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

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

14

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30

31 **Abstract**

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

43

44

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

47

48

49 **1. Introduction**

50 *Lavandula* (lavender, belongs to the family *Labiatae*, Lamiaceae) is a genus of 39
51 species, which has been largely used (either dried or as an essential oil) in folk and
52 traditional medicine (Cavanagh and Wilkinson, 2002; Denner, 2009). Lavender has been
53 used for centuries. Several studies revealed that *Lavandula* species are rich in a wide
54 range of secondary metabolites such as phenolic compounds (Areias et al, 2000; Upson
55 et al, 2000), monoterpenes, diterpenes (Politi et al, 2002), triterpenes (Topcu et al,
56 2001), sesquiterpenes (Ulubelen et al, 1988), and coumarins (Shimizu et al, 1990)
57 which are supposed to be responsible for their antimicrobial, antifungal and antioxidant
58 properties. Because of the high content of these secondary metabolites, Lamiaceae
59 family accounts for beneficial and therapeutical properties which allow a wide spectrum
60 of applications in food industry, cosmetic, perfumery and pharmaceutical preparations
61 with high industrial and commercial value (Wilkinson et al, 2003).

62 Among *Lavandula* species, *Lavandula multifida* is a short-living plant 30 to 100 cm
63 high, with a diploid genetic pool, equipped with triangular pinnatisect leaves, and blue
64 or white purple flowers which give off a strong smell. It spontaneously grows along the
65 Mediterranean coast of Egypt, Tunisia, Marocco, Algeria, Portugal and Spain, while in
66 Italy it has been found only in Calabria and in Sicily (Galesi et al, 2005). The great
67 interest for this plant is due to its essential oils and metabolites (whose antimicrobial,
68 antifungal and antioxidant properties are well-documented) and to the wide possibilities
69 of application in pharmaceutical and cosmetic industries (Ramchoun et al, 2009; Sosa et
70 al, 2005; Zuzarte et al, 2012). Unfortunaltely, the human impact on its natural habitat
71 caused the reduction and fragmentation of the populations of this plant in Southern Italy,
72 and therefore, due to the rarity and the threats to this species, it was included in the
73 “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

99 from gilthead seabream) and the bactericidal activity of the extracts on *Vibrio harveyi*,
100 *Vibrio anguillarum* and *Aeromonas salmonicida* were also checked.

101

102

103 **2. Materials and Methods**

104 *2.1. Plant extracts*

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

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

116 *2.2. Animals*

117 Thirty specimens (40.51 ± 1.47 gr weight) of the seawater teleost gilthead seabream (*S.*
118 *aurata* L.), obtained from a local farm (Murcia Spain), were kept in re-circulating
119 seawater aquaria (250 L) in the Marine Fish Facility at the University of Murcia. The
120 water temperature was maintained at 20 ± 2 °C with a flow rate of 900 L h⁻¹ and 28‰
121 salinity. The photoperiod was 12 h light: 12 h dark. Fish were allowed to acclimatise for
122 15 days before the start of the trial, where they were fed with a commercial pellet diet
123 (Skretting, Spain) at a rate of 2% body weight day⁻¹. The fish were killed after starving

124 for 24 h by using an overdose of MS-222 (Sandoz, 100 mg ml⁻¹ water). All
125 experimental protocols were approved by the Ethical Committee of the University of
126 Murcia (Permit Number A13150104).

127

128 *2.3. Head-kidney leucocyte isolation and incubation with extracts*

129 Before the dissection of the head-kidney (HK), the specimens were bled. Blood was
130 collected from the caudal vein and afterwards fish were dissected to obtain HK
131 fragments. For isolation of leucocytes to carry out the assays, HK fragments were
132 transferred to 8 ml of sRPMI [RPMI-1640 culture medium (Gibco) supplemented with
133 0.35% sodium chloride (to adjust the medium's osmolarity to gilthead seabream plasma
134 osmolarity of 353.33 mOs), 3% fetal calf serum (FCS, Gibco), 100 i.u. ml⁻¹ penicillin
135 (Flow) and 100 mg ml⁻¹ streptomycin (Flow)] (Esteban et al., 1998). Cell suspensions
136 were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100
137 µm), washed twice (400 g, 10 min), counted (Z2 Coulter Particle Counter) and adjusted
138 to 2 x 10⁷ cells ml⁻¹ in sRPMI. Cell viability was higher than 98%, as determined by the
139 trypan blue exclusion test.

140 To study the possible effects of water and ethanolic extracts on leucocyte activities,
141 aliquots of 50 µl of the obtained leucocytes suspension containing 2 x 10⁷ cells ml⁻¹ were
142 dispensed into flat-bottomed 96-well microtitre plates (Nunc). Then aliquots of 50 µl
143 well⁻¹ of water or ethanolic extracts ranging from 10, 100, 500, to 1000 µg ml⁻¹ prepared
144 in sRPMI were added. The extract aliquot was replaced by sRPMI for control samples
145 for assays with water extracts, or by 0,1% dymethyl sulfoxide (DMSO, Sigma) in RPMI
146 in case of control samples for assays with ethanolic extracts. Cells were incubated in
147 presence of the extracts for 24 h at 20 °C in an incubator with 5% CO₂ and 85%

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

150 *2.4. Leucocyte viability*

151 Aliquots of 100 μ l of leucocytes previously incubated for 24h without (control) or with
152 the plant extracts were placed in 5 ml glass tubes (Falcon, Becton–Dickinson) and 40 μ l
153 of propidium iodide (PI) ($400\mu\text{g ml}^{-1}$, Sigma) were added to each sample. The tubes
154 were gently mixed before analysis in a FACScan (Becton–Dickinson, Madrid, Spain)
155 flow cytometer with an argon-ion laser adjusted to 488 nm. Analyses were performed on
156 5000 cells, which were acquired at a rate of 300 cells s^{-1} . Data were collected in the
157 form of two-parameter side scatter (granularity, SSC) and forward scatter (size, FSC),
158 and green fluorescence (FL1) and red fluorescence (FL2) dot plots or histograms were
159 made on a computerised system. Dead cells were estimated as the percentage of cells
160 with propidium iodide (red-PI fluorescent cells).

161 *2.5. Phagocytic activity*

162 The phagocytic activity of gilthead seabream HK leucocytes was studied by flow
163 cytometry according to Esteban et al. (1998). Heat killed (30 min, 60 °C) lyophilised
164 *Saccharomyces cerevisiae*, strain S288C, were washed twice, counted and adjusted to
165 108 yeast cells/ml in sRPMI-1640. To label yeast cells with fluorescein isothiocyanate
166 (FITC, Sigma) yeast cells were incubated with 5 $\mu\text{g ml}^{-1}$ FITC at 22 °C with constant
167 stirring (40 cycles min^{-1}) and in darkness for 15 min (Rodriguez et al, 2003). After
168 labeling, free FITC was removed by washing twice in phosphate buffer saline (PBS)
169 and the yeast cells were resuspended in sRPMI-1640. FITC-labeled yeast cells were
170 acquired for flow cytometric study. The staining uniformity was examined and then the
171 yeast cell suspensions were aliquoted and stored at 4 °C.

172 Samples of 125 μ l of medium containing FITC-labeled-yeast cells and 100 μ l of HK
173 leucocytes in sRPMI were mixed, centrifuged (400 g, 5 min, 25°C), resuspended and
174 incubated at 25 °C for 60 min in dark conditions. At the end of the incubation time, the
175 samples were placed on ice to stop phagocytosis and 400 μ l of cold PBS was added to
176 each sample. The fluorescence of the extracellular yeast cells (i.e. free yeast cells and
177 yeast cells adhered to phagocytes but not ingested) was quenched by adding 50 μ l of cold
178 Trypan Blue (0.4% in PBS) per sample. Standard samples of FITC-labelled *S.*
179 *cerevisiae* yeast cells or HK leucocytes were included in each phagocytosis assay.
180 Immediately, the samples were mixed gently, acquired, and analysed in a FACScan.
181 Data were collected in the form of two-parameter side scatter (SSC) and forward scatter
182 (FSC), and green fluorescence (FL1) dot plots or histograms were made on a
183 computerized system. Fluorescence histograms represented relative fluorescence on a
184 logarithmic scale. Flow cytometer was set to analyze the phagocytic cells gated from all
185 the leucocytes because of their higher SSC and FSC values. Phagocytic ability was
186 defined as the percentage of cells with one or more ingested yeast cells (green-FITC
187 fluorescent cells) within the phagocytic cell population. The relative number of ingested
188 yeast cells per cell (phagocytic capacity) was assessed in arbitrary units from the mean
189 fluorescence intensity of the phagocytic cells.

190 *2.6. Respiratory burst activity*

191 The respiratory burst activity of seabream HK leucocytes was studied by a
192 chemiluminescence method (Bayne and Levy, 1991). Samples of 100 μ l of a PMA/
193 luminol solution [1 ng ml⁻¹ phorbol myristate acetate (PMA, Sigma) and 10⁻⁴M luminol
194 (Sigma) in HBSS with Ca²⁺ and Mg²⁺] were added to the HK leucocytes (previously
195 incubated as described above). The plate was shaken and immediately read in a
196 chemiluminometer (BMG, Fluoro Star Galaxy). Measurements were performed in 30

197 cycles of 2 minutes each. The kinetics of the reactions were analyzed and the maximum
198 slope of each curve calculated. Control samples containing leucocytes that had not been
199 incubated with the extracts were also analyzed.

200 *2.7. Peroxidase content*

201 The total peroxidase content of HK leucocytes was measured according to Quade and
202 Roth (1997). To do this, 5 μ l of HK leucocytes (previously incubated as described
203 above) were incubated for 10 min with 0.02% cetyltrimethylammonium bromide
204 (CTAB, Sigma) at 60 rpm. Afterwards, 100 μ l of 10mM 3,3',5,5'-tetramethylbenzidine
205 hydrochloride (TMB, Sigma) and 5mM H₂O₂ (both substrates prepared daily) were
206 added and after 2 min, 50 μ l of 2M sulfuric acid was also added to stop the reaction.
207 The absorbance of the samples was measured at 450 nm in a microplate reader (BMG
208 Fluostar Omega, USA). Control samples containing leucocytes that had not been
209 incubated with extracts were also analyzed.

210 *2.8. SAF-1 cell culture*

211 The established cell line SAF-1 (ECACC n 00122301) was seeded in 25 cm² plastic
212 tissue culture flasks (Nunc, Germany) cultured in L-15 Leibowitz medium (Life
213 Technologies, UK), supplemented with 10% fetal bovine serum (FBS, Life
214 Technologies), 2mM L-glutamine (Life Technologies), 100 i.u. ml⁻¹ penicillin (Life
215 Technologies) and 100 μ g ml⁻¹ streptomycin (Life Technologies). Cells were grown at
216 25 °C and with 85% humidity. Exponentially growing cells were detached from culture
217 flasks by brief exposure to 0.25% of trypsin in PBS, pH 7.2-7.4, according to the
218 standard trypsinization methods. The detached cells were collected by centrifugation
219 (1000 x g, 5 min, 25 °C) and the cell viability was determined by the trypan blue
220 exclusion test.

221 *2.9. Cytotoxicity assay on SAF-1 cell line*

222 Cytotoxicity assay was performed in quadruplicates. When SAF-1 cell lines were
223 approximately 80% confluent, they were detached from flasks culture with trypsin (as
224 described before), and aliquots of 100 μL containing 50000 cells well^{-1} were dispensed
225 in 96-well tissue culture plates and incubated (24 h, 25 °C). This cell concentration was
226 previously determined in order to obtain satisfactory absorbance values in the cytotoxic
227 assay and avoided cell overgrowth. After that, the culture medium was replaced by 100
228 $\mu\text{L well}^{-1}$ of the extracts to be tested at the appropriate dilution. Tested concentrations of
229 water and ethanol extracts ranged from 1 to 1000 $\mu\text{g mL}^{-1}$ (1, 10, 100, 1000). Cells were
230 then incubated for 24 h. Control samples received the same volume of culture medium
231 (for water extracts) or of DMSO 0,1% (for ethanolic extracts). Cells were incubated for
232 24h at 25°C and then their viability determined using the MTT assay.

233 The MTT assay is based on the reduction of the yellow soluble tetrazolium salt (3-(4,5-
234 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT, Sigma) into a blue,
235 insoluble formazan product by the mitochondrial succinate dehydrogenase (Berridge
236 and Tan, 1993; Denizot and Lang, 1986). After incubation with the leaf-extracts, SAF-1
237 cells with phosphate buffer saline solution (PBS) and 200 μLwell^{-1} of MTT (1 mg mL^{-1})
238 were added. After 4 h of incubation, the cells were washed and the formazan crystals
239 solubilized with 100 $\mu\text{L well}^{-1}$ of DMSO. Plates were shaken for 5 minutes in dark
240 conditions and the absorbance at 570 nm and 690 nm determined in a microplate reader.

241 2.10. Bacteria

242 Three pathogenic bacteria for fish (*V. harveyi*, *V. anguillarum* and *A. salmonicida*) and
243 *Escherichia coli*, as control, were used in the bactericidal assays. All bacterial strains
244 were grown from 1 mL of stock culture that had been previously frozen at -80°C. *V.*
245 *harveyi*, *V. anguillarum* and *A. salmonicida* were cultured for 48 h at 25 °C in Tryptic
246 Soy Agar (TSA, Difco Laboratories), and then inoculated in Tryptic 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 1×10^8 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*

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

279

280 **3. Results**

281

282 *3.1. Effects of leaves extracts on gilthead seabream head kidney leucocyte*

283 Head kidney leucocyte viability was tested after 24 h of incubation with water or
284 ethanolic extracts of the three tested plants in the range of concentrations from 10 to
285 1000 $\mu\text{g ml}^{-1}$ (10, 50, 100, 250, 500, 750, 1000 $\mu\text{g ml}^{-1}$). Results demonstrated that any
286 significant effect was observed on HK leucocyte viability after incubation with the cited
287 extracts, respect to the values recorded for control HK leucocytes (Fig. 1).

288 Regarding phagocytosis, incubation of HK leucocytes with water or ethanolic extracts
289 of *L. multifida* (from both Calabrian and Sicilian populations) significantly enhanced
290 their phagocytic ability when using the range of extract concentrations from 10 to 500
291 $\mu\text{g ml}^{-1}$ and 10 to 100 $\mu\text{g ml}^{-1}$, respectively (Fig. 2). However, after incubation of HK
292 leucocytes with *L. angustifolia* water extracts significant enhancements of the
293 phagocytic ability were only recorded when using highest concentrations (from 100 to
294 1000 $\mu\text{g ml}^{-1}$) (Fig. 2a). On the other hand, incubation of leucocytes with ethanolic
295 extracts from *L. angustifolia* did not affect their phagocytic ability.

296 On the contrary, significant decreases were observed in the phagocytic ability of HK
297 leucocytes after being incubated with 500 and 1000 $\mu\text{g ml}^{-1}$ of ethanolic extracts from
298 all plant tested, except for 500 $\mu\text{g ml}^{-1}$ 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 $\mu\text{g ml}^{-1}$ and 10 to 100 $\mu\text{g ml}^{-1}$ 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 phagocytic 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 $\mu\text{g ml}^{-1}$ of
309 concentration (Fig. 4). Also, ethanolic extracts from both populations of *L. multifida*
310 used at 10 and 100 $\mu\text{g ml}^{-1}$ increased the HK leucocyte respiratory burst activity, while
311 the ethanolic extracts from all plants tested at 1000 $\mu\text{g ml}^{-1}$ 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*

321 The effects of water and ethanolic extracts of *L. multifida* on cytotoxicity of SAF-1 cells
322 were also evaluated. Results from the cytotoxicity test showed that water extracts from
323 all plants tested did not alter significantly the cell viability, respect to values for control
324 samples (Fig. 6). Interestingly, incubation of SAF-1 cells with *L. multifida* S water
325 extract increased the cell viability in a significant manner at the higher concentration
326 tested ($1000 \mu\text{g ml}^{-1}$) (Fig. 6a).

327 On the contrary, incubation of SAF-1 cells with ethanolic extracts significantly affect
328 their viability, indeed showing high levels of cytotoxicity at the higher concentration
329 tested ($1000 \mu\text{g ml}^{-1}$) (Fig. 6b). Particularly, ethanolic extracts from the two populations
330 of *L. multifida* had a significantly higher level of cytotoxicity on SAF-1 cells if
331 compared to the cytotoxicity provoked on these cells after being incubated with *L.*
332 *angustifolia* ethanolic extracts (Fig. 6).

333

334 3.3. Bactericidal activity of leaves extracts

335 In this study *L. multifida* plant extracts were also tested (in comparison with the extracts
336 from the commercial species *L. angustifolia*) for their bactericidal activity against some
337 fish pathogens relevant in fish aquaculture: *V. harveyi*, *V. anguillarum* and *A.*
338 *salmonicida*, being *E. coli* used as control bacteria. Results from bactericidal assays
339 revealed that any of the assayed plant extracts neither water nor ethanolic affect bacteria
340 viability in a significant manner (Figs. 7-10). Only a slight difference in bacteria
341 viability (80% cell viability) was observed when ethanolic extracts from *L. multifida* C
342 were used to incubate *V. harveyi* bacteria cells (Fig. 7).

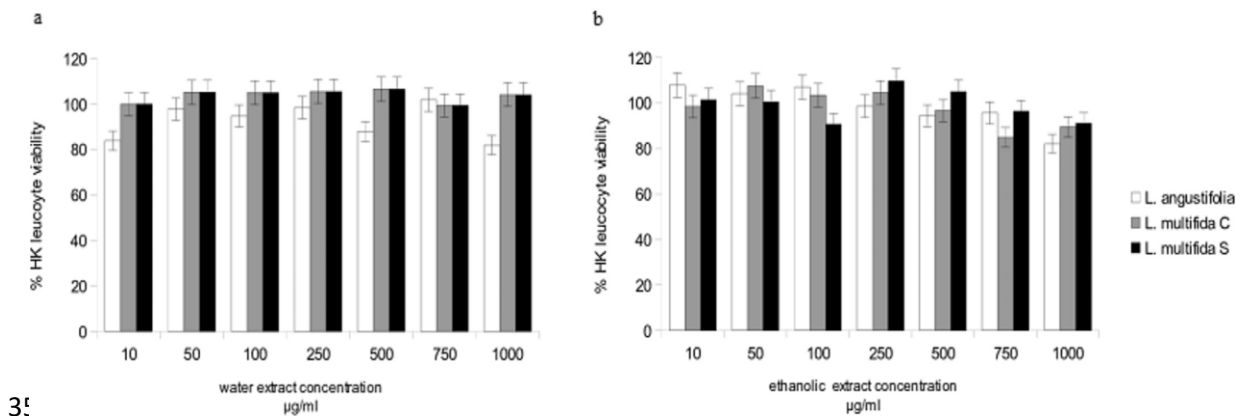
343

344

345

346 **4. Discussion**

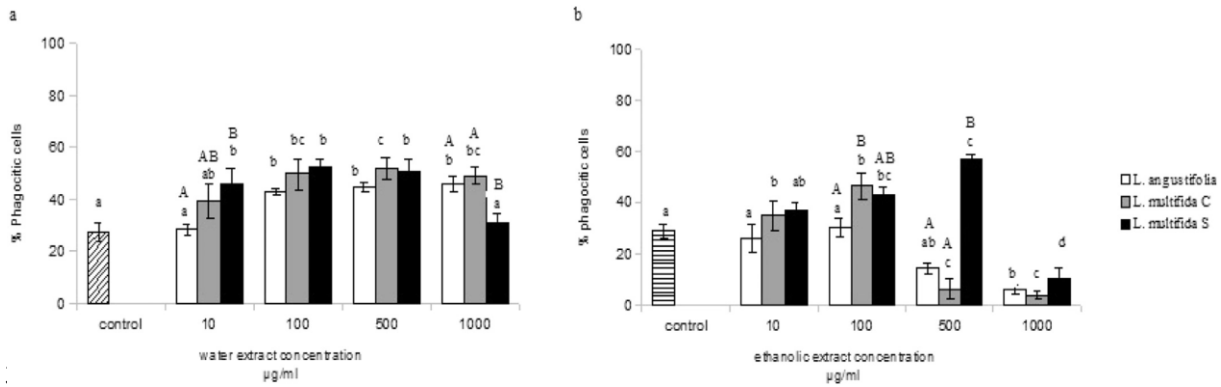
347 The massive use of antimicrobials for disease control of farmed animals is considered,
348 at present, a real hazard. Indeed food-producing animals are one of several potential
349 sources of antibiotic-resistant bacteria which may spread from animals to man via the
350 food chain (Harikrishnan et al, 2011). Furthermore, pathogens may also transfer their
351 antibiotic-resistance genes into human pathogenic bacteria thus posing a threat to
352 human health and considered as one of the main environmental problems (Alderman
353 and Hastings, 1998; Cabello, 2006; Abutbul et al, 2004; Smith et al, 1994; MacMillan,
354 2001).



355

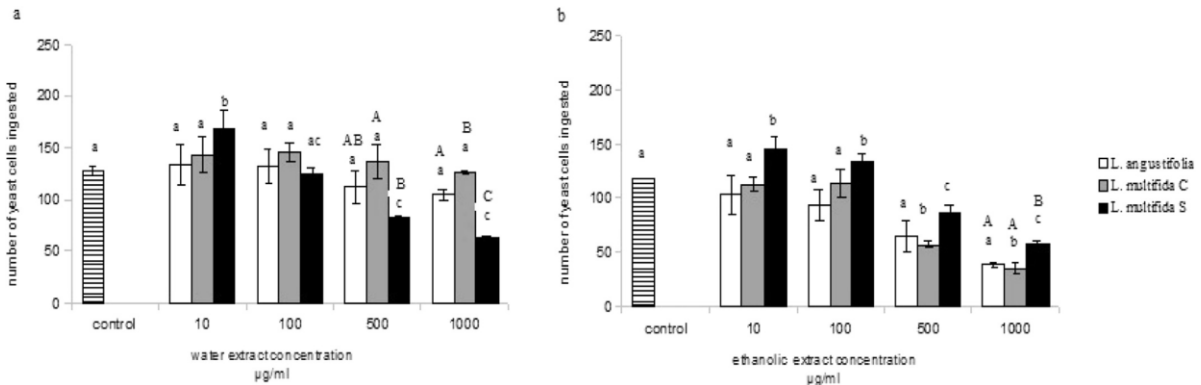
356 Fig. 1. Percentage of viability of *S. aurata* HK leucocytes after 24 h of incubation with water (a)
357 or ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian *Lavandula multifida*. *L.*
358 *angustifolia* was considered as control plant. Results of cytotoxicity are given as the percentage of
359 viability, related to the control of untreated cells (the mean optical density of untreated cells was
360 set to 100% viability). Results showed are representative of at least three independent
361 experiments and are expressed as Mean \pm Standard Error.

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



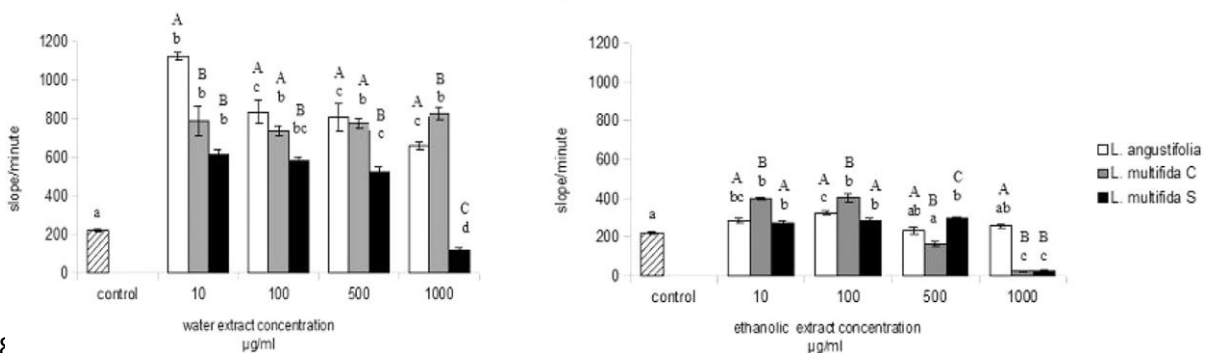
370 Fig. 2. Percentage of phagocytic cells of *S. aurata* HK leucocytes after 24 h of incubation with
 371 water (a) and ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian *Lavandula*
 372 *multifida*. *L. angustifolia* was considered as control plant. Small letters indicate significant
 373 differences between different concentrations of the same plant extract. Capital letters indicate
 374 significant differences between different plant extracts at the same treatment concentration.
 375 Differences were considered significant when $P < 0.05$. Results showed are representative of at
 376 least three independent experiments and are expressed as Mean \pm Standard Error.

377



378 Fig. 3. Phagocytic capacity of *S. aurata* HK leucocytes after 24 h of incubation with water (a) and
 379 ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian *Lavandula* *multifida*. *L.*
 380 *angustifolia* was considered as control plant. Small letters indicate significant differences between
 381 different concentrations of the same plant extract. Capital letters indicate significant differences
 382 between different plant extracts at the same treatment concentration. Differences were considered
 383 significant when $P < 0.05$. Results showed are representative of at least three independent
 384 experiments and are expressed as Mean \pm Standard Error.

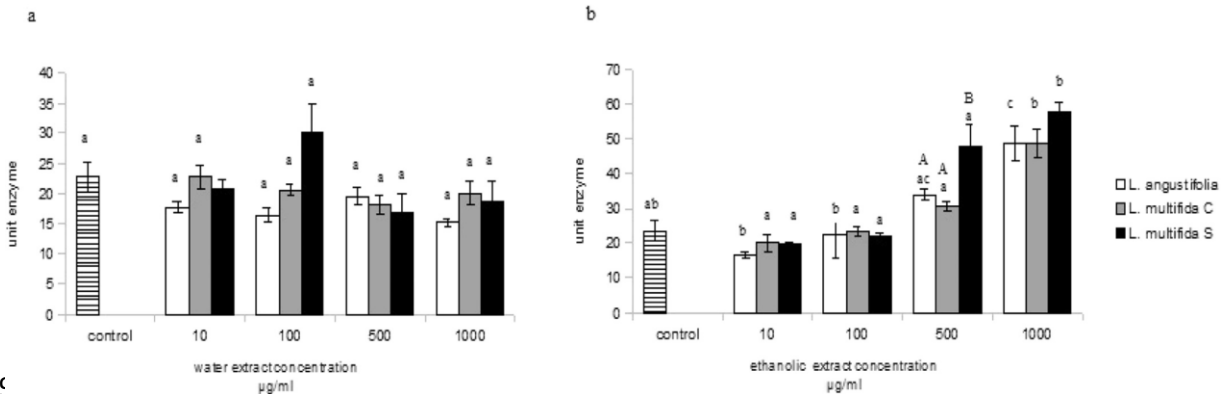
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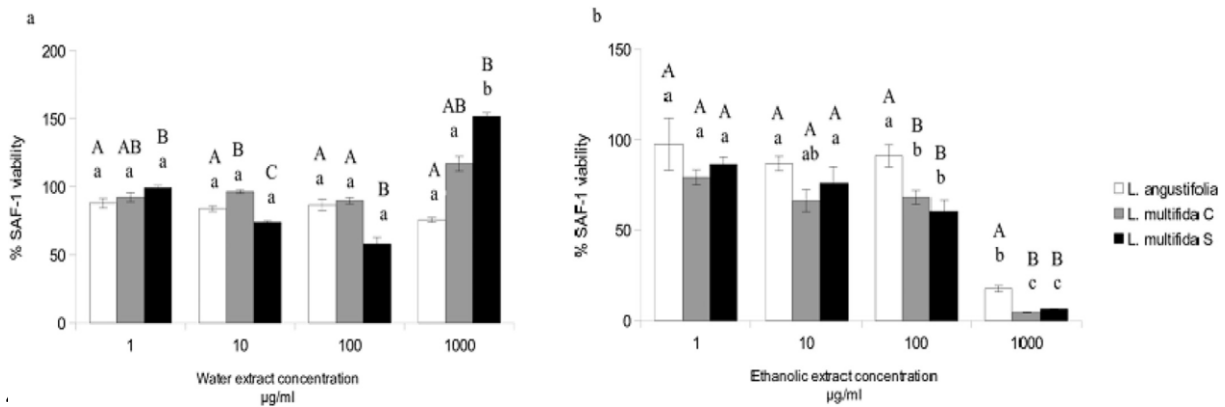
386

387 Fig. 4. Respiratory burst activity (expressed as slope/minute) of *S. aurata* HK leucocytes after
 388 24 h of incubation with water (a) and ethanolic extracts (b) obtained from leaves of Calabrian
 389 and Sicilian *Lavandula* *multifida*. *L. angustifolia* was considered as control plant. Small letters

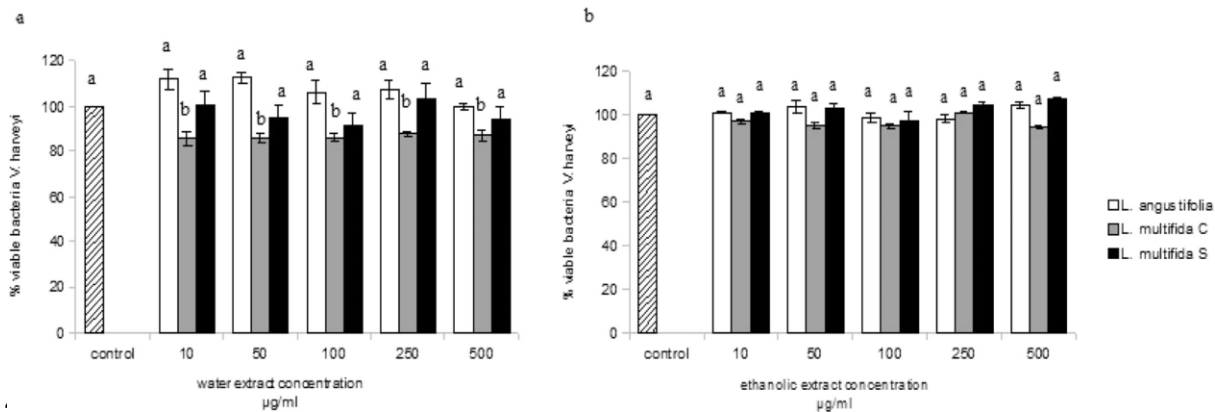
390 indicate significant differences between different concentrations of the same plant extract.
 391 Capital letters indicate significant differences between different plant extracts at the same
 392 treatment concentration. Differences were considered significant when $P < 0.05$. Results
 393 showed are representative of at least three independent experiments and are expressed as Mean
 394 \pm Standard Error



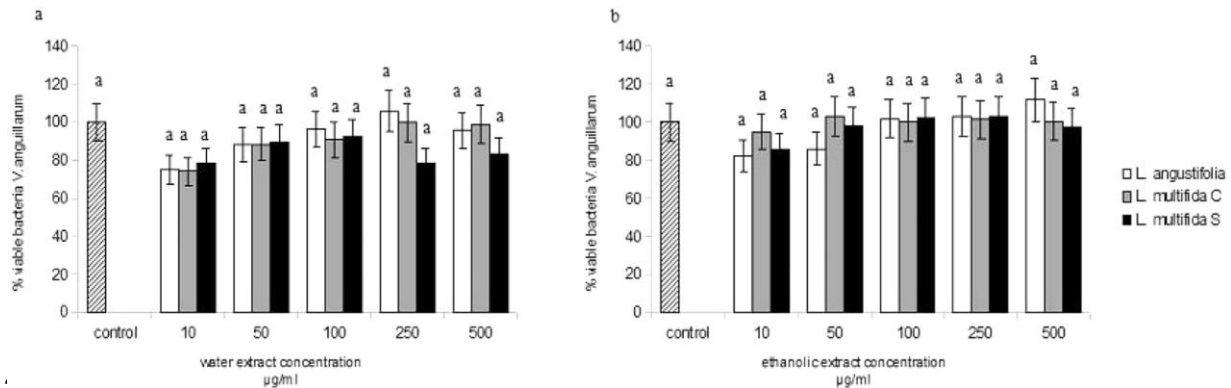
395
 396 Fig. 5. Peroxidase activity (expressed as unit enzyme) of *S. aurata* HK leucocytes after 24 h of
 397 incubation with water (a) and ethanolic extracts (b) obtained from leaves of Calabrian and
 398 Sicilian *Lavandula multifida*. *L. angustifolia* was considered as control plant. Small letters
 399 indicate significant differences between different concentrations of the same plant extract.
 400 Capital letters indicate significant differences between different plant extracts at the same
 401 treatment concentration. Differences were considered significant when $P < 0.05$.
 402 Results showed are representative of at least three independent experiments and are expressed
 403 as Mean \pm Standard Error.



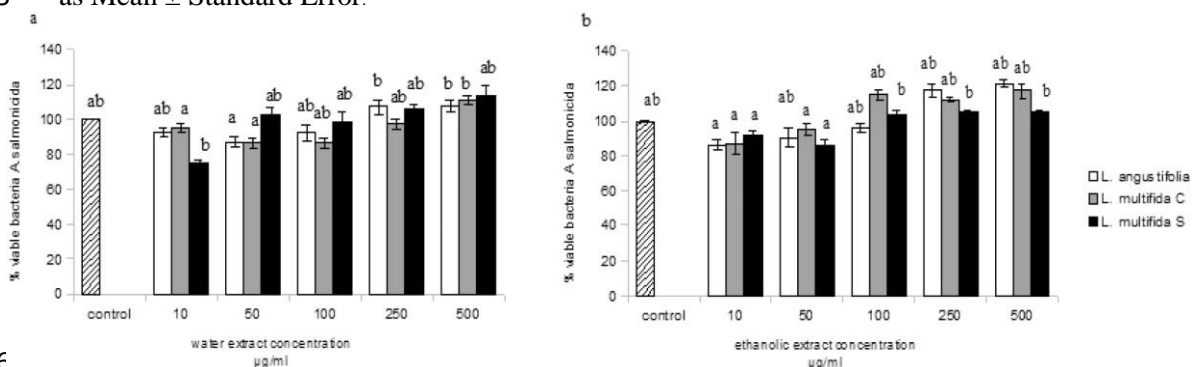
405 Fig. 6. Viability (expressed as percentage) of SAF-1 cell line after 24 h of incubation with water
 406 (a) and ethanolic extracts (b) obtained from leaves of Calabrian and Sicilian *Lavandula*
 407 *multifida*. *L. angustifolia* was considered as control plant. Small letters indicate significant
 408 differences between different concentrations of the same plant extract. Capital letters indicate
 409 significant differences between different plant extracts at the same treatment concentration.
 410 Differences were considered significant when $P < 0.05$. Results showed are representative of at
 411 least three independent experiments and are expressed as Mean \pm Standard Error.



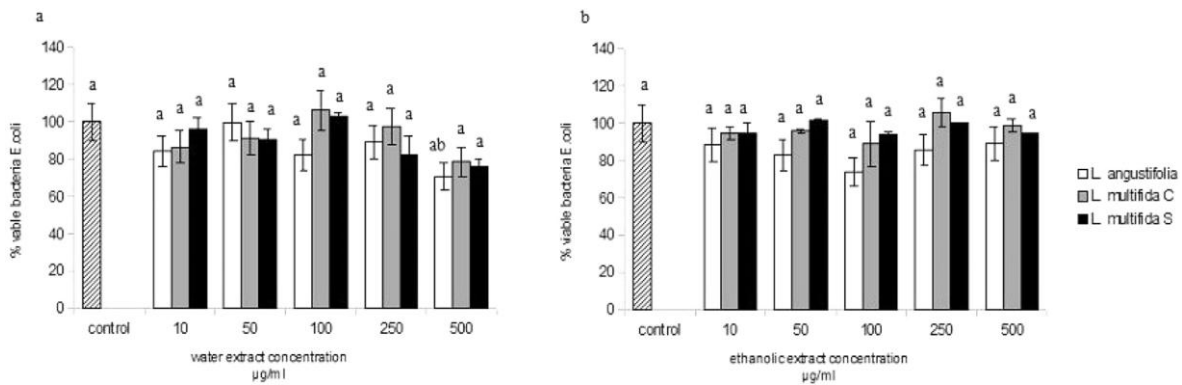
413 Fig. 7. Bactericidal activity of water (fig. 7a) and ethanolic extracts (fig. 7b) from Calabrian
 414 Sicilian *L. multifida* (indicated in the figure as C and S respectively) and from *L. angustifolia* on
 415 *Vibrio harveyi*. Small letters indicate significant differences between different concentrations of
 416 the same plant extract. Differences were considered significant when $P < 0.05$. Results showed
 417 are representative of at least three independent experiments and are expressed as Mean \pm
 418 Standard Error.



420 Fig. 8. Bactericidal activity of water (fig. 8a) and ethanolic extracts (fig. 8b) from Calabrian
 421 Sicilian *L. multifida* (indicated in the figure as C and S respectively) and from *L. angustifolia* on
 422 *Vibrio anguillarum*. Small letters indicate significant differences between different
 423 concentrations of the same plant extract. Differences were considered significant when $P < 0.05$.
 424 Results showed are representative of at least three independent experiments and are expressed
 425 as Mean \pm Standard Error.



426 Fig. 9. Bactericidal activity of water (fig. 9a) and ethanolic extracts (fig. 9b) from Calabrian
 427 Sicilian *L. multifida* (indicated in the figure as C and S respectively) and from *L. angustifolia* on
 428 *Aeromonas salmonicida*. Small letters indicate significant differences between different
 429 concentrations of the same plant extract. Differences were considered significant when $P < 0.05$.
 430 Results showed are representative of at least three independent experiments and are expressed
 431 as Mean \pm Standard Error.
 432



433
 434 Fig. 10. Bactericidal activity of water (fig. 10a) and ethanolic extracts (fig. 10b) from Calabrian
 435 and Sicilian *L. multifida* (indicated in the figure as C and S respectively) and from *L.*
 436 *angustifolia* on *Escherichia coli*. Small letters indicate significant differences between different
 437 concentrations of the same plant extract. Differences were considered significant when $P < 0.05$.
 438 Results showed are representative of at least three independent experiments and are expressed
 439 as Mean \pm Standard Error.
 440

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

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

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

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

477 Although actually there are no available data concerning the effects of lavender extracts
478 on fish immunity and their possibility of use as immunostimulant in aquaculture, some
479 scientific evidences exist about the effect of extracts from other plants belonging to
480 Lamiaceae family on innate immune response. Extracts from *Thymus guyonii*, *Salvia*
481 *verbenaca* and *Stachys circinata* (Lamiaceae family) have been tested for their
482 immunostimulant potential on the phagocytic activity in adult male mice (*Mus*

483 *musculus*) through the carbon clearance rate test, resulting in an appreciable increasing
484 in phagocytic 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,

508 without any significant effect neither on HK leucocytes viability nor on HK leucocytes
509 peroxidase, thus supporting the possibility of use of *L. multifida* L. extracts, in a well-
510 defined range of concentrations, as immunostimulant in the production of this
511 important farmed marine fish species. Immunostimulants are capable of promoting a
512 greater and more effective sustained immune response to those infectious agents
513 (viruses, bacteria, fungi, and parasites), producing subclinical disease without risks of
514 toxicity, carcinogenicity or tissue residues (Muthusamy et al, 2013). Future in vivo
515 studies could demonstrate if similar immunostimulant effects are induced after dietary
516 administration of such extracts to gilthead seabream specimens.

517 Fish cell lines are increasingly important research tools as an alternative to the
518 experimental animals. The long-term SAF-1 cell line was established in 1996 and it is a
519 fibroblast-like culture derived from gilthead seabream (Béjar et al., 1997). Since then,
520 the SAF-1 cell line has proved useful in many applications and varied studies related to
521 virology (Pérez-Prieto et al., 1999; Tafalla et al., 2004; Alonso et al., 2005; Bandín et
522 al., 2006; García Rosado et al., 2008; Cano et al., 2016), bacteriology (Acosta et al.,
523 2009; El Aamri et al., 2012), immunology (Ray et al., 2002; Pelegrín et al., 2004), and
524 more recently in nanoparticles interiorization (Trapani et al., 2015), metal toxicity
525 (Morcillo et al., 2016) or freshness and freeze-thawing of seabream fillets (Diop et al.,
526 2016) studies. Curiously, to the best of our knowledge, only one previous paper focused
527 on the immune modulatory effects of a plant extract (*Aloe arborescens*) on SAF-1 cells
528 (Picchietti et al., 2013). In the study, SAF-1 cells were treated with Aloe extract at
529 different concentrations ($1.2-4.8 \text{ mg ml}^{-1}$) and times (24-72 h). The Aloe extract
530 (1.2 mg ml^{-1}) induced a synergic effect when cells were also stimulated with LPS- or
531 poly I:C which was detected by studying some immune-related gene expression
532 (Picchietti et al., 2013).

533 Present results from the cytotoxic assay developed on SAF-1 cell line after incubation
534 with *L. multifida* extracts demonstrate no significant effects on cell viability in a limited
535 range of concentrations tested. However, the maximum doses used in our experiments
536 (1000 mg mL⁻¹) affected their viability in a significant manner, causing an increase or a
537 decrease in this parameter when SAF-1 cells were incubated with water or ethanolic
538 extracts, respectively. These data agreed with results obtained from the determination of
539 phagocytosis and respiratory burst activities: indeed ethanolic extracts from *L. multifida*
540 C and S provoked a significant decrease in the number of yeast cells ingested after
541 24 h of incubation at 1000 mg mL⁻¹ of concentration, furthermore also the percentage of
542 phagocytic cells showed a significant decrease when HK leukocytes were incubated
543 with ethanolic extracts from all plants tested during 24 h at 1000 mg mL⁻¹ of
544 concentration, and a similar trend was observed also for the respiratory burst activity.
545 Although the main components of the leaves extract have not been studied yet, the
546 analysis of the chemical composition is one of our future next step. These could allow to
547 know if differences in the biochemical components between water and ethanolic
548 extracts exist, which may explain the different properties of the extracts.

549 Finally, present results demonstrated that *L. multifida* L. extracts had no bactericidal
550 activity against the fish pathogens tested. There are no many available data about the
551 antibacterial activity of *L. multifida* L. extracts until now, and the majority of
552 antibacterial studies have been performed on its essential oils. As reported by Khadir
553 and colleagues (2016), *L. multifida* essential oils were tested against methicillin-
554 resistant *Staphylococcus aureus* (MRSA) using disc diffusion method, revealing a good
555 anti-MRSA activity, whereas the ethanolic extract was less active, thus suggesting that
556 different biological effects between leaf extracts and essential oils could be due to
557 differences in their chemical composition. In fact the chemical composition of *L.*

558 *multifida* essential oils studied by GC and GC-MS (Khadir et al, 2016) revealed that
559 carvacrol is the main component (from 27.5% to 57%), being carvacrol a monoterpenic
560 phenol responsible for many biological activities such as antimicrobial, antitumor,
561 antimutagenic, antigenotoxic, analgesic, antiinflammatory, antiparasitic, antiplatelet
562 (Baser, 2008). Instead, in a recent work ethanolic leaf extract of *L. multifida* L. from
563 Southern Italy was screened by UV spectroscopic analysis (Fazio et al, 2014), revealing
564 the presence of vitexin and flavones glucoside derivatives of hypolaetin, scutellarein,
565 luteolin, isoscutellarein, apigenin and chrysoeriol, which were reported to possess
566 mainly antiinflammatory, anticancer and antioxidant properties (Alcaraz and Hoult,
567 1985; Lin et al, 2008; Saeed et al, 2015; Choi et al, 2005). On the other hand, opposite
568 results were obtained with ethanolic extracts from *Lavandula officinalis*, *Melissa*
569 *officinalis*, *Ocimum basilicum*, *Origanum vulgare*, *Rosmarinus officinalis* and *Salvia*
570 *officinalis*, all belonging to Lamiaceae family, which were tested for antimicrobial
571 activity, exhibiting a broad spectrum of inhibitory effects on some fish pathogens like *L.*
572 *anguillarum*, *Y. ruckeri*, *P. damsela*, *L. garvieae* (Bulfon et al, 2014).

573 To conclude, *L. multifida* L. water and ethanolic leaf extracts up to 100 mg mL⁻¹ of
574 concentration increased innate immune activities of *S. aurata* HK leucocytes, more
575 concretely, their phagocytic and respiratory burst activities, but did not exert any
576 bactericidal activity on the bacterial strains tested in the present study, and did not affect
577 significantly SAF-1 cells viability up to 100 mg mL⁻¹ of concentration. Although the
578 extracts did not exert any bactericidal activity directly on the assayed bacteria strains,
579 present results suggest the possibility of use such extracts in in vivo studies in order to
580 corroborate if could be possible using those extracts in aquaculture in order to achieve
581 protection against pathogenic infections through enhancement of the innate immunity of
582 fish.

583

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