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1 **Combined** antiparasitic and anti-inflammatory effects  
2 of the natural polyphenol curcumin on turbot  
3 scuticociliatosis

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12 **Running Head:** Effects of curcumin on scuticociliatosis

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22 **Abstract**

23           The histiophagous scuticociliate *Philasterides dicentrarchi* is the aetiological  
24 agent of scuticociliatosis, a **parasitic** disease of farmed turbot. Curcumin, a polyphenol  
25 from *Curcuma longa* (turmeric), is known to have antioxidant and anti-inflammatory  
26 properties. We investigated the *in vitro* effects of curcumin on the growth of *P.*  
27 *dicentrarchi* and on the production of pro-inflammatory cytokines in turbot leukocytes  
28 activated by parasite cysteine proteases. At 100 µM, curcumin had a cytotoxic effect  
29 and completely inhibited the growth of the parasite. At 50 µM, curcumin inhibited the  
30 protease activity of the parasite and expression of genes encoding two virulence-  
31 associated proteases: leishmanolysin-like peptidase and cathepsin L-like. At  
32 concentrations between 25 and 50 µM, curcumin inhibited **the** expression of S-  
33 adenosyl-L-homocysteine hydrolase, an enzyme involved in **the** biosynthesis of the  
34 amino acids methionine and cysteine. At 100 µM, curcumin inhibited **the** expression of  
35 the cytokines tumour necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β)  
36 produced in turbot leukocytes activated by parasite proteases. Results show that  
37 curcumin has **a** dual effect on scuticociliatosis: an antiparasitic effect on the catabolism  
38 and anabolism of ciliate proteins, and an anti-inflammatory effect that **inhibits the**  
39 production of proinflammatory cytokines in the host. The **present** findings **suggest** the  
40 potential usefulness of this polyphenol in treating scuticociliatosis.

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42

43 *Keywords:*

44 *Philasterides dicentrarchi*; curcumin; proteases; S-adenosyl-L-homocysteine hydrolase;  
45 cathepsin L-like; leishmanolysin.

46

47 **Introduction**

48 Scuticociliatosis is one of the most important parasitic diseases of marine farmed  
49 flatfish to emerge in recent years (Dyková and Figueras, 1994; Sterud et al., 2000;  
50 Iglesias et al., 2001; Kim et al., 2004a). The histiophagous scuticociliate *Philasterides*  
51 *dicentrarchi* (Ciliophora, Scuticociliatia) is the main aetiological agent of  
52 scuticociliatosis in cultured turbot (Iglesias et al., 2001; Álvarez-Pellitero et al., 2004;  
53 Ramos et al., 2007). Removal of scuticociliates from water is possible using chemicals  
54 such as formalin and hydrogen peroxide (Iglesias et al., 2002; Paramá et al., 2003; Jin et  
55 al., 2010; Harikrishnan et al., 2010a,b; Budiño et al., 2012a). However, because of **the**  
56 restrictions on the use of chemicals in aquaculture and also the rapid development of the  
57 disease **at present**, there are no effective treatments for systemic infections in fish.  
58 Natural compounds such as polyphenols could possibly be used to treat  
59 **scuticociliatosis**, as **demonstrated** in the case of the polyphenols resveratrol and propyl  
60 gallate, which exert an *in vitro* anti-parasitic activity against *P. dicentrarchi* (Leiro et  
61 al., 2004, Mallo et al., 2014). Curcumin (CUR) is a polyphenol obtained from the  
62 rhizome of *Curcuma longa* L. (turmeric). CUR is used as a spice and food colouring, as  
63 a component of dyes and cosmetics and as an insect repellent; it is also used in  
64 traditional Indian medicine for its antimicrobial, antifungal and antiparasitic activities  
65 (Moghadamtousi et al., 2014; Shahiduzzaman et al., 2009). CUR has also been reported  
66 to exert immunomodulatory, antioxidant and anticarcinogenic effects (Nagajyothi et al.,  
67 2012).

68 *P. dicentrarchi* is capable of inducing severe systemic infections in turbot  
69 because of its ability to penetrate and spread through the organs of fish as a result of the  
70 production of large amounts of proteolytic enzymes (Paramá et al., 2003; 2004). As in  
71 other parasites, the cysteine proteases (the predominant proteases in this ciliate) can  
72 inactivate the protective innate immune response of the host, thus facilitating **the**

73 survival of parasite in the host during the endoparasitic phase (Kim et al., 2004b;  
74 Paramá et al., 2004; 2007a,b,c; Herrman et al., 2006; Jousson et al., 2006; Al-Marzouk  
75 and Azad, 2007; Leibowitz et al., 2009; Piazzón et al., 2011). On the other hand,  
76 parasite proteases can exacerbate **the severity** of disease by activating the host  
77 inflammatory response (Piazzon et al., 2014).

78 CUR has been shown to prevent **the** proteolytic activity in several types of cells  
79 (Siddiqui et al., 2009; Vazeille et al., 2012). It also inhibits the activity of enzymes  
80 involved in parasite metabolism, such as S-acyl-homocysteine-hydrolase (SAHH),  
81 which regulates methylation reactions, and it has been reported to be an appropriate  
82 pharmacological target in different parasitic diseases (Tanaka et al., 2004; Walker,  
83 2012). Furthermore, CUR has been reported to inhibit the host NFκB signalling  
84 pathway, which is activated during several parasite infections and interferes in  
85 inflammation and oxidative stress (Haddad et al., 2011; Cao et al., 2015). The safety  
86 profile of CUR has so far been found to be excellent (Clarke and Mullin, 2008; He et  
87 al., 2015), and the compound has shown positive effects on growth of fish in  
88 aquaculture (Manju et al., 2012; 2013; Mahfouz, 2015).

89 The main aims of the present study were to investigate the *in vitro* anti-parasitic  
90 effect of the polyphenol CUR on the scuticociliate parasite of turbot *P. dicentrarchi* and  
91 to determine whether the pharmacological activity is related to effects on the  
92 metabolism of ciliate amino acids and proteins. In addition, we also evaluated the  
93 capacity of CUR to modulate the inflammatory immune response induced in the host by  
94 proteases extracted from *P. dicentrarchi*.

95

96

## 97 **Material and Methods**

### 98 **Experimental animals and parasites**

#### 99 *Turbot*

100 Turbot *Scophthalmus maximus* of approximate weight 50 g were obtained from  
101 a fish farm in Galicia (NW Spain). The fish were distributed in 250 L tanks with  
102 recirculating seawater held at 17-18°C, under a photoperiod of 12h light/darkness and  
103 constant aeration. The fish were fed daily (**0.35g of feed / 10 g of fish / day**) with  
104 commercial pellets (Skretting, Burgos, Spain) and were acclimatized to the aquarium  
105 conditions for at least 2 weeks before the beginning of the experiments. The fish were  
106 anaesthetized with benzocaine (50 mg/L) before experimental handling (Piazzón et al.,  
107 2011).

108

#### 109 *Ciliates*

110 *Philasterides dicentrarchi* ciliates (I<sub>1</sub> and C<sub>1</sub> isolates; Budiño et al., 2011) were  
111 isolated from naturally infected turbot (obtained from a fish farm in Galicia) showing  
112 signs of scuticociliatosis. The ciliates were isolated aseptically from turbot  
113 intraperitoneal fluid, as previously described (Iglesias et al., 2001). The ciliates were  
114 cultured **axenically** at 21°C in complete sterile L-15 medium (Leibovitz, PAA  
115 Laboratories GmbH, 10% salinity, pH 7.2) containing 90 mg/L each of adenosine,  
116 cytidine and uridine, 150 mg/L guanosine, 5 g/L glucose, 400 mg/L of L- $\alpha$ -  
117 phosphatidylcholine, 200 mg/L Tween 80, 10% heat inactivated foetal bovine serum  
118 (FBS) and 10 mL/L of an antibiotic/antimycotic solution (100X, i.e. 100 units/mL  
119 penicillin G, 0.1 mg/mL streptomycin sulphate and 0.25 mg/mL amphotericin B;  
120 Sigma-Aldrich), as previously described (Iglesias et al., 2003). Virulence of the ciliates  
121 was maintained by experimentally infecting samples of fish every 6 months by

122 intraperitoneal injection with a ciliate suspension ( $5 \times 10^5$  ciliates in 200 mL of sterile  
123 physiological saline solution; 0.15M NaCl). The ciliates were then isolated from  
124 intraperitoneal fluid as previously described (Paramá et al., 2003; Leiro et al., 2008).

125

#### 126 **Ethical approval**

127 All experiments were carried out in accordance with European regulations on  
128 animal protection (Directive 86 / 609), outlined in the Declaration of Helsinki. All  
129 experimental protocols were approved by the Bioethical Committee of the University of  
130 Santiago de Compostela (Spain).

131

#### 132 **Anti-ciliate activity**

133 The anti-ciliate activity was assayed as previously described (Mallo et al., 2014).  
134 A 100 mM stock solution of CUR (Sigma-Aldrich) was prepared in dimethyl  
135 sulphoxide (DMSO) and maintained at  $-20\text{ }^{\circ}\text{C}$ , in darkness, until use. To investigate the  
136 *in vitro* anti-parasitic effect, CUR was added (at final concentrations of 25, 50 and 100  
137  $\mu\text{M}$ ) to the wells of a 96-well sterile culture plate (Corning), each containing  $15 \times 10^3$   
138 ciliates/mL in L-15 medium containing 10% FBS. The plates were incubated for 3 days  
139 at  $21\text{ }^{\circ}\text{C}$ . Aliquots (15  $\mu\text{L}$ ) of medium were removed every day from each of five  
140 replicate wells for the different treatments. The ciliates were inactivated by addition of  
141 0.25% glutaraldehyde (**Sigma-Aldrich**) before quantification in a haemocytometer  
142 (Iglesias et al., 2002). To **exclude** any possible effects of DMSO, L-15 medium  
143 containing the highest concentration of DMSO (100  $\mu\text{M}$ ) was added to five replicate  
144 wells and processed as above. The *in vitro* inhibitory concentrations ( $\text{IC}_{50}$ ) of the CUR  
145 **was** calculated using Excel software (Microsoft Office; Microsoft, Madrid, Spain), by  
146 plotting the dose response data and applying linear regression ( $y=mx+b$ ) to fit the data:

147 the values were then calculated from the equation  $IC_{50} = (0.5-b) \times \log \text{dose} / m$ , where  
148  $m$  is the slope  $y_1-y_2 / x_1-x_2$  and  $b$  is the intercept of the line.

149

### 150 **Isolation of head kidney cells**

151 Head kidney (**HK**) cells were obtained as previously described, with minor  
152 modifications (Castro et al., 2008). Briefly, **HK** was aseptically removed from  
153 anaesthetized turbot and placed in a Petri dish containing L-15 medium **with** 2% FBS,  
154 heparin (10 U/mL), penicillin G (100 U/mL) and streptomycin sulphate (0.1 mg/mL).  
155 Small pieces of head kidney were then pushed through a 100  $\mu\text{m}$  nylon mesh with the  
156 aid of a glass rod, and the resultant cell suspension was layered onto a 34%/48% v/v  
157 Percoll (**Sigma-Aldrich**) gradient. The gradients were centrifuged at 1000  $\times g$  for 30  
158 min at 4 °C. The interface cells were collected, washed once with Hanks solution  
159 (**Gibco**) at 600  $\times g$  for 10 min, resuspended in L-15 containing 2% FBS, washed again  
160 (300  $\times g$  for 5 min) and finally counted in a haemocytometer.

161

### 162 **Assays for determining protease activity**

163

#### 164 *Purification of parasite proteases*

165 Proteases were obtained as previously described (Paramá et al., 2004), with  
166 minor modifications. Briefly, the ciliates were washed three times with PBS (by  
167 centrifugation) and resuspended in 5 mL of equilibration buffer (100 mM  $\text{CH}_3\text{COONH}_4$   
168 and 10mM  $\text{CaCl}_2$ , pH 6.5). The suspensions were then sonicated on ice in a Branson W-  
169 250 sonifier (Branson Ultrasonic Corporation, USA), for 8 cycles of 10 pulses (duty  
170 cycle 50% and output intensity 4), until the ciliates ruptured. The samples were then  
171 centrifuged at 15000  $\times g$  for 10 min and filtered (0.22  $\mu\text{m}$ , Millipore, USA). The

172 proteases were purified by processing all samples in a CNBr-activated bacitracin–  
173 sepharose XK 16/20 column (GE Healthcare, USA) connected to a protein purification  
174 system (AKTApurim plus; GE Healthcare, USA). The non-retained fraction was washed  
175 with washing buffer (100mM, CH<sub>3</sub>COONH<sub>4</sub> pH 6.4) until the optical density (OD) at  
176 280 nm was basal. Proteases bound to the column were then eluted in elution buffer  
177 containing 100 mM CH<sub>3</sub>COONH<sub>4</sub>, 1 M NaCl and 25% (v/v) 2-isopropanol (pH 6.5)  
178 and collected in 2.5 mL fractions until the OD at 280 nm was basal. Samples were then  
179 dialyzed against equilibration buffer and concentrated by ultrafiltration with Amicon  
180 Ultra 10 K centrifugal filter devices (Millipore, MA, USA) and finally stored in 0.15 M  
181 PBS at -80 °C until use. The protein concentration in the extract was determined using a  
182 Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Germany), by the method of  
183 Bradford (1976) and with bovine serum albumin (Sigma–Aldrich) as standard (Khan et  
184 al., 2003; Piazzón et al., 2011).

185

#### 186 *Gelatin-FITC proteolytic activity*

187 This method was based on measurement of hydrolysis in a solution of a protein  
188 substrate (gelatin) conjugated with a fluorescent ligand (fluorescein isothiocyanate;  
189 FITC; **Sigma-Aldrich**) (Lee et al., 2003). The FITC was dissolved with the same  
190 proportion of protein (gelatin) (**1:1, Wt:Wt**) in a solution of Na<sub>2</sub>HPO<sub>4</sub> (pH 9-9.5) and  
191 incubated for 1h at room temperature (**RT**). Once coupling had taken place, excess  
192 FITC was removed by dialysis against PBS and the solution was centrifuged at 5000 x g  
193 for 10 min to eliminate precipitated protein. Aliquots were stored at -20 °C until use.

194 *P. dicentrarchi* cells were **incubated** with 50 µM CUR for 24 h, and control  
195 (untreated) cells were processed at the same time. The cells were washed twice in PBS  
196 and sonicated on ice in a Branson W-250 sonifier (Branson Ultrasonic Corporation,

197 USA), for 8 cycles of 10 pulses (duty cycle 50 %, output intensity 4) until the ciliates  
198 ruptured, as above. Samples were centrifuged at 20000 x g for 15 min at 4°C and the  
199 supernatant was used in the assay. The concentration of protein in the lysate was  
200 determined by Bradford's method (Bradford, 1976), as described above.

201 Aliquots (10 µL) of all lysates were incubated with 20 µL of PBS and 20 µL of  
202 gelatin-FITC at 21 °C for 4 h in darkness in a moist chamber. One hundred and twenty  
203 µL of a 0.6 M solution of trichloroacetic acid (TCA; **Sigma-Aldrich**) was added to each  
204 sample, and the mixtures were **incubated** for 30 min at **RT**. The samples were then  
205 centrifuged at 3000 x g for 10 min and fluorescence was measured in a fluorimeter  
206 (Flx800, Biotek, USA) at 488/520nm excitation/emission. All samples were **analyzed**  
207 in triplicate.

208

#### 209 ***SDS-PAGE-substrate***

210 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was  
211 used to study the effect of CUR on ciliate proteases. Samples were electrophoresed in a  
212 linear gel (0.1% gelatin containing 10% SDS) under non-reducing conditions, to  
213 determine the proteolytic activity. The samples were prepared as for the gelatin-FITC  
214 proteolytic activity, in loading buffer containing 62 mM Tris-HCl buffer, pH 6.8, 2%  
215 SDS and 10% glycerol. Electrophoresis was performed in a mini-vertical  
216 electrophoresis system (Hoeffer, GE Healthcare, USA) for 45 min at 200 V in  
217 electrophoresis buffer containing 25 mM Tris, 190 mM glycine and 1% SDS (pH 8.3).  
218 The gel was cut into strips containing the different samples. The gel strips were then  
219 incubated, first for 30 min in a 2.5% Triton X-100 (v/v) solution and then in 0.1 M  
220 citrate buffer, pH 4 and 0.1 mM DTT (a cysteine protease activator), for 12 h at 37 °C  
221 with constant slight agitation, to **allow the** development of the gelatin-lytic activity of

222 parasite proteases. Finally, gels were stained with Thermo Scientific GelCode Blue Safe  
223 Protein Stain (Pierce) to visualize the bands of lysed gelatin. The proteolysis bands  
224 appeared as clear bands on a blue background after destaining with water (Paramá et al.,  
225 2004; 2007 b; Piazzón et al., 2011).

226

## 227 **Quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-** 228 **PCR)**

229 **The** expression of the leishmaniolysin (Leish), cathepsin L-like (Cat) and S-  
230 adenosyl-L-homocysteine hydrolase (SAHH) genes was analysed by **qRT-PCR**. The  
231 nucleotide sequence of these genes was obtained from cDNA libraries obtained by  
232 pyrosequencing in a genome sequencer (454 LifeScience, Roche) and assembled by the  
233 Newbler software package (ver. 2.8). The assembled nucleotide sequences were  
234 translated to aa by the Translate tool available at the website of the Swiss Institute of  
235 Bioinformatics (SIB) [<http://web.expasy.org/translate/>]. The resulting aa sequences  
236 were analysed by the BLASTP program, which searches the protein database of the  
237 National Center for Biotechnology Information (NCBI)  
238 [<http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>]. Putative conserved domains  
239 detected were found in the NCBI's conserved domain database (CDD)  
240 [<http://www.ncbi.nlm.nih.gov/cdd/>] (Table 1).

241 Ciliates were incubated for 24 hours with the different concentrations of CUR  
242 (25, 50 and 100  $\mu$ M). Two ciliate isolates (I1 and C1) belonging to different haplotypes  
243 and serotypes and with different levels of virulence were used to test the gene  
244 expression levels (Budiño et al., 2011; 2012 b). In these experiments, ciliates obtained  
245 from long-term *in vitro* cultures and ciliates recently isolated from turbot ascites were  
246 compared.

247 Turbot **HK** cells were also incubated with the different treatments (100 µg/mL  
248 proteases and CUR at 25, 50 and 100 µM) for 4 hours.

249 Total RNA from 10<sup>7</sup> cells/sample was obtained with NucleoSpin ARN  
250 (Macherey-Nagel), treated with DNase I (RNase free, Thermo Scientific). **The integrity**  
251 **of RNA was assessed on a denaturing agarose gel and** the purity and final  
252 concentration **of samples** were then estimated using a NanoDrop ND-1000  
253 spectrophotometer. cDNA synthesis (25 µL/reaction) was performed by reverse  
254 transcription, with 1.25 µM random primers (Roche), deoxynucleoside triphosphates  
255 (dNTPs) (250 µM of each), 10 mM dithiothreitol (DTT), 20U of RNase inhibitor, 2.3  
256 mM MgCl<sub>2</sub> and 200U of reverse transcriptase of Moloney murine leukaemia virus,  
257 (MMLV) (Promega) in buffer containing 30 mM Tris and 20mM KCl (pH 8.3).  
258 Samples of RNA (2 µg) were used to generate cDNA (Mallo et al., 2013).

259 The **quantitative real-time PCR** reaction was performed with a reaction  
260 mixture already containing the assay buffer and dNTPs and Maxima SYBR Green  
261 qPCR Master Mix (Thermo Scientific). Primer pairs were used at a final concentration  
262 of 300 nM, and 1µL of cDNA was added to each well to a final volume of 10 µL/well,  
263 which was made up with RNase-free distilled H<sub>2</sub>O. Reaction was achieved at 95 °C for  
264 5 min followed by 40 cycles of 10s at 95 °C and 30s at 60 °C. At the end of this  
265 process, melting curve analysis was performed at 95 °C for 15s, 55 °C for 15 s, and 95  
266 °C for 15 s. The size and specificity of PCR products obtained were confirmed by 2%  
267 agarose gel electrophoresis. All reactions were carried out in a real time PCR system,  
268 Eco Real-time PCR system (Illumina). The relative quantification of gene expression  
269 was determined by the 2<sup>-ΔΔC<sub>q</sub></sup> method (Livak and Schmittgen, 2001) following minimum  
270 information guidelines to publish real-time quantitative PCR experiments (MIQUE)  
271 (Bustin et al., 2009). The following primer sequences of genes were used **for P.**

272 *dicentrarchi*: S-adenosil homocysteine hydrolase (SAHH) forward/reverse, 5'-  
273 CACATGACCATCCAAACTGC-3'/5'-TCAACGAGAATGTTGGGTCCT-3'; *P.*  
274 *dicentrarchi* Leishmanolysin-like peptidase (Leish) forward/reverse, 5'-  
275 CCCACCATCAAAGGAATCT-3'/5'- CCTTACCCTTTCCCATGAT-3'; *P.*  
276 *dicentrarchi* cathepsine- **L-like** (Cat), forward/reverse 5'-  
277 CTCTTCCGTCGATTGGGTTA-3'/5'-GGGCGTAGTTGATTCCGTTGT-3' (Table1);  
278 *P. dicentrarchi*  $\beta$ -tubulin (Tub), forward/reverse, 5'-ACCGGGGAATCTTAAACAGG-  
279 3'/5'-GCCACCTTATCCGTCCACTA-3'. Gene expression in *P. dicentrarchi* was  
280 normalized with the reference  $\beta$ -tubulin gene. For turbot expression assays, the  
281 following primer sequences were used: turbot tumour necrosis factor  $\alpha$  (TNF $\alpha$ )- NCBI  
282 accession number FJ654645- forward/reverse, 5'-TCACAGGACAAGCTGGAGTG-  
283 3'/5'-TCATCAGAGACGCGTTGAAG-3'; turbot interleukin 1  $\beta$  (IL-1B)- NCBI  
284 accession number AJ295836, forward/reverse 5'-AATGGGGCACTGAACAAAAG-  
285 3'/5'-GCCACCTTGTGGTGAACCTT-3' and turbot actin (ACT), forward/reverse, 5'-  
286 CATGTACGTTGCCATCCAAG-3'/5'-TCTCAGCAGTGGTGGTGAAG-3'. **Primer**  
287 **pairs were designed and optimized with the Primer 3 Plus program**  
288 **(<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) with a Tm of**  
289 **60 °C (Mallo et al., 2013). Host actin was used to normalize data, which were**  
290 **expressed in relative arbitrary units. The results are shown as mean values  $\pm$  the**  
291 **standard error (SE) from three trials.**

292

### 293 **Statistical analysis**

294 **The results shown in the following figures are expressed as mean values  $\pm$**   
295 **standard error. Significant differences ( $P < 0.05$ ) among CUR treatments were**  
296 **determined by one-way analysis of variance (ANOVA) assuming that the data of**

297 **experimental groups are sampled of populations with identical standard**  
298 **deviations, tested using the method of Bartlett, and also assuming that follow**  
299 **Gaussian distributions tested using the method of Kolmogorov and Smirnov,**  
300 followed by Tukey - Kramer multiple comparisons test **using the statistical program**  
301 **GraphPad Instat (GraphPad Software, USA).**

302

### 303 **Results**

304

#### 305 ***In vitro* effect of CUR on trophozoite growth**

306 The antiparasitic effect of CUR on the *in vitro* growth of *P. dicentrarchi* was  
307 determined at different concentrations (25, 50 and 100  $\mu\text{M}$ ). At the highest  
308 concentration (100  $\mu\text{M}$ ), CUR had a clear cytotoxic effect ( $P < 0.001$ ) on the ciliate  
309 (Fig. 1). The  $\text{IC}_{50}$  of CUR was  $63.48 \pm 5.77 \mu\text{M}$ , and ciliate growth was totally inhibited  
310 at 100  $\mu\text{M}$  **on the first day of culture**,  $59.25 \pm 11.65 \mu\text{M}$  on day 2, and  $64.98 \pm 6.66$   
311  $\mu\text{M}$  on day 3 (Fig. 1).

312

#### 313 **Effect of CUR on ciliate proteolytic activity**

314 Parasite proteolytic activity was affected by treatment with 50  $\mu\text{M}$  CUR for 24 h  
315 (Fig. 2a). The profile of the gelatin-lytic bands detected was almost the same in both  
316 samples (treated with 50  $\mu\text{M}$  CUR and non-treated samples, lanes 1 and 2: Fig. 2a).  
317 However, the dark bands, which represent the gelatin-lytic activity, are less intense and  
318 thinner in CUR treated-samples than in the control samples, especially in the range 40 -  
319 70 kDa (Fig. 2a).

320 The proteolytic activity measured with gelatin-FITC substrate was significantly  
321 lower in the CUR treated ciliate extract than in the corresponding control (Fig. 2b).

322

323

324

### 325 **Effect of CUR on ciliate protease gene expression**

326 Gene expression of cathepsin L-like and leishmanolysin-like peptidase was  
327 **significantly** reduced in CUR-treated ciliates, at doses of 100  $\mu\text{M}$  for the cathepsin L-  
328 **like ( $P < 0.05$ ) and** between 50-100  $\mu\text{M}$  in the case of leishmanolysin-like peptidase ( **$P$**   
329  **$< 0.01$** ) (Fig. 3 a,b).

330 Furthermore, leishmanolysin peptidase expression levels **significantly** differed  
331 ( **$P < 0.01$** ) in the different cultures and were higher in the I1 isolate recently extracted  
332 from turbot ascites than in long term *in vitro* culture of the isolate. By contrast, the  
333 levels of **leishmanolysin** expression were not different ( **$P > 0.05$** ) in the *in vitro* culture  
334 of isolate C1 and the ciliates obtained from turbot ascites (Fig. 3 c,d).

335

### 336 **Effect of CUR on SAHH gene expression**

337 Ciliates cultured for 24 hours with two different concentrations (25 and 50  $\mu\text{M}$ )  
338 of CUR, **showed a significant** decrease ( **$P < 0.01$** ) in SAHH gene expression was  
339 observed relative to the  $\beta$ - tubulin gene (Fig. 4).

340

### 341 **Anti-inflammatory effect of CUR on turbot leucocytes**

342 In this experiment, the *in vitro* effect of CUR on gene expression of two pro-  
343 inflammatory cytokines, TNF- $\alpha$  and IL-1 $\beta$ , was analysed in leukocytes isolated from  
344 the anterior kidney of turbot and incubated for 24 h with ciliate proteases (100  $\mu\text{g}/\text{mL}$ ).  
345 The results of **qRT-PCR** showed that *P. dicentrarchi* proteases activated turbot  
346 leukocytes by generating a significant increase ( **$P < 0.01$** ) in **the** expression of both

347 proinflammatory cytokines (Fig. 5). By contrast, addition of 100  $\mu$ M CUR to protease-  
348 activated leukocytes significantly inhibited ( $P < 0.01$ ) the expression of these cytokines  
349 (Fig. 5).

## 350 **Discussion**

351 The study shows for the first time that the polyphenol CUR **has** an inhibitory  
352 effect on *P. dicentrarchi* growth, indicating the potential use of this compound in  
353 treating scuticociliatosis. Although the antiparasitic potential of CUR has previously  
354 been reported (Haddad et al., 2010; Wachter et al., 2014), the antiparasitic effect of this  
355 polyphenol has never been described in a ciliate protozoa.

356 For host invasion, parasites release different molecules that **allow** them to  
357 **invade tissues** and avoid the host immune system. **Proteases** are known to be essential  
358 factors in the **disease progression** and survival of parasites as they take part in diverse  
359 processes such as invasion and degradation of host proteins as well **as** activation of host  
360 inflammation (Paramá et al., 2004; 2007 c; McKerrow et al., 2006). Specific inhibition  
361 of proteinases, by either immunoprophylaxis or chemotherapy, might **interfere with**  
362 parasite survival mechanisms (Chung et al., 2005). Although the exact mechanism  
363 whereby *P. dicentrarchi* invades the host is not known, several studies have shown that  
364 proteases may be involved in **its** invasion **capability** and pathogenicity (Paramá et al.,  
365 2004; 2007b), as described in several parasites (McKerrow, 1989; McKerrow et al.,  
366 1993; Piazzón et al., 2011; Rathore et al., 2011). Ciliate proteases have been  
367 characterized as mainly cysteine proteases **and** have been described as virulence factors  
368 **in numerous protozoan parasites** (Sajid and McKerrow, 2002; Paramá et al., 2004;  
369 2007a; Herrmann et al., 2006). Sequencing data for ciliate proteases are scarce (Seo et  
370 al., 2013), even in databases such as *MEROPS* (Rawlings et al., 2014); nevertheless,  
371 cysteine proteases appear to be the most abundant **proteases** in *P. dicentrarchi* (Paramá

372 et al., 2004; 2007a). **In the present study**, CUR **affected the** proteolytic activity of *P.*  
373 *dicentrarchi* **proteases. In vitro** treatment with 50 µM CUR significantly decreased the  
374 enzymatic activity **both** in gelatin-FITC **assay** and in gelatin-SDS gels.

375 A partial sequence of a cathepsin L-like protease has been identified in *P.*  
376 *dicentrarchi* (Shin et al., 2014). **Moreover**, cathepsin L-like cysteine proteases have  
377 been identified in other scuticociliates (such as *Uronema marinum*) and are known to  
378 play a role in adaptation to the host and to act as virulence factors in other parasites  
379 (Malagón et al., 2010). **For these reasons**, they have been proposed as a target for the  
380 development of antiparasitic treatments (Mottram et al., 1996; McKerrow et al., 1999;  
381 2008; Chung et al., 2005; Ahn et al., 2007). Indeed, several studies have focused on  
382 cathepsin-based vaccines, as in the digenean *Fasciola hepatica* (Dalton et al., 2003).  
383 Leishmanolysin peptidase, a metalloprotease present on the cell surface of protozoan  
384 parasites such as *Leishmania* and *Trypanosoma*, **is another protease of which a partial**  
385 **sequence has been identified in *P. dicentrarchi***. The activity of this metalloprotease  
386 **resulted** significantly reduced in long-term *in vitro* cultures and it has therefore been  
387 described as a **parasite** virulence factor (Joshi et al., 2002). **In agreement, in the**  
388 **present investigation** higher levels of expression of leishmanolysin peptidase mRNA  
389 **was observed in *P. dicentrarchi* I1 isolate** obtained from turbot infections. However,  
390 **mRNA expression is not altered in *P. dicentrarchi* isolate C1**, which has been found  
391 to **induce less severe scuticociliatosis** in turbot. Leishmanolysin peptidase has also  
392 been investigated as a target in the development of anti-parasitic treatments (Seo et al.,  
393 2013). Partial sequences of this protease have also been detected in ciliate parasites such  
394 as *Ichthyophthirius multifiliis* (Coyne et al., 2011), *Tetrahymena termophila* (Eisen et  
395 al., 2006), *Cryptocarion irritans* (Lokanathan et al., 2010) and *Chilodonella uncinata*  
396 (Gao et al., 2015). Additionally, **qRT-PCR** analysis with **primers for these *P.***

397 *dicentrarchi* protease revealed a **significant** decrease in **the** proteinase expression in  
398 parasite cultures treated with CUR.

399 The enzyme SAHH catalyzes the reversible hydrolysis of S-  
400 adenosylhomocysteine to homocysteine and adenosine (de la Haba and Cantoni, 1959);  
401 the **generated** homocysteine can subsequently be converted into the sulphur amino  
402 acids L-methionine and L-cysteine, which can be used in protein biosynthesis (Wirtz  
403 and Droux, 2005). CUR has shown a strong interaction with hydrophobic amino acid  
404 residues of *Plasmodium falciparum* SAHH (pfSAHH), and **bio-molecular studies**  
405 suggest that CUR may be a potent inhibitor of pfSAHH (Singh and Misra, 2009; Singh  
406 et al., 2013). Despite the high level of conservation during evolution, **and the**  
407 **consequent high similarity between human and *P. falciparum* SAHH** (Cai et al.,  
408 2009, Crowther et al., 2011), specific inhibitors that bind to **the monoacidic different**  
409 **residue of parasite SAHH** could potentially be used to develop a treatment for malaria.  
410 Although SAHH has been characterized in several protozoan parasites such as  
411 *Trichomonas vaginalis*, *Cryptosporidium parvum* and *P. falciparum* (Creedon et al.,  
412 1994; Bagnara et al., 1996; Čtrnáctá et al., 2010), very little **information** is **available**  
413 about this enzyme in ciliates (Murphy and Fall, 1985). In *P. dicentrarchi*, only one  
414 partial sequence has been identified so far. **In the present study**, CUR caused a  
415 significant dose-dependent inhibition in the expression of SAHH gene in *P.*  
416 *dicentrarchi*, indicating **that** this enzyme **can be considered** as a new line of  
417 investigation involving the treatment of scuticociliatosis.

418 The effects of cysteine proteinases isolated from *P. dicentrarchi* have been  
419 previously investigated *in vitro* in turbot, showing effects on immune system, such as  
420 increased respiratory burst, upregulation of IL-1 $\beta$  expression and prostaglandin levels,  
421 and inhibition of chemotaxis (Paramá et al., 2007a,c). It has also been suggested that *P.*

422 *dicentrarchi* proteinases may modify the turbot immune system by inducing leucocyte  
423 apoptosis (Paramá et al., 2004; 2007a,c; Piazzón et al., 2011). IL-1 $\beta$  and **TNF- $\alpha$**  are  
424 **some** of the **most important** pro-inflammatory cytokines **produced** by macrophages in  
425 response to an external agent, including parasites (Baral, 2010). CUR can overcome  
426 pro-inflammatory pathways and inhibit TNF production as well as the associated cell  
427 signalling through NF- $\kappa$ B (He et al., 2015). The known anti-inflammatory effect of  
428 CUR was demonstrated in the present study as a decrease in pro-inflammatory turbot  
429 genes **encoding for TNF $\alpha$  and IL-1 $\beta$**  driven by the ciliate proteinases. Similar results  
430 have been obtained in other fish such as Jian carp (*Cyprinus carpio* var. Jian) and Nile  
431 tilapia (*Oreochromis niloticus*), in which treatment with curcumin **reduced** the  
432 proinflammatory cytokine expression levels to normal (Cao et al., 2015; Mahfouz,  
433 2015) and had positive effects **in cultured fish** (Manju et al., 2012).

434 In summary, the study findings suggest that CUR is capable of inhibiting *in vitro*  
435 **the** growth of scuticociliate *P. dicentrarchi*. The effect is partly due to a mechanism  
436 involving the **inhibition** of ciliate proteolytic activity, demonstrating the potential  
437 therapeutic use of this polyphenol in controlling turbot scuticociliatosis, especially  
438 taking into account that **it** also displays anti-inflammatory activity in the host. The  
439 findings may lead to the development of a more environmentally friendly alternative to  
440 the use of chemicals in the treatment of scuticociliatosis.

441

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743 **TABLE AND FIGURES LEGENDS:**

744

745 **Table 1.** List of conserved domains of the Leishmanolysin, cathepsin L-like and S-  
746 adenosyl-L-homocysteine hydrolase genes obtained in the NCBI's conserved domain  
747 database (CDD). The nucleotide sequences from a cDNA library obtained by RNAseq  
748 technology, together with the corresponding translation into amino acids, are shown in  
749 the final column. The nucleotide sequences of the corresponding primers used in RT-  
750 qPCR to assess differential gene expression are highlighted in bold type and underlined.

751

752 **Figure 1.** Anti-parasitic effect of curcumin (CUR). Growth rate (line graph) of *P.*  
753 *dicentrarchi* cultured *in vitro* with L-15 supplemented with 10% FBS and with different  
754 doses of CUR (25, 50 and 100  $\mu$ M). Data are the cell counts (haemocytometer)  
755 determined **during** 3 days. The IC<sub>50</sub> values obtained are indicated on the bars in the  
756 graph. Data indicated mean  $\pm$  standard error (n= 5); \*\**P* < 0.001.

757

758 **Figure 2. a).** Gelatin/SDS-PAGE analysis of proteinase activity in *Philasterides*  
759 *dicentrarchi* total extracts treated for 24 h with (2) or without (1) curcumin (CUR; 50  
760  $\mu$ M). Gel strips were incubated for 12 h at 37 ° C with DTT. Inverted photograph with  
761 dark bands reflecting proteolytic activity. The main bands showing proteolytic activity  
762 are indicated with arrows. Mw, molecular weight markers (kDa). **b)** Fluorimetry  
763 showing *P. dicentrarchi* proteolytic activity utilizing gelatin-FITC as a substrate. Data

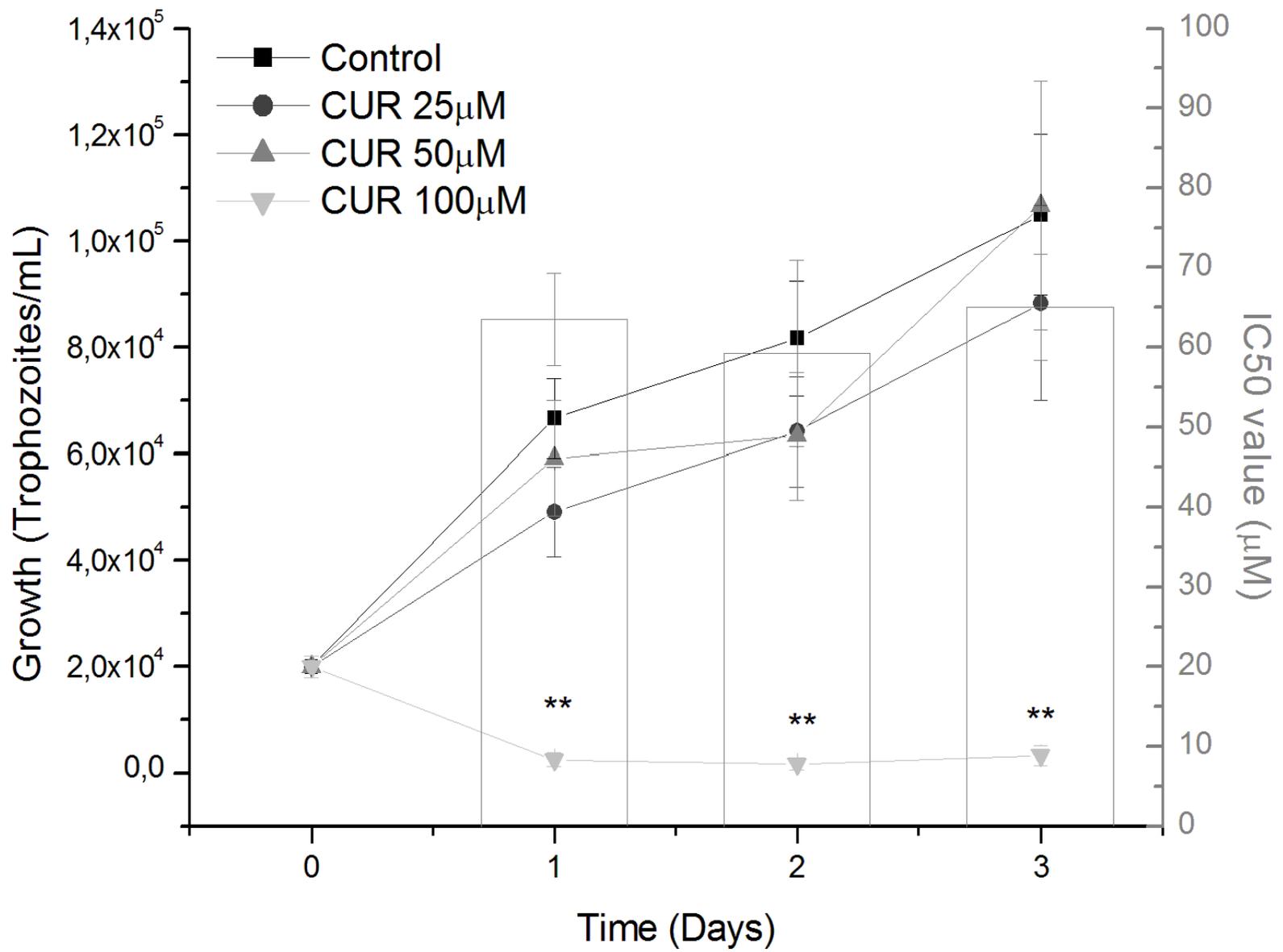
764 shown in fluorescence arbitrary units (AU), and each bar indicates the mean value  $\pm$   
765 standard error ( $n=5$ ).  $**P < 0.01$ .

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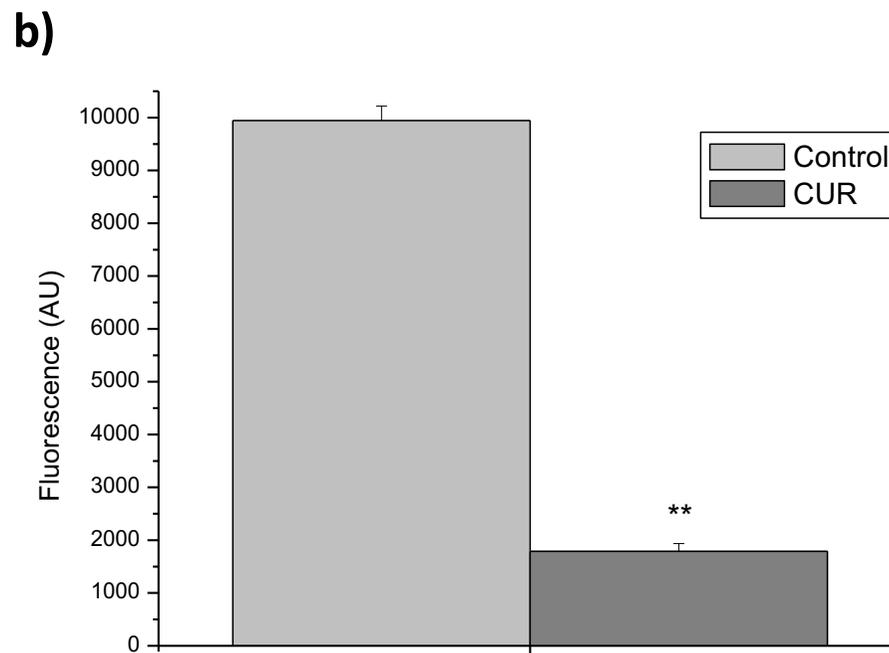
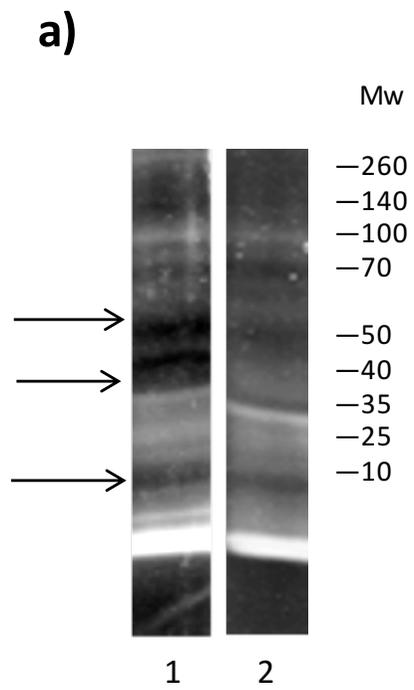
767 **Figure 3.** mRNA expression levels of proteinase genes. Assay was performed by **qRT-**  
768 **PCR** of *P. dicentrarchi* trophozoites treated for 24 h with different doses of curcumin  
769 (CUR; 25, 50 and 100  $\mu$ M). **a)** CUR effect on cathepsin L-like (Cat) levels. **b)** CUR  
770 effect on leishmanolysin peptidase (Leish) levels. **c)** mRNA levels of Leish in the  
771 isolate C1 cultured *in vitro* and obtained from infected turbot. **d)** mRNA levels of  
772 leishmanolysin peptidase in the isolate I1 cultured *in vitro* and obtained from infected  
773 turbot. All data are expressed as relative units normalized with the reference gene  $\beta$ -  
774 tubulin (Tub). Values are means  $\pm$  standard errors ( $n = 3$ ).  $*P < 0.05$ ;  $**P < 0.01$   
775 relative to control values.

776 **Figure 4.** Effect of curcumin on the expression of S-adenosyl-L-homocysteine  
777 hydrolase (SAHH), in *P. dicentrarchi*, as determined by **qRT-PCR**. Data are expressed  
778 relative to the  $\beta$ -tubulin (Tub) gene and each bar indicates the mean  $\pm$  standard error ( $n=$   
779 3).  $**P < 0.01$  relative to control (untreated) ciliates.

780 **Figure 5.** Curcumin (CUR) anti-inflammatory effect on the pro-inflammatory activity  
781 induced by *P. dicentrarchi* proteases (at 100  $\mu$ g/mL) on turbot head kidney leucocytes.  
782 Cells were incubated for 4 hours with the proteases and CUR at the concentrations  
783 indicated in the graphs, and the relative expression levels of the TNF $\alpha$  and IL-1 $\beta$   
784 mRNAs were quantified by **qRT-PCR**. Data presented are the mean values  $\pm$  standard  
785 errors ( $n=3$ ) of units expressed relative to the housekeeping  $\alpha$ -actin (ACT) gene  
786 mRNA levels. **Letters indicate significant differences between groups a-d ( $P <$**   
787 **0.05).**

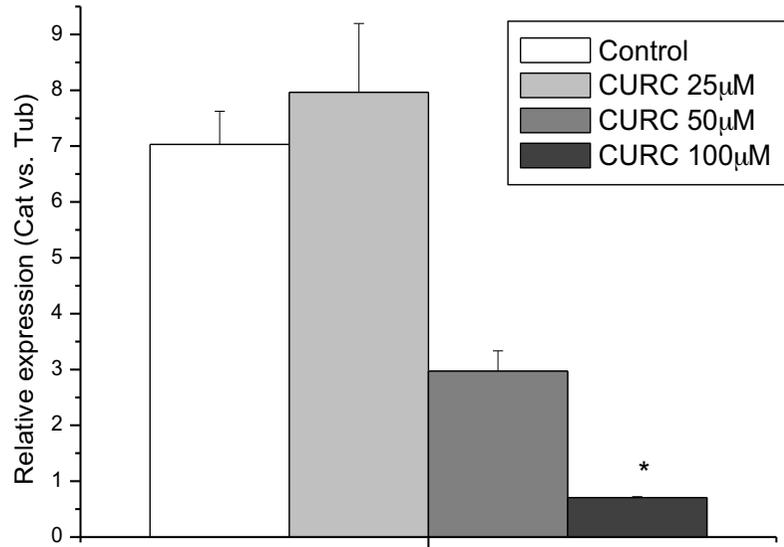


**Figure 1**

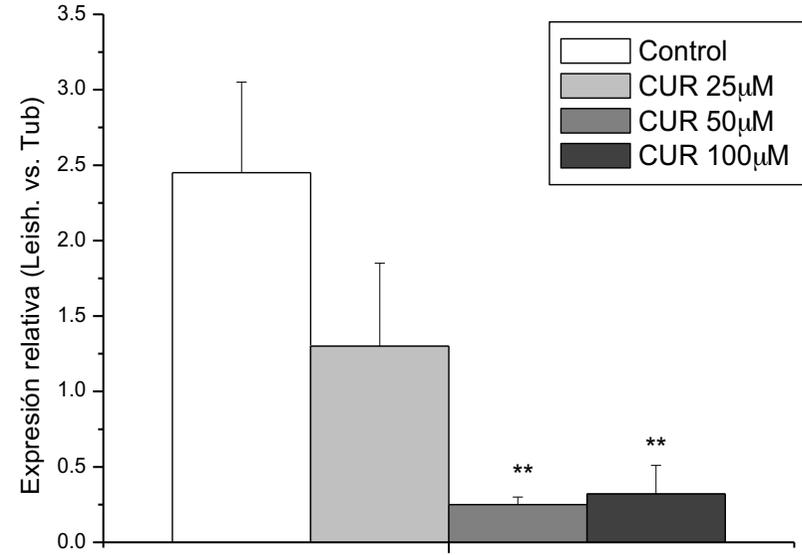


**Figure 2**

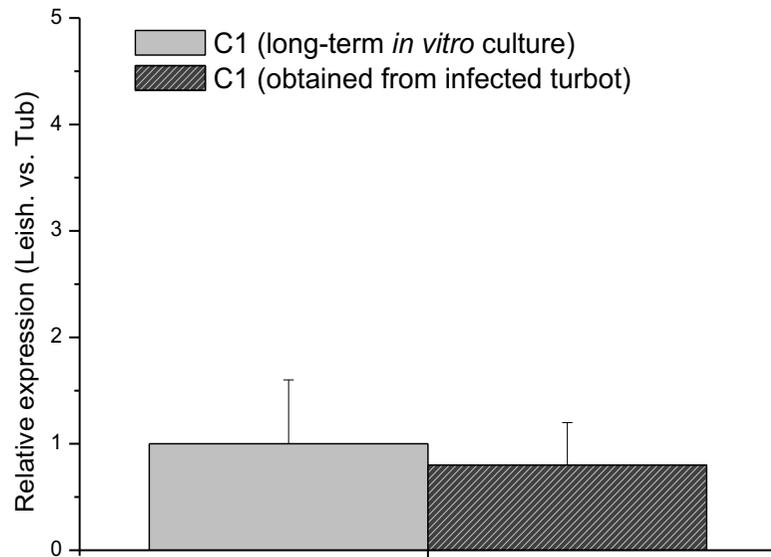
**a)**



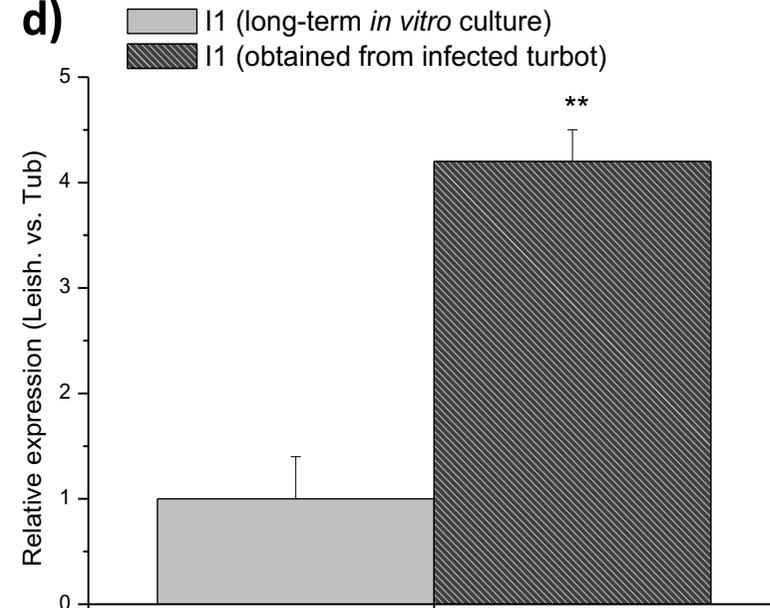
**b)**



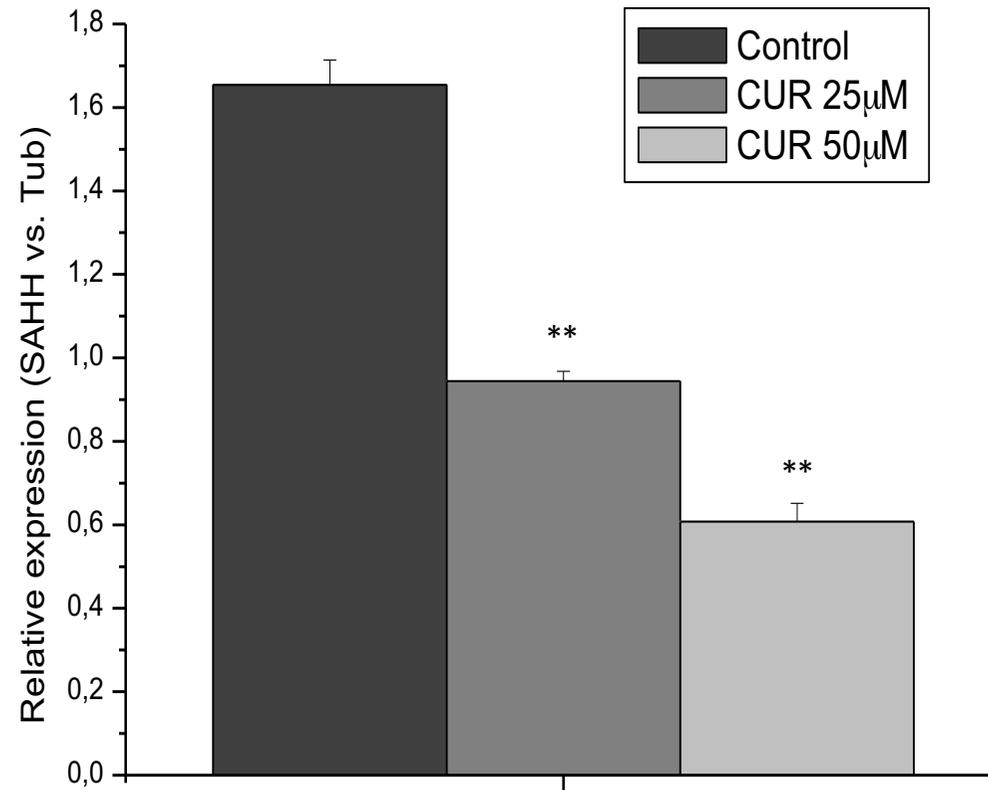
**c)**



**d)**



**Figure 3**



**Figure 4**

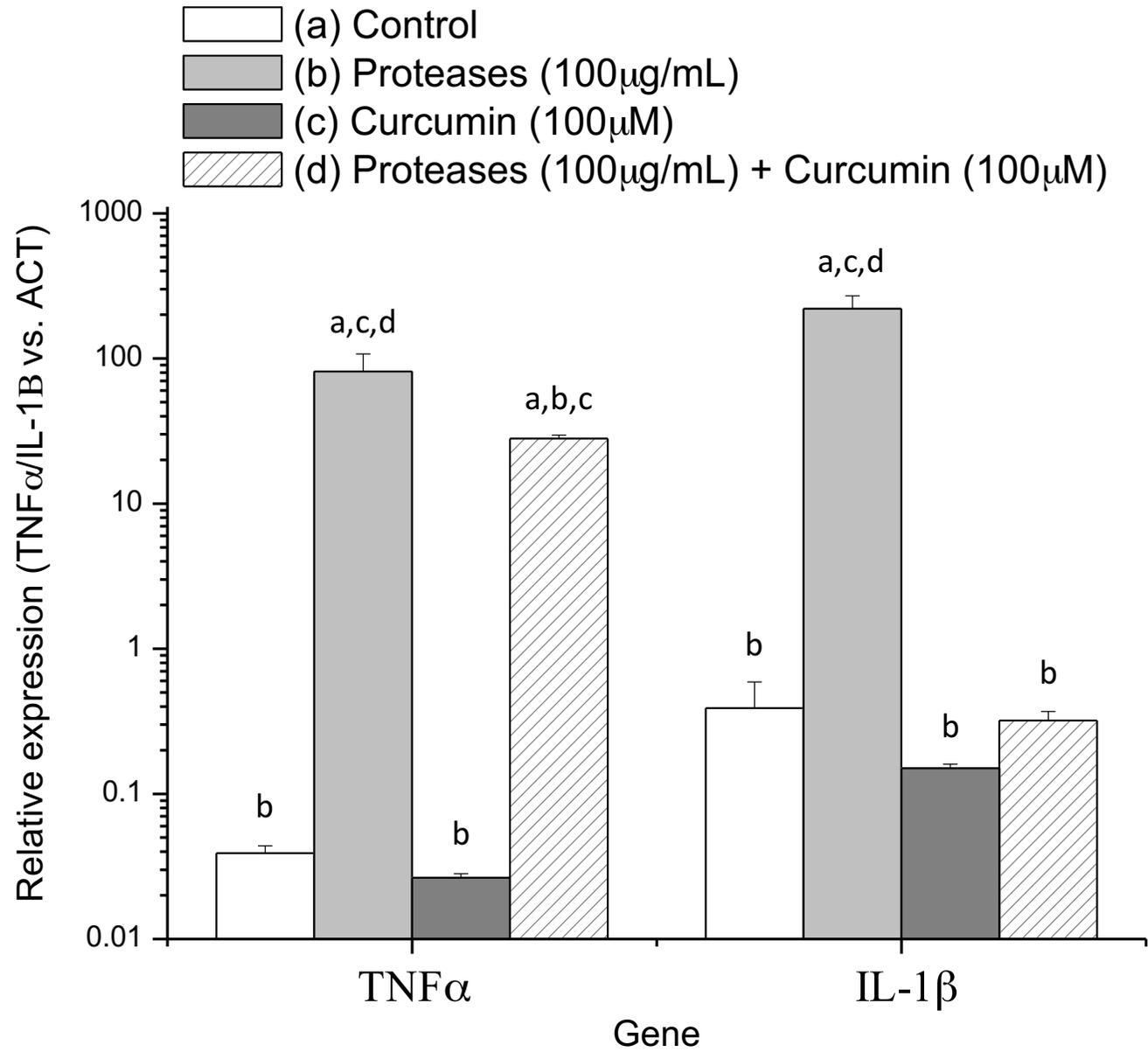


Figure 5