Combined antiparasitic and anti-inflammatory effects of the natural polyphenol curcumin on turbot scuticociliatosis

Natalia Mallo[1], Ana-Paula DeFelipe[1], Iria Folgueira[1], Rosa-Ana Sueiro[1,2], Jesús Lamas[2], José-Manuel Leiro[1]

[a]*Departamento de Microbiología y Parasitología, Instituto de Investigación y Análisis Alimentarios, Universidad de Santiago de Compostela, Santiago de Compostela, Spain.*

[b]*Departamento de Biología Celular y Ecología, Facultad de Biología, Instituto de Acuicultura, Universidad de Santiago de Compostela, Santiago de Compostela, Spain*

Running Head: Effects of curcumin on scuticociliatosis

Correspondence *JM Leiro,* Departamento de Microbiología y Parasitología, Laboratorio de Parasitología, Instituto de Investigación y Análisis Alimentarios, Universidad de Santiago de Compostela, c/ Constantino Candeira s/n, Campus Vida, 15782, Santiago de Compostela (A Coruña) *E-mail adress*: josemanuel.leiro@usc.es (J. M. Leiro)

Abstract

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

Keywords:

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

Introduction

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

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

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

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

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

Material and Methods

Experimental animals and parasites

Turbot

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

Ciliates

Philasterides dicentrarchi ciliates (I_1 and C_1 isolates; Budiño et al., 2011) were isolated from naturally infected turbot (obtained from a fish farm in Galicia) showing signs of scuticociliatosis. The ciliates were isolated aseptically from turbot intraperitoneal fluid, as previously described (Iglesias et al., 2001). The ciliates were cultured **axenically** at 21°C in complete sterile L-15 medium (Leibovitz, PAA Laboratories GmbH, 10% salinity, pH 7.2) containing 90 mg/L each of adenosine, cytidine and uridine, 150 mg/L guanosine, 5 g/L glucose, 400 mg/L of L-α-phosphatidylcholine, 200 mg/L Tween 80, 10% heat inactivated foetal bovine serum (FBS) and 10 mL/L of an antibiotic/antimycotic solution (100X, i.e. 100 units/mL penicillin G, 0.1 mg/mL streptomycin sulphate and 0.25 mg/mL amphotericin B; Sigma-Aldrich), as previously described (Iglesias et al., 2003). Virulence of the ciliates was maintained by experimentally infecting samples of fish every 6 months by

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

Ethical approval

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

Anti-ciliate activity

The anti-ciliate activity was assayed as previously described (Mallo et al., 2014). A 100 mM stock solution of CUR (Sigma-Aldrich) was prepared in dimethyl sulphoxide (DMSO) and maintained at −20 °C, in darkness, until use. To investigate the *in vitro* anti-parasit**ic** effect, CUR was added (at final concentrations of 25, 50 and 100 μM) to the wells of a 96-well sterile culture plate (Corning), each containing 15 × 10^3 ciliates/mL in L-15 medium containing 10% FBS. The plates were incubated for 3 days at 21 ºC. Aliquots (15 μL) of medium were removed every day from each of five replicate wells for the different treatments. The ciliates were inactivated by addition of 0.25% glutaraldehyde **(Sigma-Aldrich)** before quantification in a haemocytometer (Iglesias et al., 2002). To **exclude** any possible effects of DMSO, L-15 medium containing the highest concentration of DMSO (100 μM) was added to five replicate wells and processed as above. The *in vitro* inhibitory concentrations (IC_{50}) of the CUR w**as** calculated using Excel software (Microsoft Office; Microsoft, Madrid, Spain), by 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) x log 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 µm 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 *x* g for 30
158 min at 4 ºC. The interface cells were collected, washed once with Hanks solution
159 **(Gibco) at** 600 *x* g for 10 min, resuspended in L-15 containing 2% FBS, washed again
160 (300 *x* 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 CH_3COONH_4
168 and 10mM $CaCl_2$, 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 x *g* for 10 min and filtered (0.22 µm, Millipore, USA). The

proteases were purified by processing all samples in a CNBr-activated bacitracin–sepharose XK 16/20 column (GE Healthcare, USA) connected to a protein purification system (AKTAprim plus; GE Healthcare, USA). The non-retained fraction was washed with washing buffer (100mM, CH_3COONH_4 pH 6.4) until the optical density (OD) at 280 nm was basal. Proteases bound to the column were then eluted in elution buffer containing 100 mM CH_3COONH_4, 1 M NaCl and 25% (v/v) 2-isopropanol (pH 6.5) and collected in 2.5 mL fractions until the OD at 280 nm was basal. Samples were then dialyzed against equilibration buffer and concentrated by ultrafiltration with Amicon Ultra 10 K centrifugal filter devices (Millipore, MA, USA) and finally stored in 0.15 M PBS at -80 ºC until use. The protein concentration in the extract was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Germany), by the method of Bradford (1976) and with bovine serum albumin (Sigma–Aldrich) as standard (Khan et al., 2003; Piazzón et al., 2011).

Gelatin-FITC proteolytic activity

This method was based on measurement of hydrolysis in a solution of a protein substrate (gelatin) conjugated with a fluorescent ligand (fluorescein isothiocyanate; FITC**; Sigma-Aldrich**) (Lee et al., 2003). The FITC was dissolved with the same proportion of protein (gelatin) (**1:1, Wt:Wt**) in a solution of Na_2HPO_4 (pH 9-9.5) and incubated for 1h at room temperature **(RT)**. Once coupling had taken place, excess FITC was removed by dialysis against PBS and the solution was centrifuged at 5000 x *g* for 10 min to eliminate precipitated protein. Aliquots were stored at -20 ºC until use.

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

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

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

SDS-PAGE-substrate

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

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

Quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR)

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

Ciliates were incubated for 24 hours with the different concentrations of CUR (25, 50 and 100 μM). Two ciliate isolates (I1 and C1) belonging to different haplotypes and serotypes and with different levels of virulence were used to test the gene expression levels (Budiño et al., 2011; 2012 b). In these experiments, ciliates obtained from long-term *in vitro* cultures and ciliates recently isolated from turbot ascites were 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 10^7 cells/sample was obtained with NucleoSpin ARN
250 (Macherey-Nagël), treated with DNase I (RNase free, Thermo Scientific). **The integrity**
251 **of RNA was assessed on a dentaturing agarose gel and** the purity and final
252 concentration **of samples** were then estimated using a NanoDrop ND-1000
253 spectrophotometer. cDNA synthesis (25 μ**L**/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 $MgCl_2$ 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 1μ**L** 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_2O. 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 $2^{-\Delta\Delta Cq}$ 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.*

dicentrarchi: S-adenosil homocysteine hydrolase (SAHH) forward/reverse, 5'-CACATGACCATCCAAACTGC-3'/5'-TCAACGAGAATGTTGGGTCCT-3'; *P. dicentrarchi* Leishmanolysin-like peptidase (Leish) forward/reverse, 5'-CCCCACCATCAAAGGAATCT-3'/5'- CCTTACCCTTTCCCCATGAT-3'; *P. dicentrarchi* cathepsine- L-**like** (Cat), forward/reverse 5'-CTCTTCCGTCGATTGGGTTA-3'/5'-GGGCGTAGTTGATTCCGTTGT-3' (Table1); *P. dicentrarchi* β-tubulin (Tub), forward/reverse, 5′-ACCGGGGAATCTTAAACAGG-3′/5′-GCCACCTTATCCGTCCACTA-3′. Gene expression in *P. dicentrarchi* was normalized with the reference β-tubulin gene. For turbot expression assays, the following primer sequences were used: turbot tumour necrosis factor α (TNFα)- NCBI accession number FJ654645- forward/reverse, 5′-TCACAGGACAAGCTGGAGTG-3′/5′-TCATCAGAGACGCGTTGAAG-3′; turbot interleukin 1 β (IL-1B)- NCBI accession number AJ295836, forward/reverse 5′-AATGGGGCACTGAACAAAAG-3′/5′-GCCACCTTGTGGTGAAACTT-3′ and turbot actin (ACT), forward/reverse, 5′-CATGTACGTTGCCATCCAAG-3′/5′-TCTCAGCAGTGGTGGTGAAG-3′. **Primer pairs were designed and optimized with the Primer 3 Plus program (http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi) with a Tm of 60 °C (Mallo et al., 2013). Host actin was used to normalize data,** which were expressed in relative arbitrary units. The results **are** shown **as** mean values ± the standard error (SE) from three trials.

Statistical analysis

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

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

Results

In vitro effect of CUR on trophozoite growth

The antiparasitic effect of CUR on the *in vitro* growth of *P. dicentrarchi* was determined at different concentrations (25, 50 and 100 μM). At the highest concentration (100 μM), CUR had a clear cytotoxic effect **($P < 0.001$)** on the ciliate (Fig. 1). **T**he IC_{50} of CUR was 63.48 ± 5.77 μM, and ciliate growth was totally inhibited at 100 μM **on the first day of culture**, 59.25 ± 11.65 μM on day 2, and 64.98 ± 6.66 μM on day 3 (Fig. 1).

Effect of CUR on ciliate proteolytic activity

Parasite proteolytic activity was affected by treatment with 50 μM CUR for 24 h (Fig. 2a). The profile of the gelatin-lytic bands detected was almost the same in both samples (treated with 50 μM CUR and non-treated samples, lanes 1 and 2: Fig. 2a). However, the dark bands, which represent the gelatin-lytic activity, are less intense and thinner in CUR treated-samples than in the control samples, especially in the range 40 - 70 kDa (Fig. 2a).

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

Effect of CUR on ciliate protease gene expression

Gene expression of cathepsin L-like and leishmanolysin-like peptidase was **significantly** reduced in CUR-treated ciliates, at doses of 100 μM for the cathepsin L-**like (*P* < 0.05) and** between 50-100 μM in the case of leishmanolysin-like peptidase **(*P* < 0.01)** (Fig. 3 a,b).

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

Effect of CUR on SAHH gene expression

Ciliates cultured for 24 hours with two different concentrations (25 and 50 μM) of CUR, **showed** a **significant** decrease **(*P* < 0.01)** in SAHH gene expression was observed relative to the β- tubulin gene (Fig. **4**).

Anti-inflammatory effect of CUR on turbot leucocytes

In this experiment, the *in vitro* effect of CUR on gene expression of two pro-inflammatory cytokines, TNF-α and IL-1β, was analysed in leukocytes isolated from the anterior kidney of turbot and incubated for 24 h with ciliate proteases (100 μg/mL). The results **of qRT-PCR** showed that *P. dicentrarchi* proteases activated turbot leukocytes by generating a significant increase **(*P* < 0.01)** in **the** expression of both

proinflammatory cytokines (Fig. 5). By contrast, addition of 100 μM CUR to protease-activated leukocytes significantly inhibited **($P < 0.01$)** the expression of these cytokines (Fig. 5).

Discussion

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

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

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

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

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

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

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

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

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

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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 μM). Data are the cell counts (haemocytometer)
755 determined **during** 3 days. The IC_{50} values obtained are indicated on the bars in the
756 graph. Data indicated mean ± 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 μM). Gel strips were incubated for 12 h at 37 º 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 ±
765 standard error (*n*= 5). ***P* < 0.01.
766
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 μM). **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 β-
774 tubulin (Tub). Values are means ± 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 β-tubulin (Tub) gene and each bar indicates the mean ± 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 μg/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 TNFα and IL-1β
784 mRNAs were quantified by **qRT-PCR**. Data presented are the mean values ± standard
785 error**s** (n= 3) of units expressed relative to the housekeeping α-actin (ACT) gene
786 mRNA levels. **Letters indicate significant differences between groups a-d (*P* <**
787 **0.05).**

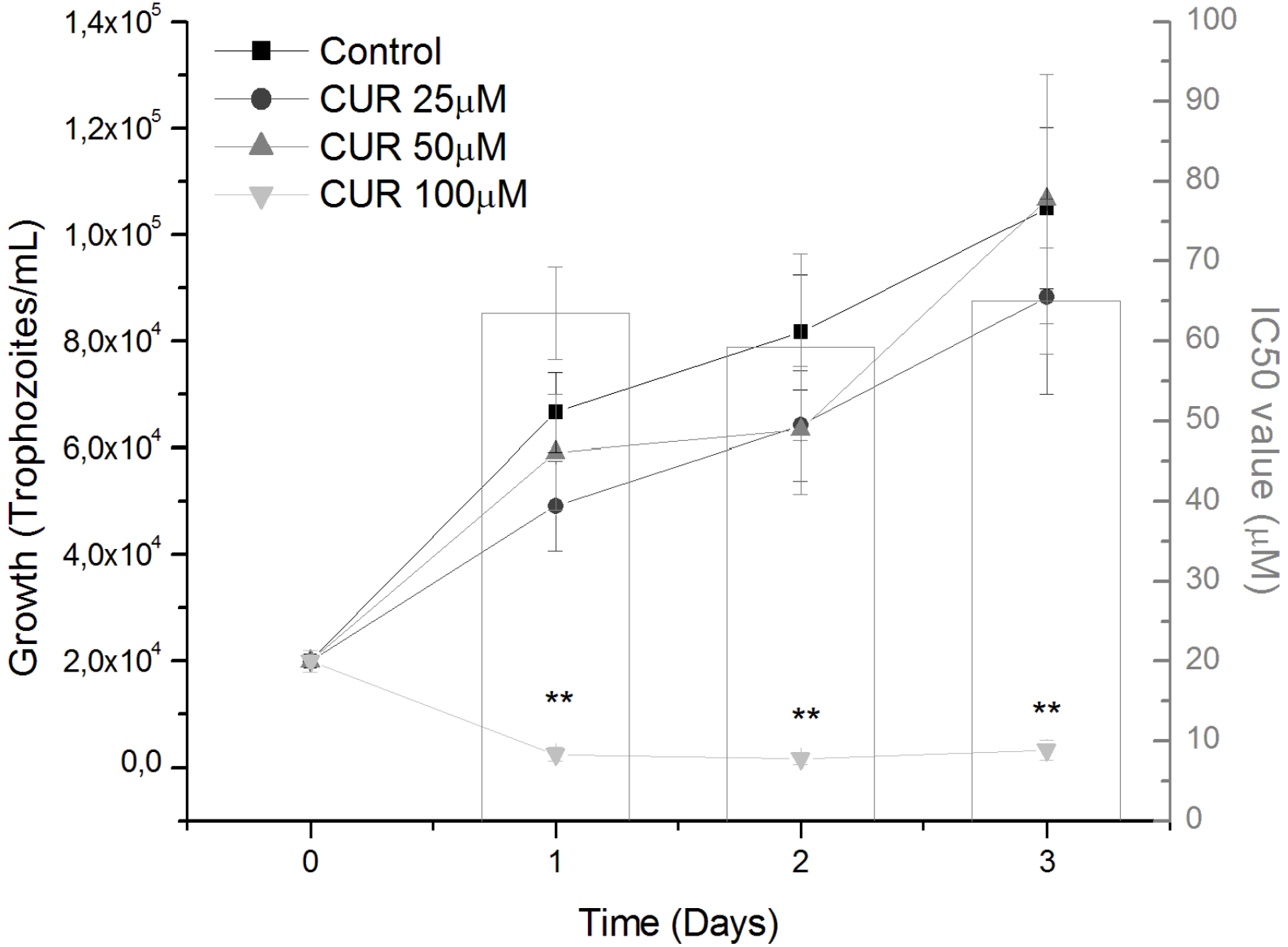
Control
CUR 25μM
CUR 50μM
CUR 100μM
Growth (Trophozoites/mL)
1,4x10^5
1,2x10^5
1,0x10^5
8,0x10^4
6,0x10^4
4,0x10^4
2,0x10^4
0,0
IC50 value (μM)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
Time (Days)
**
**
**

Figure 1

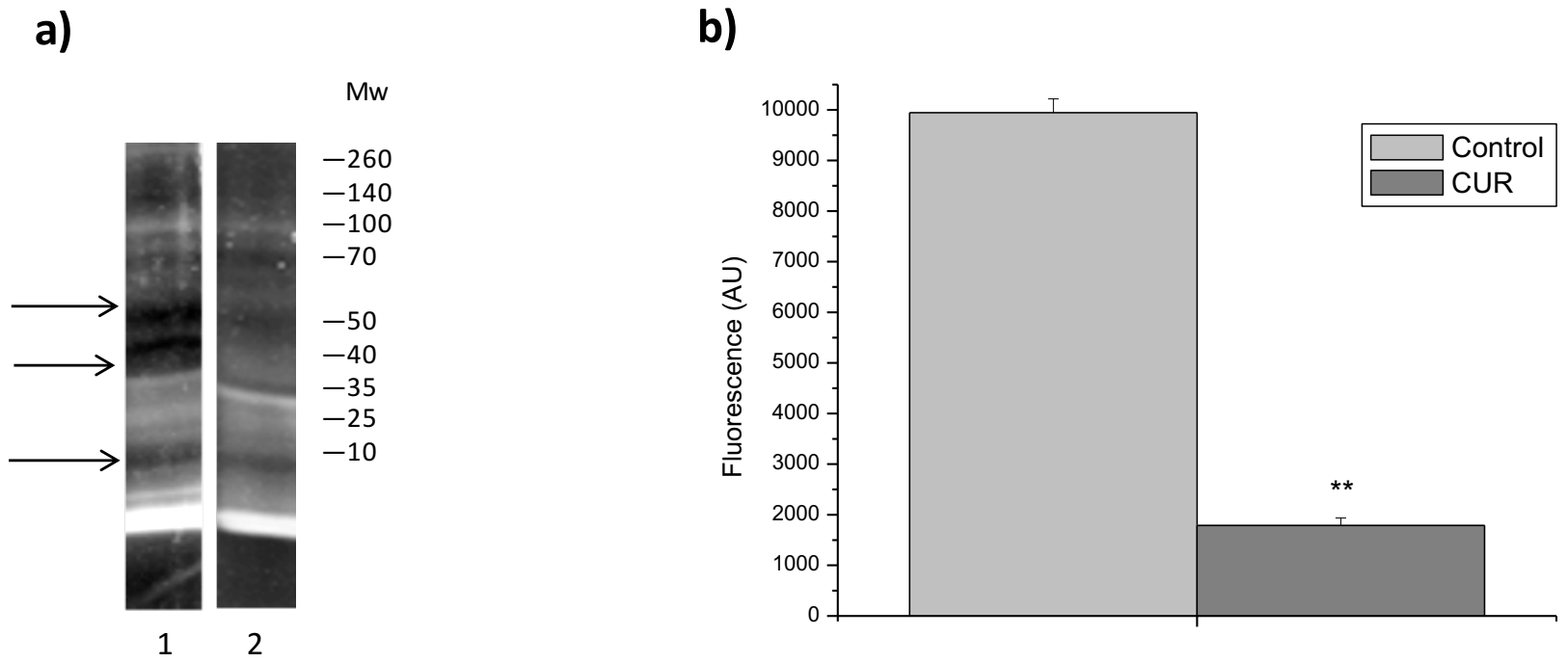
a)
Mw
260
140
100
70
50
40
35
25
10
1
2
b)
Fluorescence (AU)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Control
CUR
**

Figure 2

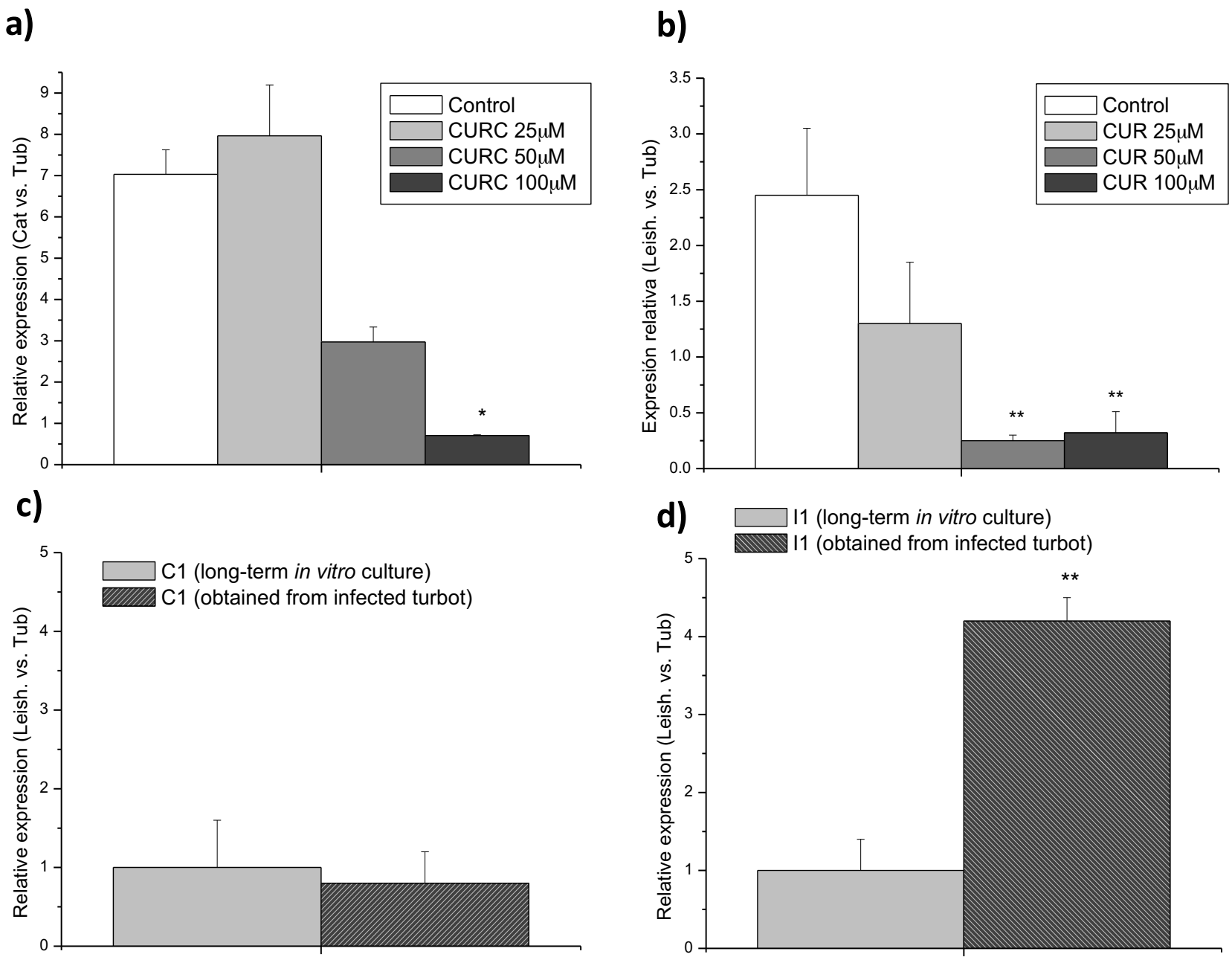
a)
Relative expression (Cat vs. Tub)
Control
CURC 25μM
CURC 50μM
CURC 100μM
*
b)
Expresión relativa (Leish. vs. Tub)
Control
CUR 25μM
CUR 50μM
CUR 100μM
**
**
c)
Relative expression (Leish. vs. Tub)
C1 (long-term in vitro culture)
C1 (obtained from infected turbot)
d)
Relative expression (Leish. vs. Tub)
I1 (long-term in vitro culture)
I1 (obtained from infected turbot)
**

Figure 3

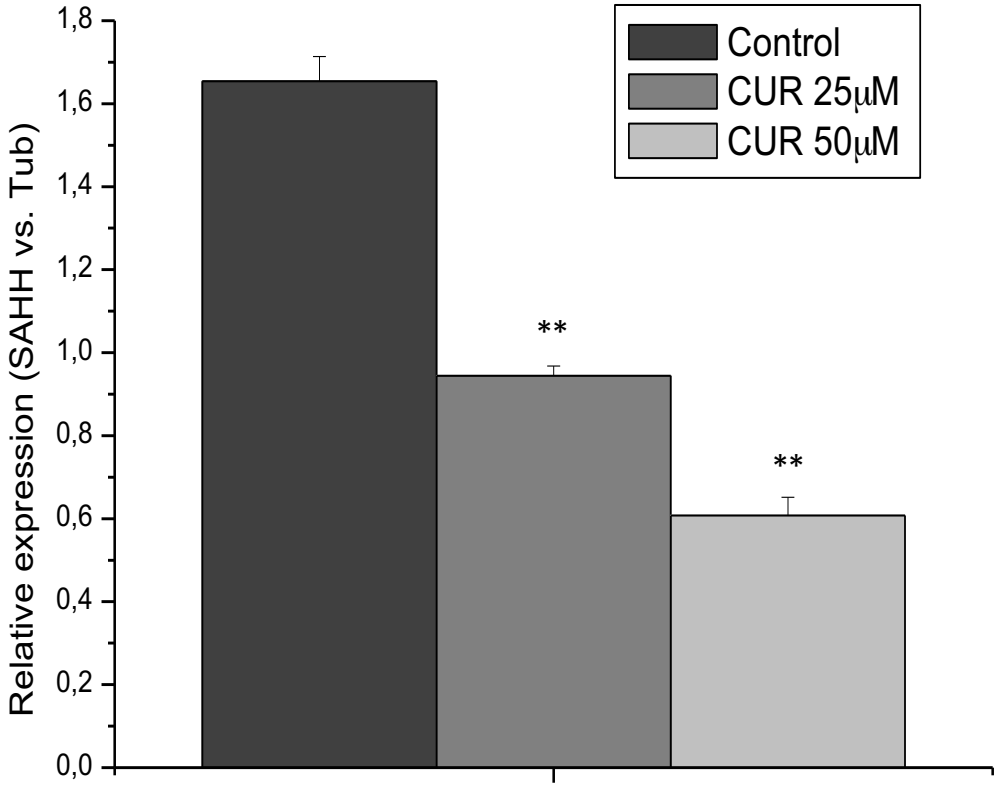
Control
CUR 25μM
CUR 50μM
Relative expression (SAHH vs. Tub)
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
**
**

Figure 4

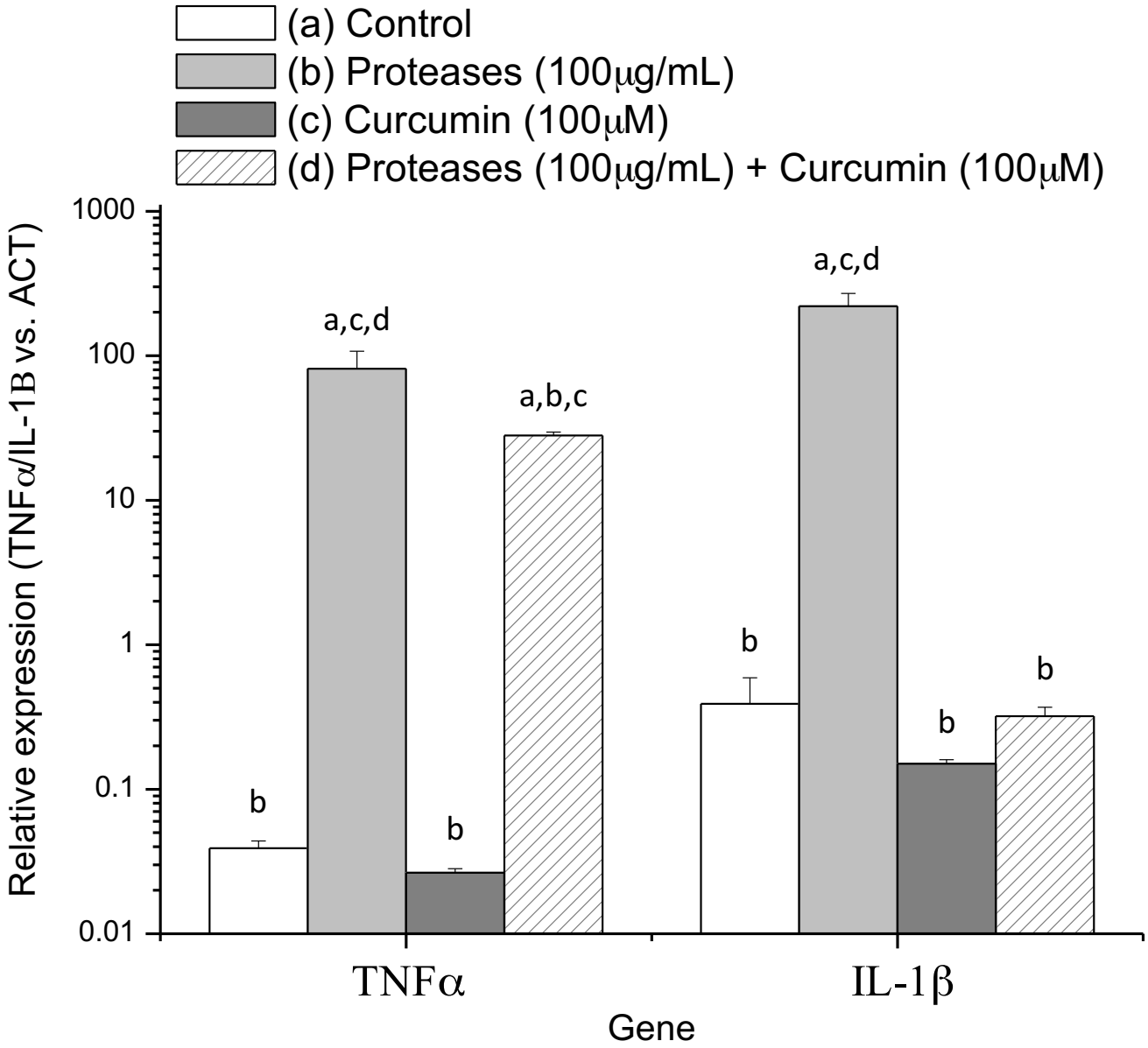
(a) Control
(b) Proteases (100μg/mL)
(c) Curcumin (100μM)
(d) Proteases (100μg/mL) + Curcumin (100μM)
Relative expression (TNFα/IL-1B vs. ACT)
1000
100
10
1
0.1
0.01
a,c,d
a,b,c
b
b
a,c,d
b
b
b
TNFα
IL-1β
Gene

Figure 5