In vitro effects of Italian *Lavandula multifida* L. leaf extracts on gilthead seabream (*Sparus aurata)* leucocytes and SAF-1 cells

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Abstract

Lavandulla multifida is very appreciated by pharmaceutical and cosmetic industries. In Italy is only found in Calabria and Sicily and, at present, urge its valorization due to its high extinction and genetic erosion risks. Possible applications of *L. multifida* extracts on fish were assayed by using gilthead seabream (*Sparus aurata*) as a marine fish model, due to its importance in fish aquaculture. The *in vitro* effects of both aqueous and ethanolic leaf extracts obtained from two Italian populations of *L. multifida* on head kidney leucocyte activities (viability, phagocytosis, respiratory burst and peroxidase content) where compared with those originated for similar extracts obtained from a widely-distributed species of *L. angustifolia*. Furthermore, the possible cytotoxic effects of all extracts on SAF-1 cells and bactericidal effects for three pathogenic bacteria for fish (*Vibrio harveyi*, *Vibrio anguillarum*, *Aeromonas salmonicida*) were also evaluated.

Keywords: *Lavandula multifida* L.; Leaf extracts; Innate immune response; *Sparus aurata*; SAF-1 cell line.

1. Introduction

Lavandula (lavender, belongs to the family *Labiatae*, Lamiaceae) is a genus of 39 species, which has been largely used (either dried or as an essential oil) in folk and traditional medicine (Cavanagh and Wilkinson, 2002; Denner, 2009). Lavender has been used for centuries. Several studies revealed that *Lavandula* species are rich in a wide range of secondary metabolites such as phenolic compounds (Areias et al, 2000; Upson et al, 2000), monoterpenes, diterpenes (Politi et al, 2002), triterpenes (Topcu et al, 2001), sesquiterpenes (Ulubelen et al, 1988), and coumarins (Shimizu et al, 1990) which are supposed to be responsible for their antimicrobial, antifungal and antioxidant properties. Because of the high content of these secondary metabolites, Lamiaceae family accounts for beneficial and therapeutical properties which allow a wide spectrum of applications in food industry, cosmetic, perfumery and pharmaceutical preparations with high industrial and commercial value (Wilkinson et al, 2003).

Among *Lavandula* species, *Lavandula multifida* is a short-living plant 30 to 100 cm high, with a diploid genetic pool, equipped with triangular pinnatisect leaves, and blue or white purple flowers which give off a strong smell. It spontaneously grows along the Mediterranean coast of Egypt, Tunisia, Marocco, Algeria, Portugal and Spain, while in Italy it has been found only in Calabria and in Sicily (Galesi et al, 2005). The great interest for this plant is due to its essential oils and metabolites (whose antimicrobial, antifungal and antioxidant properties are well-documented) and to the wide possibilities of application in pharmaceutical and cosmetic industries (Ramchoun et al, 2009; Sosa et al, 2005; Zuzarte et al, 2012). Unfortunaltely, the human impact on its natural habitat caused the reduction and fragmentation of the populations of this plant in Southern Italy, and therefore, due to the rarity and the threats to this species, it was included in the "Regional Red Lists of Italian Plants" under the status IUCN of "critically endangered"

74 in Calabria region and “endangered” in Sicily (Conti et al, 1997). For this important
75 reason, the valorization of *L. multifida* is strictly necessary due to its high extinction and
76 genetic erosion risks.
77 Currently, there is a growing interest in screening medicinal plants extracts for their
78 bactericidal, fungicidal and even immunostimulant properties, in order to exploit new
79 biocompounds of natural origin which could be employed in the prevention and/or
80 control of fish diseases in aquaculture (Reverter et al, 2014). The importance of using
81 immunostimulants in aquaculture is known since many years (Sakai, 1999) and, at
82 present, it is a topic of great interest in research. In the intensive aquaculture system,
83 application of antibiotics and chemotherapeutics as prophylactic measures has been
84 widely criticized for their negative impacts on the environment and also on fish, such as
85 immunodepression or residue accumulation in tissues, and besides this leads to the
86 development of drug resistant pathogens (Rijkers et al, 1980; Harikrishnan et al, 2009;
87 FAO, 2003; Smith et al, 1994). The use of plant extracts as immunostimulants has
88 recently received increasing attention not only because they combine lower costs with
89 the low impact on the environment, but also because they may have additional
90 physiological effects since they contain many nutrients, micronutrients as well as other
91 immunostimulant substances (Cuesta et al, 2005). Taking in considerations the needed
92 of looking for new applications of *L. multifida* extracts, and taking into account the
93 importance of gilthead seabream (*Sparus aurata*) in Mediterranean and marine fish
94 aquaculture, we developed the present study. The *in vitro* effects of leaf extracts
95 obtained from two Italian populations of *L. multifida* on head kidney leucocytes
96 activities (viability, phagocytosis, respiratory burst and peroxidase activity) were tested,
97 making a comparison with similar extracts obtained from the widely-distributed species
98 *L. angustifolia*. Furthermore, 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.

2. Materials and Methods

2.1. Plant extracts

Leaves from Sicilian *L. multifida* L. (collected from Brucoli, Siracusa, indicated in the figures as *L. multifida* S), from Calabrian *L. multifida* L. (collected from Capo dell'Armi, indicated in the figures as *L. multifida* C), and from *L. angustifolia* Miller were collected and air-dried at 50 °C until the weight was stable.

For preparation of aqueous extracts, air-dry leaves were macerated and shaked with boiling water for 4 h at 25°C, then the mixture was filtered twice using nylon net filter with a 100μm pore size, and evaporated in a rotary evaporator until dryness. Prior to be used in the assays, extracts were filtered using sterile filters of 0.22 μm of diameter. For preparation of ethanol extracts, air-dry leaves were macerated and shaked with pure ethanol (1:40, 48 h, 25 °C). The resulting mixture was then filtered-twice as described above, and concentrated by vaporizing using a rotary evaporator.

2.2. 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).

2.3. 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 8 ml 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 100 mg 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 µm), washed twice (400 g, 10 min), counted (Z2 Coulter Particle Counter) and adjusted to 2 x 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 x10^7 cells ml^{-1} were dispensed into flat-bottomed 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 RPMI in case of control samples for assays with ethanolic extracts. Cells were incubated in presence of the extracts for 24 h at 20 ºC in an incubator with 5% CO_2 and 85%

humidity. After incubation, leucocyte viability and phagocitic, respiratory burst and peroxidase activities were determined as described below.

2.4. 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, Becton–Dickinson) and 40 µl of propidium iodide (PI) (400µg ml^{-1}, Sigma) were added to each sample. The tubes were gently mixed before analysis in a FACScan (Becton–Dickinson, 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 computerised system. Dead cells were estimated as the percentage of cells with propidium iodide (red-PI fluorescent cells).

2.5. Phagocitic 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 ºC) lyophilised *Saccharomyces cerevisiae*, strain S288C, were washed twice, counted and adjusted to 108 yeast cells/ml in sRPMI-1640. To label yeast cells with fluorescein isothiocyanate (FITC, Sigma) yeast cells were incubated with 5 µg ml^{-1} FITC at 22 ºC with constant stirring (40 cycles min^{-1}) and in darkness for 15 min (Rodriguez et al, 2003). After labeling, free FITC was removed by washing 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 ºC.

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, 25ºC), resuspended and incubated at 25 ºC 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 yest 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-labelled *S. cerevisiae* yeast cells or HK leucocytes were included in each phagocytosis assay. Immediately, the samples were mixed gently, acquired, and analysed 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.

2.6. Respiratory burst activity

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

2.7. 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 10mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, Sigma) and 5mM H_2O_2 (both substrates prepared daily) were added and after 2 min, 50 µl of 2M 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.

2.8. 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), 2mM L-glutamine (Life Technologies), 100 i.u. ml^{-1} penicillin (Life Technologies) and 100 µg ml^{-1} streptomycin (Life Technologies). Cells were grown at 25 ºC and with 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 x g, 5 min, 25 ºC) and the cell viability was determined by the trypan blue exclusion test.

2.9. 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 ºC). 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 µg 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 24h at 25ºC 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 with phosphate buffer saline solution (PBS) and 200 µL$well^{-1}$ of MTT (1 mg mL^{-1}) were added. After 4 h of incubation, the cells were washed and the formazan crystals solubilized with 100µL $well^{-1}$ of DMSO. Plates were shacked for 5 minutes in dark conditions and the absorbance at 570 nm and 690 nm determined in a microplate reader.

2.10. 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,

247 Difco Laboratories), both supplemented with NaCl to a final concentration of 1% (w/v).
248 Bacteria in TSB medium were then cultured at the same temperature, with continuous
249 shaking (100 rpm) during 24 h. *E. coli* was cultured in Luria Bertani Agar (LB Agar,
250 Difco) for 48 h at 37 °C and then inoculated in Luria Bertani Broth (LB Broth, Difco).
251 *E. coli* bacteria in LB broth medium were then cultured for 24 h at 37 °C with
252 continuous shaking (100 rpm). Exponentially growing bacteria were resuspended in
253 sterile Hank's balanced salt solution (HBSS) and adjusted to 1 x 10^{8} colony forming
254 units (cfu) ml^{-1}.

255 *2.11. Bactericidal assay*

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

271 *2.12. 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 $P<0.05$.

3. Results

3.1. Effects of leaves extracts on gilthead seabream head kidney leucocyte

Head kidney 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 µg ml^{-1} (10, 50, 100, 250, 500, 750, 1000 µg ml^{-1}). Results demonstrated that any significant effect was observed on HK leucocyte viability after incubation with the cited extracts, respect to the values recorded for control HK leucocytes (Fig. 1).

Regarding phagocytosis, incubation of HK leucocytes with water or ethanolic extracts of *L. multifida* (from both Calabrian and Sicilian populations) significantly enhanced their phagocitic ability when using the range of extract concentrations from 10 to 500 µg ml^{-1} and 10 to 100 µg ml^{-1}, respectively (Fig. 2). However, after incubation of HK leucocytes with *L. angustifolia* water extracts significant enhancements of the phagocitic ability were only recorded when using highest concentrations (from 100 to 1000 µg ml^{-1}) (Fig. 2a). On the other hand, incubation of leucocytes with ethanolic extracts from *L. angustifolia* did not affect their phagocytic ability.

296 On the contrary, significant decreases were observed in the phagocitic ability of HK
297 leucocytes after being incubated with 500 and 1000 µg ml^{-1} of ethanolic extracts from
298 all plant tested, except for 500 µg ml^{-1} ethanolic extract of *L. multifida* S which
299 increased their phagocytic ability (Fig. 2b).
300 A similar trend was observed in the phagocytic capacity because HK leucocytes
301 incubated with 10 µg ml^{-1} and 10 to 100 µg ml^{-1} water and ethanolic extracts from *L.*
302 *multifida* S, respectively, showed an increased phagocytic capacity (Fig. 3). Instead,
303 water and ethanolic extracts from *L. angustifolia* did not affect significantly the
304 phagocitic capacity of gilthead seabream HK leucocytes, respect to control samples.
305 Regarding respiratory burst, incubation of HK leucocytes with water extracts from all
306 populations of *Lavandula* tested increased this activity at all concentrations tested in a
307 significant manner, in comparison with the control group, except for *L. multifida* S
308 water extract which decreased significantly this activity when using at 1000 µg ml^{-1} of
309 concentration (Fig. 4). Also, ethanolic extracts from both populations of *L. multifida*
310 used at 10 and 100 µg ml^{-1} increased the HK leucocyte respiratory burst activity, while
311 the ethanolic extracts from all plants tested at 1000 µg ml^{-1} significantly decreased the
312 respiratory burst activity of HK leucocytes compared to the control samples (Fig. 4).
313 The observed enhancements of the respiratory burst activity of leucocytes was always
314 higher after incubation with water extracts than with ethanolic extracts.
315 No significant variations in the peroxidase content of HK leucocytes were observed
316 after being incubated neither with aqueous nor with ethanolic extracts from the three
317 plants tested in the present work, respect to the values recorded for control samples
318 (Fig. 5).
319
320 *3.2. Effects of leaves extracts on SAF-1 cell line*

The effects of water and ethanolic extracts of *L. multifida* on cytotoxicity of SAF-1 cells were also evaluated. 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. 6). 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 µg ml^{-1}) (Fig. 6a).

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 µg ml^{-1}) (Fig. 6b). 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. 6).

3.3. Bactericidal activity of leaves extracts

In this study *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. 7-10). 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. 7).

4. Discussion

The massive use of antimicrobials for disease control of farmed animals is considered, at present, a real hazard. Indeed food-producing animals are one of several potential sources of antibiotic-resistant bacteria which may spread from animals to man via the food chain (Harikrishnan et al, 2011). Furthermore, pathogens may also transfer their antibiotic-resistance genes into human pathogenic bacteria thus posing a threat to human health and considered as one of the main environmental problems (Alderman and Hastings, 1998; Cabello, 2006; Abutbul et al, 2004; Smith et al, 1994; MacMillan, 2001).

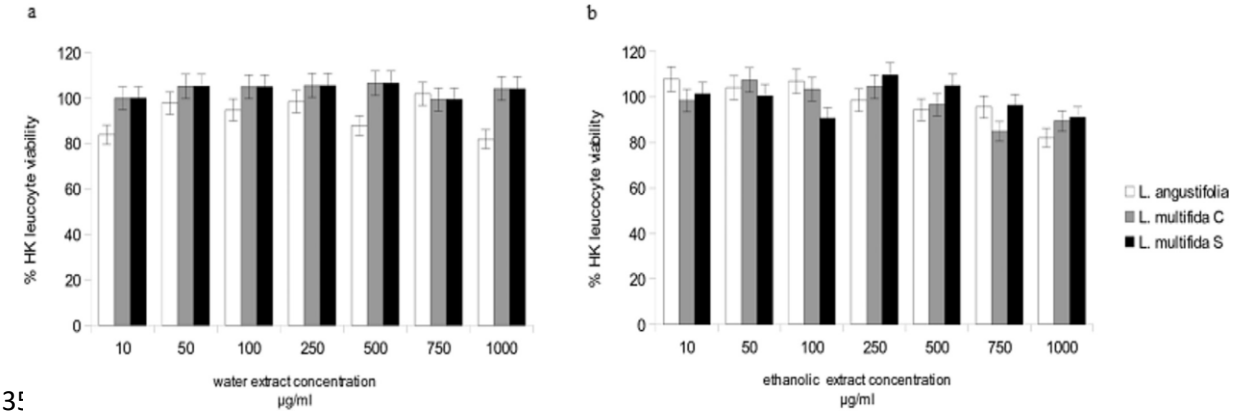

Fig. 1. Percentage of viability of S. aurata HK leucocytes after 24 h of incubation with water (a) or ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian Lavandula multifida. L. angustifolia was considered as control plant. Results of cytotoxicity are given as the percentage of viability, 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.

Regarding farmed fish, while some immunostimulants cannot be used due to their high cost and/or limited effectiveness (Ringo et al, 2012), treatment with medicinal plants is gaining attention due to the fact that they may be a potentially beneficial alternative to antibiotics. Firstly, these plants mitigate many of the side effects associated with the use of chemicals and antibiotics (Madhuri et al, 2012). Furthermore, treatment of bacterial diseases with different herbs has been safely used in organic agriculture, veterinary and human medicine (Madhuri et al, 2012; Bairwa et al, 2012).

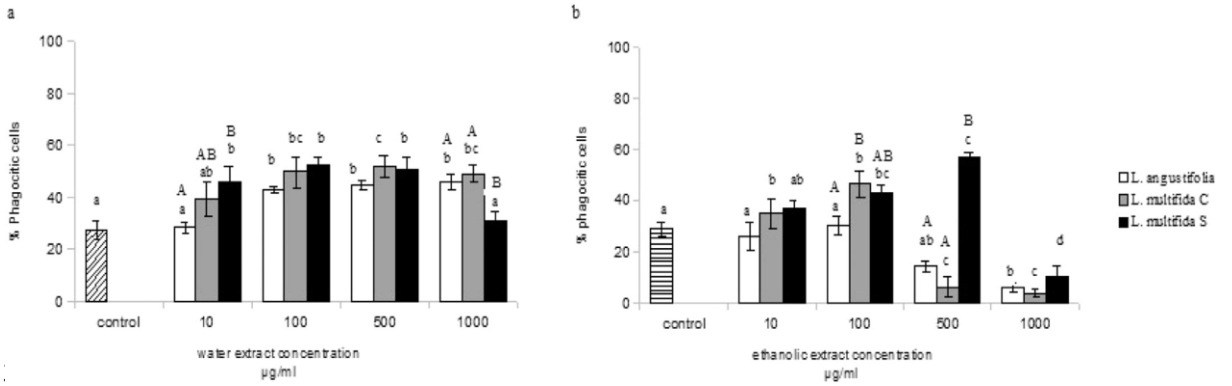

Fig. 2. Percentage of phagocytic cells of S. aurata HK leucocytes after 24 h of incubation with water (a) and ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian Lavandula multifida. L. angustifolia was considered as control plant. Small letters indicate significant differences between 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.

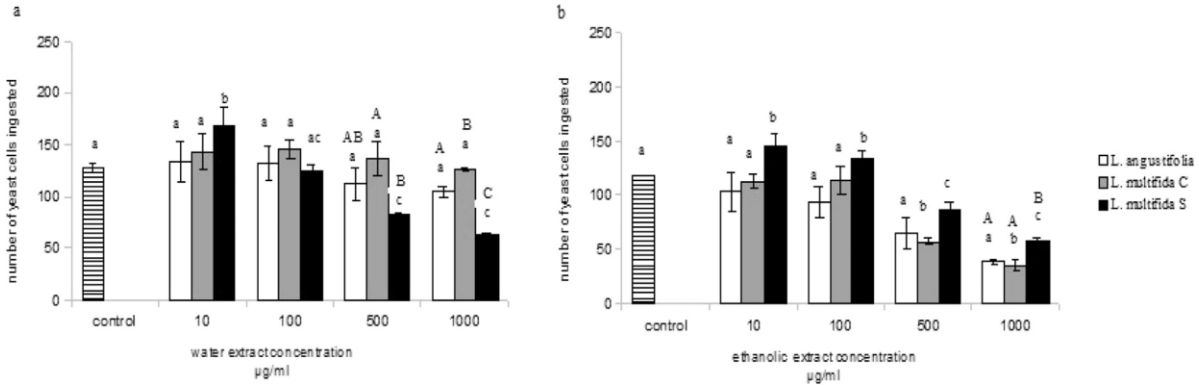

Fig. 3. Phagocytic capacity of S. aurata HK leucocytes after 24 h of incubation with water (a) and ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian Lavandula multifida. L. angustifolia was considered as control plant. Small letters indicate significant differences between different 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.

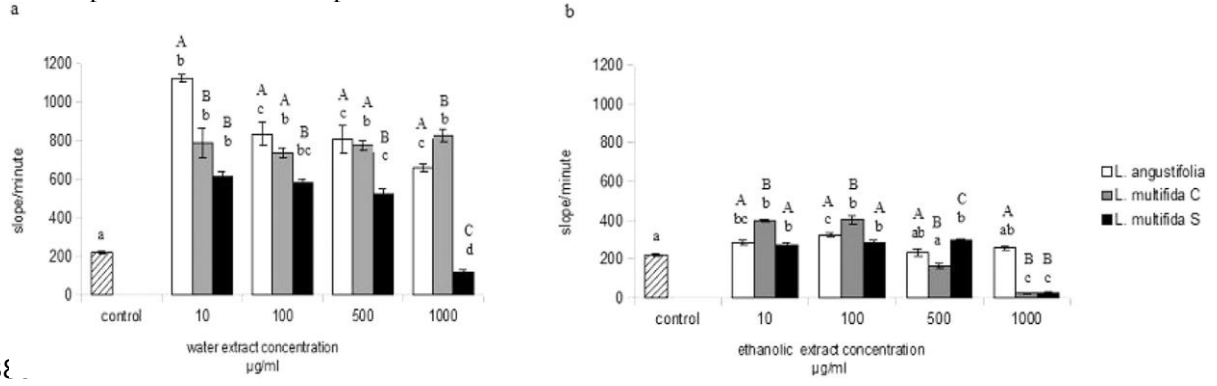

Fig. 4. Respiratory burst activity (expressed as slope/minute) of S. aurata HK leucocytes after 24 h of incubation with water (a) and ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian Lavandula multifida. L. angustifolia was considered as control plant. Small letters

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

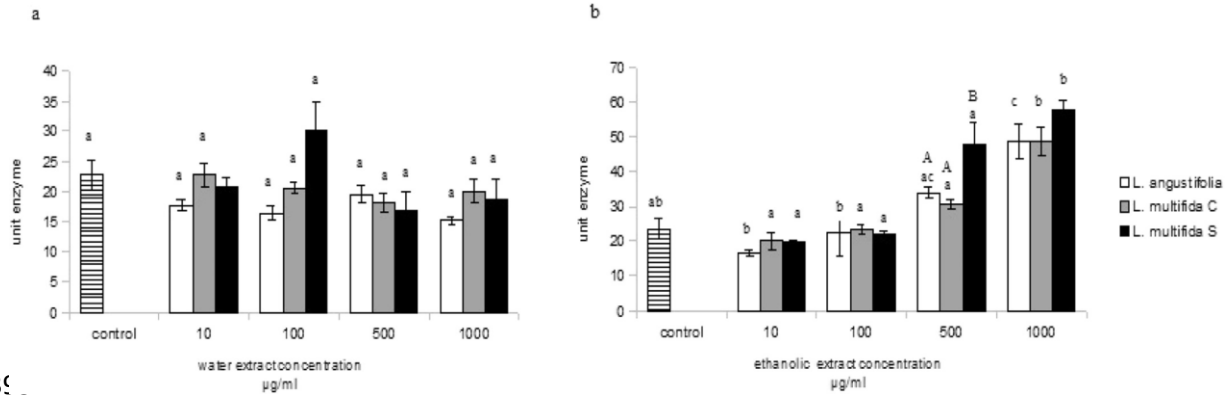

Fig. 5. Peroxidase activity (expressed as unit enzyme) of S. aurata HK leucocytes after 24 h of incubation with water (a) and ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian Lavandula multifida. L. angustifolia was considered as control plant. Small letters indicate significant 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.

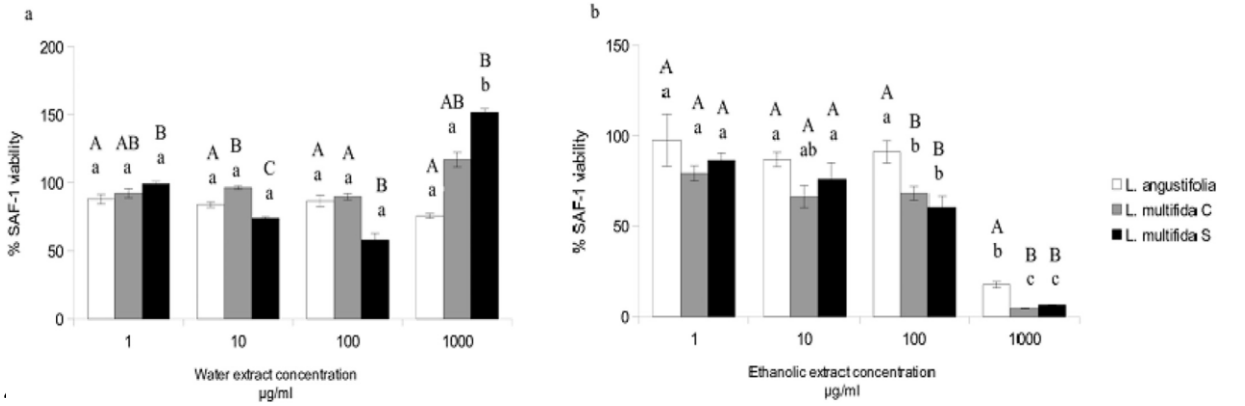

Fig. 6. Viability (expressed as percentage) of SAF-1 cell line after 24 h of incubation with water (a) and ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian Lavandula multifida. L. angustifolia was considered as control plant. Small letters indicate significant differences between 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.

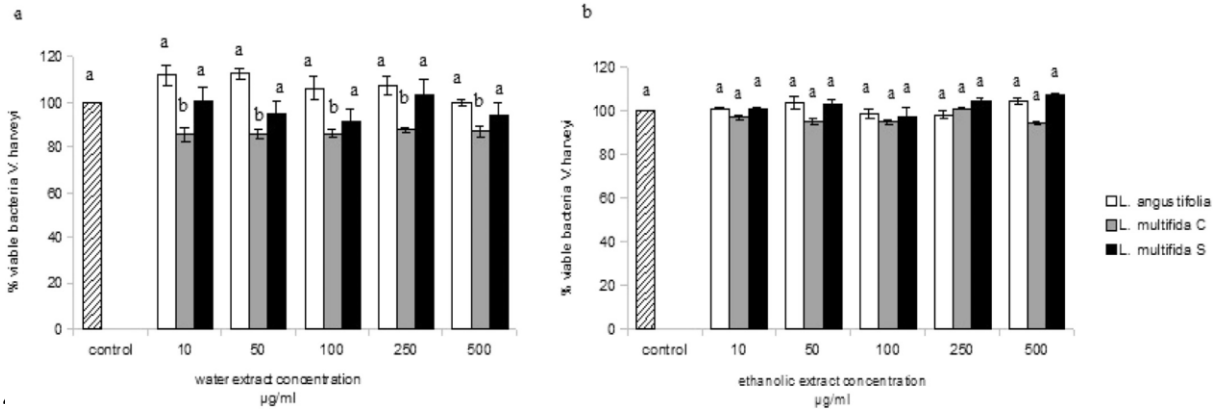

Fig. 7. Bactericidal activity of water (fig. 7a) and ethanolic extracts (fig. 7b) from Calabrian and Sicilian L. multifida (indicated in the figure as C and S respectively) and from L. angustifolia on Vibrio harveyi. Small letters indicate significant differences between different concentrations of the 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.

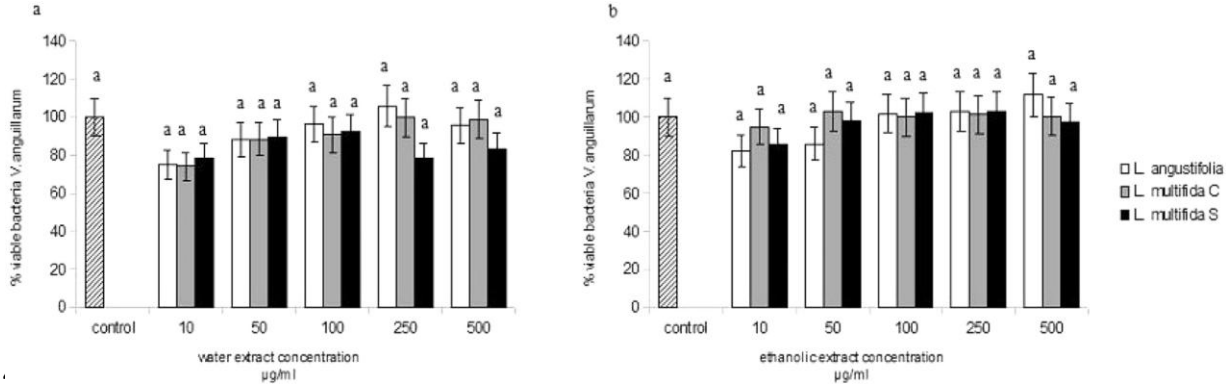

Fig. 8. Bactericidal activity of water (fig. 8a) and ethanolic extracts (fig. 8b) from Calabrian and Sicilian L. multifida (indicated in the figure as C and S respectively) and from L. angustifolia on Vibrio anguillarum. Small letters indicate significant differences between different concentrations 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.

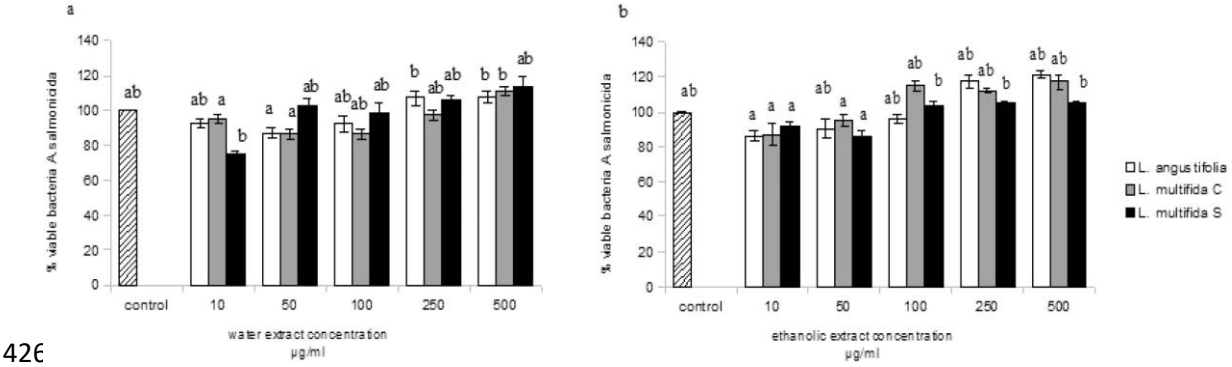

Fig. 9. Bactericidal activity of water (fig. 9a) and ethanolic extracts (fig. 9b) from Calabrian and Sicilian L. multifida (indicated in the figure as C and S respectively) and from L. angustifolia on Aeromonas salmonicida. Small letters indicate significant differences between different concentrations 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.

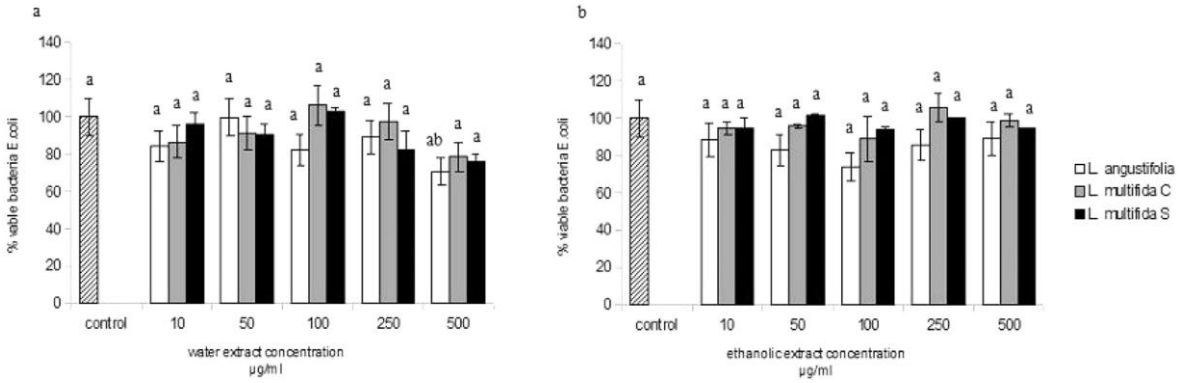

Fig. 10. Bactericidal activity of water (fig. 10a) and ethanolic extracts (fig. 10b) from Calabrian and Sicilian L. multifida (indicated in the figure as C and S respectively) and from L. angustifolia on Escherichia coli. Small letters indicate significant differences between different concentrations 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.

Lately, the use of new and non-purified immunostimulants has received many attentions, and an essential prerequisite is that any new potential immunostimulant should always be respectful with the environment (Sakai et al, 1999). Currently increased consumer demand of quality in farmed fish and shellfish, news efforts in order to avoid the possible pollutants, use of antibiotics and/or carcinogens during the production process urged. In this scenario, plants or their byproducts are preferred since they content numerous compounds like phenols, polyphenols, alkaloids, quinones, terpenoids, lectines and polypeptides many of which have been shown to be very effective alternatives to antibiotics, chemicals, vaccines, and other synthetic compounds (Harikrishnan et al, 2011). Since herbs have little side effects and are easily degradable, various studies have investigated the effect of plant products on innate and adaptive immune response and to prevent and control fish and shellfish diseases (reviewed by Valladão et al, 2015). Fruits, leaves, seeds, flowers, roots and barks extracts from many plants have been reported to have immunomodulator porperties (NagaPreethi and Rajeshwari, 2014). Concomitantly, natural plant products promote different activities in animals like growth, appetite stimulation, immunostimulation, aphrodisiac, antistress and antimicrobial properties due to the already mentioned numerous active principles

present in them (Reverter et al, 2014). The necessity of looking for new natural substances with potential application in fish aquaculture due to their immunostimulant properties or biocides activities against potential pathogenic microbes move us to carry out the present work.

Among plants belonging to Lavandula genus, L. multifida represents a remarkable species because of its wide utilization in traditional medicine since ancient times. Most of the studies performed to date focused on the antibacterial, antifungal and antioxidant properties of its essential oils, as well as on their biochemical constituents.

It is well known that biological and therapeutic properties of medicinal plants are closely related to their chemical compounds; due to the differences in solubility in water or in alcohol, each class of these chemical compounds has a preferred effective extraction method which facilitate getting out the chemicals out of the plant and into the herbal remedy that is being prepared, and this is the reason why water and ethanolic extracts are obtained in order to analyze the different biological and therapeutic properties (Hashemi et al, 2008). Many plant species are known to synthesize many bioactive secondary metabolites like terpenoids, alkaloids, glycoside, phenolic compounds, polysaccharides, flavonols and tannins which possess various biological properties. Furthermore, it is well-known that phytomedicines are inexpensive, eco-friendly and easy to be prepared (Hashemi et al, 2008).

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 effect of extracts from other plants belonging to Lamiaceae family on innate immune response. Extracts from *Thymus guyonii*, *Salvia verbenaca* and *Stachys circinata* (Lamiaceae family) have been tested for their immunostimulant potential on the phagocitic activity in adult male mice (*Mus*

483 *musculus*) through the carbon clearance rate test, resulting in an appreciable increasing
484 in phagocitic index in animals when administered plant extracts by intraperitoneal
485 injection (Nassar et al, 2015). Also, leaf extracts from *Ocimum sanctum* (Lamiaceae
486 family) have been tested for their effect on the innate and specific immune responses
487 and disease resistance against *Aeromonas hydrophila* in *Oreochromis mossambicus*,
488 resulting in an important immunostimulatory effect when leaf extracts were
489 administered orally and intraperitoneally (Logambal et al, 2000). Similarly, dietary
490 administration of peppermint (*Menta piperita,* Lamiaceae family) promoted growth
491 performance and increased the main hematological and immune humoral parameters in
492 fry Caspian white fish (*Rutilus frisii kutum*) (Adel et al, 2015). Furthermore *Zataria*
493 *multiflora* (Lamiaceae) exerted immunostimulatory effects on some immunological
494 factors such as antibody titers, total white blood cells and serum bactericidal activity
495 when its essential oils were dietary administered in *Cyprinus carpio* (Soltani et el,
496 2010). Present results corroborate, firstly that both aqueous and ethanolic extracts of *L.*
497 *multifida* have any negative impact on HK leucocyte viability at the concentrations and
498 incubation times tested in the present study. Furthermore, and more interesting, our
499 results demonstrate that *L. multifida* L. extracts increased HK leucocytes phagocytosis
500 and respiratory burst activities, thus supporting the possibility of use of *L. multifida* L.
501 extracts as immunostimulant in the production of this important farmed marine fish
502 species. Also, respiratory burst activity showed a significant increase when HK
503 leucocytes were treated with water and ethanolic extracts from all populations of
504 Lavandula, although a negative effect was observed when using ethanolic extracts at
505 the maximum concentration tested (1000 mg mL^{-1}), being this similarly observed for the
506 phagocytic ability. Present results show that L. multifida L. extracts positively affect
507 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). Future in vivo studies could demonstrate if similar immunostimulant effects are induced after dietary administration of such extracts to gilthead seabream specimens.

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 (Béjar et al., 1997). Since then, the SAF-1 cell line has proved useful in many applications and varied studies related to virology (Pérez-Prieto et al., 1999; Tafalla et al., 2004; Alonso et al., 2005; Bandín et al., 2006; García Rosado et al., 2008; Cano et al., 2016), bacteriology (Acosta et al., 2009; El Aamri et al., 2012), immunology (Ray et al., 2002; Pelegrín 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. Curiously, to the best of our knowledge, only one previous paper focused on the immune modulatory effects of a plant extract (*Aloe arborescens*) on SAF-1 cells (Picchietti et al., 2013). In the study, SAF-1 cells were treated with Aloe extract at different concentrations (1.2-4.8 mg ml^{-1}) and times (24-72 h). The Aloe extract (1.2 mg ml^{-1}) induced a synergic effect when cells were also stimulated with LPS- or poly I:C which was detected by studying some immune-related gene expression (Picchietti et al., 2013).

Present results from the cytotoxic assay developed on SAF-1 cell line after incubation with L. *multifida* extracts demonstrate no significant effects on cell viability in a limited range of concentrations tested. However, the maximum doses used in our experiments (1000 mg mL^{-1}) affected their 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. Although the main components of the leaves extract have not been studied yet, the analysis of the chemical composition is one of our future next step. These could allow to know if differences in the biochemical components between water and ethanolic extracts exist, which may explain the different properties of the extracts.

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 (2016), *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, 2016) 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, antitumor, antimutagenic, antigenotoxic, analgesic, antiinflammatory, antiparasitic, antiplatelet (Baser, 2008). Instead, in a recent work ethanolic leaf extract of *L. multifida* L. from Southern Italy was screened by UV spectroscopic analysis (Fazio et al, 2014), revealing the presence of vitexin and flavones glucoside derivates of hypolaetin, scutellarein, luteolin, isoscutellarein, apigenin and chrysoeriol, which were reported to possess mainly antiinflammatory, anticancer and antioxidant properties (Alcaraz and Hoult, 1985; Lin et al, 2008; Saeed et al, 2015; Choi et al, 2005). On the other hand, opposite results were obtained with ethanolic extracts from *Lavandula 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 study, and did not affect significantly SAF-1 cells viability up to 100 mg $mL^{-1}$of concentration. Although the extracts did not exert any bactericidal activity directly on the assayed bacteria strains, present results suggest the possibility of use such extracts in in vivo studies in order to corroborate if could be possible using those extracts in aquaculture in order to achieve protection against pathogenic infections through enhancement of the innate immunity of fish.

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