amilase (EC 3.2.1.1) was determined at 546 nm as described by Coombe et al. (1967). At 0.3 ml of 1% starch solution was added 0.3 ml of seed-extract; the mixture was incubated in water bath at 30°C for 30 minutes. After 0.6 ml of colour reagent was added and the mixture was boiled for 5 minutes. At the end, 2.5 ml of distillate water were added and the final mixture was cooled for 30 minutes.

Isocytrate-lyase activity (ICL EC 4.1.3.1) was determined according to Bajracharya and Schopfer (1979). At 0.8 ml of buffer K-phosphate 0.1M (pH 7.6) were added: 0.15 ml of phenylhydrazine 33 mM, 0.15 ml of dithiothreitol 50mM, 0.15 ml of magnesium chloride 220 mM and 0.1 ml of seed extract. The mixture was incubated at 25°C for 5 minutes, and after 0.15 ml of isocytrate 175 mM was added. The kinetic was recorded at 334 nm for 180 seconds.

Glucose 6-Phosphate Dehydrogenase (G6P-DH EC.1.1.1.49) activity was determined at 340 nm as reduction of NADP to NADPH. At 0.8 ml of Tris Hcl 86mM were added 0.1

ml of seed-extract, and 0.1 ml of NADP 0,4 mM (freshly prepared) and of 0.1 ml of glucose 6-phosphate 1,2 mM (De Meillon et al, 1990).

Superoxide dismutase (SOD EC 1.15.1.1) activity was estimated by recording the decrease in absorbance of formazan produced from NBT, at 560 nm (Dhindsa et al., 1981). The mixture reaction contained:1.5 ml of 0.1 M potassium-phosphate buffer (pH 7.5) containing EDTA, 15 µl of 13 mM methionine, 15 µl of 50 µM nitroblue tetrazolium, 50 µl extract. Reaction was started by adding 150 µl of 2 mM riboflavin and placing the tubes under two fluorescent lamps for 15 minutes. A complete reaction mixture without extract gave the maximal color after the irradiation, and served as control; while a non-irradiated complete reaction mixture served as 151 a blank. Reaction was stopped by switching off the light and putting the tubes into dark at room temperature.

Catalase activity (CAT, EC 1.11.1.6) was determined according to Beaumont et al. (1990) by monitoring the disappearance of H_2O_2 at 240 nm and calculated by using its extinction coefficient (ε) = 0.036 mM^{-1} cm^{-1}. The reaction mixture contained 1 ml potassium phosphate buffer (50 mM, pH 7.0), 40µl enzyme extract and 5 µl H_2O_2.

Peroxidase activity (POX, EC 1.11.1.7) was determined as reduction in guaiacol concentration by reading the absorbance at 436 nm continuously for 90 seconds. The reaction mixture contained 1 ml potassium phosphate buffer (0.1 M, pH 7.0), 20 µl guaiacol, 40 µl enzyme extract and 15 µl H_2O_2. POX activity was quantified by the amount of tetraguaiacol formed using its extinction coefficient (ε) = 25.5 mM^{-1} cm^{-1} (Panda et al., 2003).

Dehydroascorbate reductase (DHA-Rd, EC 1.8.5.1) activity was assayed following the increase in absorbance at 265 nm owing to the reduced glutathione (GSH) dependent production of ASC (Doulis et al., 1997). The reaction mixture contained 0.1 M K-phosphate buffer pH 6.5, 1 mM GSH and 1 mM DHA (ε =14 $mM^{-1}cm^{-1}$).

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined from the decrease in absorbance at 290 nm, due to oxidation of ascorbate. The reaction mixture was 0.1 M

K-phosphate buffer pH 6.5, 90 mM H_2O_2 and 50 mM ascorbate (Amako et al., 1994). The decrease in absorbance was recorded continuously for 90 seconds (extinction coefficient 14 $mM^{-1}cm^{-1}$).

Glutathione Reductase (GR, EC 1.6.4.2) activity was assayed following the oxidation rate of NADPH at 340 nm (ε = 6.2 mM^{-1} cm^{-1}). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.0), 20 mM GSSG, 2 mM $NADPH_2$, 350 µl H_2O and 50 µl enzyme extract.

Ascorbate (ASC) and dehydroascorbate (DHA) were determined by Kampfenkel (1995). Seeds (1 g) were homogenized in 5% metaphosphoric acid. The homogenate was centrifuged at 13 000 g and the supernatant was used for ASC and DHA analysis.

The effects of treatments on final germination were analysed by fitting factorial generalized linear model (GLM) to the germination data. A statistical analysis was performed with the software SYSTAT v. 8.0 software (SPSS Inc.) using one-way Anova, followed by LSD test to evaluate significant differences in enzymatic activities and antioxidant pathway within species.

Antioxidant and flavonoid profile experiments

- ✓ *Leaf collection and extraction*

The plants of *L. multifida* were collected between March and May 2014 from Capo dell'Armi (Reggio Calabria, Italy). Fresh leaves (0.5 g) were ground using a chilled mortar and pestle, and homogenized in 0.1M phosphate buffer solution (pH 7.0) containing 100 mg soluble polyvinylpolypyrrolidone (PVPP) and 0.1 mM EDTA. The homogenate was filtered through two layers of muslin cloth and centrifuged at 12,000g for 20 min at 4 °C. The same extraction procedures have been performed with fresh leaves of *Lavandula angustifolia* MILLER. The resulting supernatant was used for all assays. All enzyme activities were measured at 25 °C on a UV/VIS spectrophotometer.

- ✓ *Determination of total proteins and enzyme assays*

Determinations of total proteins and the activities of catalase, peroxidase, dehydroascorbate reductase and ascorbate peroxidase were carried out as previously reported for seed analysis.

- ✓ *Total Phenols*

Total phenols were measured by the Box method (Singleton et al, 1999), using Folin–Ciocalteau reagent. The extract from fresh leaves was obtained in H_2O (1:4) and the absorbance was recorded against blank at 765 nm. Tannic acid was used as a reference standard and the results were expressed as microgram tannic acid equivalent (µg TAET/g fresh weight).

- ✓ *Reduced Glutathione*

The reduced glutathione (GSH) was assayed using the method of Jollow et al (1974). Fresh leaves were homogenized in 3% CCl3COOH at 4 °C and the homogenate was centrifuged at 1000g for 10 min at 4 °C. The absorbance was measured at 412 nm and related to a calibration curve of GSH solutions (0 – 500 µg/ml).

- ✓ *Ascorbic and Dehydroascorbic acid*

For ascorbic and dehydroascorbic acid (ASC and DHA), fresh leaves (0.5 g) were homogenized using a chilled mortar in 5% metaphosphoric acid at 4 °C. The homogenate was centrifuged at 39,200g at 4°C and the supernate was used for the determination of ASC and DHA using the method of Law et al (1983).

- ✓ *Total Antioxidant Capacity*

Total antioxidant capacity was measured by the method of Prieto et al (1999). Fresh leaves were homogenized in 50% MeOH and were centrifuged at 4 °C at 12,000g for 20 min; the supernatant was used to measure the absorbance at 546 nm to express the total antioxidant capacity as µg ascorbic acid/g fresh weight.

- ✓ *Tocopherols*

Tocopherols were assayed using the method of Prieto et al (1999). Fresh leaves were homogenized in hexane, the homogenate was centrifuged at 4 °C at 12000g for 20 min; the supernatants were used as described by the method. The absorbance was recorded at 695 nm against an appropriate blank.

- ✓ *Total Carotenoids*

About 50 mg of fresh leaves were incubated in 2.5 ml of 100% EtOH at dark for 24 h at 4 °C; after incubation, the samples were centrifuged for 10 min at 6000g. For carotenoid determination, the absorbance of supernatants was recorded at 649, 665, and 470 nm, and total carotenoid concentrations were obtained using Lichtenthaler's equations (Lichtenthaler, 1987).

- ✓ *Anthocyanins*

For anthocyanin assay, 20 mg of fresh leaves were incubated in 0.5 ml of a MeOH:HCl solution (99:1); samples were centrifuged at 4 °C for 10 min at 6000g and the supernatant was used. The absorbance was recorded at 530 and 657 nm, and anthocyanin content was calculated using the following equation:

[A530nm - (0.025 x A657nm) X ml extract]/g fresh weight.

- ✓ *Tartaric Acid Ester*

Tartaric acid esters were assayed according to Romani et al (1996): the absorbance was recorded at 320 nm against a blank and caffeic acid was used as standard.

- ✓ *RP-DAD-HPLC identification of Flavonoid Components*

Reverse phase-diode array detector-high performance liquid chromatography (RP-DAD-HPLC): Shimadzu system (Shimadzu Ltd., Canby, Oregon, USA), consisting of a LC-10AD pomp system, a vacuum degasser, a quaternary solvent mixing, a SPD-M10A diode array detector, a Rheodyne 7725i injector. Separation of each compound was performed on a 250 mm 9 4.6 mm i.d., 5 lm Discovery C18 column (Supelco, Bellefonte, PA, USA), equipped with a 20 mm 9 4.0 mm guard column. The column was placed in a column oven set at 25 °C. The injection loop was 20 µl and the flow rate was 1.0 ml/min. The spectroscopic determinations have been carried out with UV-1800 CE spectrophotometer (Shimadzu).

The identification of flavonoid components have been carried out with the HPLC system. The mobile phase consisted of a linear gradient of solvent A (MeOH/AcOH/ H_2O 18:1:1) in 2% acidified H_2O (AcOH/H2O 2:98) as follows: 25 – 100% (0 – 20 min), 100% (20 – 25 min), and 100 – 25% (25 – 35 min). UV-Vis spectra were recorded between 200 and 600 nm, and simultaneous detection by diode array was performed at 278 and 325 nm.

- ✓ *Determination of Carvacrol, Carvacrol Methyl-ether and Thymol*

HPLC experiments were performed by a Shimazu system. The mobile phase was an isocratic combination of MeCN/ H_2O (50:50) with a flow rate of 1.0 ml/min. The temperature of the column was 25 °C. Before injection, the samples were filtered with a 0.45 µm filter. UV/VIS spectra were recorded between 200 and 600 nm, and simultaneous detection by diode array was performed at 278 nm for identification of monoterpene phenol derivatives.

- ✓ *Acid Hydrolysis*

Hydrolysis was carried out on the samples according to the procedure reported by Hertog et al (1992). About 6M HCl (10 ml) in a MeOH (25 ml)/ H_2O (10 ml) solution was added to 5 ml of extract to give a solution of 1.2M HCl in 50% aqueous MeOH. Ascorbic acid (50 mg) was added as antioxidant. After refluxing at 90°C for 20 h under stirring, the solution was allowed to cool at room temperature, the solvents were evaporated under reduced pressure, and the residue was suspended in 10 ml H_2O/DMF (1:1). The mixture was filtered through an Iso-Disc P-34 membrane (Sigma-Aldrich, St. Louis, Missouri, USA) and analyzed by HPLC. UV/VIS spectra were recorded between 200 and 600 nm, and simultaneous detection by diode array was performed at 278 and 325 nm.

Cytotoxicity and cytoprotection experiments

- ✓ *Plant collection and preparation of the extracts*

L. multifida leaves from 1 to 1.5 cm long were collected from Capo dell'Armi (Reggio Calabria, Calabria), during spring-summer time. The experiments described in this section were carried out in parallel with leaves of *L. multifida* collected from Siracusa (Sicily) and with leaves of the commercial and widespread species *Lavandula angustifolia* (*L. officinalis*).

The leaves were air-dried at 50ºC until constant weight and processed to obtain aqueous and ethanolic extracts. For the preparation of aqueous extracts, air-dry leaves were macerated and soaked in boiling water for 4 h at 25°C, then the supernatant was collected and the remaining pellet was resuspended in boiling water and reincubated (24 h, 25°C). Thus, both supernatants were pooled, and the resultant mixture was filtered twice as described above, and then evaporated in a rotary evaporator (Buchi Rotavapor R-215) until dryness.

To prepare the ethanolic extracts, air-dried leaves were macerated and shaken with pure ethanol (1:40) for 48 h at 25°C. The resulting mixture was then filtered twice using a nylon net filter with a 100µm mesh size, and concentrated in a rotary evaporator (Buchi Rotavapor R-215).

Prior to being used in the assays, the extracts were filtered using sterile filters of 0.22 µm of diameter.

- ✓ *HeLa cells culture*

Mammalian cancer cell line, HeLa, was grown from 1 mL aliquots of stock culture that had been previously frozen at -170°C in liquid nitrogen. The cells were grown at 37°C in a 95% air/5% CO2 humidified atmosphere, in EMEM culture medium, supplemented with 10% foetal bovine serum (FBS, Life Technologies), 2 mM L-glutamine (Life Technologies), 100 i.u. ml-1 penicillin (Life Technologies) and 100 µg ml-1 streptomycin (Life Technologies). Exponentially growing cells were detached from culture flasks by brief exposure to 0.25% trypsin in PBS, pH 7.2-7.4, according to the standard trypsinization methods. Cell viability was determined by the Trypan blue exclusion test.

- ✓ *HeLa cells cytotoxicity*

Cytotoxicity assays were performed in quadruplicate in 96-well tissue culture plates (Nunc). When cell cultures were confluent, the cells were detached and aliquots of 100 µL containing 1.0 x 10 ^ 4 cells well-1 were dispensed and incubated at 37°C and 5% CO2 for 24 h. Afterwards, the culture medium was replaced by 100µL well-1 of the assayed extracts at the different tested concentrations: 1, 10, 25, 50, 100, 250, 500 and 1000 µg mL-1. Controls received the same volume of culture medium (for aquaeous extracts) or of dimethyl sulfoxide 0.1% (DMSO for ethanolic extracts). Cells were incubated with the extracts for 2 and 24 h at 37°C and 5% CO2, and cell viability was determined by using the MTT assay.

The MTT assay is based on the reduction of the yellow soluble tetrazolium salt (MTT, Sigma) to a blue, insoluble formazan product by the mitochondrial succinate dehydrogenase (Berridge and Tan, 1993; Denizot and Lang, 1986). After exposure of the leaf-extracts, cells were washed with PBS solution and 200µL/well containing 1 mg mL-1 of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were added. After 4h of incubation at 37°C, cells were washed again and the formazan crystals solubilized with 100µL/well of dymethil sulfoxide (DMSO, Sigma). Plates were shacked for 5 min in dark conditions and the absorbance at 570 nm and 690 nm was determined in a microplate reader (BMG Fluostar Omega, USA).

✓ *Antioxidant cytoprotection of extracts on HeLa cell line*

The antioxidant cytoprotective assay was performed as described below, according to the method of Botta et al (2014) with some modifications. Firstly, HeLa cells were exposed to different concentrations of H_2O_2 (Merck) to establish the concentration that decreased the cell viability by 50% (IC50). When cell cultures were confluent, cells were detached and aliquots of 100 µL containing 1.0 x 10 4 cells well-1 were dispensed into 96-well tissue culture plates (Nunc) and incubated at 37°C and 5% CO_2 for 24 h. Then, cells were incubated for 24h with extract concentrations below IC50 of each extract before being treated for 2h with IC50 of H_2O_2. This experiment allowed assessment of the ability of the extracts to offer cytoprotection before a stressing event such as exposure to hydrogen peroxide.

The antioxidant cytoprotective assay described above was also performed after pretreating cells for 2h with IC50 of H_2O_2, followed by incubation for 24 h with extract concentrations below the IC50. In this way it was possible to evaluate the ability of the extracts to offer protection after the oxidative stress (post-oxidative stress cytoprotection).

The cytotoxicity results are given as the percentage of viability of treated cells, compared with that of the control consisting of untreated cells (taken to be 100%). The protective effect of the extracts against the oxidative stress induced by hydrogen peroxide was calculated as the increase in viability expressed as a percentage compared with the hydrogen peroxide-treated controls. Results are expressed as Mean ± Standard Error of at least 3 independent experiments.

✓ *Kinetic assay for catalase of the extract*

The CAT kinetic was determined by the degradation of H_2O_2 using the method described by Aebi (1983). The reaction mixture in a quartz cuvette contained 50 mM phosphate buffer (pH 7.0), 40 mM of H_2O_2 and 50 µl of leaf-extract. The H_2O_2 degradation was monitored at 240 nm for 30 seconds at 25°C and expressed as Abs/min.

✓ *Determination of total phenolic content and flavonoid content*

Total phenol content was determined using the Folin–Ciocalteu method (Singleton et al, 1999). Briefly, 0.1 mL of leaf extract was mixed with 0,5 mL Folin–Ciocalteu reagent (diluted 10 times with distilled water) and 1 ml of distilled water. After 5 min, 1 mL of sodium carbonate solution (7.5%) was added and the mixture was shaken and then incubated for 1 h in the dark at room temperature. The absorbance of the resulting solution was measured at 765 nm. The phenol content was expressed in terms of milligrams of gallic acid equivalents per gram of dry weight (mg GAEg-1 DW).

Flavonoid content in leaf extract was estimated according to the aluminium chloride colorimetric method of Djeridane et al (2006). In this method, 1 mL of diluted leaf extract was mixed with 1 mL of 2% $AlCl_3$ methanolic solution. After incubation at room temperature for 15 min, the absorbance was measured at 430 nm. Flavonoids were calculated from a calibration curve of rutin and expressed as milligrams of rutin equivalents per gram of dry weight (mg REg-1 DW).

Immunostimulant and bactericidal assays

- ✓ *Plant extracts*

Water and ethanolic leaf extracts from Calabrian *L. multifida* have been prepared as indicated in the above section "*Cytotoxicity and cytoprotection experiments".* The experiments have been performed in parallel with leaves of *L. multifida* collected from Siracusa (Sicily) and with leaves of the commercial and widespread species *Lavandula angustifolia* (*L. officinalis*).

- ✓ *Animals*

Thirty specimens (40.51 ± 1.47 gr weight) of the seawater teleost gilthead seabream (*S. aurata* L.), obtained from a local farm (Murcia Spain), were kept in re-circulating seawater aquaria (250 L) in the Marine Fish Facility at the University of Murcia. The water temperature was maintained at 20 ± 2 °C with a flow rate of 900 L h^{-1} and 28% salinity. The photoperiod was 12 h light: 12 h dark. Fish were allowed to acclimatise for 15 days before the start of the trial, where they were fed with a commercial pellet diet (Skretting, Spain) at a rate of 2% body weight day^{-1}. The fish were killed after starving for 24 h by using an overdose of MS-222 (Sandoz, 100 mg mL^{-1} water). All experimental protocols were approved by the Ethical Committee of the University of Murcia (Permit Number A13150104).

✓ *Head-kidney leucocyte isolation and incubation with extracts*

Before the dissection of the head-kidney (HK), the specimens were bled. Blood was collected from the caudal vein and afterwards fish were dissected to obtain HK fragments. For isolation of leucocytes to carry out the assays, HK fragments were transferred to 8mL of sRPMI [RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride (to adjust the medium's osmolarity to gilthead seabream plasma osmolarity of 353.33 mOs), 3% fetal calf serum (FCS, Gibco),100 i.u. mL^{-1} penicillin (Flow) and 100mg mL^{-1} streptomycin (Flow)] (Esteban et al, 1998). Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 mm), washed twice (400 g,10 min), counted (Z2 Coulter Particle Counter) and adjusted to 2 * 10^7 cells mL^{-1} in sRPMI. Cell viability was higher than 98%, as determined by the trypan blue exclusion test. To study the possible effects of water and ethanolic extracts on leucocyte activities, aliquots of 50 µL of the obtained leucocytes suspension containing 2 * 10^7 cells mL^{-1} were dispensed into flatbottomed 96-well microtitre plates (Nunc). Then aliquots of 50 µL $well^{-1}$ of water or ethanolic extracts ranging from 10, 100, 500, to 1000 µg mL^{-1} prepared in sRPMI were added. The extract aliquot was replaced by sRPMI for control samples for assays with water extracts, or by 0,1% dymethil sulfoxide (DMSO, Sigma) in sRPMI in case of control samples for assays with ethanolic extracts. Cells were incubated in presence of the extracts for 24 h at 20 ° C in an incubator with 5% CO2 and 85% humidity. After incubation, leucocyte viability and phagocytic, respiratory burst and peroxidase activities were determined as described below.

✓ *Leucocyte viability*

Aliquots of 100 µL of leucocytes previously incubated for 24h without (control) or with the plant extracts were placed in 5 mL glass tubes (Falcon, BectoneDickinson) and 40 µL of propidium iodide (PI) (400 µg mL^{-1}, Sigma) were added to each sample. The tubes were gently mixed before analysis in a FACScan (BectoneDickinson, Madrid, Spain) flow cytometer with an argon-ion laser adjusted to 488 nm. Analyses were performed on 5000 cells, which were acquired at a rate of 300 cells s^{-1}. Data were collected in the form of two-parameter side scatter (granularity, SSC) and forward scatter (size, FSC), and green fluorescence (FL1) and red fluorescence (FL2) dot plots or histograms were made on a computerized system. Dead cells were estimated as the percentage of cells with propidium iodide (red-PI fluorescent cells).

✓ *Phagocytic activity*

The phagocytic activity of gilthead seabream HK leucocytes was studied by flow cytometry according to Esteban et al (1998). Heat killed (30 min, 60 °C) lyophilized *S. cerevisiae*, strain S288C, were washed twice, counted and adjusted to 10^8 yeast cells mL^{-1} in sRPMI-1640. To label yeast cells with fluorescein isothiocyanate (FITC, Sigma) yeast cells were incubated with 5 µg mL^{-1} FITC at 22 °C with constant stirring (40 cycles min^{-1}) and in darkness for 15 min (Rodriguez et al, 2003). After labeling, free FITC was removed bywashing twice in phosphate buffer saline (PBS) and the yeast cells were resuspended in sRPMI-1640. FITC-labeled yeast cells were acquired for flow cytometric study. The staining uniformity was examined and then the yeast cell suspensions were aliquoted and stored at 4 °C. Samples of 125 µL of medium containing FITC-labeled-yeast cells and 100 µL of HK leucocytes in sRPMI were mixed, centrifuged (400 g, 5 min, 25 °C), resuspended and incubated at 25 °C for 60 min in dark conditions. At the end of the incubation time, the samples were placed on ice to stop phagocytosis and 400 µL of cold PBS was added to each sample. The fluorescence of the extracellular yeast cells (i.e. free yeast cells and yeast cells adhered to phagocytes but not ingested) was quenched by adding 50 µL of cold Trypan Blue (0.4% in PBS) per sample. Standard samples of FITC-labeled *S. cerevisiae* yeast cells or

HK leucocytes were included in each phagocytosis assay. Immediately, the samples were mixed gently, acquired, and analyzed in a FACScan. Data were collected in the form of two-parameter side scatter (SSC) and forward scatter (FSC), and green fluorescence (FL1) dot plots or histograms were made on a computerized system. Fluorescence histograms represented relative fluorescence on a logarithmic scale. Flow cytometer was set to analyze the phagocytic cells gated from all the leucocytes because of their higher SSC and FSC values. Phagocytic ability was defined as the percentage of cells with one or more ingested yeast cells (green-FITC fluorescent cells) within the phagocytic cell population. The relative number of ingested yeast cells per cell (phagocytic capacity) was assessed in arbitrary units from the mean fluorescence intensity of the phagocytic cells.

- ✓ *Respiratory burst activity*

The respiratory burst activity of seabream HK leucocytes was studied by a chemiluminescence method (Bayne and Levy, 1991). Samples of 100 mL of a

PMA/luminol solution [1 ng mL^{-1} phorbol myristate acetate (PMA, Sigma) and 10^{-4} M luminol (Sigma) in HBSS with Ca^{2+} and Mg^{2+}] were added to the HK leucocytes (previously incubated as described above). The plate was shaken and immediately read in a chemiluminometer (BMG, Fluoro Star Galaxy). Measurements were performed in 30 cycles of 2 min each. The kinetics of the reactions were analyzed and the maximum slope of each curve calculated. Control samples containing leucocytes that had not been incubated with the extracts were also analyzed.

- ✓ *Peroxidase content*

The total peroxidase content of HK leucocytes was measured according to Quade and Roth (1997). To do this, 5 µL of HK leucocytes (previously incubated as described above) were incubated for 10 min with 0.02% cetyltrimethylammonium bromide (CTAB, Sigma) at 60 rpm. Afterwards, 100 µL of 10 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, Sigma) and 5 mM H2O2 (both substrates prepared daily) were added and after 2 min, 50 µL of 2 M sulfuric acid was also added to stop the reaction.

The absorbance of the samples was measured at 450 nm in a microplate reader (BMG Fluostar Omega, USA). Control samples containing leucocytes that had not been incubated with extracts were also analyzed.

- ✓ *SAF-1 cell culture*

The established cell line SAF-1 (ECACC n 00122301) was seeded in 25 cm^2 plastic tissue culture flasks (Nunc, Germany) cultured in L-15 Leibowitz medium (Life Technologies, UK), supplemented with 10% fetal bovine serum (FBS, Life Technologies), 2 mM Lglutamine (Life Technologies), 100 i.u. mL^{-1} penicillin (Life Technologies) and 100 µg mL^{-1} streptomycin (Life Technologies). Cells were grown at 25 °C under humidified atmosphere (85% humidity). Exponentially growing cells were detached from culture flasks by brief exposure to 0.25% of trypsin in PBS, pH 7.2-7.4, according to the standard trypsinization methods. The detached cells were collected by centrifugation (1000 rpm, 5 min, 25 °C) and the cell viability was determined by the trypan blue exclusion test.

- ✓ *Cytotoxicity assay on SAF-1 cell line*

Cytotoxicity assay was performed in quadruplicates. When SAF-1 cell lines were approximately 80% confluent, they were detached from flasks culture with trypsin (as described before), and aliquots of 100 µL containing 50000 cells $well^{-1}$ were dispensed in 96-well tissue culture plates and incubated (24 h, 25 °C). This cell concentration was previously determined in order to obtain satisfactory absorbance values in the cytotoxic assay and avoided cell overgrowth. After that, the culture medium was replaced by 100 µL $well^{-1}$ of the extracts to be tested at the appropriate dilution. Tested concentrations of water and ethanol extracts ranged from 1 to 1000 mg mL^{-1} (1, 10, 100, 1000). Cells were then incubated for 24 h. Control samples received the same volume of culture medium (for water extracts) or of DMSO 0.1% (for ethanolic extracts). Cells were incubated for 24 h at 25 °C and then their viability determined using the MTT assay.

The MTT assay is based on the reduction of the yellow soluble tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) (MTT, Sigma) into a blue,

insoluble formazan product by the mitochondrial succinate dehydrogenase (Berridge and Tan, 1993; Denizot and Lang, 1986). After incubation with the leaf-extracts, SAF-1 cells were washed with phosphate buffer saline solution (PBS) and 200 µL $well^{-1}$ of MTT (1 mg mL^{-1}) were added. After 4 h of incubation, cells were washed again and the formazan crystals solubilized with 100 µL $well^{-1}$ of DMSO. Plates were shacked (5 min, 100 rpm) in dark conditions and the absorbance at 570 nm and 690 nm determined in a microplate reader.

✓ *Bacteria*

Three pathogenic bacteria for fish (*V. harveyi*, *V. anguillarum* and *A. salmonicida*) and *Escherichia coli*, as control, were used in the bactericidal assays. All bacterial strains were grown from 1 mL of stock culture that had been previously frozen at 80 ° C. *V. harveyi*, *V. anguillarum* and *A. salmonicida* were cultured for 48 h at 25 ° C in Triptic Soy Agar (TSA, Difco Laboratories), and then inoculated in Triptic Soy Broth (TSB, Difco Laboratories), both supplemented with NaCl to a final concentration of 1% (w/v). Bacteria in TSB medium were then cultured at the same temperature, with continuous shaking (100 rpm) during 24 h. *E. coli* was cultured in Luria Bertani Agar (LB Agar, Difco) for 48h at 37 °C and then inoculated in Luria Bertani Broth (LB Broth, Difco). *E. coli* bacteria in LB broth medium were then cultured for 24h at 37°C with continuous shaking (100 rpm). Exponentially growing bacteria were resuspended in sterile Hank's balanced salt solution (HBSS) and adjusted to 1 * 10^8 colony forming units (c.f.u.) mL^{-1}.

✓ *Bactericidal assay*

Bactericidal activity was determined following the method of Stevens et al (1991) with some modifications. Samples of 20 µL of water or ethanolic leaf-extracts previously adjusted to 20, 100, 200, 500, 1000 µg mL^{-1} were added in quadruplicate wells of a U-shaped 96-well plate (Nunc). Hank's balanced solution was added to some wells instead of the extracts and served as positive control. Aliquots of 20 µL of the bacteria previously cultured were added and the plates were incubated for 2.5 h at 25 ° C (in case of *V. harveyi*, *V. anguillarum* and *A. salmonicida*), or at 37 °C (in case of *E. coli*). After that, 25 µL of MTT (1 mg mL^{-1}) were added to each well and the plates were newly incubated for 2h (at the appropriate temperature taken into account the assayed bacteria) to allow the formation of formazan. Plates were then centrifuged (2000g, 10 min), being the precipitates dissolved in 200 µL of DMSO and transferred to a flat-bottom 96 well-plate. The absorbance of the dissolved formazan was measured at 560 nm. Bactericidal activity was expressed as percentage of no viable bacteria, calculated as the difference between absorbance of bacteria surviving compared to the absorbance of bacteria from positive controls (100%).

- ✓ *Statistical analysis*

Results showed are representative of at least three independent experiments and are expressed as Mean ± Standard Error. All assays related to leucocyte activities were performed in duplicate and results were expressed as Mean ± Standard Error for each group (three fish per group). Data were analyzed by one-way analysis of variance (ANOVA), and Tukey post-hoc test was performed in order to make a multiple comparison between experimental groups. Differences were considered statistically significant when $P < 0.05$.

RESULTS AND DISCUSSION

Germination experiments

In this paragraph, the evaluation of the germination capacity of Calabrian *Lavandula multifida* under different environmental conditions (temperature and photoperiod) is described, in comparison with Spanish *L. multifida,* a very widespread plant in different Spanish regions. Germination capacity was assessed at 15°C, 20°C, 25°C and 30°C, under a photoperiod of 12 hours light/dark. Experiments were also performed at dark, at 20°C and 25°C, to evaluate the importance of the light on germination process.

Also, the activity of some hydrolyzing enzymes involved in germination and the antioxidative pathway in seeds of Calabrian *L. multifida* are analysed and discussed, in comparison with Spanish *L. multifida*. Among hydrolyzing enzymes, alpha-amylase, isocytrate lyase, and glucose 6-phosphate dehydrogenate activities are analyzed and discussed. Regarding the antioxidative pathway in seeds, superoxide dismutase and catalase activities, as well as peroxidase, dehydroascorbate reductase, ascorbate peroxidase and gluthatione reductase activities are discussed. Also, ascorbic acid and dehydroascorbate content in dry seeds is presented.

The aim is the understanding of the environmental adaptability and of the germination strategies of Calabrian *L. multifida*, with the objective of defining possible conservation programs for the population reinforcement and reintroduction in Southern Italy.

Germination percentage of Calabrian *L. multifida* seeds did not show significant differences between 20°C and 25°C; but it strongly decreased at 30°C (Figure 1). Spanish *L. multifida* seeds showed a similar trend under the same temperature conditions (Figure 1), even if the maximum percentage of germination of Calabrian *L. multifida* (about 25%) was significantly lower than that of *L. multifida* from Spain (about 70%), and also the speed of germination was significantly lesser in Calabrian *L. multifida* seeds if compared to Spanish *L. multifida*, in all treatments.

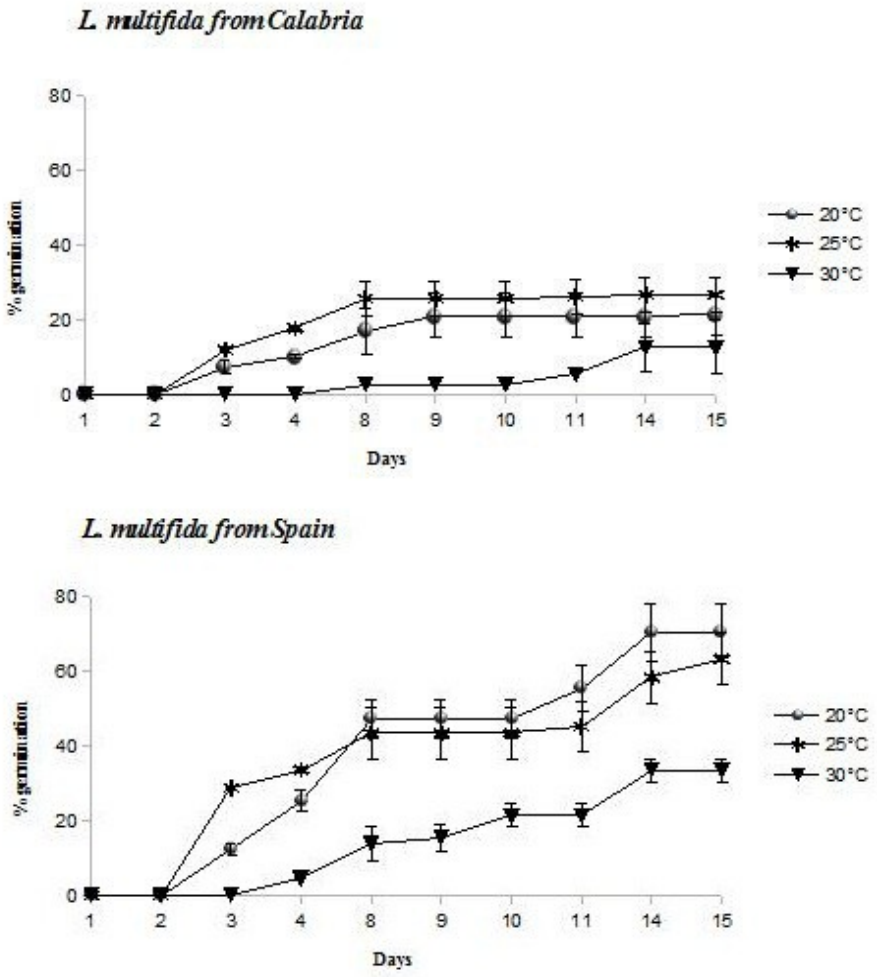


Figure 1. Germination percentages of Calabrian *L. multifida* and Spanish *L. multifida* seeds incubated at 20°C, 25°C and 30°C under a photoperiod of 12 hours light/12 hours dark. Data were expressed as mean ± standard error.

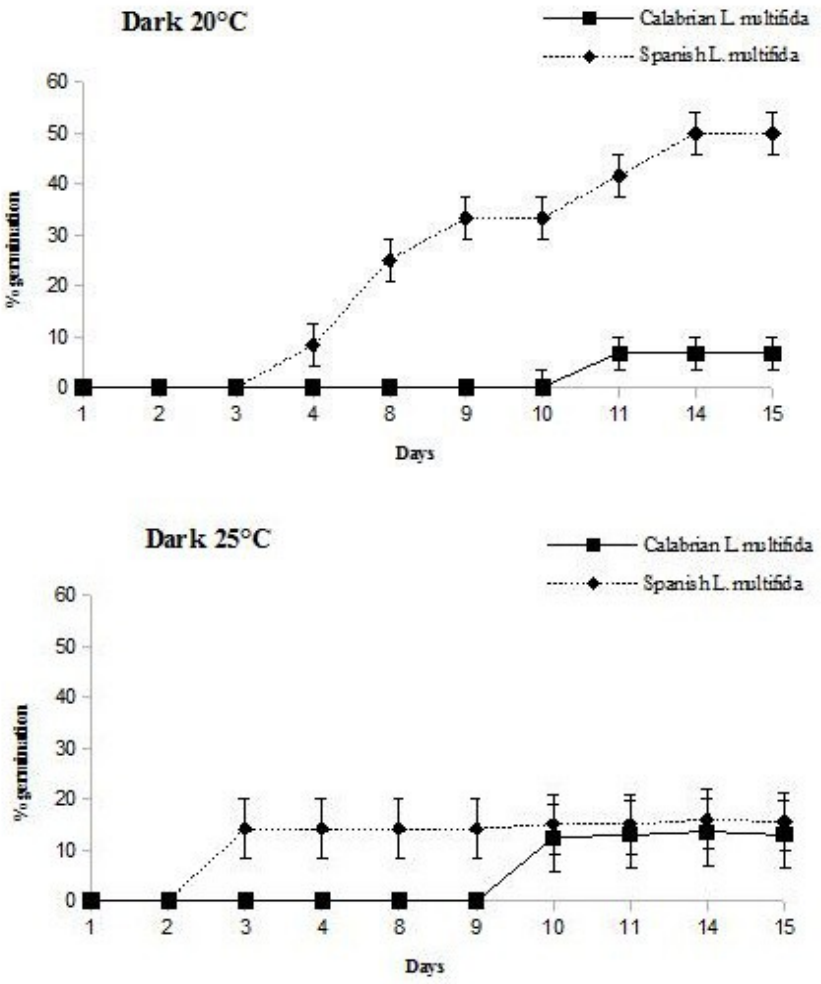


Figure 2. Germination percentages of Calabrian *L. multifida* and Spanish *L. multifida* seeds incubated at 20°C and 25°C under dark conditions. Data were expressed as mean ± standard error.

Germination in both species was strongly affected when the seeds were incubated in the dark. Particularly, a delay in germination process until the 9th day of incubation was observed in Calabrian *L. multifida*. (Figure 2) and also total germination was reduced, with a maximum germination percentage of about 13% for the seeds incubated at 25°C and about 6% for the seeds incubated at 20°C. Conversely, no delay in the germination process was observed in Spanish *L. multifida* seeds (Figure 2), and germination percentage was significantly higher than that of Calabrian *L. multifida* seeds at 20°C, in the dark.

These results showed that temperature and photoperiod differently affect germination process in the two populations of *L. multifida*. Particularly, Calabrian *L. multifida* was more sensitive than Spanish and this different resistance may be important for the environmental adaptability and the distribution of this species. Both *L. multifida* provenances have the maximum germination at 20 and 25°C, with 12 h light/dark alternating, and these findings are in agreement with what has been observed for the Spanish populations of this species (Estrelles et al. 2004). Instead, there are significant differences in germination percentages. This different germination performance can be linked to specific characteristics of the populations, as reported by Menges (1991) who pointed out that seeds of small populations, at limit of their distribution area, have fewer germinative capacity.

The activities of some hydrolyzing enzymes like alpha-amylase and isocytrate lyase (ICL) were assayed on seed extracts of both *L. multifida* populations at the end of stratification (0 day) and four days after sown (4 days). The activity of α-amylase declined during four days in Spanish *L. multifida* while it did not change in the Calabrian lavandula seeds (Table I).

	Calabrian *L. multifida*		Spanish *L. multifida*	
	0 days	4 days	0 days	4 days
α-amylase	178.12 ± 6.7 a	164.35 ± 8.4 a	349.04 ± 10.2 a	160.35 ± 7.5 b
ICL	0.75 ± 0.03 a	0.40 ± 0.02 a	0.32 ± 0.08 b	0.64 ± 0.01a
G6P-DH	0.63 ± 0.02 b	4.71 ± 0.03 a	n.d.	2.65 ± 0.02 a

Table I. Activities of hydrolyzing enzymes in seeds at 0 and 4 days after sown. α-amylase activity was expressed as μg maltose per mg of protein. ICL and G6P-DH activities were expressed as enzyme units (U) per mg of protein. One unit of enzyme was defined as the amount of enzyme necessary to decompose 1 nmoL of substrate per min at 25°C. Data were expressed as mean ± standard error. Small letters indicate significant differences within species. Significance level was set at $P<0.05$.

Utilization of seed reserves is one of the important physiological and biochemical process associated with germination and α-amylase enzyme is responsible for initiating the mobilization of starch in germinating seeds. The development of this activity is important for providing sugars, the main source of energy during the early development of the plant. In Spanish population, the activity of α-amylase decreased over time suggesting a more efficient mobilization and use of reserves compared to Calabrian *L. multifida*.

The activity of Isocytrate lyase was very low and showed an opposite trend between two species (Table I). In Calabrian *L. multifida,* ICL activity lowered during 4 days, while in Spanish *L. multifida* seeds, this activity doubled at time 4 compared to time 0. Isocytrate lyase (ICL) represents a key-enzyme involved in glyoxylate cycle which is essential to convert lipids into carbohydrates during the germination. Lipids are not generally considered to be quantitatively important respiratory substrate in plants

(Eastmond et al. 2000). Also for both *L. multifida* populations, the low activities of ICL suggest that, during germination, carbohydrates are mainly originated from starch degradation, through amylase action, rather than from malate produced by glyoxylate cycle through gluconeogenesis.

One of the first changes upon imbibition is the resumption of respiratory activity that reflects the oxidation of carbohydrates via the respiratory pathway. Infact, glucose 6-phosphate dehydrogenase (G6PDH) is an enzyme of the oxidative pentose-phosphate pathway (OPPP) and it plays an important role in the regulation of germination, at resumption of respiratory activity, providing the cell with reducing power (NADPH). (Come et al., 1988; Perino & Come, 1991). In both populations, G6PDH activity increased overtime during germination, with the highest values in Calabrian one 4 days after sown (Table I). The balance between glycolysis and the OPPP ensures that the seed, during germination, is supplied with the necessary levels of reducing power, ATP and carbon skeletons. The significant difference of enzyme activities involved in the mobilization of energetic reserves between the two *L. multifida* populations may explain the diverse germination speed and germination percentages. However, the relationship between enzyme activity and germination is not always well defined, in fact it is difficult to identify how much of the metabolism occurring during germination is actually necessary for the radicle emergence per se, rather than in preparation for post-germinative events and this means a limited mobilization of reserves during germination compared to reserve utilization following germination (Garnczarska & Wojtyla 2008; Nonogaki et al. 2010).

The reactivation of metabolism may provide an important source of reactive oxygen species (ROS) (Garnczarska & Wojtyla, 2008), in fact germination and ROS accumulation appear to be linked so that seed germination success may be closely associated with internal ROS contents and activities of ROS-scavenging systems (Gomes & Garcia, 2013). ROS regulation might be achieved by changes in concentration of low molecular mass antioxidants, such as ascorbate and glutathione, and by different responses of antioxidative enzymatic systems. As reported for dry orthodox seeds of several angiosperms, the activities of two ROS scavenger enzymes,

superoxide dismutase (SOD) and catalase (CAT), are very low in seeds of both lavandula populations but they suddenly raised during germination (Wojtyla et al. 2006). Results for the two *L. multifida* populations under study showed that ROS-scavenging systems are activated in both populations. SOD and CAT activities increased and the highest activities were detected in Spanish *L. multifida,* after 14 days (Figure 3). The coupled activities of SOD and CAT are of particular importance in maintaining intracellular redox homoeostasis of seeds during germination; SOD acts as the first line of defence converting the toxic superoxide radical into H_2O_2 and CAT detoxifies H_2O_2 to H_2O. Peroxidase (POX) activity, differently from SOD and CAT, was significantly higher in Calabrian than in Spanish *L. multifida* (Figure 3). Peroxidases are a group of nonspecific enzymes that catalyze the oxidation of a wide variety of substrates, using hydrogen peroxide as electron donor, they are involved in several metabolic processes and not exclusively in mechanisms of defence (Biles and Martyn, 1993). Dry seeds of both populations contained moderate amount of ascorbate while DHA was mainly accumulated (Table II). Seeds, at the end of stratification, showed a significant DHA-Rd activity, in particular the Spanish population (Figure 4). This may confirm the role postulated for DHA reduction in producing an initial ascorbic acid supply during germination (De Tullio and Arrigoni, 2003; Hameed et al, 2014). DHA-Rd and APX activities increased, for both populations, up to 14 days (Figure 4). Ascorbate peroxidase (APX) and dehydroascorbate reductase (DHA Rd) are enzymes of ascorbate-glutathione cycle. The increase in DHA-Rd activity, for both populations, over time means a high supply of ascorbate for APX. In fact, the activity of APX was very low in seeds, but gradually increased contributing in H_2O_2 removal and catalyzing the DHA production. According to APX and DHA Rd activities, also GR activity increased over time to reduce oxidized GSSG to GSH. In seeds, glutathione (GSH) could be involved as an antioxidant in direct reactions with free radicals, or in cooperation with ascorbate in the ascorbate-glutathione cycle (Tommasi et al, 2001; Szalai et al, 2009). In all pathways, GSH is oxidized to GSSG and must be rapidly reduced by GR activity. Glutathione Reductase activity (GR) was undetectable in seeds and then increased over time, and the values were similar for both Calabrian and Spanish populations of *L. multifida* (Figure 4).

Figure 3

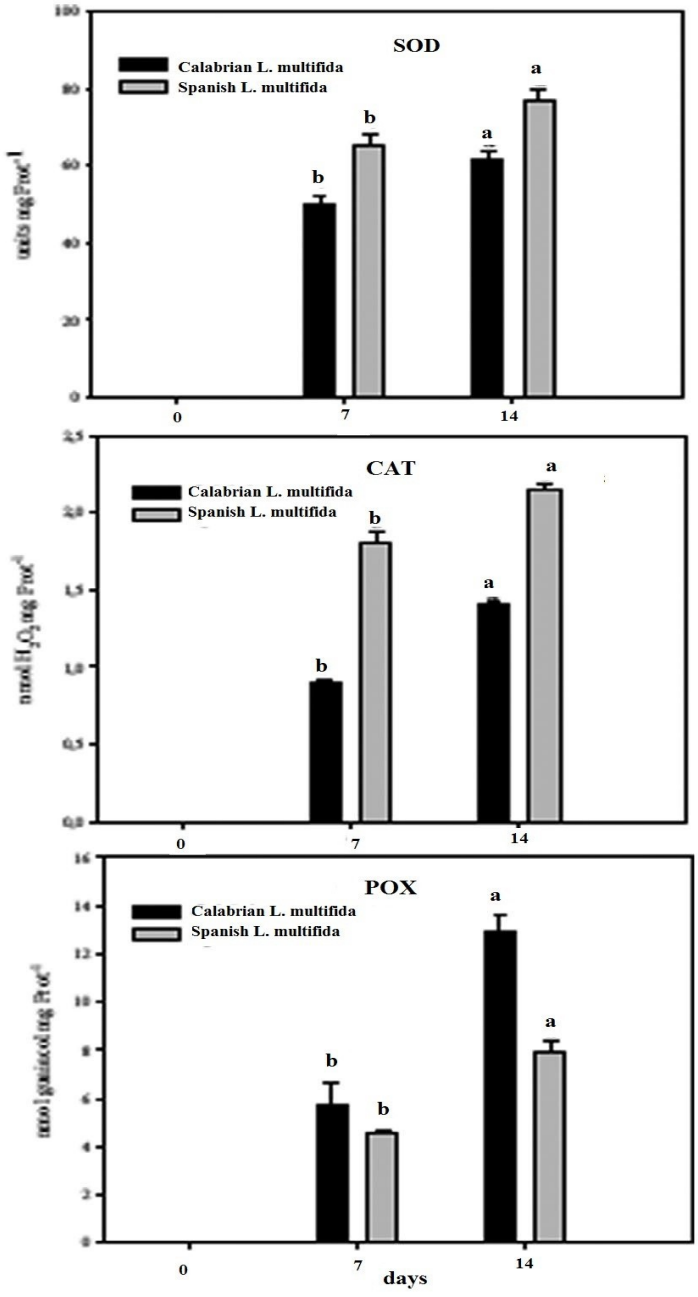


Figure 3. SOD, CAT and POX activity in Calabrian and Spanish *L. multifida* seeds at 0, 7 and 14 days after sown. Data were expressed as mean ± standard error. Small letters indicate significant differences within species. Significance level was set at $P<0.05$.

Figure 4

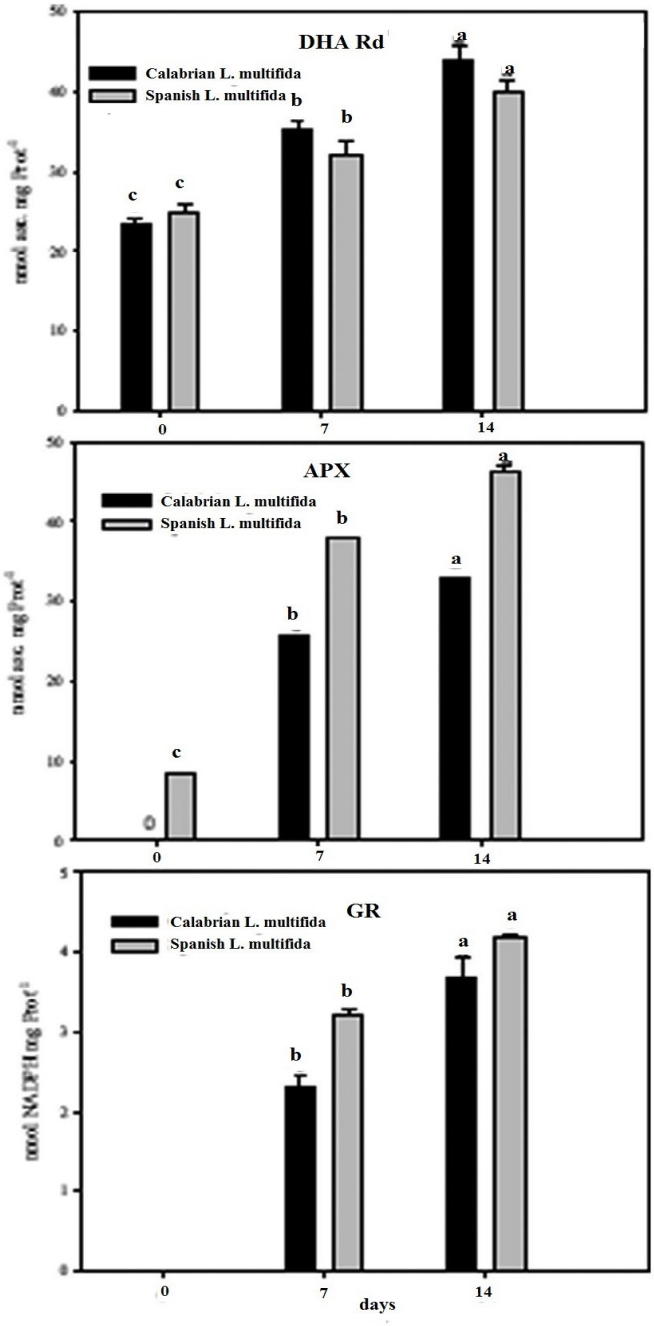


Figure 4. DHA-Rd, APX and GR activity in Calabrian and Spanish L. multifida seeds at 0, 7 and 14 days after sown. Data were expressed as mean ± standard error. Small letters indicate significant differences within species. Significance level was set at $P<0.05$.

	Calabrian *L. multifida*	Spanish *L. multifida*
ASC	23.12 ± 0.96 b	34.87 ± 9.10 a
DHA	120.59 ± 4.35 b	212.15 ± 29.68 a
ASC/DHA	0.19	0.16

Table II. Ascorbic acid (ASC) and Dehydroascorbate (DHA) content in dry seeds. ASC was expressed as μg ascorbic acid g^{-1} F.W.; DHA was expressed as μg dehydroascorbic acid g^{-1} F.W. Small letters indicate significant differences between species. Significance level was set at $P<0.05$.

Results showed that to prevent oxidative damage, the two populations of Lavandula possess a battery of antioxidant enzymes and also antioxidant compounds that are differently involved in seed germination and after emergence of radicle (Smirnoff and Wheeler, 2000; Miller et al, 2010; Foyer and Noctor, 2011). Although ROS were long considered hazardous molecules, their function as cell signalling compounds is now well established. In seeds, ROS have important roles in endosperm weakening, mobilization of seed reserves and may also function as messengers or transmitters of environmental cues during germination (Kwak et al, 2006). It is important that antioxidant systems maintain intracellular redox homoeostasis, preventing the accumulation of toxic amounts of ROS while allowing ROS-signalling function. Therefore, the different activation of antioxidative systems during germination between Calabrian and Spanish populations of *L. multifida* can be also considered as metabolic adaption preparing the seeds to conditions occurring after emergence of radicle. Furthermore, the different behaviour may in part explain the different ability of these two species to respond to external cues. Calabrian population of *L. multifida* shows lower germination ability in comparison with Spanish population. Results from enzymatic assays further confirmed the different behaviour of seeds of the two *L. multifida* populations during germination and early seedling development. Differences

between two populations in antioxidative molecules and enzymes and their activation during germination perform a crucial function in regulation of ROS concentration, but can be also considered a metabolic adaption related to high germination capacity and vigorous seedling development and then can also explain the difference in environmental adaptability of two populations of *L. multifida*. The lower germination ability of peripheral populations, such as the Calabrian *L. multifida*, can be related to the small size of populations and associated inbreeding depression (Lammi et al, 1999).

L. multifida, as a rare and endangered plant in Southern Italy, needs specific actions for the conservation of its populations. Propagation from seed is a viable method for the ex situ conservation of peripheral populations of *L. multifida*, and considering germination requirements, it can be profitably realized only by research facilities or specialized centres.

Antioxidant and flavonoid profile experiments

In the present paragraph, the analysis of some enzymatic and non enzymatic antioxidants and phenolic composition in leaf extract of Calabrian *L. multifida* are presented and discussed, in comparison with the commercial and widespread species *Lavandula angustifolia* (*L. officinalis*). The activities of catalase, peroxidase, dehydroascorbate reductase and ascorbate peroxidase are considered among enzymatic antioxidants, while total antioxidant capacity, tocopherols, ascorbic and dehydroascorbic acid, reduced gluthatione, total phenols, total carotenoids, anthocyanins and tartaric acid esters derivatives are presented among non enzymatic antioxidants. Moreover, the presence of carvacrol, carvacrol methyl-ether, thymol and flavonoid components determined in leaves of Calabrian *L. multifida* are presented.
The aim is to evaluate the ability of Calabrian *L. multifida* to optimize survival strategies in its natural habitat and to withstand environmental stress. Also, the objective is to show possible differences in antioxidant contents between Calabrian *L.multifida* and the commercial species *L. angustifolia*, as well as to improve the knowledge of bioactive compounds present in leaves of Calabrian *L. multifida* which could be helpful for possible pharmaceutical and therapeutical applications.

In fresh leaves of both Lavandula species, Catalase (CAT) activity had very low levels, especially in *L. multifida* (Table III) likely because the plants were in their natural habitat. In fact, catalase activity is generally low under normal growth conditions, while it increases during stress for ROS scavenging (Apel and Hirt, 2004; Muscolo et al, 2011; Lopez-Huertas et al, 2000; Mitler, 2002). Results showed that Peroxidase (POX) activity was significantly higher in leaves of *L. multifida* than in *L. angustifolia* (Table III). POX are involved in several metabolic processes and also in the mechanisms of defense, as they use H_2O_2 as electron donor but, unlike CAT (present only in peroxisomes), are mainly present in cytosol, vacuole, and cell wall (Hamid and Rehman, 2009). Also, the activities of Ascorbate Peroxidase (APX) and

Dehydroascorbate Reductase (DHA Rd), enzymes involved in ascorbate-glutathione cycle, were significantly higher in *L. multifida*, when compared with *L. angustifolia* (Table III). Both enzymes are involved, during normal metabolism, in the control and production of overall ROS level (Apel and Hirt, 2004).

Enzymatic Activities (U/mg Prot)		
	L. angustifolia	*L. multifida*
CAT	7.10 ± 0.3 a	0.00 ± 0.0 b
POX	7.21 ± 1.0 a	61.4 ± 7.4 b
APX	12.81 ± 2.9 a	198.9 ± 21.9 b
DHA-Rd	10.61 ± 0.4 a	28.50 ± 1.3 b

Table III. Analysis of enzymatic antioxidants in leaves of *L. angustifolia* and *L. multifida* grown under natural conditions. Data were expressed as mean ± standard error. Small letters indicate significant differences between species. Significance level was set at $P < 0.05$.

As regards the antioxidant molecules, according to the results on APX and DHA Rd activities in *L. multifida* leaves, the content of reduced glutathione (GSH) and ascorbic acid was significantly greater in comparison with the *L. angustifolia* extract, while dehydroascorbic acid level was similar in both species (Table IV). Ascorbate and glutathione represent the major cellular redox buffers for the maintenance of redox homeostasis in cells under both optimal and stress conditions (Foyer and Noctor, 2005; Szalai et al, 2009). In *L. multifida*, the significant content of ascorbic acid was further supported by high levels of total antioxidant capacity (TAC). Ascorbic acid also has an additional role in protecting or regenerating oxidized carotenoids or lipophilic α-tocopherol radical (Davey et al, 2000). The results showed similar tocopherol amount in extracts of both species, instead carotenoids were significantly more abundant in *L. multifida* leaves (Table IV). Tocopherols are lipid-soluble antioxidants and are considered essential components of the plastid antioxidant network (Shao et al, 2008; Shahidi and Wanasundara, 1992). Carotenoids are known for their radical scavenging properties and also for their important beneficial properties for human health and in preventing diseases (Kähkönen et al, 1999).

Non-enzymatic antioxidants		
	L. angustifolia	*L. multifida*
Total antioxidant capacity [mg ascorbic acid/g F.W.]	1.7 ± 0.1 a	3.2 ± 0.1 b
Tocopherols [µg tocopherol/g F.W.]	413.6 ± 11.1 a	391.0 ± 11.4 a
Ascorbic acid (ASC) [µg ascorbic acid/g F.W.]	23.9 ± 0.4 a	47.9 ± 0.7 b
Dehydroascorbic acid (DHA) [µg dehydroascorbic acid/g F.W.]	85.9 ± 5.4 a	84.2 ± 6.3 a
Reduced glutathione (GSH) [µmoles GSH/g F.W.]	33.5 ± 2.1 a	76.4 ± 2.2 b
Total Phenols [µg TAET/g F.W.]	572.5 ± 4.5 a	262.8 ± 6.5 b
Total carotenoids [mg/g F.W.]	13.3 ± 1.4 a	30.3 ± 2.5 b

Table IV. Analysis of non enzymatic antioxidants in leaves of *L. angustifolia* and Calabrian *L. multifida* grown under natural conditions. Data were expressed as mean ± standard error. Small letters indicate significant differences between species. Significance level was set at $P < 0.05$.

Among phenols, tartaric acid ester derivative content was significantly lower in *L. multifida* than in *L. angustifolia* leaves (Table V), an opposite trend was evident for anthocyanins, with the highest amount in *L. multifida* (Table V). Flavonoids, in particular, anthocyanin pigments provide protection from DNA cleavage, enzyme inhibition, inflammatory processes, and lipid peroxidation (Larson, 1988; Rao and Rao, 2007). However, to properly evaluate the antioxidant power, we must consider the synergic contribution of antioxidants, such as ascorbate, carotenoids, glutathione, flavonoids, rather than the content of a single antioxidant (Ramirez-Tortosa et al, 2001; Barreca et al, 2011).

	L. angustifolia	Calabrian *L. multifida*
Anthocyanins [µg anthocyanin g/F.W.]	6.5 ± 0.3 a	14.5 ± 0.1 b
Tartaric acid esters derivatives [µg caffeic acid g/F.W.]	20.8 ± 0.5 a	4.3 ± 0.1 b

Table V. Quantification of anthocyanins and tartaric acid esters derivatives in fresh leaf extracts of *L. angustifolia* and Calabrian *L. multifida*. Data were expressed as mean ± standard error. Small letters indicate significant differences between species. Significance level was set at $P < 0.05$.

On the whole, these results show a different amount and composition in antioxidant pathways between the two species. In fact, *L. multifida* leaves contain relevant quantity of enzymatic and non enzymatic antioxidants suggesting a remarkable ability to withstand environmental stress and optimize survival strategies in its natural habitat.

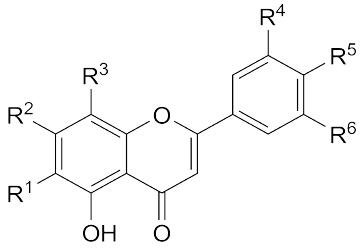
The above results suggest that Calabrian *Lavandula multifida* L. may be a good potential source of antioxidant substances for human health.

In order to shed some light on the secondary metabolites, present in this extract, we performed a RP-DAD-HPLC separation of phenolic monoterpene derivatives of cymene, flavone, and flavanone derivatives. As widely reported, natural monoterpene phenol derivative of cymene (such as thymol and carvacrol, as well as their derivatives) are active secondary metabolites with several useful biological properties (Suntres et al, 2015). Isocratic HPLC determination has been performed to identify the presence of carvacrol (tR = 8.9), carvacrol Me-ether (tR = 9.3), and thymol (tR = 10.5) in the MeOH extract of fresh leaves of *L. multifida* L. The results showed that the peak of carvacrol was present and well defined, while both carvacrol Me-ether and thymol were absent. As far as flavones and flavanones are concerned, they are characterized by the presence of a skeleton composed of two aromatic rings (commonly designated as A and B), which are connected through a pyrone ring (C) in flavones or a di-hydropyrone ring in flavanones (Gattuso et al, 2007). This class of natural products has been, for long time, utilized as the principal active constituents of several drugs, gaining researchers' attention due to their several biological activities (Skibola and Smith, 2000; Veer et al, 2000; Middleton et al, 2000; Rice-Evans et al, 1996).

Two main bands were easily individuated: band I (300 – 380 nm) due to absorption of ring B and band II (240 – 280 nm) due to absorption of ring A. Moreover, the position of these bands supplied unique information about the kind of flavonoid and its substitution pattern (Bonaccorsi et al, 2005; Bonaccorsi et al, 2008). Keeping in mind that both flavanones and flavones have strong absorptions in the 270 – 280 nm region, whereas in the 320 – 330 nm regions only flavones show absorption, a comparison of the chromatograms recorded with detection at 278 and 325 nm allowed for an easy and unequivocal discrimination of the two classes of compounds.

The inspection of each peak and the relative spectrum revealed the presence of only flavones derivatives. In addition, the compounds were studied by UV spectroscopy, by their acid hydrolysis, and subsequent analysis of the aglycones and sugars (Harborne, 1998). In Figure 5, the structures of the identified compounds and their relative t_R are depicted. Compound 1 was characterized by the presence of well-defined bands of absorbance, with a maximum at 269 and 338 nm, which suggested a flavone skeleton

attributable to apigenin. The time of elution and the position of the band suggest the presence of substituent groups on the base skeleton. Moreover, peak 1 remained unaltered following acid hydrolysis, indicating the presence of a C-sugar bond in the molecule, while the other peaks decreased due to the presence of O-sugar bonds. In fact, only C-glycoside compounds are acid hydrolysis-resistant, while O-glycoside ones are easily broken down by the same treatment. On the basis of the excellent agreement of these data with those present in the literature and co-chromatography with authentic standard, compound 1 has been identified as vitexin (apigenin- 8-C-glucoside). Following the same observations, compounds 2 – 7 were identified as the 7-glucoside derivatives of hypolaetin, scutellarein, luteolin, isoscutellarein, apigenin, and chrysoeriol, respectively. These identifications were confirmed by co-chromatography with authentic standard and comparison with literature data (Upson et al, 2000). Compound 8 showed the spectrum of an apigenin derivative, characterized by the presence of OH group probably in the position on C(8). Based on the comparison with literature data and co-chromatography with authentic standard compound 8 was identified as isoscutellarein 8-O-glucoside. In our inspection of chromatographic separation, we also found the presence of the apigenin (9).



Flavone-*C*-glucoside (**1**), flavones-*O*-glycosides (**2** – **8**), and flavone (**9**)

	t_R	R^1	R^2	R^3	R^4	R^5	R^6	Name
1	13.2	H	OH	Glu	H	OH	H	Vitexin
2	13.9	H	Glu	OH	H	OH	OH	Hypolaetin-7-*O*-glucoside
3	14.1	OH	Glu	H	H	OH	H	Scutellarein-7-*O*-glycoside
4	14.2	H	Glu	H	OH	OH	H	Luteolin-7-*O*-glucoside
5	15.4	H	Glu	OH	H	OH	H	Isoscutellarein-7-*O*-glycoside
6	15.7	H	Glu	H	H	OH	H	Apigenin-7-*O*-glucoside
7	15.9	H	Glu	H	MeO	OH	H	Chrysoeriol-7-*O*-glucoside
8	17.5	H	OH	Glu	H	OH	H	Isoscutellarein-8-*O*-glucoside
9	20.5	H	OH	H	H	OH	H	Apigenin

Figure 5. Structures and t_R of the flavonoids (1 – 9) found in MeOH extracts of *Lavandula multifida* L. fresh leaves.

On the whole, the results showed a different amount and composition of some enzymatic and nonenzymatic antioxidants in leaf extracts of Calabrian *Lavandula multifida* in comparison with the commercial species *Lavandula angustifolia* MILLER (*Lavandula officinalis*), suggesting a remarkable ability of this plant to optimize survival strategies and withstand environmental stressful conditions of its natural habitat. The chromatographic characterization of MeOH extract of *L. multifida* leaves has allowed the identification of carvacrol and nine flavones derivatives not previously reported in this plant (apigenin-8-C-glucoside, apigenin, and 7-O-glucoside derivatives of hypolaetin, scutellarein, luteolin, isoscutellarein, apigenin, and chrysoeriol).

These results represent an important contribution to the characterization of Calabrian *L. multifida* as natural source of secondary metabolites with antioxidant properties, which have potential beneficial effects on the human health.

Cytotoxicity and cytoprotection experiments

In this paragraph, the cytotoxic and cytoprotective effects of water and ethanolic leaf extracts of Calabrian *L. multifida* (between 10 µg/mL and 1000 µg/mL) on HeLa cell line are presented and discussed, in comparison with leaf extracts of Sicilian *L. multifida* and of the commercial species *L. angustifolia.* The aim is to demonstrate the ability of *L. multifida* leaf extracts to offer cytoprotection on HeLa cells against chemically-induced oxidative stress, and to demonstrate the possibility to incorporate these extracts in antioxidant formulations for application in dermopharmaceutical and cosmetic fields.

- ✓ *Cytotoxic effects on HeLa cells*

The cytotoxicity test allowed to measure viable cells and dead cells after the treatment of HeLa cells with extracts of L. multifida sp. Cell viability can be defined as the number of healthy cells in a sample, and determines the amount of cells that are living or dead (regardless of phase around the cell cycle), based on a total cell sample. HeLa cells were incubated for 2 and 24 hours with different leaf extracts of Lavandula sp. and cell viability was tested by the MTT assay. The results are presented in Fig. 6a-b (incubation with *L. angustifolia* extracts), Fig. 7a-b (incubation with Calabrian *L. multifida* extracts, indicated as L. multifida C) and Fig. 8a-b (incubation with Sicilian *L. multifida* extracts, indicated as L. multifida S). Incubation of HeLa cells with the two highest concentrations tested of the ethanolic extracts (500 µg/mL and 1000µg/mL) from both Calabrian and Sicilian *L. multifida* produced cytotoxicity after 24 hours. Cell viability was 19.4 % ± 0.7 with the *L. multifida* C extract and 9.9 % ± 1.0 with the *L. multifida* S extract. However, the ethanolic extracts from the commercial species *L. angustifolia* led to a decrease in HeLa cell viability of 50 % after 24 hours' incubation. The IC50 values (concentration of the extracts that reduces the cell viability by 50%)

showed that the *L. multifida* ethanolic extracts had a higher cytotoxic effect against HeLa cells than the commercial species at the highest concentrations tested after 24 hours of incubation: 473.51 ± 23.6 µg/mL and 682.41 ± 34.1 µg/mL (for L. multifida C and L. multifida S, respectively), and 940.33 ± 37.5 µg/mL for *L. angustifolia* extract. Curiously, water extracts from the selected plant species had the opposite effect on HeLa cells, since they seemed to boost cell proliferation and increase the viability of HeLa cells in a dose-dependent manner. The SC50 value (concentration of extract which causes a stimulation of growth or increase in the cell viability in 50% of the cell population) obtained for the incubation of HeLa cells with water extracts of the commercial species *L. angustifolia* was 834.62 ± 41.7 µg/mL while the SC50 of L. multifida C and L. multifida S were 394.1 ± 19.7 µg/mL and 479.4 ± 24.9 µg/mL, respectively.

Fig. 6a

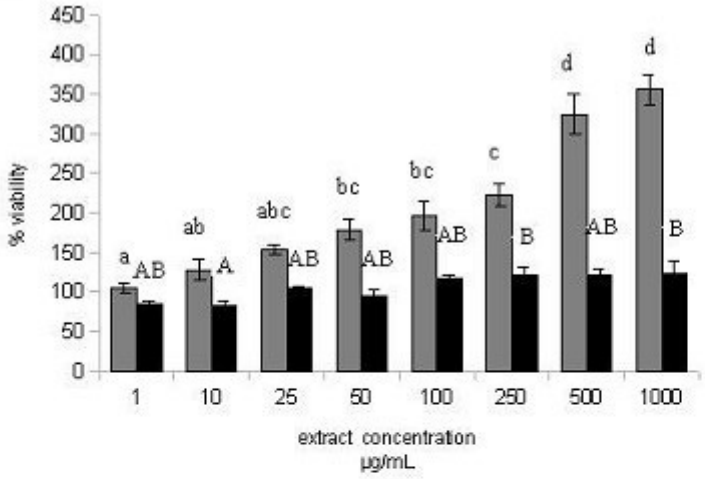


Fig. 6b

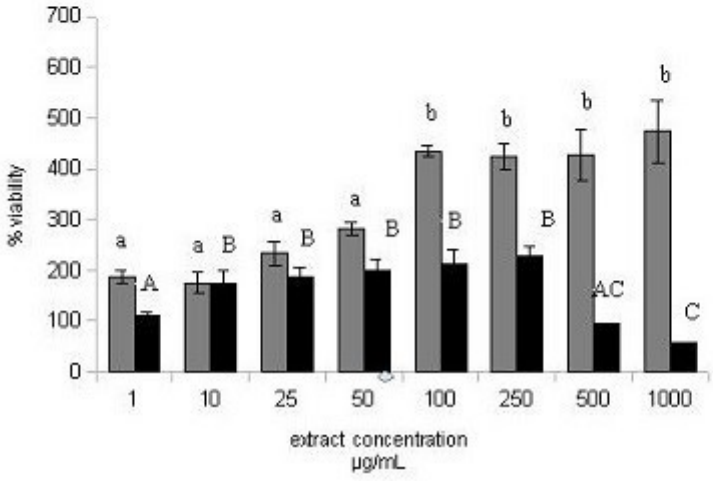


Figure 6 a-b. Percentage of Hela cells viability after incubation with water (grey bars) and ethanolic extracts (black bars) from *L. angustifolia* after 2h (Fig. 6a) and 24h (Fig. 6b) of incubation. Small letters *a, b* and *c* indicate statistically significant differences between different treatment concentration of the water extract, while capital letters (A, B, C) indicate statistically significant differences between different treatment concentration of the ethanolic extract in a post/hoc Tukey analysis ($p<.05$). Results are given as the percentage of viability related to the control of untreated cells (the mean optical density of untreated cells was set to 100 % viability). Data showed are representative of three independent experiments, and are represented as Mean ± Standard Error.

Fig. 7a

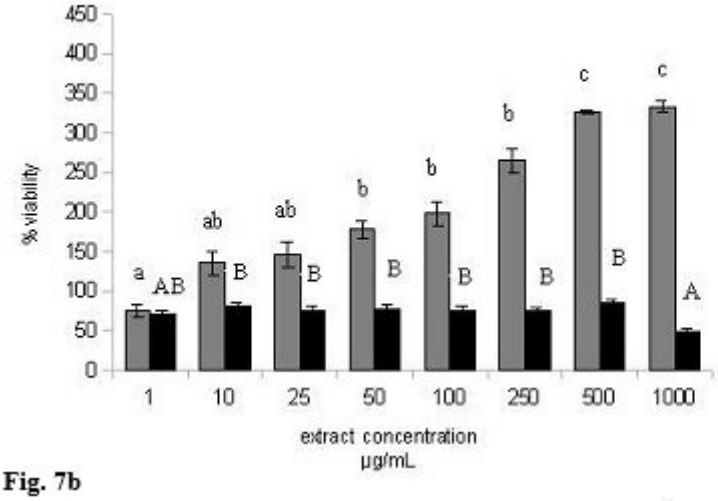


Fig. 7b

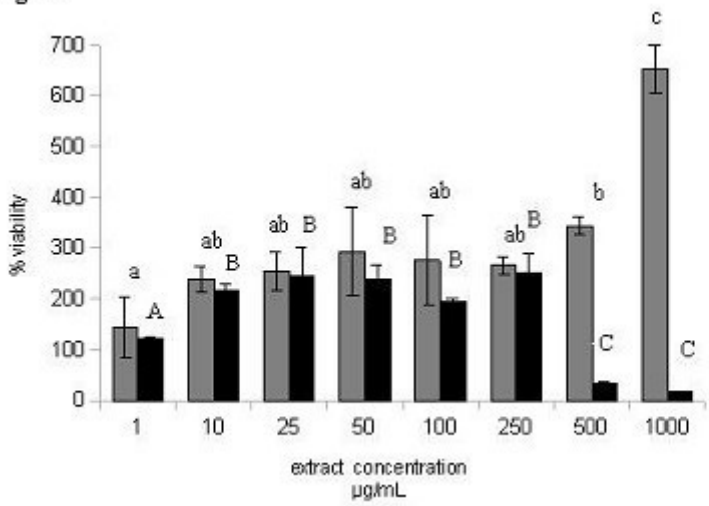


Figure 7 a-b. Percentage of Hela cells viability after incubation with water (grey bars) and ethanolic extracts (black bars) from *L. multifida C* after 2h (Fig. 7a) and 24h (Fig. 7b) of incubation. Small letters *a, b* and *c* indicate statistically significant differences between different treatment concentration of the water extract, while capital letters (A, B, C) indicate statistically significant differences between different treatment concentration of the ethanolic extract in a post/hoc Tukey analysis ($p<.05$). Results are given as the percentage of viability related to the control of untreated cells (the mean optical density of untreated cells was set to 100 % viability). Data showed are representative of three independent experiments, and are represented as Mean ± Standard Error.

Fig. 8a

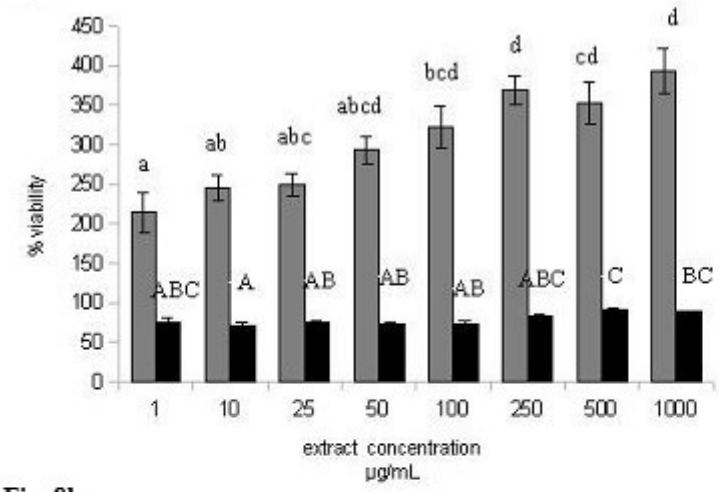


Fig. 8b

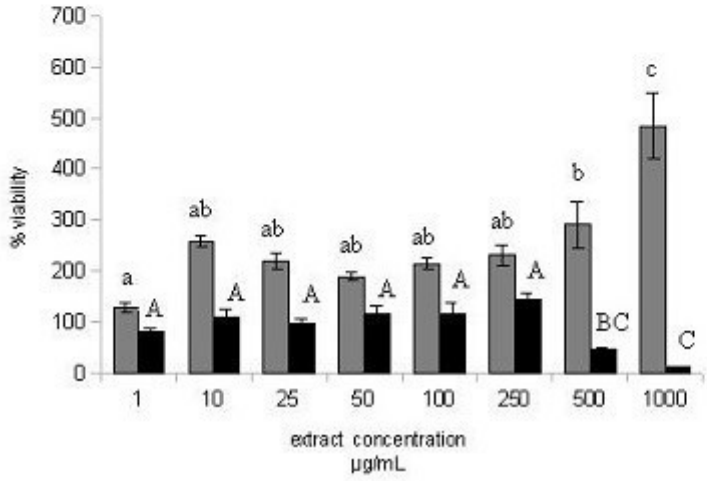


Figure 8 a-b. Percentage of Hela cells viability after incubation with water (grey bars) and ethanolic extracts (black bars) from *L. multifida S* after 2h (Fig. 8a) and 24h (Fig. 8b) of incubation. Small letters *a, b* and *c* indicate statistically significant differences between different treatment concentration of the water extract, while capital letters (A, B, C) indicate statistically significant differences between different treatment concentration of the ethanolic extract in a post/hoc Tukey analysis ($p<.05$). Results are given as the percentage of viability related to the control of untreated cells (the mean optical density of untreated cells was set to 100 % viability). Data showed are representative of three independent experiments, and are represented as Mean ± Standard Error.

HeLa cell line is the first continuous cell line used worldwide for scientific and medical research, because its steady growth makes it an excellent model for almost every process that occurs in human cells (Masters, 2002). Results obtained from cytotoxic test on HeLa cells suggest two different possibility of application for ethanolic and water extracts of *L. multifida*. The reasons for these opposite effects on cell viability could be due to the differences in the biochemical composition and properties of the two extracts used. Furthermore, the results seem to suggest that in both plant extracts there are substances which have a direct effect on the HeLa cell cycle. The present results using the HeLa cell viability agree with those previously obtained using the same cell line after incubation with essential oil, ethanol and petroleum ether extracts from *L. angustifolia.* It was observed that cell growth was inhibited in a concentration and time-dependent manner after 48 h in all these extracts, while the water extracts had no such effect (Tayarani-Najaran et al, 2014). Moreover, in a rat insulinoma cell line (INS-1E) incubated with *Leonurus sibiricus* (Lamiaceae family) water extracts for 24 and 48 h, the number of cells significantly increased compared to the untreated-control cells, supposing a direct effect of water extract constituents on cell proliferation (Schmidt et al, 2013). However, opposite results were obtained when SW742 (colon cancer cell line), MKN45 (gastric cancer cell line) and Hodgkin's lymphoma cells were treated with water extracts from *L. angustifolia* flowers, a significant decrease in cell viability being observed in these cases (Dalilan et al, 2013). These results suggest that different active molecules or different concentrations of the same molecules but at different concentration are present in different parts of this plant (e.g. leaves and flowers). Although this has not been investigated in *L. multifida* extracts until now, an analysis of the chemical composition of essential oils from the leaves and flowers of *L. ssp. stoechas* pointed to differences in the bioactive constituents present in the essential oils from each part of the plant, leading to significant differences in their respective biological activities (Angioni et al, 2006). To the best of our knowledge, there are no previous studies reporting the cytotoxic effects of Italian *L. multifida* leaf-extracts on human cell lines, and most of the studies performed to date have focused on the antibacterial, antifungal and antioxidant properties of *L. multifida* essential oils, as well as on their biochemical constituents (Chograni et al, 2010; Benbelaid et al, 2012;

Zuzarte et al, 2012; Sellam et al, 2013; Soro et al, 2014). The resulting observations can explain why these extracts had been widely used in traditional medicine since ancient times. Essential oils from *L. multifida* collected in Algeria had a remarkable bactericidal effect (Benbelaid et al, 2012). Similarly, essential oils from *L. multifida* collected in Portugal showed fungicidal activity (Zuzarte et al, 2012), while essential oils from *L. multifida* collected from Morocco are used by local people to treat digestive and respiratory infections and suggested the possibility that they may serve as a model for new antibacterial drugs (Soro et al, 2014). Furthermore, *L. multifida* essential oils collected from the South of Morocco displayed strong antioxidant activity as a result of their radical scavenging ability (Sellam et al, 2013). Another study used ethanolic and acqueous extracts from the aerial parts of Moroccan *L. multifida* to investigate their topical antinflammatory activity in inhibiting cCroton oil-induced ear edema in mice, revealing a dose-dependent antinflammatory activity (Sosa et al, 2005). Furthermore, Ramchoun and coworkers (2009) investigated the antioxidant and hypolipidemic effects of polyphenol rich-extracts from Moroccan *L. multifida,* observing good antioxidant and anti-hemolysis activity and an improvement in erythrocyte membrane stability, opening up new possible applications at the anti-atherosclerotic process level.

However, other plant species belonging to Lamiaceae family have been investigated for their cytotoxic and genotoxic effects. For example, *L. stoechas* extracts from flowers significantly reduced the mitotic index (number of dividing cells) at overdosis concentrations (80g/L and 120 g/L), inducing chromosome and mitotic aberrations, pole deviation and micronuclei in *Allium cepa* root tip meristem cells, causing cytotoxicity probably via inducing membrane damage (Çelik and Aslantürk, 2007). Lavender (*L. officinalis*) acqueous extracts affected cell proliferation and inhibited cell growth through necrosis in MKN45 cell line, pointing to the interesting anticancer characteristics of the extracts (Zamanian et al, 2011). Also, the antiproliferative effect of *L. angustifolia* essential oils on human mouth epidermal carcinoma (KB) and murine leukemia (P388) cell lines was assessed using the MTT assay, the oils showing their good potential for cancer treatment (Manosroi et al, 2006). Acetone extracts from *L. spica* L. and *L. x intermedia* L. were investigated for their cytotoxic activity on the

HeLa and Vero cell lines, the cytotoxic effect on Vero cell line being stronger than that on HeLa (Berrington and Lall, 2012). These results suggest that the cytotoxic activity depends not only on the type of extract used but also on the tumour cell line used in the assays.

✓ *Antioxidant cytoprotective assay*

Overnight incubation of HeLa cells with ethanolic extracts from *L. multifida* or *L. angustifolia* at concentrations between 50 and 300 µg/mL (below the IC50), before exposure to H_2O_2, resulted in a significant modulation of HeLa cell viability (Fig. 9 a-b). A significant increase in cell viability was observed after incubation of HeLa cells with ethanolic extracts from both plant species at all tested concentrations (Fig. 9a), compared with control cells treated with IC50 H_2O_2 concentration. Similar results were obtained when HeLa cells were incubated overnight with water extracts at concentrations ranging from 10 to 1000µg/mL (Fig. 9b). However, a dose dependent effect was not observed, and no significant differences were detected between the different plant extracts at the tested concentrations. Preincubation of HeLa cells for 2h with H_2O_2 IC50 and then incubation for 24h with ethanolic or water extracts did not offer cytoprotection in any case (data not shown), meaning that the extracts can only have the desirable prevention effect if used before H_2O_2. For these assays, the protective effect of the extracts was calculated as the percentage of viability increase referred to hydrogen peroxide-treated controls. The percentage of protection ranged from 14,4 to 38,9% for the water extracts from *L. multifida* and *L. angustifolia*, and from 23,8 to 53,3% in case of ethanolic extracts of all plant extracts from *L. multifida* and *L. angustifolia* (Table VI).

% Cytoprotection of ethanolic extracts			
Concentration (µg/ml)	***L. angustifolia***	***L. multifida C***	***L. multifida S***
50	31,8 ± 1,6	29,6 ± 1,5	35,9 ± 1,8
100	28,8 ± 1,4	27,3 ± 1,4	37,4 ± 1,9
200	43,0 ± 2,1	32,6 ± 1,6	36,9 ± 1,8
300	44,8 ± 2,2	23,8 ± 1,2	53,3 ± 2,7
% Cytoprotection of water extracts			
Concentration (µg/ml)	***L. angustifolia***	***L. multifida C***	***L. multifida S***
10	36,1 ± 1,8	37,0 ± 1,9	24,1 ± 1,2
100	14,4 ± 0,7	36,0 ± 1,8	15,2 ± 0,8
500	21,0 ± 1,0	38,0 ± 1,9	23,0 ± 1,2
1000	22,0 ± 1,1	38,9 ± 1,9	47,4 ± 2,4

Table VI. Percentage of cytoprotection of ethanolic and water extracts obtained from *L. angustifolia*, and from Calabrian and Sicilian *L. multifida* (indicated in the table as L. multifida C and S). The percentage of cytoprotection was calculated as the difference between the percentage of viability of cells pretreated with extracts and then treated with IC50 H_2O_2 and the percentage of viability of cells treated with IC50 H_2O_2 but not pretreated with extracts.

Fig. 9a

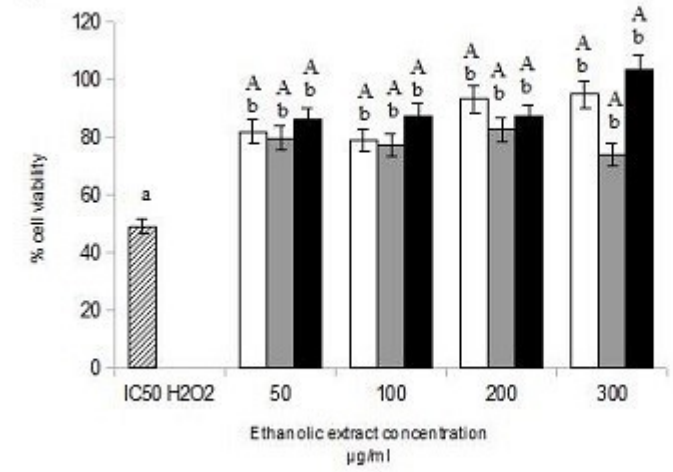


Fig. 9b

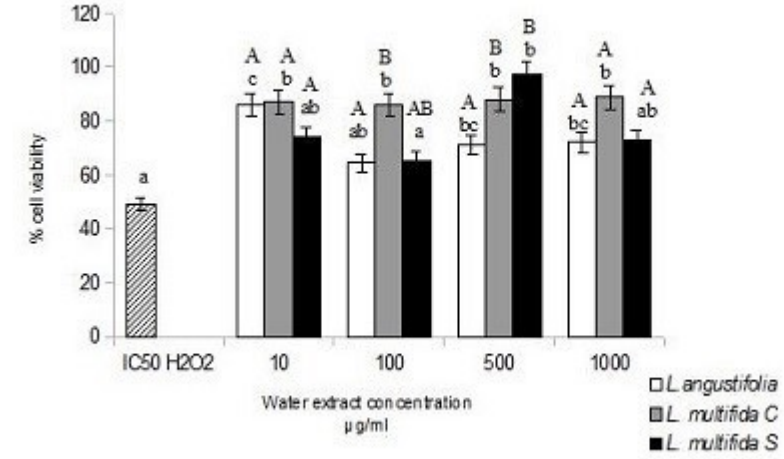


Figure 9 a-b. Percentage of cell viability after treatment with ethanolic (9a) and water extracts (9b) from Calabrian and Sicilian *L. multifida* (indicated in the figure as L. multifida C and S) and from *L. angustifolia* referred to hydrogen peroxide-treated control. Small letters indicate significative differences between each plant extract treatment and hydrogen peroxide-treated control. Capital letters indicate significative differences between different plant extracts at the same treatment concentration in a post/hoc Tukey analysis. P-value was set as P<.0,05.

The search for natural plant-derived compounds aims to obtain antioxidants of natural origin which could protect cells from oxidative damage and so act as alternatives to synthetic antioxidants; in this respect, plants are recognized as important sources of natural antioxidants (Na and Bae, 2011). For the first time the cytoprotective effects of leaf extracts obtained from Calabrian *L. multifida* have been investigated, in comparison with leaf extracts obtained from Sicilian *L. multifida* and from *L. angustifolia*. The antioxidant capacity of the extracts on HeLa cells was determined using H_2O_2 as an oxidant agent. Several studies have demonstrated that cell lines can be considered very sensitive models for studying stress-induced cell damage as well as for understanding the action mechanism of drugs (Piga et al, 2005; Guan et al, 2006; Mandraju et al, 2008). The present results related to the antioxidant cytoprotective assay show that treatment of cultured HeLa cells with water or ethanolic extracts of *L. multifida* exerts a significant cytoprotective effect against a chemically-induced condition of oxidative stress.

Similar results were reported for *L. viridis* extracts, whose neuroprotective effects were assessed against oxidative damage induced by H_2O_2 in A172 human astrocyte cells. *L. viridis* extracts protected astrocytes against H_2O_2 and reduced intracellular ROS accumulation (Costa et al, 2013). Such extracts may therefore have beneficial effects against oxidative damage associated with neurodegenerative diseases. Extracts from *L. coronopifolia* were also assessed for their protective potential in oxidative stress-mediated cell death induced by ethanol, a known hepatotoxin in human hepatocellular carcinoma (HepG2) cells. The results showed that pretreatment of cells for 24 h with these extracts significantly inhibited ROS generation, thus showing hepatoprotective activity against ethanol-induced damage in HepG2 cells (Farshori et al, 2015). Similarly, when the protective effect of phenolic-enriched extracts from *L. dentata* was assessed in HepG2 cells, they were seen to offer effective cytoprotection against ROS produced by potassium dichromate incubation, the percentage of protection being between 20 and 30% (Pereira et al, 2013). Until now, the cytoprotective effect of *L. multifida* extracts from Southern Italy has never been studied in a cell model. The protective effects of the extracts against the oxidative injury, caused by exogenous H_2O_2,

could be due to the capacity of the extracts to scavenge intracellular ROS production but not to neutralize H_2O_2 itself.

- ✓ *Kinetic assay for catalase of the extract*

To further deepen the underlying mechanism of the antioxidant activity, kinetic assays for catalase were carried out. In order to establish whether or not incubation with the extracts offered cytoprotection by directly neutralizing the oxidative agent used in the assay (H_2O_2), the kinetic for catalase of each extract used was determined spectrophotometrically. No degradation of the H_2O_2 by any of the tested extracts was recorded, the Abs min^{-1} being zero in all cases (data not shown). These results suggest that the protective effect that the extracts conferred to the HeLa cells was not caused by the neutralizer effect of the extracts on H_2O_2, suggesting that other intracellular mechanisms are involved in the cytoprotective effect.

✓ *Determination of total phenolic content and flavonoid content*

As showed in the table below (Table VII) *L. angustifolia* water and ethanolic extracts of *L. angustifolia* have the highest content of phenols and flavonoids, if compared with *L. multifida* extracts obtained from *L. multifida* populations. Both total phenols and flavonoids showed had higher concentrations in ethanolic extracts than in water extracts in all plants tested. Anyway, no significant differences were observed in the amount of total phenols and flavonoids between the two populations of *L. multifida*, neither in ethanolic nor in water extracts.

	TOTAL PHENOLS (mg GAE g^{-1} DW)		**FLAVONOIDS** (mgRE g^{-1} DW)	
	Ethanolic extract	Water extract	Ethanolic extract	Water extract
L. Multifida C	51.27 ± 0.38 a*	22.45 ± 0.11 a	19.69 ± 0.56 a*	9.030 ± 0.16 a
L. Multifida S.	46.55 ± 0.51 a*	25.01 ± 0.30 a	21.02 ± 0.11 a*	11.54 ± 0.34 a
L.Angustifolia	89.94 ± 0.11 b*	36.26 ± 0.38 a	29.02 ± 0.42 b	20.47 ± 1.00 b

Table VII. Quantitative determination of Total Phenols and Flavonoids in ethanolic and water extracts obtained from Calabrian and Sicilian L. multifida. L. angustifolia was used as control plant. Small letters indicate significant differences between the different plant species in the same extract (ethanolic or water) in a post/hoc Tukey analysis, while * indicates significant differences between ethanolic and water extract in the same plant species. P-value was set as $P<0.05$.

It is known that natural phenolic compounds are able to regulate the intracellular redox balance through a variety of processes, so the cytoprotective effect of *L. multifida* leaf-extracts may reflect the presence of these compounds in the used extracts. Accordingly to this, and as reported in the Table VII, the higher amount of flavonoids and total phenols in ethanolic extracts from *L. multifida* may explain the higher percentage of cytoprotection which characterizes the ethanolic extracts comparing to the water extracts, this percentage ranging from 23,8 to 53,3% in case of ethanolic extracts, and from 14,4 to 38,9% in case of water extracts. Infact, flavonoids are able to exert a strong cytoprotective effect in correlation with their ability to act as classical electron (or hydrogen) donating antioxidants in vitro (Potapovich and Kostyuk, 2003), to reduce lipid peroxide production, to improve the activity of antioxidant enzymes, and to reduce DNA damage and inhibit apoptosis (Ishige et al, 2001). The cytoprotective effect of flavonoids has also been demonstrated by measuring the degree of protection against H_2O_2–induced damage in human Jurkat cells (Zhang et al, 2006), in Hep-G2 cells (Cheng et al, 2016), and in PC12 cells (Dajas et al, 2003). Also, the potential of phenols in affording protection to cellular DNA from an oxidative challenge has been assessed in a neuroblastoma IMR-32 cell line and in the histiocytic lymphoma U937 cell line, showing significant neuroprotective efficacy (Young et al, 2008). Anyway, even if many studies on the cytoprotective potential characterized the flavonoids as direct scavengers of free radical species, thereby reducing the levels of oxidative damage to cell biomolecules, and attenuating the overall loss of cell function (Cheng et al, 2016; Dajas et al, 2003; Potapovich and Kostiuk, 2003; Schroeter et al, 2001), some other studies highlighted the potentially toxic effects due to the excessive presence of flavonoids, such as cytotoxic agents, mutagens, prooxidants, inducers of apoptosis, and inhibitors of key enzymes involved in hormone metabolism (Skibola and Smith, 2000; Susanti et al, 2007; Wätjen et al, 2005). This could explain the cytotoxic effect that high concentrations of the ethanolic extracts (1000 µg/mL) from all plants tested exerted on Hela cells, resulting in a strong decrease in the percentage of living Hela cells (Fig. 1b, 2b, 3b). Supporting this, high concentrations of flavonoids induced cytotoxicity, DNA strand breaks, oligonucleosomal DNA fragmentation and caspase activation in H4IIE rat

hepatoma cells, confirming that flavonoids can give cytoprotection on one hand and induce cellular damage on their own on the other hand (Wätjen et al, 2005).

The model of oxidative stress in cultured cells used in this study represents a suitable method for evaluating the antioxidant defence mechanisms of cells and also for assaying the possible chemopreventive effects of plant extracts.

The present section provides information on the cytotoxic effects of *L. multifida* extracts on HeLa cell viability, and describes the differences in cell viability shown by HeLa cells when incubated with ethanolic/water extracts from *L. multifida*. The results suggest different possibilities of application of water and ethanolic extracts, perhaps due to the differences in their biochemical composition. Also, the ability of the *L. multifida* extracts to protect HeLa cells against exogenous H_2O_2 induced-oxidative damage has been demonstrated, thus suggesting that such leaf-extracts could be considered as a source for new active principles in drug design for new antioxidant formulations.

Immunostimulant and bactericidal assays

In the present paragraph, the effects of water and ethanolic leaf extracts (between 10 µg/mL and 1000 µg/mL) of Calabrian *L. multifida* on innate immune system of Sparus aurata (phagocytosis, respiratory burst activity, peroxidase activity) are discussed. Also, the determination of cytotoxic effects of water and ethanolic extract (between 1 µg/mLand 1000 µg/mL) of Calabrian *L. multifida* on SAF-1 cell line is presented. Moreover, the bactericidal effects of water and ethanolic leaf extracts (between 10 µg/mL and 500 µg/mL) of Calabrian *L. multifida* on some fish pathogens (Vibrio harveyi, Vibrio anguillarum, Aeromonas salmonicida) are discussed. All these assays were performed making a comparison with water and ethanolic leaf extracts of Sicilian *L. multifida* and *L. angustifolia*. The aim is to support the possibility to use extracts of Calabrian *L. multifida* as immunostimulant in aquaculture production of Sparus aurata, considering the importance of Sparus aurata in Mediterranean diet and in marine fish aquaculture.

Before performing the experiments to assess the effects of *L. multifida* extracts on innate immune system of *Sparus aurata*, the determination of the head kidney leucocyte viability was performed. Cell viability can be defined as the number of healthy cells in a sample, and determines the amount of cells that are living or dead (regardless of phase around the cell cycle), based on a total cell sample. Head kidney leucocyte viability was tested using propidium iodide (PI, Phenanthridinium, 3,8-diamino-5-[3-(diethylmethylammonio)propyl]-6-phenyl-, diiodide). PI is a red fluorescent intercalating which binds to DNA by intercalating between the bases without sequence preference. As PI is not able to cross the membrane of live cells, it's commonly used to detect dead cells in a population. The information derived from the cell viability assay allowed to know whether the cell population that has been exposed to the leaf extracts was healthy or dying, actively dividing or in stasis.

Head kidney (HK) leucocyte viability was tested after 24 h of incubation with water or ethanolic extracts of the three tested plants in the range of concentrations from 10 to 1000 mg mL^{-1} (10, 50, 100, 250, 500, 750, 1000 mg mL^{-1}). Results demonstrated that HK leucocyte viability was not affected after incubation with all extracts, respect to the values recorded for control HK leucocytes (Fig. 10 a-b).

Fig. 10a

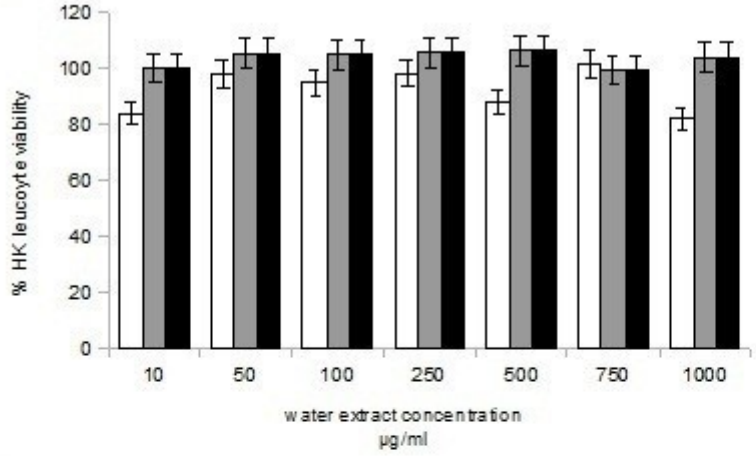


Fig. 10b

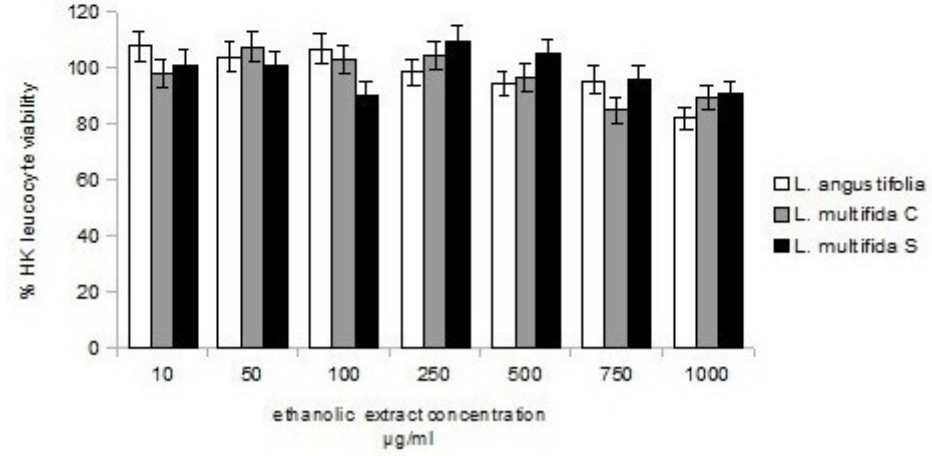


Fig. 10 a-b. Percentage of viability of S. aurata HK leucocytes after 24 h of incubation with water (a) or ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian Lavandula multifida. L. angustifolia was considered as control plant. Results of cytotoxicity are given as the percentage of viability, related to the control of untreated cells (the mean optical density of untreated cells was set to 100% viability). Results showed are representative of at least three independent experiments and are expressed as Mean ± Standard Error.

The immune system of fish is physiologically similar to that of higher vertebrates, despite certain differences. Particularly, in fish the innate response represents an essential component in combating pathogens due to the limitations of the adaptative immune system, their limited repertoire of antibodies and the slow proliferation, maturation and memory of their lymphocytes (Whyte, 2007). Infact, fish are free-living organisms from early embryonic stages of life and depend on their innate immune system for survival (Rombout et al, 2005).

Among the various components involved in fish innate response, phagocytosis is one of the most important. The main cells involved in phagocytosis in fish are neutrophils and macrophages (Secombes and Fletcher, 1992), specifically dedicated to the recognition and elimination of non self material. Particularly, these cells remove bacteria mainly by the production of reactive oxygen species during a respiratory burst. In addition, neutrophils possess myeloperoxidase in their cytoplasmic granules, which in the presence of halide and hydrogen peroxide kills bacteria by halogenation of the bacterial cell wall. Moreover, these cells have lysozymes and other hydrolytic enzymes in their lysosomes (Fischer et al, 2006).

In the present thesis, the phagocitosis process is expressed in two different forms: phagocitic ability and phagocitic capacity. The phagocitic ability represents the percentage of cells wich have ingested yeast cells within the phagocitic cell population, while the phagocitic capacity represents the relative number of ingested yeast cells per phagocitic cell. While the former refers to the phagocitic cell population in total, the latter refers to the phagocitic cell, considered individually.

Incubation of HK leucocytes with water or ethanolic extracts of *L. multifida* (from both Calabrian and Sicilian populations) significantly enhanced their phagocytic ability when using the range of extract concentrations from 10 to 500 mg mL^{-1} and 10-100 mg mL^{-1}, respectively (Fig. 11 a-b). However, after incubation of HK leucocytes with *L. angustifolia* water extracts significant enhancements of the phagocytic ability were only recorded when using highest concentrations (from 100 to 1000 mg mL^{-1}) (Fig. 11a). On

the other hand, incubation of leucocytes with ethanolic extracts from *L. angustifolia* did not affect their phagocytic ability.

On the contrary, significant decreases were observed in the phagocytic ability of HK leucocytes after being incubated with 500 and 1000 mg mL^{-1} of ethanolic extracts from all plant tested, except for 500 mg mL^{-1} ethanolic extract of *L. multifida* S which increased their phagocytic ability (Fig. 11b).

Fig. 11a

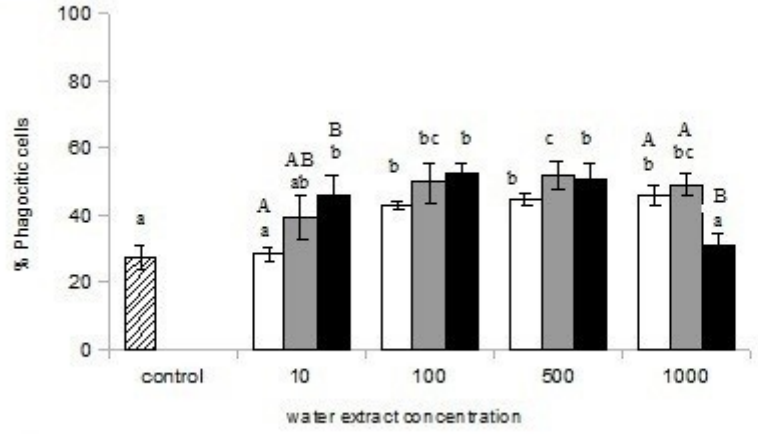


Fig. 11b

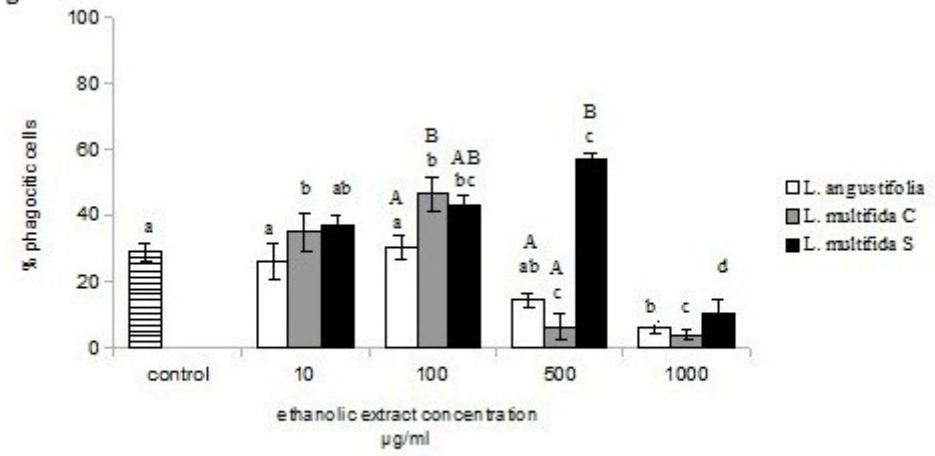


Fig. 11 a-b. Percentage of phagocytic cells of S. aurata HK leucocytes after 24 h of incubation with water (a) and ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian Lavandula multifida. L. angustifolia was considered as control plant. Small letters indicate significant differences between different concentrations of the same plant extract. Capital letters indicate significant differences between different plant extracts at the same treatment concentration. Differences were considered significant when $P < 0.05$. Results showed are representative of at least three independent experiments and are expressed as Mean ± Standard Error.

A similar trend was observed in the phagocytic capacity because HK leucocytes incubated with 10 mg mL^{-1} and 10-100 mg mL^{-1} water and ethanolic extracts from *L. multifida* S, respectively, showed an increased phagocytic capacity (Fig. 12 a-b). Instead, water and ethanolic extracts from *L. angustifolia* did not affect significantly the phagocytic capacity of gilthead seabream HK leucocytes, respect to control samples.

Fig. 12a

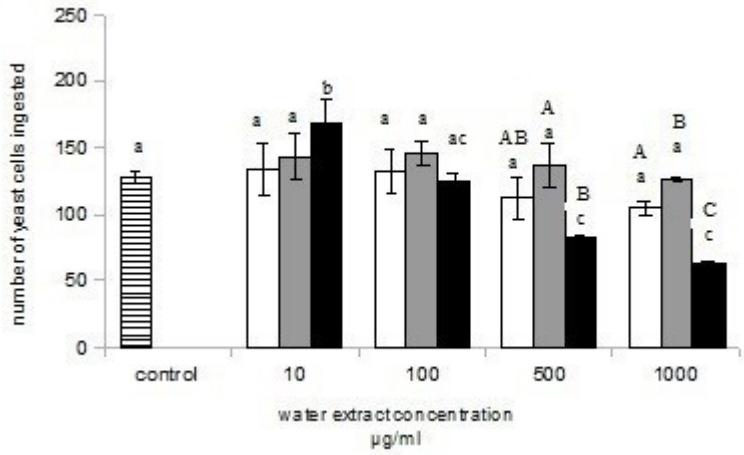


Fig. 12b

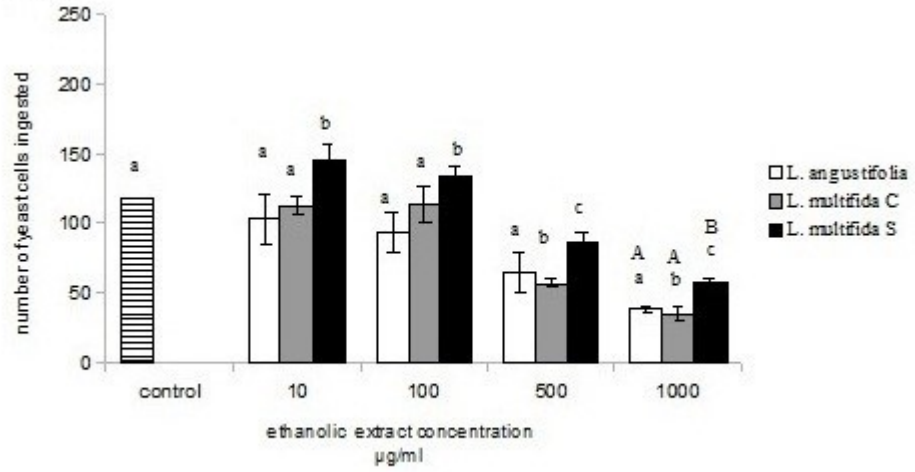


Fig. 12 a-b. Phagocytic capacity of S. aurata HK leucocytes after 24 h of incubation with water (a) and ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian Lavandula multifida. L. angustifolia was considered as control plant. Small letters indicate significant differences between different concentrations of the same plant extract. Capital letters indicate significant differences between different plant extracts at the same treatment concentration. Differences were considered significant when $P < 0.05$. Results showed are representative of at least three independent experiments and are expressed as Mean ± Standard Error.

Once bacteria are phagocited by fish leucocytes, the activation of a host's NADPH-oxidase increases oxygen consumption leading to the generation of ROS such as superoxide anions, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Roch, 1999). All these reactive oxygen species are bactericidal and represent a process known as respiratory burst (Secombes and Fletcher, 1992).

Regarding this, incubation of HK leucocytes with water extracts from all populations of Lavandula tested increased HK leucocyte respiratory burst activity at all concentrations tested in a significant manner, in comparison with the control group, except for *L. multifida* S water extract which decreased significantly this activity when using at 1000 mg mL^{-1} of concentration (Fig. 13 a-b). Also, ethanolic extracts from both populations of *L. multifida* used at 10 and 100 mg mL^{-1} increased the HK leucocyte respiratory burst activity, while decreased the above cited activity compared to the control samples at 1000 mg mL^{-1} (Fig. 13 a-b). The observed enhancements of the respiratory burst activity of leucocytes were always higher after incubation with water extracts than with ethanolic extracts.

Fig. 13a

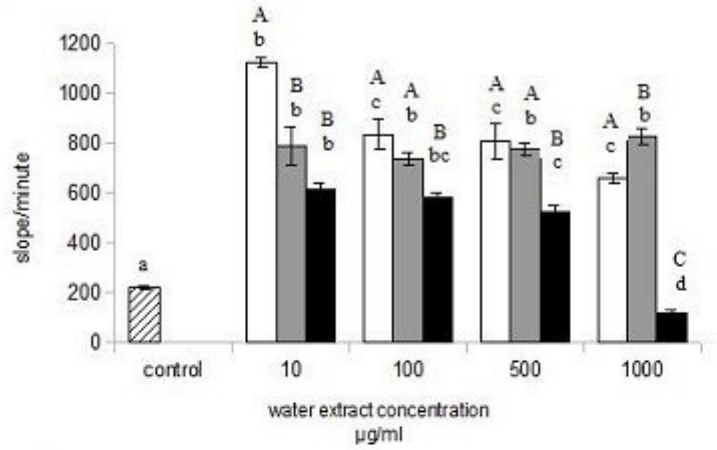


Fig. 13b

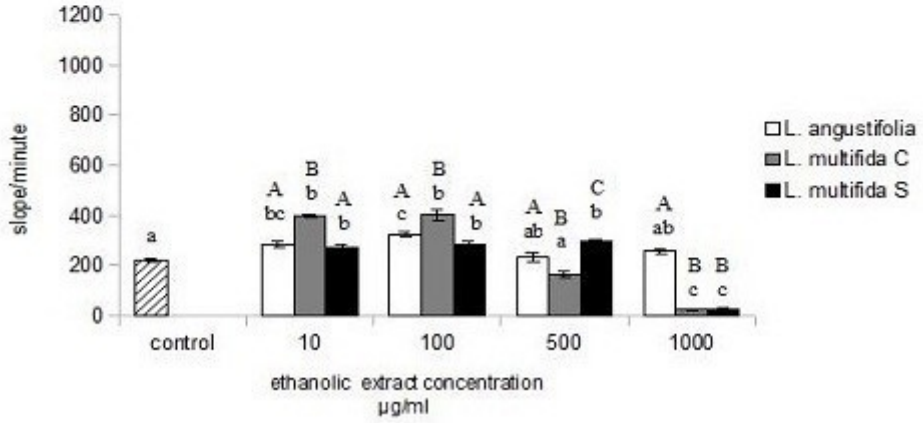


Fig. 13 a-b. Respiratory burst activity (expressed as slope/minute) of S. aurata HK leucocytes after 24 h of incubation with water (a) and ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian Lavandula multifida. L. angustifolia was considered as control plant. Small letters indicate significant differences between different concentrations of the same plant extract. Capital letters indicate significant differences between different plant extracts at the same treatment concentration. Differences were considered significant when $P < 0.05$. Results showed are representative of at least three independent experiments and are expressed as Mean ± Standard Error.

After phagocytosis of microorganisms, leucocytes also release lysosomal enzymes into the phagosome, such as peroxidases (myeloperoxidase and eosynophil peroxidase) which have been widely reported to act as important microbicidal agents in mammals. These peroxidases, in the presence of the H_2O_2 produced during respiratory burst and halide ions, give rise to chlorine and chloramines mediated by the peroxidase-H_2O_2 -chloride oxidative systems (Quade and Roth, 1997; King et al, 1997; Klebanoff, 1998; Ellis, 1999; Hachiya et al, 2000). In addition to this mechanism of intracellular toxicity, these peroxidases and H_2O_2 can be released by exocytosis from the cell, where they are toxic to adjacent microorganisms (Klebanoff, 1998). Therefore, peroxidase activity is assumed to be one of the good markers of leucocyte activation (Rodriguez et al, 2003).
Anyway, some slender variations in the peroxidase content of HK leucocytes were observed after being incubated with water extracts of L. multifida S at 100 µg/mL, and with ethanolic extracts of the three species at 1000 µg/mL, respect to the values recorded for control samples (Fig. 14 a-b).

Fig. 14a

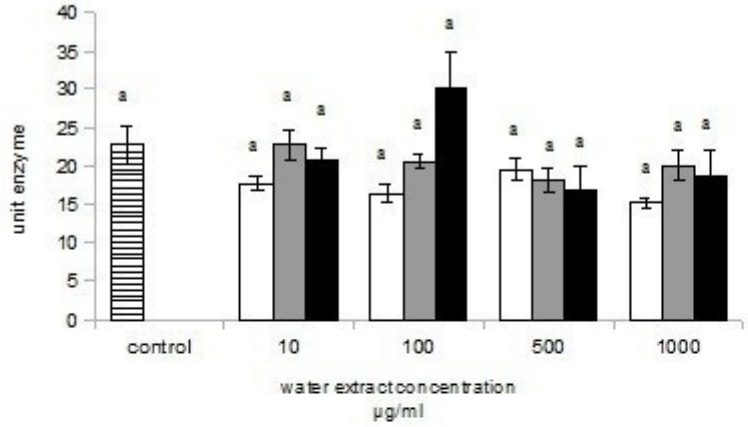


Fig. 14b

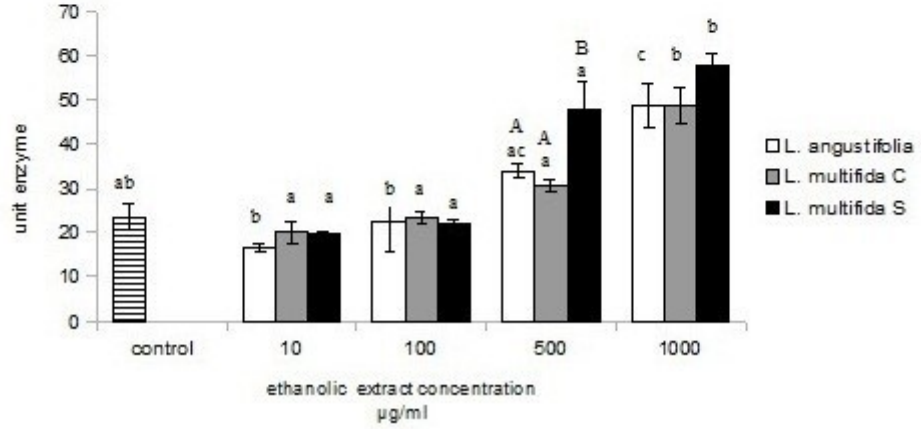


Fig. 14 a-b. Peroxidase activity (expressed as unit enzyme) of S. aurata HK leucocytes after 24 h of incubation with water (a) and ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian Lavandula multifida. L. angustifolia was considered as control plant. Small letters indicate significant differences between different concentrations of the same plant extract. Capital letters indicate significant differences between different plant extracts at the same treatment concentration. Differences were considered significant when $P < 0.05$. Results showed are representative of at least three independent experiments and are expressed as Mean ± Standard Error.

The effects of water and ethanolic extracts of *L. multifida* on viability of SAF-1 cells were also evaluated.

Fish cell lines are increasingly important research tools as an alternative to the experimental animals. The long-term SAF-1 cell line was established in 1996 and it is a fibroblast-like culture derived from gilthead seabream (Bejar et al, 1997). Since then, the SAF-1 cell line has proved useful in many applications and varied studies related to virology (Perez-Prieto et al, 1999; Tafalla et al, 2004; Alonso et al, 2005); Bandin et al, 2006; Garcia-Rosado et al, 2008; Cano et al, 2016), bacteriology (Acosta et al, 2009; El Aamri et al, 2012), immunology (Ray et al, 2002; Pelegrin et al, 2004), and more recently in nanoparticles interiorization (Trapani et al, 2015), metal toxicity (Morcillo et al, 2016) or freshness and freeze-thawing of seabream fillets (Diop et al, 2016) studies. Results from the cytotoxicity test showed that water extracts from all plants tested did not alter significantly the cell viability, respect to values for control samples (Fig. 15). Interestingly, incubation of SAF-1 cells with *L. multifida* S water extract increased the cell viability in a significant manner at the higher concentration tested (1000 mg mL^{-1}) (Fig. 15a).

On the contrary, incubation of SAF-1 cells with ethanolic extracts significantly affect their viability, indeed showing high levels of cytotoxicity at the higher concentration tested (1000 mg mL^{-1}) (Fig. 15b). Particularly, ethanolic extracts from the two populations of *L. multifida* had a significantly higher level of cytotoxicity on SAF-1 cells if compared to the cytotoxicity provoked on these cells after being incubated with *L. angustifolia* ethanolic extracts (Fig. 15). So, the maximum doses used in our experiments (1000 mg mL^{-1}) affected the viability in a significant manner, causing an increase or a decrease in this parameter when SAF-1 cells were incubated with water or ethanolic extracts, respectively. These data agreed with results obtained from the determination of phagocytosis and respiratory burst activities: indeed ethanolic extracts from L. multifida C and S provoked a significant decrease in the number of yeast cells ingested after 24 h of incubation at 1000 mg mL^{-1} of concentration, furthermore also the percentage of phagocytic cells showed a significant decrease when HK leukocytes were incubated with ethanolic extracts from all plants tested during 24 h at 1000 mg mL^{-1} of concentration, and a similar trend was observed also for the respiratory burst activity.

Fig. 15a

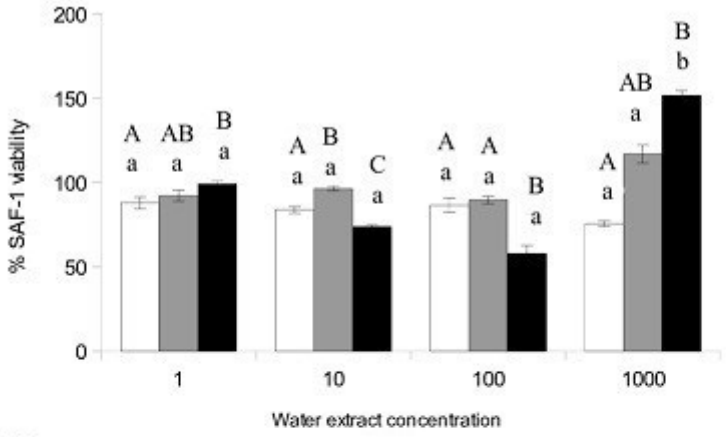


Fig. 15b

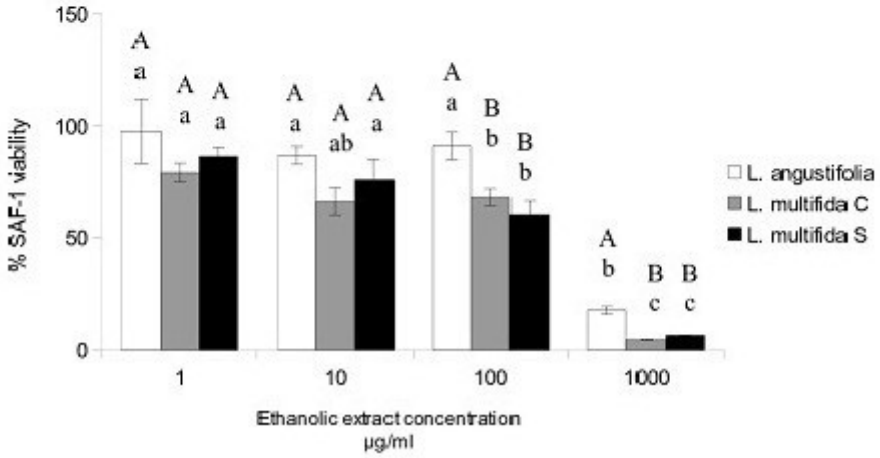


Fig. 15 a-b. Viability (expressed as percentage) of SAF-1 cell line after 24 h of incubation with water (a) and ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian Lavandula multifida. L. angustifolia was considered as control plant. Small letters indicate significant differences between different concentrations of the same plant extract. Capital letters indicate significant differences between different plant extracts at the same treatment concentration. Differences were considered significant when $P < 0.05$. Results showed are representative of at least three independent experiments and are expressed as Mean ± Standard Error.

L. multifida plant extracts were also tested (in comparison with the extracts from the commercial species *L. angustifolia*) for their bactericidal activity against some fish pathogens relevant in fish aquaculture: *V. harveyi*, *V. anguillarum* and *A. salmonicida*, being *E. coli* used as control bacteria. Results from bactericidal assays revealed that any of the assayed plant extracts neither water nor ethanolic affect bacteria viability in a significant manner (Figs. 16-19). Only a slight difference in bacteria viability (80% cell viability) was observed when ethanolic extracts from *L. multifida* C were used to incubate *V. harveyi* bacteria cells (Fig. 16 a-b).

Fig. 16a

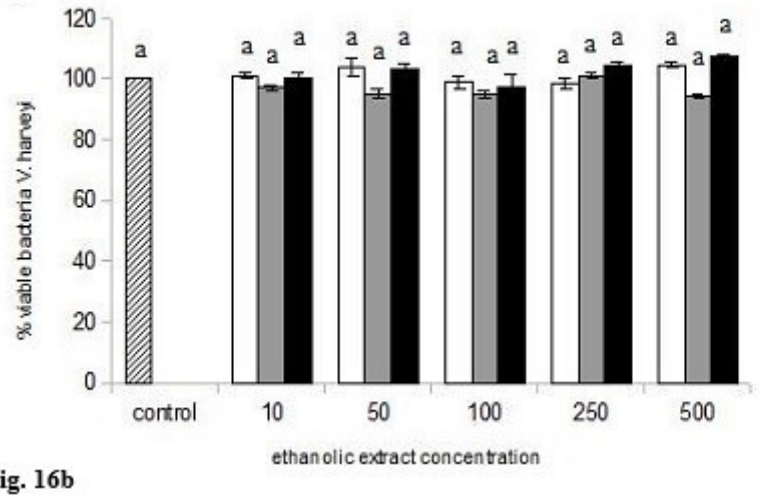


Fig. 16b

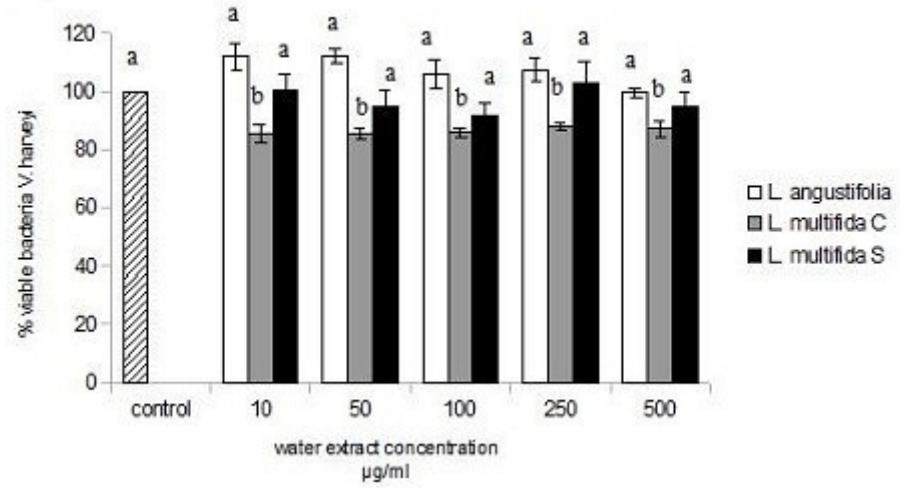


Fig. 16 a-b. Bactericidal activity of water (a) and ethanolic extracts (b) from Calabrian and Sicilian L. multifida (indicated in the figure as C and S respectively) and from L. angustifolia on Vibrio harveyi. Small letters indicate significant differences between different concentrations of the same plant extract. Differences were considered significant when $P < 0.05$. Results showed are representative of at least three independent experiments and are expressed as Mean ± Standard Error.

Fig. 17a

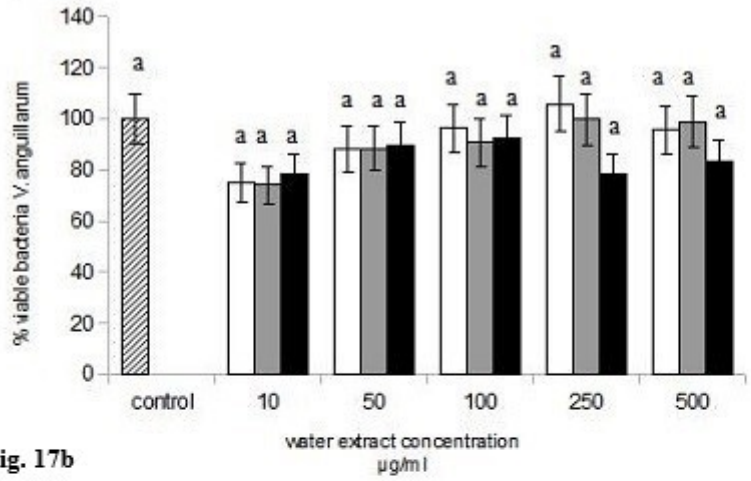


Fig. 17b

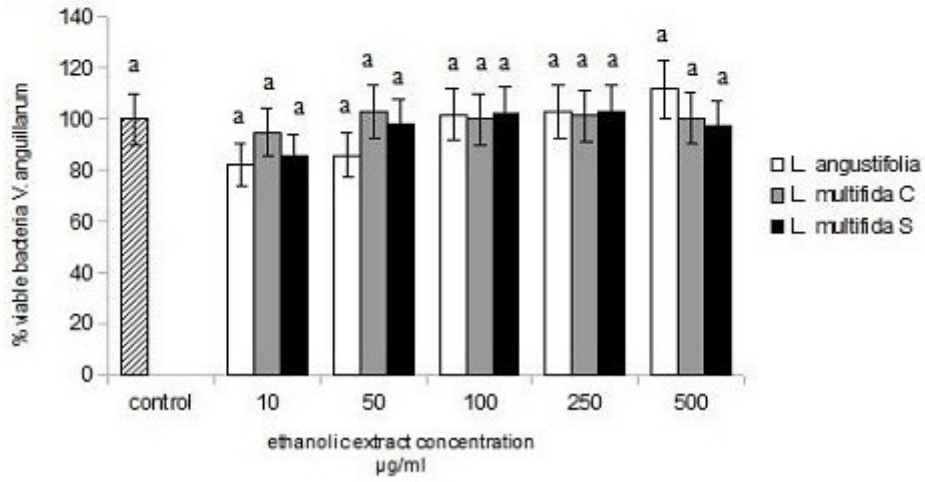


Fig. 17 a-b. Bactericidal activity of water (a) and ethanolic extracts (b) from Calabrian and Sicilian L. multifida (indicated in the figure as C and S respectively) and from L. angustifolia on Vibrio anguillarum. Small letters indicate significant differences between different concentrations of the same plant extract. Differences were considered significant when $P < 0.05$. Results showed are representative of at least three independent experiments and are expressed as Mean ± Standard Error.

Fig. 18a

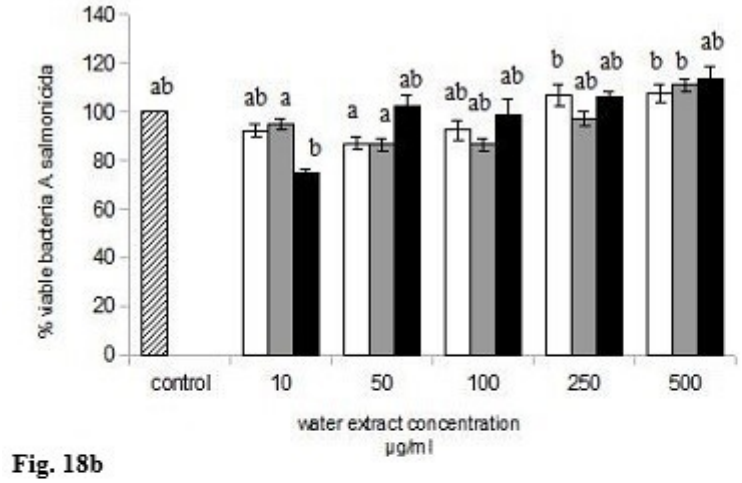


Fig. 18b

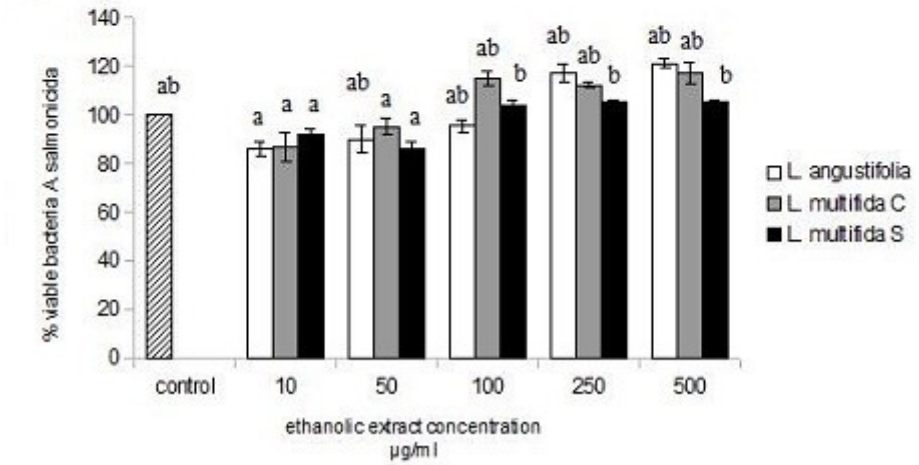


Fig. 18 a-b. Bactericidal activity of water (a) and ethanolic extracts (b) from Calabrian and Sicilian L. multifida (indicated in the figure as C and S respectively) and from L. angustifolia on Aeromonas salmonicida. Small letters indicate significant differences between different concentrations of the same plant extract. Differences were considered significant when $P < 0.05$. Results showed are representative of at least three independent experiments and are expressed as Mean ± Standard Error.

Fig. 19a

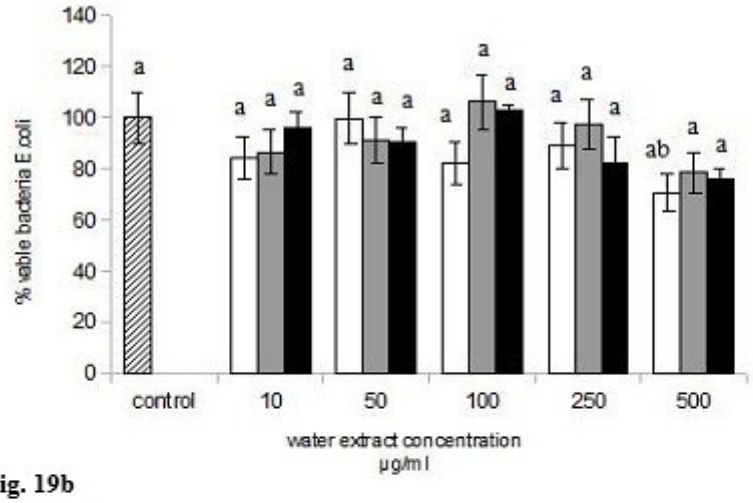


Fig. 19b

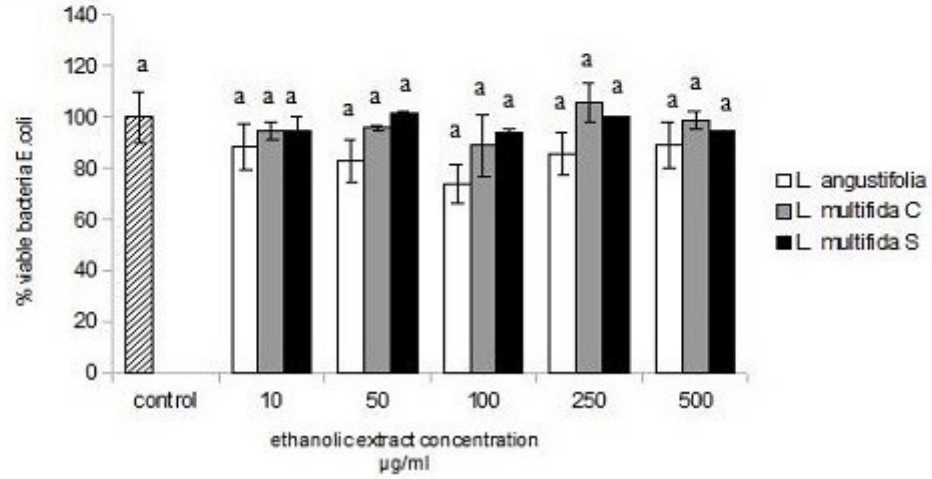


Fig. 19 a-b. Bactericidal activity of water (a) and ethanolic extracts (b) from Calabrian and Sicilian L. multifida (indicated in the figure as C and S respectively) and from L. angustifolia on Escherichia coli. Small letters indicate significant differences between different concentrations of the same plant extract. Differences were considered significant when $P < 0.05$. Results showed are representative of at least three independent experiments and are expressed as Mean ± Standard Error.

Although actually there are no available data concerning the effects of lavender extracts on fish immunity and their possibility of use as immunostimulant in aquaculture, some scientific evidences exist about the immunostimulant effect of extracts from other plants belonging to Lamiaceae family (Nassar et al, 2015; Logambal et al, 2000; Adel et al, 2015; Soltani et al, 2010).

Results of this study show, firstly, that both aqueous and ethanolic extracts of *L. multifida* have any negative impact on HK leucocyte viability at the concentrations and incubation times tested. Noteworthy, results demonstrate an important increase in HK leucocytes phagocytosis and respiratory burst activities after treatment with such extracts: particularly, water and ethanolic extracts from both populations of *L. multifida* L. significantly enhanced the HK leucocyte phagocytic ability, although a negative effect was observed after treatment with ethanolic extracts from all plants at the maximum concentration tested (1000 μg mL^{-1}); on the other hand, extracts from only one population of *L. multifida* (L. multifida S) increased the HK leucocytes phagocytic capacity. Also, respiratory burst activity showed a significant increase when HK leucocytes were treated with water extracts from all the populations under study at all concentrations tested except for Sicilian *L. multifida* at 1000 μg/mL, and with ethanolic extracts at 10 μg/mL and 100 μg/mL of concentration from Calabrian and Sicilian *L. multifida.* However, a negative effect on respiratory burst activity was observed when using ethanolic extracts at the maximum concentration tested (1000 μg mL^{-1}), being this similarly observed for the phagocytic ability. Present results show that *L. multifida* L. extracts positively affect phagocytosis and respiratory burst activity of gilthead seabream HK leucocytes, without any significant effect neither on HK leucocytes viability nor on HK leucocytes peroxidase, thus supporting the possibility of use of *L. multifida* L. extracts, in a well-defined range of concentrations, as immunostimulant in the production of this important farmed marine fish species. Immunostimulants are capable of promoting a greater and more effective sustained immune response to those infectious agents (viruses, bacteria, fungi, and parasites), producing subclinical disease without risks of toxicity, carcinogenicity or tissue residues (Muthusamy et al, 2013).

Finally, present results demonstrated that *L. multifida* L. extracts had no bactericidal activity against the fish pathogens tested. There are no many available data about the antibacterial activity of *L. multifida* L. extracts until now, and the majority of antibacterial studies have been performed on its essential oils. As reported by Khadir and colleagues (2014) *L. multifida* essential oils were tested against methicillin-resistant Staphylococcus aureus (MRSA) using disc diffusion method, revealing a good anti-MRSA activity, whereas the ethanolic extract was less active, thus suggesting that different biological effects between leaf extracts and essential oils could be due to differences in their chemical composition. In fact the chemical composition of *L. multifida* essential oils studied by GC and GC-MS (Khadir et al, 2014) revealed that carvacrol is the main component (from 27.5% to 57%), being carvacrol a monoterpenic phenol responsible for many biological activities such as antimicrobial, antitumoral, antimutagenic, antigenotoxic, analgesic, antiinflammatory, antiparasitic, antiplatelet (Baser, 2008). On the other hand, opposite results were obtained with ethanolic extracts from *L. officinalis, Melissa officinalis, Ocimum basilicum, Origanum vulgare, Rosmarinus officinalis* and *Salvia officinalis*, all belonging to Lamiaceae family, which were tested for antimicrobial activity, exhibiting a broad spectrum of inhibitory effects on some fish pathogens like *L. anguillarum, Y. ruckeri, P. damselae, L. garvieae* (Bulfon et al, 2014).

To conclude, *L. multifida* L. water and ethanolic leaf extracts up to 100 mg mL^{-1} of concentration increased innate immune activities of *S. aurata* HK leucocytes, more concretely, their phagocytic and respiratory burst activities, but did not exert any bactericidal activity on the bacterial strains tested in the present thesis, and did not affect significantly SAF-1 cells viability up to 100 mg mL^{-1} of concentration. Present results suggest the possibility of use such extracts in "in vivo" studies in order to corroborate the use of those extracts in aquaculture to achieve protection against pathogenic infections through enhancement of the innate immunity of fish.

CONCLUSIONS

Lavandula multifida L. represents a plant species growing in the Western Mediterranean Basin, with a threatened peripheral population in Southern Italy. Calabrian *L. multifida* is a rare and endangered plant, due to its small, isolated and fragmented populations.

The need of conservation of this rare and endangered species required the study of its environmental adaptability. The study of germination strategies explained the metabolic adaptation and the ability of Calabrian *L. multifida* to respond to external environmental conditions and aimed to find out the specific actions for the preservation of its populations. Indeed, these experiments support the possibility of utilization of propagation from seeds under controlled environmental conditions as a viable method for the ex-situ conservation of Calabrian *L. multifida.*

L. multifida is able to survive in hot and arid climatic conditions on poorly evolved limestone soils. Calabrian *L. multifida* leaves contain relevant quantity of enzymatic and non enzymatic antioxidants, suggesting a remarkable ability of this plant to optimize survival strategies in its natural habitat and to withstand environmental stress. Moreover, the analysis of the flavonoid composition confirm that Calabrian *L. multifida* leaves represent a good potential source of antioxidant substances for human health.

The richness in health promoting compounds of Calabrian *L. multifida* led to investigate the cytotoxic and cytoprotective effects of its leaf extracts on Hela cell line, demonstrating the ability of such extracts to protect HeLa cells against chemically-induced oxidative damage. Furthermore, possible applications of Calabrian *L. multifida* leaf extracts as immunostimulant in aquaculture were investigated using Sparus aurata as a marine fish model, showing the ability of these extracts to stimulate in vitro the fish innate immune system and to improve fish defense against pathogenic infections.

The emerging results suggest the possibility of considering leaf extracts of Calabrian *L. multifida* as a potential source for new active principles in drug design for new antioxidant formulations, as well as the possibility to use such extracts as potential immunostimulant in aquaculture production.

ACKNOWLEDGEMENTS

This PhD research was supported in part by Progetto LAMULCA “Progetti comprensoriali per la salvaguardia del patrimonio regionale”, and in part by Spanish Ministry of Economy and Competitiveness cofunded by the European Regional Development Funds (ERDF/FEDER) (grant number AGL2014-51839-C5-1-R) and the Fundacion Seneca de la Region de Murcia (Grupo de Excelencia, grant number 19883/GERM/15). I want to thank University of Tuscia (Viterbo) for my PhD grant.

I would like to express my deepest gratitude to my supervisor, Professor Panuccio Maria Rosaria for the continuous support of my Ph.D. study and research, for her motivation, patience, knowledge, encouragement and good suggestions.

Also thanks to Prof. Esteban Maria Angeles, Prof. Cuesta Alberto and Dr. Cerezuela Rebeca, who allowed me to join their research team as intern in the department of Cell Biology (University of Murcia, Spain), giving me access to their laboratories and research facilities to carry on my experimental work on human and animal cell lines.

I want to thank all the technicians of Tissue and Cell Culture Laboratories of SAI section (Servicio de Apoyo a la Investigacion) of University of Murcia for their excellent contribution in the maintenance of laboratory equipment and support to research users.

I want to thank Prof. Muscolo Adele, Prof. De Santis Diana and Prof. Poiana Marco, who represented the doctoral internal committee and evaluated my PhD progress during three years, giving me advice and suggestions to improve my doctoral work.

Thanks to the external referees of this PhD thesis, Professor Bonaccorsi Paola (University of Messina, Italy) and Professor Guardiola Abellan Francisco (University of Porto, Portugal) for the deep examination and for the best suggestions to improve this PhD thesis.

Thanks to Prof. Spampinato Giovanni and Doctor Musarella Carmelo (University Mediterranea of Reggio Calabria) who supported me in the search of Calabrian and Sicilian *L. multifida* 'in situ', and helped in the plant species identification.

Thanks to Prof. Barreca Davide and to Dr. Papalia Teresa (Department of Chemical, Pharmacological and Biological Sciences, University of Messina) for their contribution in the chemical characterization of leaf extracts.

I want to thank my family and all the people who gave me the spiritual support and encouragement I needed during my PhD course and during the writing of this thesis.
A special thank to my daughter, *Sophia*.

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