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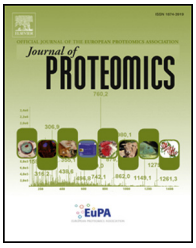

The flavonoid (–)-epicatechin affects cytoskeleton proteins and functions in *Entamoeba histolytica*☆

Verónica Bolaños[a], Alfredo Díaz[a], Jacqueline Soto[a], Mario A. Rodríguez[c], Cesar López-Camarillo[d], Laurence A. Marchat[a,b], Esther Ramírez-Moreno[a,b,*]

[a]Posgrado en Biomedicina Molecular, ENMyH, Instituto Politécnico Nacional, México City 07320, México
[b]Doctorado en Biotecnología, ENMyH, Instituto Politécnico Nacional, México City 07320, México
[c]Departamento de Infectómica y Patogénesis Molecular, CINVESTAV-IPN, México City A.P. 14-740, México
[d]Posgrado en Ciencias Genómicas, Universidad Autónoma de la Ciudad de México, México City 03100, México

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ABSTRACT

Human amoebiasis is an intestinal disease with a global distribution. Due to reports of parasite resistance or susceptibility reduction to metronidazole treatment, there is a renewed interest for the search of new molecules with antiamoebic activity. The flavonoid (–)-epicatechin that was isolated from the Mexican medicinal plant *Geranium mexicanum* HBK has an *in vitro* activity against *E. histolytica* trophozoites, however its molecular effects have been poorly documented. Using a proteomic approach based on two-dimensional gel electrophoresis and mass spectrometry (ESI-MS/MS) analysis, we evidenced that *E. histolytica* cytoskeleton proteins exhibit differential abundance in response to (–)-epicatechin treatment. Moreover, functional assays revealed modification on pathogenic mechanisms associated with cytoskeleton functionality, namely, adhesion, migration, phagocytosis and cytolysis. Consequently, these data suggested that (–)-epicatechin could affect virulence properties of this human pathogen.

Biological significance

This work contributes with some advances in the action mechanisms involved in the antiamoebic effect of the flavonoid (–)-epicatechin. We found that this flavonoid has an unusual effect on trophozoites growth that is dependent of its concentration. Additionally, we reported that (–)-epicatechin affects mainly amebic cytoskeleton proteins, which results in alteration on important virulence mechanisms, like adhesion, migration, phagocytosis and cytolysis. This study provides new knowledge about a potential alternative therapy directed to the treatment of amoebiasis.

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Abbreviations: IC50, 50% inhibitory concentration.

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* *Corresponding author at:* Laboratorio de Biomedicina Molecular 2, Escuela Nacional de Medicina y Homeopatía-IPN, Guillermo Massieu Helguera No. 239, La Escalera, Ticoman. México, City, C. P. 07320, México. Tel./fax: +52 57 29 60 00x55538.
E-mail address: estherramirezmoreno@yahoo.com (E. Ramírez-Moreno).

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1. Introduction

The protozoan parasite *Entamoeba histolytica* is the causal agent of human amoebiasis. This intestinal disease has a global distribution although infections are more frequent in developing tropical countries from Latin America, Asia and Africa. Prevalence rate can reach 50% in endemic countries and *E. histolytica* is estimated to cause more than 100,000 deaths per year [1]. The most common treatment for amoebiasis is metronidazole, but there are some reports about parasites exhibiting drug resistance or reduction of susceptibility to metronidazole treatment [2,3]. Nowadays, there is a renewed interest for the discovery of new molecules with antiamoebic activity.

In their search for new therapeutic antiprotozoal alternatives from Mexican medicinal plants used in the treatment of diarrhoea and dysentery, Calzada et al. [4] isolated the flavan-3-ol, (–)-epicatechin, from *Geranium mexicanum* HBK. *In vitro* susceptibility tests evidenced that (–)-epicatechin shows a 50% growth inhibitory concentration (IC50) of 1.9 μg/ml against *E. histolytica* trophozoites after incubation for 48 h at 37 °C. This molecule possesses the basic structure of flavonoids, with two rings of phenyls (A and B) linked by a C ring of pyran; additionally, it has an –OH group in position 3 of the C ring, typical of the flavanol group [5]. The biological activity of (–)-epicatechin has been demonstrated in other protozoan parasites. Alanis et al. [6] tested seven compounds obtained from *Rubus coriifolius*, a medicinal plant used by Mexican Maya communities to treat bloody diarrhea, and found that (–)-epicatechin was the main responsible for the *in vitro* activity against *E. histolytica* and *Giardia lamblia* trophozoites; interestingly, its activity was comparable to emetine. Mendonca-Filho et al. [7] evaluated the *in vitro* leishmanicidal effects of the polyphenolic-rich extract from *Cocos nucifera Linn*, which contains a high amount of (–)-epicatechin, and found that 10 μg/ml was the minimal inhibitory concentration to completely abrogate parasite growth in infected macrophages. Results also suggested an irreversible injury of parasite metabolism and the induction of nitric oxide synthesis by murine macrophages, which enhance the potential of killing mechanisms of these cells. There are also some reports about (–)-epicatechin efficacy *in vivo*, using mouse models for experimental infection of *G. lamblia* [8] and *Trypanosoma cruzi* [9].

Despite its interesting antiprotozoal activity, the molecular mechanisms underlying the effects of (–)-epicatechin on protozoan parasites have been poorly documented. Our group recently reported that this flavonoid induces nuclear and cytoplasmic changes in *E. histolytica* trophozoites treated with the IC50 of 1.9 μg/ml [10]. In order to gain insights on the action mechanisms involved in the anti-amoebic effect of (–)-epicatechin, here we carried out a two dimensional gel-based proteomic analysis to evidence changes in global protein expression profile of *E. histolytica* trophozoites treated with (–)-epicatechin. Our results suggested the modulation of several proteins, mainly cytoskeleton proteins. As a consequence, migration, adhesion, phagocytosis and cytolytic capacities were changed, which could affect the pathogenic processes of this human pathogen.

2. Materials and methods

2.1. *E. histolytica* trophozoites culture

E. histolytica trophozoites, HM1-IMSS strain, were axenically grown in TYI-S-33 medium, supplemented with 20% bovine serum and harvested in the log phase of growth [11].

2.2. Cell growth and viability assays

The (–)-epicatechin flavonoid purchase from Sigma-Aldrich Co. (St. Louis, Mo.) was dissolved in 3 μl DMSO and 997 μl complete TYI-S-33 medium in sterile conditions to obtain a final concentration of 1 μg/μl. *E. histolytica* trophozoites (1.5×10^4) were incubated in 10 ml complete TYI-S-33 medium for 48 h at 37 °C in the presence of increasing (–)-epicatechin concentrations ranging from 0.96 to 3.84 μg/ml, corresponding to 3.3 to 13.2 μM. Then, cells were harvested by cooling and centrifugation at 500 × g for 5 min at 4 °C; pellets were suspended in 2 ml phosphate-buffered saline (PBS) and amoebas were counted in a Neubauer chamber.

For cell viability assays, *E. histolytica* trophozoites (1.5×10^5) grown for 48 h at 37 °C in the presence of 3.3 to 13.2 μM (–)-epicatechin concentrations were mixed with 100 μl trypan blue stain exclusion (0.4%) and viable trophozoites were determined using a Neubauer chamber.

Experiments were performed by triplicate and results are expressed as mean ± standard deviation (SD). Cells grown in complete TYI-S-33 medium and 0.05% DMSO were used as controls in all experiments. Cell growth and viability were determined as the number of trophozoites in each treatment condition in relation to the number of trophozoites grown in complete medium, and expressed in percentage.

2.3. Cytotoxicity assays

Human epithelial colorectal adenocarcinoma cells (Caco-2: HTB-37 ATCC, USA) were cultured in a 96-well microplate (3.0×10^4 cells/well) containing Advance Minimum Essential Medium (MEM, Gibco) supplemented with 5% fetal bovine serum, 200 mM glutamine (Gibco), 0.0125 % penicilin and 0.02% streptomycin. Cultures were maintained in a 5% CO_2 humidified atmosphere at 37 °C for 24 h. After eliminating medium, confluent Caco-2 cells were incubated in fresh medium with 8.2 μM (–)-epicatechin or 0.05% DMSO for 48 h. Then, supernatants were collected, centrifuged at 500 × *g* for 5 min, and transferred to a microtiter plate (50 μl/well). Lactate dehydrogenase (LDH) levels were measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) following manufacturer recommendations. Briefly, 50 μl substrate mix was added to each well and plates were incubated in the dark for 30 min at 22 °C. Then, 50 μl stop solution was added, and absorbance was quantified in a spectrophotometer at a 492 nm wavelength. Cells incubated with free medium and treated with 9% Triton X-100 were used as negative and positive controls, respectively. Cytotoxicity was determined from absorbance values and expressed as: [(cells treated with (–)-epicatechin or DMSO – cells in free medium)/(cells of positive control – cells of

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negative control)] × 100. Experiments were performed twice by triplicate and results were expressed as mean ± SD.

2.4. MTT assays

Caco-2 cells were cultured in a 96-well microplate (3.0×10^4 cells/well) as described above. Then, confluent cells were incubated in 100 μl fresh medium containing 8.2 μM (–)-epicatechin or 0.05% DMSO at 37 °C. At 48 h, medium was removed and cells were incubated with 1 mM MTT at 37 °C for 4 h. After eliminating MTT, formazan dye crystals were solubilized in 100 μl DMSO for 5 min. Absorbance was measured in a spectrophotometer at 570 nm wavelength. Viability was determined from absorbance values and expressed as: [(cells treated with (–)-epicatechin or DMSO – DMSO)/(cells in free medium – DMSO)] × 100. Experiments were performed twice by triplicate and results were expressed as mean ± SD.

2.5. Protein extraction

Trophozoites (15×10^6) grown in TYI-S-33 medium or in the presence of 8.2 μM (–)-epicatechin for 48 h at 37 °C were lysed in 100 mM Tris (1 ml) in the presence of 30 μl Complete proteases inhibitor Cocktail (Roche), as well as E64 100 mM (1 ml) and 15 μl PMSF (100 mM). Samples were frozen in dry ice for 5 min and unfrozen by mixing three times. Then, they were centrifuged at 15,000 g (14,000 rpm) for 5 min at 4 °C, supernatant was retrieved and cleaned using the ReadyPrep 2D Clean Up kit (Bio-Rad) according to the manufacturer´s protocol. Then, protein pellets were dissolved in 100 μl sample buffer (8 M urea, 4% CHAPS, and 80 mM dithiotreitol [DTT]) and protein concentration was determined using the Bradford method. Protein integrity was assessed by 10% SDS-PAGE and Coomassie blue staining.

2.6. Two-dimensional differential in gel electrophoresis (2-D DIGE)

Protein samples (400 μg) obtained from *E. histolytica* trophozoites grown without or with 8.2 μM (–)-epicatechin for 48 h at 37 °C were mixed with 1 μl bromophenol blue (1%) and 250 μl rehydration solution (8 M urea, 4% CHAPS, 2% ampholines pH 4–7, 0.002% bromophenol blue, 80 mM DTT), and loaded onto 13 cm Immobiline DryStrips (linear pH gradient 4.0–7.0, GE Healthcare) that were passively hydrated for 18 h. Then, proteins were isoelectrically focused using the Ettan IPGphor 3 GE HealthCare in four steps: an initial gradient from 1 to 500 V at 500 V/h, followed by a gradual increase from 500 to 1000 V at 800 V/h, and from 1000 to 8000 V at 11,300 V/h. Finally, a hold step at 5400 V/h was applied. Next, samples were successively reduced with 2% DTT and alkylated with 2.5% iodoacetamide in equilibrium solution (6 M urea, 2% SDS, 0.375 M Tris–HCl pH 8.8, 20% glycerol) for 10 min at room temperature. For the second dimension analysis, samples were separated on 12% SDS-PAGE for 7 h at 150 V/400 mA in a vertical electrophoresis system. After electrophoresis, gels were stained with Bio-Safe colloidal Coomassie Blue (Biorad) according to manufacturer´s protocols. Finally, gels were fixed with 30% ethanol–10% acetic acid. Three independent biological experiments were performed to assess the reproducibility of the assay.

2.7. Detection and identification of proteins with differential abundance

Images from 2-D gels were documented in an IMAGEMASTER Amersham GE Life Sciences scanner. The Melanie 7.0 software was used to visualize, explore and analyze 2D electrophoresis gel images, in order to detect differential spots between images corresponding to trophozoites grown without or with 8.2 μM (–)-epicatechin. Briefly, spot detected in gels from treated and untreated trophozoites were submitted to boundary tool and densitometry analysis, and spot quantities were normalized to remove variations non-related to expression changes in spot intensity. The criterion of a differential abundance of any particular protein between both experimental groups was set as at least a 2-fold change in spot volume between matched sets in triplicates, according to Student's t test ($p < 0.05$).

Selected spots were excised from Bio-Safe colloidal Coomassie Blue stained gels and analyzed by Tandem mass spectrometry (LC/ESI-MS/MS) at the Laboratorio Universitario de Proteómica, IBT/UNAM, Cuernavaca, Mexico, in order to determine protein identity. Searches were conducted using the National Center for Biotechnology Information non-redundant database (NCBInr, http://www.ncbi.nih.gov). A protein 'hit' was accepted as a valid identification when MS/MS spectrum matched at the 95% level of confidence ($p < 0.05$). Ion score is $-10*\text{Log}(P)$, where P is the probability that the observed match is a random event. The threshold ion score in the above conditions was 41 for $p < 0.05$.

2.8. Western blot assays

Protein extracts (60 μg) from *E. histolytica* trophozoites grown in the absence or presence of 8.2 μM (–)-epicatechin for 48 h at 37 °C were resolved by 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were blocked with 5% non fat milk in PBS-Tween 0.05% for 1 h at 37 °C. The detection of selected proteins was performed with the following primary antibodies: rabbit anti-amoebic myosin II antibody (1:750 dilution), mouse monoclonal anti-human actin antibody (1:900 dilution). Mouse polyclonal antibodies against the *E. histolytica* housekeeping lectin (1:750 dilution) were used as internal control. Membranes were incubated with primary antibodies overnight at 4 °C. Goat anti-rabbit IgG (H + L) Horseradish Peroxidase Conjugate and goat Anti-Mouse IgG (H + L) Horseradish Peroxidase Conjugate antibodies (Invitrogene) (1:2000, 1 h at 37 °C) were used as secondary antibodies. Proteins were revealed by the 3,3'-diaminobenzidine (Sigma Aldrich) method. Bands were submitted to densitometric analysis, assays were performed by duplicated. Lectin data were used to normalize myosin II and actin data in each condition.

2.9. Adhesion assays

2.9.1. Adhesion of trophozoites to fixed Caco-2 cells monolayers

Caco-2 cells (2.0×10^4 cells/well) were cultured in a 24-well microplate containing MEM medium supplemented with 5% fetal bovine serum and antibiotics as described. Cultures were maintained in a 5% CO_2 humidified atmosphere at 37 °C, changing medium every third day, until the formation of a monolayer. Cells were counted and monolayers were washed

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twice with sterile PBS and covered with 1 ml serum-free TYI-S-33 immediately before adding the suspension of amoebae previously grown in 8.2 μM (–)-epicatechin for 48 h at 37 °C (one amoeba per four Caco-2 cells). After 1 h at 37 °C, medium was removed and kept; monolayers were washed twice with serum-free TYI-S-33 medium to remove additional non adherent trophozoites [12]. The number of adherent trophozoites was expressed as the difference between the initial number of amoebae added in each well and the number of non-adherent trophozoites determined by Trypan blue assay. Trophozoites grown in complete TYI-S-33 medium and 0.05% DMSO were used as controls. Experiments were performed by triplicate and results were expressed as mean ± SD.

2.9.2. Erythrocyte binding affinity assay

Erythrocytes binding to trophozoites were evaluated as described Voigt et al. [13] with some modifications. Trophozoites (2.0×10^5 in 0.2 ml) previously treated with 8.2 μM (–)-epicatechin for 48 h at 37 °C were mixed with washed human erythrocytes (2.0×10^7 in 0.2 ml). Cellular interaction (1:100) was incubated on ice for 15 min. After that, cells were fixed with 4% parafomaldehyde (1 ml) for 30 min at 37 °C. Free erythrocytes were removed by centrifugation and washing, the pellet of trophozoites was resuspended and stained by the Novikoff method [14,15], using 1 ml 3,3-diaminobenzidine (Sigma, 2 mg/ml) in 2-amino-2-methyl-propanediol (Merck, 0.05 M, pH 9.7 buffer) for 5 min at 37 °C. After washing and suspending in 100 μl of PBS pH 6.8, cellular suspension was placed on microscopic slide and visualized through a Nikon Eclipse 80i microscope (20–100× magnification) connected to the Nis Elements Advanced Research Software, Nikon Version 3.0. The number of attached erythrocytes per amoeba was counted in randomly selected fields (100 trophozoites). Trophozoites grown in complete TYI-S-33 medium and 0.05% DMSO were used as controls. Experiments were performed by triplicate and results were expressed as mean ± SD.

2.10. Cell migration assays

E. histolytica trophozoites (5.0×10^4) previously treated with 8.2 μM (–)-epicatechin for 48 h at 37 °C were placed in serum-free TYI-S-33 medium on the upper compartment of the Transwell chamber (Corning) with 6.5 mm diameter and 8 μm pore size polycarbonate membrane, whereas the lower chamber was loaded with complete TYI-S-33 medium. After 3 h at 37 ºC, the number of trophozoites that have migrated into the lower chamber was determined using Trypan blue assay [16,17]. Trophozoites grown in 0.05% DMSO or complete medium were used as controls. Experiments were performed by triplicate and results were expressed as mean ± SD.

2.11. Tissue culture monolayer destruction assays

Caco-2 cells (1.4×10^5 cells/well) were cultured in a 24-well plate as described above. Monolayers were washed with phosphate saline solution pH 7.4 and trophozoites grown with 8.2 μM (–)-epicatechin for 48 h at 37 °C were added to yield an infection ratio of 1 trophozoite:4 Caco-2 cells, in a final volume of 1 ml TYI-S-33 medium. Plates were incubated 5 min at 37 °C with 5% CO_2 and cytolysis was quantified using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) as described above. Trophozoites grown in complete TYI-S-33 medium and 0.05% DMSO were used as controls. Experiments were performed by triplicate and results were expressed as mean ± SD.

2.12. Erythrophagocytosis assays

2.12.1. Microscopic evaluation

Trophozoites grown with 8.2 μM (–)-epicatechin for 48 h at 37 °C were incubated with human erythrocytes for 5 and 10 min at 37 °C as described above. Following fixing and washing steps, engulfed erythrocytes were stained by the Novikoff method as described above [14,15]. The cellular suspension was placed on microscopic slide and visualized through a Nikon Eclipse 80i microscope (20–100× magnification). The number of engulfed erythrocytes per amoeba was counted in randomly selected fields (100 trophozoites). Results were expressed as a phagocytic index, which is the percentage of amebic trophozoites that had engulfed erythrocytes multiplied by the average number of erythrocytes ingested per ameba [18]. Trophozoites grown in complete TYI-S-33 medium and 0.05% DMSO were used as controls. Experiments were performed by triplicate and results were expressed as mean ± SD.

2.12.2. Spectrophotometric evaluation

Internalized hemoglobin in trophozoites was quantified by a colorimetric method [13]. Amoebas grown with 8.2 μM (–)-epicatechin for 48 h at 37 °C (2.0×10^5) and human erythrocytes (2.0×10^7) were incubated for 10 min at 37 °C. After centrifugation, cells were resuspended in 1 ml cold distilled water for bursting non-ingested erythrocytes. After centrifugation, the pellet was resuspended in 1 ml concentrated formic acid (Sigma), absorbance was measured at 400 nm with a spectrophotometer. Trophozoites grown in complete TYI-S-33 medium and 0.05% DMSO were used as controls. Experiments were performed by triplicate and results were expressed as mean ± SD.

2.13. Statistical analysis

Statistical analysis was performed using one-way ANOVA test and the Dunnett´s and Bonferroni test through the computer program GraphPad Prism 5.01. Statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Trophozoites growth and viability are affected by (–)-epicatechin treatment

In order to corroborate the effects of (–)-epicatechin previously reported by Calzada et al. [4], trophozoites were incubated with increasing concentrations of drug for 48 h at 37 °C to evaluate *E. histolytica* growth and viability. Interestingly, (–)-epicatechin at 3.3, 4.9, and 6.6 μM caused a reduction in cell number by about 25.77% ± 2.47%, 20.51% ± 2.25% and 27.17% ± 2.44%, respectively; the best inhibitory effect of 74.48% ± 3.16% ($p < 0.05$) was

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obtained using 8.2 μM (–)-epicatechin, while higher concentrations (9.9 and 11.5 μM) only reduced cell number by 53.79% ± 2.37% and 43.89% ± 2.94%, respectively. Notably, the highest concentration (13.2 μM) did not have a significant inhibitory effect in comparison with control cells growing in TYI-S-33 medium and DMSO (Fig. 1A). Trypan blue assays confirmed that 8.2 μM (–)-epicatechin concentration was the most effective dose against *E. histolytica* trophozoites, reducing cell viability by about 75% in comparison with control cells growing in TYI-S-33 medium and DMSO. Again, the highest concentration (13.2 μM) did not have any effect on trophozoite viability (Fig. 1B).

3.2. (–)-Epicatechin treatment does not affect Caco-2 cells

Growth and viability assays showed that 8.2 μM (–)-epicatechin has a ~70% inhibitory effect on amoeba. To evidence whether this concentration also affects human cells, we first evaluated the toxicity on Caco-2 cells using the CytoTox 96® Non-Radioactive Cytotoxicity kit (Promega) (Fig. 2A). Interestingly, 8.2 μM (–)-epicatechin only exhibited 2.06% ± 3.30% cytotoxicity on Caco-2 cells, which is not significant in comparison with control cells growing in MEM medium and DMSO. We also performed MTT assays to evaluate Caco-2 cells viability in response to (–)-epicatechin (Fig. 2B). Results evidenced that cell viability was not significantly modified in the presence of 8.2 μM (–)-epicatechin (113% ± 20.90%) in comparison with control cells. Taken altogether, these data showed that 8.2 μM (–)-epicatechin specifically inhibits growth and viability of trophozoites, without affecting Caco-2 cells. Therefore, this dose was considered as the most effective dose against *E. histolytica* trophozoites and used in the subsequent experiments.

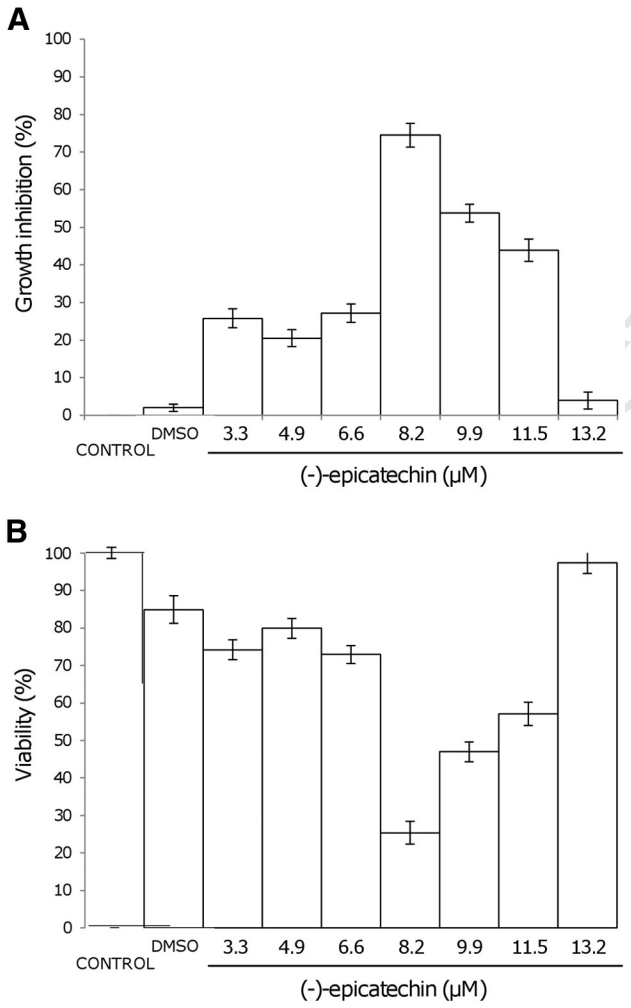

Fig. 1 – Effect of (–)-epicatechin on *E. histolytica* growth and viability. Trophozoites were incubated with different concentrations of (–)-epicatechin for 48 h at 37 °C. Growth inhibition was determined by cellular counting (A) and viability by trypan blue assay (B). Experiments were performed by triplicate. Data correspond to mean values ± SD and are expressed in percentage in relation to the number of trophozoites grown in complete medium.

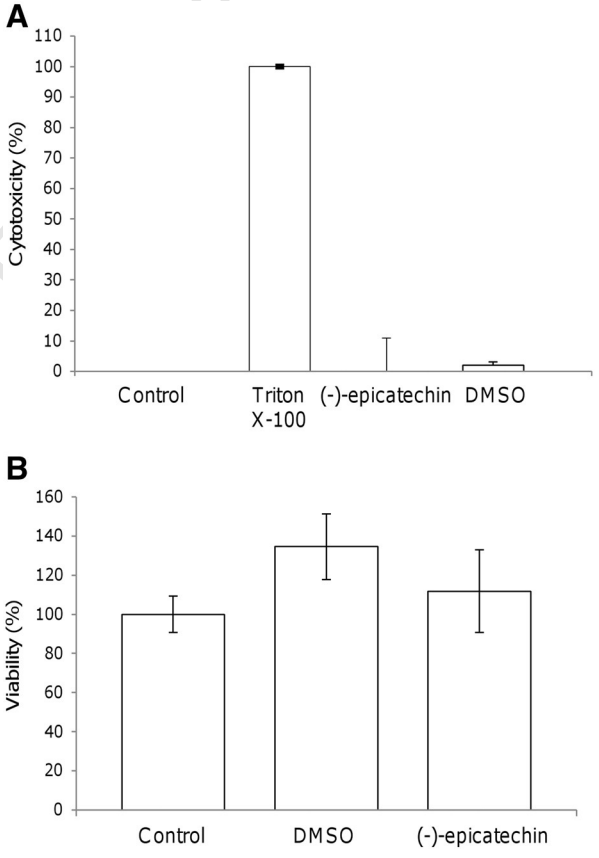

Fig. 2 – Effect of (–)-epicatechin against Caco-2 cells. A) Cytotoxicity assays. Caco-2 cells were incubated with different concentrations of (–)-epicatechin for 48 h at 37 °C and cytotoxic effect was determined using the CytoTox 96® Non-Radioactive Cytotoxicity kit (Promega). Cells growing in MEM, Triton X-100 or DMSO were used as control. B) Viability assays. Caco-2 cells were incubated with different concentrations of (–)-epicatechin for 48 h at 37 °C and viability was evaluated through MTT assays. Cells growing in MEM or DMSO were used as control. Experiments were performed by triplicate. Data correspond to mean values ± SD and are expressed in percentage in relation to the number of cells grown in MEM medium.

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3.3. (–)-Epicatechin treatment affects the abundance of several proteins in trophozoites

To gain insights in the molecular mechanisms underlying the anti-amoebic effect of (–)-epicatechin, we carried out a two dimensional gel-based proteomic analysis to compare the global protein expression profile of *E. histolytica* trophozoites in response to 8.2 μM (–)-epicatechin treatment. Since proteomic profiles corresponding to both control groups (trophozoites growing in complete TYI-S-33 medium or with 0.05% DMSO) were similar [19], trophozoites growing in standard medium were used as control group in this proteomic approach. The proteomic profiles obtained were highly reproducible in the different experiments. Fig. 3A shows two representative gels from untreated (up) and (–)-epicatechin treated trophozoites (down). The comparative analysis of 2-D gels allowed the detection of 51 spots with a differential abundance between both conditions. Among them, 30 were up-modulated and 21 were down-modulated in trophozoites treated with (–)-epicatechin. Magnification of eight selected spots and densitometry analysis are shown in Fig. 3B and C. After LC/ESI-MS/MS tandem mass spectrometry analysis and NCBI database search, these modulated proteins were identified; their identity and function, accession number, sequence coverage, Mascot score, and MS/MS peptide sequence are summarized in Table 1. Up-modulated proteins included the heat shock protein 70, myosin II heavy chain, actin, glyceraldehyde-phosphate dehydrogenase and a hypothetical protein. Down-modulated proteins were fructose-1,6-biphosphate aldolase, alpha-actinin and an hypothetical protein. Interestingly, alpha-actinin, myosin II and actin are proteins of the cytoskeleton, while fructose-1,6-biphosphate aldolase and glyceraldehyde-phosphate dehydrogenase are involved in the energetic metabolism. Proteomic results were validated by western blot assay using rabbit anti-amoebic myosin II and mouse monoclonal anti-actin antibodies (Fig. 4A). Results confirmed that myosin II and actin were about 1.6-fold over-expressed in response to 8.2 μM (–)-epicatechin treatment (Fig. 4B), which were in agreement with the differential abundance detected in proteomic profiles.

3.4. (–)-Epicatechin treatment modifies adhesion and migration capacity of *E. histolytica* trophozoites

Proteomic analysis evidenced that the abundance of various proteins of the cytoskeleton, including alpha-actinin, myosin II heavy chain and actin proteins, was modulated in response to 8.2 μM (–)-epicatechin treatment. These changes could affect several functions of *E. histolytica* trophozoites cytoskeleton that are important for parasite survival and virulence, such as adhesion and migration. To test this hypothesis, we first performed adhesion assays using 3×10^4 trophozoites

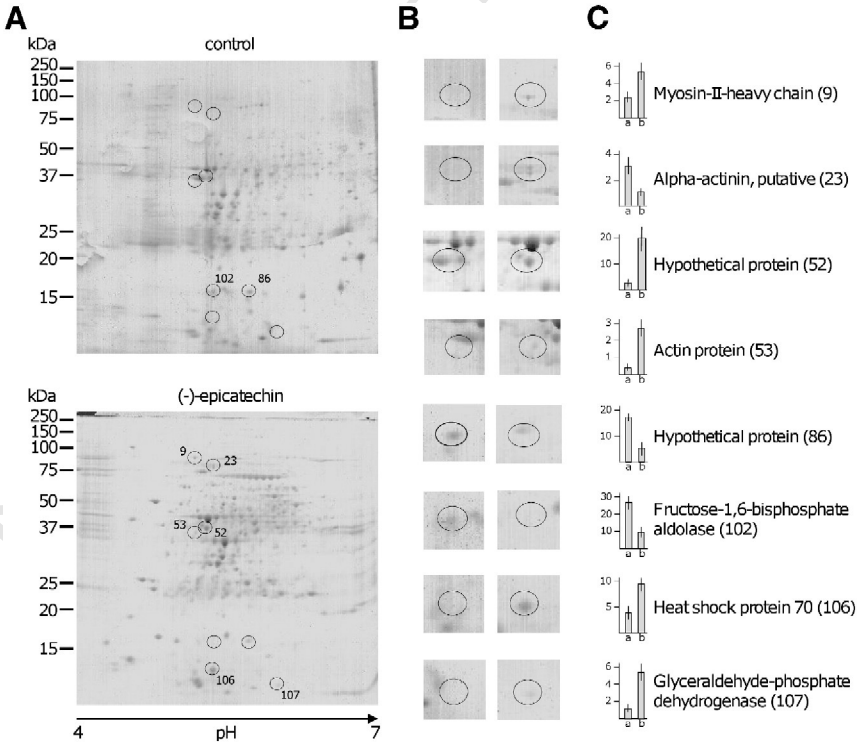

Fig. 3 – Proteomic analysis of *E. histolytica* in response to **(–)-epicatechin treatment. Trophozoites were incubated with 8.2 μM (–)-epicatechin for 48 h at 37 °C and proteins were submitted to two-Dimensional Differential in Gel Electrophoresis. A) Representative 2-D DIGE gels of *E. histolytica* trophozoites untreated (up) or treated with 8.2 μM (–)-epicatechin. Circles show differential spots between both conditions. B) Magnification of eight selected spots in A) Left, control cells; right; cells treated with (–)-epicatechin. C) Densitometry analysis of selected spots in each condition (a, control; b, with (–)-epicatechin). Experiments were performed by triplicate. Data correspond to mean values ± SD. Spots were determined as differentially abundant according to Student's t test ($p < 0.05$).**

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Table 1 – Modulated proteins in response to (–)-epicatechin treatment of trophozoites.

Protein (Spot number)	Accession number[a]	Fold change	Score	No. of matched peptides	Molecular mass/pI	Sequence coverage (%)	MS/MS peptide sequence
Up-modulated proteins							
Myosin II heavy chain (9)	C4LU72	2.7	1.32	2	246,709.8/ 5.2	1.2	^{1366}AALSEEIDQANEK ^{1532}DNQSAEEELNELR
hypothetical protein (52)	C4M126	7.5	3	3	37,759.0/ 5.1	15.3	^{97}ILNEIVKPLEALVK^{110} ^{126}SINSHNALVTAAEK^{139} ^{226}AVLDQFVVTHIELSTIIADVSQR^{248}
actin protein (53)	B1N2P0	10	89	1	42,042/5.26	5	^{290}LYGNIVLSGGTSMYPGINTR
heat shock protein 70 (106)	C4M3S5	2.25	1172	7	71,782/5.32	20	^{81}SDPAIQNDMK ^{116}KFTPEEISSMVLTK ^{130}MKETAESFVGK ^{144}NAVITCPAYFNDSQRQATK ^{163}DAGTIAGMNVMRIINEPTAAAIAYGLDKK ^{197}NVLIFDLGGGTFDVSLLAIDDGVFEVK ^{224}ATNGDTHLGGEDFDNRLVNHFIAEFK
glyceraldehyde-phosphate dehydrogenase (107)	C4LVR9	5.5	125	1	32,216/7.15	8	^{149}VINNEFGIVEGLMTTIHATTATQK
Down-modulated proteins							
Alpha-actinin (23)	B0E673	2.8	2.6	1	50,833.3/ 5.2	3.2	^{61}TGIVLINFFEIISK
hypothetical protein (86)	C4LWF5	3.4	3.52	3	15,976.9/ 5.4	30.3	^{44}NFFFDQGTQAVYCK^{57} ^{63}ATATAVTDSIAIK^{75} ^{94}GAGGKPHSVVFGDSSER^{110}
Fructose-1,6-bisphosphate aldolase (102)	C4LXD7	2.7	5.59	5	36,216/6.1	25.8	^{92}GVPISLNLDHGANFQICK ^{145}FGVTVEGELGVLSGVEDDVAAAEHVFTDPK ^{175}DVEAFV ^{182}KDTGVDALAISIGTSHGAYK ^{209}IRLDILHEVEK

[a] UniproKnowledge database.

with Caco-2 cells and erythrocytes. Results showed that only 46.94% ± 2.73% of trophozoites previously treated with 8.2 μM (–)-epicatechin were able to adhere to the monolayer of Caco-2 intestinal cells. In contrast, 91.33% ± 2.73% and 75% ± 2.73% of trophozoites grown in medium or 0.05% DMSO, respectively, were found adhered to the target cells (Fig. 5A, B).

Similarly, erythrocyte binding affinity assay showed that 39.33% of (–)-epicatechin treated trophozoites were able to bind at least one erythrocyte, while this percentage increased to 50.66% and 49.33% in control groups (Fig. 5C and D).

Then, we used the Transwell chamber system to evaluate the migration capacity of trophozoites. As shown in Fig. 5E

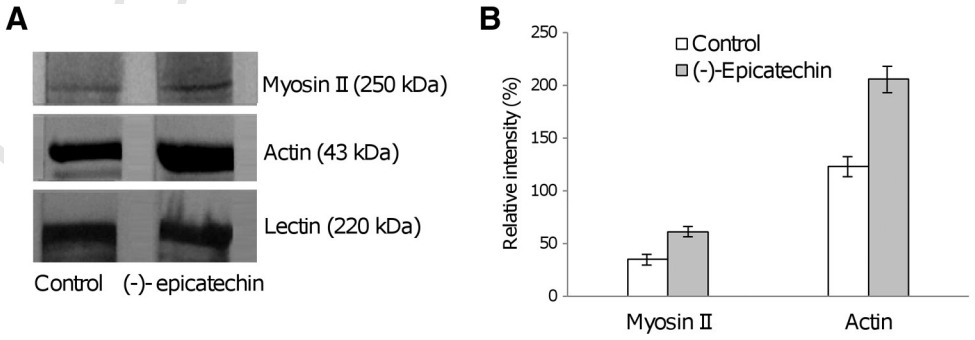

Fig. 4 – Expression of Myosin II heavy chain and actin in *E. histolytica* in response to (–)-epicatechin treatment. Trophozoites were incubated with 8.2 μM (–)-epicatechin for 48 h at 37 °C; proteins were separated through SDS-PAGE, electrotransferred to a nitrocellulose membrane and submitted to Western blot assays. A) Immunodetection of myosin II heavy chain and actin using specific antibodies. Lectin was used as an internal loading control. B) Densitometry analysis of bands in A. Pixels corresponding to lectin were used to normalize data in each condition. Experiments were performed by triplicate. Data correspond to mean values ± SD.

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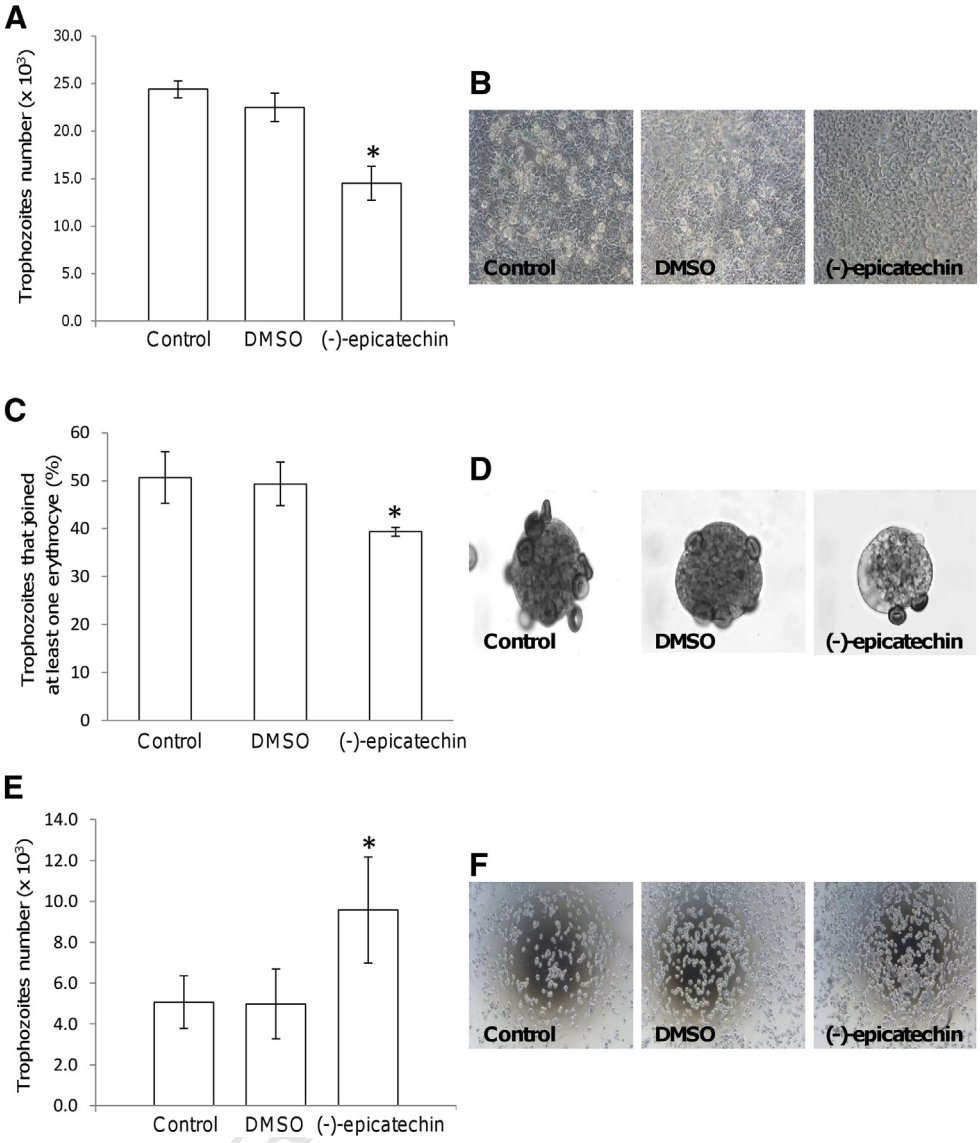

Fig. 5 – Effect of (–)-epicatechin on adhesion and migration of *E. histolytica*. A) Caco-2 cells adhesion assay. Trophozoites previously treated with 8.2 μM (–)-epicatechin were incubated with Caco-2 cells for 1 h at 37 °C and the number of adherent cells was determined by Trypan blue assay. B) Representative photographs showing trophozoites adhered to Caco-2 cells in each condition. C) Erythrocytes adhesion assay. Trophozoites previously treated with 8.2 μM (–)-epicatechin were incubated with erythrocytes for 15 min on ice and the number of adherent erythrocytes was determined by staining with Novikoff method and microscopic observation. D) Representative photographs showing erythrocytes adhered to trophozoites in each condition. E) Migration assay. Trophozoites previously treated with 8.2 μM (–)-epicatechin were loaded on the upper compartment of the Transwell chamber (Corning). After 3 h at 37 ºC, the number of trophozoites that have migrated into the lower chamber was determined using Trypan blue assay. F) Representative photographs showing trophozoites that have migrated to the lower chamber of the Transwell chamber. Experiments were performed by triplicate. Data shown in A, B and C correspond to mean values ± SD.

and F, 9578 ± 2567 (19.16%) trophozoites incubated with 8.2 μM (–)-epicatechin were able to migrate to the lower compartment, whereas only 5068 ± 1291 (10.13%) and 4974 ± 2232 (9.92%) migratory cells were found in control trophozoites growing in medium and 0.05% DMSO, respectively.

Taken altogether, our data indicated that (–)-epicatechin treatment negatively affects adhesion mechanism in trophozoites, while migration was increased.

3.5. *(–)-Epicatechin treatment modifies cytolysis and phagocytosis capacity of E. histolytica trophozoites*

Because adhesion and migration capacities were affected, we hypothesized that (–)-epicatechin may affect cytolytic and phagocytic capacity of trophozoites. Therefore, we evaluated the effect of trophozoites on Caco-2 cells using the CytoTox 96® Non-Radioactive Cytotoxicity kit (Promega) (Fig. 6A). Surprisingly, we observed that cytolytic activity

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was reduced by 25% and 21.5% in trophozoites treated with 8.2 μM (–)-epicatechin in comparison with control cells growing in medium and DMSO, respectively.

Then, we investigated if (–)-epicatechin treatment affects the erythrophagocytosis capacity of trophozoites by two complementary methods. Microscopic evaluation showed that the phagocytic index corresponding to the 5 min interaction of (–)-epicatechin treated trophozoites with erythrocytes, slightly increased in comparison with control groups. However, differences were not statistically significant (Fig. 6B and C). When the interaction was maintained for 10 min, the number of engulfed erythrocytes was so high in all groups that it was impossible to count them (Fig. 6C). Therefore, we decided to perform a spectrophotometric assay to better characterize the phagocytosis capacity of trophozoites. Interestingly, results evidenced a significant increase in the amount of internalized hemoglobin in trophozoites grown in the presence of 8.2 μM (–)-epicatechin in comparison with control cells growing in medium and DMSO (Fig. 6D).

Taken altogether, these data indicated that (–)-epicatechin treatment negatively affects the cytolytic capacity of trophozoites, while phagocytosis was increased.

4. Discussion

The development of novel anti-amoebic drugs is essential to be able to control the emergence of drug resistant *E. histolytica* trophozoites. In these searches, compounds from medicinal plants are of particular interest. The flavonoid (–)-epicatechin

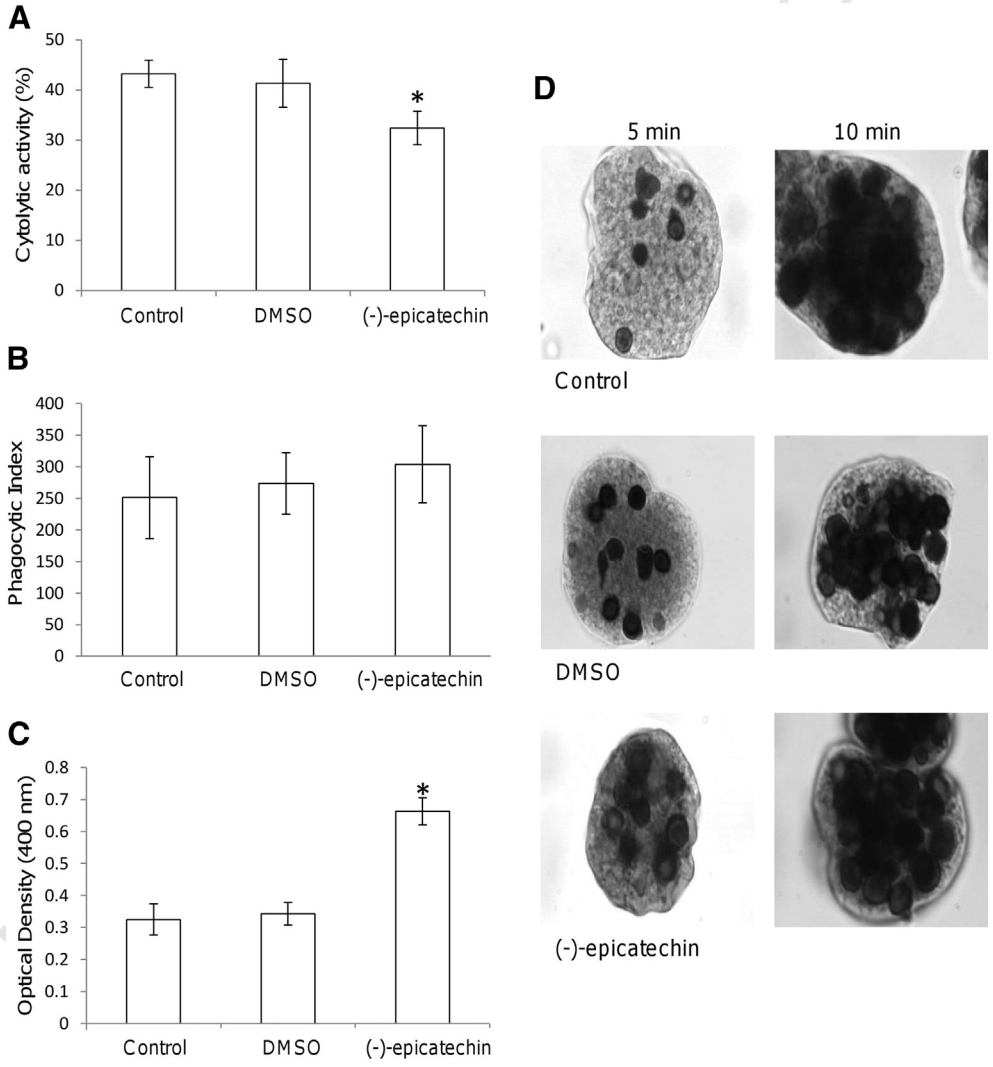

Fig. 6 – Effect of (–)-epicatechin on cytolytic and phagocytic capacity of *E. histolytica*. A) Trophozoites previously treated with 8.2 μM (–)-epicatechin were incubated with Caco-2 cells for 5 min at 37 °C and cytolysis was quantified using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). (B) Amoebas were incubated for 5 min with human erythrocytes in a relation of 1:100, and engulfed erythrocytes were determined by microscopic observation. C) Amoebas were incubated for 10 min with human erythrocytes as described and internalized hemoglobin in trophozoites was quantified at 400 nm. D) Representative photomicrographs showing erythrocytes engulfed by trophozoites at 5 and 10 min of interaction. Trophozoites grown in complete TYI-S-33 medium and 0.05% DMSO were used as controls. Experiments were performed by triplicate and results were expressed as mean ± SD.

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isolated from *Geranium mexicanum* roots was demonstrated to have an important antiamoebic activity *in vitro* [4], producing changes on nucleus and cytoplasm of *E. histolytica* trophozoites, but the action mechanism underlying this effect has been poorly understood. Looking for a new insight on the effect of (–)-epicatechin on *E. histolytica* trophozoites, we evaluated different concentrations of this molecule on trophozoites growth and viability in order to find the most effective concentration. We observed that the inhibitory effect of (–)-epicatechin increased with drug concentration, with a maximum inhibitory effect of 70% at a concentration 8.2 μM. Surprisingly, at higher (–)-epicatechin concentrations (9.9, 11.5 and 13.2 μM) the inhibitory effect on growth rate diminishes progressively. The dual effect of flavonoid has been reported on mammal cells, which argues that although the antioxidant activity of these molecules is well recognized, they can also display a prooxidant activity under certain conditions such as high doses or in the presence of metal ions [20–22]. Wätjem et al. [21] reported that flavonoids like quercetin and fisetin have protective effects at concentrations as low as 10–25 μM, whereas they induce cytotoxicity at higher concentrations (50–250 μM). Our results suggested that a low concentration of (–)-epicatechin (8.2 μM) inhibits *E. histolytica* trophozoites growth. It would be interesting to evaluate higher (–)-epicatechin concentrations to determine if the inhibitory effect can be reverted and the drug can promote trophozoites growth showing a true dual effect.

Interestingly, cytotoxicity assays showed that (–)-epicatechin is not toxic for mammal cells at the most effective dose of 8.2 μM, which led us to study the molecular effect of (–)-epicatechin on *E. histolytica* trophozoites. The proteomic analysis of trophozoites treated with the most effective growth inhibitory concentration of (–)-epicatechin (8.2 μM) evidenced the differential abundance of several protein spots. MS/MS spectra with low coverage showed 100% identity with *E. histolytica* proteins, 100% query coverage, and low or no identity with proteins of other organisms, which support the accuracy of protein identification. We found the deregulation of two enzymes that participate into the fourth and sixth steps of glycolysis, namely, fructose-1,6-bisphosphate aldolase and glyceraldehyde-phosphate dehydrogenase. Fructose-1,6-bisphosphate aldolase catalyzes the aldol cleavage of fructose-1,6-bisphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate; meanwhile, glyceraldehyde-phosphate dehydrogenase catalyzes the reversible oxidative phosphorylation of d-glyceraldehyde 3-phosphate into d-glycerate 1,3-bisphosphate [23,24]. Alteration of *E. histolytica* glycolytic pathway can be related with the increase in the number of glycogen deposits previously reported by our group [10]. The overexpression of Heat shock protein 70, which has been previously reported in *E. histolytica* trophozoites exposed to high oxygen environment [25], could be a response to the potential prooxidant properties of (–)-epicatechin. Interestingly we observed that three cytoskeleton proteins were affected by (–)-epicatechin, namely, myosin II heavy chain, actin and alpha-actinin. Myosin II is necessary for cytokinesis, control of cell shape, and maintenance of cell polarity, activities which are related to cellular motion [26]. Some studies have suggested a role for the actin–myosin complex in capping and uroid formation in *E. histolytica*, which has been postulated to participate in parasitic defense mechanisms against the host immune response, because it eliminates complement and specific antibodies bound to the amoeba surface [27]. Actin is one of the most abundant proteins in cells. In *E. histolytica*, actin polymerizes to form filaments and interacts with other proteins at specific sites to perform several cellular processes, such as adhesion, secretion of proteases and directed locomotion [28]. *E. histolytica* has two alpha-actinins isoforms. Both isoforms are able to form dimers, as well as to bind and crosslink actin filaments in a calcium-sensitive manner [29,30]. These proteins also have been implicated in the infectious mechanism of the parasite. It has been suggested that one of the alpha-actinins, or possibly both, can interact with the intracellular C-terminus of the Gal/GalNAc lectin that is required for adhesion [31–33]. It is probable that the down regulation of alpha-actinin that we observed on (–)-epicatechin treated trophozoites can be related with adhesion reduction to erythrocytes and Caco-2 cells.

There are different evidences pointing out that alterations on *E. histolytica* cytoskeleton proteins can dramatically modify cellular functions and, therefore, parasite pathogenicity. Overexpression of the last 579 amino acids of the myosin II heavy chain (that is essential for myosin II filament formation) produced a cytoskeleton-deficient *E. histolytica* strain that is characterized by inhibition of capping and uroid formation, abnormal motility, and therefore, changes in parasite virulence [34].

On the other hand, overexpression of myosin IB, a member of the myosin I family of motor proteins that interacts with actin and alpha-actinin and is involved in phagocytosis in *E. histolytica*, enhances cytoplasm viscosity and reduces erythrophagocytosis, indicating that an excess of myosin IB might lead to a denser actin network without inducing polymerization of new actin filaments [13,35]. To our knowledge, there are no reports about the overexpression of actin in *E. histolytica*, however the overexpression of beta-actin in myoblast has been shown to have a marked effect on cell morphology, producing striking effects in motility, increasing cell speed to almost double that in control cells [36,37]. Consistently, our results showed that the higher abundance of actin and myosin II heavy chain and the lower abundance of alpha-actinin in trophozoites treated with (–)-epicatechin are associated to changes in trophozoites properties related to cytoskeleton functions. Particularly, trophozoites treated with (–)-epicatechin exhibited a significant reduction in adhesion and cytolysis, while a significant increase in migration and phagocytosis was observed.

Cells require attachment sites on extracellular matrices in order to reorganize their cytoskeleton and initiate protrusions that are important for migration. However, the establishment of contact sites cannot be too avid or the cells, once attached, are less able to detach and move. Cellular migration is a mechanism that depends on interplay between forces of attachment and detachment, it is also intrinsically linked to cellular adhesiveness [38,39]. Therefore, (–)-epicatechin could be reducing adhesion forces and promoting migration by facilitating detachment of trophozoites on fibronectin matrix. This effect may involve different physical and/or biochemical mechanisms, through the regulation of integrins, receptor expression, receptor-ligand affinity, proteins phosphorylation, and changes of intracellular Ca^{2+} levels, among others.

Reducing adhesion forces may favor migration by facilitating detachment in models of high adhesivity. Calof and Lander [40]

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reported that laminin and merosin act as antiadhesion molecules in olfactory cells attaching to otherwise highly adhesive substrata; this reduction in attachment, in turn, results in the disassembly of focal contacts and the onset of migration.

Surprisingly, in trophozoites treated with (–)-epicatechin, we found that even when the adhesion is decreased there is an increase in phagocytic capacity, suggesting that (–)-epicatechin can enhance phagocytosis by a mechanism independent of the adhesion.

In a model for invasion, it has been shown that parasite motility is essential for establishment of infectious *foci*, while the adhesion to host cells modulates the distribution of trophozoites in the liver and their capacity to migrate in the hepatic tissue [41]. Therefore it is possible that trophozoites treated with (–)-epicatechin have changes in their virulence.

Taken altogether, our data show that (–)-epicatechin has an unusual dose–response effect on *E. histolytica* trophozoites that is dependent on its concentration. The action mechanism of (–)-epicatechin antiamoebic effect can be related to alteration in the abundance of glycolytic enzymes, stress oxidative proteins, and mainly with cytoskeleton proteins, modifying important pathogenicity mechanisms such as adhesion, migration, cytolysis and phagocytosis.

Further experiments should help to know if the changes produced by (–)-epicatechin on *E. histolytica* trophozoites could contribute to avoid or modify the course of infection in an *in vivo* infection model.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

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