Hydrogenosome metabolism is the key target for antiparasitic activity of resveratrol against *Trichomonas vaginalis*

Natalia Mallo, [a] Jesús Lamas, [b] and José M. Leiro[a,*]

Departamento de Microbiología y Parasitología, Laboratorio de Parasitología, Instituto de Investigación y Análisis Alimentarios, Universidad de Santiago de Compostela, Santiago de Compostela, Spain[a]; Departamento de Biología Celular y Ecología, Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain[b]

SHORT TITLE: Hydrogenosomal activity in *Trichomonas* treated with resveratrol

Journal: Antimicrobial Agents and Chemotherapy

[*]Corresponding author
Laboratorio de Parasitología,
Instituto de Investigación y Análisis Alimentarios,
c/ Constantino Candeira s/n,
15782, Santiago de Compostela (La Coruña), Spain
Tel: +34 981 56 31 00
Fax: + 34 881 81 60 27
E-mail address: josemanuel.leiro@usc.es

ABSTRACT

Metronidazole (MDZ) and related 5-nitroimidazoles are the recommended drugs for treatment of trichomoniasis; a sexually transmitted disease caused by the protozoan parasite *Trichomonas vaginalis*. However, novel treatment options are needed, as recent reports claim resistance to these drugs in *T. vaginalis* isolates. In this study, we analyzed for the first time the *in vitro* effects of the natural polyphenol resveratrol (RESV) on *T. vaginalis*. At concentrations between 25-100 μM, RESV inhibited the *in vitro* growth of *T. vaginalis* trophozoites; doses of 25 μM exerted a cytostatic effect and higher doses exerted a cytotoxic effect. At these concentrations, RESV caused inhibition of the specific activity of a 120 kD [Fe]-hydrogenase (Tvhyd). RESV did not affect Tvhyd gene expression and only upregulated pyruvate ferredoxin oxidoreductase (a hydrogesomal enzyme) gene expression at a high dose (100 μM). At doses of 50-100 μM, RESV also caused overexpression of heat shock protein 70 (Hsp70), a protective protein found in the hydrogenosome of *T. vaginalis*. The results demonstrate the potential of RESV as an antiparasitic treatment for trichomoniasis and suggest that the mechanism of action involves induction of hydrogenosomal dysfunction. In view of the results, we propose the hydrogenosomal metabolism as a key target in the design of novel antiparasitic drugs.

Keywords**:** *Trichomonas vaginalis,* resveratrol, hydrogenosome, hydrogenase, heat shock protein 70, antiparasitic activity

INTRODUCTION

Trichomoniasis is known as the most common non-viral sexually transmitted disease (STD) in the world (1). An ancient extracellular obligate parabasalian flagellate, *Trichomonas vaginalis*, which colonizes the human urogenital tract, causes the disease. Trichomoniasis is a frequent source of vaginitis (about 4 to 35% of vaginitis diagnosed in symptomatic women) and may evolve from an asymptomatic to a chronic inflammatory disease (2, 3, 4). The disease can contribute to premature rupture of membranes during pregnancy, preterm birth, low birth weight, and may facilitate HIV acquisition (5). Currently, metronidazole (MZD) and other 5-nitroimidazoles (tinidazole, ornidazole and secnidazole), which are potent drugs against infections caused by anaerobic or microaerophilic microorganisms, are the only recommended drugs for standard treatment of *T. vaginalis* infection. However, resistance of *T. vaginalis* to MZD, allergic reactions and failure to remedy the infection with two consecutive courses of treatment have been reported (4, 6-9). Studies have shown that at least 5% of clinical cases of trichomoniasis are caused by parasites that are resistant to the above drugs. Because of the lack of approved alternative treatments, the only option for patients with resistant infections is to use higher and sometimes toxic doses of MDZ, which leads to an increase in the occurrence of side effects (6). The reliance on a single class of drugs for treating *T. vaginalis* infections may be problematic if resistance to nitroimidazole becomes widespread in *T. vaginalis* strains. Hence, studies to find new safe agents that are efficacious in the treatment and prevention of refractory trichomoniasis are clearly essential.

Natural products research provides a wide variety of lead structures that are used by the pharmaceutical industry as templates in the development of new drugs that are more effective and have fewer or no undesirable side effects than current treatments (10-12). Resveratrol (RESV) is a natural major phytoalexin (a functionally defined class of

71 secondary metabolites), which is produced *de novo* by plants in response to stress factors,
72 such as pathogen attack, and which promotes disease resistance (13). RESV, which is
73 found naturally in grapes and red wine (14), acts as a precursor for stilbene compounds
74 of higher fungotoxicity that accumulate in grapevine as a result of infection or stress (15).
75 RESV has also been shown to be active against bacteria, fungi, protozoa and viruses (16-
76 22). In addition to its antimicrobial activity, RESV has also attracted attention on the
77 basis of its health benefits to humans, which include anti-inflammatory effects, as well as
78 reduced risk of cardiovascular disease, cancer, obesity, diabetes and neurodegenerative
79 diseases, and also extension of lifespan by mimicking the caloric restriction effect (23).
80 *T. vaginalis* is an amitochondrial anaerobic parasite that possesses a
81 hydrogenosome (a double membrane-bound organelle involved in catabolic processes,
82 including glycolysis, which produce energy and excrete molecular hydrogen, thus aiding
83 redox balance) (24). The pathway of oxidative decarboxylation of pyruvate in
84 hydrogenosomes is responsible for metabolic activation of 5-nitroimidazole drugs, such
85 MDZ, used to treat trichomoniasis (25). Hydrogenosomes are considered an excellent
86 drug target because their metabolic pathway is distinct from those found in mitochondria
87 and thus medicines directed at these organelles will probably not affect the host cells (26).
88 In this study, we evaluated, for the first time, the *in vitro* effects of RESV on *T.*
89 *vaginalis*, and we describe some of the potential biochemical targets of hydrogenosomal
90 metabolism involved in the antiparasitic activity.
91
92
93 **MATERIAL AND METHODS**
94 **Parasites.** The *Tv1* isolate of *T. vaginalis* was obtained from a female patient suffering
95 from vaginal trichomoniasis attending the Gynaecology service at the Santiago de

96 Compostela University Hospital Complex (Spain). Parasites were cultured axenically *in*
97 *vitro* in modified Diamond´s medium (MDM) (27). The parasites were cultured at 35ºC
98 in 15 mL culture plates completely filled with the medium, which contained (w/v) 2%
99 trypticase, 1% yeast extract, 0.5 maltose, 0.1% L-ascorbic acid, 0.1% L-cysteine, 0.1%
100 KCl, 0.1% $KHCO_3$, 0.1% KH_2PO_4, 0.1% K_2PO_4, 0.02% $FeSO_4$ (pH 6.2) and was
101 supplemented with 10% (v/v) heat-inactivated bovine serum. Cells were grown to late log
102 phase (1-2x10^6 cells/mL) and harvested by centrifugation (200 x *g*) for all further
103 manipulations

104 ***In vitro* growth assays**. The effects of RESV and MDZ on the *in vitro* growth of
105 *T. vaginalis* were determined as previously described, with minor modifications (18). For
106 all experiments, stock solutions of RESV and MDZ were prepared in DMSO, to a
107 concentration of 100 mM, and stored away from light at -80 °C. In order to investigate
108 their effects on *T. vaginalis*, RESV and MDZ were added to wells of sterile 24-well
109 culture plates (Corning) containing 7x10^4 trophozoites / well in 1 mL of MDM with the
110 different concentrations of the drugs (25, 50 and 100 µM for RESV and 6.25, 12.5, 25,
111 50 and 100 µM for MDZ). Control wells containing DMSO at the highest concentration
112 used were included in each plate. The plates were then incubated for 2 days at 35°C in a
113 container under vacuum. The number of ciliates was determined daily by counting the
114 number in 10 µL aliquots of the medium removed from each well, in a haemocytometer.

115 **Preparation of cell crude extracts (CEs).** *T. vaginalis* trophozoites (10^7) were
116 harvested by centrifugation (200 x *g* for 5 min at 4ºC) and washed twice with Dulbecco's
117 phosphate buffered saline (DPBS; pH 7.0) supplemented with calcium and magnesium,
118 discarding the supernatant. Cells were lysed by adding 200 µl of ultrapure water
119 containing 25 mM metrizamide (Sigma-Aldrich) and 1 mM of phenylmetahnesulfonyl

120 fluoride (PMSF) to the pellet for 15 min at 4ºC. Proteins were quantified using the Bio-
121 Rad DC assay.

122 **Spectrophotometric assays for [Fe]-hydrogenase (Tvhyd) activity.** Tvhyd
123 activity on the CEs was quantified spectrophotometrically. Reduced methyl viologen
124 (MV) and a proton (buffer solution) were used as the electron donor and acceptor,
125 respectively, to measure the proton reduction activity ($2H^+ + 2e^- \rightarrow H_2$). The reaction
126 buffer contained 20 mM sodium dithionite and 1 mM MV dissolved in DPBS. N_2 gas was
127 bubbled though the reaction solution for 10 min in tubes with over-flange stoppers.
128 Reaction solution (1.4 mL) was preheated to 37ºC and transferred to a cuvette, which was
129 then covered with a silicone plug. The reaction was started by the injection of 0.1 mL of
130 CE with an insulin syringe, and it was monitored at 604 nm in a spectrophotometer
131 (Jenway, UK) equipped with temperature control. One enzymatic unit of Tvhyd was
132 defined as the amount of enzyme required to reduce 1 μmol of MV (equivalent to the
133 production of 1 μmol of H_2 per min from reduced MV). The specific activity was defined
134 as the number of enzymatic units per milligram of protein.

135 **Tvhyd activity on native polyacrylamide gel electrophoresis (Native PAGE).**
136 Aliquots (475 μg) of CEs proteins were dissolved in 200 μl of native protein loading
137 sample buffer (0.1 M Tris-HCl, pH 6.8; 20% glycerol, 0.2% bromophenol blue) and 30
138 μL of sample was loaded onto 12.5% nondenaturing polyacrylamide gel in a buffer
139 containing 25 mM Tris-HCl, 192 mM glycine (pH 8.3) and 25 mM metrizamide. After
140 electrophoresis, the lane for activity staining was cut out and soaked in basal buffer
141 containing 5 mM MV and 5 mM sodium dithionite previously bubbled for 10 min with
142 N_2 gas. The N_2 gas was then removed and replaced with hydrogen by bubbling H_2 gas
143 through the buffer containing the gel until the Tvhyd band activities were revealed. The
144 staining was fixed by adding of 1 mg/ml of 2,3,5-triphenyltetrazolium chloride to the

145 buffer and incubating the gel for 15 min at room temperature; the gel was then thoroughly
146 washed with deionized water. The bands exhibiting hydrogenase activity stained red due
147 to the formation of a red precipitate.

148 **Functional hydrogenosome alterations.** In order to determine if RESV and
149 MDZ causes alterations in the hydrogenosome functionality, 2 x 10^6 *T. vaginalis*
150 trophozoites were incubated with MDM alone or with RESV (50 and 100 mM) or MDZ
151 (6.25 and 12.5 mM) for 2 h at 37ºC. The trophozoites were permeabilized with 0.1 mM
152 of digitonin and incubated for 2 min at 37ºC. The cells were then washed twice by
153 centrifugation (200 x *g* for 5 min) before being resuspended in 3 ml of MDM containing
154 500 nM MitoTracker ® Deep Red FM (Molecular Probes) and incubated for 30 min at
155 37 ° C in the dark. After two washes with DPBS by centrifugation, the pellet was
156 resuspended in 100 µl of DPBS, and the fluorescence was measured in a microplate
157 fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA) (excitation / emission =
158 644/665 nm). In all experiments, the florescence produced by spontaneous oxidation of
159 the probe was subtracted from the fluorescence values obtained for the experimental
160 samples. The trophozoites were fixed with 2-4% formaldehyde for 15 min and washed
161 twice with DPBS to permeabilize them prior to visualization by fluorescence microscopy.
162 Samples fixed in this way can be used for immediate viewing in a fluorescence
163 microscope (excitation / emission = 644/665 nm) or stored in the dark for several days.

164 **Hydrogenosomal membrane potential (Δψm).** The hydrogenosomal membrane
165 potential (Δψm) of *T. vaginalis* was determined using the JC-1 kit (Molecular Probes)
166 with the cationic fluorescent probe 5,5',6,6'-tetrachloro-1, 1',3,3'-
167 tetraethylbenzimidazolcarbocyanine iodide. JC-1 can selectively enter hydrogenosomes
168 and, according to the magnitude of the hydrogenosomal membrane potential, change its
169 oligomeric state, thereby allowing it to fluorescence (28). The assay was carried out as

follows: *T. vaginalis* trophozoites were suspended in culture medium ($5x10^5$ per 100 µL) containing RESV (at concentrations of 0, 25, 50 and 100 mM) or MDZ (at concentrations of 0, 3.125, 6.25 and 12.5 mM) and incubated in 96-well cell culture plates for 2 h at 37ºC in a container to which vacuum was applied. After this period, 10 µL of the probe (diluted 1:10 in MDM) was added to each well and trophozoites were incubated at 37ºC in darkness for 30 minutes. The RESV and MDM were then removed by centrifuging the plates (200 x *g* for 5 min), and the trophozoites were washed twice with 200 µl of the previously prepared assay buffer and suspended in 100 µL of the same buffer. Finally, emitted fluorescence was measured immediately in a microplate fluorimeter to detect FITC (excitation/ emission = 485/535 nm). In all experiments, the florescence produced by spontaneous oxidation of the probe was subtracted from the fluorescence values obtained for the experimental samples.

Assay of intracellular reactive oxygen species (ROS) production. Intracellular production of ROS was evaluated with the fluorescent probe 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (carboxy-DCFDA, Fluka). A stock solution of probe (10 mM) was prepared in dimethyl sulphoxide (DMSO; Sigma) and stored in the dark at −80 °C until use. The flagellates obtained from cultures were centrifuged at 200 x*g* for 5 min and then resuspended in MDM (5×10^6 cells/ml). For the assay, 100 µl of the flagellate solution were added to each well of 96-well flat-bottom microtitre plates along with 1 µl of a 1:10 dilution (in DPBS) of stock solution of the probe in DMSO (final concentration of DMSO: 10 µM). To determine the effect of RESV and MDZ on intracellular ROS production, 1 µl of different dilutions of the drugs being tested was also added to provide the final concentrations used (0, 25, 50 and 100 µM for RESV and 0, 3.125, 6.25 and 12.5 µM for MDZ). The plates were then incubated for 1 h at 37 °C. Fluorescence was measured in a microplate fluorescence reader (excitation/emission =

490/525 nm). In all experiments, the florescence produced by spontaneous oxidation of the probe was subtracted from the fluorescence values obtained for the experimental samples.

Real-time reverse transcriptase polymerase chain reaction (RT-qPCR).

Total RNA from *T. vaginalis* trophozoites (10^7 cells/sample) was isolated with a NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany), in accordance with the manufacturer's instructions. The resulting RNA was dried and dissolved in diethylpyrocarbonate (DEPC)-treated RNase-free water at a concentration of 1 µg/ml. cDNA synthesis (25 µl/reaction) was achieved with 1.25 µM random hexamer primers (Roche), 250 µM of each deoxyribonucleotide triphosphate (dNTP), 10 mM DTT, 20 U of RNase inhibitor, 2.5 mM $MgCl_2$, 200 U of MMLV (murine leukaemia virus) reverse transcriptase (Promega) in 30 mM Tris and 20 mM KCl, pH 8.3, and 2 µg of sample RNA.

PCR was performed with gene-specific primers: pyruvate ferredoxin oxidoreductase D (pfoD) gene (forward/reverse primer pair, 5′-TCTCCGTTCTTGATCGTTCC-3′/5′-TGTTGTCGAAGACAGCCTG-3′, GenBank of NCBI accession number HQ657201) and the *T. vaginalis* [Fe]-hydrogenase A (TvHydA) gene (forward/reverse primer pair, 5′-ATTATGCCATGCACACGAAA-3′/5′-ACACCACCAGTTGCACAAAA-3′, GenBank of NCBI accession number U19897). A parallel PCR with primers for β-tubulin (tub2) gene (forward/reverse primer pair, 5′-TACTCCATCGTCCCATCTCC-3′/5′-CCGGACATAACCATGGAAAC-3′, GenBank NCBI accession number L05469) was used as a reference gene for RT-qPCR. Primer sets were designed and optimized using the Primer 3Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) based on the default parameters. PCR reactions (20 µL) contained 10 µL Maxima SYBR Green qPCR Master

220 Mix (Thermo Scientific), the primer pair at 300 nM, 1 µL of cDNA, and RNAase-
221 DNAase free water. PCR reactions were subjected to 95ºC for 5 min, followed by 40
222 cycles at 95ºC for 10 s and 60ºC for 30 s. This was followed by melting curve analysis at
223 95ºC for 15 s, 55ºC for 15 s and 95ºC for 15 s. The specificity and size of PCR products
224 for each gene were confirmed by gel electrophoresis. All PCRs were performed in an
225 Eco^{TM} Real-Time PCR System (Illumina). Relative quantification of gene expression
226 was determined by the $2^{-\Delta\Delta Cq}$ method (29) by using software conforming to MIQE
227 (Minimum Information for Publication of Quantitative Real-Time PCR experiments)
228 guidelines (30).
229 **SDS-PAGE and Immunoblot analysis**. Sodium dodecyl sulphate
230 polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 12.5% linear gel
231 (31, 32). The CEs samples were reduced and denatured by incubation for 5 min at 100 ºC
232 with 62 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol and 0.02 M
233 dithiothreitol (DTT). Electrophoresis was performed in a mini-vertical electrophoresis
234 system (Hoefer, USA) for 45 min at a constant 200 V in electrode buffer containing
235 25 mM Tris, 190 mM glycine and 1% SDS (pH 8.3).
236 Western blot analysis was performed as previously described, with minor modifications
237 (33). Following electrophoresis, reduced CEs were immunoblotted at 15 V for 35 min to
238 Immobilon-P transfer membranes (0.45 µm; Millipore, USA) in a trans-blot SD transfer
239 cell (Bio-Rad, USA) with the electrode buffer containing 48 mM Tris, 29 mM glycine,
240 0.037% SDS and 20% methanol, pH 9.2. Membranes were washed with Tris buffer saline
241 (TBS; 50 mM Tris, 0.15 M NaCl, pH 7.4), stained with Ponceau S (to verify protein
242 transfer), transfer-blocked for 2 h at room temperature with TBS containing 0.2% Tween
243 20 and 5% non-fat dry milk, washed in TBS, incubated for 1 h with a 1:500 dilution of
244 rabbit polyclonal antibody raised against highly conserved sequence in amino acids 342-

641 mapping at the C-terminus of heat shock protein (Hsp) 70 of human origin (Santa Cruz Biotechnology, USA) and finally incubated for 1h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig (Dakopatts; dilution 1:2000). Immunodetection of Hsp70 proteins was carried out with an enhanced luminol-based chemiluminescent substrate for detecting HRP (ECL Western blotting substrate, Thermo Scientific, USA), following the manufacturer's instructions. Digital images were obtained after exposing the membrane to a chemiluminiscent imaging system (FlorChem FC2, USA) equipped with automatic image capture software. Finally, the bands in the digitized images were quantified by densitometry analysis (ImageMaster Total Lab, ver. 2.00; Amersham-Pharmacia-Biotech).

Data presentation and statistical analysis. The results are expressed as means ± standard error of the mean (S.E.M.). The data were examined by one-way analysis of variance (ANOVA) followed by Tukey–Kramer test for multiple comparisons, and differences were considered significant at $\alpha = 0.05$. The concentration of drugs that caused 50% inhibition of the response (IC_{50}) was estimated by nonlinear regression analysis (GraphPad Prism software, San Diego, CA, U.S.A.) from the concentration–response curves obtained in each case (34).

RESULTS

Effect of RESV on *in vitro* growth of *T. vaginalis*. The effects of RESV (0, 25, 50 and 100 μM) and MDZ (0, 6.25-100 μM) on the *in vitro* growth kinetics of *T. vaginalis* trophozoites were compared (Fig. 1). At all concentrations tested, RESV caused a significant decrease in the *in vitro* growth, which was detected on day 1 of culture. However, while the concentrations of 50 and 100 μM significantly decreased the number of trophozoites (relative to the control) and appeared to be cytotoxic, the lowest

concentration of RESV used (25 μM) significantly inhibited cell growth, indicating a cytostatic activity (Fig. 1A). The kinetics of antiparasitic activity of MDZ against *T. vaginalis* was very similar to that of RESV, although the former appeared to be slightly more toxic. At concentrations above 6.25 μM, MDZ produced a decrease in the number of parasites in culture from 24h, thus displaying cytocidal activity; at the lowest concentration tested, the drug produced cytostatic activity (Fig. 1B). The mean inhibition values (IC_{50}) obtained for the RESV were 32 μM on day 1 and 25 μM on day 2, and the mean IC_{50} values for MDZ were 3.2 μM on day 1 and 4.25 μM on day 2 of culture.

Effect of RESV on Tvhyd activity. The specific activity of Tvhyd was quantified by spectrophotometric assay, and the effect of RESV (25 and 50 μM) on the enzyme activity was determined. The effect of MDZ (3.125 and 6.25 μM) on Tvhyd activity was also determined. At the concentrations tested, both RESV and MDZ produced a significant dose-dependent decrease in Tvhyd activity, with a mean IC_{50} of 35.6 and 3.2 μM, respectively (Fig. 2A, B).

In a parallel experiment, the effect of RESV and MDZ (100 and 12.5 μM, respectively) on the Tvhyd activity was also tested qualitatively, on native SDS-PAGE (Fig. 2C). The bands indicating Tvhyd activity were detected when the CEs were applied to SDS-PAGE and the resulting gels were stained with MV. Native SDS-PAGE revealed the presence of a single band of 120 kD, which was stained when CEs of untreated trophozoites were incubated anaerobically with MV and H_2 gas. However, the band disappeared when the gels containing the CEs were incubated in the presence of 12.5 μM MDZ or 100 μM RESV (Fig. 2C).

Effect of RESV and MDZ on energetic state of the hydrogenosome. The effect of RESV and MDZ on the energetic state of the hydrogenosome of *T. vaginalis* was analyzed using cell-permeant MitoTracker® Deep Red probes. The *T. vaginalis*

295 trophozoites incubated with the probe were fluorescent, often showing intracytoplasmic
296 fluorescent granules of shape and size compatible with hydrogenosomes (Fig. 3B).
297 Fluorescence levels of trophozoites incubated with MitoTracker ® Deep Red FM
298 probe were quantified by fluorometry. Both trophozoites incubated with RESV (50 and
299 100 μM) or MDZ (5.12 to 6.25 μM) exhibited significantly higher, dose dependent
300 fluorescence than untreated controls (Fig. 3A).
301 **Effect of RESV and MDZ on ROS production and on the Δψm of**
302 **hydrogenosome.** The *T. vaginalis* trophozoites were initially incubated under
303 microaerobic conditions and the intracellular ROS production was determined using the
304 fluorescent probe carboxy-DCFDA, in a fluorometric assay. Addition of RESV (25, 50
305 and 100 μM) or MDZ (3.125, 5.12 and 6.25 μM) did not significantly affect intracellular
306 ROS production (Fig. 4A). In a second experiment, the JC-1 fluorescent probe was used
307 to analyze the effect of RESV on the Δψm in treated and untreated *T. vaginalis*
308 trophozoites after incubation for 2 h with the drugs in MDM medium. In this case, there
309 was a significant dose-dependent decrease in the Δψm in the trophozoites incubated for
310 2 h with MDZ or RESV (Fig. 4B).
311 **Influence of RESV on hydrogenosome enzyme gene expression.** The effect of
312 RESV and MDZ on the expression of two keys genes within the hydrogenosome
313 metabolism of *T. vaginalis* was evaluated: the TvhydA gene and the gene encoding the
314 enzyme PFO responsible for pyruvate oxidation (pfoD gene). Although RESV (100 μM)
315 did not affect TvhydA gene expression, MDZ (3.125 and 6.25 μM) had a significant dose-
316 dependent effect on expression of this gene (Fig. 5). However, treatment with RESV (100
317 μM) increased pfoD gene expression, and MDZ (3.125 and 6.25 μMm) also significantly
318 increased the levels of expression of this gene (Fig. 5).

Effect of RESV on Hsp70 expression. Finally, the effect of RESV and MDZ on Hsp70 protein expression in *T. vaginalis* trophozoites was analyzed by western blotting. The results indicate a dose dependent increase in the protein expression of these chaperones in the trophozoites of *T. vaginalis* treated with RESV (50-100 μM) or MDZ (3.125 and 6.25 μM) (Fig. 6).

DISCUSSION

The 5-nitroimidazole drugs, of which MDZ is the most commonly prescribed, are the only effective drugs approved for treatment of trichomoniasis (6, 35). MDZ is considered to be cost-effective because it is inexpensive; generally efficient in eliminating *T. vaginalis* infection, has favourable pharmacokinetic and pharmacodynamics properties, and exerts minor adverse effects (8). Resistance to MDZ is frequently reported and cross-resistance among the family of 5-nitroimidazole drugs is common, leaving no alternative treatments, so that some cases remain unresolved (35). Alternative treatments for trichomoniasis are also needed for individuals who are allergic to MDZ (36, 37). The reliance on a single class of antimicrobial drugs for treating *T. vaginalis* infections may also increase the emergence of resistance, and studies of the novel therapeutics options for treatment and prevention of refractory trichomoniasis are therefore essential (5). This study demonstrates that *in vitro* treatment with RESV significantly inhibits growth of *T. vaginalis* and displays a very similar kinetic antiparasitic activity to that produced by the reference drug MDZ, although with slightly lower toxicity to the parasite. Mechanistic studies in cells *in vitro* have almost invariably used concentrations of RESV in the range 10^{-5} to 10^{-4} M to establish the therapeutic efficacy of this compound (38). Some studies have analyzed the *in vitro* antitrichomonal activity of some natural extracts from medicinal plants displayed good antitrichomonal activity, with IC_{50} values ranging

between 5.6 and 8.0 μg/ml (39-43). These values are similar to those obtained for RESV in this study, but much higher than those obtained with MDZ, the antiprotozoal drug used as positive control, which in the present study yielded a slightly higher value than in other studies (44).

The hydrogenosome, which is a spherical or slightly elongated structure of diameter 0.5-1.0 μm, is usually associated with cytoskeletal structures as the axostyle and costa in trichomonads (45). This unusual organelle, which is found in *T. vaginalis*, is an anaerobic form of mitochondrion (46-47) and produces H_2 during ATP synthesis (46). In *T. vaginalis*, fermentative energy metabolism relies on the highly oxygen-sensitive enzymes pyruvate-ferredoxin oxidoreductase (PFO), which is an iron-sulphur protein that converts pyruvate to acetyl-CoA (48), and Tvhyd (49). Hydrogenases (cytochrome c_3 oxidoreductase, EC 1.18.99.1) are classified, on the basis of the metal content of their dinuclear catalytic centres, as [NiFe], [Fe] or [NiFeSe] hydrogenases (50). There is evidence that Tvhyd belongs to the [Fe]-hydrogenases, which serves as terminal electron acceptors, evolving molecular hydrogen (51, 52). The present results demonstrate that both MDZ and RESV cause dose dependent inhibition of H_2 production by *T. vaginalis* hydrogenosome, as determined by measurement of specific activity by photochemically reduced MV.

Two closely related Tvhyd genes, TvhydA and TvhydB, have previously been characterized from *T. vaginalis*, and one of these contains a 50-kDa protein (53). Other authors subsequently isolated a gene encoding a putative 64 kDa-like [Fe]-hydrogenase from *T. vaginalis* (54). We analyzed the presence Tvhyd in CEs by SDS-PAGE under native conditions, and we found a single protein band of about 120 kDa. Most of the Tvhyd characterized so far consisted of at least two subunits (55), which may indicate that the Tvhyd has a dimeric structure, which is consistent with the sizes predicted by

369 genetic analysis (54). As in the spectrophotometric assay, native SDS-PAGE shows that
370 both MDZ and RESV inhibit hydrogenase activity. Most studies establish that the
371 antiparasitic mechanism of MDZ is related to the generation of cytotoxic anion radicals
372 (nitro radicals), which bind transiently to DNA, disrupt the DNA and cause cell death (4,
373 25). It has been established that the electrons required for MDZ activation are released
374 from pyruvate and malate by the activity of PFO enzyme and are transferred to the drug
375 by a low-redox-potential carrier, ferredoxin (Fd), which is reduced to its cytotoxic nitro
376 radical anion (25). In the hydrogenosome, electrons released in the PFO reaction are
377 accepted by Fd, which is subsequently reoxidized by Tvhyd and, in this reaction, electrons
378 are coupled to protons to form H_2 (56). MDZ acts as an electron sink in *T. vaginalis* by
379 capturing the electrons generated by PFO, which are then transported by Fe [2Fe-2S] to
380 the drug and not to their natural acceptor, Tvhyd. MDZ thus effectively competes for
381 electrons with the Tvhyd and consequently, activation of the drug is reflected by reduced
382 H_2 production by hydrogenosomes (57). Several mechanisms are potentially responsible
383 for the RESV-induced inhibition of H_2 production in hydrogenosomes: 1) competition
384 with Tvhyd for electrons (like MDZ), 2) inhibition of PFO activity and prevention of the
385 generation of electrons that are transferred to the Tvhyd, 3) inhibition of the transport of
386 electrons from the Fd, and 4) direct inhibition of Tvhyd. Because RESV and MDZ
387 inhibited H_2 production in the band corresponding to Tvhyd in native SDS-PAGE gels,
388 the effects of RESV on H_2 production must involve competition for electrons generated
389 by PFO or to enzyme inhibition. Although it is known that RESV may act as a radical-
390 scavenging antioxidant via its chemical repair free radicals (56), there is no evidence for
391 any ability to compete for electrons as described for MDZ (58). Therefore, in view of the
392 present results, it could be inferred that RESV acts primarily as an inhibitor of Tvhyd. It
393 has been suggested that iron may regulate hydrogenosomal activity through

hydrogenosomal enzyme expression and Δψm (59). Therefore, it is possible that the inhibitory effect of RESV on Tvhyd enzyme may involve Fe transport as it has been shown that the RESV blocks Fe transport and can act as an enzyme inhibitor (60).

Comentado [FdB1]: Podríamos obviar esto

MitoTracker® Deep Red FM is a far red-fluorescent dye (abs/em ~644/665 nm) that stains mitochondria and hydrogenosomes in live cells and can be used for *in vivo* determination of the mass and localization of the hydrogenosome in *T. vaginalis* (61, 62). We noted that both RESV and MDZ significantly increased the fluorescence emitted by the parasite hydrogenosomes incubated with MitoTracker ® Deep Red FM, suggesting that both compounds induce changes in hydrogenosomal metabolism in *T. vaginalis* trophozoites. This increase in fluorescence may be related to the induction of oxidative stress, a phenomenon that has also been detected in other parasites treated with RESV (63). Thus, our previous studies on parasitic ciliates indicate that treatment with RESV yielded a significant increase in intracellular ROS production and that oxidative damage may preferentially affect the stability and function of enzymes containing iron-sulphur clusters, such as complexes II and III (63-65). However, the present results indicate that either RESV or MDZ did not significantly increased ROS production in *T. vaginalis*. *T. vaginalis* has a microaerophilic lifestyle and uses redox antioxidant systems to counter the detrimental effects of oxygen and express a wide range of genes encoding for defence molecules, including superoxide dismutases, thioredoxin reductases, peroxiredoxins and rubrerytrins (4), which may eliminate the small amount of ROS produced.

JC-1 is a fluorescent lipophilic and cationic probe that can be used to determine the mitochondrial and hydrogenosomal Δψm (28, 66). Use of the JC-1 probe revealed that RESV and MDZ induced a loss of Δψm, suggesting also that these agents produced a metabolic perturbation of the hydrogenosome. Δψm may be altered by deregulation of intracellular ionic charges (eg. H^+), caused by alterations in Tvhyd activity. In ciliate

parasites, RESV also generates a collapse and dysfunction of mitochondrial Δψm accompanied by a significant increase in intracellular Ca^{2+} levels (65).

Several studies have correlated MDZ resistance with deregulation of hydrogenosomal enzyme gene expression (67). Thus, some studies have shown a significant reduction in the PFO and Tvhyd transcription levels in strains of *T. vaginalis* that are resistant to MDZ (68). The present results indicate that MDZ has an inductive effect on gene expression of PFO (like RESV), at the highest dose used, and also on the expression of the gene encoding the Tvhyd enzyme in our strain of *T. vaginalis*. Therefore, the increased expression of both enzymes may be related to the maintenance of homeostasis in the hydrogenosome in parasites treated with both compounds.

Comentado [FdB2]: Esta parte creo que iría mejor antes y relacionarla con la actividad de la TvHyd) y dejar el final para hablar sobre el estrés energético y los daños.

The results obtained in the present study suggest that RESV and MDZ caused a high degree of bioenergetic stress in treated parasites. Like mitochondria, hydrogenosomes of *T. vaginalis* contain heat shock proteins, Hsp70, Hsp60, and Hsp10 (69). The primary function of the chaperone 70 families is to maintain mitochondrial homeostasis and quality control (70). Overexpression of Hsp70 under the treatment may be related to an attempt by the parasite to aid hydrogenosomal survival by protecting against induced damage caused by the drugs.

In conclusion, this study confirms the *in vitro* anti-trichomonal activity of RESV, demonstrating that the antiparasitic mechanism of this polyphenol occurs through induction of hydrogenosomal metabolism alteration. This effect on the trichomonal energy metabolism leads to a profound dysfunction of hydrogenosome, which has deleterious effects on the parasite.

ACKNOWLEDGEMENTS

443 This study was financially supported by grants AGL2010-21219/ACU and AGL2011-
444 29927-C02-01 from the Comisión Interministerial de Ciencia y Tecnología (CICYT;
445 Spain).
446
447 **RERERENCES**
448 1. **Miller M, Liao Y, Gomez AM, Gaydos CA, D'Mellow D.** 2008. Factors associated
449 with the prevalence and incidence of *Trichomonas vaginalis* infection among African
450 American women in New York City who use drugs. J. Infect. **197**:503-509.
451 2. **Anderson MR, Klink K, Cohrssen A.** 2004. Evaluation of vaginal complaints. J.
452 Anim. Med. Assoc. **291**:1368-1379.
453 3. **Azimi H, Fallah-Tafti M, Karimi-Darmiyan M, Abdollahi M.** 2011. A
454 comprehensive review of vaginitis phytotherapy. Pak. J. Biol. Sci. **14**:960-966.
455 4. **Harp DF, Chowdhury I.** 2011. Trichomoniasis: Evaluation to execution. Eur. J.
456 Obstet. Gynecol. Reprod. Biol. **157**:3-9.
457 5. **Kirkcaldy RD, Augostini P, Asbel LE, Bernstein KT, Kerani RP, Mettenbrink**
458 **CJ, Pathela P, Schwebke JR, Secor WE, Workowski KA, Davis D, Braxton**
459 **J,Weinstock HS.** 2012. *Trichomonas vaginalis* antimicrobial drug resistance in 6 US
460 cities, STD Surveillance Network, 2009-2010. Emerg. Infect. Dis. **18**:939-943.
461 6. **Cudmore SL, Delgaty KL, Hayward-McClelland SF, Petrin DP, Garber GE.** 2004.
462 Treatment of infections caused by metronidazole-resistance *Trichomonas vaginalis*.
463 Clin. Microbiol. Rev. **17**:783–93.
464 7. **Das S, Huengsberg M, Shahmanesh M.** 2005. Treatment failure of vaginal
465 trichomoniasis in clinical practice. Int. J. STD. AIDS **16**:284–286.
466 8. **Löfmark S, Edlund C, Nord CE.** 2010. Metronidazole is still the drug of choice for
467 treatment of anaerobic infections. Clin. Infect. Dis. **50**(Suppl1):S16-S23.

9. **Muzny C, Barnes A, Mena L.** 2012. Symptomatic *Trichomonas vaginalis* infection in the setting of severe nitroimidazole allergy: successful treatment with boric acid. Sex Health. **9**:389-391.

10. **Borris RP.** 1996. Natural products research: Perspectives from a major pharmaceutical company. J. Ethnopharmacol. **51**:29-38.

11. **Butler MS.** 2004. The role of natural products in drug discovery. J. Nat. Prod. **67**:2141-2153.

12. **Koehn FE, Carter GT.** 2005. Rediscovering natural products as a source of new drugs. Discov. Med. **5**:159-164.

13. **Chang X, Heene E, Qiao F, Nick P.** 2011. The phytoalexin resveratrol regulates the initiation of hypersensitive cell death in *Vitis* cell. PLoS One. 6:e26405.

14. **Siemann EH, Creasy LL.** 1992. Concentration of the phytoalexin resveratrol in wine. Am. J. Enol. Vitic. **43**:49–52.

15. **Jeandet P, Douillet-Breuil AC, Bessis R, Debord S, Sbaghi M, Adrian M.** 2002. Phytoalexins from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. J. Agric. Food Chem. **50**:2731-2741.

16. **Chan MM.** 2002. Antimicrobial effect of resveratrol on dermatophytes and bacterial pathogens of the skin. Biochem. Pharmacol. **63**:99–104.

17. **Jung HJ, Hwang IA, Sung WS, Kang H, Kang BS, Seu YB, Lee DG.** 2005. Fungicidal effect of resveratrol on human infectious fungi. Arch. Pharm. Res. **28**:557–560.

18. **Leiro J, Arranz JA, Parama A, Alvarez MF, Sanmartin ML.** 2004. *In vitro* effects of the polyphenols resveratrol, mangiferin and (−)- epigallocatechin-3-gallate on the scuticociliate fish pathogen *Philasterides dicentrarchi*. Dis. Aquat. Org. **59**:171–174.

493 19. **Leiro J, Cano E, Ubeira FM, Orallo F, Sanmartin ML.** 2004. *In vitro* effects of
494 resveratrol on the viability and infectivity of the microsporidian *Encephalitozoon*
495 *cuniculi*. Antimicrob. Agents Chemother. **48**:2497–2501.
496 20. **Docherty JJ, Fu MM, Stiffler BS, Limperos RJ, Pokabla CM, DeLucia AL.** 1999.
497 Resveratrol inhibition of herpes simplex virus replication. Antivir. Res. **43**:145–155.
498 21. **Kedzierski L, Curtis JM, Kaminska M, Jodynis-Liebert J, Murias M.** 2007. *In*
499 *vitro* antileishmanial activity of resveratrol and its hydroxylated analogues against
500 *Leishmania major* promastigotes and amastigotes. Parasitol. Res. **102**:91-97.
501 22. **Son IH, Chung IM, Lee SJ, Moon HI.** 2007. Antiplasmodial activity of novel
502 stilbene derivatives isolated from *Parthenocissus tricuspidata* from South Korea.
503 Parasitol. Res. **101**:237-241.
504 23. **Nakata R, Takahashi S, Inoue H.** 2012. Recent advances in the study on resveratrol.
505 Biol Pharm Bull. **35**:273-279.
506 24. **Embley TM, van der Giezen M, Horner DS, Dyal PL, Foster P.** 2003.
507 Mitochondria and hydrogenosomes are two forms of the same fundamental organelle.
508 Phil. Trans. R. Soc. Lond. B **358**:191-203.
509 25. **Kulda J.** 1999. Trichomonads, hydrogenosomes and drug resistance. Int. J. Parasitol.
510 **29:**199-212.
511 26. **Benchimol M.** 2008. The hydrogenosome as a drug target. Curr Pharm Des. **14**:872-
512 881.
513 27. **Diamond LS.** 1957. The establishment of various trichomonads of animals and man
514 in axenic cultures. J. Parasitol. **43**:488-490.
515 28. **Vilela RC, Benchimol M.** 2012. *Trichomonas vaginalis* and *Tritrichomonas foetus*:
516 interaction with fibroblasts and muscle cells –new insights into parasite-mediated host
517 cell cytotoxicity. Mem. Inst. Oswaldo Cruz **107**:720-727.

518 29. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using
519 real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods **25**:402–408.
520 30. **Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller**
521 **R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT.** 2009. The
522 MIQE guidelines: minimum information for publication of quantitative real-time PCR
523 experiments. Clin Chem 55:611-622.
524 31. **Laemmli EK.** 1970. Cleavage of structural proteins during the assembly of the head
525 of bacteriophage T_4. Nature **227**:680-685.
526 32. **Paramá A, Iglesias R, Álvarez MF, Leiro J, Ubeira FM, Sanmartín ML.** 2004.
527 Cysteine proteinase activities in the fish pathogen *Philasterides dicentrarchi*
528 (Ciliophora: Scuticociliatida). Parasitology **128**:541–548.
529 33. **Paramá A, Castro R, Lamas J, Sanmartín ML, Santamarina MT, Leiro J.** 2007.
530 Scuticociliate proteinases may modulate turbot immune response by inducing
531 apoptosis in pronephric leucocytes. Int. J. Parasitol. **31**: 87-95.
532 34. **Álvarez E, Campos-Toimil M, Justiniano-Basaran H, Lugnier C, Orallo F.** 2006.
533 Study of the mechanisms involved in the vasorelaxation induced by (-)-
534 epigallocatechin-3-gallate in rat aorta. Br. J. Pharmacol. 147:269-280.
535 35. **Dunne RL, Dunn LA, Upcroft P, O'Donoghue PJ, Upcroft JA.** 2003. Drug
536 resistance in sexually transmitted protozoan *Trichomonas vaginalis*. Cell. Res. **13**:
537 239-249.
538 36. **Bachmann LH, Hobbs MM, Seña AC, Sobel JD, Schwebke JR, Krieger**
539 **JN, McClelland RS, Workowski KA.** 2011. *Trichomonas vaginalis* genital
540 infections: progress and challenges. Clin. Infect. Dis. **53** (Suppl. 3):SW160-S172.
541 37. **Secor WE.** 2012. *Trichomonas vaginalis*: treatment questions and challenges. Expert.
542 Rev. Ant. Infect. Ther. **10**: 107-109.

38. **Scott E, Steward WP, Gescher AJ, Brown K.** 2012. Resveratrol in human cancer chemoprevention –choosing the 'right' dose. Mol. Nutr. Food Res. **56**: 7-13.

39. **Calzada F, Yépez-Mulia L, Tapia-Contreras A.** 2007. Effect of Mexican medicinal plant used to treat trichomoniasis on *Trichomonas vaginalis* trophozoites. J. Ethnopharmacol. **113**:248-251.

40. **Arthan D, Sithiprom S, Thima K, Limmatvatirat C, Chavalitshewinkoon-Petmitr P, Svasti J.** 2008. Inhibitory effects of Thai plants beta-glycosides on *Trichomonas vaginalis*. Parasitol. Res. **103**:443-448.

41. **Cantillo-Ciau Z, Moo-Puc R, Quijano L, Freile-Pelegrín Y.** 2010. The tropical brown alga *Lobophora variegata*: a source of antiprotozoal compounds. Mar. Drugs **8**:1292-1304.

42. **Frasson AP, dos Santos O, Duarte M, da Silva Trentin D, Giordani RB, da Silva AG, da Silva MV, Tasca T, Macedo AJ.** 2012. First report of anti-*Trichomonas vaginalis* activity of the medicinal plant *Polygala decumbens* from the Brazilian semi-arid region, Caatinga. Parasitol Res. **110**:2581-2587.

43. **Moraes ME, Cunha GH, Bezerra MM, Fechine FV, Pontes AV, Andrade WS, Frota Bezerra FA, Moraes MO, Cavalcanti PP.** 2012. Efficacy of the *Mentha crispa* in the treatment of women with *Trichomonas vaginalis* infection. Arch Gynecol Obstet. **286**:125-130.

44. **Crozet MD, Botta C, Gasquet M, Curti C, Rémusat V, Hutter S, Chapelle O, Azas N, De Méo M, Vanelle P.** 2009. Lowering of 5-nitroimidazole's mutagenicity: towards optimal antiparasitic pharmacophore. Eur. J. Chem. **44**:653-659.

45. **Benchimol M.** 1999. The hydrogenosome. Acta Microscopica **8**:1-22.

46. **Lindmark DG, Müller M.** 1973. Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Tritrichomonas foetus*, and its role in pyruvate metabolism. J. Biol. Chem. **248**:7724-7728.

47. **Embley TM, Horner DA, Hirt RP.** 1997. Anaerobic eukaryotic evolution: hydrogenosomes as biochemically modified mitochondria. Trends Ecol. Evol. **12**: 437-441.

48. **Williams KP, Leadlay PF, Lowe PN.** 1990. Inhibition of pyruvate: ferredoxin oxidoreductase from *Trichomonas vaginalis* by pyruvate and its analogues. Comparison with the pyruvate decarboxylase component of the pyruvate dehydrogenase complex. Biochem. J. **268**:69-75.

49. **Page-Sharp MC, Behm CA, Smith GD.** 1976. *Tritrichomonas foetus* and *Trichomonas vaginalis* the pattern of inactivation of hydrogenase activity by oxygen and activities of catalase and ascorbate peroxidase. Microbiology **142**:207-211.

50. **Frey M.** 2002. Hydrogenases: hydrogen-activating enzymes. Chem. Biochem. **3**:153-160.

51. **Payne MJ, Chapman A, Cammarck R.** 1993. Evidence for an [Fe]-type hydrogenase in the parasitic protozoan *Trichomonas vaginalis*. FEBS Lett. **317**:101-104.

52. **Müller M.** 1993. The hydrogenosome. J. Gen. Microbiol. **139**:2879-2889.

53. **Bui ET, Johnson PJ.** 1996. Identification and characterization of [Fe]-hydrogenases in the hydrogenosome of *Trichomonas vaginalis.* Mol. Biochem. Parasitol. **76**:305-310.

54. **Horner DS, Foster PG, Embley TM.** 2000. Iron hydrogenases and the evolution of anaerobic eukaryotes. Mol. Biol. Evol. **17**:1695-1709.

591 55. **Ueno Y, Kurano N, Miyachi S.** 1999. Purification and characterization of
592 hydrogenase from the marine green alga, *Chlorococcum littorale*. FEBS Lett. **443**:144-
593 148.
594 56. **Hrdý I, Cammack R, Stopka P, Kulda J, Tachezy J.** 2005. Alternative pathway of
595 metronidazole activation in *Trichomonas vaginalis* hydrogenosomes. Antimicrob
596 Agents Chemother **49**:5033-5036.
597 57. **Lloyd D, Kristensen B.** 1985. Metronidazole inhibition of hydrogen production *in*
598 *vivo* in drug-sensitive and –resistant strains of *Trichomonas vaginalis*. J. Gen.
599 Microbiol. **131**:137-140.
600 58. **Karlsson J, Emgard M, Brundin P, Burkitt MJ.** 2000. Trans-resveratrol protects
601 embryonic mesencephalic cells from tert-butyl hydroperoxide: electron paramagnetic
602 resonance spin trapping evidence for a radical scavenging mechanism. J. Neurochem.
603 **75**:141-150.
604 59. **Kim Y-S, Song H-O, Choi I-H, Park S-J, Ryu J-S.** 2006. Hydrogenosomal activity
605 of *Trichomonas vaginalis* cultivated under different iron conditions. Korean J.
606 Parasitol. **44**:373-378.
607 60. **Kim M, Song E.** 2010. Iron transport by proteoliposomes containing mitochondrial
608 F(1) F(0) ATP synthase isolated from rat heart. Biochimie **92**:333-342.
609 61. **Agnello M, Morici G, Rinaldi AM.** 2008. A method for measuring mitochondrial
610 mass and activity. Cytotechnology 56:145-149.
611 62. **Emelyanov VV, Goldberg AV.** 2011. Fermentation enzymes of *Giardia intestinalis*,
612 pyruvate: ferredoxin oxidoreductase and hydrogenase do not localize to its mitosomes.
613 Microbiology **157**:1602-1611.
614 63. **Lamas J, Morais P, Arranz JA, Sanmartín ML, Orallo F, Leiro J.** 2009.
615 Resveratrol promotes an inhibitory effect on the turbot scuticociliate parasite

Philasterides dicentrarchi by mechanisms related to cellular detoxification. Vet. Parasitol. **161**:307-315.

64. **Solans A, Zambrano A, Rodríguez M, Barrientos A.** 2006. Cytotoxicity of a mutant huntingtin fragment in yeast involves early alterations in mitochondrial OXPHOS complexes II and III. Hum. Mol. Genet. **15**:3063-3081.

65. **Morais P, Piazzon C, Lamas J, Mallo N, Leiro JM.** 2012. Effect of resveratrol on oxygen consumption by *Philasterides dicentrarchi*, a scuticociliate parasite of turbot. Protist (in press, http://dx.doi.org/10.1016/j.protis.2012.07.002).

66. **Vilela R, Menna-Barreto RF, Benchimol M.** 2010. Methyl jasmonate induces cell death and loss of hydrogenosomal membrane potential in *Trichomonas vaginalis*. Parasitol. Int. **59**: 387-393.

67. **Mead JR, Fernández M, Romagnoli PA, Secor WE.** 2006. Use of *Trichomonas vaginalis* to evaluate correlation of gene expression and metronidazole resistance. J. Parasitol. **92**:196-199.

68. **Land KM, Delgadillo-Correa MG, Tachezy J, Vanacova S, Hsieh CL, Sutak R, Johnson PJ.** 2004. Targeted gene replacement of a ferredoxin gene in *Trichomonas vaginalis* does not lead to metronidazole resistance. Mol. Microbiol. **51**:115-122.

69. **Bui ET, Bradley PJ, Johnson PJ.** 1996. A common evolutionary origin for mitochondria and hydrogenosomes. Proc. Natl. Acad. Sci. U S A. **93**:9651-9656.

70. **Yang H, Zhou X, Liu X, Yang L, Chen Q, Zhao D, Zuo J, Liu W.** 2011. Mitochondrial dysfunction induced by knockdown of mortalin is rescued by Parkin. Biochem. Biophys. Res. Commun. **410**:114-120.

FIGURE LEGENDS

Figure 1. Effect of resveratrol (RESV), at concentrations of 25, 50 and 100 μM (A), and metronidazole (MDZ), at concentrations of 6.25, 12.5, 25, 50 and 100 μM (B), on the *in vitro* kinetic of growth of *T. vaginalis*. Each point on the lines represents the mean ± standard error of the number of trophozoites / mL (n = 5). Asterisks indicate statistically significant differences between treated groups and untreated controls: * $P < 0.01$.

Figure 2. Tvhyd activity in cell extracts (CEs) from *T. vaginalis* strain Tv1: the Tvhyd activity was measured by monitoring the oxidation of reduced methyl viologen (MV) in CEs from *T. vaginalis* treated with resveratrol (RESV) at 25 and 50 μM (A) and metronidazole (MDZ) at 3.125 and 6.25 μM (B). Histogram bars show mean ± standard error (n= 5). Asterisks indicate statistically significant differences between the treated groups and the untreated controls: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. C) Tvhyd-activity in native stained gel after sodium dodecyl sulphate polyacrylamide

electrophoresis (PAGE) of *T. vaginalis* CE. Lanes containing CE were incubated in presence of 12.5 μM MDZ, 100 μM RESV or without drugs (lane 0). Lane 0 show a stained band of a size of approximately 120 kD (arrow) that disappears in the lanes incubated with both drugs. MW: molecular weight markers in kD.

Figure 3. (A) Effect of resveratrol (RESV) at 50 and 100 μM and metronidazole (MDZ) at 6.25 and 12.5 μM on energetic state of the *T. vaginalis* hydrogenosome by using the cell-permeant MitoTracker probe, MitoTracker® Deep Red FM. Results are expressed in arbitrary units of fluorescence and the bars show ± standard error (n= 5). Asterisks indicate statistically significant differences with respect to control (*$P < 0.01$). B) Detection of *T. vaginalis* hydrogenosomes by using fluorescence microscopy in cells stained with Mitotracker Red: hydrogenosomes appear intensely stained red (arrows).

Figure 4. A) Intracellular reactive oxygen species (ROS) production by *T. vaginalis* trophozoites incubated in presence of resveratrol (RESV) at 25, 50 and 100 μM and metronidazole (MDZ) at 3.125, 6.25 and 12.5 μM. Prooxidant levels were measured using the oxidation sensitive fluorescent probe 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate. B) Effect of RESV at 25, 50 and 100 mM and MDZ at 3.125, 6.25 and 12.5 μM on membrane potential in *T. vaginalis* hydrogenose, quantified by using the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolcarbocyanine iodide (JC-1); the results are expressed in arbitrary units of fluorescence. Histogram bars show mean ± standard error (n= 5). Asterisks indicate statistically significant differences between the treated groups and the untreated controls: *$P < 0.05$; **$P < 0.01$.

691 **Figure 5.** Expression level of ferredoxin oxidorreductase (PfoD) and hydrogenase
692 (TvhydA) genes after exposure to resveratrol (RESV, 50-100 μM) and metronidazole
693 (MDZ, 3.125-6.25 μM), measured by retrotranscriptase-real time PCR (RT-qPCR) using
694 β-tubulin (btub2) gene as reference for gene expression normalization. Analysis of
695 relative gene expression was on by the $2^{-\Delta\Delta Cq}$ method and each vertical bar represent the
696 mean ± standard error. Asterisks indicate statistically significant differences between the
697 treated groups and the untreated controls: *$P < 0.05$; **$P < 0.01$.
698
699 **Figure 6.** A) Western-blot analysis of cell lysates (CEs) of *T. vaginalis* incubated with
700 anti-heat shock protein (Hsp) 70 antibodies. *T. vaginalis* trophozoites were incubated in
701 the presence of resveratrol (RESV), at 50 and 100 μM, and metronidazole (MDZ), at
702 3.125 and 6.25 μM; (-) control without the addition of anti-Hsp70 antibody. B)
703 Densitometric analysis of the immunostained CEs from A (see Materials and Methods
704 for description of bands quantification).