In vitro effects of *Origanum vulgare* leaf extracts on gilthead seabream (*Sparus aurata* L.) leucocytes, cytotoxic, bactericidal and antioxidant activities

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35 **Abstract**

36 *Origanum vulgare* is a well-known medicinal plant that has been used since ancient
37 times as an additive in foods and cosmetic preparations. The possible application of *O.*
38 *vulgare* extracts in fish was assessed by using gilthead seabream (*Sparus aurata*) as a
39 marine fish model due to its importance in aquaculture. The *in vitro* effects of aqueous
40 and ethanolic leaf extracts of *O. vulgare* were tested in order to observe any
41 immunostimulant, cytotoxic, bactericidal or antioxidant properties. The results showed
42 that medium or high concentration of aqueous extracts and low concentrations of
43 ethanolic extract, increased head kidney leucocyte activities as well as the number of
44 SAF-1 cells. However, moderate to high concentrations of ethanolic extracts decreased
45 both leucocyte activities and the number of viable SAF-1 cells, suggesting some
46 possible toxic effect towards them. Only the highest concentration of the aqueous
47 extract and medium to high concentrations of the ethanolic extracts showed cytotoxic
48 activity against the tumor PLHC-1 cell line. Bactericidal activity was only detected
49 against *Vibrio harveyi, V. anguillarum* and *Photobacterium damselae* when using the
50 highest concentration of aqueous extract and moderate to high concentrations of
51 ethanolic extract. Finally, both plant extracts presented antioxidant activity particularly
52 the aqueous extract. Overall, the results suggest that both extracts (when used at the
53 appropriate concentration) have immunostimulant, cytotoxic, bactericidal and
54 antioxidant properties, making *O. vulgare* an interesting candidate for incorporation as
55 additive in functional diets for farmed fish.

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58 **Keywords:** *Origanum vulgare*; leave extracts; immunostimulants; cytotoxicity;
59 bactericidal activity; antioxidants; gilthead seabream (*Sparus aurata* L.); teleosts;
60 aquaculture.

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69 **1. Introduction**

70 Global fish consumption and aquaculture activity have increased in recent decades, and
71 this industry is one of the fastest-growing animal food producing sectors [1], with Spain
72 being the biggest producer in the European Union [2]. However, the super-intensive
73 practices developed in fish farms have led to problems, including environmental harm
74 (e.g. bad water quality), increased number of opportunistic microorganisms and stress
75 conditions. Such negative situations may compromise fish growth and health and make
76 animals more susceptible to infections and diseases, resulting in substantial economic
77 loss [3]. Furthermore, in recent years the use of antibiotics to treat and control fish
78 diseases has been banned in the EU because they can accumulate in fish tissues and give
79 rise to resistant bacteria. However, disease prevention is important in order to preserve a
80 sustainable aquaculture, both environmentally and economically. Prophylactic methods
81 based on stimulation of the fish immune system have been successfully used for this
82 purpose and have become an integrated part of the management of modern aquaculture
83 processes [4]. At present, the main prophylactic measures available for farmed fish
84 include vaccination, probiotics and immunostimulation [5].

85 Oregano or Pot Marjoram (*Origanum vulgare)* is a well-known plant used worldwide
86 since ancient times in traditional and folk medicine [6]. Oregano is the most important
87 and variable species of this genus and is widespread throughout the world and is
88 particularly abundant in the Mediterranean area [7-9], Eurasia and the North of Africa
89 [10]. Many studies have demonstrated that the plant presents a wide variety of
90 secondary metabolites, most of them phenolic compounds such as flavonoids,
91 terpenoids, phenolic acids and alkaloids, and fatty acids among others [10-13], which
92 are the principal components responsible for its activities and allows its use not only in
93 traditional medicine, but also in foods and cosmetic preparations [13,14]. Among the
94 pharmacological properties demonstrated for the compounds present in *O. vulgare* are
95 antibacterial, antivirus, anti-inflammation and antioxidant activities [6,15]. For all these
96 reasons, *O. vulgare* could be considered *a priori* as a good source of new natural
97 compounds to treat, prevent and/or control fish diseases in aquaculture.

98 At present, there is intense and active research into natural products with
99 immunostimulant or biocidal activities for fish [16]. In this sense, medicinal plants are a
100 promising alternative to antibiotics, for several reasons, including all the beneficial
101 properties that their biological compounds present in other animals or even in human
102 beings, the low negative impacts on fish, the environment and the human health, low

103 cost and eco-friendly origin [3,17-19]. In addition to the immunostimulant properties, it
104 has also been demonstrated that many medicinal plants are also able to have other
105 positive effects on fish, such as the stimulation of fish growth, weight gain and early
106 maturation of cultured species [20].
107 There are many *in vivo* studies in which plants, their extracts or their essential oils have
108 been used as additives in animal feed, particularly fish [13,17-19]. However, to the best
109 of our knowledge, there are very few studies about the *in vitro* effect of these plants,
110 extracts or essential oils on fish. The present study was undertaken taking into account
111 all these considerations (including the prohibition of antibiotics, their possible
112 replacement by medicinal plants, the abundance of *O. vulgare* and its bioactive
113 compounds). The aim was to evaluate the *in vitro* effects of leaf extracts (both aqueous
114 and ethanolic) obtained from *O. vulgare* on gilthead seabream (*S. aurata* L.) head
115 kidney leucocyte activities (viability, phagocytosis, respiratory burst and peroxidase).
116 Gilthead seabream was selected as a representative species of marine aquaculture.
117 Furthermore, the possible cytotoxic activity of such extracts on SAF-1 cells (cell line
118 obtained from *S. aurata* fibroblasts) and PLHC-1 cell line (hepato-carcinoma obtained
119 from *Poeciliopsis lucida*, topminnow) and the bactericidal activity against three
120 bacterial pathogens for fish (*Vibrio harveyi, V. anguillarum* and *P. damselae*) were also
121 evaluated. Finally, the antioxidant activity of the extracts was determined. The results of
122 the selected properties of the oregano extracts (immunostimulant, cytotoxic, bactericidal
123 and antioxidant) are discussed and suggest that this plant may be considered an
124 interesting candidate for incorporation as additive in functional diets for farmed fish.
125
126
127 ## 2. Material and methods
128
129 *2.1. Plant extracts*
130 Dried leaves of *O. vulgare* were bought in a local market (Murcia, Spain). Leaves were
131 crashed until to be powder. One g of powder and 40 mL of water or absolute ethanol
132 were used for extract´s preparations [21]. To prepare the aqueous extracts, leaves were
133 macerated and shaken with boiling water for 4 h at 25 ºC. The mixture was filtered
134 twice using a nylon net filter with a 100 mm pore size, and evaporated in a rotary
135 evaporator (Buchi Rotavapor R-215) until dryness. Prior to use in the assays, the
136 extracts were filtered using sterile filters of 0.22 mm diameter. For the preparation of

137 ethanolic extracts, dry leaves were macerated and shaken with pure ethanol (1:40, 48 h,
138 and 25 ºC). The resulting mixture was then filtered twice as described above, and
139 concentrated by vaporizing using a rotary evaporator.
140
141 *2.2. Animals*
142 Five specimens (52.75 ± 3.62 g weight) of the seawater teleost gilthead seabream (*S.*
143 *aurata* L.), obtained from a local farm (Murcia, Spain), were kept in re-circulating
144 seawater aquaria (250 L) in the Marine Fish Facilities at the University of Murcia. The
145 water temperature was maintained at 20 ± 2 ºC with a flow rate of 900 L h^{-1} and 28‰
146 salinity. The photoperiod was 12 h light:12 h dark. Fish were allowed to acclimatize for
147 15 days before the start of the trial, where they were fed with a commercial pellet diet
148 (Skretting, Spain) at a rate of 2% body weight day^{-1}. The fish were killed after starving
149 for 24 h by using an overdose of MS-222 (Sandoz, 100 mg mL^{-1} water). All
150 experimental protocols were approved bythe Ethical Committee of the University of
151 Murcia.
152
153 *2.3. Head-kidney leucocyte isolation and incubation with extracts*
154 Before the dissection of the head-kidney (HK), the specimens were bled. Blood was
155 collected from the caudal vein and afterwards fish were dissected to obtain HK
156 fragments, isolating the leucocytes according to Esteban et al. [22]. Briefly, HK were
157 cut into small fragments and transferred to 12 mL of sRPMI [RPMI-1640 culture
158 medium (Gibco) supplemented with 0.35% sodium chloride (to adjust the medium's
159 osmolarity to gilthead seabream plasma osmolarity of 353.33 mOs), 3% foetal calf
160 serum (FCS, Gibco), 100 i.u. mL^{-1} penicillin (Flow) and 100 mg mL^{-1} streptomycin
161 (Flow)]. HK leucocytes were obtained by forcing fragments of the organ through a
162 nylon mesh (mesh size 100 mm), washed twice (400 g, 10 min), counted in an
163 automatic counting chamber (BioRad) and adjusted to $2x10^7$ cells mL^{-1} in sRPMI. Cell
164 viability was determined by the trypan blue exclusion test.
165 To study the possible effects of aqueous and ethanolic extracts on HK leucocyte
166 activities, aliquots of 50 µL of the HK leucocyte suspension containing $2x10^7$ cells mL^{-1}
167 were dispensed into glass tubes (Falcon, Becton Dickinson) to ascertain viability and
168 phagocytic activity, 50 µL into a flat-bottomed 96-well plates to assess respiratory burst
169 activity and 5 µL into a flat-bottomed 96-well plates for peroxidase activities.
170 Afterwards, aliquots of 50 µL of aqueous or ethanolic extracts (0.002, 0.2, 1 and 2 mg

171 mL^{-1} prepared in sRPMI) were added to each glass tubes for viability and phagocytic
172 activity assays. Aliquots of 50 µL of the extracts were added to each well of flat-
173 bottomed 96-well plates to check respiratory burst activity and aliquots of 5 µL of the
174 extracts were added to each well of flat-bottomed 96-well plates for peroxidase activity.
175 The extract aliquots were replaced by sRPMI on control samples for those assays
176 developed with aqueous extracts. On the other hand, extracts were replaced by 1%
177 dimethyl sulfoxide (DMSO, Sigma) in sRPMI in the case of control samples for assays
178 carried out with ethanolic extracts. Cells were incubated in the presence of the extracts
179 for 24 h at 21 ºC in an incubator with 5% CO_2 and 85% humidity. After incubation, HK
180 leucocyte viability, phagocytic, respiratory burst and peroxidase activities were
181 determined as described below.
182
183 *2.4. Leucocyte viability*
184 Leucocyte viability was studied adding 50 µL of propidium iodide (PI) (400 mg mL^{-1},
185 Sigma) to each 100 µL aliquot of HK leucocytes (previously incubated with the
186 extracts, as described above). The tubes were gently mixed before analysis in a
187 FACScan (Becton Dickinson, Madrid, Spain) flow cytometer with an argon-ionlaser
188 adjusted to 488 nm. Analyses were performed on 5,000 cells, which were acquired at a
189 rate of 300 cells s^{-1}. Data were collected in the form of two-parameter side scatter
190 (granularity, SSC) and forward scatter (size, FSC), and green fluorescence (FL1) and
191 red fluorescence (FL2) dot plots or histograms were made on a computerized system.
192 Dead cells were estimated as the percentage of cells with propidium iodide (red-PI
193 fluorescent cells). A quantitative study of the flow cytometric results was made using
194 the statistical option of the Lysis Software Package (Becton Dickinson).
195
196 *2.5. Phagocytic activity*
197 The phagocytic activity of gilthead seabream HK leucocytes was studied by flow
198 cytometry according to Esteban et al. [23]. Heat killed (30 min, 60 ºC) lyophilized *S.*
199 *cerevisiae*, strain S288C, were washed twice, counted and adjusted to 10^8 yeast cells
200 mL^{-1} in sRPMI-1640. To label yeast cells with fluorescein isothiocyanate (FITC,
201 Sigma) they were incubated with 5 mg mL^{-1} FITC at 22 ºC with constant stirring (40
202 cycles min^{-1}) and in darkness for15 min [24]. After labelling, free FITC was removed
203 by washing twice in phosphate buffer saline (PBS) and the yeast cells were resuspended
204 in sRPMI-1640. FITC-labeled yeast cells were acquired for flow cytometric study. The

205 staining uniformity was examined and then the yeast cell suspensions were aliquoted
206 and stored at -80 ºC.
207 Phagocytosis samples consisted of 60 µl of labelled-yeast cells and 100 µl of HK
208 leucocytes (previously incubated as described above). Samples were mixed, centrifuged
209 (400 g, 5 min, and 22 °C), resuspended and incubated at 22 °C for 30 min. At the end of
210 the incubation time, samples were placed on ice to stop phagocytosis and 400 µl ice-
211 cold PBS was added to each sample. The fluorescence of the extracellular yeasts was
212 quenched by adding 50 µl ice-cold trypan blue (0.5% in PBS). Standard samples of
213 FITC-labelled *S. cerevisiae* or HK leucocytes were included in each phagocytosis assay.
214 All samples were analysed in a flow cytometer (Becton Dickinson) with an argon-ion
215 laser adjusted to 488 nm. Analyses were performed on 5,000 cells, which were acquired
216 at a rate of 300 cells s^{-1}. Data were collected in the form of two-parameter side scatter
217 (granularity) (SSC) and forward scatter (size) (FSC), and green fluorescence (FL1) dot
218 plots or histograms were made on a computerised system. The fluorescence histograms
219 represented the relative fluorescence on a logarithmic scale. The cytometer was set to
220 analyse the phagocytic cells showing the highest SSC and FSC values. Phagocytic
221 ability was defined as the percentage of cells with one or more ingested bacteria (green-
222 FITC fluorescent cells) within the phagocytic cell population. The relative number of
223 ingested yeast cells per cell (phagocytic capacity) was assessed in arbitrary units from
224 the mean fluorescence intensity of the phagocytic cells.
225
226 *2.6. Respiratory burst activity*
227 The respiratory burst activity of HK leucocytes was studied by a chemiluminescence
228 method [25]. Briefly, 100 µl of HBSS (Hank's balanced salt solution, Gibco) containing
229 1 mg mL^{-1} phorbol myristate acetate (PMA, Sigma) and 10^{-4} M luminal were added to
230 the 100 µl of HK leucocytes (previously incubated as described above). The plates were
231 shaken and immediately read in a chemiluminometer (BMG, FluoStar Galaxy).
232 Measurements were performed in 30 cycles of 2 min each. The kinetics of the reactions
233 were analyzed and the maximum slope of each curve was calculated. Control samples
234 containing leucocytes that had not been incubated with the extracts were also analyzed.
235
236 *2.7. Peroxidase activity*
237 The total peroxidase activity of HK leucocytes was measured according to Quade and
238 Roth [26]. To do this, HK leucocytes (previously incubated as described above) were

239 incubated for 10 min with 0.002% cetyltrimethylammonium bromide (CTAB, Sigma) at
240 60 rpm. Afterwards, 100 µL of 10 mM 3,3',5,5'-tetramethylbenzidine hydrochloride
241 (TMB, Sigma) and 5 mM H_2O_2 (both substrates prepared daily) were added and after 2
242 min, 50 µL of 2 M sulfuric acid was also added to stop the reaction. The absorbance of
243 the samples was measured at 450 nm in a microplate reader (BMG Fluostar Omega,
244 USA). Control samples containing leucocytes that had not been incubated with extracts
245 were also analyzed.
246
247 *2.8. Cytotoxic activity*
248 The established cell line SAF-1 (ECACC nº 00122301) was seeded in 25 cm^2 plastic
249 tissue culture flasks (Nunc, Germany) in L-15 Leibowitz medium (Life Technologies,
250 UK), supplemented with 10% fetal bovine serum (FBS, Life Technologies), 2 mM L-
251 glutamine (Life Technologies), 100 i.u. mL^{-1} penicillin (Life Technologies) and 100 mg
252 mL^{-1} streptomycin (Life Technologies). Cells were grown at 25 ºC in a humidified
253 atmosphere (85% humidity). Exponentially growing cells were detached from culture
254 flasks by brief exposure to trypsin (0.25% in PBS, pH 7.2-7.4), according to the
255 standard trypsinization methods. The detached cells were collected by centrifugation
256 (200 g, 5 min, 25 ºC) and cell viability was determined by the trypan blue exclusion
257 test.
258 The established cell line PLHC1 (ATCC® CRL2406™) was seeded in 25 cm^2 plastic
259 tissue culture flasks in Minimum essential medium (Eagle) with 2 mM L-glutamine and
260 Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential
261 amino acids, 1.0 mM sodium pyruvate, 95% and fetal bovine serum, 5% (Life
262 Technologies, UK). Cells were grown at 30 ºC in a humidified atmosphere (85%
263 humidity) and 5% CO_2. Exponentially growing cells were detached from the culture
264 flasks by brief exposure to of trypsin (0.05% in PBS, pH 7.2-7.4), according to the
265 standard trypsinization methods. The detached cells were collected by centrifugation
266 (200 g, 5 min, 30 ºC) and the cell viability was determined by the trypan blue exclusion
267 test.
268 A cytotoxicity assay of each cell type was performed in five replicates at each
269 concentration of each extract. When cell lines were approximately 80% confluent, cells
270 were detached from the flasks culture with trypsin (as described before), and aliquots of
271 100 µL containing 50,000 cells $well^{-1}$ were dispensed into 96-well tissue culture plates
272 and incubated (24 h, at the temperature for each cell line). This cell concentration was

273 previously determined in order to obtain satisfactory absorbance values in the cytotoxic
274 assay and to avoid cell overgrowth. After that, the culture medium was replaced by 100
275 µL well^{-1} of the extracts to be tested at the appropriate dilution. The tested
276 concentrations of aqueous and ethanolic extracts were 0.001, 0.05, 0.1, 0.25, 0.5, 0.75
277 and 1 mg mL^{-1}. Control samples received the same volume of culture medium (for the
278 aqueous extracts) or DMSO 1% (for the ethanolic extracts). Cells were incubated for 24
279 h and then their viability was determined using the MTT assay, which is based on the
280 reduction of the yellow soluble tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-
281 diphenyltetrazolium bromide) (MTT, Sigma) to a blue, insoluble formazan product by
282 mitochondrial succinate dehydrogenase [27,28]. After incubation with the plant extracts,
283 cells were washed with phosphate buffer saline solution (PBS) and 200 µL well^{-1} of
284 MTT (1 mg mL^{-1}) were added. After 4 h of incubation, cells were washed again and the
285 formazan crystals were solubilized with 100 µL well^{-1} of DMSO. Plates were shaken (5
286 min, 100 rpm) in dark conditions and the absorbance at 570 nm and 690 nm determined
287 in a microplate reader (BMG Fluostar Omega, USA).
288
289 *2.9. Bactericidal activity*
290 Three pathogenic bacteria for fish (*V. harveyi*, *V. anguillarum* and *P. damselae*) were
291 used in the bactericidal assay. All bacterial strains were grown from 1 mL of stock
292 culture that had been previously frozen at -80 ºC. The three bacteria were cultured for
293 48 h at 25 ºC in Triptic Soy Agar (TSA, Difco Laboratories), and then inoculated in
294 Triptic Soy Broth (TSB, Difco Laboratories), both supplemented with NaCl to a final
295 concentration of 1% (w/v). Bacteria in TSB medium were then cultured at the same
296 temperature, with continuous shaking (100 rpm) for 24 h. Exponentially growing
297 bacteria were resuspended in sterile PBS and adjusted to 10^8 colony forming units
298 (c.f.u.) mL^{-1}.
299 Bactericidal activity was determined following the method of Stevens et al. [29] with
300 some modifications. Samples of 20 µL of aqueous or ethanolic extracts previously
301 adjusted to 0.002, 0.1, 0.2, 0.5, 1, 1.5, 2 mg mL^{-1} were added (in six replicates) to the
302 wells of a flat-bottomed 96-well plate. PBS solution was added to some wells instead of
303 the extracts and served as positive control. Aliquots of 20 µL of the previously cultured
304 bacteria were added and the plates were incubated for 5 h at 25 ºC. Then, 25 µL of MTT
305 (1 mg mL^{-1}) were added to each well and the plates were newly incubated again for 10
306 min at 25 ºC to allow the formation of formazan. Plates were then centrifuged (2,000 g,

307 10 min), and the precipitates dissolved in 200 µL of DMSO were transferred to a flat-
308 bottom 96-well plate. The absorbance of the dissolved formazan was measured at 570
309 nm. Bactericidal activity was expressed as percentage of non-viable bacteria, calculated
310 as the difference between absorbance of surviving bacteria compared to the absorbance
311 of bacteria from positive controls (100%).
312
313 *2.10. Antioxidant activity*
314 The antioxidant power of aqueous or ethanolic extracts were analysed by the 2,2'-azino-
315 bis-3-(ethylbenzothiazoline-6-sulphonic acid) (ABTS) method described by Cano and
316 Acosta [30], which is based on the ability of the antioxidants in the sample to reduce the
317 radical cation of ABTS, as determined by the decolouration of ABTS·+, and measuring
318 the quenching of the absorbance at 730 nm. This activity is calculated by comparing the
319 values of the sample with a standard curve of ascorbic acid and expressed as ascorbic
320 acid equivalents (mmol) mg protein^{-1}. Samples of 50 µL of aqueous or ethanolic
321 extracts previously adjusted using PBS to 0.1, 0.5 and 1 mg mL^{-1} were added to 950 µL
322 of cation ABTS·+ and the decrease of absorbance was measured in a spectrophotometer
323 (BOECO S-22 UV/Vis, Germany) using as blank of reaction PBS. The samples were
324 analyzed in triplicate.
325
326 *2.11. Statistical analyses*
327 The results are expressed as means ± SEM. The normality of the variables was
328 confirmed by the Shapiro–Wilk test and homogeneity of variance by the Levene test.
329 Statistical differences among the four groups of treatments were assessed by one-way
330 ANOVA analyses, followed by the Tukey or Games Howell test, depending on the
331 homogeneity of the variables. The significance level was 95% in all cases ($P<0.05$). All
332 the data were analysed by the computer application SPSS for Windows® (version 15.0,
333 SPSS Inc., Chicago, USA).
334
335
336 **3. Results**
337
338 *3.1. Effects of leaf extracts on head kidney leucocyte activities*
339 The viability of the cells as well as the phagocytic, respiratory burst and peroxidase
340 activities of gilthead seabream HK leucocytes were studied after 24 h of incubation with

341 aqueous or ethanolic extracts in a range of concentrations from 0.001 to 1 mg mL^{-1}
342 (0.001, 0.1, 0.5, 1 mg mL^{-1}). The obtained results were compared with control samples
343 (cells incubated in culture medium without extracts). The results showed that only the
344 most concentrated (1 mg mL^{-1}) ethanolic extract decreased HK leucocytes viability with
345 respect to the control group to a statistically significant degree (Fig. 1).
346 As regard phagocytosis, aqueous extracts significantly increased the phagocytosis
347 ability of HK leucocytes with respect to control leucocytes, but not at the highest
348 concentration of 1 mg mL^{-1} (Fig. 2A). In the case of ethanolic extracts, the lowest
349 concentration (0.001 mg mL^{-1}) also increased the phagocytosis activity while 1 mg mL^{-1}
350 decreased it, both with respect to the control samples (Fig. 2B). On the other hand, only
351 the incubation of leucocytes with 0.5 mg or 1 mg mL^{-1} of aqueous extracts and 1 mg
352 mL^{-1} of ethanolic extract significantly decreased their phagocytic capacity compared
353 with the leucocytes from the control group (Fig. 2C, 2D).
354 Respect to respiratory burst activity, incubating leucocytes with 1 mg mL^{-1} of aqueous
355 extract or with 0.1 mg mL^{-1} or higher concentrations of ethanolic extracts significantly
356 decreased this activity in HK leucocytes compared with the control group (Fig. 3A, 3B).
357 Finally, no significant variations were recorded in the peroxidase activity of HK
358 leucocytes after incubation with either of the studied extracts at any tested
359 concentrations (Fig. 3C, 3D).
360
361 *3.2. Effects of leaf extracts on SAF-1 and PLHC-1 cell lines*
362 The possible cytotoxic effects of aqueous and ethanolic extracts were evaluated on
363 SAF-1 and PLHC-1 cells after 24 h of incubation with different concentrations of
364 extracts ranging from 0.001 to 1 mg mL^{-1} (0.001, 0.05, 0.1, 0.25, 0.5, 0.75, 1 mg mL^{-1}),
365 comparing the results with the control group, which consisted of cells incubated only
366 with medium. The results showed that the incubation of SAF-1 cells with leaf extracts
367 ranging from 0.001 to 0.25 mg mL^{-1} did have no significant effect on cell viability.
368 However, when SAF-1 cells were incubated with 0.5 mg mL^{-1} aqueous extract or more
369 concentrated, significant increases were detected in SAF-1 cells viability (Fig. 4A).
370 Regarding the ethanolic extracts, no significant effects were recorded on SAF-1 cells
371 viability after incubation with concentrations ranging from 0.001 to 0.75 mg mL^{-1}.
372 However, SAF-1 cell viability fell significantly when cells were incubated with 1 mg
373 mL^{-1}, respect to control leucocytes (incubated only with medium) (Fig. 4B).

374 The viability of the PLHC-1 cell line was not significantly affected by the incubation
375 with aqueous extracts ranging from 0.001 to 0.25 mg mL^{-1}. However, the viability of
376 these cells significantly decreased after incubation with 0.5 mg mL^{-1} or higher
377 concentrations (Fig. 5A). Similarly, no significant effects in cell viability were recorded
378 when PHLC-1 cells were incubated with ethanolic extracts ranging from 0.001 to 0.1
379 mg mL^{-1}, respect to control cells (Fig. 5B). However, significant decreases were
380 recorded in cell viability when cells were incubated with 0.25 mg mL^{-1} or higher
381 concentrations of ethanolic extracts.
382
383 *3.4. Bactericidal activity of leaf extracts*
384 In this study *O. vulgare* leaf extracts were also tested for their bactericidal activity
385 against three important pathogens for fish aquaculture (*V. harveyi*, *V. anguillarum* and
386 *P. damselae*). In the case of *V. harveyi*, aqueous extracts promoted their growth in a
387 dose-dependent manner, with respect to control samples. The observed differences were
388 statistically significant after incubation of the bacteria with aqueous extracts ranging
389 from 0.05 to 1 mg mL^{-1} (Fig. 6A). No significant differences were recorded in the
390 bactericidal activity of *O. vulgare* against *V. anguillarum* and *P. damselae* with the
391 exception of the highest concentration tested (1 mg mL^{-1}) which produced bacterial
392 activity against *P. damselae* (Fig. 6B, 6C). In the case of the ethanolic extracts,
393 significant dose-dependent bactericidal activity was detected against the three pathogen
394 bacteria used in the present work, starting from 0.5 mg mL^{-1} for *V. harveyi* (Fig. 6D)
395 and from 0.25 mg mL^{-1} for *V. anguillarum* and *P. damselae* (Fig. 6E, 6F) respect control
396 samples.
397
398 *3.5. Antioxidant activity of leaf extracts*
399 Finally, the antioxidant activity of *O. vulgare* leaf extracts was determined in a range of
400 concentrations from 0.1 to 1 mg mL^{-1} (0.1, 0.5 and 1 mg mL^{-1}). Interestingly, both
401 aqueous and ethanolic extracts showed antioxidant activity in a dose-dependent manner,
402 the aqueous extracts having more antioxidant activity than the ethanolic (Fig. 7).
403
404
405 **4. Discussion**
406 The use of immunostimulants (a chemical, drug, stressor or action that enhances the
407 defence mechanisms or immune response [31]) in the fish diet has been a common

408 practice for many years [32]. Among immunostimulants, many medicinal plants can be
409 considered as good candidates for use in farmed fish, mainly due their known beneficial
410 effects on humans [3,17-19] . Indeed, such plants have numerous bioactive components
411 which are considered as a powerful alternative to the use of antibiotics [6,15,19]. In
412 recent years, many studies have described the good results obtained with use of different
413 medicinal plants and their *in vivo* effects on fish [3,17-19, reviewed by 33]. It was these
414 good results obtained using medicinal plants that led us to undertake the present work
415 using *O. vulgare*.
416 Oregano was selected for its recognised valuable properties in humans and also due to
417 its abundance. In traditional medicine, this plant has been used to treat a wide range of
418 diseases [13,15,34-36]. Many properties as a tonic, muscle relaxant, antioxidant,
419 cytotoxic, detoxifying, antimicrobial, anti-inflammatory, antimutagenic, antidiabetic
420 [6], antinociceptive [37] and nephroprotective [38] activities, among others, have been
421 described for this plant [10,39]. The present work focuses on the possible application of
422 this plant as immunostimulant, cytotoxic, bactericidal or antioxidant agent on farmed
423 fish, and attempts to select the most appropriate extract for each situation. The results
424 will help to design future research efforts dissected at the inclusion of the extracts as
425 feed additive for fish.
426 It is well known that the biological activities attributed to a plant are related to the
427 whole chemical compounds present in them [13,40]. These compounds, which are
428 secondary metabolites, can be obtained from the whole plant or from a specific part of a
429 plant, including fruits, seeds, flowers, roots, stems and leaves [41]. The chemical
430 composition of plants depends on many factors [40,42] and, although the presence and
431 concentration of the different components may vary according to the part of the plant, it
432 has been demonstrated that they are usually more varied and presented in higher
433 concentration in the aerial parts of the plants [41,43,44]. This was the main reason for
434 choosing the leaves of *O. vulgare* to obtain the extracts used in the present work.
435 It is also known that the active compounds present in plants differ as regards solubility,
436 and that the chemical compounds extracted from a plant depend on the time of
437 extraction, solvent used and the method of extraction [40]. In the present work we tested
438 different concentrations of aqueous and ethanolic extracts in a range from 0.001 to 1 mg
439 mL^{-1}, the same concentrations as used with *Lavandula sp.* plant extracts, also in gilthead
440 seabream HK leucocytes and the SAF-1 cell line [45], in order to facilitate comparison
441 among of the obtained results.

442 In general, the aqueous extracts of oregano contain high amounts of phenolic
443 compounds and flavonoids, including chlorogenic, caffeic, p-coumaric, ferulic,
444 rosmarinic and ursolic acids among others, which confer antioxidant activity [13,37,40].
445 However, in the ethanolic extracts carvacrol and thymol are the most abundant
446 monoterpenes although p-cymene, l-octocosanol and phytol are also present. These
447 substances are responsible for the rest of the activities demonstrated for oregano
448 although they also have an antioxidant effect [6,15,46]. Besides this, oregano presents a
449 peculiar composition of fatty acids [10-13], principally α-linolenic, linoleic and palmitic
450 acids [13]. Fatty acids are very important in the normal growth and in health
451 maintenance because they are able of improving the immune status and acting
452 preventing diseases [47]. In the present study these fatty acids could also be responsible
453 for the immunostimulant effects on gilthead seabream leucocytes. Furthermore, this
454 plant contains high levels of vitamins including A,C, E and B-complex, which also
455 present immunostimulant properties and high antioxidant activity [46]. Future studies
456 could be developed to determine the effects of these bioactive compounds on the fish
457 metabolism.
458 Several works have studied the immune parameters of various fish species after
459 intraperitoneal injection or the dietary administration of plant extracts. In general,
460 treated fish showed increased humoral (e.g. plasma protein, lysozyme and complement
461 activity) and cellular (phagocytic and respiratory burst activities) immune activities [48-
462 50]. However, there are very few studies that have tested the *in vitro* effects of plant
463 extracts on fish immune cells [45,51]. We carried out this *in vitro* study in an attempt to
464 reduce the number of animals used in research activities. The present work studies the
465 immunostimulant effect of oregano extracts on gilthead seabream HK leucocytes.
466 Firstly, we verified the effects on cell viability so that those plant extracts with a
467 negative impact on cells could be discarded. The present results corroborate that only
468 the most concentrated ethanolic extract used (1 mg mL^{-1}) significantly decreased HK
469 leucocyte viability, since it was toxic for cells. These results underline the fact that both
470 the concentration and type of extract are crucial for obtaining good results. Afterwards,
471 the effects of such extracts were studied on the main innate cellular immune activities.
472 Phagocytosis is a self-protective reaction against infection and invasion of the animal
473 body by foreign substances, and is considered a crucial mechanism for limiting the
474 growth of fish pathogens [52]. In fact, the phagocytic activity is considered the main
475 cellular activity of the innate immunity and recent studies have revealed

476 that phagocytosis is also crucial for tissue homeostasis and remodelling [53].
477 Phagocytosis in the head kidney of gilthead seabream is carried out mainly by
478 specialized cells, the monocyte-macrophages and granulocytes [22]. In the present work
479 we determined two parameters of phagocytosis, the phagocytic ability, which is the
480 percentage of cells that interiorized the target (yeast cells in the present work) and the
481 phagocytic capacity, which reflects the quantity of test particles interiorized by each
482 phagocytic cell. The results demonstrated that the incubation of leucocytes with both
483 tested extracts increased their phagocytic ability, with the exception of 1 mg mL^{-1}
484 ethanolic extracts, which induced a decrease in this activity and also in the phagocytic
485 capacity, probably due to a decrease in the number of viable leucocytes, as previously
486 indicated. However, the stimulant properties of the extracts disappeared when using the
487 highest concentration tested. More specifically, the decreased phagocytic capacity was
488 detected when leucocytes were incubated with 0.5 mg mL-1 or 1 mg mL^{-1} aqueous
489 extracts. In general, positive effects of phagocytic activity of leucocytes were recorded
490 after incubation of the cells with plant extracts. For example, the effects of some plant
491 extracts on phagocytosis have also been reported on mammals. Different plant extracts
492 were tested a large range of dilutions as used in Homeopathy on the phagocytosis
493 carried out by human granulocytes. The high stimulatory action was observed
494 when extracts from *Uvae Ursi* and *Saponaria* were tested. A moderate stimulatory
495 action was found when using extracts from *Echmaceea*, *Aleo* and *Prumis* [54].
496 Similarly, enhanced phagocytic activity was also demonstrated after incubation of
497 seabream leucoytes with extracts from *Lavandula* sp. [45]. More studies are needed to
498 understand which substances present in the extracts may be responsible for these results.
499 During the phagocytosis of microorganisms, phagocytes increase their oxygen
500 consumption, which generates superoxide anion and hydrogen peroxide through the
501 activity of an NADPH-oxidase. Afterwards, these oxygen metabolites give rise to other
502 reactive oxygen species that have a strong anti-microbial activity, but which may also
503 cause some damages *in situ* by destroying surrounding tissue and inducing apoptosis in
504 other immune cells. Furthermore, although less studied, granulocyte peroxidases are
505 lysosomal proteins stored in cytoplasmic granules that are also involved in antimicrobial
506 mechanisms. Peroxidases possess antimicrobial activity via hypohalous acid production
507 [55] and are released into the extracellular space during degranulation [23,56]. Only
508 depressing effects were observed in gilthead seabream HK leucocyte respiratory burst
509 activity after incubation with *O. vulgare* extracts. A better understanding of the

510 molecular phenomena involved in the regulation of NADPH oxidase in fish could help
511 us to understand how the respiratory burst, which is also a crucial effector mechanism
512 of fish immunity, is regulated. Besides the above, no significant variations were
513 detected in the peroxidase activity of leucocytes after incubation with the oregano
514 extracts. These results suggest that the effect of the extracts depended on the leucocyte
515 activity tested, the type of the extract and the extract concentration. The results also
516 indicate the range of concentrations of aqueous and ethanolic extracts of *O. vulgare*
517 optimum which can be used as immunostimulants without the risk of toxicity for the
518 cells when used in *in vitro* studies. To the best of our knowledge, there is only one
519 previous paper about the *in vitro* effects of the oregano extracts on cells. The study
520 demonstrated that the use of oregano ethanolic extracts improved the post-thawed
521 quality of semen. Authors suggested that perhaps the observed improvements were due
522 to increasing in antioxidant enzyme activity and reduction in lipid peroxidation [57].
523 Fish cell lines are increasingly significant research tools as alternatives to the use of
524 experimental animals. The SAF-1 cell line is a fibroblast-like culture obtained in 1996
525 from gilthead seabream fin and it has a doubling time of 2 days [58]. To the best of our
526 knowledge, this is the first study of the *in vitro* effects of oregano extracts on a fish cell
527 line. Incubation of the cells with aqueous extracts increased cell viability, while
528 incubation with ethanolic extracts had the opposite effect, decreasing viability. These
529 results agree with those obtained in a previous study developed by our research group
530 that focused on SAF-1 cell viability after incubation with extracts of three different
531 species of *Lavandula.* The results indicated that SAF-1 cell line viability was not
532 significantly affected after incubation with *L. multifida* extracts. However, the
533 maximum doses used in the experiment (1 mg mL^{-1}) affected the viability in a
534 significant manner, causing an increase or a decrease in this parameter when SAF-1
535 cells were incubated with aqueous or ethanolic extracts, respectively [45]. The present
536 work allowed us to compare the effects of oregano extracts on the viability of a primary
537 culture of HK leucocytes and on a cell line obtained from the same fish species. The
538 results demonstrate that moderate or high concentrations of ethanolic extracts have
539 cytotoxic effects while aqueous extracts are able to increase SAF-1 cell viability. In
540 both cell types (leucocytes and SAF-1 cells), ethanolic extracts at moderate or high
541 concentrations were toxic for cells. Regarding the increase of mitosis and viability of
542 the SAF-1 cells observed after incubation with the aqueous extracts of oregano, it could
543 be interesting to study whether dietary administration of such extracts or of the whole

544 leaves could be used to treat wounds or scarring of the fish tissues after injury, taking
545 into account that the SAF-1 cells were obtained from fin [58].
546 Regarding the cytotoxic activity of oregano extracts on the PLHC-1 cell line, only the
547 highest concentration of the aqueous extracts tested and low-moderate to high
548 concentrations of the ethanolic extracts showed cytotoxic activity. Our results agree
549 with those of similar studies, where both aqueous and ethanolic extracts of leaves from
550 oregano showed dose-dependent cytotoxic activity against different human tumour cell
551 lines, including HeLa cells (human cervical cancer), and RD (rhabdomyo sarcoma),
552 CACO2 (colon adenocarcinoma) and A549 (human lung adenocarcinoma) cell lines
553 [37,40,59-62]. Phenolic compounds including flavonoids, phenolic acids and especially
554 the terpenoids carvacrol and thymol are the main compounds present in the extracts
555 responsible for this activity and, it is known that, in general, they are much more
556 abundant in ethanolic extracts [6,61].
557 The ability of some herbs and seaweeds to inhibit the activity of bacterial fish pathogens
558 is of great interest [63-67]. However, our knowledge about the bactericidal activity of
559 oregano as a natural treatment for fish bacterial pathogens is limited. Therefore, another
560 objective of the present study was to study the effects of *O. vulgare* extracts against
561 three opportunistic pathogenic bacteria, *V. harveyi*, *V. anguillarum* and *P. damselae*
562 (formerly *V. damselae*) [68-70], which were selected because they are responsible for
563 infections that affect a variety of marine animals, including fish, crustaceans, molluscs
564 and cetaceans, and also humans. Furthermore, Vibrionaceae represents the major cause
565 of mortality in farmed marine species [70,71]. In this respect, past results demonstrate
566 that Gram-positive marine bacteria are generally more susceptible to herbal extracts
567 than Gram-negative marine Vibrionaceae [67]. Nevertheless, some herbals act as
568 inhibitors of the quorum-sensing pathways in *Vibrio* sp. [72]. It is important to
569 underline that in the present study, both extracts showed bactericidal activity against the
570 three fish pathogen tested in a dose-dependent manner. Again, our results demonstrated
571 that ethanolic extracts showed higher bactericidal activity than aqueous extracts. Low-
572 medium and high concentrations of ethanolic extracts showed bactericidal activity
573 against the three pathogens tested, while only the highest concentration of the aqueous
574 extract showed activity against *P. damselae*. The present results agree with other studies
575 that demonstrated that ethanolic extracts have stronger bactericidal activity than
576 aqueous ones [15,61,73,74]. The different bactericidal activity observed in this study
577 between aqueous and ethanolic extracts may be due to the different components

578 extracted in each case. Among the most abundant components present in oregano
579 leaves, flavonoids, phenolic acids and specially terpenoids present bactericidal activity
580 and are much more abundant in ethanolic than in aqueous extracts [6,15]. When ethanol,
581 methanol and hexane extracts from *Ocimum basilicum* were investigated for their *in*
582 *vitro* antimicrobial properties against 146 microbial organisms including aquaculture
583 pathogens, the hexane extract showed a stronger and broader spectrum of antibacterial
584 activity [75]. This suggests that hexane extracts of oregano might exhibit even higher
585 bactericidal activity than the activities obtained in the present work.
586 As regards *V. harveyi*, the aqueous extracts did not show any bactericidal activity
587 against this bacterium, and the number of viable bacteria increased in a dose-dependent
588 manner. Previous studies indicated that aqueous extracts of oregano had no [15,73] or
589 low [12,15,76-78] bactericidal activity against different human bacteria. Furthermore,
590 the available results seem to indicate that the temperature is also important for the
591 composition of the obtained extracts and may be a very significant factor in terms of
592 bactericidal activity. In this sense, an aqueous decoction was prepared by boiling
593 oregano leaves in sterile distilled water for 15 minutes and the obtained extract did not
594 have bactericidal activity [78,79]. However, when an aqueous infusion was prepared by
595 soaking *the oregano* leaves in sterile distilled water for two days with occasional
596 shaking, the obtained infusion had bactericidal activity against many bacteria (including
597 *E. coli*, *P. aeruginosa, P. mirabilis, A. hydrophila, E. aerogenes, S. marcescens, S.*
598 *dysenteriae, Staphylococcus sp., Klebsiella sp., Micrococcus sp., Bacillus sp.,*
599 *Salmonella sp., Citrobacter sp.* and *Flavobacterium sp.*) [12,78,79].
600 Finally, due the high intensity of cultivation that takes place in fish farms, fish can
601 suffer stress and produce reactive oxygen radicals which can cause diseases, infections
602 and even death. Furthermore, the use of natural antioxidants is emerging as an effective
603 methodology for controlling rancidity and limiting its deleterious consequences on fish
604 [80]. Due to this fact, the incorporation of antioxidant compounds in the fish feed is
605 increasingly important. It is known that herbal compounds have the ability to inhibit the
606 generation of oxygen anions and to scavenge free radicals. The antioxidant effect of
607 herbal compounds has been shown to be similar to that of superoxide dismutase, metal-
608 ion chelators and xanthine oxidase inhibitors [72]. Both tested extracts of oregano
609 showed a dose-dependent antioxidant activity, which was particularly evident in the
610 aqueous extracts. Previous studies have shown the antioxidant activity of aqueous and
611 ethanolic extracts depended on the technique used [12,15,40,61,73,81]. The antioxidant

612 activity of oregano extracts is attributable to phenolic compounds, including terpenoids,
613 flavonoids, phenolics acids and vitamins present on them [15,40,73].
614 To conclude, the oregano bioactive principles present in aqueous and ethanolic extracts
615 have many interesting properties (immunomodulator, cytotoxic, bactericidal and
616 antioxidant) which make them very attractive for use in fish aquaculture. Their use will
617 reduce the side effects of applying synthetic compounds. However, further research is
618 needed to determine the effects of the active compounds on the fish metabolism.
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620

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841 **Figures**

842

843 **Fig. 1**. Viability (expressed as percentage) of *S. aurata* HK leucocytes after 24 h of
844 incubation with aqueous (A) or ethanolic (B) extracts obtained from leaves of *O.*
845 *vulgare*. White bars represent incubation of cells with medium (control group).
846 Cytotoxicity results are given as the percentage of viability, compared with untreated
847 cells (the mean optical density of untreated cells was set at 100% viability). The results
848 are representative of at least three independent experiments and are expressed as mean ±
849 SEM (n=5). Different letters denote significant differences between treatment groups
850 ($p < 0.05$).

851

852 **Fig. 2.** Phagocytic ability (expressed as percentage) and phagocytic capacity (expressed
853 as aleatory units) of *S. aurata* HK leucocytes after 24 h of incubation with aqueous (A,
854 C) or ethanolic (B, D) extracts obtained from leaves of *O. vulgare*. White bars represent
855 incubation of cells with medium alone (control group). The results showed are
856 representative of at least three independent experiments and are expressed as mean ±
857 SEM (n=5). Different letters denote significant differences between treatment groups
858 ($p < 0.05$).

859

860 **Fig. 3.** Respiratory burst activity (expressed as slope $minute^{-1}$) and peroxidase activity
861 (expressed as unit 10^7 leucocytes) of *S. aurata* HK leucocytes after 24 h of incubation
862 with aqueous (A, C) or ethanolic (B, D) extracts obtained from leaves of *O. vulgare*
863 (A,C). White bars represent incubation of cells with medium alone (control group). The
864 results are representative of at least three independent experiments and are expressed as
865 mean ± SEM (n=5).

866

867 **Fig. 4.** Cytotoxic activity (expressed as percentage of viable cells) of SAF-1 cell line
868 after 24 h of incubation with aqueous (A) or ethanolic (B) extracts obtained from leaves
869 of *O. vulgare*. White bars represent incubation of cells with medium alone (control
870 group). The results are representative of at least three independent experiments and are
871 expressed as mean ± SEM (n=6). Different letters denote significant differences
872 between treatment groups ($p < 0.05$).

873

874 **Fig. 5.** Cytotoxic activity (expressed as percentage of viable cells) of PLHC-1 cell line
875 after 24 h of incubation with aqueous (A) or ethanolic (B) extracts obtained from leaves
876 of *O. vulgare*. White bars represent incubation of cells with medium alone (control
877 group). The results are representative of at least three independent experiments and are
878 expressed as mean ± SEM (n=6). Different letters denote significant differences
879 between treatment groups ($p < 0.05$).
880
881 **Fig. 6.** Bactericidal activity (expressed as percentage) of aqueous (A, B, C) and
882 ethanolic (D, E, F) extracts obtained from leaves of *O. vulgare*. White bars represent
883 incubation of cells with medium alone (control group). The results are representative of
884 at least three independent experiments and are expressed as mean ± SEM (n=6).
885 Different letters denote significant differences between treatment groups ($p < 0.05$).
886
887 **Fig. 7.** Antioxidant activity (expressed as equivalents of ascorbic acid) of aqueous (A)
888 or ethanolic (B) extracts obtained from leaves of *O. vulgare*. White bars represent
889 incubation of cells with medium alone (control group). The results are representative of
890 at least three independent experiments and are expressed as mean ± SEM (n=6).
891 Different letters denote significant differences between treatment groups ($p < 0.05$).
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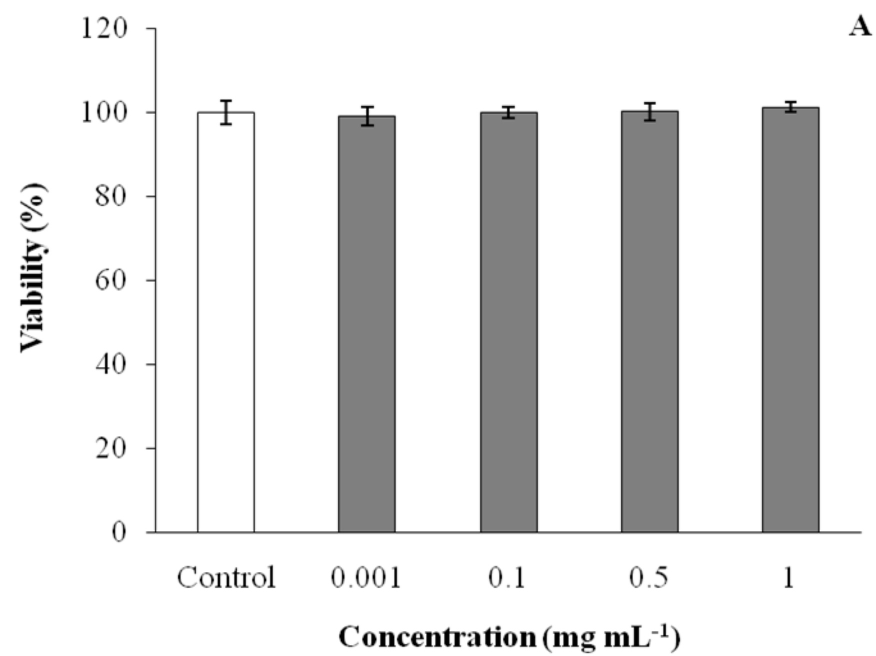

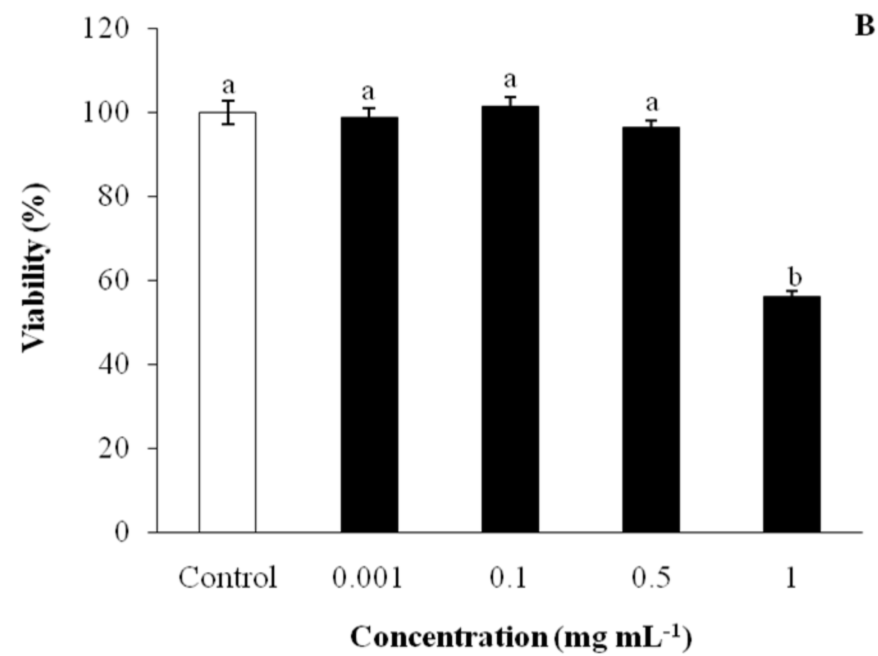

Figure 1

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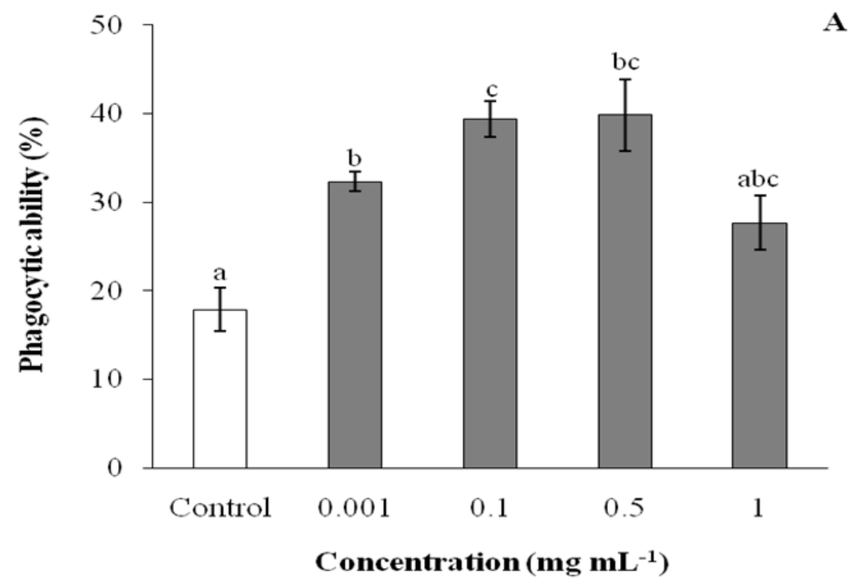

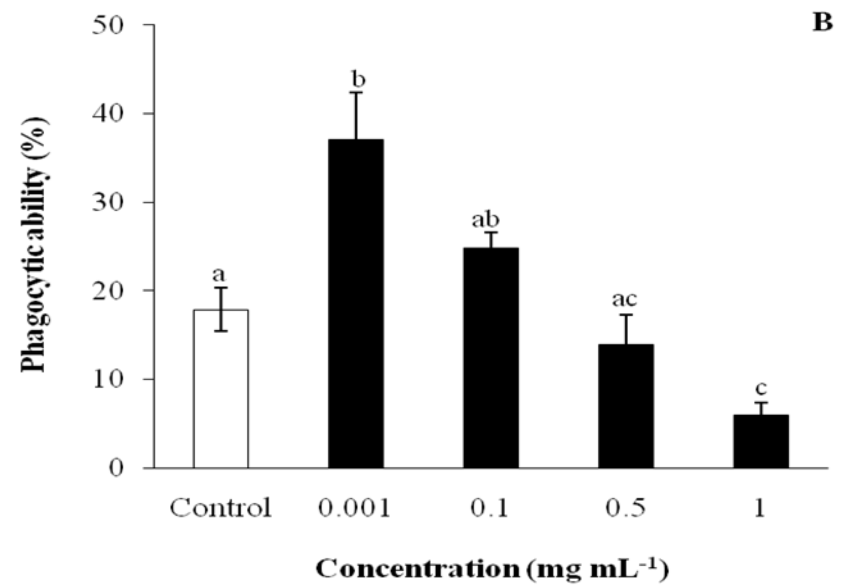

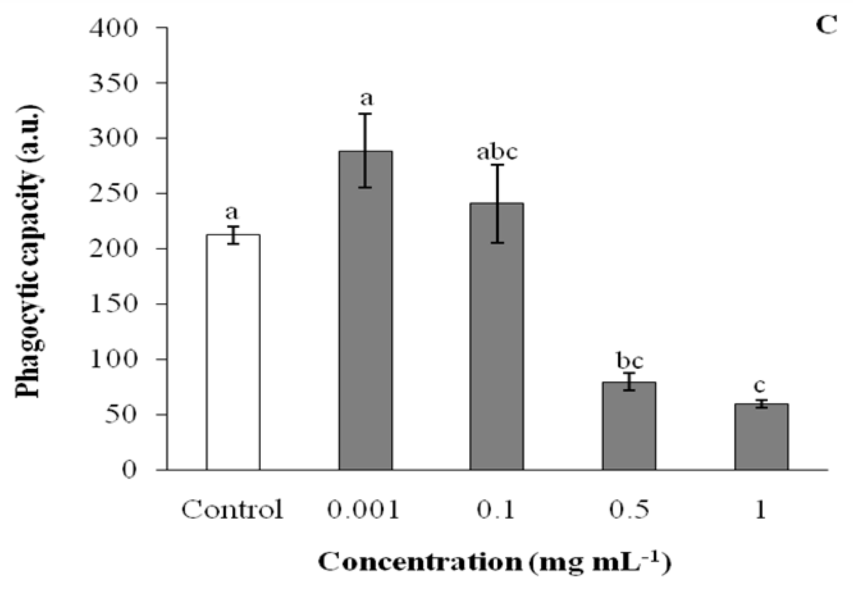

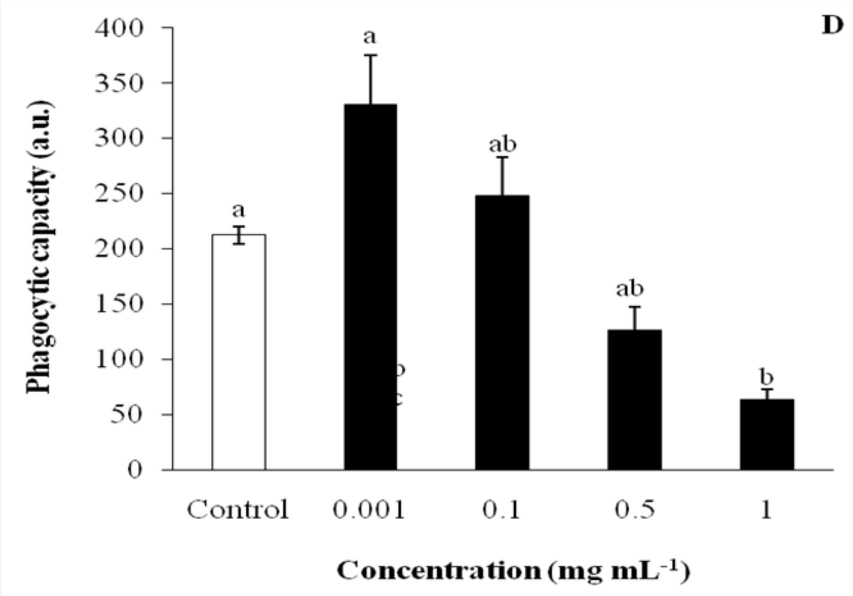

Figure 2

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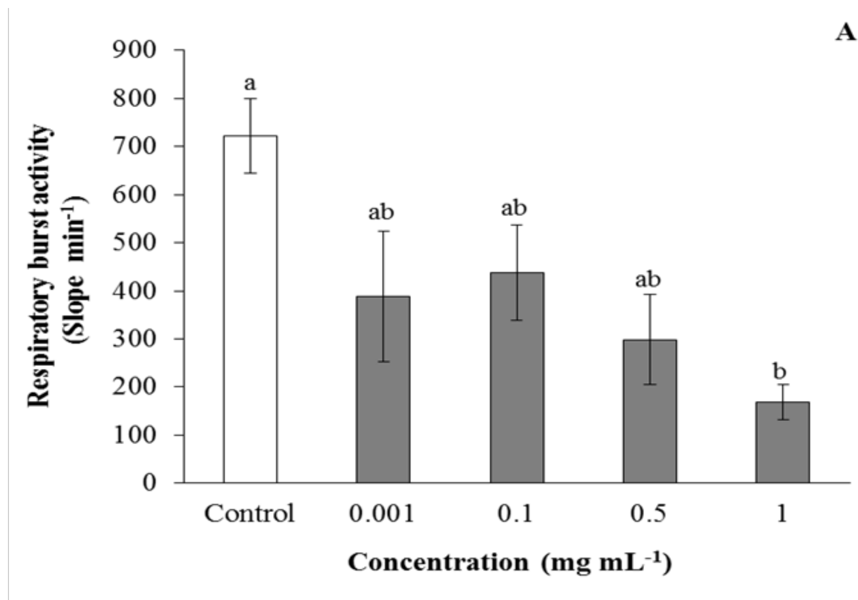

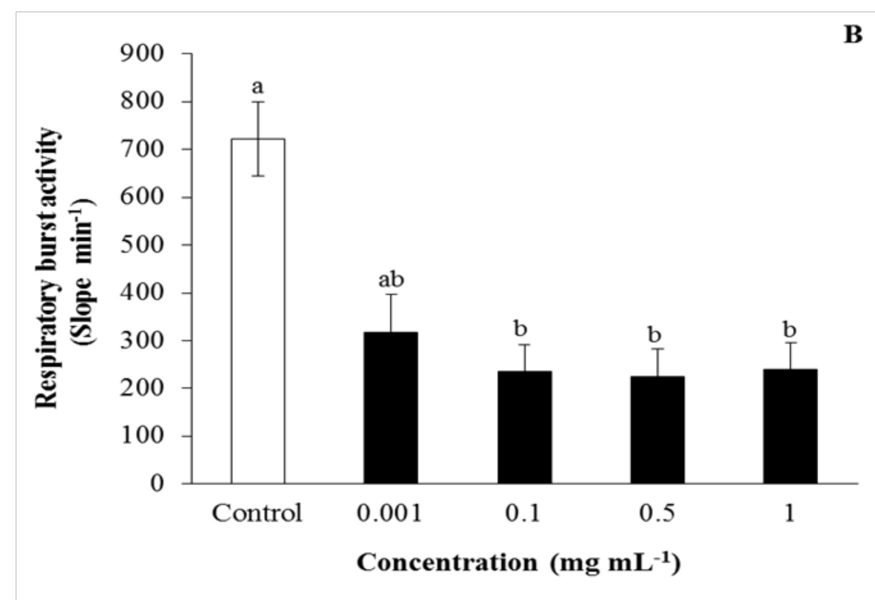

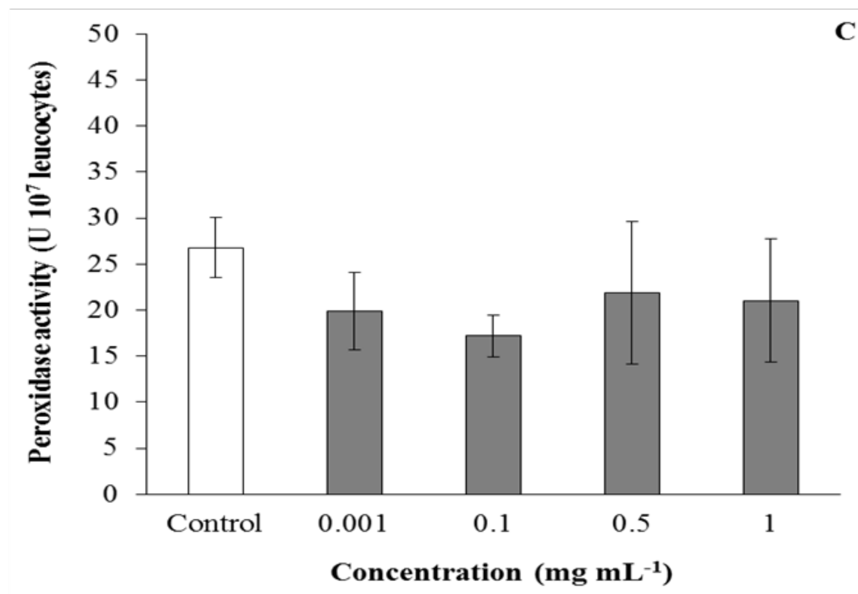

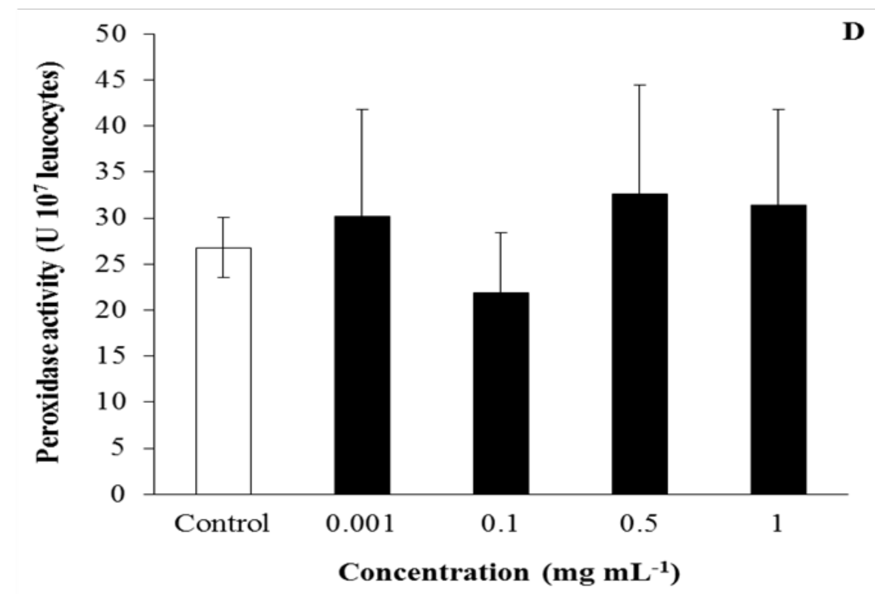

Figure 3

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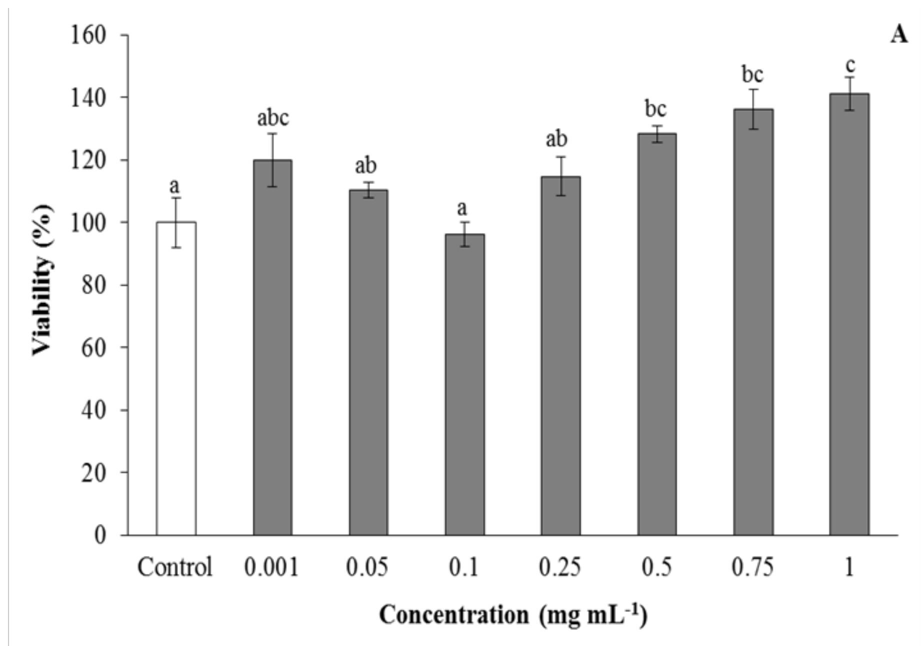

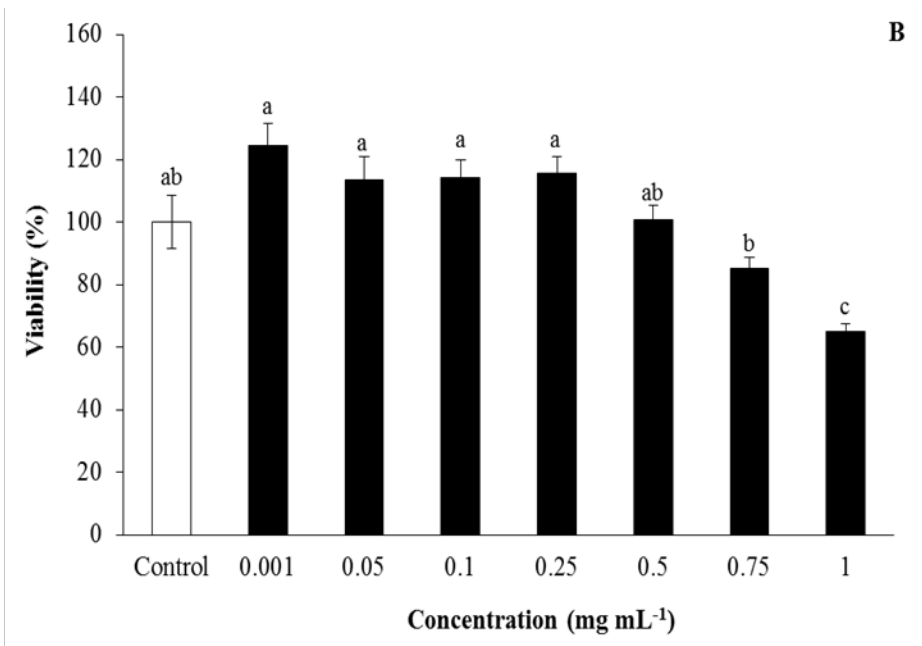

Figure 4

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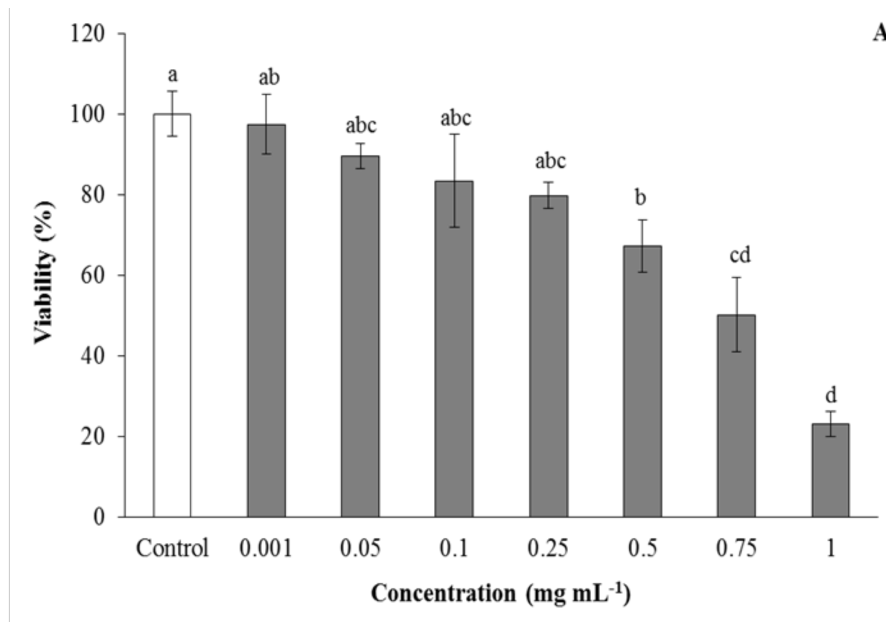

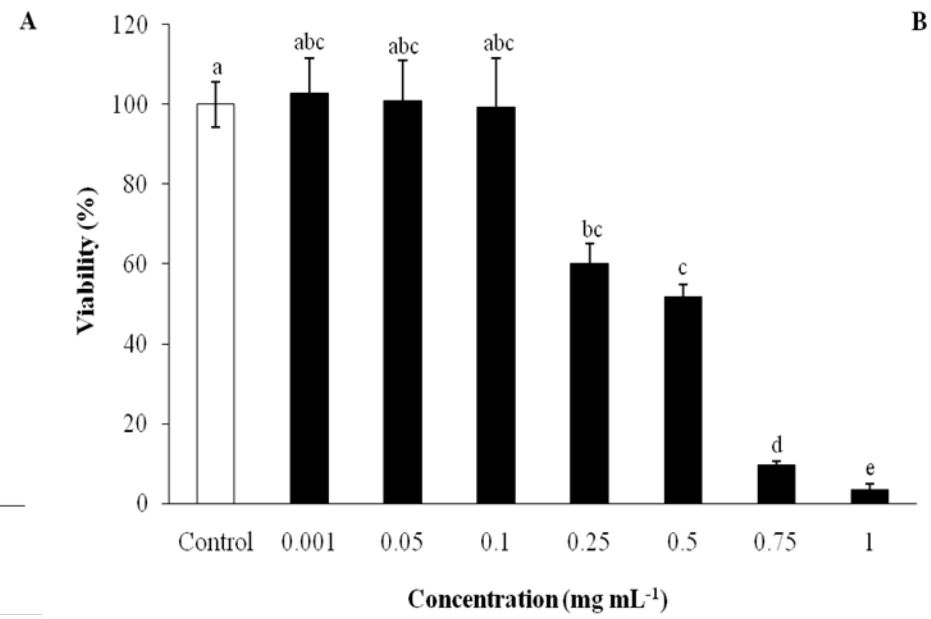

Figure 5

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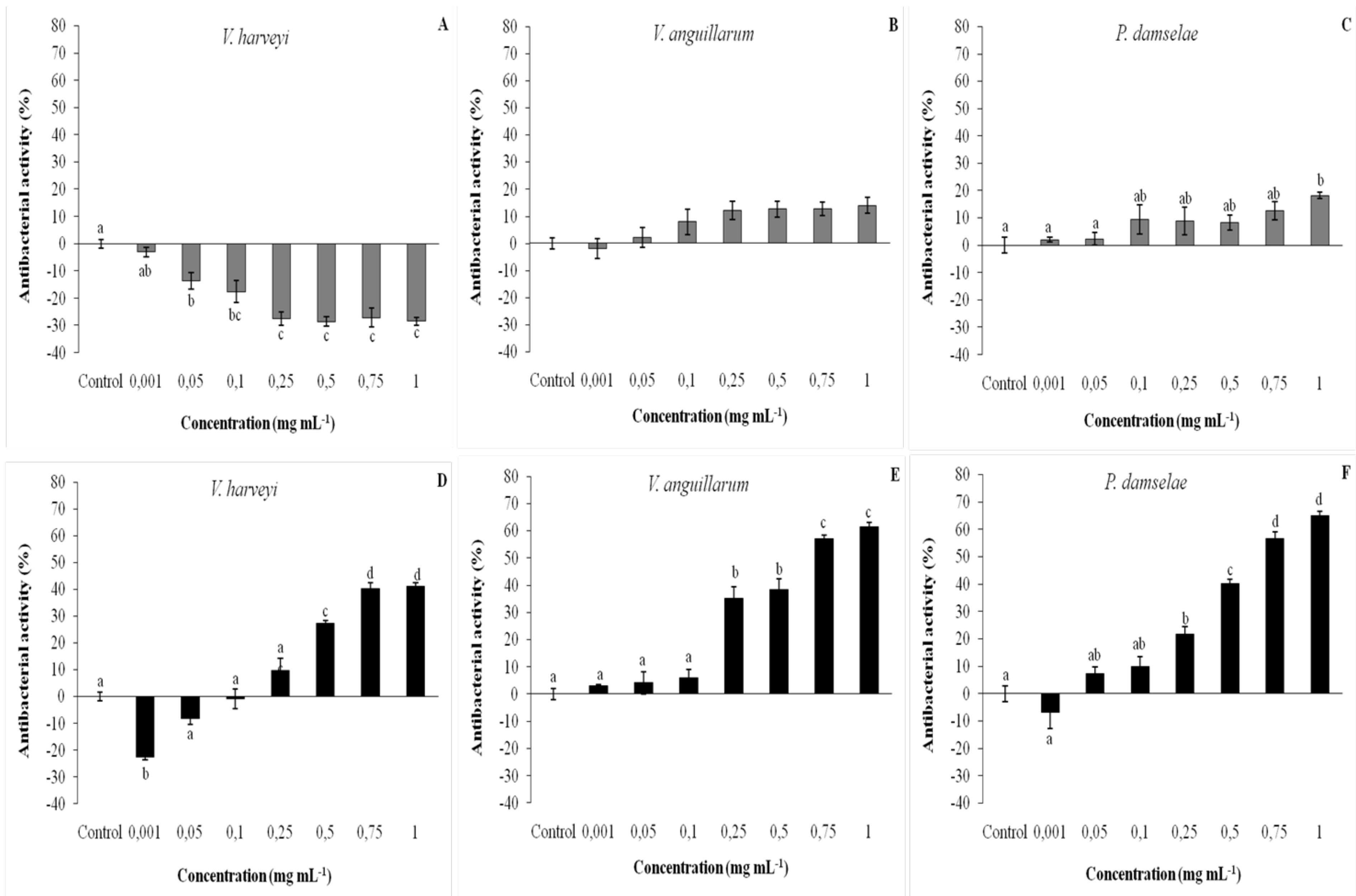
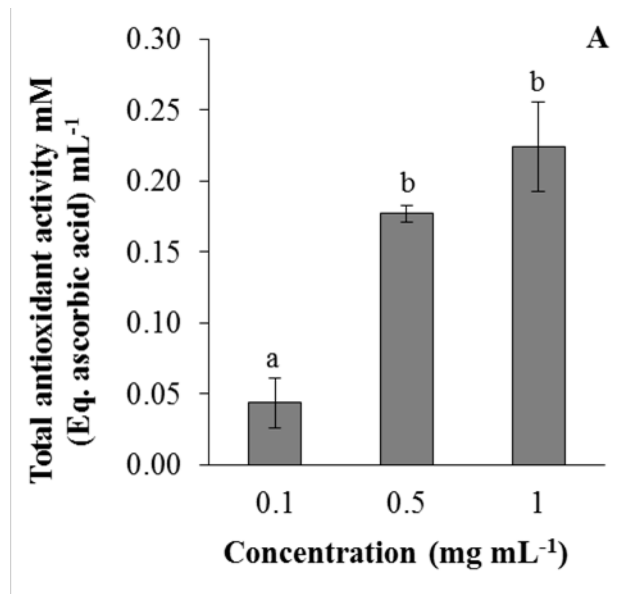


Figure 6



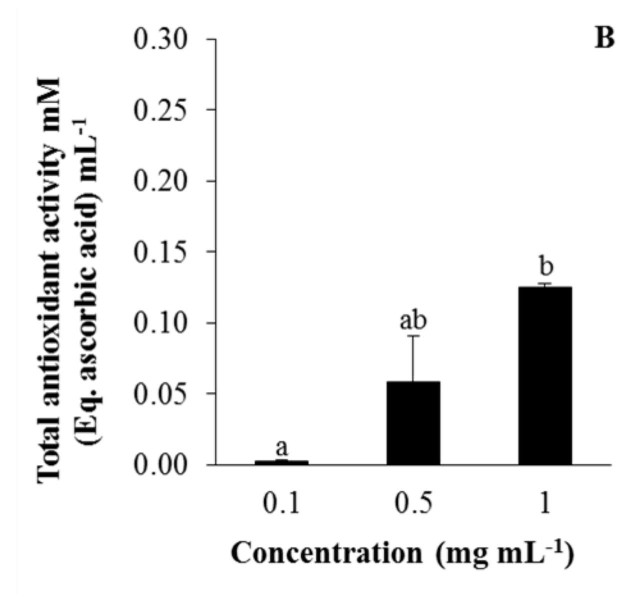


Figure 7

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